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Variations in lipophilic and vacuolar flavonoids among European *Pulicaria* species

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Four European *Pulicaria* species, *P. odora, P. paludosa, P. sicula* and *P. vulgare,* were analysed for their surface and vacuolar constituents for comparison with previous data obtained for *P. dysenterica*. Each species had a distinct flavonoid pattern with notable differences between leaf and inflorescence. 6-Hydroxyflavonols were the major lipophilic components in all of the species and tissues except in the leaves of *P. paludosa* and *P. vulgare,* where scutellarein 6-methyl ether was the main constituent. In the leaves of *P. sicula* a more unusual flavone, 6-hydroxyluteolin 5,6,7,3′,4′-pentamethyl ether, was a major component. *Pulicaria odora* was distinguished by the presence of a series of methylated 6-hydroxykaempferol derivatives including a 3,5,6,7,4′-pentamethyl ether. Quercetagetin hexamethyl ether occurred in both tissues of *P. sicula* together with the 3,7,3,4′-tetra methyl ether and other quercetagetin derivatives, which were 5-methylated. Quercetagetin 3,7,3′-methyl ether was present in all species except *P. odora*. Flavonol glucuronides were characteristic vacuolar constituents of all the taxa studied. Two rare glycosides, patuletin and 6-hydroxykaempferol 6-methyl ether 7-glucuronides were identified in the inflorescence of *P. odora*. *Pulicaria vulgaris*, a rare plant of southern England, had the vacuolar flavonoid profile most similar to the other more abundant British plant, *P. dysenterica*.

Keywords: *Pulicaria odora*; *P. paludosa*; *P. sicula*; *P. vulgaris*; Compositae; Surface and vacuolar flavonoids; Methylated 6-hydroxyflavones and flavonols; Flavonol glycosides; Chemotaxonomy

1. Introduction

Pulicaria Gaertner is a genus of the Compositae, tribe Inuleae, containing ca. 80 species with a distribution from Europe into North Africa and Asia. There are only five European taxa, of which *P. dysenterica* (L.) Benth. (common fleabane), is the most widespread and is locally abundant in damp places in the UK. *Pulicaria vulgaris* Gaertner (small fleabane) occurs in S. England, S. Sweden and S. Russia southwards, while *P. sicula* (L.) Moris and *P. paludosa* Link are found only in the Mediterranean region, the latter being restricted to the Iberian peninsula. Another Mediterranean species, *P. odora* (L.) Reichenb., also occurs in Portugal.

A previous publication reported the geographical variation in the surface flavonoids of *P. dysenterica* and identified the major flavonoid constituents of leaf and disc florets as quercetagetin 3,7,3′-trimethyl ether and 6-hydroxykaempferol 3,7-dimethyl ether (Williams et al., 2000). The leaf and flower tissue of one race additionally contained quercetagetin 3,7,3′,4′-tetramethyl ether and 6-hydroxykaempferol 3,7,4′-trimethyl ether. The only vacuolar flavonoid component found was quercetin 3-glucuronide in all tissues. These results differ from those of two earlier investigations of *P. dysenterica*, where quercetagetin 3,7,4′-trimethyl ether and kaempferol 3-glucoside were found in flowers (Schulte et al., 1968), whilst 6-hydroxykaempferol 3,6,7-trimethyl ether (penduletin), 6-hydroxykaempferol and quercetagetin-3,7-dimethyl ethers, scutellarein,

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6-hydroxykaempferol 3-methyl ether 6-glucoside and the coumarin aesculetin were reported in leaf tissue (Pares et al., 1981). The only other European species that has been analysed for flavonoids is *P. paludosa*. In the aerial parts of this plant San Feliciano et al. (1989) identified 5,6,8-trihydroxy-7,4′-dimethoxyflavone, scutellarein 7,4′-dimethyl ether and 6-hydroxykaempferol 3,6-dimethyl ether. *Pulicaria paludosa* is closely related to and may be conspecific with *P. arabica* from N. Africa, which has been collected only once in Crete. However, the flavonoids that have been reported from *P. arabica* include different and more highly methylated derivatives, e.g. quercetagetin 3,5,6,7,4′- and 3,5,6,7,3′-pentamethyl ethers together with quercetagetin 3′,4′-dimethyl ether (Melek et al., 1988) and in a second report quercetagetin 3,5,7,3′-tetramethyl ether and its 3,5,7-trimethyl ether as well as quercetagetin 3,7-dimethyl ether and quercetin 3-glucoside and 3-glucuronide (El-Negoumy et al., 1982).

The present study was undertaken in order to complete the flavonoid survey of the leaves and inflorescences of the remaining four European *Pulicaria* species, including *P. paludosa*, for vacuolar and lipophilic constituents and to compare the results with those obtained previously for *P. dysenterica*. This paper describes the presence of some thirty-seven compounds (**1**–**37**) in various *Publicaria* species and their chemotaxonomic significance.

