

## The essential oil of the protected species: *Thymus praecox* ssp. *penyalarensis*

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### Abstract

The essential oil of a protected Spanish species, *Thymus praecox* ssp. *penyalarensis*, was firstly analyzed [Gas Chromatography-Mass Spectrometry (GC-MS)] from wild populations during the phenological stages of flowering and fruiting. In flowering, the yield was 1.2%, and the major components were identified as thymol (18.5%), *p*-cymene (14.6%), carvacrol (11.6%), and  $\gamma$ -terpinene (10.1%). In fruiting, the yield was 0.9% and the major components resulted to be *p*-cymene (19.0%), carvacrol (16.5%), and borneol (10.5%). An experimental cultivation of the taxa was made also in order to analyze its essential oil as well. The yield was 1.3%, and the main compounds were thymol (19.1%), *p*-cymene (17.0%), and  $\gamma$ -terpinene (12.3%). Finally, the composition of the essential oil of this plant is discussed in relation to chemotaxonomic and biogeographical aspects.

**Keywords:** endemism, essential oil, GC-MS, threatened species, *Thymus praecox* ssp. *penyalarensis*

### Introduction

Most works that have dealt with the chemical composition of essential oils of plants of the genus *Thymus* (Lamiaceae) have been centred on *Thymus vulgaris* L., because of its extensive use in many countries as an aromatic and medicinal plant due to its antioxidant, antimicrobial and anti-inflammatory activity [1-3]. Nevertheless, the chemical composition of some species of that genus is practically unknown, in spite of being protected species or endemic plants of interest environmentally. That is the case of *Thymus praecox* ssp. *penyalarensis* (Pau) Rivas Martínez, Fernández González & Sanchez-Mata. This is a thyme that lives exclusively in the highest peaks of the Sierra de Gredos (Sistema Central, Spain) between 1800 and 2400 m a.s.l., carpeting cracks in siliceous rocks. It is listed in the Catalogue of Threatened Species of the Extremadura Autonomous Community [4] under the category of "Special Interest". On that basis, the present work was undertaken with the aim of contributing the first analysis of its essential oil production and to assess its interest from both the chemotaxonomic and applied points of view.

### Material and methods

#### Plant material

Several specimens of *Th. praecox* ssp. *penyalarensis* were collected at Pico Calvitero, Caceres, Spain (2200 m a.s.l., 30TTK56) in 2004, first in the flowering stage and then in the fruiting stage. Voucher samples (HSS <sup>11842/11843/11848</sup>) were deposited in the Herbarium HSS of the La Orden-Valdesequera Research Centre, Extremadura Local Government. On the other hand, seeds were collected from the same population. A germination test was performed under the ISTA [5] protocol. Seedlings were sown to produce an experimental cultivation in the Research Center La Orden, situated in Guadajira, (232 m a.s.l., 29SPD90), Badajoz, Spain, and several specimens was collected in the flowering stage in 2007.

#### Isolation of volatile components

The extraction of the essential oils was carried out by hydro-distillation according to the method proposed by the European Pharmacopoeia [6]. The essential oil sample obtained was used to estimate the essential oil yield (mL/100 g) and to determine its percentage composition.

#### Gas chromatography (GC)

The analytical GC was carried out on a Varian 3300 gas chromatograph fitted with a fused methyl silicone DB-1 column (50 m × 0.25 mm, 0.25  $\mu$ m film thickness). Temperature was programmed from 95°C to 240°C at 4°C min<sup>-1</sup>. Injection was performed at 250°C in the split mode (1:100). Nitrogen was used as the carrier gas (1.5 mL min<sup>-1</sup>). Flame ionization

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detection (FID) was performed at 300°C. Injection for all the samples was 0.5 µL of 1% solution of essential oil in diethyl ether, in the split mode (1:100).

