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Original Article

Evaluation of Growth, Essential Oil Content and Composition in Four Thyme Species under Dryland Farming System in Zagheh, Rangeland, Khoramabad, Iran

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Abstract

In order to study the variation of four thyme species for aerial parts biomass, essential oil content and composition, a study was carried out in Zagheh, Rangeland, Khoramabad, Iran. Seeds of four species of *Thymus pubescens* Boiss. & Kotschy ex Celak., *Thymus daenensis* Celak. *Thymus daenensis* subsp. lancifolius (Celak.) Jalas and *Thymus kotschyanus* Boiss. & Hohen. were collected from their natural habitat in Lourestan province, Iran. Seeds sown in Jiffy pots in glasshouse in September 2016 and the seedlings were transferred to the field in October 2016 using Randomized Complete Block Design (RCBD) with 3 replications. Each unit of the experiment consists of 40 plants in two rows with 50 cm distance between rows and 50 cm between plants within rows. Data were collected for aerial parts biomass, morphological traits and essential oil percentages over two years 2017-2018. The essential oils of all samples were obtained by hydro-distillation, in flowering stage and the essential oil components were identified using gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS). Considerable variation was found between species for fresh and dry weight of aerial parts biomass, plant height, root length and essential oil content. The mean values of aerial parts biomass, crown diameter and essential oil in *T. daenensis* were higher than other species. For the root length, the higher value was obtained in *T. pubescens*. The lower and higher essential oil content with average values of 1.53% and 1.95% were obtained in *T. pubescens* and *T. daenensis* subsp. lancifolius, respectively. The results indicated that the major components in the essential oil were carvacrol (5.8, 18.5, 42.4 and 33.5%) and thymol (78.9, 51.9, 36.19 and 42.7%) for *T. daenensis, T. kotschyanus, T. daenensis* subsp. lancifolius and *T. pubescens*, respectively.

Keywords: *Thymus pubescens, T. lacnifolius, T. daenensis* subsp. *lancifolius, T. kotschyanus,* Drought

Introduction

Lamiaceae family, a large and diverse family of about 200 genera and 3200 species is distributed in the world [1]. *Thymus* genus belongs to Lamiaceae family containing almost 215 species of perennial forbs and small shrubs native to the Mediterranean region and also grows in some parts of southern Europe, Africa and some parts of Asia [2]. This genus is represented in the Iranian flora by 18 species [3], four of which (*Thymus carmanicus* Jalas, *T. daenensis and T. daenensis* subsp. lancifolius, *Thymus persicus* (Ronniger ex Rech.f.) Jalas *and Thymus trautvetteri* Klokov & Des.-Shost.) are endemic of Iran [4].

In Iran, the aerial parts biomass of thymes is more widely used as herbal tea, flavoring agents (condiment and spice) and medicinal plants [2]. It also is used as tonic, carminative, digestive, antispasmodic, anti-inflammatory, antitussive, expectorant and for the treatment of colds in Iranian traditional medicine [1]. The antimicrobial properties are often due to their phenol content and presence of thymol and carvacrol in its oils [5].

Thymes have abundance stems, relatively short and woody which gives a pulvinate crown to this species

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along with robust and dense roots playing a key role in soil stabilization and also preventing from water erosion in mountainous and sharp slope regions [6].

Medicinal plants, unlike other crops that are in the stress conditions may produce more active components in dry conditions and thereby increase their economic efficiency. Lebaschy and Sharifi reported that the secondary metabolite production in plants fluctuated with changing environmental conditions and water stress is a major factor for the synthesis of natural products [7]. Sabih *et al.* in assessing *Cymbopogon martini* (Roxb.) W.Watson found that oil production and plant growth are influenced by environmental factors such as drought stress [8]. The environmental factors as (temperature, drought, soil properties) could influence thymus species essential oil composition [9]. Miguel *et al.* found a high variability in the essential oil composition in *T. carnosus*, that it amount was related to harvesting stage [10]. Khorshidi *et al.* in investigating phenological stages on essence oil of *T. daenensis* found the higher essence percentage in full blooming stage ranged from 2.9 to 3.4% [11]. Sefidkon et al., reported that the beginning of flowering is the best stage for obtaining the highest oil content and the thymol percentage of *Thymus vulgaris* L. [12]. Safaei *et al* (2012) in *T. daenensis* obtained the highest aerial dry biomass and oil yield at a full flowering stage and the highest oil content at 50% flowering [13].