2. Results and discussion

2.1. Identification of surface flavonoids

Four *Pulicaria* species, *P. odora, P. paludosa, P. sicula* and *P. vulgaris*, were analysed for their lipophilic leaf and inflorescence flavonoid constituents. The compounds were isolated by briefly dipping the whole leaf or inflorescence in acetone; this indicated that these flavonoids were external and not vacuolar. During these analyses 19 major flavonoids were detected by TLC and HPLC, and these are listed in Table 1 together with their chromatographic data. Two more compounds (**9** and **14**), found previously in *P. dysenterica* only (Williams et al., 2000), are also presented in this table. Details of the purification and characterisation of the

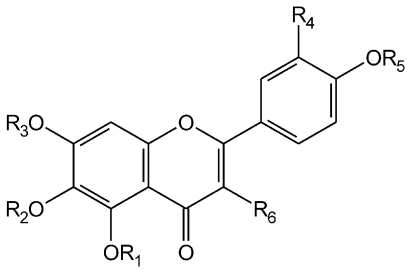
Table 1
HPLC and R_f data for surface flavonoids found in five European *Pulicaria* species[a]

Flavonoid	HPLC R_t (min)	HPLC UV λ_{max} (nm)	Colour in UV/NH_3	TLC $R_f \times 100$ in:	
				Tol:HOAc, 4:1 silica gel	30%HOAc cellulose
Flavone					
Scutellarein					
6-methyl ether (**1**)	6.7	273, 338	Dk/DkY	12	18
dimethyl ether (**2**)[b]	5.6	274, 330	G/G	06	23
6-Hydroxyluteolin (**3**)	3.8	285, 348	Dk/Dk	06	10
6-methyl ether (**4**)	5.4	255, 272, 350	Dk/DkY	06	07
7-methyl ether (**5**)	6.4	255, 283, 351	Dk/Dk	08	13
trimethyl ether (**6**)[b]	5.9	270, 335	G/G	06	23
5,6,7,3′,4′-pentamethyl ether (**7**)	13.9	255, 271*sh*, 350	B/B	83	50
Flavonols					
6-hydroxykaempferol					
6-methyl ether (**8**)	5.8	266, 367	Dk/DkY	10	10
3,7-dimethyl ether (**9**)	6.3	280, 342	Dk/Dk	12	27
3,7,4′-trimethyl ether (**10**)	11.7	280, 340	Dk/Dk	42	43
3,6,7,4′-tetramethyl ether (**11**)	15.4	271, 339	Dk/Dk	49	61
3,5,6,7,4′-pentamethyl ether (**12**)	14.7	239, 263, 331	B/B	31	76
Quercetagetin					
6-methyl ether (**13**)	4.8	255, 374	Dk/DkY	02	07
3,7-dimethyl ether (**14**)	5.0	259, 280, 353	Dk/Dk	03	18
3,7,3′-trimethyl ether (**15**)	6.7	260*sh*, 280, 353	Dk/Dk	16	30
3,7,3′,4′-tetramethyl ether (**16**)	9.8	257, 281, 353	Dk/Dk	28	22
3,5,7,3′-tetramethyl ether (**17**)	6.1	255*sh*, 270*sh*, 340	B/B	28	24
3,5,6,3′,4′-pentamethyl ether (**18**)	8.9	245, 255, 267*sh*, 339	B/B	18	61
3,5,6,7,3′,4′-hexamethyl ether (**19**)	12.5	241*sh*, 250, 338	B/B	36	74
Dihydroflavonols					
20	6.6	293	None	20	nd
21	7.3	288	None	20	nd

sh = shoulder, B = fluorescent blue, Dk = dark absorbing, Y = yellow, G = green.

[a] For details of HPLC conditions see Section 4.

[b] Not completely identified.



	R_1	R_2	R_3	R_4	R_5	R_6
1	H	Me	H	H	H	H
7	Me	Me	Me	OMe	Me	H
10	H	H	Me	H	Me	OMe
11	H	Me	Me	H	Me	OMe
12	Me	Me	Me	H	Me	OMe
15	H	H	Me	OMe	H	OMe
16	H	H	Me	OMe	Me	OMe
19	Me	Me	Me	OMe	Me	OMe

Fig. 1. Structures of the major surface flavones and flavonols found in species of *Pulicaria*.

flavonoids are given in the Experimental. All the major constituents were either 6-hydroxyflavonols (derivatives of 6-hydroxykaempferol and quercetagetin, see Fig. 1) or 6-hydroxyflavones (derivatives of scutellarein and 6-hydroxyluteolin, see Fig. 1). Seven of these compounds, 6-hydroxyluteolin 6-methyl ether (**4**) and 7-methyl ether (**5**), 6-hydroxykaempferol 6-methyl ether (**8**) and 3,7-dimethyl ether (**9**), quercetagetin 6-methyl ether (**13**), 3,7-dimethyl ether (**14**) and 3,7,3′-trimethyl ether (**15**), were identified by chromatographic and spectroscopic comparison with flavonoids isolated and reported previously from *Tanacetum* species and *P. dysenterica* (Williams et al., 1999, 2000). Standards of scutellarein 6-methyl ether (**1**) and 6-hydroxyluteolin (**3**) were also available for comparison. The identification of the remaining compounds is described below.

