CHEMOTAXONOMIC STUDY ON *THYMUS XTOLETANUS* LADERO AND ITS PARENTAL SPECIES

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ABSTRACT

We analysed the essential oils of cultivated material of *Thymus xtoletanus* Ladero and its parents, *Th. mastichina* (L.) L. and *Th. villosus* subsp. *lusitanicus* (Boiss.) Coutinho, with seeds from the same locality in the centre of the Iberian Peninsula. The essential oil of *Th. xtoletanus*, which had not been analyzed previously, presented 1,8-cineole as the major component (25.5%), as was also the case for *Th. mastichina* (76.1%). Other components with a major presence in *Th. xtoletanus* and *Th. villosus* subsp. *lusitanicus* were [(*Z*)-β-ocimene (8.1%); camphor (4.5%); sabinene (3.2%); α-pinene (2.8%)], and [(*Z*)-β-ocimene (4.1%), camphor (9.8%), sabinene (2.8%), α-pinene (7.1%)], respectively. However, in the hybrid the components β-phelandrene (14.5%), limonene (6.9%), (*E*)-γ-bisabolene (3.5%), and viridiflorol (3.3%) stood out, inter alia, whereas their presence in the parents was limited. The study confirms the hybrid origin of *Th. xtoletanus* as intermediate between *Th. mastichina* and *Th. villosus* subsp. *lusitanicus*.

KEY WORDS: *Thymus xtoletanus*, *Thymus mastichina*, *Thymus villosus* subsp. *lusitanicus*, hybrid, chemotaxonomy, cultivation.

INTRODUCTION

Thymus xtoletanus Ladero is an endemism to the Iberian Peninsula. It was described from the Villuercas highlands (Cáceres, Spain) (Ladero 1970). It is a procumbent shrub of 10-50 cm, growing on acid soils whose structural support are quartzites, shales or sands, commonly associated with areas that have problems of erosion. The species appears at 1000-1400 m altitude as a constituent of the heather and cistus scrub which is the substitution stage of deciduous oak forest, and may also appear in the understorey of evergreen *Quercus rotundifolia* Lam. woodland. Its natural populations are currently in a phase of expansion (Blanco et al. 2007). They arise in the vicinity of populations of *Th. mastichina* (L.) L. and *Th. villosus* subsp. *lusitanicus* (Boiss.) Coutinho. Its morphology is intermediate between both of them. This fact has to be interpreted as evidence of its being a hybrid. Indeed, hybridization is common among thymes. In the Iberian Peninsula alone, more than 60 hybrids between taxa of this genus have been described (Morales 1995; Sanchez et al. 2004).

A notable character that *Th. xtoletanus* shares with *Th. mastichina* is the morphology of the mature leaves. With *Th. villosus* subsp. *lusitanicus*, it shares the form of the flower bracts and the form and arrangement of the teeth of the calyx. Other characters, such as the presence of cilia on the leaves, vary considerably from one population to another of *Th. xtoletanus*, so much so that three different nothomorphs have even been considered for the species (Ladero 1970).

Chemotaxonomy has proven to be an effective tool to confirm or reject a taxon's hybrid nature by comparison with its putative parents (Tzakou and Constantinidis 2005). In the case of thymes, there have been antecedents of this type of analysis in *Th. xenicensis* Blanca (Sáez 1995), *Th. xmonrealensis* Pau ex R. Morales nothosubsp. *garcia-vallejoi* Sánchez-Gómez Alcaraz & Sáez (Sáez 1995), *Th. xarandanus* Willk. (Soriano 1997), *Th. xviciosoi* Pau x Morales (Salgueiro et al. 1993), and *Th. xmourae* Paiva & Salgueiro (Salgueiro et al. 2000a).

Herewith we report the first chemotaxonomic study of *Thymus xtoletanus*, and of *Th. mastichina* and *Th. villosus* subsp. *lusitanicus* as its putative parents. The aim is to determine the hybrid nature of the taxon based on knowledge of its chemical composition.

MATERIAL AND METHODS

Plant material

Seeds of *Th. mastichina* and *Th. villosus* subsp. *lusitanicus* were collected in the municipality of Alía, in the county of Las Villuercas, Cáceres province, Spain (30SUJ16). The seeds were subjected to a germination test following the ISTA (1999) protocol. Seedlings that emerged in the test were used to produce an experimental crop in the research centre Centro de Investigación La Orden-Valdesequera in Guadajira municipality, Badajoz province, Spain (29SPD90).

One of the individuals produced in the culture of *Th. villosus* subsp. *lusitanicus* was found to be *Th. xtoletanus*. When this individual reached some 25 cm in height and 30 cm diameter cover, it was multiplied vegetatively to obtain an experimental crop.

