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FREE FLAVONOID AGLYCONES FROM *THYMUS HERBA BARONA* AND ITS MONOTERPENOID CHEMOTYPES

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Key Word Index—*Thymus herba barona*; Labiatae; flavonoid aglycones; chemotaxonomy.

Abstract—From the diethyl ether extracts of the aerial parts of *Thymus herba barona*, the flavanones eriodictyol, naringenin and the flavones luteolin, sorbifolin, thymusin, cirsiliol, apigenin, sideritoflavone, cirsimaritin, cirsilineol, xanthomicrol, 8-methoxycirsilineol and genkwanin were isolated and characterized. This is the first report of sorbifolin and cirsiliol in the genus *Thymus*. The distribution of these flavonoids in the seven terpenoid chemotypes of *T. herba barona* growing in Corsica was studied and the chemotaxonomic implications of this data discussed.

INTRODUCTION

In recent years many papers describing the free flavone aglycones from *Thymus* species have been published [1–9]. Within this genus, such flavonoids are the most useful of all the phenolic compounds as taxonomic markers. Thus, the presence of lipophilic flavones has been used recently to support the exclusion of *T. capitatus* from the genus and its inclusion in the genus *Thymbra* [10]. However, the previous chemical investigations have included very few species from section *Serpyllum* [7, 9], a group which has been reported to be taxonomically difficult [11]. Therefore, a flavonoid study of *T. herba barona* Loisel., a species belonging to this section (subsection *Pseudopiperellae*, Jalas [12]), was of particular interest. Moreover, as the samples of this species growing in Corsica represented seven different terpenoid chemotypes [13], a detailed flavonoid analysis of each could be useful for chemotaxonomic purposes.

RESULTS AND DISCUSSION

In this study all the extracts were obtained according to the previously described method for the study of free flavonoid aglycones from *Mentha* × *piperita* [14]. The results thus obtained can only be compared with those from authors who have used lipophilic solvents. In this respect, Ferreres *et al.* [4] and Barberan *et al.* [15] have strongly emphasized the importance of the properties of the solvents used in extraction.

Structural analysis of flavonoid aglycones

The aerial parts of *T. herba barona* (carvacrol chemotype) were twice extracted with diethyl ether. The flavonoids were isolated from these extracts by preparative TLC (see Experimental). The existence and location of free hydroxyls and the supplementary substitution at C-6, or C-6 and C-8, were deduced from the UV-visible spectrophotometric studies (Table 1) with classical reagents [16–20]. In some cases, the mass spectrum or the ^{1}H or ^{13}C NMR spectrum of the aglycone were carried out to complete the characterization (see Table 1 and Experimental). Finally, chromatographic comparisons (TLC and HPLC) of all compounds against authentic samples confirmed each identification.

Amongst the thirteen free identified aglycones, were two flavanones, eriodyctiol (**1**) and naringenin (**2**). Except for the phloroglucinol-based flavones, luteolin (**3**), apigenin (**7**) and genkwanin (**13**), all the other compounds were substituted at C-6 or at C-6 and C-8. The additional substituent at C-6 was either a methoxyl, cirsiliol (**6**), cirsimaritin (**9**) and cirsilineol (**10**), or a hydroxyl group, sorbifolin (**4**). On the A-ring of all the 6,8-disubstituted flavone derivatives, the substituent at C-8 was a methoxyl group and that at C-6 was either a hydroxyl, thymusin (**5**) or a methoxyl group, sideritoflavone (**8**), xanthomicrol (**11**) and 8-methoxycirsilineol (**12**). Thus, these structural identifications showed that *T. herba barona* has the capacity to produce external flavonoids with different A-ring substitution patterns and that the most important flavone characteristics of the tribe Saturejeae (subfamily Nepetoideae [21]) i.e. the presence of 5,6-dihydroxy-7,8-dimethoxyflavone, also occurred in this species. Three particular chemical features must be emphasized. First, the finding of sorbifolin (**4**) and cirsiliol (**6**) for the first time within the genus *Thymus*. Secondly, the co-occurrence of 5,6-dihydroxy-7,8-dimethoxyflavone derivative (**5**) and 5,6-dihydroxy-7-methoxy-

Table 1. UV values of the naturally occuring flavonoids in methanol and after addition of the classical shift reagents