Compound **10**, 6-hydroxykaempferol 3,7,4′-trimethyl ether, was identified tentatively in our previous study of *P. dysenterica* (Williams et al., 2000). We have now carried out MS and UV spectral analysis of **10**. APCI-MS in positive mode gave the protonated molecule $[M+H]^+$ at m/z 345 (MW = 344) expected for a flavone with two hydroxyls and three methoxyl groups. The HPLC UV λ_{max} at 280 and 340 nm is typical of a 6-hydroxykaempferol derivative with a free 6-hydroxyl and 3-methylation. In santin, the 3,6,4′-trimethyl ether isomer, the short wave band is at 270, and in derivatives with a free 3-hydroxyl the long wave band is always 355 or longer. The absence of sodium acetate and borate shifts in the UV spectral analysis confirmed that the 7-hydroxyl was blocked and the absence of an *ortho*-dihydroxy B-ring, respectively. The positive aluminium chloride shift indicated that the 5-hydroxyl was free and the depressed absorbance of the long wave band on addition of alkali that the 4′-position was occupied.

The structure of 6-hydroxykaempferol 3,6,7,4′-tetramethyl ether (**11**), previously found together with **10** in *P. dysenterica* (Williams et al., 2000), is now confirmed. Thus, positive APCI-MS of **11** gave the expected $[M+H]^+$ at m/z 359 (MW = 358) for a flavone with one hydroxyl and four methoxyl groups. As with **10** the UV spectral analysis indicated methoxyls at the 3-, 7- and 4′-positions and a free 5-hydroxyl. The short wave band at 273 nm, rather than at 280 as in **10**, indicated the presence of the third methoxyl at 6, the only remaining possible position. Demethylation of **11** gave 6-hydroxykaempferol 3,7,4′-trimethyl ether (**10**), detected by HPLC analysis of the products. The data also agreed with literature values (Sachdev and Kulshreshtha, 1983).

The only new compound identified in the present study was the fully methylated 6-hydroxykaempferol 3,5,6,7,4′-pentamethyl ether (**12**). This substance appeared fluorescent blue in UV light, a property typical of 3,5-dimethylated flavonols and 5-methylated flavones. 5-Methylation also causes a reduction in HPLC R_t and a hypsochromic shift in the UV spectrum. APCI-MS of **12** gave a protonated molecule $[M+H]^+$ at m/z 373 (MW = 372) indicative of a flavone with five methoxyl groups. The absence of any shifts in the UV spectral analysis confirmed that there were no free hydroxyl groups in the molecule. The structure was confirmed by demethylation to give both **10** and **11**, identified by HPLC analysis.

Four related quercetagetin derivatives, the fully methylated 3,5,6,7,3′,4′-hexamethyl ether (**19**), the 3,5,7,3′-tetramethyl (**17**), 3,5,6,3′,4′-pentamethyl (**18**) and 3,7,3′,4′-tetramethyl (**16**) ethers, which are known compounds, but not previously isolated from European *Pulicaria* species, were identified by similar procedures and by comparison with literature data. The remaining compounds **2**, **6**, and **7** were fluorescent blue or green in UV, suggesting that the 5-hydroxyl is methylated. Flavones **2** and **6** were partially characterised by APCI-MS and HPLC R_t and UV spectra (Table 1 and Experimental) as a scutellarein dimethyl ether (**2**) and a 6-hydroxyluteolin trimethyl ether (**6**), whereas ES-MS, HPLC R_t and UV spectra of **7** (MW 372) suggest it to be the fully methylated 6-hydroxyluteolin 5,6,7,3′,4′-pentamethyl ether. There was insufficient amounts for NMR analysis to determine the position of the methoxyl groups in **2** or **6**.

