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Comparative phytochemical and morphological analyses of three Italian *Primula* species

Gelsomina Fico [a,*], Graziella Rodondi [a], Guido Flamini [b], Daniele Passarella [c], Franca Tomé [a]

[a] *Dipartimento di Biologia, Università degli Studi di Milano, via Celoria 26, 20133 Milano, Italy*
[b] *Dipartimento di Chimica Bioorganica e Biofarmacia, Università degli Studi di Pisa, via Bonanno 33, 56126 Pisa, Italy*
[c] *Dipartimento di Chimica Organica e Industriale, Università degli Studi di Milano, via Venezian 21, 20133 Milano, Italy*

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Abstract

The taxonomy of alpine *Primula* species has long been in dispute because of high morphologic variability and several hybridisations. In *Primula* species, the trichome height and the colour of hair-tips are usually indicated as diacritic characters, but in our experience this is not adequate. The present study, focused on *Primula auricula*, *Primula daonensis* and *Primula hirsuta*, therefore proposes the use of other morphologic trichome parameters (size and dimensional ratio of stalk, neck and gland head). Phytochemical investigations about the flavonoid composition (epicuticular and vacuolar) of leaves, as taxonomic markers, have also been performed. We report the isolation and identification of two new flavonol glycosides, isorhamnetin 3-*O*-(2,6-di-*O*-β-D-glucopyranosyl-β-D-glucopyranoside) (**1**) and kaempferol 3-*O*-(2-*O*-α-L-rhamnopyranosyl-6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) (**2**) and of eight known flavonoids. Size and dimensional ratio of the three trichome elements (stalk, neck and glandular head) are typical for each species analysed. The flavonoid profile well characterise the entities under study. Three different profiles have been obtained with both vacuolar and epicuticular flavonoids.

The morphologic and phytochemical markers proposed in this work seem to be parameters which significatively discriminate the species under study.

Keywords: *Primula auricula*; *Primula daonensis*; *Primula hirsuta*; Primulaceae; Phytochemistry; Morphology; Taxonomy; Flavonoid; Trichome

1. Introduction

The *Primula* L. genus is the most important one in the Primulaceae family and includes, considering the latest evaluations, 426 species, mainly located in the temperate and cold regions of the northern hemisphere and in the tropical mountains.

The systematic studies on European *Primula* L. genus started in the middle of 19th century, and are based on morphological (Duby, 1844; Pax, 1889; Lüdi, 1926; Smith and Fletcher, 1948), cytological (Bruum, 1932), caryologic (Wanner, 1943), biochemical (Müeller, 1915; Brunswik, 1922; Hegnauer, 1969), cytotaxonomic (Kress, 1963, 1989) and palynologic (Spanovschy, 1962) analyses. Recently the monographs of Richards (1993, 2003) investigates the *Primula* genus all over the world.

The systematics of the alpine *Primula* species belonging to the sect. *auricula* Duby has long been in dispute, because of high morphological variability, several hybridizations and frequent description of new species and subspecies (Banfi and Ferlinghetti, 1993; Moser, 1998; Prosser and Scortegagna, 1998; Jeßen and Lehmann, 2005).

Difficulties to distinguish among closely related *Primula* sect. *auricula* species have recently prompted biomolecular studies (Scudiero et al., 1980; Zhang and Kadereit, 2004)

* Corresponding author. Tel.: +39 0250314770; fax: +39 0250314764.
E-mail address: gelsomina.fico@unimi.it (G. Fico).

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and critical and nomenclatural revisions (Zhang and Kadereit, 2005). The major taxonomic characters useful to differentiate *Primula* species are leaf morphology (shape, margins, glandular hair, farinas), flower colour, seed coat structure and size and shape of calyx and bract (Valentine and Kress, 1972; Pignatti, 1982; Zhang and Kadereit, 2004).

With regard to the secondary metabolite composition, rhizome and roots are rich in saponins (Calis et al., 1992; Ahmad and Shah, 1993; Yayli, 2001; Morozowska, 2004), responsible for medicinal plant properties (Della Loggia, 1993).

The aerial parts are rich in flavonoids. Nestler (1904) by his investigations on allergic reactions caused by irritant compounds in glandular trichomes of *P. obconica*, was one of the early pioneers in the field of trichome chemistry and one of the first scientists to use trichome metabolites for plant taxonomy. Wollenweber characterized a great number of aglycone flavonoids and he defined some typical patterns within the *Primula* genus (Wollenweber and Dietz, 1981; Wollenweber, 1984). Moreover some papers dealt with flavonoid composition in *P. vulgaris*, *P. elatior*, and *P. veris* (Karl et al., 1981; Petitjean-Freytet et al., 1993; Huck et al., 2000; Budzianowski et al., 2005). As a phytochemical contribution to the studies of this genus, we report the isolation and identification of two new flavonol glycosides, **1** and **2** and of eight known flavonoids, mostly isolated for the first time from *Primula* (*P. auricula*, *P. daonensis* and *P. hirsuta*).