Compounds	MeOH			$AlCl_3$			$AlCl_3$ + HCl			NaOMe		
1: eriodictyol*		286			309			307				
5,7,3',4'-tetrahydroxy		(1.00)			(1.00)			(1.00)				
flavanone		320			375			375			322	
		(0.30)			(0.17)			(0.16)			(1.00)	
2: naringenin†		288			310			309			324	
5,7,4'-trihydroxy		(1.00)			(1.00)			(1.00)			(1.00)	
flavanone		325			381			369			394	
		(0.43)			(0.24)			(0.30)			(0.12)	
3: luteolin	238sh	253	266		272	300sh	261	274	299		266	328
5,7,3',4'-tetrahydroxy	(0.78)	(0.89)	(0.85)		(0.86)	(0.37)	(0.98)	(1.00)	(0.75)		(0.89)	(0.40)
flavone	291		349		328	420		356	383			400
	(0.59)		(1.00)		(0.22)	(1.00)		(0.94)	(0.91)			(1.00)
4: sorbifolin		284			262	302		262	301		277	308
5,6,4'-trihydroxy-7-methoxy		(0.84)			(0.33)	(0.87)		(0.45)	(0.91)		(0.42)	(0.51)
flavone		333				366			359			378
		(1.00)				(1.00)			(1.00)			(1.00)
5: thymusin*·†		295			311			284	310		324	
5,6,4'-trihydroxy-7,8-dimethoxy		(0.99)			(0.82)			(0.56)	(0.88)		(0.50)	
flavone		334			368				359		383	
		(1.00)			(1.00)				(1.00)		(1.00)	
6: cirsiliol		255	275	275	287sh	338sh	263	281	295sh		270	
5,3',4'-trihydroxy-6,7-dimethoxy		(0.69)	(0.82)	(0.97)	(0.74)	(0.31)	(0.98)	(0.99)	(0.90)		(0.92)	
flavone		343				424			368			402
		(1.00)				(0.53)			(1.00)			(1.00)
7 apigenin		268			277	301		277	301		275	324
5,7,4'-trihydroxy		(0.86)			(0.86)	(0.90)		(0.93)	(0.98)		(0.69)	(0.57)
flavone		334			368	380		342	379			392
		(1.00)			(1.00)	(0.82)		(1.00)	(0.76)			(1.00)
8: sideritoflavone*·†		257	279		278	305	262	287	301		268	
5,3',4'-trihydroxy-6,7,8-trimethoxy		0.71)	(0.83)		(0.90)	(0.68)	(0.65)	(0.78)	(0.77)		(0.73)	
flavone		345				436			363		408	
		(1.00)				(1.00)			(1.00)		(1.00)	
9: cirsimaritin*·†		274		262sh	281	300	262sh	285	299		270	284sh
5,4'-dihydroxy-6.7-dimethoxy		(0.75)		(0.57)	(0.73)	(0.73)	(0.61)	(0.3)	(0.84)		(0.47)	(0.27)
flavone		331				362			352		388	
		(1.00)				(1.00)			(1.00)		(1.00)	
10: cirsilineol	250	275		260	282	297sh	265	284	296		268	
5,4'-dihydroxy-6,7,3'-trimethoxy	(0.65)	(0.79)		(0.63)	(0.75)	(0.66)	(0.66)	(0.82)	(0.74)		(1.00)	
flavone		337				372			361		403	
		(1.00)				(1.00)			(1.00)		(1.00)	
11: xanthomicrol*·†·‡		282	292	262sh	286	310	262sh	282	310		252	274
5,4'-dimethoxy-6,7,8-trimethoxy		(0.86)	(0.68)	(0.41)	(0.59)	(0.71)	(0.41)	(0.64)	(0.82)		(0.42)	(0.46)
flavone		330			360	416sh		352	408			392
		(1.00)			(1.00)	(0.27)		(1.00)	(0.31)			(1.00)
12: 8-OMe cirsilineol*	254	282		262	295	305	263	288	304		270	
5,4'-dihydroxy-6,7,8,3'-tetramethoxy	(0.69)	(1.00)		(0.64)	(0.83)	(0.87)	(0.63)	(0.85)	(0.94)		(0.77)	
flavone		341			371	418sh		362	418sh		411	
		(0.98)			(1.00)	(0.44)		(1.00)	(0.42)		(1.00)	
13: genkwanin		267			276	300		276	300	253sh	267	295sh
5,4'-dihydroxy-7-methoxy		(0.80)			(0.79)	(0.75)		(0.81)	(0.78)	(0.41)	(0.45)	(0.25)
flavone		329			346	381		343	380			386
		(1.00)			(1.00)	(0.82)		(1.00)	(0.76)			(1.00)

The relative A is presented for λ_{max} for each compound using the highest peak as 100% (1.00) of each spectrum. sh :shoulder.
*EIMS recorded.
†^{1}H NMR spectrum recorded.
‡^{13}C NMR spectrum recorded.

flavone (sorbifolin, **4**) demonstrated in the present study was previously observed only in two other *Thymus* species, *T. piperella* from Spain [3] and *T. satureoides* from North Africa [6]. Finally, the flavonoid pattern of *T. herba barona* is characterized by the absence of 4′-*O*-methylated compounds.