#### Gas chromatography-mass spectrometry (GC-MS)

GC-MS was carried out on a Hewlett-Packard 5890 gas chromatograph fitted with a fused silica SE-30 capillary column (50 m × 0.22 mm, 0.25 µm film thickness), coupled to a HP 5971A mass selective detector. Column temperature was programmed from 70°C to 220°C at 4°C min<sup>-1</sup>, and helium was used carrier gas. Mass spectra were recorded in the scan mode at 70 eV.

Most constituents were tentatively identified by GC by comparison of their retention indices with those of authentic standards available in the author's laboratory or with retention indices in close agreement with references [7-9]. Further identification was achieved by GC/MS. The mass spectral fragmentation patterns were compared with those stored (Wiley built-in library) in the spectrometer data base.

## Results

The chromatograms showed the major components of the essential oil to be, in the flowering stage, thymol (18.5%), *p*-cymene (14.6%), carvacrol (11.6%), and  $\gamma$ -terpinene (10.1%). Other components that reached high percentages (in the range 5-10%) were borneol, sabinene, (E)-caryophyllene, and 1,8-cineol. During the fructification the major components were *p*-cymene (19.0%), carvacrol (16.5%), and borneol (10.5%). In the range of 5-10% we found  $\gamma$ -terpinene, 1,8-cineol, and sabinene. The percentage of thymol (4.1%) is much lower than those corresponding to flowering stage (18.5%) and to cultivated material (19.1%). The composition varied from flowering to fruiting, as also did the yield, which declined from 1.25% to 0.94% (Tab. 1).

## Discussion

Many of the components of the thymes essential oils have very interesting biological activities, as is the case of those we found in the greatest proportions in the plant of the present study. In particular, these components were thymol, carvacrol, and *p*-cymene, all of them compounds exhibiting antimicrobial [4], insecticidal [10], anti-inflammatory [11], antispasmodic [12] and antioxidant [13] activities. There is no evidence that this plant has had either medicinal or culinary uses, probably due to the inaccessibility of its natural populations. Due to the chemical composition of this plant, that makes it a potential source of interesting products, and also for its threatened species character, we considered prioritary its domestication. Results obtained in cultivation were satisfactory, for the seedlings had an optimum development in the essay plot, although the climate conditions were clearly warmer than in the original wild population.

Concerning composition, in flowering the chemical composition of the essential oil in the wild material reached values similar to those obtained with cultivated material at the same phenological stage. We must outline that the obtained percentage of carvacrol (3.4%) in cultivated material was much lower than those corresponding to wild material either at flowering (11.6%) or at fruiting (16.5%) stage. Yield was 1.3%. No significant differences were observed, although carvacrol reached very lower levels in comparison with those obtained from wild material (cultivated 3.4%; wild 11.6%).

From a conservation perspective, the knowledge of the phytochemistry of this plant adds to its botanical interest and the importance of its status of legal protection. In this respect we must outline that the compounds of a general pathway for the biosynthesis of aromatic monoterpenes in genus *Thymus* [14], have a high degree of representation with regard to the total sample analyzed, with values over 49% (Tab. 2). We can highlight that *p*-cymene, the main precursor of the oxygenated

**Tab. 1** Percentage oil composition and yield of the essential oil of *Th. praecox* ssp. *penyalarensis*.

Compound	RI	RTM	Wild plant		Cultivated plant
			Flowering stage	Fruiting stage	Flowering stage
$\alpha$ -thujene	926	4.93	0.7	1.0	1.7
$\alpha$ -pinene	933	5.07	2.3	2.8	3.1
camphene	952	5.32	3.2	4.8	3.9
sabinene	985	5.66	6.5	5.7	6.2
$\beta$ -pinene	990	5.79	1.8	1.3	1.2
myrcene	995	5.93	0.3	0.2	0.4
3-octanol	999	5.93	0.9	1.1	1.5
$\alpha$ -phellandrene	1007	6.19	0.3	0.2	0.4
d-3-carene	1012	6.3	<i>t</i>	<i>t</i>	0.1
$\alpha$ -terpinene	1018	6.41	2.7	2.3	5.8
<i>p</i> -cymene	1020	6.59	14.6	19.0	17.0
limonene	1027	6.65	1.1	1.5	2.1
$\beta$ -phellandrene	1027	6.65	1.9	3.8	3.3
1,8-cineol	1028	6.74	5.2	9.1	5.6
(Z)- $\beta$ -ocimene	1039	6.9	0.8	1	1.2
$\gamma$ -terpinene	1050	7.23	10.1	9.2	12.3
cis-sabinene hydrate	1062	7.37	0.8	0.2	0.2
terpinolene	1080	7.77	<i>t</i>	<i>t</i>	<i>t</i>
linalool	1122	7.93	0.2	<i>t</i>	0.4
camphor	1151	9.01	<i>t</i>	0.1	0.2