Different climatic conditions make different reaction of medicinal plants. Iran with average annual precipitation of 240 mm is considered as arid regions among the countries worldwide. A wide range of agricultural lands in dryland areas have low efficiency coupled with low production. The low efficiency pastures in large parts of Iran are lacking production capacity. Many plant species with low water requirements have great economic benefits for entering the cropping pattern. The need for comprehensive research and proper utilization of medicinal plants for pharmaceutical and cosmetic industries, health and accelerating food is essential. The aim of this study was to assess the response of four thyme species for yield, morphological traits and essential oil content and oil compounds in dryland farming system

Material and Methods

This study was conducted at Zagheh Rangeland Research Station in 35 km northeast of Khorramabad with latitude 33°28′48″ N and longitude 48°21′0″ E with an average altitude of 1960 m. In the study area, relative humidity was 54%, average annual precipitation and temperature were 613 mm and 18.4 °C, respectively. The number of frost days was 119 days, and annual evaporation was 1183 mm. The soil texture of the area was silty clay with acidity, pH=7.2 to 7.7. The dominant species of the area are *Bromus tomentellus* Boiss., *Agropyron trichophorum* (Link) K.Richt. *and Festuca ovina* L. and the vegetation cover of area consists of a mixture of annual and perennial herbaceous Forbs.

Seeds of four species of *Thymus pubescens, T. daenensis, T. lacnifolius* and *T. kotschyanus* were collected from their natural habitat in the Lourestan province in the west of Iran (Table 1). Seeds sown in Jiffy pots in outdoor of glasshouse in September 2016 and the seedlings was transplanted in a field in the early development stage (7 leaves) as spaced plant under a dryland farming system in October 2016 in Zagheh, Rangeland station, Khoramabad, Iran. The experiment layout was Randomized Complete Blocks Design (RCBD) with three replications. Each unit of the experiment consists of 40 plants in two rows with 50 cm distance between rows and 50 cm between plants within rows. In order to use rainwater, the seedlings were transplanted in the bottom of the furrows. No fertilizer was added at any time. Weeds were controlled mechanically by 2 times during growth period in the establishment year.

Data were collected over two years of 2017 and 2018 for the following morphological traits at full flowering stage: Fresh and dry weight of aerial parts biomass as (g/plant), crown diameter (cm), plant height (cm), leaf area (cm^2) and root length (cm).

The essential oil was obtained using the hydro-distillation method with a Clevenger type apparatus. Each sample containing 10 plants per plots. The plants were harvested at full flowering stage. The samples dried in shade and ground. The materials of each species (80 g in three replications) were placed in a 2.5-liter round-bottomed flask containing 1.5 L of water and distilled for 3h. Then, the oil weight was recorded according to the Hungarian plant pharmocopy letter [14] as follows:

$$\text{Essential oil\%} = \frac{\text{Essential oil weight (g)}}{\text{Aerial dry biomass weight (g)}}$$

Essential oil yield was calculated by essential oil% × aerial biomass (g/plant).

