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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.

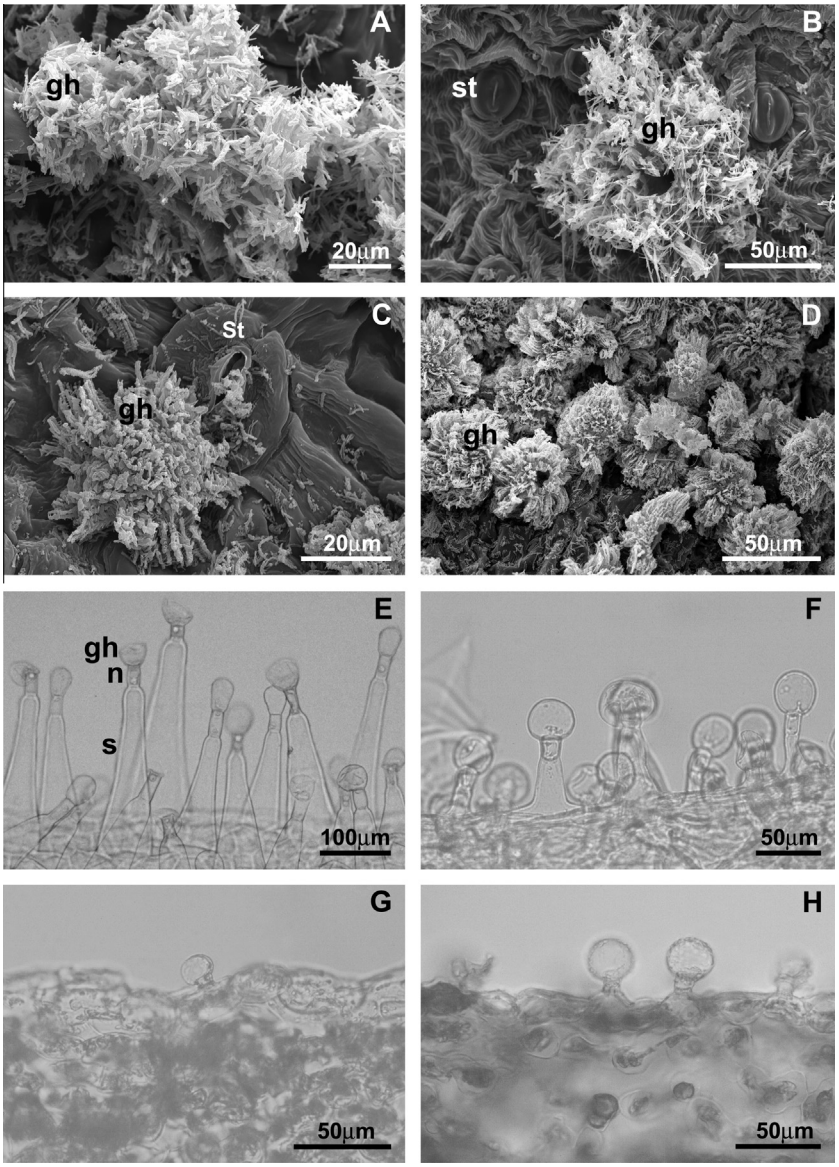


Fig. 1. SEM (A–D) and LM (E–H) micrographs showing the morphology of farinose exudates and glandular leaf trichomes of *P. albenensis* (A and E), *P. auricula* (B and F), *P. farinosa* (C and G) and *P. halleri* (D and H). *Abbreviations:* n, neck; gh, glandular head; s, stalk; st, stoma.

