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Morphological and Chemical Diversity of Thymus Daenensis Celak (Lamiaceae) in Iran

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Research Article

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ABSTRACT

Thymus is taxonomically a very complex genus with a high rate of hybridization and introgression among sympatric species. The purpose of present study was to find out the relationship between morphological traits and essential oils components in 20 populations of Thymus daenensis Celak in the identical condition. The collected seeds from overall Iran planted in the greenhouse, and were transferred to the farm. A total of 60 morphological traits were measured and 11 important components of essential oils were compared within 74 individuals of 20 populations. Data analysis using ANOVA, PCA and cluster showed that the populations can be divided into two distinctive types based on their chemical and morphological traits. They contain over 70% thymol to over 70% carvacrol in their essential oils, and so might be divided into two subspecies with further studies.

INTRODUCTION

Traditional medicine has maintained its importance in the world, and its use is rapidly gaining in popularity in industrialized countries. To cure illnesses and ailments almost 80% of the global population use traditional medicine, mainly medicinal plants. Today, according to the World Health Organization, 20,000 medicinal and aromatic plants are used in the world [1]. Long term experience has shown that the local populations of the flowering plants are highly variable. This diversity includes the morphological properties and hidden differences such as chromosomes, allozyme, natural products and etc. [2]. The diversity can be detected in a gradual diversification forms as well as a mosaic of ecotypes [3]. The thyme genus shows the morphological and chemical polymorphism from subfamily Nepetoidae in Lamiaceae family [4]. Many species of thyme have phenolic terpenes that content over 10% of thymol and carvacrol. However, there are many species of thyme that the compounds are absent or less than 10% and therefore the Thymus species is divided into two phenolic and non-phenolic types based on these combinations. P-Cymene and γ-Terpinene are the second groups of important compounds in thyme genus. Other hydrocarbon compounds of the thyme's essential oils by values of more than 10% as follows: Linalool, Geraniol, 1,8 –Cineole tried out chemical diversity of 71 Spain's populations of wild thyme from different climatic regions and proclaimed that pure and hybrid populations were completely separated by morphometric and anatomical assessment [5]. Hartvig conducted a research on the population complex of Thymus teucrioides Boiss. et spruner and recognized three new taxa [6]. Hadj Ali evaluated essential oils and genetic diversity of eight natural populations of Thymus algeriensis Boiss. et Reut and concluded that the chemical and genetic diversity of populations is accordance with geographical distances of isolated populations, although, the chemical diversity of the populations was more than variety of RAPDs molecular markers [7]. Babalar affirmed that planting different populations in the same conditions diminish environmental effects in the development of growth characteristics, consequently variety and amount of ingredients and the genetic variation of ecotypes were clearly revealed. They evaluated the morphological diversity and essential oils yields of ten populations of thyme and concluded that were belonged to four groups. They observed significant differences between the different characteristics [8]. According to Jakko Jalas Classification, Thymus daenensis Celak. Belongs to the subsection Kotschyani, from the section Serpyllum in thymus Genus [9].

Thymus daenensis Celak is native to Iran and widespread from north-west, west, center, south-west to east of the country. It has been reported from Azerbaijan, Zanjan, Kurdistan, Hamedan, Lorestan, Chahar mahal-o-bakhteyari, Esfahan, Fars, Khorasan, Gum, Semnan, Tehran and Qazvin provinces and traditionally, it is one of the most important species that is used as herbal and culinary purposes in Iran.

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MATERIALS AND METHODS

Plant Material

This study was conducted on essential oils and morphological diversity of 74 individuals from 20 populations of Thymus daenensis Celak **(Table 1)**. The thyme seeds were collected from all over **(Figure 1)**.

Figure 1. Seeds collection regions. The codes of accessions were the Irano-Turanian region of Iran.

Table 1. Geographical characteristics of the populations (all specimens kept in the herbarium of agriculture and Natural Resources Research Center of Zanjan and Khwarizmi University Herbarium).