Essential Oil Analysis Using GC and GC-MS

The essential oil was analyzed by GC and GC/MS [15] using a 6890 Agilent gas chromatograph equipped with HP-Innowax fused silica capillary column (30 m, 0.25 mm, film thickness 0.25 mm). The oven temperature was held at 50 °C for 5 min and then increased from 50 °C to 220 °C at a rate of 8 °C/min. Injector and detector (FID) temperatures were 250 °C and 250 °C, respectively. Helium was used as the carrier gas at a flow rate of 1.3 ml/min. Diluted samples (1/100 in chloroform, v/v) of 2.0 ml were injected in the split/splitless (5:1 split) mode. Quantitative data were obtained electronically from FID area percent data. GC/MS analysis was performed using an Agilent system consisting a model 6890 with 5973 mass selective detector equipped with HP-Innowax fused silica capillary column (30 m 0.25 mm, film thickness

0.25 mm). For GC/MS detection, an electron ionization system, with ionization energy of 70 eV, was used. Helium was carrier gas, at a flow rate of 1.3 ml/min. The oven temperature programming was the same with GC analysis. Injector and MS transfer line temperatures were set at 220 °C and 250 °C, respectively. Diluted samples (1/100 in chloroform, v/v) of 2.0 ml were injected in the split/splitless (5:1 split) mode. Identification of oil components was accomplished based on comparison of their retention times with those of authentic standards and by comparison of their mass spectral fragmentation patterns (WILLEY and NIST database/ Chem Station data system).

The components of the essential oils were identified by comparison of their mass spectra with those of a computer library or with authentic compositions and confirmed by comparison of their retention indices, either with those of authentic compositions or with data published in the literature [16, 17, 18]. Mass spectra from the literature were also compared [16]. The retention indices were calculated for all volatile constituents using a homologous series of n-alkanes.

Statistical Analysis

The combine analysis over two years was performed for agronomical traits and the essential oli yield. For essential oil compounds, the data of the 22 compounds were subjected to one-way ANOVA between four thyme species. Duncan multiple range test (DMRT) method used for comparing treatment means using SAS9 software

Results

The results of analysis of variance (ANOVA) showed significant differences among species for aerial fresh weight, root length ($p<0.01$), aerial dry weight, plant height, leaf area, oil content and oil yield ($p<0.05$). There were no significant differences between species for crown diameter (Table 2). The effect of year was significant for all of the traits except oil percentage. The species by year interaction effects were significant for all of the traits except leaf area and oil content (Table 2). In comparisons between years, the higher values of all of the traits except oil percentage were obtained in the second year (Table 2). It is expected since all thyme species are perennial and in such species, during the establishment year, the plant growth is slow and weak, but, they grow faster in the second year. Therefore, the reliable data were obtained in the second year (Table 4 and Fig. 1).

The species x year interaction effects were significant for all of agronomic traits except leaf area and oil content, indicating that species had different performance in each year (Fig. 1). For example, for aerial fresh and dry weight, crown diameter and oil yield, there were no significant differences between species in the first year, but, there were significant differences for all of the traits in the second year (Fig. 1). Therefore, due to non-significant differences between species in the first year, the mean comparisons were made between species only in the second year (Table 4 and Fig. 1). Results showed that for aerial fresh and dry wright, *T. daenensis*, *T. daenensis* subsp. lancifolius and *T.pubescens* had the highest production and ranked in class a. For crown diameter, the highest and lowest values of 40.67 and 34.33 cm were observed in *T. daenensis* and *T. kotschyanus*, respectively. For the leaf area, the highest value of 17.25 cm^2/p was obtained in *T. daenensis* subsp. lancifolius that was significantly higher than the other species. For stem length, *T. daenensis* with average values of 21.33 cm had longer stem in the second year that was significantly higher than that for the other species. For root length, the highest (43 cm) and lowest (32 cm) values were observed in *T. pubescens* and *T. kotschyanus*, respectively (Table 4 and Fig 1).

The higher and lower essential oil content with average values of 1.57% and 1.95% were observed in *T. pubescens* and *T. daenensis* subsp. lancifolius, respectively. There was no significant difference, between *T. pubescens* (1.96%) and *T. daenensis* (1.93%) for essential oil content (Table 4). However, due to higher aerial dry weight, the *T. daenensis* with an average value of 0.72 milligram per plant (mg/p) had higher oil production (Table 4).