These data are consistent with a isorhamnetin skeleton. Furthermore, the signals of three anomeric sugar protons were clearly visible as two broad singlets at δ 4.71 and 4.40 and as a doublet (J = 7.3 Hz) at δ 5.25. At higher fields, two three-proton doublets (δ 1.06, J = 5.9 and δ 0.98, J = 6.1 Hz) were present. This situation led us to hypothesize the presence of two deoxysugars. Analysis of the ^{13}C NMR spectrum permitted us to identify the three sugar units as two α-rhamnopyranose residues and a β-galactopyranose one. By HMBC experiments, it was possible to determine position 3 as the glycosilation site of the trisaccharide moiety and to determine the relative linkage sites of the sugar units. NMR data of the sugar moiety were practically identical with those of compound **11**. Therefore, compound **13** was identified as isorhamnetin 3-*O*-α-rhamnopyranosyl-(1→3)-*O*-[α-rhamnopyranosyl-(1→6)]-*O*-β-galactopyranoside, a new natural compound (Table 2).

Compound **14** showed the same NMR data as above for its sugar moiety and a very similar pattern for the aglycone part. The most notable difference, apart from some shifts in the position of the signal of ring B carbons, was the lack of the methoxyl resonance. This permitted to identify its structure as quercetin 3-*O*-α-rhamnopyranosyl-(1→3)-*O*-[α-rhamnopyranosyl-(1→6)]-β-galactopyranoside, a new natural product, also confirmed by 2D-NMR data (Table 2).

According to its NMR data, compound **15** was identified as kaempferol 3-*O*-β-glucopyranosyl-(1→2)gentiobioside, already isolated from *Primula maximowiczii* (Qu et al., 2008) (Table 2).

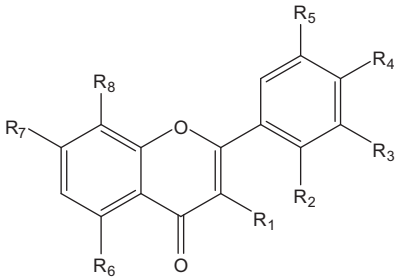
Compound **16** was identified as 4′-*O*-(β-glucopyranosyl)-3′-hydroxyflavone on the basis of its NMR data. This flavonoid had been previously isolated from *P. faberi* (Zhang et al., 1993).

In these three species with farina, 3-*O*-glycosides of kaempferol, isorhamnetin and quercetin are present in the tissues. The sugar moiety consists of trisaccharides based on glucose, rhamnose, and galactose. Monoglycosides of 2′ or 3′ hydroxyflavone (Table 2) are also present; glucose is linked in position 4′ or 3′, respectively. These compounds are shown in Table 2 in comparison with those obtained from *P. auricula* in our previous work (Fico et al., 2007).

Volatile compounds

The volatile compounds emitted by the four *Primula* species leaves were investigated, as well as the volatile compounds emitted by the flower (corolla and calyx) of *P. halleri.*

Table 1
Epicuticular flavonoids of *Primula* species.



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Species	Ref. num.	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
P. farinosa	**1**	OH	H	H	H	H	OH	H	H
P. albenensis	**2**	H	H	H	H	H	H	H	H
P. auricula									
P. farinosa									
P. halleri									
P. halleri	**3**	H	H	H	H	H	OH	H	H
P. farinosa									
P. halleri	**4**	H	H	H	H	H	OH	OH	H
P. halleri	**5**	H	H	H	H	H	H	OH	OH
P. albenensis	**6**	H	OH	H	H	H	H	H	H
P. auricula									
P. farinosa									
P. halleri									
P. albenensis	**7**	H	H	H	OH	H	H	H	H
P. auricula									
P. farinosa									
P. halleri									
P. halleri	**8**	H	OH	H	H	OH	H	H	H
P. farinosa	**9**	H	H	H	H	H	OH	H	OH

In the headspace around the leaves of these *Primula* species, 45 chemicals were identified, accounting for 97.6–99.7% of total volatiles (Table 3).

With the exception of *P. albenensis*, sesquiterpene hydrocarbons were the most represented (96.9%, 86.0%, 85.5% for *P. auricula*, *P. farinosa*, *P. halleri*, respectively).

