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CHEMICAL CHARACTERIZATION OF BIOACTIVE VOLATILE MOLECULES OF FOUR *THYMUS* SPECIES USING NANOSCALE INJECTION METHOD

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This paper is devoted to an investigation carried out on the volatile molecules of air-dried leaves of four species of *Thymus* species: *Thymus persicus, Thymus eriocalyx, Thymus daenensis subsp. daenensis and Thymus serpyllum* L. growing wild in Lorestan area in the western part of Iran. The species were obtained through hydro-distillation process and analyzed by using nanoscale injection method in GC and GC/MS. Four species were compared to determine the similarities and differences among their volatile compounds. Sixty-five constituents representing 98.05%, 89.70%, 96.45% and 93.08% of essential oils were identified respectively. The major constituents noted were as *Thymus daenensis subsp. daenensis*: cis-Sabinene hydrate (9.2%), α-Terpineol (13.18%) and Caervacrol (12.38%), *Th. Eriocalyx*: 1-Borneol (10.46%) and Thymol (66.34%), *Th. Percicus*: Limonene (11.62%), Thymol (10.38%) and Carvacrol (25.71%), *Th. serpyllium*: α-Pinene (12.2%) and Carvacrol (14.94%). The complex array and differing abundances of these compounds among the *Thymus* species under the investigation suggest that they may provide useful characters in understanding the phylogenetic relationships among closely related species.

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Keywords: Thymus, Labiatae, Essential oil, nanoscale injection, Thymol, Carvacrol

1. Introduction

The genus *Thymus* L. is a well known aromatic perennial herb originated from Mediterranean region. Among 215 species of this genus grown in the world, 14 species are distributed in Iranian flora [1- 3]. *Thymus* species are well known as medicinal plants because of their biological and pharmacological properties. In traditional medicine, the leaves and flowering parts of *Thymus* species are widely used as tonic and herbal tea, flavouring agents (condiment and spice), antiseptic, antitussive and carminative as well as treating colds [4,5].

Thymus oils and extracts are widely used in pharmaceutical, cosmetic and perfume industry as well as for the purpose of flavoring and preservation of several food products [6]. The genus *Thymus* L. (Labiatae) consists of about 11 species in west of Iran which *Thymus Fallaxs, Thymus daenensis subsp. daenensis, Thymus transcaneasicus, Thymus persicus, Thymus fedtschenjoi, Thymus migricus, Thymus caucasicus, Thymus eriocalyx, Thymus tranutvetteri and Thymus kotshyanus* are endemic [5]. The Iranian popular name for the genus is "Avishan" [1, 7].

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Thymus species are commonly used as flavoring agents, spice and medicinal plants. Recent studies have showed that *Thymus* species have strong antibacterial, antifungal, antiviral, antiparasitic, spasmolytic and antioxidant activities [5, 7-8].

The genus Thymus has made it one of the most popular plants throughout the entire world due to its volatile constituents. Therefore, there is a considerable research interest in the compositional analysis of *Thymus* essential oils obtained from the aerial parts of the plant [5]. The essential oil substances are thymol, carvacrol, p-cymene, β - pinene, γ-terpinene, β-caryophyllene, 1-borneol, 1, 8-cineole, etc [9, 10]. The aromatic and medicinal properties of the genus *Thymus* have made it one of the most popular plants throughout the entire world. It is believed that a part of these activities is due to its volatile constituents. That is why there is a considerable research interest towards the compositional analysis of Thymus essential oils [3]. Many studies on composition of essential oils from different Thymus species have been carried out, one of which is *T. kotschyanus*. The published results reveal that major volatile constituents obtained from the aerial parts of the plant are thymol, carvacrol, p-cymene, γ-terpinene, β-caryophyllene, etc. [11]. Contrary to *T. kotschyanus*, the study concerning the composition of *T. daenensis* oil is very limited. In a previous investigation on *T. daenensis* Celak. subsp. lancifolius (Celak.) Jalas oil are reported thymol (73.9%), carvacrol (6.7%), p-cymene (4.6%), β-bisabolene (1.5%), terpinen-4-ol (1.4%), borneol (1.1%) and spathulenol (1.0%) as the main constituents [9, 12].