Since flavonoids have been employed intensively to solve taxonomic questions (Lim et al., 1999; Fico et al., 2003; Walkama et al., 2003; Joshi, 2003; Marin et al., 2004; Wang et al., 2004; Ferreira et al., 2005), in this work we have studied both flavonoid aglycones associated with secretory structures and glycosides which, being water soluble, are located in the vacuole. The aim of this work is to investigate whether these secondary metabolites and the size and dimensional ratio of stalk, neck and gland head of leaf trichomes, could be used to distinguish *Primula* taxa.

2. Results

2.1. Trichome types, morphology and distribution on the leaves

In *Primula* species aerial organs present glandular hairs: the upper and lower leaf epidermis of all the *Primula* taxa examined are densely covered with trichomes which produce copious sticky secretions in *P. daonensis* and *P. hirsuta* and exudates as crystalline deposit named ''farina'' in *P. auricula*. The secretions of the three species produce a strong aromatic smell, which still remains also when the plant is dry. The secretory heads of *P. daonensis* are variously coloured from red to brown, while *P. hirsuta* ones are yellow-hyaline. *P. auricula* leaves present such an intense farina deposition, especially concentrated on the leaf margin (Fig. 1A), so that their colour becomes almost white: this is particularly evident on the young leaves, before and immediately after distension. Moreover in *P. auricula* farinas are so abundant to hide the trichomes: only the head remains outstanding (Fig. 1B). At the scanning electron microscope (SEM), farinas appear arranged around the gland of each trichome looking like needles randomly extruded (Fig. 1C).

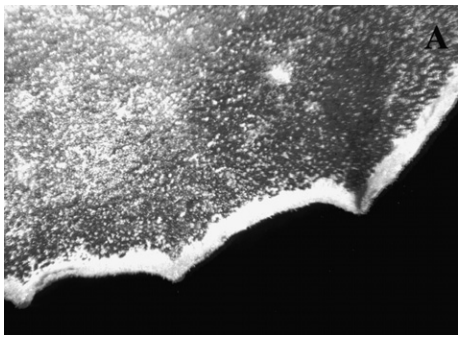


Fig. 1A. *P. auricula*: leaf, with characteristic farina deposition along the margin.

At LM (light microscope) fully developed capitate trichomes consist of an unicellular glandular head supported by a stalk, which consists in a neck composed of one rectangular cell and a base formed by 5–8 cells. The three stud-

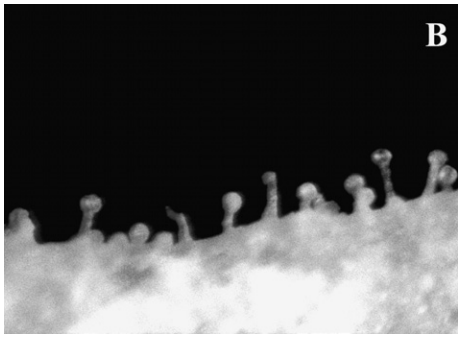


Fig. 1B. Trichomes of the leaf margin covered with farinas.

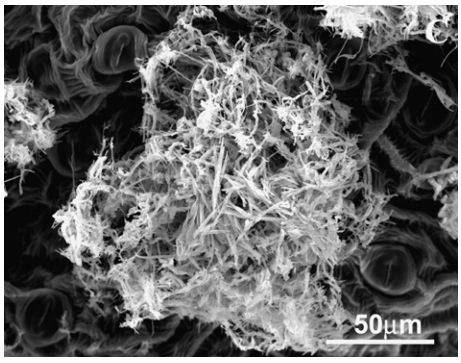


Fig. 1C. *P. auricula*: SEM micrograph of farinas extruded around the trichome gland.

ied species show some typical aspects of size and dimensional ratio of the trichome elements as follows:

- *P. auricula* has a very short stalk, a rectangular neck and a quite spherical gland (Fig. 1D);
- *P. hirsuta* has a long conical stalk, a square neck and a round gland (Fig. 1E);
- *P. daonensis* has a short stalk, a very long and rectangular neck, a big and round gland (Fig. 1F).

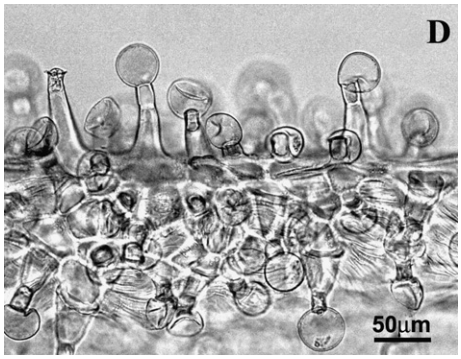


Fig. 1D. *P. auricula*: LM micrograph of trichomes with very short stalks and quite spherical glands.

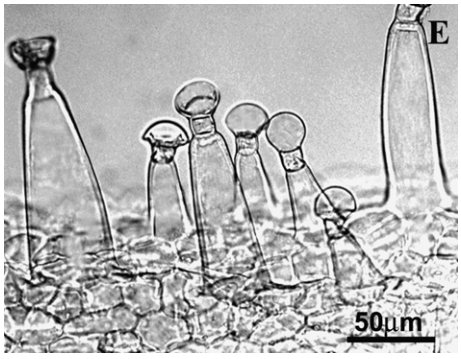


Fig. 1E. *P. hirsuta*: LM micrograph of trichomes with long conical stalks, square necks and round glands.