In *P. albenensis* 13 chemicals were identified. Five of them are exclusive to this species: paeonal (98.0%), α-thujene (0.1%), terpinolene (0.1%), 2′-hydroxyacetophenone (0.2%) and 4′-methoxyacetophenone (0.3%).

In *P. auricula*, the 19 substances emitted were almost entirely sesquiterpene hydrocarbons (96.9%). The compounds exclusive to this species were present only in very low amounts: isocaryophyllene (0.9%), *(E)*-β-farnesene (0.4%) and γ-muurolene (0.5%). The main components were β-caryophyllene (28.1%), germacrene D (22.3%), β-elemene (14.3%) and α-cubebene (10.6%).

A slight percentage of an oxygenated sesquiterpene, caryophyllene oxide (0.9%), was also found.

In *P. farinosa*, 9 chemicals out of the 26 identified were exclusive to this species. Sesquiterpene hydrocarbons (86.0%) were the prevailing volatiles. The main compounds were γ-gurjunene (20.6%), also a connotative substance, germacrene D (18.7%) and β-caryophyllene (16.7%). Smaller quantities of non-terpene derivatives (7.8%) and monoterpene hydrocarbons (4.5%) were found.

In *P. halleri*, 17 volatiles were identified, 6 of which were typical of this species. In addition to sesquiterpenes (85.5%), also non-terpene derivatives (8.1%) were detected, as well as a small quantity of exclusive phenylpropanoid methyl eugenol (4%). The compounds emitted in higher percentages were γ-himachalene (27%), also exclusive of this species, germacrene D (13.1%), β-elemene (12.2%) and β-caryophyllene (9.1%).

In the headspace around calyx and corolla of *P. halleri*, 26 chemicals were identified, accounting for 92.1–97.5% of total volatiles (Table 4). The substances emitted in higher percentages were non-terpenes derivatives (53.3% and 60.8% in calyx and corolla, respectively) and sesquiterpene hydrocarbons (44.2% and 25.3% in calyx and corolla, respectively), whereas oxygenated sesquiterpenes (2.2% only from corolla) and apocarotenoids (3.8 in corolla) were less represented. Monoterpene hydrocarbons, oxygenated monoterpenes and phenylpropanoids were completely absent.

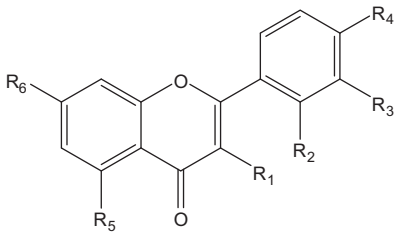
The comparison between corolla and calyx showed that they have nine compounds in common. Only one, β-gurjunene (3.2%), was specific to the calyx, while 16 chemicals were found only in corolla: inter alia, the oxygenated sesquiterpene kessane (2.2%), and the apocarotenoid hexahydrofarnesylacetone.

Discussion

Morphology

Farinose exudate morphology is the same in all the species investigated and the base model of glandular hairs morphology does not show any variation. The size of each single cell of the

Table 2
Tissue flavonoids of *Primula* species.



. Species	Ref. Num.	R_1	R_2	R_3	R_4	R_5	R_6
P. farinosa	**10**	H	OH	O-Gal	H	H	H
	11	ORha(1→3)Rha(1→6)Gal	H	H	OH	OH	OH
	12	ORha(1→3)Rha(1→6)Glc	H	H	OH	OH	OH
P. halleri	**13**	ORha(1→3)Rha(1→6)Gal	H	OCH_3	OH	OH	OH
	14	ORha(1→3)Rha(1→6)Gal	H	OH	OH	OH	OH
	15	OGlc(1→2)Glc(1→6)Glc	H	H	OH	OH	OH
P. albenensis	**16**	H	H	OH	O-Glc	H	H
P. auricula		OGlc(1→2)Glc(1→6)Glc	H	OCH_3	OH	OH	OH
		OGlc(1→2)Glc(1→6)Glc	H	OH	OH	OH	OH

Table 3
Volatiles of fresh leaves of *Primula* species sampled by SPME.

