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Farinose alpine *Primula* species: Phytochemical and morphological investigations

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ABSTRACT

This work investigated the epicuticular and tissue flavonoids, the volatiles and the glandular trichome structure of the leaves of four species of *Primula* L. that grow in the Italian Eastern Alps. *Primula albenensis* Banfi and Ferlinghetti, *P. auricula* L., *P. farinosa* L., *P. halleri* Gmelin produce farinose exudates that are deposited on the leaf surface as filamentous crystalloids.

In addition to compounds already known, a new flavone, the 3,5-dihydroxyflavone, was isolated from the acetone extract of leaf farinas and three new flavonol glycosides, 3′-*O*-(β-galactopyranosyl)-2′-hydroxyflavone, isorhamnetin 3-*O*-α-rhamnopyranosyl-(1→3)-*O*-[α-rhamnopyranosyl-(1→6)]-*O*-β-galactopyranoside, quercetin 3-*O*-α-rhamnopyranosyl-(1→3)-*O*-[α-rhamnopyranosyl-(1→6)]-*O*-β-galactopyranoside, were isolated from the MeOH extract of the leaves. All the structures were elucidated on the basis of their ^{1}H and ^{13}C NMR data and 2D NMR techniques, as well as on HPLC–MS. The leaf-volatiles emitted by these *Primula* species were mainly sesquiterpene hydrocarbons, with the exception of *P. albenensis*, which produced almost exclusively a non-terpene derivative; *P. halleri* flowers were also examined and the volatiles emitted by the flower parts (corolla and calyx) were compared with the corresponding leaves.

Introduction

The genus *Primula* L. belongs to the Primulaceae family and includes more than 400 species of both annual and perennial herb plants distributed in temperate and cold regions of the Northern hemisphere and in tropical mountains. All the species are characterised by a rosette of sessile or petioled basal leaves. The flowers, usually on top of a scape, are gathered in large or contracted/capituliform umbels. The fruits are usually indehiscent capsules containing many seeds. The glands of some *Primula* species produce farinas and/or exudates. The species studied are the only alpine *Primula* taxa living on Italian territory that show a leaf farina deposit (Banfi and Ferlinghetti, 1993; Pignatti, 1982).

The relation between glandular trichome morphology and exudate type has been previously investigated by Bhutia et al., 2012; Fico et al., 2007; Higuchi et al., 1999; Vitalini et al., 2011.

Most of the available literature on *Primula* species phytochemistry explores the activities of the saponins, which can be found particularly in the hypogeal parts, because they are the compounds with the main known pharmacological properties (Ahmad et al., 1993; Calis, 1992; Coran and Mulas, 2012; Della Loggia, 1993; Morozowska and Wesołowska, 2004; Müller et al., 2006; Okršlar et al., 2007).

Flavonoid content of some *Primula* species have been investigated in previous studies: *Primula vulgaris* (Harborne, 1968); *P. pulverulenta* (Wollenweber et al., 1988a, 1989); *P. polyantha* (Saito et al., 1990); *P. macrophylla* (Ahmad et al., 1991); *P. officinalis* (Karl et al., 1981); *P. elatior* (Petitjean-Freytet, 1993); *P. faberi* (Zhang et al., 1993); *P. denticulata* (Tokalov et al., 2004; Wollenweber et al., 1990); *P. veris* (Budzianowski et al., 2005; Huck et al., 1999, 2000); *P. hirsuta*, *P. auricula* and *P. daonensis* (Fico et al., 2007); *P. maximowiczii* (Qu et al., 2008); *P. spectabilis* (Vitalini et al., 2011); exudate flavonoids of *Primula* spp. (Bhutia et al., 2011, 2012, 2013; Bhutia and Valant-Vetschera, 2012; Valant-Vetschera et al., 2009).

Data from literature show that *Primula* genus is characterised by mono-, di- and triglycosylated flavonols, which glycone consists

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mainly of galactose, glucose, and rhamnose linked to the aglycone preferentially in 3-position. Flavonoids in the free form, mainly flavones and flavonols, are present as well.

The aim of this work is the characterisation of the four species through the analysis of epicuticular and tissue flavonoids, volatile compounds and glandular trichome morphology of the leaves. The volatile compounds emitted by the flower (calyx and corolla) of *P. halleri* were also studied.