Table 2
Surface flavonoid constituents of five European *Pulicaria* species

Flavonoid	*Pulicaria* species									
	dysenterica[a]		*odora*		*paludosa*		*sicula*		*vulgaris*	
	L	In	L	In	L	In	L	In	L	In
Flavones										
Scutellarein										
6 -ME (**1**)	–	–	–	–	++	–	–	–	++	–
DiME (**2**)[b]	–	–	–	–	(+)	–	–	–	–	–
6-Hydroxyluteolin (**3**)	–	–	–	–	(+)	–	–	–	–	–
6- ME (**4**)	–	–	–	–	(+)	–	–	–	–	–
7-ME (**5**)	–	–	–	–	(+)	–	–	–	–	–
Tri ME (**6**)[b]	–	–	–	–	(+)	–	–	–	–	–
5,6,7,3′,4′ -PentaME (**7**)	–	–	–	–	–	–	+	–	–	–
Flavonols										
6-Hydroxykaempferol										
6- ME (**8**)	–	–	–	(+)	–	–	–	–	–	–
3,7- DiME (**9**)	+	+	–	–	–	–	–	–	–	–
3,7,4′-TriME (**10**)	(+)[c]	–	–	++	–	–	–	–	–	–
3,6,7,4′-TetraME (**11**)	–	–	+	++	–	–	–	–	–	–
3,5,6,7,4′-PentaME (**12**)	–	–	+	++	–	–	–	–	–	–
Quercetagetin										
6-ME (**13**)	–	–	–	(+)	–	–	–	–	–	–
3,7-DiME (**14**)	(+)[c]	–	–	–	–	–	–	–	–	–
3,7,3′-TriME (**15**)	++	++	–	–	–	++	+	++	++	++
3,7,3′,4′-TetraME (**16**)	(+)[c]	–	–	–	–	–	+	++	–	+
3,5,7,3′-TetraME (**17**)	–	–	–	–	(+)	–	(+)	(+)	–	–
PentaME (**18**)[b]	–	–	–	(+)	–	–	(+)	(+)	–	–
3,5,6,7,3′,4′-HexaME (**19**)	–	–	–	–	–	–	++	++	–	–
Dihydroflavonols										
Methylated derivatives (**20**,**21**)	–	–	(+)	(+)	–	–	–	–	–	–

++ = Major constituent , + = minor constituent, (+) = trace constituent, L = leaf, In = inflorescence, ME = methyl ether.
[a] Data from Williams et al.(2000).
[b] Compound not completely identified
[c] Present only in some races of *P. dysenterica*.

2.2. Distribution of surface flavonoids

The distribution of compounds **1**–**21** in the European species of *Pulicaria* is presented in Table 2. The surface flavonoids of *Pulicaria* species, as in other Compositae, usually occur in complex mixtures composed of major and minor components. Quercetagetin 3,7,3′-trimethyl ether (**15**), one of the characteristic leaf and disc floret surface components of *P. dysenterica,* was present as a major constituent of all the taxa surveyed except *P. odora*. The other *P. dysenterica* constituents, 6-hydroxykaempferol and quercetagetin 3,7-dimethyl ethers (**9, 14**), were not detected in any other European species. Each species has a distinctive flavonoid profile and there are some differences between the leaf and inflorescence patterns.

The simplest flavonoid profiles were encountered in *P. vulgaris*, where the major leaf components were scutellarein 6-methyl ether (**1**) and quercetagetin 3,7,3′-trimethyl ether (**10**) and those of the inflorescence were **10** and quercetagetin 3,7,3′,4′-tetramethyl ether (**16**).

In *P. sicula* a complex mixture containing a series of highly methylated quercetagetin derivatives were present on the leaf and inflorescence surfaces including the fully methylated hexamethyl ether (**19**) and 3,7,3′,4′-tetramethyl ether (**16**). Two further fluorescent blue (in UV light) quercetagetin derivatives were detected in the *P. sicula* isolates, a penta- (**18**) and a tetramethyl ether (**17**), which were present in small amount. The leaf additionally produced a fully methylated flavone, sinensetin (6-hydroxyluteolin 5,6,7,3′,4′-pentamethyl ether, **7**), which has been reported previously from several other members of the Compositae e.g. from aerial parts of *Ageratum conyzoides* (González et al., 1991).

The main leaf component of *P. paludosa* was scutellarein 6-methyl ether (**1**) with traces of other 6-hydroxyflavones, including three 5-methylated compounds, a scutellarein dimethyl ether (**2**), a 6-hydroxyluteolin trimethyl ether (**6**) and quercetagetin 3,5,7,3′-tetramethyl ether (**17**). By contrast the major inflorescence

Table 3
The vacuolar flavonoid constituents of leaves and inflorescences of five European *Pulicaria* species