Constituents	l.r.i.	*P. albenensis* leaves	*P. auricula* leaves	*P. farinosa* leaves	*P. halleri* leaves
α-Thujene	928	0.1	–	–	–
p-Cymene	1028	–	–	2.1	–
Limonene	1032	0.1	–	0.9	–
γ-Terpinene	1063	0.1	–	1.0	–
Terpinolene	1090	0.1	–	–	–
p-Cymenene	1091	–	–	0.5	–
Phenylethyl alcohol	1112	–	–	–	4.4
2′-Hydroxyacetophenone	1164	0.2	–	–	–
α-Cubebene	1351	–	10.6	0.6	–
4′-Methoxyacetophenone	1352	0.3	–	–	–
α-Copaene	1377	–	5.1	2.3	–
β-Bourbonene	1385	0.1	1.5	–	–
β-Cubebene	1390	–	–	0.7	–
β-Elemene	1392	0.1	14.3	5.7	12.2
Methyl eugenol	1403	–	–	–	4.0
Isocaryophyllene	1406	–	0.9	–	–
cis-α-Bergamotene	1416	–	–	0.8	–
β-Caryophyllene	1419	0.3	28.1	16.7	9.1
β-Gurjunene	1432	–	–	–	1.9
β-Copaene	1433	–	0.9	1.1	1.3
trans-α-Bergamotene	1438	–	–	3.9	2.0
Paeonal	1439	98.0	–	–	–
α-Guaiene	1441	–	–	2.0	1.3
α-Himachalene	1450	–	–	2.8	–
α-Humulene	1456	–	5.4	2.2	–
(E)-β-Farnesene	1460	–	0.4	–	–
Alloaromadendrene	1462	–	–	1.3	3.7
cis-Muurola-4(14),15-diene	1463	–	0.5	0.6	–
(E)-2-Dodecen-1-ol	1469	–	–	–	2.0
γ-Gurjunene	1475	–	–	20.6	–
γ-Himachalene	1478	–	–	–	27.0
γ-Muurolene	1478	–	0.5	–	–
Germacrene D	1482	0.1	22.3	18.7	13.1
β-Selinene	1487	–	0.4	–	1.6
valencene	1494	–	0.7	1.1	3.5
Viridiflorene	1496	–	–	1.0	–
α-Muurolene	1502	–	0.5	0.7	–
(E,E)-α-Farnesene	1506	–	2.5	–	6.8
trans-γ-Cadinene	1513	0.1	0.5	0.8	–
7-*epi*-α-Selinene	1520	–	–	0.4	–
δ-Cadinene	1524	0.1	1.8	2.0	2.0
Caryophyllene oxide	1582	–	0.9	–	–
n-Hexadecane	1600	–	–	7.8	–
n-Heptadecane	1700	–	–	–	1.7
Monoterpene hydrocarbons		0.4	0.0	4.5	0.0
Sesquiterpene hydrocarbons		0.8	96.9	86.0	85.5
Oxygenated sesquiterpenes		0.0	0.9	0.0	0.0
Phenylpropanoids		0.0	0.0	0.0	4.0
Non-terpene derivatives		98.5	0.0	7.8	8.1
Total identified		99.7	97.8	98.3	97.6

capitate trichome differs among species. Capitate trichomes can be divided into two types based on the dimension of stalk: long-stalked capitate trichomes of *P. albenensis* and *P. auricula* and short-stalked capitate trichomes of *P. farinosa* and *P. halleri*.

Summarising, the characters that, in different combinations, define each species are: (1) distribution of farinas and glandular trichomes (uniform distribution on both leaf surfaces, upper surface and leaf margins, lower surface), (2) colour of farinas (white or yellow), (3) size and shape of glandular trichomes stalk and head.