In order to compare the present results with those of Hernandez *et al.* [7], a quantitative evaluation of the free aglycones in ether leaf extracts of *T. herba barona* (carvacrol chemotype) and from *T. vulgaris* (section Thymus) was obtained using previously described methods. Hernandez *et al.* [7] defined *T. vulgaris* as a species with a high level of free aglycones. This comparison shows that *T. herba barona* also produces a large quantity of excreted flavonoids unlike the other species of section *Serpyllum* examined previously [7]. Such a high level may be explained by 'the influence of ecological factors on the excretion of flavonoids' [7]. Therefore, it may be significant that the other species previously analysed from section *Serpyllum*, *T. pulegioides*, *T. praecox* and *T. nervosus* grow in alpine habitats whereas *T. herba barona*, although located at middle altitude (800 m), grows in xeric habitats.

Flavonoids from the T. herba barona *terpenoid chemotypes*

Previous analyses of *T. herba barona* terpenoid patterns have revealed the existence of seven chemotypes, namely those producing: carvone (**I**), α-terpenyl acetate (**II**), carvacrol (**III**), thymol (**IV**), linalool (**V**), dihydrocarvone (**VI**) and geraniol (**VII**) [13]. In the present study the diethyl ether flavonoid extracts of these different chemotypes were analysed by reverse-phase HPLC (see Experimental). The results are reported in Table 2 and show the relative amounts of each compound from the different extracts.

A detailed comparison of these data with those previously described for other *Thymus* species using an analogous extraction method [7] showed that all the terpenoid chemotypes share the flavonoid characteristics of the genus *Thymus*, particularly the presence of thymusin (**5**), cirsimaritin (**9**), cirsilineol (**10**), xanthomicrol (**11**) and 8-methoxycirsilineol (**12**). Paying particular attention to the flavonoid pattern, especially to the 5,6-dihydroxyflavone derivatives, Hernandez *et al.* [7] suggested the division of the genus into two subgenera, the first containing only *T. piperella* and the second comprising the rest of the species. The present results are in accordance with such a division since *T. herba barona* does not synthesize 5,6-dihydroxylated and 4′-methoxylated flavone derivatives, which are characteristic of *T. piperella* (Table 3).

Another distinguishing feature is the absence of thymonin in *T. herba barona*. This compound was detected in three other members of section *Serpyllum* (subsection *Alternantes*, *T. pulegioides*, subsection *Pseudomarginati*, *T. praecox* and *T. nervosus*) and, within the subgenus Group II suggested by Hernandez *et al.* [7], it is present in all the sections except section *Pseudothymbra* (Table 3). However, although *T. herba barona* and the species of section *Pseudothymbra* are similar in not producing thymonin, the latter can be distinguished by the presence of 4′-methoxy derivatives of 6-*O*-(5-desmethylsinensetin) and 6,8-di-*O*-substituted flavones (5-desmethylnobiletin), which are absent from *T. herba barona* and *T. micantes* (Table 3).

In addition, the flavonoid data were studied by normalized principal component analysis (nPCA). The distribution matrix was made up of 13 rows (free flavonoid aglycones) and 7 columns (terpenoid chemotypes). The first two axes account for 82% of total inertia (F1: 59.4%; F2 = 23.4%; Fig. 1(a). In Fig. 1(c) the terpenoid chemotypes are graphically represented as a function of their coordinates in the principal plane (F1 × F2) of the nPCA. In Fig. 1b, the most discriminative pattern along axis 1 is positively represented by compounds **8** (sideritoflavone), **11** (xanthomicrol), **12** (8-methoxycirsilineol) and **13** (genkwanin) and negatively by compounds **1** (eriodyctiol), **2** (naringenin), **3** (luteolin), **4** (sorbifolin) and **7** (apigenin); on axis 2, the main contribution is supported positively by **6** (cirsiliol) and negatively by **9** (cirsimaritin). On this basis, the terpenoid chemotypes of *T. herba barona* can be organized into three flavonoid groups. The geraniol chemotype (**VII**) constitutes group **A**. Its flavone pattern is essentially defined by large relative amounts of phloro-glucinol-based flavanones and flavones, and sorbifolin and a very low amount of xanthomicrol; it is of note that a similar flavone composition was found by Adzet *et al.* [9] in a methanolic extract of *T. willkommii*