Flavonoid glycoside	Colour in UV/NH_3	*Pulicaria* species									
		dysenterica		*odora*		*paludosa*		*sicula*		*vulgaris*	
		L	In	L	In	L	I	L	In	L	In
Quercetin											
3-Glucuronide (**24**)	Dk/Y	++	++	–	(+)	+	+	+	+	++	+
3-Glucoside (**25**)	Dk/Y	–	–	–	(+)	+	+	+	+	–	+
3-Galactoside (**26**)	Dk/Y	–	–	–	–	+	+	+	+	–	+
7-Glucuronide (**27**)	Y/Y	–	–	–	–	–	–	–	+	–	–
7-Glucoside (**28**)	Y/Y	–	–	–	+	(+)	(+)	–	(+)	–	–
3-Rutinoside (**29**)	Dk/Y	–	–	–	–	+	+	–	–	–	–
3-Rhamnosylgluctoside (**30**)	Dk/Y	–	–	–	–	+	+	–	–	–	–
3-Diglucuronide (**31**)	Dk/Y	–	–	–	–	–	+	–	+	–	–
Isorhamnetin											
3-Glucoside (**32**)	Dk/Y	–	–	–	–	–	(+)	–	–	–	–
3-Galactoside (**33**)	Dk/Y	–	–	–	–	–	(+)	–	–	–	–
3-Rhamnosylglucoside (**34**)	Dk/Y	–	–	–	–	–	(+)	–	–	–	–
3-Rhamnosylgalactoside (**35**)	Dk/Y	–	–	–	–	–	(+)	–	–	–	–
Patuletin											
7-Glucoside (**36**)	Y/Y	–	–	–	++	–	–	–	–	–	–
6-Hydroxykaempferol 6-methyl ether											
7-Glucoside (**37**)	Y/Y	–	–	–	++	–	–	–	–	–	–

++ = major constituent, + = minor component, (+) = trace constituent, – = not detected, L = leaf, In = inflorescence.

flavonoid was a flavonol, quercetagetin 3,7,3′-trimethyl ether (**15**); flavones were not detected.

Pulicaria odora differed from all the other European species in producing a series of highly methylated 6-hydroxykaempferol derivatives as the major surface flavonoid constituents of both leaf and inflorescence, including the new fully methylated 3,5,6,7,4′-pentamethyl ether (**12**). Another component of this plant, 6-hydroxykaempferol 3,6,7,4′-tetramethyl ether (**11**), has been recorded previously from the aerial parts of *Dodonaea viscosa* (Sapindaceae) by Sachdev and Kulshreshtha (1983). The 3,7,4′-trimethyl ether of 6-hydroxykaempferol (**10**) was first reported from *P. dysenterica* (Williams et al., 2000) but not completely characterised. This compound was found in the leaf of *P. odora* in sufficient quantity to confirm its structure (see earlier and Section 4). Only traces of quercetagetin derivatives were detected in *P. odora* and there was also evidence of two possible flavanonols (**20** and **21**) in the exudates. The latter compounds appeared as minor peaks in the HPLC analysis (R_ts 6.6 and 7.33, respectively) with typical dihydroflavonol UV absorbance (293 and 288, respectively) and when the partially purified extracts were chromatographed on paper in water, they could be seen as pink spots of high R_f when treated with zinc powder and 5 M HCl.

Dihydrokaempferol (**22**), dihydroquercetin (taxifolin) (**23**) and their 7-methyl ethers have been reported from *P. undulata*, a species which has a distribution from N. Africa to Saudi Arabia, The Yemen and Oman (Abdel-Moqib et al., 1989). Dihydroquercetin has an R_t of 3.8, suggesting that the compounds in *P. odora* may be methylated derivatives, possibly the 7-methyl ethers of taxifolin and 6-hydroxykaempferol. 6-Hydroxykaempferol 7-methyl ether together with the corresponding 3,7-, 5,7-, 7,4′- and 3,4′-dimethyl ethers have been reported from *Eupatorium* species (Herz et al., 1972) in another tribe of the Compositae, the Eupatorieae.

2.3. Vacuolar flavonoids

After removal of the lipophilic surface flavonoids with acetone, the vacuolar flavonoid constituents of the four European *Pulicaria* species were isolated from 80% methanolic leaf and inflorescence extracts using multiple preparative paper chromatography (see Section 4 for details of purification and characterisation). The results of these analyses are given in Table 3 together with previous data for *P. dysenterica* from Williams et al. (2000). Flavonol glycosides were found to be the characteristic flavonoid constituents of all the species surveyed. No flavone glycosides were detected. Quercetin 3-glucuronide (**24**), the only vacuolar flavonoid constituent of both tissues of *P. dysenterica* (Williams et al., 2000), was present also in leaves and inflorescences of *P. paludosa, P. sicula* and *P. vulgaris* with only a trace in the inflorescence of *P. odora*. Otherwise the flavonoid glycoside patterns for each species were quite distinct.

