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Essential Oil Content and Composition of *Thymus migricus* Klokov & Desj-Shost. Affected by Plant Growth Stage and Wild Habitat Altitude

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Abstract

Essential oil content and composition of air dried aerial parts of wild growing Thymus migricus Klokov. & Desj-Shost. plants were evaluated in response to different phenological stages (pre-flowering or vegetative, flowering and post-flowering stages) and wild habitat altitude (2000 and 2100m above mean sea level). The results revealed that the highest essential oil content (0.34%) belonged to the flowering stage at 2000m altitude. GC and GC/MS analysis of volatile oils showed that α-terpineol (3.4-21.8%), thymol (3.4-19.4%), 1,8-cineol (1.9-14.5%) and carvacrol (2.5-16.1%) were the major oil components. However, carvacrol was determined in plant samples from 2000m altitude during vegetative stage. Geranial (3.5-12.4%), geranyl acetate (2-10.8%), borneol (1.4-8.3%), geraniol (0.5-8.3%), p-cymene (1.8-8.1%), and camphor (0.7-6.7%) were the other major oil components with scattered distribution between treatment combinations. Taking into account the essential oil content, it seems that flowering stage at 2000m altitude was the most appropriate harvest stage for this plant from its wild habitat. α-Terpineol proportional percentage was the highest during vegetative and full-flowering stage.

Keywords: *Thymus migricus*, Altitude, Phenological stage, GC/MS, α-Terpineol, Thymol, Carvacrol

Introduction

Thymus (Lamiaceae) is a polymorphic genus distributed in an area covering Mediterranean region to Europe, West Asia, North Africa and Canary Island [1]. In Iranian flora, it is represented by 14 species, two of which are endemic. These species are mainly distributed in North and West of Iran [2,3]. *Thymus migricus* Klokov & Desj-Shost. is a well-recognized aromatic herb with frequent occurrence in high altitude wild habitats in Northwest Iran [3]. *T. migricus* is a perennial herb, wooden in base, sub-erect, much branding, flowering stems 4-13 cm, slender, often branched apically, leaves triangular to ovate both surfaces covered sparsely with glandular trichomes, oil dots numerous and reddish; flowering period, June-July [1,3].

T. migricus plant and its preparations specially essential oil extracted from herbaceous aerial parts of this plant and other *Thymus* species have traditionally been used as carminative, anti-tussive, anti-convulsant, diuretic, digestive, antiseptic and expectorant [4,5]. Additionally, this plant has been used as spice for flavoring many types of foods.

Antimicrobial especially strong antifungal activities have been reported for essential oil of *Thymus* species describes their wide application in pharmaceutical, food and hygienic industries as flavoring, aromatic and preservative agent [4,5].

Several internal and external factors affect secondary metabolites pool of medicinal plants. Apart from species, genetic make-up and biochemical potential of volatile oil bearing plants, volatile constituents biosynthesis and accumulation is closely related with carbon assimilation, primary metabolism as well as diverse biochemical pathways normally ongoing in plants. Aforementioned biological activities and relations are extremely affected by plants growing environment and its related criteria mainly geographical and climatological factors of growing habitat [6,7].

There is considerable research interest toward the essential oil compositional analysis of *Thymus* species from different parts of the world. Several distinct studies have been reported thymol and carvacrol as the predominant volatile oil component of different *Thymus* species [8-12].

Despite these comprehensive studies on different *Thymus* species there is scarce information on the chemical profiling of *T. migricus* essential oil. Husnu Can Baser *et al.* [13] reported that carvacrol, thymol and linalool were the main volatile oil components of *T. migricus* from Anatolian region in Turkey.

T. migricus is a hardy species in *Thymus* taxa commonly adapted to high growing altitudes. Furthermore, harvesting from natural habitats supplies the majority of needs for this plant. Study of the effects of growing habitat altitude on volatile oil components biosynthesis and accumulation of aromatic plants is a major research era especially on wild growing un-naturalized plants.