The crops of *Th. mastichina*, *Th. villosus* subsp. *lusitanicus*, and *Th. xtoletanus* were harvested in the flowering stage (2007-06-01). They were dried in an airy room in darkness, and then conserved for one month in paper bags. Vouchers were deposited in the Herbarium of the Centro de Investigación La Orden-Valdesequera, Junta de Extremadura (*Th. mastichina* HSS 41860; *Th. villosus* subsp. *lusitanicus* HSS 15418; *Th.* x*toletanus* HSS 15419).

Isolation of volatile components

The extraction of the essential oils was carried out by means of hydrodistillation according to the method proposed by the European Pharmacopoeia (Council of Europe 1996). The essential oil sample obtained was used to estimate the essential oil yield (mL/100 g) and to determine its percentage composition.

Gas chomatography

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 µm film thickness). Temperature was programmed from 95°C to 240°C at 4°C min^{-1}. Injection was performed at 250°C in the split mode (1:100). Nitrogen was used as the carrier gas (1.5 mL min^{-1}). Flame ionization 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 chomatography-mass spectrometry

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^{-1}, 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 (Adams 2001; Joulain and König 1998; Swigar and Silverstein 1981). 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 AND DISCUSSION

Table 1 lists the results for the chemical composition of the three species: *Th. villosus* subsp. *lusitanicus*, *Th. xtoletanus*, and *Th. mastichina*.

The majority component in the case of *Th. mastichina* was 1,8-cineole (76.1%). At far lower percentages there appeared β-myrcene (7%), α-terpineol (4.1%), camphene (3.9%), *trans*-dihydro-α-terpineol (1.7%), linalool (1.4%), and limonene (1.1%). This thyme is endemic to the Iberian Peninsula, and is widely distributed within that area. Three types have been described for it: the 1,8-cineole type (Morales 1986; Carvalho 1994; Faleiro et al. 1999; Miguel et al. 1999a, b, 2003), the linalool type (Gaviña et al. 1974; Miguel et al. 2003), and an intermediate type (García et al. 1984; Tomei et al. 1995). The present study population could be included in the so-called 1.8-cineole type, although the percentage that we found for this majority component (76.1%) is among the highest recorded for this species.

For *Th. villosus* subsp. *lusitanicus*, the principal components quantitatively were linalool (49.6%), camphor (9.8%), *cis*-sabinene hydrate (9.0%), α-pinene (7.1%), camphene (5.6%), (*Z*)-β-ocimene (4.1%), and sabinene (2.8%). This thyme is also endemic to the Iberian Peninsula, but its range is far smaller (Estremadura and Beira Litoral in Portugal, and specific zones of Ciudad Real, and Cáceres in Spain). Its essential oil has a high degree of chemical polymorphism, with various types having been described for both the Portuguese (linalool; linalool/terpinen-4-ol/trans-sabinene hydrate; linalool/1,8-cineole; geranyl acetate/geraniol; geranyl acetate/geraniol/1,8-cineole; Salgueiro et al. 2000b) and the Spanish (camphor/1,8-cineole; camphor/1,8-cineole/linalool; Pérez-Alonso and Velasco-Negueruela 1984) populations. A recent study (Morales and López-González 2008) has revealed that the populations studied in the province of Toledo (Pérez-Alonso and Velasco-Negueruela 1984; Morales 1986) and having the type camphor/borneol correspond to a different taxon: *Th. villosus* subsp. *velascoi* R. Morales & G. López.

The present study population stands out for its high linalool content (49.6%), followed by camphor and *cis*-sabinene hydrate with values below 10%. These results are novel because the linalool component had not previously been found to exceed 18.2% in the Spanish or 24.5% in the Portuguese populations.

TABLE 1. The composition of the essential oils of *Thymus villosus* subsp. *lusitanicus*, *Thymus xtoletanus* and *Thymus mastichina*.