Table 4
A summary of the surface and vacuolar flavonoid constituents in five European *Pulicaria* species

Flavonoid	*Pulicaria* species									
	dysenterica		*odora*		*paludosa*		*sicula*		*vulgaris*	
	L	In	L	In	L	In	L	In	L	In
Flavones										
Scutellarein derivs	–	–	–	–	++	–	–	–	++	–
6-Hydroxyluteolin derivs	–	–	–	–	(+)	–	+	–	–	–
5-Methylation present	–	–	–	–	(+)	–	+	–	–	–
Flavonols										
6-Hydroxykaempferol derivs	+	+	++	++	–	–	–	–	–	–
Quercetagetin derivs	++	++	–	(+)	(+)	++	++	++	++	++
5-Methylated 6-hydroxykaempferol derivs	–	–	+	++	–	–	–	–	–	–
5-Methylated quercetagetin derivs	–	–	–	(+)	(+)	–	++	++	–	–
Dihydroflavonols	–	–	(+)	(+)	–	–	–	–	–	–
Flavonol glycosides										
Quercetin 3-glucuronide	++	++	–	(+)	+	+	+	+	++	+
3-O-Glycosides	+	+	–	(+)	+	+	+	+	+	+
7-O-Glycosides	–	–	–	+	(+)	(+)	–	+	–	–
Quercetin glycosides	++	++	–	+	+	+	+	+	++	+
Isorhamnetin glycosides	–	–	–	–	–	(+)	–	–	–	–
Galactosides present	–	–	–	–	+	+	+	+	–	+
Patuletin 7-glucoside	–	–	–	++	–	–	–	–	–	–
6-Hydroxykaempferol 6-methylether 7-glucoside	–	–	–	++	–	–	–	–	–	–

++ = major constituents, + = minor components, (+) = trace constituents, derivs = derivatives, L = leaf, In = inflorescence.

The most complex patterns were seen in *P. paludosa* with quercetin 3-glucoside (**25**), 3-galactoside (**26**) and 7-glucoside (**28**), rutin (**29**) and the corresponding 3-rhamnosylgalactoside (**30**) in the leaf. The inflorescence additionally contained quercetin 3-diglucuronide (**31**) and isorhamnetin 3-glucoside (**32**), 3-galactoside (**33**), 3-rutinoside (**34**) and 3-rhamnosylgalactoside (**35**). *Pulicaria sicula* had a similar basic profile but differed in the absence of rutin, quercetin 3-rhamnosylgalactoside and isorhamnetin derivatives and was the only species to produce quercetin 7-glucuronide (**27**), which co-occurred with the 7-glucoside in the inflorescence. *Pulicaria odora* was unique in producing the rare 7-glucosides of patuletin (quercetin 6-methyl ether) (**36**) and 6-hydroxykaempferol methyl ether (**37**), which are fluorescent yellow in UV light. The species with the pattern most similar to that of *P.dysenterica* was *P. vulgaris*, the only difference being in the inflorescence profile where quercetin 3-glucosides and 3-galactosides were identified in addition to quercetin 3-glucuronide in the latter.

3. Conclusions

A summary of the combined surface and vacuolar flavonoid data for the five European *Pulicaria* species is presented in Table 4 to enable the main similarities and differences between the species to be seen more clearly. The following conclusions can be drawn.

1. The vacuolar and surface flavonoid data show different affinities between the species. For example, *P. vulgaris* has a glycoside pattern which is similar to *P. dysenterica* in the absence of 7-glycosides, but the major leaf surface constituent in *P. vulgaris* is a flavone (scutellarein 6-methyl ether, **1**), which was otherwise found only in *P. paludosa*
2. *Pulicaria odora* is confirmed as the chemically most distinct species with highly methylated 6-hydroxykaempferol derivatives, including 5-methylation and the unusual 7-glucosides of patuletin and 6-hydroxykaempferol 6-methyl ether (**36**, **37**).
3. Methylation of the 5-hydroxyl of flavonols and sometimes of flavones was found in all species except *P. dysenterica* and *P. vulgaris*.
4. 7-Methylation is characteristic of all five *Pulicaria* species studied, in common with many other genera of the tribe Inuleae. In most other tribes of the Compositae 6- and/or 6,8-methylation is more usual (Wollenweber, 1994).
5. The presence of large quantities of surface flavonoids, especially those with 5-methylation, may be an adaptation to the warmer climates and higher UV light levels to which some of the more southerly and coastal *Pulicaria* species are exposed. The aerial parts of another species,

P. arabica, have also been reported to be rich in surface flavonoids including 5-methylated quercetagetin derivatives (Ahmed et al., 1988).

6. Flavonol glucuronides are characteristic vacuolar constituents of European *Pulicaria* species.

4. Experimental

4.1. General

Electrospray (ES) mass spectra were analysed on a Quattro II mass spectrometer (Micromass, Manchester, UK) using an infusion pump with samples dissolved in acetronitrile/water 1:1. Direct introduction ES in positive mode: capillary 3.6 kV, cone 32 V, source temp. 120 °C, desolvation temp. 300 °C, mass range 100–800 and scan speed of 5 s. Direct introduction ES in negative mode: capillary 3.0 kV, cone 40 V, source temp. 120 °C, desolvation temp. 250 °C, mass range 100–800 and scan speed of 5 s. Positive ion atmospheric pressure chemical ionization (APCI) mass spectra were obtained with a quadrupole ion-trap instrument (Finnigan LCQ) using a vaporiser temperature of 550 °C, sheath and auxiliary nitrogen gas pressures of 80 and 20 psi, a needle current of 5 μA, and a heated capillary temperature of 150 °C. Samples were introduced via an HPLC. HPLC/DAD was carried out using a Waters 600 multisolvent delivery system in conjunction with a Waters photodiode-array detector.