Row	Populations Code	Province	Altitude	Latitude	Longitude
1	A4	Markazi	1900	34˚ 59` 17˝	49˚ 19` 40˝
2	A5	Qazvin	2000	36˚ 34` 00˝	49˚ 55` 00˝
3	A14	Zanjan	1740	36˚ 16` 18˝	48˚ 27` 20˝
4	A18	Qazvin	1500	36˚ 26` 00˝	50˚ 07` 00˝
5	A21	Kurdistan	2400	35˚ 25` 00˝	46˚ 52` 00˝
6	A25	Tehran	2200	35˚ 49` 14˝	51˚ 56` 40˝
7	A31	Tehran	2100	36˚ 05` 21˝	50˚ 55` 45˝
8	A33	Khorasan	1800	36˚ 24` 54˝	57˚ 17` 36˝
9	A35	Yazd	2200	31˚ 37` 04˝	54˚ 09` 16˝
10	A43	Lorestan	2312	33˚ 11` 00˝	49˚ 30` 00˝
11	A48	Lorestan	1900	33˚ 25` 00˝	48˚ 40` 00˝
12	A49	Esfahan	2500	32˚ 39` 13˝	49˚ 54` 27˝
13	A50	Zanjan	1900	36˚ 32` 30˝	48˚ 23` 30˝
14	A52	West Azerbaijan	1340	36˚ 55` 43˝	45˚ 56` 67˝
15	A54	West Azerbaijan	1389	36˚ 55` 12˝	45˚ 22` 45˝
16	A56	Kerman	2400	30˚ 54` 25˝	56˚ 47` 35˝
17	A61	Markazi	2404	34˚ 11` 38˝	49˚ 29` 41˝
18	A62	Lorestan	1820	33˚ 46` 00˝	48˚ 28` 00˝
19	A66	Semnan	2564	36˚ 27` 15˝	53˚ 50` 00˝
20	A70	West Azerbaijan	1487	37˚ 17` 8˝	45˚ 07` 14˝

They were planted in the greenhouse in winter 2008. Next, they were transferred to the farm in the spring. The seedlings have been planted at the kheyrabad station of agricultural and natural resources research center of Zanjan province. This station is located in 47˚ 48`N and 31˚ 36`E, altitude 1770 meters, the long-term average minimum and maximum temperatures is 2.1 and 17.3°C respectively, 142 frost days, sandy clay and clay sandy soil, pH 7.5-8 and EC less than 1MS / cm, Long-term average annual precipitation 284.5 mm. The seedlings were grown on the same conditions for three years in the field.

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Essential Oils and Morphometric Data

A total of 60 morphological and inflorescence traits were measured and 11 important components of essential oils were compared within 74 individuals of 20 populations **(Table 2)**. Assessment of morphological traits was performed on planted and herbarium specimens. The herbarium specimens were prepared from cultivated plants according to the standard methods of botany. The specimens were identified by the Flora Iranica, Flora of Iran and Thymus and Satureja species of Iran and finally compared to previous known specimens [9-11].

Table 2. Studied characteristics in Thymus daenensis.

Studied Characteristics in Thymus daenensis			
Characters	**Comments**		
Vegetative form	Creeping stems with root=0+	Straight stems without rooting= 1	
Branching status	condensed together=1 comparatively patulous=2 patulous=3		
Reproductive characters			
Start of flowering phase	The number of days from March 21 to start of flowering phase		
50% flowering phase	The number of days from March 21 to 50% flowering phase		
The peduncle length	Without peduncle =0 The length of the cyme peduncle(mm)		
Inflorescence status	Compact=0 Slightly widespread=1 Widespread=2		
Pedicel length	Without pedicel =0 The maximum length of the pedicel (mm)		
Calyx shape	Cuneate=0 Tubular=1		
Calyx length	The length of Calyx (mm)		
Calyx length without dent	The length of Calyx without dent (mm)		
Lower dent length	The length of lower Calyx dent (mm)		
Lower sepal dent status	Smooth and without bent =0	Recurved =1	
The size of lower sepal dent	Longer than the upper dent=0	The same size as upper dent=1	
The width of the upper sepal dent	Wider than the sepals tube=0 The same size as tube=1		
Form of the upper sepal dent	3 short triangular teeth=0	3 lanceolate teeth=1	
Type of gender	Hermaphrodite=0 Just the female=1 Just male flowers=2		
Sepal color	Green=0	Purple or purple streaks=1	
Bract color	Green=0	Purple or purple streaks=1	
Bract shape	Awl-shaped=0	Linear=1	Both of them=2
External indumentum of sepals	Indumentum The same as leaves=0 pilosa=1 villous=2 Glabrous=3		
Petal color	Purple=0 Pink to red =1 White to purple=2 Bicolor(outside and inside different)=3		
Petals length	The length of Petals (mm)		
Stamens status to each other	Two tall and two short=0 the same size=1 Without a male flowers=2		
Stamens status as to stigma	Without stamen=0 Shorter than the stigma=1 The same size as stigma2= Longer than the stigma=3 various=4		
Anther color	Without stamen=0 Violet=1 Yellow or orange=2 Pink to Purple=3		
Style status to petals	Longer than the petal=1 The Same size as the petal=2 Shorter than petal=3 Every two or three status=4		
Petals status to sepals	The same size as or slightly longer than sepals=1 Significantly longer than sepals=2		
Vegetative characters			
Flowering stem length (cm)	The average of 5 flowering stem length to cm		
The second internode length of flowering stem	The second internode length of bottom of the inflorescence(mm)		
The third internode length of flowering stem	The third internode length of bottom of the inflorescence(mm)		