For Essential oil compositions, the means of identifying essential oil compositions and their retention indices in four thyme species over two years is presented in Table 5. The differences between species were significant in 17 out of 22 compositions. In the other word, there was no significant differences between species for α-thujene, β-pinene, 1,8-cineole, terpinolene and methyl ether carvacrol.

The results indicated that the major compounds in the essential oil were thymol and carvacrol. For thymol the higher values of 78.85% and 51.90 % were obtained in *T. daenensis* and *T. kotschyanus*, and the lower values of 36.19 % and 42.73% in *T. daenensis* subsp. lancifolius and *T. pubescens*, respectively. In contrast, for carvacrol the higher values of 42.37% and 33.52% were obtained in *T. daenensis* subsp. lancifolius and *T. pubescens*, and the lower values of 5.76% and 18.45% in *T. daenensis* and *T. kotschyanus*, respectively.

For γ-terpinene higher and lower values of 5.79% and 2.57% were obtained in *T. kotschyanus* and *T. daenensis*, respectively. For ρ-cymene, the higher and lower values of 5.91% and 2.93% were obtained in *T. kotschyanus* and *T. daenensis*, respectively. For E-caryophyllene, the higher and lower values of 2.85% and 0.8% were obtained in *T. daenensis* and *T. pubescens*, respectively.

Table 1 Name of thyme species and their geographical position for seed collection

Name of species	Name of seed collection area	Longitude	Latitude	Altitude masl
Thymus kotschyanus	Aleshtar -Persak	48° 20' 56" E	33°48'24" N	1834
Thymus pubescens	Bourojerd- Absardeh	48° 41' 08" E	33°45'11" N	1752
Thymus daenensis subsp. lancifolius	Khramsbad-Zaghah	48° 40' 26" E	33°29'11" N	1973
Thymus daenensis	Khramsbad-Zaghah	48° 40' 26" E	33°29'11" N	1973

Table 2 Analysis of variance and mean of squares of aerial parts biomass, morphological traits and essential oil in four thyme species over two years (2017 and 2018)

Source	DF	Fresh Weight	Dry Weight	Crown Diameter	Plant Height	Leaf Area	Root Length	Oil %	Oil Yield
Species (S)	3	370.77**	91.86*	8.56	3.81*	3.81*	42.49**	2.19*	0.051*
Replication	2	101.38	8.84	38.54	5.37**	2.22	4.50*	12.6**	0.031
Error1	6	80.99	33.09	8.10	0.99	1.479	2.59	0.52	0.019
Year (Y)	1	7141.5**	2179.3**	4266.6**	570.37*	865.8**	4620.9**	0.01	0.65**
S x Y	3	174.27*	48.83*	12.33*	1.82*	0.73	21.47**	0.14	0.031*
Error2	8	48.33	14.57	2.29	0.58	0.29	0.92	0.18	0.011
Total	23	-	-	-	-	-	-	-	-
CV%		18.79	19.88	6.35	5.05	5.48	4.17	7.62	30.98

*, **: significant at P= 0.05 and 0.01 levels, respectively.

Table 3 Mean comparisons between years (2017 and 2018) for aerial parts biomass, morphological traits and essential oil in four thyme species under dryland farming system in Zagheh, Rangeland, Khoramabad.