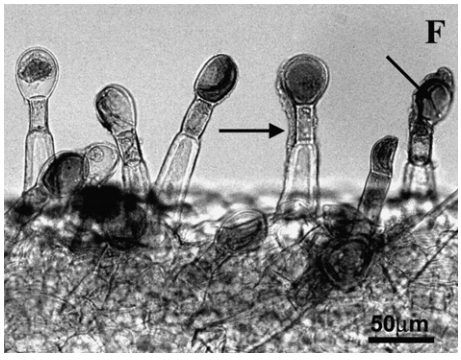


Fig. 1F. *P. daonensis*: LM micrograph of trichomes with short stalks, long and rectangular necks, bulb-shaped glands. The secretions are sticky and fluid (arrows).

2.2. Flavonoid isolation and identification

From $CHCl_3$–MeOH and MeOH leaf extracts of three different species of the genus *Primula* two new flavonols glycosides, isorhamnetin 3-*O*-(2,6-di-*O*-β-D-glucopyranosyl-β-D-glucopyranoside) (**1**) and kaempferol 3-*O*-(2-*O*-α-L-rhamnopyranosyl-6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) (**2**) were isolated. The structural elucidation of these compounds was deduced on the basis of their ^{1}H- and ^{13}C NMR spectral data, including those derived from 2D NMR, as well as on HPLC–MS results.

Compound **1** was obtained as a yellow powder; its mass spectrum, measured in the negative mode, showed a quasimolecular peak $[M-H]^-$ at *m/z* 801, corresponding to the molecular formula $C_{34}H_{42}O_{22}$, also supported by elemental analysis. The ^{13}C NMR spectrum showed 34 resonances, sorted by DEPT experiments into one CH_3, 3 CH_2, 20 CH and 10 quaternary C. In the ^{1}H NMR spectrum obtained in DMSO-d_6, two coupled doublets (δ 6.06 and 6.32, $J = 1.8$ Hz), typical of the two *meta*-related H-6 and H-8 protons of ring A of a flavonoid unit were present. Ring B showed an ABM system (δ 8.01, *d*, $J = 1.9$ Hz; δ 7.54, *dd*, $J = 8.4$ and 1.9 Hz; δ 6.87, *d*, $J = 8.4$ Hz). Furthermore, a three-proton methoxyl singlet was observed at δ 3.82. This group was linked to the 3′ carbon as demonstrated by the typical methoxylation shifts experienced by the other carbons of ring B and by COLOC experiments. This permitted to identify the flavonoid nucleus as isorhamnetin. Moreover, three one-proton doublets were present at δ 5.40 ($J = 7.3$ Hz), δ 4.20 ($J = 7.1$ Hz) and δ 4.06 ($J = 7.4$ Hz). This suggested the presence of three sugar units having a β-configuration because of the *J* values due to *trans*-diaxial interactions. The placement of the sugar chain at C-3 was determined on the basis of the typical glycosilation shifts that occurred with respect to isorhamnetin: downfield shifts of C-2 and C-4 (ca. 9 and 3 ppm, respectively) and an upfield shift of C-3 (ca. 2 ppm). Furthermore, COLOC experiments permitted to confirm this placement and to determine the interglycosidic linkages. In particular, the second sugar unit was linked to C-6 of the inner one, while the outer unit resulted linked to C-2 of the second one. All the chemical shifts were in good agreement with a glucosyl(1 → 2)gentiobioside moiety (16). Therefore, **1** was identified as isorhamnetin 3-*O*-(2,6-di-*O*-β-D-glucopyranosyl-β-D-glucopyranoside). This structure is also supported by its mass spectrum where, besides the quasimolecular peak at *m/z* 801, there were peaks at *m/z* 639 $[M-H-162]^-$, *m/z* 477 $[M-H-324]^-$ and *m/z* 315 $[M-H-486]^-$, due to the loss of one, two and three glucose units, respectively.

Compound **2** was purified as an amorphous yellowish powder. The ESI mass spectrum exhibited the ion peak at *m/z* 726 consistent with a molecular formula of $C_{32}H_{38}O_{19}$. The ^{13}C NMR spectrum showed 32 resonances and in particular DEPT experiments permitted to

recognize one CH_3, two CH_2, 20 CH and nine quaternary C. In obtained DMSO-d_6, the signals at δ 5.66 (*d*, $J = 7.5$ Hz), 5.25 (*d*, $J = 1.3$ Hz) and at 4.10 ($J = 7.2$ Hz) revealed the presence of three sugar moieties that present in two cases the β-configuration and in the other one the α-configuration. Complete assignments of each sugar proton system were achieved by considering TOCSY and ^1H–^1H COSY spectra. The presence of a glucose unit was confirmed by the large vicinal coupling among ring protons due to the *trans*-diaxial orientation. The ^1H NMR spectrum showed two coupled doublets with a very small *J*-value, typical of the two *meta*-related H-6 and H-8 protons of the A ring of the flavonoid unit and an AA′BB′ system (δ 8.09 and 6.91, $J = 8.9$ Hz) that is due to the B ring of the same flavonoid unit. The sugar moiety was found to be linked to C-3 as consequence of the same reason that we described above. By this way the flavonoid unit was recognized as kaempferol with the a glycosidic bond at position 3. The interglycosidic linkages were confirmed by HMBC experiments. In particular the glucose unit, that was directly connected to kaempferol, was linked through position 2 to rhamnose and through position 6 to xylose.