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The leaves size on the stem	Isomorph=1 The bottom leaves of the inflorescence larger=2 The bottom leaves of the inflorescence smaller=3				
The status of petiole and bottom of leaf	Petiole absent=0 Simple short pile=1 Simple long pile=2 Ciliate=3 mixture of Simple pile and ciliated=4				
Leaf shape	linear elliptic=0 linear ovate =1 Ovate =2				
Leaf status	Thin=0	Thick=1			
Leaf edges	Revolute= 0	Smooth=1			
The condition of the leaf tip	Obtuse =0	Acute =1			
Leaf width	The average width of 4 or 5 median leaf (mm)				
Leaf length	The average length of 4 or 5 median leaf (mm)				
The length ratio to width of the leaf	Ratio of length each leaf to width of its:				
The status and type of leaves indumentums	Sparse simply Pubescent =0 Multicellular hirsute=1 Glabrous=2				
Petiole status	Unpetiolate=0	The maximum length of the petiole (mm)			
Gland color	Green or colorless =0 Orange=1 Red=2				
Stem indumentum status	Glabrous=0 Downwards pubescent=1 Wide-spreading pubescent=2				
Stem indumentum size	Glabrous=0	Shorter than the stem width=1		The size same as stem =2	
Stem glands status	No gland=0	Scarce and scattered glands=1		Numerous glands=2	
Vein status of the intermediate leaves					
Lateral veins known or unknown	Unknown=0	Partially known=1		Known=2	
Lateral veins marginal or not.	Not marginal=0		Marginal=1		
The veins number of intermediate leaves	1 pair=0	2 pairs=1	2 or 3 Pairs=2	3 Pairs=3	4Pairs =4
vein status of the bottom leaves of Inflorescence					
Lateral veins known or unknown	Unknown=0	Partly known=1		Known=2	
Lateral veins marginal or not.	Not marginal=0		Marginal=1		
The veins number of intermediate leaves	1 pair =0	2 pairs=1	2 or 3 Pairs=2	3 Pairs=3	4Pairs =4
important components of essential oils					
The main components of essential oils	p-cymene 1,8-cineol δ-terpinene Linalool Thymol carvacrol Borneol Geraniol α-Terpineol Geranylacetate α-Terpinyl acetate				

Extraction of Essential Oils

The shoots were prepared from planted thyme at 50% flowering phase in order to extraction of essential oils. They were dried at room temperature for two weeks. Extraction of essential oils was conducted by using Clevenger device and water distillation method.

GC/MS Analysis

Gas Chromatography was model Varian 3400 Connected to a Saturn II mass spectrometer. Spectra detected by their retention indices and normal hydrocarbon injection (C7-C25) in the same conditions, and then they were calculated by injecting the essential oils. The separation and identification of essential oils components was done by GC/MS.