4.2. Plant material

All the *Pulicaria* species were grown from seed in the glasshouses of The School of Plant Sciences, University of Reading, and the identity of the resulting plant verified by Dr. Nicholas Hind of The Royal Botanic Gardens, Kew. Voucher specimens have been lodged in RNG. The seed sources were: *P. odora* (L.) Reichenb., Trás-os-Montes (Alto Douso), Portugal supplied by the University of Porto, Portugal; *P. paludosa* Link, wild collection grown at Hortus Botanicus, Coimbra, Portugal; *P. sicula* (L.) Moris, cult. from wild coll. 936, leg. Royl 6801, NE-coast, La albufera, Mallorca, Spain by BG of Berlin-Dahlem, Germany and *P. vulgaris* Gaertner, cult. Nr 9.81 64/95 1998154 from BG, Marburg, Germany and cult.150, Szent István, Univ. BG, Gödöllo, Hungary.

4.3. Extraction and chromatography of lipophilic flavonoids

Lipophilic flavonoids were removed from the surface of leaves and inflorescences by briefly dipping the whole tissues in Me_2CO. The concentrated Me_2CO extracts were dissolved in MeOH and separated by multiple silica gel TLC in toluene:HOAc, 4:1, followed by purification by PPC in 30% HOAc and H_2O where needed. HPLC/DAD analysis of the isolates was carried out using a C18-phenyl 250×4.6 (i.d.) mm reversed phase column at 25° using a linear gradient of 40%A–60%B to 100%B over 20 min at a flow rate of 1 ml min^{-1} with UV detection at 260 and 350 nm. Solvent A = 2% aqueous acetic acid and solvent B = MeOH, H_2O, HOAc = 18:1:1. The LC-APCI-MS was carried out with a Merck LiChrospher 250×4.0 (i.d.) mm (5 μm particle size) C18 reversed phase column using a 20 min linear gradient of 25–100% MeOH in 1% aqueous HOAc at 1 ml min^{-1}.

4.4. Identification of lipophilic flavonoids

The known constituents **1**, **3**, **4**, **5**, **8**, **9**, **13**, **14**, and **15** were identified by comparison (UV spectroscopy, colour in UV on TLC, R_f values, HPLC R_t values) with marker compounds available from previous studies (Williams et al., 1999, 2000) and our marker collection. Data are given in Table 1. The remaining compounds were identified by a combination of UV spectroscopy using shift reagents (Mabry et al., 1970), APCI-MS (Grayer et al., 2000, 2001), ES-MS, R_t (HPLC), R_f (TLC), colour in UV/NH_3 and demethylation (see later).

4.4.1. 6-Hydroxykaempferol 3,5,6,7,4′-pentamethyl ether (*12*) from P. odora

HPLC and R_f data: Table 1. Fluorescent blue in UV/NH_3. APCI-MS (+ve) $[M+H]^+$ m/z 373 (MW 372). UV λ_{max} MeOH (nm) 266, 325; +NaOAc 266, 329; +H_3BO_3 267, 329; +NaOH 325; NaOH +HCl 330; +$AlCl_3$ 266, 328; $AlCl_3$ + HCl 266, 330. The lack of any shifts indicates a fully methylated compound. Demethylation with pyridinium chloride for 2 h gave 6-hydroxykaempferol 3,6,7,4′-tetramethyl ether (**11**) and 3,7,4′-trimethyl ether (**10**), determined by HPLC comparison with **11** and **10**, respectively and with previous findings (Williams et al., 2000).

4.4.2. 6-Hydroxykaempferol 3,6,7,4′-tetramethyl ether (*11*) from P. odora

HPLC and R_f data: Table 1. Dark to dark in UV/NH_3. APCI-MS (+ve) $[M+H]^+$ m/z 359 (MW 358). UV λ_{max} MeOH (nm) 273, 335; +NaOAc 274, 336; +H_3BO_3 274, 336; +NaOH 249, 299, 376′ depressed; +NaOH +HCl 272, 338; +$AlCl_3$ 235, 305, 361; +$AlCl_3$ 282, 302, 357. Demethylation gave 6-hydroxykaempferol 3,7,4′-trimethyl ether (HPLC comparison with **10**).