Mazandarani and Rezaie [14] reported that altitude had considerable effects on principle volatile oil constituents of *T. carmanicus*. Wild growing environment related geographical and climatological factors had significant effect on *T. vulgaris* essential oil quantity and quality [15].

Plant growth stage is another influential factor affects secondary metabolites content and composition of medicinal and aromatic plant [6,16,17]. Several studies carried out on various *Thymus* species demonstrating the importance of suitable plant growth stage and/or harvest time on oil yield and its chemical composition [18,19]. Senatore [20] reported that post-flowering was the best harvest stage for obtaining the maximum content of volatile phenolic constituents of *T. pulegioides* from Italy. Molado *et al.* [21] noted that seasonal and plant growth stage variations had substantial effects on *T. zygis* volatile oil biosynthesis and accumulation. Pre-flowering stage has been considered as the optimum harvesting stage regarding essential oil and carvacrol content of *T. vulgaris* from Iran [22]

As mentioned above, in spite of several reports on the effects of environmental factors on different *Thymus* species, information about the chemical composition analysis of *T. migricus* volatile oil affected by extrinsic factors is lacking. Therefore, the aim of the present experiment was to evaluate the essential oil content and composition of *T. migricus* plant affected by plant growth stage and wild growing habitat altitude from Northwest Iran for the first time.

Material and Methods

Plant material: Aerial parts of *Thymus migricus* were collected from a natural habitat (Bardkesh village: 37° 12′ 30″ N, 45° 12′ 15″ E, slope side: Northeast, slope percent: 20-30%) located at East of Urmia city towards Mahabad in Northwest Iran. Plant materials were harvested during three growing stages (vegetative or pre-flowering, flowering and post-flowering stage) from two altitudes (2000 and 2100 m above mean sea level).

Essential oil extraction: 20 grams of air dried and grinded plant materials were subjected to hydrodistillation by an all-glass Clevenger type apparatus for 2 hrs. Extracted yellow colored essential oils were dried over anhydrous Na_2SO_4 and kept at refrigerator in sealed dark glass vials until analysis. Essential oil content was expressed as volume per weight (v/w) based on plant material air-dried weight. Extraction was carried out in triplicate and data were subjected to statistical analysis.

GC and GC/MS: The analysis of the oils were carried out using a Shimadzu 17A gas chromatograph equipped with an apolar DB-5 (95% dimethyl polysiloxane) capillary column (30m × 0.25 mm i.d. and 0.25μm film thickness). Oven temperature was set at 40^0C for 3 min, then programmed until 200^0C at a rate of 4^0C/min and finally increased at the rate 40^0C/min to 280^0C, isothermal at the temperature for 3 min. The carrier gas was helium with constant flow rate of 0.8 ml/min. Injection mode, split; split ratio 1:40, Injector and detector temperatures were 240^0C and 260^0C, respectively. GC/MS analysis were performed on a Shimadzu 17A GC interfaced with a Shimadzu QGF 5050 mass selective detector. The chromatographic conditions were the same as described above. The MS operating parameters were as follows: Ionization potential, 70 eV and acquisition mass range: 50-450 amu.

Identification and quantification of components: Relative percentage amounts of the essential oil constituents were evaluated from total peak area (TIC) by apparatus software. Identification of components in the volatile oil was based on the comparison of their retention indices and mass spectral data with those of the authentic compounds and by computer matching with NIST and WILEY library as well as by comparison of the mass spectral data with those reported in the literature [12, 13,23].

Statistical analysis: Analysis of variance for essential oil content was carried out by MSTATC software based on completely randomized block design as factorial combination. Mean comparison was evaluated by Duncan's multiple range test. Graph was drawn by Excel (Microsoft office 2003).