Statistical Analysis

Analysis variance (ANOVA) of all chemical and morphological data was performed prior to principal component analysis (PCA) and cluster analysis. It helps to selection of variable and invariable characters of populations of Thymus daenensis Celak. (Data not shown). Cluster analysis was performed by Squared Euclidean distance with the Ward method, which was analyzed using Statistical Package for the Social Sciences (SPSS, version 16.0). PCA was carried out by the use of correlation matrix and the variables consisted of chemical and morphological data, which were analyzed by jmp11software (versions 2013).

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RESULTS AND DISCUSSION

Data were analyzed by ANOVA on all traits of 74 individuals of 20 populations from Thymus daenensis. The results indicated averages of 49 traits and 9 chemicals compounds have significant differences in thyme populations. Cluster analysis was performed by WARD method to determine the chemical similarity between the studied populations of Thymus daenensis. **Figure 2** shows the dendrogram generated by this method.

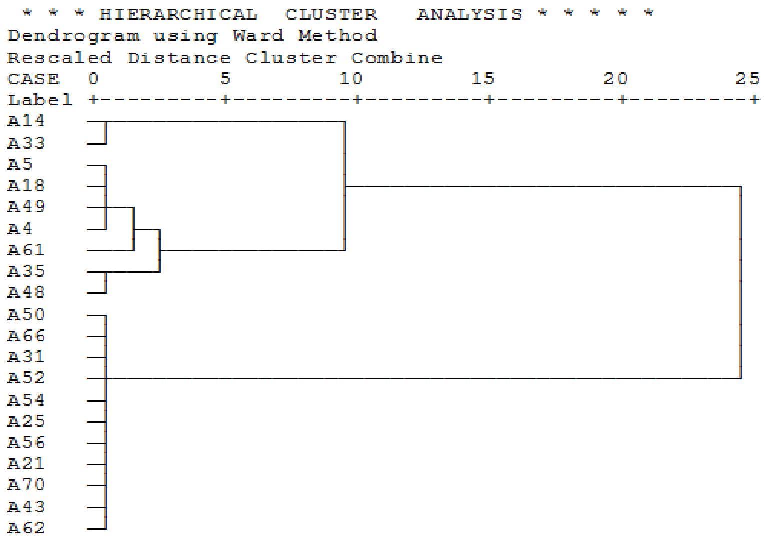

Figure 2. Dendrogram on essential oil ingredient of populations of Thymus daenensis.

The dendrogram has two main clusters at 25. This means that the studied populations of Thymus daenensis are divided into two main groups according to the chemical composition of essential oils. The first group contains over 60% thymol and low carvacrol including populations A62 and A43 of Lorestan, populations A70, A52 and A54 of West Azerbaijan, population A21 of Kurdistan, population A56 of Kerman, populations A25 and A31 of Tehran, population A66 of Semnan and population A50 of Zanjan Provinces. Subsequently, the second group is divided into two subgroups at 10. One of them has over 70% carvacrol and low thymol, including population A14 of Zanjan and population A33 of Khorasan. Other subgroup is divided into two clusters at 3. One of the groups consists of populations A35 and A48 contain thymol less than the first group and low carvacrol and also population A61 with 36% thymol and lack of carvacrol. Other group contains less than 20% thymol and carvacrol in the chemical composition of essential oils, so these are special intermediate types including population A4 of Markazi, populations A5 and A18 of Qazvin and population A49 of Esfahan provinces **(Figure 3)**.