To the best of our knowledge, the essential oil of the leaf of this plant in the Lorestan region of west of Iran has not been considered before. Thus it was thought that it is worthwhile to identify the chemical constituents of their essential oil from this region. The maters on hand of this study focus on four species of the *Thymus* genus (*Thymus persicus, Thymus eriocalyx, Thymus daenensis subsp. daenensis and Thymus serpyllum* L) detailed analysis of the essential oils of leaves growing wild in Lorestan state in the south west of Iran by capillary GC and GC-MS with the determination of the percentage bioactive and fragrant molecules by nano scale injection [13, 14].

2. Material and methods

2.1 Plant materials

The fresh leaves of all spices of thymus were collected from Highland of Zagros Mountain in Lorestan province in the West of Iran at an altitude of ca. 1600-2200 m in April 2007 and were dried in the shade (at room temperature). The plants were identified and authenticated by Dr. Nasser Akbari at the Faculty of Agriculture, University of Lorestan. Voucher specimens were deposited in the Herbarium of Research Institute of Forest and Rangeland Tehran.

2.2 Extraction of the essential oil

The essential oil of all air-dried samples (100 g) was isolated by hydro-distillation for 2-4 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia [15]. The distillated oils were dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4 °C until analysis. The oils were yellow in color and had distinct sharp odor.

2.3 Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC/MS):

GC analyses were carried out on a Shimutzu 17A gas chromatograph equipped with a FID and a BP-10 (non-polar) capillary column (30 m × 0.32 mm; 0.25 μm film thickness). The oven temperature was held at 60 °C for 3 min then programmed at 5°C /min to 260 °C. Other operating conditions were as follows: Carrier gas He, at a flow rate of 5 ml/min; injector temperature 230°C; detector temperature 245 °C; split 40, column flow ratio, 1:8ml/min. The amount of the sample injected was 1.0 nL (diluted 1.0 μL of sample in 1000 ml of n-pentane, v/v) in the splitless mode.

GC/MS analyses were performed on a Shimutsu 17A GC coupled with Shimutsu QP5050A Mass system. The operating conditions were the same conditions as described above but the carrier gas was He. Mass spectra were taken at 70 eV. Mass range was from m/z 50–500 amu. Quantitative data was obtained from the electronic integration of the FID peak areas.

2.4 Identification of bioactive and fragrant components:

The components of the oil were identified with the comparison of their mass spectra and retention indices with those published in the literature [16] and presented in the MS computer library (WILEY 229.L and NIST 1998). Quantitative data was obtained from FID area percentages without the use of correction factors.

3. Results and discussion

The hydro-distillation of the leaves of four *Thymus* species (*Thymus persicus, Thymus eriocalyx, Thymus daenensis subsp. daenensis and Thymus serpyllum* L) gave pale yellow and green oil with a yield of 3.1% ± 0.1, 1.3±06, 2.00± 0.7 and 1.2±0.8 (v/w) respectively on dry weight basis. The general chemical profiles of the tested oils, the percentage content of the individual components and retention indices are given in Table 1. In the oil of *T. Persicus* 26, *T. eriocalyx* 15, *T. daenensis subsp. daenensis* 42 and *T. serpyllum L.* 20 components were identified representing about 98.05%, 89.70%, 96.45% and 93.08% of the total detected constituents, respectively. The most abundant components of *Thymus daenensis subsp. daenensis*, were: cis-Sabinene hydrate (9.2%), α-Terpineol (13.18%) and Caervacrol (12.38%). *T. daenensis* is similar to other members of the genus in that the major component is Carvacrol. The leaf essential oil of T*hymus eryocalyx* was composed mostly of oxygenated monoterpenoids (91.87%) (Table 2).