Epicuticular flavonoids

Flavonoid compounds isolated on leaf surfaces are flavones hydroxylated in positions 5, 7, 8 on the A ring and positions 2′, 4′, 5′ on the B ring.

Flavone, 2′-hydroxyflavone and 4′-hydroxyflavone are present in all the species analysed.

On the qualitative point of view, *P. halleri* shows the richest profile, presenting flavones with hydroxylation in different positions, both on A and B rings. *P. farinosa* is characterised by 3,5-dihydroxyflavone, a new compound. *P. albenensis* and *P. auricula* do not show any distinctive compound.

The compounds found in the present work are exclusively hydroxylated, while those isolated in the past by Valant-Vetschera et al. (2009) and Wollenweber (1974) are partly methoxylated. Compared to the compounds isolated in the present study, in the works above mentioned, hydroxylation in position 5 in the A ring and in position 2′ on the B ring was the most frequent. The differences may be due to the different geographical origin of the specimens considered, as evidenced by literature (Marin et al., 2003).

Tissue flavonoids

Tissue flavonoids are flavonol-3-*O*-glycosydes based upon the aglycones kaempferol, quercetin or isorhamnetin, or flavones

Table 4
Volatiles of *P. halleri* sampled by SPME.

Constituents	l.r.i.	*Primula halleri* leaves	*Primula halleri* calyxes	*Primula halleri* corollas
1-Undecene	1093	–	5.2	2.5
Phenylethyl alcohol	1112	4.4	–	–
n-Dodecane	1200	–	–	1.1
Decanal	1205	–	–	0.9
1-Decanol	1273	–	–	1.8
1-Tridecene	1293	–	–	5.9
2-Undecanone	1294	–	–	1.8
n-Tridecane	1300	–	–	4.7
1-Undecanol	1371	–	–	4.5
β-Elemene	1392	12.2	9.7	9.3
n-Tetradecane	1400	–	18.1	7.6
Methyl eugenol	1403	4.0	–	–
β-Caryophyllene	1419	9.1	–	0.9
β-Gurjunene	1432	1.9	3.2	–
β-Copaene	1433	1.3	–	–
trans-a-Bergamotene	1438	2.0	–	–
α-Guaiene	1441	1.3	–	0.6
Alloaromadendrene	1462	3.7	–	–
(E)-2-Dodecen-1-ol	1469	2.0	10.9	7.0
γ-Himachalene	1478	27.0	26.5	9.5
Germacrene D	1482	13.1	–	–
β-Selinene	1487	1.6	–	2.1
1-Pentadecene	1491	–	3.1	1.7
Valencene	1494	3.5	4.8	2.4
n-Pentadecane	1500	–	12.1	16.5
(E,E)-a-Farnesene	1506	6.8	–	–
7-*epi*-a-Selinene	1520	–	–	0.5
δ-Cadinene	1524	2.0	–	–
Kessane	1531	–	–	2.2
1-Tridecanol	1570	–	–	0.6
1-Hexadecene	1591	–	–	2.0
n-Hexadecane	1600	–	3.9	1.1
n-Heptadecane	1700	1.7	–	–
n-Octadecane	1800	–	–	1.1
Hexahydrofarnesylacetone	1848	–	–	3.8
Sesquiterpene hydrocarbons		85.5	44.2	25.3
Oxygenated sesquiterpenes		0.0	0.0	2.2
Apocarotenoids		0.0	0.0	3.8
Phenylpropanoids		4.0	0.0	0.0
Non-terpene derivatives		8.1	53.3	60.8
Total identified		97.6	97.5	92.1

showing a 3′ or 4′-B ring glycosylation. The sugars are glucose, galactose and rhamnose, both as mono- and trisaccharides.