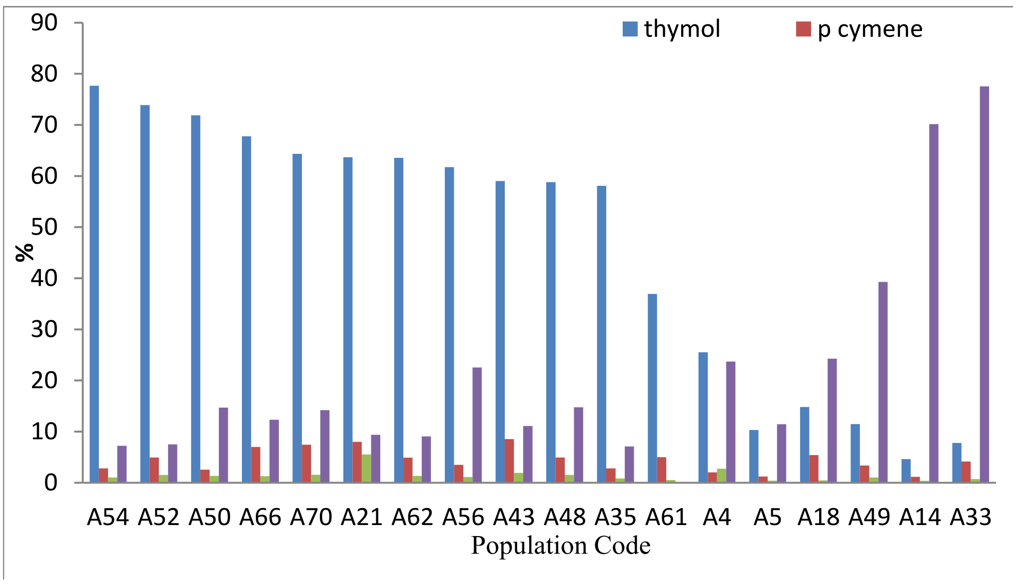

Figure 3. Variation of the most important compositions of essential oil of populations of Thymus daenensis.

Sàez and Stahl-Biskup demonstrated that chemical polymorphism in *T. hyemalis* and *T. baeticus* populations were closely related to the high variability of ecological conditions in southeast Spain [12,13]. In contrast, Salgueiro et al. remarked that different essential oils chemotypes can be found in the same habitat, which suggested that the environmental effect is only partially responsible for different chemical composition.

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A simple comparison between essential oils of the different regions populations shows that the populations A4 and A61 of Markazi Province are similar regards with ρ-cymene, 1,8-cineol, δ-terpinene, Linalool and Borneol quantities. But the other materials are differed so that the population A4 has considerable quantities of carvacrol, α-Terpineol and Geranylacetate, while the population A61 was a lack of them and instead, they possess thymol. As a matter of fact, they were two different chemotype of thymol and carvacrol of *Thymus daenensis*.

The populations A14 and A50 were identified as the two chemotype of thymol and carvacrol from Zanjan province. The population A50 contains over 71% thymol and 15% carvacrol without geraniol, but the population A14 has about 5% thymol, 70% carvacrol and 14% geraniol. Three populations A43, A48 and A62 from Lorestan province show homogeneous population in terms of the type and quantity of essential oil components. Three populations A52, A54 and A70 from West Azerbaijan province are very similar to Lorestan populations with high thymol and low carvacrol. The populations A5 and A18 are from Qazvin province. They were not much different. However, they are intermediate types because of low thymol and carvacrol. Six populations were studied from Kurdistan (A21), Khorasan (A33), Yazd (A35), Isfahan (A49), Kerman (A56) and Semnan (A66) provinces. The Khorasan province's population was the only carvacrol type with over 77% carvacrol and 8% thymol, four other populations belongs to thymol types and the Isfahan population was an intermediate type similar to the Qazvin populations. Since, they have approximately 11% thymol and 39% carvacrol. This is in agreement with finding of Loziene and Venskutonis (2005) which demonstrated that Thymus pulegioides plants are chemically stable, predetermined genetically by performing cloning experiments in uniform environmental conditions. However, they found that certain genotypes were more susceptible to environmental influence than others. Furthermore, Hernandez et al. identified that different species of Thymus growing under the same ecological conditions differed not only in the types but also in the relative amounts of exudates flavonoids.