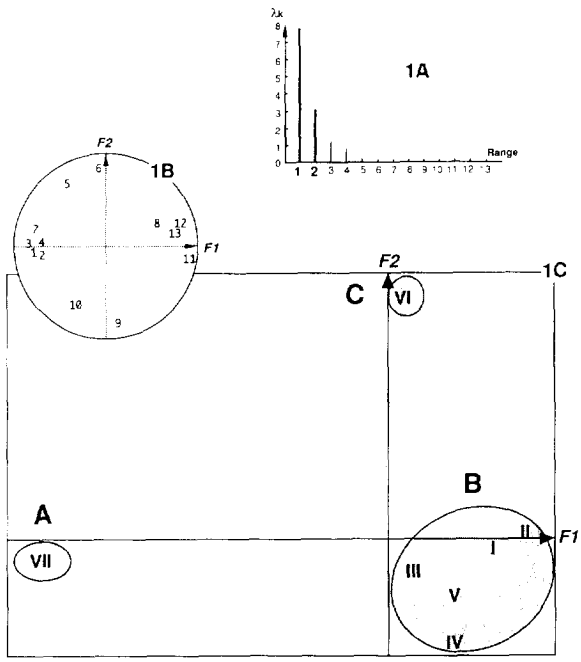

Fig. 1. Normalized principal component analysis (nPCA), corresponding to number of terpenoid chemotypes I–VII and to 13 free flavone aglycones (**1–13**) (a): Eigenvalues of the nPCA (b): correlation circle of the flavone aglycones on the F1/F2 axes; (c): factorial map of the terpenoid chemotypes on the F1/F2 axes.

Table 2. Percentages of free flavone aglycones from *Thymus herba barona* obtained by HPLC analyses of terpenoid chemotypes

		Terpenoid chemotypes						
		I	II	III	IV	V	VI	VII
Compounds	R_t (HPLC)	Carvone	α-Terpenyl acetate	Carvacrol	Thymol	Linalool	Dihydrocarvone	Geraniol
1: eriodictyol	14.47	0.1	0.6	1.6	0.5	0.7	1.3	9.2
2: naringenin	20.56	0.3	0.4	0.5	0.4	0.7	0.7	5
3: luteolin*	25.36	1	2.2	3.6	2	1.4	4	19.9
4: sorbifolin*		0.3	0.6	0.8	0.6	0.4	1.1	5
5: thymusin*	29.33	4.7	5.4	9	5.9	7.1	19	14.8
6: cirsiliol*		2.2	1	2.1	1.2	1.5	5.3	2.3
7: apigenin	31.98	2.2	0.4	2.2	1	1.8	5.6	13.6
8: sideritoflavone	34.14	4.8	15.9	8.8	11.5	11.8	16.1	3.6
9: cirsimaritin	36.22	13.5	10.4	19.4	22.8	21.3	4	14.8
10: cirsilineol	37.1	0.5	2.8	2	4.2	2.9	0.4	4.3
11: xanthomicrol	43.03	59.4	43.5	43	40.1	39.3	31	6
12: 8-OMe cirsilineol	44.73	5.3	8.7	3.5	5.9	8.4	7.5	1
13: genkwanin	49.79	5.6	8	3.3	3.7	1.8	4	0.3

R_t: Retention times (minutes).

*Determination of percentages: see Experimental.

Table 3. Free flavone aglycones discriminating the European *Thymus* sections

			Thymus									
			Group I*					Group II*				
		Section							*Serpyllum*			
		Subsection							*Pseudo*	*marginati*		
		Species	*Piperella*	*Mastichina*	*Micantes*	*Thymus*	*Hyphodromi*	*Alternates*	*praecox*	*nervosus*	*Pseudo piperellae*	*Pseudothymbra*
Substitution of flavonoid aglycones		Trivial name									*herba barona*	
OH	OMe											
5,6	7,4′	Ladanein	+	–	–	–	–	–	–	–	–	–
5,6	7,3′,4′		+	–	–	–	–	–	–	–	–	–
5,6	7,8,4′	Pebrellin	+	–	–	–	–	–	–	–	–	–
5,6	7,8,3′,4′		+	–	–	–	–		–		–	–
5,6,4′	7	Sorbifolin	–	–	–	–	–	–	–	–	+	–
5,6,4′	7,8	Thymusin	–	+	+	+	+	+	+	+	+	+
5,6,4′	7,8,3′	Thymonin	–	+	+	+	+	+	+	+	–	–
5,3′	6,7	Cirsiliol	–	–	–	–	–	–	–	–	+	–
5	6,7,3′,4′	5-Desmethyl sinensetine	–	+	–	+	+	+	+	+	–	+
5	6,7,8,3′,4′	5-Desmethyl nobiletin	–	+	–	+	+	+	+	+	–	+

*See Ref. [7].