Compound	RI	RTM	A%	B%	C%
α-thujene	926	4,91	0.7	0.6	-
α-pinene	934	5.05	7.1	2.8	0.3
camphene	949	5.3	5.6	2.2	3.9
sabinene	979	5.65	2.8	3.2	0.1
1-octen-3-ol	981	5.7	t	-	-
β-pinene	985	5.75	0.7	1.3	0.8
β-myrcene	991	5.86	0.4	0.2	7.0
α-phelandrene	1006	6.19	-	-	t
α-terpinene	1018	6.39	0.4	0.5	0.3
p-cymene	1020	6.53	0.3	1.3	0.5
limonene	1025	6.62	0.1	6.9	1.1
β-phelandrene	1025	6.69	0.2	14.5	t
1,8-cineole	1026	6.69	0.2	25.5	76.1
(*Z*)-β-ocimene	1038	6.91	4.1	8.1	0.1
(*E*)-β-ocimene	1051	7	t	-	t
γ-terpinene	1060	7.17	0.6	0.4	0.6
cis-sabinene hydrate	1066	7.35	9.0	0.2	0.2
cis-linalool oxide	1078	7.44	0.8	0.1	-
trans-linalool oxide	1090	7.75	0.9	-	-
terpinolene	1091	7.77	-	t	t
trans-sabinene hydrate	1096	7.82	-	-	t
linalool	1102	7.93	49.6	3.4	1.4
nonanal	1104	8.01	t	-	-
1-octen-3-yl acetate	1110	8.09	t	t	-
α-campholenal	1124	8.32	t	-	-
cis-verbenol	1140	8.89	-	t	-
camphor	1148	9.01	9.8	4.5	0.2
trans-dihydro-α-terpineol	1165	9.42	-	-	1.7
borneol	1172	9.44	1.9	0.2	0.1
terpinen-4-ol	1174	9.67	0.5	0.9	0.9
p-cymen-8-ol	1181	9.8	t	0.1	-
l-α-terpineol	1188	9.94	t	2.7	4.1
cis-dihydrocarvone	1205	10.09	t	-	-
myrtenol	1206	10.1	-	0.7	-
verbenone	1209	10.2	-	1.3	-
p-cymen-7-ol	1286	11.80	t	-	-
thymol	1290	12.1	t	t	-
carvacrol	1298	12.23	t	t	-
thymol acetate	1365	12.75	-	-	0.2
carvacrol acetate	1372	13.4	-	-	0.1
geranyl acetate	1400	14	-	0.1	-
β-bourbonene	1405	14.23	0.1	t	t
β-elemene	1410	14.32	0.1	t	-
α-cedrene	1414	14.43	0.2	-	-
α-gurjunene	1415	14.75	-	t	-
(*E*)-caryophyllene	1423	14.98	0.7	1.5	t
β-gurjunene	1443	15.34	0.1	0.0	-
γ-elemene	1443	15.54	-	-	t
α-humulene	1458	15.68	t	t	0.1
allo-aromadendrene	1463	15.85	t	0.1	-
γ-muurolene	1465	16.25	0.2	1.1	-
β-selinene	1480	16.56	t	t	-
β-bisabolene	1494	16.7	t	0.4	-
γ-cadinene	1510	16.87	0.2	2.8	t
d-cadinene	1520	17.02	t	t	-
(*E*)-γ-bisabolene	1522	17.35	-	3.5	-
elemol	1542	17.53	1.2	t	0.2
(*E*)-nerolidol	1558	17.74	1.0	0.1	-
ledol	1560	17.95	0.2	t	-
germacrene D-4-ol	1572	18.13	t	t	-
spathulenol	1575	18.16	-	-	t
caryophyllene oxide	1578	18.32	t	2.4	t
viridiflorol	1585	18.53	t	3.3	0.2
1-epi-cubenol	1610	18.89	0.1	0.2	-
γ-eudesmol	1635	19.37	0.3	2.7	-
epi-α-mouurolol	1644	19.62	t	0.2	-
cubenol	1645	20.08	t	t	-
β-eudesmol	1649	20.41	-	-	t
α-eudesmol	1652	20.63	-	-	t
α-cadinol	1653	20.68	-	-	t
Yield (mL/100 g)			2.0	2.3	2.9

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

A – *Thymus villosus* subsp. *lusitanicus*; **B** – *Thymus* **x***toletanus*; **C** – *Thymus mastichina*.

With respect to our analysis of the essential oil of *Th. xtoletanus*, we must outline the components 1,8-cineole (25.5%), β-phelandrene (14.5%), (*Z*)-β-ocimene (8.1%), and limonene (6.9%). There are no published antecedents for the chemistry of this essential oil, so this is the first study.

Similarities between *Th. xtoletanus* and its parents there exist. The majority component of *Th. xtoletanus* was 1,8-cineole (25.5%), as it also was for *Th. mastichina* (76.1%). Moreover, the significant presence in the hybrid of other components [(*Z*)-β-ocimene (8.1%), camphor (4.5%), sabinene (3.2%), α-pinene (2.8%)] was similar to those found in *Th. villosus* subsp. *lusitanicus* [(*Z*)-β-ocimene (4.1%), camphor (9.8%), sabinene (2.8%), α-pinene (7.1%)].

Nevertheless, there was a significant presence of some components in *Th. xtoletanus* which only present very low levels in its parents. These were: β-phelandrene (14.5%), limonene (6.9%), (*E*)-γ-bisabolene (3.5%), viridiflorol (3.3%), γ-cadinene (2.8%), γ-eudesmol (2.7%) caryophyllene oxide (2.4%), and γ-muurolene (1.1%).

One therefore can infer from these results that the morphologically detected hybrid character of the plant is also reflected in the chemical composition of its essential oil. The study confirms the hybrid origin of *Th. xtoletanus* as intermediate between *Th. mastichina* and *Th. villosus* subsp. *lusitanicus*.

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