Table 1. Chemical composition of essential oil of four Thymus species

Componenets[a]	RI[b]	*Th. daenensis* %[c]	*Th. Eriocalyx* %[c]	*Th. Persicus* %[c]	*Th. Serpyllium* %[c]
Tricyclene	920	tr	tr	–	–
α-Thujene	927	0.24	–	–	–
α-Pinene	937	–	–	1.14	12.2
Camphene	950	–	–	1.33	tr
Sabinene	969	–	tr	0.56	–
β-Pinene	971	2.31	–	1.02	–
Myrcene	985	–	–	–	0.18
α-Phellandrene	990	–	–	0.86	–
Trans-Ocimene	995	6.62	–	–	–
(+)-3-Carene	1005	–	–	1.04	–
α-Terpinene	1014	0.96	–	2.54	0.21
1-Limonene	1021	–	–	0.10	–
p-Cymene	1023	–	–	–	2.54
cis-Osimenol	1025	–	–	0.87	–
1,8-Cineole	1026	6.17	3.07	5.24	0.76
Limonene	1027	–	–	11.62	–
β-Ocimene	1028	–	–	–	–
p-Mentha-1,5,8-Triene	1043	0.30	–	–	tr
γ-Terpinene	1048	–	–	5.63	–
trans-Sabinene hydrate	1050	–	–	7.78	–

Componenets[a]	RI[b]	*Th. daenensis* %[c]	*Th. Eriocalyx* %[c]	*Th. Persicus* %[c]	*Th. Serpyllium* %[c]
cis-Sabinene hydrate	1056	9.20	–	1.05	0.07
Camphor	1058	2.82	–	tr	–
Isoborneol	1081	2.21	–	–	–
L-Linalool	1084	–	1.01	1.22	–
α-Terpinolene	1085	–	–	1.05	0.82
Linalool	1087	5.15	–	–	0.10
Terpinene-4-ol	1089	2.86	–	–	–
α-Terpineol	1110	7.27	–	–	–
L-Camphor	1112	–	–	3.61	–
1-α-Terpineol	1120	0.58	–	–	tr
(-)-α-Terpineol	1134	5.34	–	–	–
1-Borneol	1141	–	10.46	4.07	–
Isopulegone	1156	0.28	–	–	–
Terpineol	1159	–	0.83	–	–
Carvacrol methyl ether	1165	0.93	0.61	1.11	0.28
Geranial	1171	0.10	–	tr	tr
Bornyl asetate	1183	0.25	–	–	–
β-Bourbonene	1272	0.13	–	–	–
β-Elemene	1281	0.32	–	–	–
Thymol	1285	4.23	66.34	10.38	7.39
Carvacrol	1293	17.38	7.50	25.71	14.94
Thymyl asetae	1300	0.51	–	–	–
Carvacryl acetate	1305	–	–	tr	0.31
β-Caryophyllene	1308	4.26	–	2.50	0.59
Isocaryophyllene	1319	0.15	–	–	–
α-Humulene	1333	0.58	–	–	–
Gemacrene-D	1360	1.60	–	–	–
Bicyclo Germacrene	1372	2.11	–	–	tr
β-Bisabolene	1386	0.90	0.88	–	–
γ-Muuorolene	1397	0.14	–	–	–
δ-Cadinene	1403	tr	–	–	–
trans-Caryophyllene	1414	–	tr	–	–
cis-α-Bisabolene	1418	1.54	–	–	–
Ethyl cinamate	1432	–	2.05	–	tr
Geranyl Butyrate	1435	0.25	–	–	–
Caryophyllene oxide	1450	1.44	2.96	2.56	–
trans-Carvyl propionate	1483	tr	–	–	–
cis-Bisabolene	1509	–	1.75	1.65	–
α-Bisabolol	1540	tr	–	–	–
Tetradecanal	1567	tr	–	–	–
Spathulenol	1577	–	0.67	1.59	–

Componenets[a]	RI[b]	*Th. daenensis* %[c]	*Th. Eriocalyx* %[c]	*Th. Persicus* %[c]	*Th. Serpyllium* %[c]
Juniper Camphor	1590	1.25	–	0.97	–
Benzyl Benzoate	1609	tr	–	–	–
Geranyl Caproate	1709	tr	–	–	–
α- Terpinyl Butanoate	1715	tr	–	–	–
Total		96.45%	89.70%,	98.05	93.08%

tr=traces(≤ 0.06).