Results and Discussion

The results showed that the effects of growth stage, altitude and interactive effects of growth stage X altitude were significant ($p \leq 0.01$) on volatile oil content of *T. migricus*. Mean comparison for the interactive effects showed that the highest (0.34%) essential oil content belonged to the flowering stage at 2000 m altitude. Flowering stage at 2100m altitude had less oil (0.28%) compared to above treatment. The lowest amount of oil (0.09%) was obtained at vegetative stage from an altitude of about 2100m. In both altitudes, flowering stages had higher volatile oil content compared with pre and post flowering stages. In total, effect of growing stage on oil content was stronger than altitude.

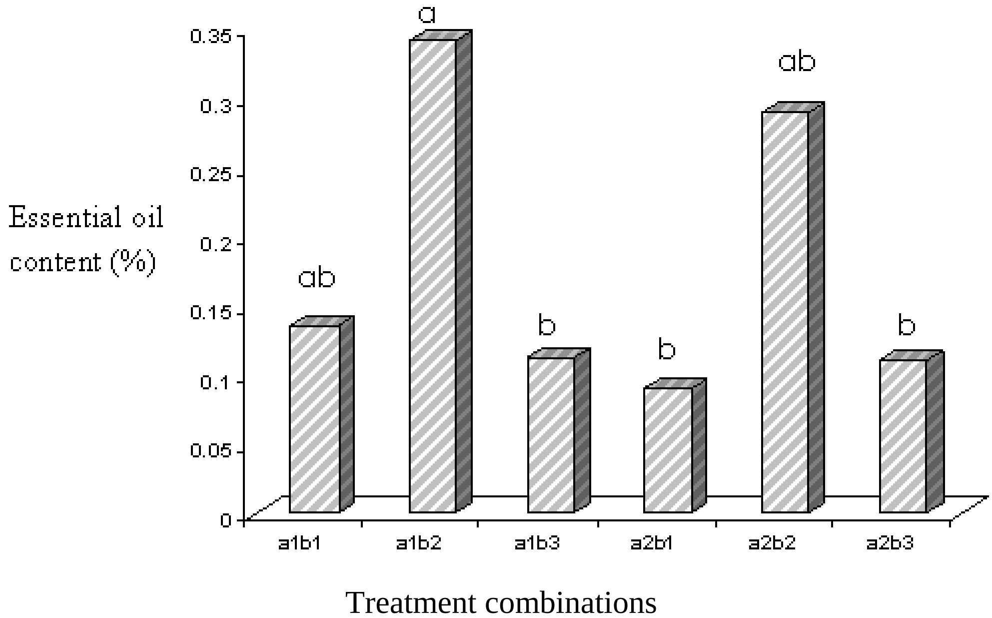

Figure 1. Mean comparison for the interactive effects of growth stage and altitude on essential oil content of *Thymus migricus*

b_1: Pre-flowering or vegetative stage, b_2: Flowering stage and b_3: Post-flowering stage

a_1: 2000m altitude and a_2: 2100m altitude

Different letters on bars show significant difference ($P \leq 0.01$) between treatment combinations based on Duncan's multiple range test.

Taking into account the oil content, there are some differences between our findings and reports of other scientists regarding different *Thymus* species. However, essential oil content of *T. migricus* plant studied in the present experiment can be considered as moderate-rich oil according to the category of Husnu Can Baser [12]. It seems that the appropriate conditions for maximum essential oil extraction of *T. migricus* plants is harvesting of plants from 2000m altitude during full-flowering stage. This finding is similar with several reports on other *Thymus* species [9-18].

The results obtained from the chemical analysis of volatile oils of *T. migricus* are reported in table1. In total, forty nine components were identified in the essential oils of six treatment combinations accounting for 80.2 to 95.4 % of total oils. The data shows that there are meaningful differences between treatment combinations regarding the type and proportional percentage of volatile oil components.