(section *Serpyllum*). The dihydrocarvone chemotype (**VI**), specific to *T. herba barona* within the genus *Thymus*, constitutes group **B** which is characterized by a high percentage of cirsiliol and a low percentage of cirsimaritin. In group **C** (terpenoid chemotypes **I**–**V**), the main compounds are always xanthomicrol and cirsimaritin, while the phloro-glucinol-based flavones and flavanones are weakly represented.

The finding of three distinct flavonoid patterns within the seven terpenoid chemotypes confirms the inherent chemical variability in *T. herba barona*. Previously defined as a paleo-endemic species, going back probably to the origin of the tertiary period and separated from the original area by the maritime barrier [22]. *T. herba barona* is now considered as an apo-endemic species [23], growing in Majorca ($2n = 28$ [24]), Sardinia ($2n = 84$) and Corsica ($2n = 56$).

EXPERIMENTAL

Plant material. The different samples of *T. herba barona* were collected from plants in full flower. Chemotypes **I** (carvone) and **VI** (dihydrocarvone): Cap Corse, Mandriale (altitude 790 m); chemotype **II** (a-terpenyl acetate): Asco (1500 m); chemotype **III** (carvacrol): Sermano (800 m); chemotype **IV** (thymol): Venaco (1200 m); chemotype **V** (linalool) Asco (1500 m); chemotype **VII** (geraniol): Venaco (1200 m).

The spectrophotometric determinations of flavone amounts and extraction of free flavone aglycones have been described previously [14].

Isolation and purification of flavonoids. Dried and powdered Et_2O leaf extracts ($\times 3$) were combined. The Et_2O was removed at room temp. and the residue resuspended in MeOH. The crude extract was filtered and sepd by Polyamide DC6 TLC using toluene–petrol (bp 100–140°)–MeCOEt–MeOH, 5:2:1:1, into 10 bands detected under UV light. From each band, the following compounds were isolated: band 1, R_f 0.03: luteolin; band 2, R_f 0.06: eriodictyol + apigenin; band 3 R_f 0.11: sorbifolin; band 4, R_f 0.13: cirsiliol + naringenin; band 5, R_f 0.23: thymusin; band 6, R_f 0.25: sideritoflavone; band 7, R_f 0.36: cirsimaritin + genkwanin; band 8, R_f 0.5: cirsilineol; band 9, R_f 0.53: xanthomicrol; band 10, R_f 0.62: 8-OMe cirsilineol. Bands 2 and 7 were scraped off, eluted with MeOH, rechromatographed on Polyamide DC6 with toluene–MeCOEt–MeOH, 4:2:1 and gave respectively, band 2, lower band: eriodictyol, upper band: apigenin; band 7: lower band: genkwanin, upper band: cirsimaritin. All the compounds were purified by Sephadex LH 20 CC. The identification of flavonoids was based on chromatographic comparisons with authentic samples (R_t HPLC, see Table 2) and confirmed by comparison with published spectral data for all compounds [16–20] and in some cases by EIMS and/or 1H NMR (see Table 1).

Xanthomicrol (**11**). ^{13}C NMR (DMSO-d_6 with TMS as int. standard): δ164.3 (C-2), 102.3 (C-3) (attribution by 2D XH CORR), 182.4 (C-4), 145.1 (C-5), 135.8 (C-6), 152.3 (C-7), 132.6 (C-8), 148.5 (C-9), 106.1 (C-10), 120.3 (C-1′), 128.4 (C-2′, C-6′), 116.3 (C-3′, C-5′), 162.3 (C-4′).

The HPLC analysis conditions were previously described [14]. Peaks were detected at 340 nm and the percentage of each peak was delimited and calculated with the aid of Station KONTRON data system 450 except for two split peaks, 3 and 4 on the one hand and 5 and 6 on the other hand (see Table 2). In these cases, the relative amounts given by the system data for the doublet were shared by measuring the respective height of each split peak on the HPLC chromatogram and their relative percentage calculated.

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