As shown in Table 2, each species is characterised by a different chromatographic profile, since there are no shared compounds among the four species investigated. More precisely, we noticed a great chemical affinity among the isolated compounds in each species. Flavonol derivatives show indeed the same type of aglycones (kaempferol, quercetin and isorhamnetin). Moreover, the triglycosylated compounds often have in common the linkage position on the aglycone and their structure: e.g. Rha-Rha-Gal triglycoside is present in both *P. farinosa* and *P. halleri*, while a Glc-gentiobioside triglycoside can be found both in *P. auricula* and *P. halleri*.

Only a few studies have investigated the tissue flavonoid composition of *Primula* genus (see 'Introduction' section). An overview of these studies has pointed out that the tissue flavonoids, which characterize flowers and leaves of the species belonging to *Primula* genus, are mostly flavonols based upon aglycones mainly represented by kaempferol, quercetin and isorhamnetin. Sometimes also limocitrin and tamarixin have been characterised. Among flavones, apigenin and luteolin are the most represented structures. Tissue flavonoids are mono-, di- and triglycosides, while the most represented sugars are glucose and rhamnose, often linked to form gentiobiose, neohesperidose and sophorose, as well as mannose, galactose and xylose. The most observed derivatives have the sugar moiety linked to the A ring or in the position 3, although *P. spectabilis* shows glycosylation almost exclusively on position 7, or seldom C-glycosilation on positions 6 and 8.

Along with flavonols, flavone derivatives methoxylated, hydroxylated or glycosylated in position 7, 4′ can be found. B ring glycosylation is less frequent and, so far, was observed in *P. faberi* only. In this study, though, it was also observed in *P. albenensis* and *P. farinosa*, both in positions 3′ and 4′.

We have discussed these results from a chemical point of view only, since from the existing data and those we obtained it is still almost impossible to determine the taxonomic relationships existing among the species.

This is mainly because the data we have are little and were obtained, from heterogeneous sources: the plants part inspected are sometimes the leaf, sometimes the flower, or, in some cases, the aerial parts.

Volatiles

We investigated the volatile compounds spontaneously emitted by glandular hairs of fresh leaves of the four species and, in the case of *P. halleri*, the compounds emitted by the flower parts (corolla and calyx) compared with the corresponding leaves.

The headspace leaf analysis showed that most of the 45 identified compounds are sesquiterpene hydrocarbons, but there are also non-terpene derivatives.

In *P. albenensis* leaves, was found almost exclusively paeonal. In *P. auricula* leaves there were only sesquiterpenes. *P. farinosa* leaves contained also a small amount of monoterpene hydrocarbons (4.5%), which are missing in the other species (only traces in *P. albenensis*). The phenylpropanoid methyl eugenol (4%) was found only in *P. halleri*.

Concerning sesquiterpenes, β-caryophyllene, β-elemene and germacrene D were the main constituents shared by the *bouquet* of the species investigated.

Regarding leaf-flower comparison in *P. halleri*, 10 substances were identified in calyxes, 25 in corollas and 17 in leaves. Calyxes released non-terpene derivatives (53.3%) and sesquiterpene hydrocarbons (44.2%), whereas corollas emitted mostly non-terpene derivatives (60.8%), followed by sesquiterpenes (25.3%) and apocarotenoids (3.8%).

In *P. halleri*, corolla produces 25 compounds, of which 13 are exclusive to the corolla itself, 5 can be found both in corolla and calyx and 4 compounds, β-elemene, *(E)*-2-dodecen-1-ol, γ-himachalene and valencene, are present in all the three parts considered.

Experimental

Plant material

Aerial parts of *Primula* species were sampled from randomly selected individuals collected in the Alps in Lombardy (Italy) and have been determined according to Banfi and Ferlinghetti (1993) and Pignatti (1982). Ten specimens have been taken out of every species considered and five leaves have been examined for each entity. Voucher specimens were deposited at the Dipartimento di Bioscienze, Università degli Studi di Milano (Italy) (Table 5). All the leaves were collected at flowering time.