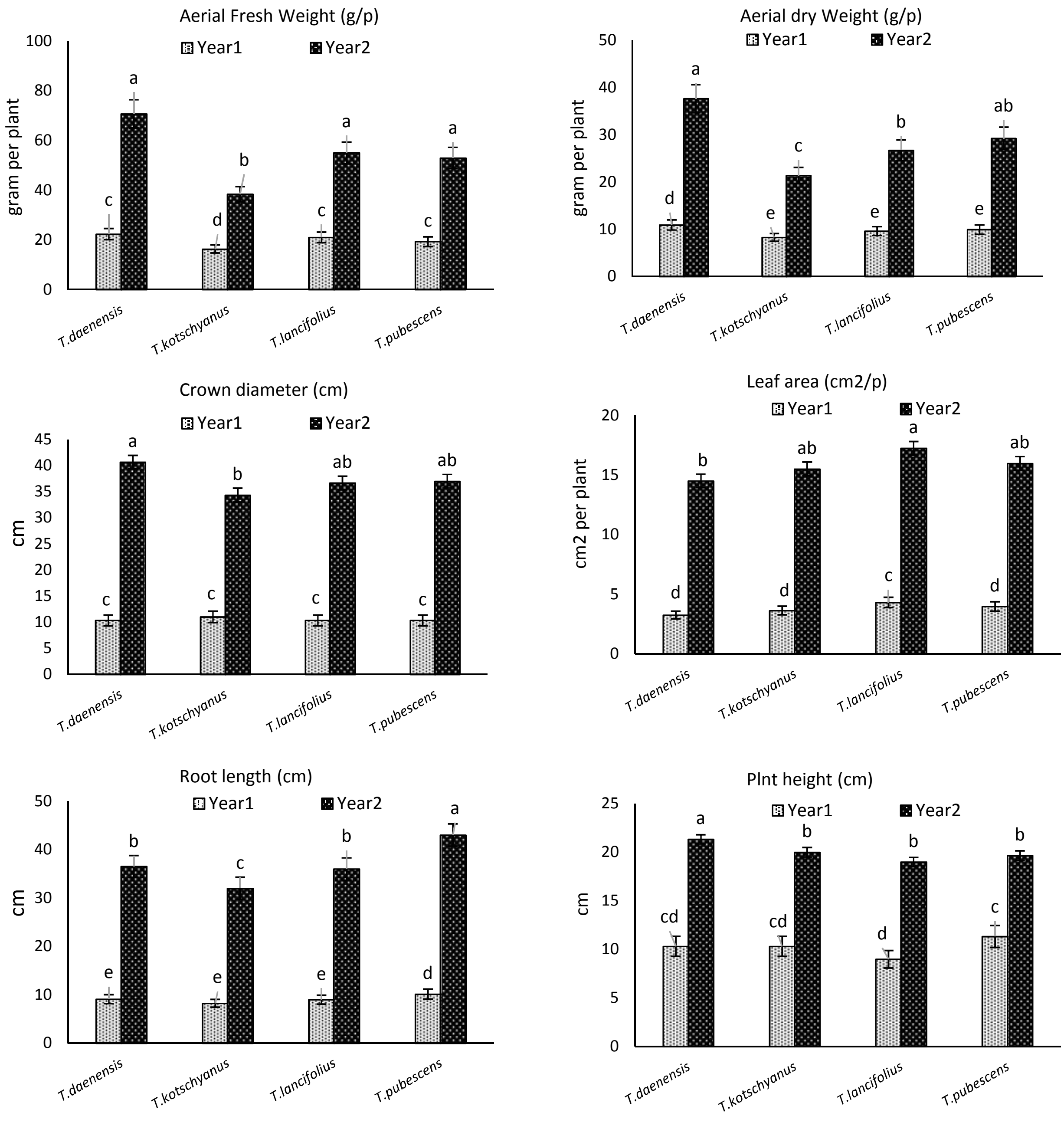
Year	Fresh weight g/p	Dry weight g/p	Crown Diameter (cm)	Leaf area cm^2/p	Plant height cm	Root length (cm)	Oil %	Oil yield mg/p
Year1	19.75 b	9.675 b	10.5 b	3.79 b	10.25 b	9.125 b	1.77 a	0.171 b
Year2	54.25 a	28.73 a	37.16 a	15.80 a	20.01 a	36.87 a	1.78 a	0.502 a