4.4.3. 6-Hydroxykaempferol 3,7,4′-trimethyl ether (*10*) from P. odora

HPLC and R_f data: Table 1. Dark to dark in UV/NH_3. APCI-MS (+ve) $[M+H]^+$ m/z 345 (MW 344). UV λ_{max} MeOH (nm) 284, 339; +NaOAc 286, 331; +H_3BO_3 290, 339; +NaOH 295, 370; +NaOH+HCl decomp.; +$AlCl_3$ 305, 364; +$AlCl_3$ +HCl 302, 360.

4.4.4. Quercetagetin 3,5,6,7,3′4′-hexamethyl ether (**19**) from P. sicula

HPLC and R_f data: Table 1. ES-MS (+ve) $[M+H]^+$ m/z 403 (MW 402; hexamethoxyflavone). UV λ_{max} MeOH (nm) 265′, 335; +NaOAc 265′, 335; $+H_3BO_3$ 265′, 335; +NaOH 265′, 335; +NaOH 265′, 335; $+AlCl_3$ 265, 335; $+AlCl_3$ +HCl 265′, 335. UV/NH_3 fluorescent blue. Similar values to literature data (Ahmed et al., 1989).

4.4.5. Quercetagetin 3,7,3′,4′-tetramethyl ether (**16**) from P. sicula

HPLC R_t 9.8. HPLC UV λ_{max} (nm) 257, 281, 353. R_f data: Table 1. Dark to dark in UV/NH_3. ES-MS (−ve) $[M-H]^-$ m/z 373 (MW 360). ES-MS (+ve) m/z 375 $[M+H]^+$ and 397 $[M+Na]^+$, (MW 374; trihydroxy-trimethoxyflavone). The dark colour in UV light and the UVλ_{max} at 281 confirm that the 5- and 6-positions are free and the 7-position is not, respectively.

4.4.6. Quercetagetin 3,5,7,3′-tetramethyl ether (**17**) from P. paludosa

HPLC and R_f data: Table 1, suggest it is a flavonol methylated at the 3- and 5-positions. Fluorescent buff/yellow in UV/NH_3. APCI-MS (+ve) $[M+H]^+$ m/z 375 (MW 374) indicates a dihydroxy-tetramethoxyflavone. Data agree with lit. data for **17** from *P. arabica* (El-Negoumy et al., 1982).

4.4.7. 6-Hydroxyluteolin 5,6,7,3′,4′-pentamethyl ether (**7**) from P. sicula

HPLC R_t 13.9; HPLC UV λ_{max} (nm) 257, 272, 348. R_f data: Table 1. Fluorescent blue in UV/NH_3. ES-MS (+ve) $[M+H]^+$ m/z 373 (MW 372), indicating a pentamethoxyflavone. Previously reported from *Ageratum conyzoides* (Gonzalez et al., 1991).

4.4.8. Scutellarein dimethyl ether (**2**), a trace constituent from P. paludosa

HPLC and R_f data: Table 1. APCI-MS (+ve) $[M+H]^+$ m/z 315 (MW 314) indicating a dihydroxy-dimethoxyflavone. These data plus the green colour in UV/NH_3 (methylation of the 5-hydroxyl of a 6-hydroxy-flavone) and UV λ_{max} (nm) at 274, 330 suggest that **2** is a scutellarein dimethyl ether with methylation at the 5- and 7-positions. This flavonoid has not been reported before.

4.4.9. 6-Hydroxyluteolin trimethyl ether (**6**), a trace constituent from P. paludosa

HPLC and R_f data: Table 1. APCI-MS (+ve) $[M+H]^+$ m/z 345 (MW 344) indicating a dihydroxy-trimethoxy-flavone, possibly a 6-hydroxyluteolin trimethyl ether. Green colour in UV/NH_3 and UVλ_{max} (nm) 270, 335 suggest methylation at the 5-, 7- and 3′- or 4′-positions.

4.5. Vacuolar flavonoids

The water soluble vacuolar flavonoids were extracted with hot 80% MeOH from leaf and inflorescence tissues after removal of the surface flavonoids by dipping in Me_2CO. The flavonoid glycosides were isolated from the concd MeOH extracts by two different methods: (1) PPC in BAW (*n*-BuOH:HOAc:H_2O, 4:1:5, top layer) followed by further PPC of the isolated bands in 15% HOAc, H_2O and /or BEW (*n*-BuOH:EtOH:H_2O, 6:1:2.2) or (2) multiple 2D PPC in BAW and 15%HOAc with further purification in H_2O. All the flavonoid glycosides isolated (**22–35**) were known compounds. Their identities were determined by standard procedures: HPLC R_t comparison with authentic markers, UV spectral analysis, acid hydrolysis to aglycone and sugar and TLC R_f comparison or where possible co-TLC in four solvents (BAW, !5% HOAc, H_2O and CAW (1:1) or BEW) with standard markers. The presence of glucuronic acid was confirmed by the mobility of the glycoside on paper electrophoresis at pH 4.4 in acetate/formic acid buffer at 30 v/cm for 2 h.

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