Table 1: Effects of plant growth stage and wild habitat altitude on essential oil constituents of *Thymus migricus* Klokov & Desj-Shost. from Iran**.**

No.	**Compound**	**RI_{exp}**	**a_1b_1**	**a_1b_2**	**a_1b_3**	**a_2b_1**	**a_2b_2**	**a_2b_3**
1	α-Thujene	930	0.8	-	-	0.4	-	0.9
2	α-Pinene	939	-	1.2	0.5	-	-	-
3	(Z)-Ocimene	950	-		3.5	2.7	3.1	-
4	α-Fenchene	953	2.8	-	-	-	-	-
5	Camphene	954	1	0.7	2	1.1	1.4	5.5
6	Sabinene	975	-	0.3	0.4	0.7	0.5	0.5
7	β-Pinene	979	-	0.3	0.5	0.4	0. 5	-
8	Myrcene	991	2.6	1.4	1.6	4.6	2.9	0.8
9	α-Phellandrene	1003	-	-	0.1	-	-	0.5
10	α-Terpinene	1017	-	-	0.7	0.3	0.2	1
11	ρ-Cymene	1025	2.8	4	8.1	1.8	5.2	-
12	Limonene	1029	0.8	0.6	-	1.6	1.1	-
13	1,8-Cineole	1031	3.1	4.7	5.8	1.9	3.7	14.5

14	(E)-β-Ocimene	1050	4.1	0.9	0.6	1.6	-	1.2
15	γ-Terpinene	1050	6.1	1.5	3.4	2.3	1.7	4.9
16	(Z)-Sabinene hydrate	1070	1.3	2.6	4.3	3.2	3	3.1
17	Terpinolene	1089	-	-	0.2	0.9	0.9	-
18	Linalool	1097	5.4	5	6.8	-	-	2
19	(E)-Sabinene hydrate	1098	-	0.4	0.9	0.3	0.5	0.9
20	(E)-Limonene oxide	1142	-	-	0.3	-	-	-
21	Geijerene	1143	-	-	-	-	-	0.5
22	Camphor	1146	0.7	2.6	6.7	2.2	2.6	3.3
23	Pinocarvone	1165	-	-	0.6	-	-	0.5
24	Borneol	1169	-	2.4	7.7	-	1.4	8.3
25	Terpinene-4-ol	1177	1.8	1.9	-	3.5	2	-
26	α-Terpineol	1189	17.9	14.7	12	21.8	21.6	3.4
27	Carvacrol methyl ether	1245	1.3	0.3	1.9	-	1.1	7.1
28	Z-Citral	By mass	-	1.4	-	-	2.1	-
29	Anethole	1253	-	0.3	-	-	-	-
30	Geraniol	1253	-	3.6	0.5	2.7	8.3	-
31	Geranial	1267	-	4.3	-	12.4	3.5	-
32	Bornyl acetate	1289	-	1	1.3	2.2	1.2	-
33	Thymol	1290	19.4	13.1	6	7.5	6.6	3.4
34	Carvacrol	1299	-	2.5	9.7	0.8	1.8	16.1
35	Thymyl acetate	1352	-	-	-	-	-	0.9
36	Neryl acetate	1362	0.8	0.2	0.2	-	0.3	-
37	Geranyl acetate	1382	-	9	2	10.8	9.5	-
38	β-Bourbonene	1388	-	1.1	0.5	-	1	0.6
39	(E)-Caryophyllene	1419	2.8	3.1	1.5	3.3	2.6	2.8
40	Alloaromadendrene	1460	-	0.2	-	-	-	0.4
41	β-Selinene	1484	-	0.3	-	-	-	-
42	Germacrene D	1485	1	1.9	0.4	1.8	1.1	0.4
43	Bicyclogermacrene	By mass	1	1.1	0.4	1.1	0.8	2.3
44	δ-Cadinene	1523	0.6	0.4	-	-	-	0.7
45	Spathulenol	1578	-	-	-	-	-	1.2
46	Elemol	1550	-	-	0.2	-	-	-
47	Caryophyllene oxide	1583	1	4.9	3.9	1.3	3.8	3.2
48	Dillapiole	1621	0.6	0.4	0.4	-	0.3	-
49	δ-Cadinol	1649	-	0.2	0.2	-	-	0.7
	Total identified		80.2	95	95.4	95.1	95	91.4

b_1: Pre-flowering or vegetative stage, b_2: Flowering stage and b_3: Post-flowering stage
a_1: 2000m altitude and a_2: 2100m altitude
Compounds are reported according to their elution order on non-polar column.