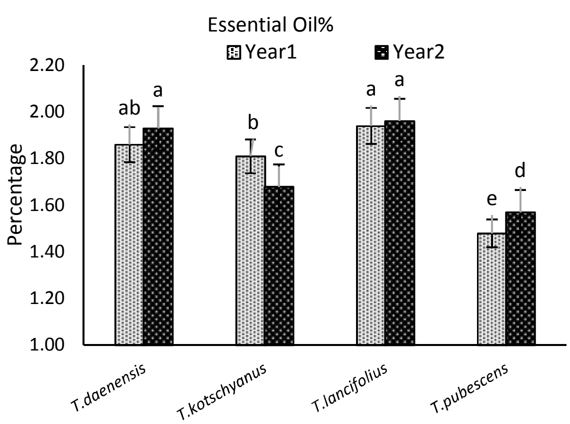
Means of column followed by same letters have no significant differences based on Tukey method

Table 4 Means of species for aerial parts biomass, morphological traits and essential oil content under dryland farming system in Zagheh, Rangeland, Khoramabad, in the second year (2018).

Species name	Fresh weight g/p	Dry weight g/p	Crown Diameter (cm)	Leaf area cm^2/p	Plant height cm	Root length cm	Oil %	Oil yield mg/p
T.daenensis	70.67 a	37.60 a	40.67 a	14.50 b	21.33 a	36.50 b	1.93 a	0.72 a
T.kotschyanus	38.33 b	21.37 b	34.33 b	15.50 b	20.00 b	32.00 c	1.68 b	0.34 c
T. daenensis subsp. lancifolius	55.00 ab	26.73 ab	36.67 ab	17.25 a	19.00 b	36.00 b	1.96 a	0.52 b
T.pubescens	53.00 ab	29.23 ab	37.00 ab	15.97b	19.67 b	43.00 a	1.57 b	0.43 b

Means of column followed by same letters have no significant differences based on Tukey method





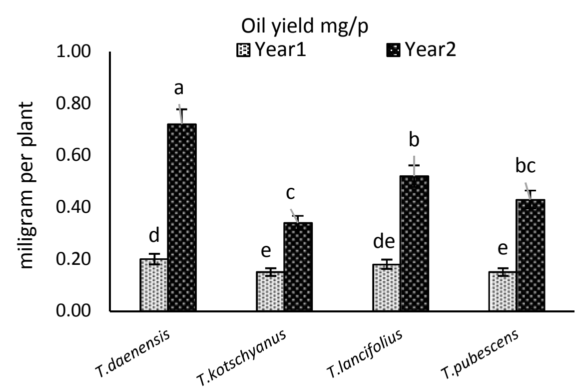


Fig. 1 Means of species by year interactions for aerial parts biomass, morphological traits and essential oil content in four thyme species over two year under dryland farming system in Zagheh, Rangeland, Khoramabad

Journal of Medicinal Plants and By-products (2021) 1: 59-65

Table 5 Means of the essential oil compositions in four thyme species under dryland farming system in Zagheh, Rangeland, Khoramabad, over two years (2017 and 2018).

Oil composition	(RI)	%				
		T. daenensis	*T. kotschyanus*	*T. daenensis* subsp. lancifolius	*T. pubescens*	F test
α-thujene	934	0.40	0.39	0.42	0.74	ns
α-pinene	946	0.37 d	0.75 c	1.34 a	1.09 b	**
Camphene	954	0.12 b	0.40 ab	0.51 ab	0.65 a	*
β-pinene	980	0.42	0.31	0.76	0.00	ns
myrcene	993	0.25 b	0.53 ab	0.27 b	0.98 a	*
α-phellandrene	1005	0.08 b	0.09 b	0.19 a	0.21 a	*
α-terpinene	1015	0.74 b	1.08 a	0.92 ab	1.15 a	*
p-cymene	1024	2.93 b	5.91 a	4.01 ab	4.50 ab	**
Limonene	1029	0.15 b	0.24 ab	0.28 a	0.21 ab	*
1,8-cineole	1031	0.99	1.32	1.11	1.28	ns
γ-terpinene	1058	2.57 b	5.79 a	4.07 a	4.36 a	*
cis-sabinene hydrate	1072	0.13 c	2.40 a	0.88 b	0.98 b	**
Terpinolene	1090	0.28	0.12	0.17	0.20	ns
Linalool	1099	0.20 a	0.00 c	0.10 b	0.16 b	*
Camphor	1148	0.03 b	0.26 a	0.19 a	0.21 a	*
Borneol	1170	0.00 c	0.63 a	0.16 c	0.48 a	**
Terpinen-4-ol	1179	0.95 b	3.90 a	2.66 a	3.29 a	**
α-terpineol	1190	0.13 c	0.42 a	0.29 b	0.34 ab	**
Methyl ether carvacrol	1247	0.29	0.52	0.24	0.38	ns
Thymol	1292	78.85 a	51.90 b	36.19 c	42.73 c	**
Carvacrol	1302	5.76 c	18.45 b	42.37 a	33.52 a	**
E-caryophyllene	1424	2.85 a	1.45 b	1.42 b	0.80 c	**
Total		98.49	96.86	98.55	97.43	