The sugar moiety was almost superimposable to that of compound **1** and it was found to be linked to C-3 as above described. Therefore, compound **2** was identified as kaempferol 3-*O*-(2-*O*-α-L-rhamnopyranosyl-6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) (Table 1).

Besides to **1** and **2,** the known compounds, quercetin 3-*O*-(glucosyl(1 → 2)gentiobioside) (**3**), kaempferol 3-*O*-neohesperidoside (**4**), isorhamnetin 3-*O*-neohesperidoside (**5**), tamarixin (**6**), isorhamnetin 3-*O*-glucopyranoside (**7**), isorhamnetin 3-*O*-(2-*O*-α-L-rhamnopyranosyl-6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) (**8**), quercetin 3-*O*-neohesperidoside (**9**), 7,2′- dihydroxyflavone 7-*O*-β-D-glucopyranoside (**10**), were isolated and identified by spectral methods in comparison with literature data (Vidal et al., 1989; Kruthiventi and Krishnaswamy, 2000) (Tables 2 and 3).

The chromatograms of the flavonoids in the exudate from *P. auricula, P. daonensis* and *P. hirsuta* are shown in Fig. 2A–C. Each chromatogram was characterized by the presence of one main peak, with R_t respectively of 35.34, 36.69 and 37.75 min.

The comparison of spectra of these peaks indicated that the three species are characterized by different compounds (Fig. 3A–C).

Traditionally the taxonomic value of trichomes, in diagnostic key, to distinguish different *Primula* species is correlated to their presence/absence or their overall length (Pignatti, 1982), or density and colour of hair-tips (Tutin et al., 1993). Our LM analysis enhanced the differences in shape, size and dimensional ratio of the trichome elements: stalk, neck and glandular head, making the trichome micro morphology a diagnostic element more stringent than the overall length of trichomes, or the hair-tip colour, to solve taxonomic questions.

Table 1
NMR data of new compounds **1** and **2**

Position	**1** (DMSO-d_6)		**2** (CD_3OD)	
	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
Aglycone				
2		155.2		166.8
3		133.3		135.4
4		176.9		180.2
5		161.0		162.2
6	6.06, *d*, 1.8 Hz	99.2	6.18, *d*, 2.0 Hz	~98[a]
7		163.8		163.9
8	6.32, *d*, 1.8 Hz	94.1	6.39, *d*, 2.0 Hz	~93[a]
9		156.6		159.4
10		103.4		106.9
1′		122.0		124.0
2′	8.01, *d*, 1.9 Hz	115.2	8.09, *d*, 8.9 Hz	117.0
3′		146.8	6.91, *d*, 8.9 Hz	133.1
4′		149.5		159.3
5′	6.87, *d*, 8.4 Hz	113.2	6.91, *d*, 8.9 Hz	133.1
6′	7.54, *dd*, 1.9, 8.4 Hz	121.0	8.09, *d*, 8.9 Hz	117.0
OMe	3.82 *s*	55.7		
	3-Glc		3-Glc	
1	5.40, *d*, 7.3 Hz	101.3	5.66, *d*, 7.5 Hz	101.3
2	3.28[b]	74.4	3.64, *dd*, 7.5, 8.9 Hz	80.8
3	3.22[b]	76.1	3.58, *t*, 8.9 Hz	79.6
4	3.08, *t*, 9.0 Hz	70.1	3.32–3.40, *m*	72.8
5	3.51[b]	76.1	3.42–3.47, *m*	78.3
6	3.40[b]	67.8	3.59, *dd*, 6.5, 11.8 Hz	70.4
	3.82, *d*, 11.4 Hz		3.93, *dd*, 1.8, 11.8 Hz	
	6″-Glc		2″-Rha	
1	4.20, *d*, 7.1 Hz	101.6	5.25, *d*, 1.3 Hz	103.5
2	3.03, *m*	82.3	4.03, *dd*, 1.3, 3.4 Hz	73.3
3	3.21[b]	75.7	3.82, *dd*, 3.4, 9.6 Hz	73.2
4	3.07, *m*	69.7	3.32–3.40, *m*	74.9[c]
5	2.61, *m*	76.1	4.06–4.15, *m*	70.8
6	3.34[b] 3.50[b]	60.4	1.04, *d*, 6.2 Hz	18.4
	2‴-Glc		6″-Xyl	
1	4.06, *d*, 7.4 Hz	104.4	4.10, *d*, 7.2 Hz	105.9
2	2.51[d]	74.4	3.03, *dd*, 7.2, 8.8 Hz	75.6
3	3.03, *m*	75.7	3.12, *t*, 8.8 Hz	78.2
4	2.98, *m*	69.7	3.32–3.40, *m*	71.9[c]
5	2.97, *m*	77.4	3.68, *dd*, 5.2, 11.4 Hz 2.91, *dd*, 10.0, 11.4 Hz	67.4
6	3.52[c] 3.64[c]	61.0		

[a] Broad signals assigned on the basis of Hetcorr.
[b] Signal superimposed by H_2O, attributed by COLOC or HSQC.
[c] Interchangeable signals.
[d] Signal superimposed by DMSO-d_6, attributed by COLOC or HSQC.