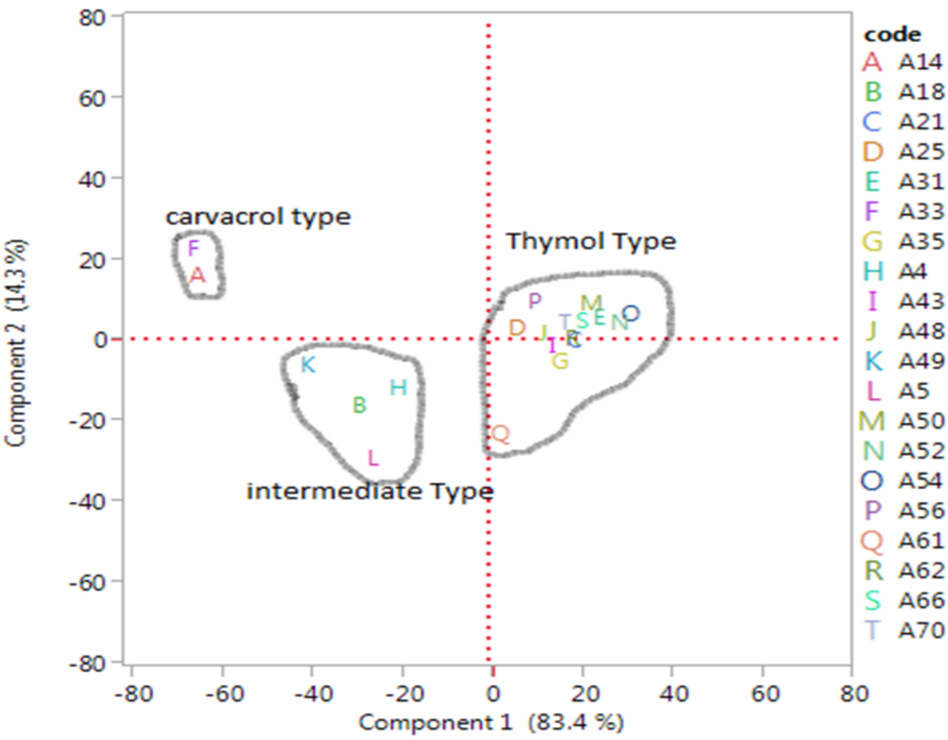

Figure 4. Principal component analysis (PCA) of chemical data from the different populations of *Thymus daenensis*.

Principal component analysis (PCA) was performed to investigate the correlation between morphological and chemical between the studied populations **(Table 3)**. **Figures 4 and 5** show the ordination diagram based on two main components. The first component has an 83.4% variance and variance of second component is 14.3%. This graph shows that the two populations A14 and A33 together form a group as carvacrol type and also populations A18, A4, A49 and A5 make a group as intermediate type and the rest of them are placed in a group as thymol type. It is interesting that the cluster analysis based on the essential oils components by Ward method also suggested that these populations have formed distinct clusters **(Figures 2 and 4)**.

PCA has identified the factors that were responsible for the clustering of the different populations of *Thymus daenensis*. Review of these figures and summary of correlation table demonstrated that the maximum length of stem, Peduncle length, average length and width of leaves, the ratio of length to width of leaves, Length petals, Petals status to sepals and Gland color characters are highly correlated with the separated chemotypes. Unfortunately, geographical distribution does not show significant correlation with the other traits. It seems that in the studied populations does not exist a special correlation between the distribution regions and the other traits.

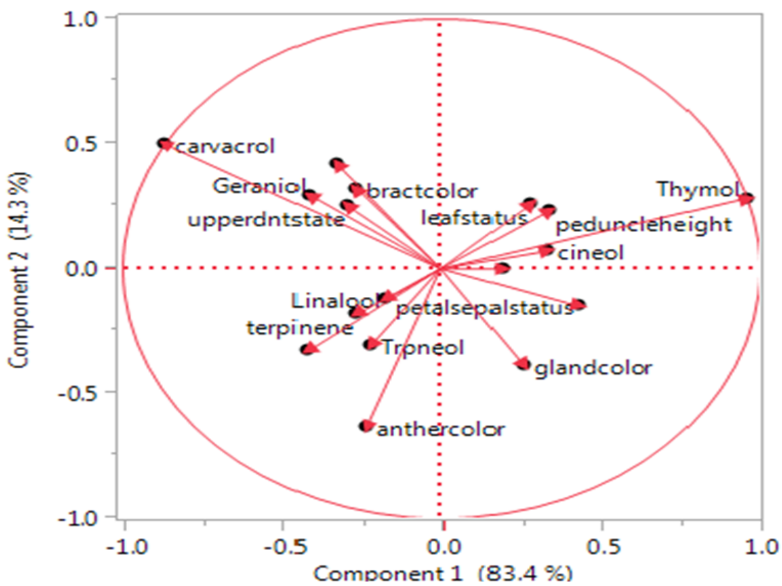

Figure 5. Principal component analysis (PCA) of morphological data from the different populations of *Thymus daenensis*.