Means of rows followed by same letters have no significant differences based on Tukey method

Discussion

The highest value of root length was observed in *T. pubescens* that was significantly higher than that for other the species. The plant longer root could be an advantage for absorption, the soil moisture in dryland farming. Amini Dehaghi and Babaee reported the increased drought stress at Thyme species causes increasing the root volume, root dry weight and root length [19]. Ranjbar *et al.,* in *Thymus transcaucasicus* Ronniger reported that the root length increased in drought condition [20].

For essential oil content, the higher values of 1.96% and 1.93% were obtained in *T. pubescens* and *T. daenensis*, respectively. These values were lower than the values of (2.9 to 3.4%) that reported in *T. daenensis* in full blooming stage [11]. There are some reports indicating the benefit of mild stress for increasing essential oil. Pourmeidani *et al.* (2017) found a higher oil yield in *T. kotschyanus* in a mild stress than that for control [21]. Similarly, Nouraei *et al.* in the same species, found a higher oil content in moderate stress (60% FC) [22].

For essential oil compositions the differences between species were significant in 17 out of 22 compositions. Thymol and carvacrol were the major components in the essential oil of all species. We obtaned, the higher value of thymol (52.9%) in *T. kotschyanus* that was higher than those reported in publications [23, 24, 25 and 26]. In *T. daenensis* subsp. lancifolius we found the thymol value of 36.19% that it was higher than (16.4%) that reported by Amiri (2012) [23], but lower than the value (73.9%) that reported by Sadjadi and Khatamsaz [27]. In *T. daenensis* subsp. lancifolius we found the carvacrol value of 42.37% that was similar to Amiri [23], but much higher than that reported by Sadjadi and Khatamsaz (6.7%) [27]. In our study the carvacrol value was (18.45) for *T. kotschyanus*. It was similar to that reported Rustaiyan [24] and Rasooli and Mirmostafa [25]. But, lower values reported by Sefidkon *et al* [26].

According to the results, there was an inverse relationship between thymol and carvacrol as the most important chemical components of thyme essential oil in different species. This evidence could be used for breeding species for these chemical compositions. Although our results are in agreement with some mentioned reports, but there are considerable quantitative and qualitative variations between genotypes within species. These variations in the essential oil composition might have arisen from environmental factors (climatic, seasonal variation, geographical, and geological factors) [8,9].

The higher values of γ-terpinene (5.79%) and ρ-cymene (5.91%), were obtained in *T. kotschyanus* and the higher values of E-caryophyllene (2.85%) was obtained in *T. daenensis*

The results indicated that the major components in the essential oil were carvacrol (5.76, 18.45, 42.37 and 33.52%) and thymol (78.85, 51.90, 36.19 and 42.73%) for *T. daenensis*, *T.kotschyanus*, *T. daenensis* subsp. lancifolius and *T.pubescens*, respectively. There was considerable variation between the species. For thymol, carvacrol, terpinene, ρ-cymene, E-caryophyllene and linalool and, etc. Therefore, due to the useful variations, selection to breeding improved new varieties of *T. daenensis* for domestication and cultivation in dryland farming system in Lourstan province rangelands are recommended.

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