The phytochemical analysis has pointed out three different flavonoid profiles for the species under study (Table 4). The identified flavonol compounds are 3-*O*-glycosides, represented by derivatives of kaempferol, quercetin, isorham-

Table 2
^{1}H NMR data of known compounds **3**–**10**

Position	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Aglycone								
3								6.90, *s*
5								7.24, *br d*, 9.0 Hz
6	6.12, *d*, 1.8 Hz	6.16, *d*, 1.9 Hz	6.18, *d*, 1.9 Hz	6.23, *d*, 1.9 Hz	6.18, *d*, 2.0 Hz	6.02, *d*, 1.9 Hz	6.17, *d*, 1.8 Hz	7.56, *br d*, 8.0 Hz
8	6.41, *d*, 1.8 Hz	6.38, *d*, 1.9 Hz	6.38, *d*, 1.9 Hz	6.44, *d*, 1.9 Hz	6.42, *d*, 2.0 Hz	6.21, *d*, 1.9 Hz	6.37, *d*, 1.8 Hz	7.53, *br s*
2'	7.66, *br s*	8.01, *d*, 8.9 Hz	7.98, *d*, 1.8 Hz	7.66, *br s*	7.94, *d*, 1.9 Hz	7.91, *d*, 1.8 Hz	7.51, *d*, 2.1 Hz	
3'		6.86, *d*, 8.9 Hz						7.62, *br d*, 8.0 Hz
4'								7.78, *br t*, 7.8 Hz
5'	6.80, *d*, 8.4 Hz	6.86, *d*, 8.9 Hz	6.91, *d*, 8.5 Hz	7.00, *d*, 8.9 Hz	6.87, *d*, 8.7 Hz	6.88, *d*, 8.5 Hz	6.81, *d*, 8.5 Hz	7.50, *m*
6'	7.62 *br d*, 8.4 Hz	8.01, *d*, 8.9 Hz	7.54, *dd*, 1.8, 8.5 Hz	7.51, *br d*, 8.9 Hz	7.50, *dd*, 1.9, 8.8 Hz	7.43, *dd*, 1.8, 8.5 Hz	7.59, *dd*, 2.1, 8.5 Hz	8.02, *br d*, 7.3 Hz
OMe			3.97, *s*	3.92, *s*	3.95, *s*	3.83, *s*		
	3-Glc	3-Glc	3-Glc			3-Glc	3-Glc	7-Glc
1	5.30, *d*, 6.8 Hz	5.70, *d*, 7.2 Hz	5.89, *d*, 7.2 Hz	5.27 *d*, 7.4 Hz	5.29, *d*, 7.6 Hz	5.70, *d*, 7.4 Hz	5.64, *d*, 7.4 Hz	4.88 *d*, 7.0 Hz
2	3.26[a]	3.59[a]	3.63[a]	3.48[a]	3.50[a]	3.61[a]	3.60[a]	3.32[a]
3	3.24[a]	3.57[a]	3.52[a]	3.44[a]	3.44[a]	3.58[a]	3.53[a]	3.38[a]
4	3.09, *t*, 8.7 Hz	3.53[a]	3.51[a]	3.37[a]	3.31[a]	3.48[a]	3.53[a]	3.24[a]
5	3.50[a]	3.26[a]	3.24[a]	3.22[a]	3.23[a]	3.60[a]	3.24[a]	3.42[a]
6	3.40[a]	3.50[a]	3.51[a]	3.69[a]	3.70[a]	3.71[a]	3.52[a]	3.49[a]
	3.79, *d*, 11.8 Hz	3.71, *br d*, 11.1 Hz	3.76, *br d*, 12.1 Hz	3.61[a]	3.58[a]	4.12, *br d*, 11.1 Hz	3.73, *br d*, 11.8 Hz	3.75, *br d*, 11.5 Hz
	6″-Glc	2″-Rha	2″-Rha			2″-Rha	2″-Rha	
1	4.14, *d*, 7.4 Hz	5.21, *br s*	5.19, *br s*			5.02, *br s*	5.06, *br s*	
2	3.00 *m*	4.02, *br d*, 3.3 Hz	3.95, *br d*, 3.0 Hz			4.00, *br d*, 3.6 Hz	3.96, *br d*, 3.1 Hz	
3	3.21[a]	3.78, *dd*, 3.3, 9.1 Hz	3.76, *dd*, 3.0, 8.9 Hz			3.67[a]	3.76, *dd*, 3.1, 8.9 Hz	
4	3.02 *m*	3.30[a]	3.28[a]			3.33[a]	3.33[a]	
5	2.65 *m*	4.00 *m*	3.97 *m*			3.92 *m*	3.99 *m*	
6	3.36[a] 3.49[a]	0.72, *d*, 6.1 Hz	0.62, *d*, 6.1 Hz			0.65, *d*, 6.1 Hz	0.75, *d*, 6.1 Hz	
	2‴-Glc					6″-Glc		
1	4.00, *d*, 7.5 Hz					4.05, *d*, 7.2 Hz		
2	2.52[b]					3.01, *dd*, 7.2, 8.9 Hz		
3	3.00, *m*					3.10, *t*, 8.9 Hz		
4	2.98, *m*					3.15, *t*, 8.9 Hz		
5	2.99, *m*					2.86, *m*		
6	3.48[a]					3.41[a]		
	3.65[a]					3.58[a]		