Tab. 1 (continued)

Compound	RI	RTM	Wild plant		Cultivated plant
			Flowering stage	Fruiting stage	Flower stage
borneol	1168	9.53	7.5	10.5	4.3
terpinen-4-ol	1194	9.7	<i>t</i>	<i>t</i>	0.1
<i>p</i> -cymen-8-ol	1199	9.8	<i>t</i>	<i>t</i>	0.1
$\alpha$ -terpineol	1216	9.95	0.3	<i>t</i>	0.5
thymyl-methyl ether	1246	11.05	0.2	0.1	0.6
carvone	1252	11.46	<i>t</i>	<i>t</i>	<i>t</i>
<i>p</i> -cymen-7-ol	1290	11.92	<i>t</i>	<i>t</i>	<i>t</i>
thymol	1295	12.26	18.5	4.1	19.1
carvacrol	1309	12.46	11.6	16.5	3.4
$\alpha$ -terpenyl acetate	1359	13.19	0.1	<i>t</i>	0.3
thymyl acetate	1365	13.44	0.2	<i>t</i>	0.1
eugenol	1366	13.57	<i>t</i>	<i>t</i>	0.1
carvacryl acetate	1381	13.83	<i>t</i>	0.5	<i>t</i>
(E)-caryophyllene	1418	15.06	5.3	3.8	3.2
$\alpha$ -trans-bergamotene	1434	15.22	<i>t</i>	<i>t</i>	0.1
aromadendrene	1440	15.41	<i>t</i>	<i>t</i>	<i>t</i>
(Z)- $\beta$ -farnesene	1445	15.55	<i>t</i>	<i>t</i>	<i>t</i>
$\alpha$ -humulene	1458	15.7	0.3	0.1	0.2
(E)- $\beta$ -farnesene	1461	15.78	<i>t</i>	<i>t</i>	<i>t</i>
allo-aromadendrene	1465	15.86	<i>t</i>	<i>t</i>	0.1
germacrene D	1503	16.09	<i>t</i>	<i>t</i>	<i>t</i>
germacrene A	1497	16.57	0.2	<i>t</i>	0.1
$\beta$ -bisabolene	1511	16.71	1.5	0.8	0.6
$\beta$ -sesquiphellandrene	1520	17.01	0.2	<i>t</i>	0.1
spathulenol	1575	18.19	<i>t</i>	<i>t</i>	0.2
caryophyllene oxide	1579	18.32	0.6	0.3	0.4
epi- $\alpha$ -mouurolol	1639	19.31	0.1	<i>t</i>	<i>t</i>
<b>Essential oil yield (mL/100 g)</b>			1.2	0.9	1.3

RI – retention index according to *n*-paraffins on DB-1 column; RTM – retention time in GLC-MS; *t* – trace (<0.1%).

derivatives, has been transformed in thymol and carvacrol on flowering more easily in the field (wild material) than under cultivation.