Twelve compounds were common between treatments. α-Terpineol (3.4-21.8%), thymol (3.4-19.4%) and 1,8-cineol (1.9-14.5%) were the predominant common constituents of volatile oils. Other common components with significant amounts were camphor (0.7-6.7%), γ-terpinene (1.5-6.1%), camphene (0.7-5.5%), caryophyllene oxide (1-4.9%), myrcene (0.8-4.6%), (Z)-sabinene hydrate (1.3-4.3%) and (E)-caryophyllene (1.5-3.3%).

Carvacrol (2.5-16.1%), geranial (3.5-12.4%), geranyl acetate (2-10.8%), borneol (1.4-8.3), geraniol (0.5-8.3%), *p*-cymene (1.8-8.1%), carvacrol metyl ether (0.3-7.1) and linalool (2-6.8) were other un-common major components with highlighted amounts. Vegetative (21.8%) and flowering (21.6%) stages at 2100m altitude had the highest amounts for α-terpineol as the predominant component of all oils. Post-flowering stage at 2100m had about 6 fold lower amounts for α-terpineol compared to above mentioned treatments. The highest and the least amounts for thymol belonged to vegetative stage at 2000m altitude (19.4%) and post-flowering stage at 2100m (3.4%), respectively. Carvacrol had the highest content (16.1%) during post-flowering stage at 2100m altitude. Interestingly, despite previous reports [8,9,12,13] for several *Thymus* species, flowering stage at both altitudes had minor amounts for this compound.

Like two previous components, p-cymene, linalool, camphor, borneol, camphene, 1,8-cineol and carvacrol methyl ether had their greatest amounts during post-flowering stage. *α*-Fenchone and spathulenol were compounds exclusive of flowering period at 2000m and post-flowering stage at 2100m, respectively. (Z)-Citral (1.4-2.1%) was only belonged to flowering period at both altitudes. Geranyl acetate was another compound with highlighted amounts during flowering stage. (Z)-Ocimene and terpinene-4-ol were other components possessed by special treatment combinations. (E)-β-Ocimene (4.1%) had notable amounts under vegetative stage at 2000m altitude. Regarding the chemical profile and principle components of volatile oils, it seems that there are considerable quantitative differences between treatment combinations. Moreover, some qualitative and quantitative distinctions were detected between the present experiment and previous reports on *T. migricus* and other related *Thymus* species [8,9,12,13].

These chemical profile and major constituents differences indicating that different plant growing stages and environmental conditions strongly affect assimilation potential of plants and hence influence the primary and secondary metabolism relationship of plants for compartmentalization of different biochemical pathways leading to a wide variety of oil components [24]. In the present experiment, chemical variations were mainly due to the differences in plant growth stages.

In conclusion, the chemical composition of the volatile oil of *T. migricus* plants spontaneously growing in high altitudes in Northwest Iran was characterized by the presence of high amounts of *α*-terpineol, thymol, carvacrol and 1,8-cineol. Flowering stage at 2000m altitude had the highest essential oil content. However, in the light of aforementioned and other major components, essential oil derived from all treatment combinations has potential applicability in related industries. Overall, *T. migricus* plants studied in the present experiment can be a hopeful and accessible source of *α*-terpineol, thymol, carvacrol, 1,8-cineol and other principle components for substituting other sources of these volatile compounds or for submitting the great demands of pharmaceutical, food and other industries for these high-valued volatile components. However, this claim needs comprehensive studies about the effects of other intrinsic and extrinsic factors on essential oil of this plant.

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