[a] Signal superimposed by H_2O, attributed by COLOC or HSQC.
[b] Signal superimposed by DMSO-d_6, attributed by COLOC or HSQC.

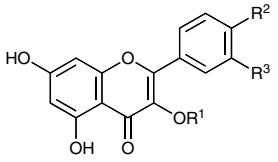
netin and tamarixin and the sugar moieties include glucose, rhamnose, xylose and di- and three-saccharides based on these sugars. The sole isolated flavone was 7,2′-dihydroxyflavone 7-*O*-glucopyranoside.

The first profile characterize *P. auricula* and shows the presence of flavonols with three sugar units. The sugar moiety was represented by glucose, the aglycones were quercetin and isorhamnetin.

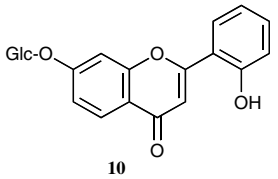
Table 3
^{13}C NMR data of known compounds **3**–**10**

Position	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Aglycone								
2	156.1	161.0	158.4	157.3	159.0	155.1	156.0	155.6
3	133.6	134.0	134.4	133.9	134.4	132.2	132.8	105.9
4	177.4	178.9	179.3	178.6	178.7	176.5	177.2	177.0
5	161.1	163.0	163.2	162.3	163.2	161.0	161.2	116.0
6	98.6	98.1	99.8	98.9	98.0	98.5	98.3	118.4
7	164.2	165.5	165.7	164.8	165.6	165.0	164.3	162.6
8	93.6	94.2	94.6	93.9	94.2	94.2	93.5	113.6
9	156.4	157.4	158.5	157.7	157.6	156.6	156.2	147.0
10	104.2	104.9	105.0	104.2	104.5	104.2	103.9	125.1
1′	121.2	123.1	123.5	122.1	122.9	121.7	121.1	123.3
2′	116.3	133.3	114.6	113.9	114.8	113.2	115.9	148.4
3′	144.7	115.8	150.5	147.3	149.8	149.3	144.9	118.4
4′	148.5	159.9	148.4	149.2	147.6	146.8	148.4	134.2
5′	115.1	115.8	116.0	115.0	116.1	115.1	115.1	125.5
6′	121.4	133.3	123.6	122.0	123.4	121.0	121.6	124.8
OMe			57.0	56.9	57.1	55.8		
	3-Glc	3-Glc	3-Glc	3-Glc	3-Glc	3-Glc	3-Glc	7-Glc
1	100.9	100.3	100.8	101.3	101.1	100.7	100.5	101.2
2	74.2	79.9	80.4	74.4	74.3	82.3	79.3	73.3
3	76.1	79.0	78.9	76.7	76.5	77.5	77.4	75.9
4	69.6	71.7	71.9	70.6	70.3	70.5	70.6	69.8
5	76.1	78.2	78.4	77.0	77.2	76.1	77.2	77.3
6	68.1	62.4	62.6	61.3	61.0	67.8	60.9	60.8
	6″-Glc	2″-Rha	2″-Rha			2″-Rha		
1	101.5	103.1	102.8			102.5	103.0	
2	82.1	72.4	72.4			68.6	71.8	
3	75.7	72.3	72.4			68.2	71.7	
4	69.4	74.1	74.0			71.7	73.0	
5	76.0	69.9	69.9			65.3	68.2	
6	60.5	17.6	17.4			17.0	17.2	
	2‴-Glc					6″-Glc		
1	104.0					103.2		
2	74.2					73.4		
3	75.7					76.6		
4	69.6					69.7		
5	77.3					76.2		
6	61.0					60.7		

Table 4
Flavonoid composition of *Primula* species



R^1	R^2	R^3
Primula auricula		
β-D-glucopyranosyl(1 → 2)gentiobioside (**1**)	OH	OCH_3
β-D-glucopyranosyl(1 → 2)gentiobioside (**3**)	OH	OH