[a] RI(retention index) measured relative to n-alkanes (C_6–C_{24}) on the BP-10 capillary column

[b]Compounds listed in order of their RI

[c] %, Relative percentage obtained from peak area

The major components of *Th. erio*calyx are: 1-Borneol (10.46%), Thymol (66.34%), Carvacrol (7.5%). The most abundant compounds of *Thymus percicus*: are: Limonene (11.62%), Thymol (10.38%), Carvacrol (25.71%). The bulk of the leaf oil of *Thymus percicus* was made up of sesquiterpene hydrocarbons (4.05%) and monoterpene hydrocarbons (61.04%) with oxygenated sesquiterpenoids (5.12%) making up the remainder (Table 2). *Thymus percicus* also differs from other *Thymus* species reported in the literature because of the presence of carvacrol. The leaf essential oil of *Thymus serpyllium* was largely made up of oxygenated monoterpenoids (23.78%) and with smaller amounts of sesquiterpene hydrocarbons (0.54%), and monoterpene hydrocarbons (15.95%) (Table 2). α-Pinene (12.2%), Thymol (7.39%) and Carvacrol (14.94%) are the major compounds in the essential oil of *Thymus serpyllium* L.

In particular, oxygenated monoterpenes were the most abundant compound group of the oils 61.15, 91.87, 61.04 and 23.78% respectively. Compared to reported essential oil compositions of different *Thymus* species, investigations on their biological activities are still scarce.

Table 2. Class compositions of four Thymus species essential oils

Terpenes	*Th. daenensis*	*Th. Eriocalyx*	*Th. Persicus*	*Th. Serpyllium*
Monoterpene hydrocarbons	10.48	-	26.89	15.95
Oxyganated monoterpene	61.15	91.87	61.04	23.78
Sesquitepene hydrocarbons	12	2.68	4.15	0.59
Oxyganated sesquiterpenes	2.75	3.63	5.12	-
Total	86.34	98.18	97.20	40.32

Here are some biological properties and the application of major components from essential oil of four investigated *Thymus* species:

Carvacrol: is used as disinfectant in organic synthesis, anti-infective, anthelmintic.

Thymol: is used as disinfectant in oral hygiene products, starting material for the production of racemic menthol. Meanwhile it has antibacterial, antifungal and antiseptic activities.

Limonene: is used as fragrance material for perfuming household products and as component of artificial essential oils.

α-Terpineol: with its typical lilac odor is one of the most frequently used fragrance substances. It is stable and inexpensive, and is used in soaps and cosmetics.

1-Borneol: is used in the reconstitution of the essential oils in which it occurs naturally and it is a natural insect repellent

***α*-Pinene:** is used as a fragrance substance to improve the odor of industrial products such as insecticides, antiseptics.

1,8-Cineole: can be used internally as a flavoring (e.g. in oral hygiene products) and medicine ingredient at very low doses, typical of many volatile oils, also is is toxic if ingested at high doses

With regard to the previously reported contents of the essential oil of *Thymus species*, it is interesting to point out that there are no important qualitative differences between the present work and those studies but there are some quantitative differences indicating that environmental factors strongly influence its chemical compositions [10, 11, 17]. The oils of the four investigated species are rich in monoterpene phenols (especially, Thymol and Carvacrol) and due to this high phenol content, they can be considered as substitutes for Thymus in our study. Most of the antimicrobial activity in essential oils from *Thymus* genus appears to be associated with phenolic compounds (Thymol and Carvacrol). These results agree with those reported by other authors (Table 3)

Table 3. Percentage of Thymol and Carvacrol in various species of Thymus.

Thymus species	Thymol%	Carvacrol%
T. kotschyanus[10]	38.0	14.2
T. pubescens[18]	37.9	14.1
T. carmanicus[19]	40.8	24.8
T. carnosus[20]	27.2	-
T. fedtschenkoi[18]	31.8	24.3
T. transcaspicus[21]	56.4	(7.6%)
T. daenensis celak. subsp. Lancifolius[11]	73.9	6.7

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