Results

Glandular trichomes, morphology and distribution

In *P. albenensis*, sparse glandular hairs may cover the entire leaf on both surfaces, producing a thin cover of farina. In *P. auricula,* glandular hairs can be mainly found on the leaf margin. The farinose exudates of both *P. albenensis* and *P. auricula* are white. In *P. farinosa* and *P. halleri*, glandular hairs can be found only on the lower surface of the leaf. In both species, the exudates form a thick coating, respectively white and yellow.

Under the scanning electron microscope (SEM), the glandular hair morphology is completely masked by the extruded farina that appears deposited around the gland of each trichome, in the shape of needles randomly extruded (Fig. 1A–D).

Under the light microscope (LM), fully developed capitate trichomes show an unicellular glandular head and a monoseriate stalk, which consists of a rectangular neck and a cylindrical/conical base (Fig. 1E–H). In *P. albenensis,* the lower stalk cell is cylindrical and very long (about 130 μm), and the secretory head is bulb-shaped (about 35 per 25 μm) (Fig. 1E). In *P. auricula* the lower stalk cell is conical/pyramidal, about 40 μm long, and the head is round, about 35 μm in diameter (Fig. 1F). *P. farinosa* and *P. halleri* have short-stalked capitate trichomes with a very short conical stalk cell and a globoid head. The glandular head of *P. halleri* (about 25–30 μm in diameter) is twice as big as that of *P. farinosa* (about 15–20 μm in diameter) (Fig. 1G and H).

Isolation and identification of epicuticular flavonoids

Nine flavones were isolated from the leaf farina acetone extract of the four species: the new compound 3,5-dihydroxyflavone (**1**) in *P. farinosa* and the already known flavone (**2**) (Weller et al., 1953), 5-hydroxyflavone (primuletin) (**3**) (Geissman, 1962), 5,7-dihydroxyflavone (chrysin) (**4**) (in *Pinus* spp., Lindstedt, 1949a,b, 1950), 7,8-dihydroxyflavone (**5**) (in *Tridax procumbens,* Abubakar et al., 2012), 2′-hydroxyflavone (**6**) (Bouillant et al., 1971), 4′-hydroxyflavone (**7**) (in *Sophora* spp., Ruiz et al., 1999), 2′,5′-dihydroxyflavone (**8**) (Wollenweber et al., 1988b), 5,8-dihydroxyflavone (primetin) (**9**) (Tokalov et al., 2004).

The ^{13}C NMR spectrum of compound (**1**) showed 13 signals, sorted by DEPT experiments into 6 CH and 7 quaternary C. In the ^{1}H NMR spectrum obtained in DMSO-d_6, a 1-H singlet was present at δ 12.53, indicating the presence of an unsubstituted OH linked to the C-5 carbon. Furthermore, a two-proton double doublet (δ 8.16, J = 6.6 and 1.8 Hz) and a three-proton multiplet suggested the presence of an unsubstituted B-ring of a flavonoid. The A-ring was substituted only on the C-5 because of the typical proton pattern signals: two doublets (δ 6.65 and 7.25, J = 8.8 Hz) and a double doublet (δ 7.09, J = 8.8 and 8.8 Hz), all integrating for one proton. These data permitted to identify compound (**1**) as 3,5-dihydroxyflavone; the identification was confirmed by ^{13}C NMR data (Table 1). Compounds **2**–**9** were identified by NMR and ESI-MS experiments and compared to literature data (Table 1).

Isolation and identification of tissue flavonoids

Three compounds were isolated from the MeOH leaf extract of *P. farinosa* (30 g of dried leaves/5.1 g of extract): the new flavonol glycoside 3′-*O*-(β-galactopyranosyl)-2′-hydroxyflavone (t_R 18.81, 8.2 mg) (**10**); the known compound kaempferol 3-*O*-α-rhamnopyranosyl-(1→3)-*O*-[α-rhamnopyranosyl-(1→6)]-*O*-β-galactopyranoside (t_R 15.55, 4.8 mg) (**11**), never found in *Primula* genus before; and clitorin (t_R 20.55; 5.6 mg) (**12**), which is quite a widespread compound in nature and had already been isolated from *Primula maximowiczii* (Qu et al., 2008).