From the biogeographical point of view, we must emphasize that within the *Th. praecox* species there exist several subspecies. In the northernmost zones of Europe, reaching up to the Arctic Circle, there exist *Th. praecox* ssp. *arcticus* (E. Durand), Jalas. This differs from the rest in the type of pilosity and the size of the leaves and calyx [15]. Its essential oil polymorphism has been studied, and different number of chemotypes have been described on material from Britain, Iceland, Greenland, and Norway [16-20], revealing that this polymorphism is more distinctive in the southern than in the northern regions. The most frequent chemotypes were the acyclic monoterpenic ones: hedycarol, linalool/linalyl acetate, germacra-1(10),4-dien-6-ol. Opposite to this, the other published cases of subspecies from the more southerly latitudes of the zones around the Mediterranean sea present a higher frequency of phenolic monoterpenes (*p*-cymene, thymol, carvacrol) – a situation that is similar to that found in the present work. For example, in the mountains of Central and Southern Europe, there exists *Th. praecox* ssp. *polytrichus* A. Kerner ex Borb Jalas, similar to the previous subspecies, except for some differences in the size of the cauline leaves [15]. Bischof-Deichnik et al. [21], in an analysis of samples from 16 sites in the Austrian and Italian

Alps, found 12 types of oils, with the thymol type being the most frequent.

*Th. praecox* ssp. *skorpilii* (Velen.) Jalas is a subspecies of the Northern and Central Balkans in which various morphological varieties have been described [15,22] that coincide with differences in chemical composition and geographic distribution. Baser et al. [23] studied its essential oil in Turkey. The var. *laniger*, endemic of C. Anatolia, with villous leaves, had high percentages of thymol (7.75-41.38%) and carvacrol (7.64-10.51%). The var. *skorpilii*, which has a somewhat broader area of distribution and glabrous leaves, showed high percentages of geraniol (24.21%)  $\alpha$ -terpinyl acetate (22.67%), and geranyl acetate (9.31%).

Another close subspecies studied by Baser et al. [23] was *Th. praecox* ssp. *grossheimii* var. *grossheimii*, for which high percentages were found for thymol (26.64%) and *p*-cymene (24.88%). This is a thyme that lives in Northeastern and Southeastern Anatolia, the Caucasus, and Northwestern Iran, and differs from the foregoing in the shape and size of the basal and cauline leaves [22].

The subspecies studied in Turkey and the Alps, and also those ones analyzed in the present work, belong to zones of the world with a Mediterranean-type macrobioclimate [24], in spite of being found in the highest zones of great mountain ranges (Oromediterranean subtype). The Arctic subspecies,

**Tab. 2** Compounds involved in the biosynthetic pathway of aromatic monoterpenes in *Th. praecox* ssp. *penyalarensis*.

Compound	Wild plant		Cultivated plant
	Flowering stage	Fruiting stage	Flowering stage
<i>p</i> -cymene	14.6	19.0	17.0
$\gamma$ -terpinene	10.1	9.2	12.3
<i>p</i> -cymen-8-ol	<i>t</i>	<i>t</i>	0.1
thymyl-methyl ether	0.2	0.1	0.6
<i>p</i> -cymen-7-ol	<i>t</i>	<i>t</i>	<i>t</i>
thymol	18.5	4.1	19.1
carvacrol	11.6	16.5	3.4
thymyl acetate	0.2	<i>t</i>	0.1
carvacryl acetate	<i>t</i>	0.5	<i>t</i>
<b>Total</b>	55.2	49.4	52.5

*t* – trace (<0.1%).

located in temperated to circumpolar zones, is adapted to temperated to extreme cold year round, with a temperated to boreal macrobioclimate.

The Mediterranean macrobioclimate is not only warmer than the temperated or boreal, but also involves summer drought and a greater degree of climatic variation over the course of the year. Over the course of evolution, this could have represented a selective pressure that may be related to the variability of chemotypes in the Mediterranean subspecies, dominating the phenolic terpenes chemotypes. That contrasts with the chemotype variability of the temperated or boreal zones, which are less climatically variable over the course of the year and have not adaptations to support summer droughts, as is typical in the Mediteranean.

Essential oil polychemismus is a widespread phenomenon in the genus *Thymus* all over the continent [25], and from the foregoing, it can be derived that the chemical composition, which has been described here for the first time, has biological, taxonomic, and evolutionary significance, and represents a new opportunity to consider plants as both heritage and resource.

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