Table 3. Summary of the correlation between the studied traits.

List	pcymene	1,8-cineol	δ-terpinene	Linalool	Thymol	carvacrol	Borneol	Geraniol	α-Terpineol	Geranyl acetate
Max L sepal dent	-0.057	0.097	0.073	0.192	-0.446	0.197	-0.134	-0.182	0.401	0.436
Peduncle height	-0.069	0.188	-0.001	-0.151	0.442	-0.175	-0.19	-0.219	-0.082	-0.061
Leaf status	0.247	0.206	-0.106	-0.081	0.337	-0.116	-0.104	-0.094	-0.272	-0.204
Anther color	-0.346	-0.338	-0.159	-0.09	-0.409	-0.112	-0.217	-0.065	0.046	-0.091
Petal sepal status	0.217	0.252	0.128	-0.002	0.388	-0.446	-0.402	-0.321	0.1	0.216
Upper dent state	-0.083	-0.142	0.074	-0.209	-0.227	0.379	0.214	0.26	-0.11	-0.175
Bract color	0.212	0.5	0.2	0.111	-0.175	0.393	0.07	-0.098	-0.095	0.134
Gland color	0.118	-0.081	-0.261	-0.355	0.141	-0.419	-0.45	-0.342	-0.334	-0.283
Max petal	-0.129	-0.154	0.138	-0.462	0.239	-0.17	-0.129	-0.213	-0.139	-0.116
Pedicel margin	-0.353	0.027	0.012	0.236	0.051	-0.071	-0.01	-0.148	0.515	0.328
Vein character	0.128	0.082	-0.502	0.213	-0.149	0.096	0.054	0.083	0.396	0.266
Mealeaf wide	0.135	-0.021	0.048	0.198	0.24	0.024	0.421	-0.058	0.052	-0.075
Number vein	0.405	0.166	0.111	0.036	-0.239	0.306	0.353	0.239	-0.103	0.107
Stem cover status	-0.241	0.022	0.066	0.324	-0.066	-0.025	0.191	0.017	0.388	0.159
branching	0.42	0.21	-0.002	0.001	0.049	-0.25	0.047	0.217	-0.142	0.028
Stamin status	0.55	0.47	0.066	0.265	0.087	0.121	0.199	-0.185	-0.174	-0.076
Peduncle status	-0.224	-0.42	0.157	-0.278	0.168	0.002	-0.042	-0.229	-0.282	-0.275

CONCLUSION

In conclusion, Thymus daenensis has two main chemotypes in Iran, which are characterized by the following features:

1- Thymol chemotype contains more than 60% thymol and less than 20%carvacrol in the essential oils, stem up to 37 cm, peduncle to 4 mm, Leaf length to 25 mm, leaf width to 5.5 mm, Petal length up to 9 mm, Petals are usually longer than sepals.

2- Carvacrol chemotype contains less than 10% thymol and more than 70% carvacrol in the essential oils, stem up to 28 cm, without peduncle, Leaf length to 22 mm, leaf width to 4.5 mm, Petal length up to 7 mm, Petals are the same size as or slightly longer than sepals.

These two chemotypes can be introduced as two new subspecies of *Thymus daenensis* by additional investigation, particularly at genome level. The intermediate types are probably hybrid between these two chemotypes that require further investigation and, of course, genome level.

This study has revealed that essential oils compositions can provide valuable information to resolve taxonomically complex and morphologically polymorphic taxa such as Thymus. There are many variations in chemical and morphological characteristics of populations of *Thymus daenensis* in Iran. Hence, their thymol and carvacrol can be considerably varied between 78% - 10% in relation to their special morphological characteristics. The results are consistent with other researches. The populations derived from the same regions were evidently composed of different chemotypes of *Thymus daenensis*. In contrast, the populations collected from different regions could produce the same essential oils compositions under the same condition. Thus, the results indicated that the chemotypes are different and were two distinctive types of thymus daenensis with specific characteristics in Iran.

Overall, this study has identified interesting relationships among essential oils and morphology. Further studies would benefit by using molecular fingerprinting and gene mapping techniques along with chemical data to elucidate the relationships among the different populations and species in these regions.

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