Two new flavonol glycosides were isolated from the MeOH leaf extract of *P. halleri* (20 g of dried leaves/3 g of extract): isorhamnetin 3-*O*-α-rhamnopyranosyl-(1→3)-*O*-[α-rhamnopyranosyl-(1→6)]-*O*-β-galactopyranoside (t_R 19.27, 4.9 mg) (**13**)) and quercetin 3-*O*-α-rhamnopyranosyl-(1→3)-*O*-[α-rhamnopyranosyl-(1→6)]-*O*-β-galactopyranoside (t_R 13.51, 5.3 mg) (**14**). From the same extract kaempferol 3-*O*-β-glucopyranosyl-(1→2)gentiobioside (t_R 12.48, 5.6 mg) (**15**), an already known flavonol glycoside, was also isolated.

The known flavonol glycoside 4′-*O*-(β-glucopyranosyl)-3′-hydroxyflavone (t_R 20.19, 10.5 mg) (**16**) was found in the MeOH leaf extract of *P. albenensis*.

The structural elucidation of these compounds was deduced on the basis of their ^{1}H and ^{13}C NMR data, including those derived from 2D-NMR, as well as from HPLC–MS results.

Compound **10** was obtained as an amorphous white solid. The negative ESI-MS spectrum returned a quasimolecular peak at m/z 415.3 $[M-H]^-$ and a fragment at m/z 253.2. The loss of 162 mass units from the molecular ion and a signal at δ 61.69 ppm, shown by APT to represent a CH_2 group, suggested a sugar moiety. The NMR spectrums were obtained in CD_3OD in order to avoid the overlap between the protons of the sugar moiety and the protons related to the water of DMSO-d_6. The combination of ^{1}H NMR, COSY, HMBC, HSQC and NOESY experiments presented a typical flavonoid pattern related to 2′,3′-disubstituted-flavone. HMBC signal between proton H-1″ of the sugar moiety and C-3′ of the flavonoid skeleton indicated the presence of the sugar unit to C-3′ of the aglycone and the OH-group at position 2′. The signal in ^{1}H NMR spectrum at 4.98 ppm was assigned to the anomeric proton (H-1″) with a coupling constant J = 7.9 Hz indicating a β-configuration. NOE signals between H-1″ and H-3″, H-1″ and H-5″, and H-3″ and H-4″ of the sugar moiety indicated the presence of a β-galactose. Therefore, compound **10** was identified as 3′-*O*-(β-galactopyranosyl)-2′-hydroxyflavone (Table 2).

On the basis of its NMR data, compound **11** was identified as kaempferol 3-*O*-α-rhamnopyranosyl-(1→3)-[α-rhamnopyranosyl(1→6)]-*O*-β-galactopyranoside, previously isolated by *Jasminum officinale* L. var. *grandiflorum* (Zhao et al., 2007) (Table 2).

Compound **12** was identified as clitorin (Kazuma et al., 2003; Nahrstedt et al., 2006) (Table 2).

Compound **13** was obtained as a yellowish powder that appeared on TLC as a yellow spot after treatment with Naturstoffreagenz A-PEG. The negative ESI–MS spectrum showed a quasimolecular peak $[M-H]^-$ at 769 m/z, corresponding to the molecular formula $C_{34}H_{42}O_{20}$. The ^{13}C NMR spectrum showed 31 signals, sorted by DEPT experiments into 20 CH, 1 CH_2, 3 CH_3 and 10 quaternary C. In the ^{1}H NMR spectrum obtained in DMSO-d_6, a three-proton ABM system was present (δ 7.92, d, J = 1.8 Hz; 7.39, dd, J = 8.8 and 1.8 Hz; 6.80, d, J = 8.8 Hz), typical of a 3′,4′-disubstituted ring B of a flavonoid nucleus. Ring A showed two coupled doublets (δ 6.08 and 6.29, d, J = 1.8 Hz), due to the two *meta*-related H-6 and H-8 protons. Moreover, a three-proton singlet was present at δ 3.80, indicating the presence of an aromatic methoxyl group. The linkage of this group on the carbon 3′ was confirmed by the typical shifts experienced by the other carbons of ring B and by HMBC experiments.