10

7,2′-dihydroxyflavone 7-*O*-β-D-glucopyranoside

Primula daonensis		
Neohesperidoside (**5**)	OH	OCH_3
β-D-glucopyranoside (**6**)	OCH_3	OH
β-D-glucopyranoside (**7**)	OH	OCH_3
2-*O*-α-L-rhamnopyranosyl-6-*O*-β-D-glucopyranosyl-β-D-glucopyranoside (**8**)	OH	OCH_3
Neohesperidoside (**9**)	OH	OH
Primula hirsuta		
2-*O*-α-L-rhamnopyranosyl-6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside (**2**)	OH	H
Neohesperidoside (**4**)	OH	H

Table 5
Primula samples

Samples (according to Flora d'Italia)	Locality	Herbarium No.
P. auricula	Monte Alben (Bergamo – 1650–1750 m)	Pa-101
P. daonensis	Passo del Gavia (Brescia – 2500 m)	Pd-101
P. hirsuta	Alpe Gera (Sondrio – 1700 m)	Ph-101

The second profile typified *P. daonensis* and it is characterised by the presence of flavonols with no, one, two and three sugar units. The sugar moiety was represented by glucose and rhamnose, the aglycones were quercetin, isorhamnetin and tamarixin.

The third profile typical of *P. hirsuta* is characterised by the presence of flavonols with two and three sugar units represented by glucose, rhamnose and xylose; the aglycone was exclusively kaempferol.

P. auricula, *P. daonensis* and *P. hirsuta* are therefore species having each one a well defined and exclusive composition: these *taxa* presented in fact species-specificity in their vacuolar profiles. The spectral data showed that also the composition of exudates and farina was different for the three species.

Therefore, it can be concluded that the morphological results are supported by the chemical data and permit to characterize the three *Primula* species.

On the basis of the above results, the mycromorphological (regarding the fine structure of trichomes) and phytochemical investigations (regarding epicuticular and vacuolar flavonoids) can be therefore a useful tool for future works concerning taxonomic studies on this genus.

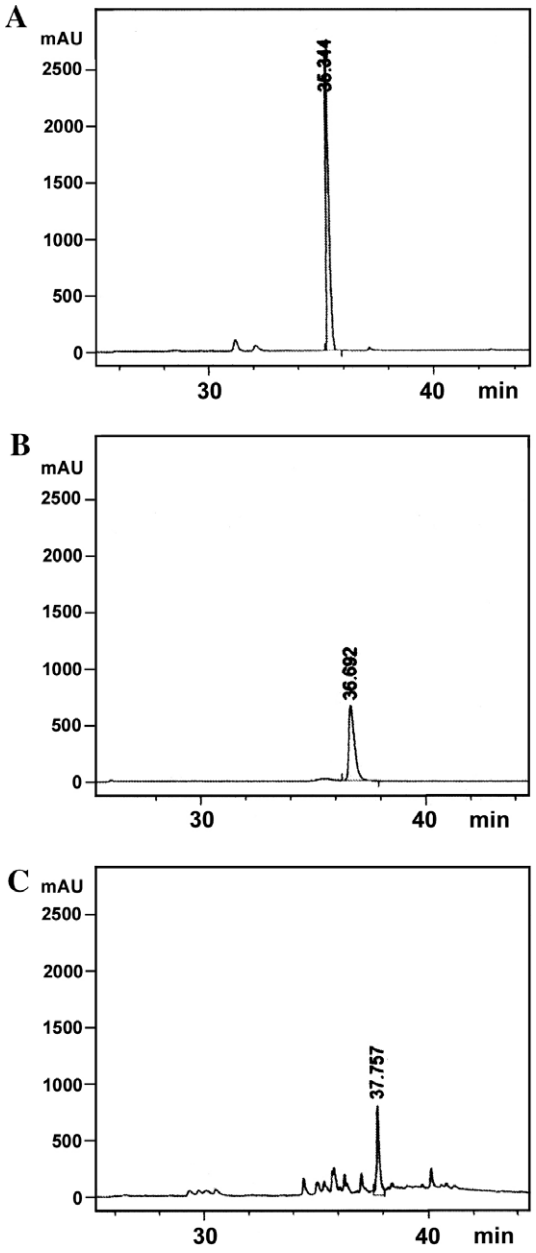


Fig. 2. Chromatograms of *P. auricula* farina (A) and *P. daonensis* (B) and *P. hirsuta* (C) exudates.

3. Experimental

3.1. Plant material

Three populations of *Primula* genus belonging to the species *P. auricula* L., *P. daonensis* Leiyb. and *P. hirsuta* All., were collected in the Alps of Lombardia (Italy) during summer 2002 and have been determined according to Pignatti (1982). The voucher specimens are deposited in the Dipartimento di Biologia, Università di Milano. Table 5 shows localities and identification numbers of samples.

3.2. Morphological analysis

For morphological observations, fresh young leaves (approx. 5–15 mm in lenght) were used. Epidermal tissues, cut from superior margin, were cleared using sodium hypochlorite 5% and observations were made using LEICA DM light microscopy (LM).

For scanning electron microscopy (SEM), pieces of 5×5 mm marginal leaves were fixed in 2.5% glutaraldehyde in 50 mM cacodylate buffer at pH 7.2 for 3 h, washed in cacodylate buffer and dried at room temperature for a week, mounted on aluminium stubs, coated with a thin layer of gold and examined in a CAMBRIDGE SEM STEREOSCAN 360.

3.3. Phytochemical analysis

The dried powdered leaves were defatted with *n*-hexane and successively extracted with $CHCl_3$, $CHCl_3$-MeOH (9:1), and MeOH, each solvent for three times. The methanol extract was submitted to RP-HPLC on a Merck LiChrospher 100 RP-18 column (5 μm, 250×4 mm, flow rate 1.3 ml/min) with binary gradient elution (A: H_2O (pH 3.5 with HCl); B: ACN; gradient: 0–10 min 88% A, 10–15 min 82% A, 30 min 55% A, 35–42 min 100% B, minimum re-equilibration time between two injections: 10 min). The detection range was 250–360 nm. The concentration of samples was 100 mg/ml and the injection volume was 15 μl.

The analytical chromatographic analyses were performed with a Merck-Hitachi L 6200 system with a photo diode array detector Hewlett Packard 1040, controlled by HP-Chemstation (Hewlett–Packard) software.

After removal of the solvent, the $CHCl_3$:MeOH and MeOH extracts were separately chromatographed on a Sephadex LH-20 column, using MeOH as eluent, to obtain fractions, combined together into further fractions according to TLC analyses [Silica 60 F_{254} gel-coated aluminium sheets; eluent: *n*-BuOH–CH_3COOH–H_2O (60:15:25)]. The flavonoid-containing fractions, selected using NTS-PEG (Naturstoffe Reagenz A-Polyethylenglycol) as reagent, were submitted to RP-HPLC on a C_{18} μ-Bondapak column (300×7.8 mm, flow rate 2.5 ml min^{-1}) with MeOH–H_2O (40:60) to yield compound **1**–**10**. In particular the new compounds **1** (10 mg) and **2** (7 mg) were obtained respectively by 26.6 g and 11.3 g of leaves (dry weight).

All the isolated compounds were submitted to NMR spectroscopic measurements with Bruker AC 400 (400 MHz) apparatus using CD_3OD or DMSO-d_6 as solvents, and the chemical shifts were expressed in δ (ppm) referring to solvent peaks: δ_H 3.31 or 2.49 and δ_C 49.0 or 39.5, respectively. The HPLC–MS and UV–vis spectra were performed on a HP 1090L instrument equipped with a Diode Array Detector, managed by a HP 9000 workstation interfaced with a HP 1100 MSD API-electrospray unit. Melting points (uncorrected) were determined with a Kofler apparatus.

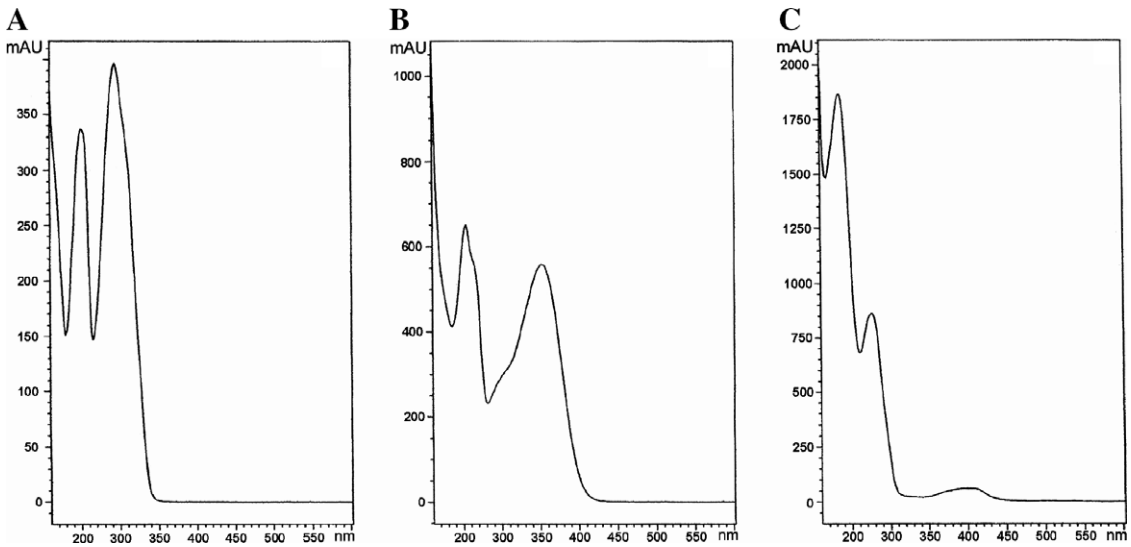


Fig. 3. Spectrum of the main compounds present in farina and exudates of *P. auricula*(A), *P. daonensis*(B) and *P. hirsuta* (C).

Epicuticular flavonoids have been extracted after fresh leaves immersion in *n*-hexane, for 3 min. The extracts were analysed by HPLC and by spectral library comparison.

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