Morphological analysis

Specimens for LM were preserved in a mixture of 37% formaldehyde, glacial acetic acid, 50% ethanol (5:5:90 by volume). Pieces of leaves were then washed in distilled water and leaf sections of approximately 5 μm were cut using a razor blade; alternatively, leaves were peeled by microscope tweezers. Epidermal tissues and leaf sections were cleared using sodium hypochlorite 5%, washed in distilled water and mounted in a 90% solution of glycerine. Semi-permanent slides were examined and photographed with a LEICA DM light microscope.

For SEM morphological observations, leaves were air dehydrated and sputter-coated with gold. The samples were examined using a Cambridge Stereoscan 90 instrument.

Phytochemical analysis

Fresh leaves were rinsed with acetone in order to extract the epicuticular flavonoids. The extract was then chromatographed on a Sephadex LH-20 column, using MeOH-CH_3COCH_3 (9:1) as eluent for *P. auricula* e *P. farinosa* and EtOH as eluent for *P. albenensis* and *P. halleri*, to obtain fractions combined together according to TLC analyses [Silica 60 F_{254} gel-coated aluminium sheets; eluent: $C_6H_5CH_3$–$CH_3COOC_2H_5$–HCOOH (50:40:10)]. Some fractions were subjected to purification by flash column chromatography, using *n*-hexane–EtOAc at different ratio (Table 6). These techniques allowed the isolation of compounds **1–9**. For the identification, the NMR and Mass spectrums of the isolated flavonoids have been compared to literature data (Looker et al., 1984; Park et al., 2007; Wollenweber et al., 1988b; Yoon et al., 2011).

After removing farinas, the dried and powdered leaves of *Primula* species were defatted with *n*-hexane and successively extracted with CH_2Cl_2, CH_2Cl_2–MeOH (9:1) and MeOH, three times for each solvent. The MeOH 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 256–350 nm. The concentration of samples was 100 mg/ml and the injection volume was 100 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 extracts were separately chromatographed on a Sephadex LH-20 column, using MeOH or EtOH as eluent, to obtain fractions, combined together 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 (Naturstoffreagenz A-Polyethylenglycol) as spray 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 compounds **10–16**.

All the isolated compounds were submitted to NMR spectroscopic measurements with Bruker AC 400 (400 MHz) and Bruker Advance II 250 (250 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. ESI mass spectra were recorded on Thermo Fischer Finningan LCQ Advantage Mass Spectometer.

New compounds

*3,5-Dihydroxyflavone (**1**):* amorphous white solid; ^{1}H NMR (250 MHz, DMSO-*d6*): δ 6.65 (1H, d, *J* = 8.8 Hz, H-6), 7.09 (1H, dd, *J* = 8.8 Hz 8.8 Hz, H-7), 7.25 (1H, d, *J* = 8.8 Hz, H-8), 7.61 (2H, m, H-3′ H-4′ H-5′), 8.16 (2H, dd, *J* = 6.6.8 Hz 1.8 Hz, H-2′ H-6′), 12.53 (1H, s, OH-5); ^{13}C NMR (62.5 MHz, DMSO-*d6*): δ 105.5 (C-8), 110.1 (C-6), 110.7 (C-10), 126.7 (C-2′ C-6′), 129.2 (C-3′ C5′), 130.8 (C-1′), 132.3 (C-4′), 132.8 (C-7), 138.2 (C-3), 151.0 (C-9), 163.8 (C-2), 165.7 (C-5), 183.4 (C-4); ESI-MS: 254.0 [M +], 277.0 [M + Na+].

*3′-O-(β-galactopyranosyl)-2′-hydroxyflavone (**10**):* amorphous white solid; ^{1}H NMR (400 MHz, CD_3OD): δ 3.45 (1H, m, H-4″), 3.51 (1H, m, H-5″), 3.53 (1H, s, H-3″), 3.57 (1H, m, H-2″), 3.76 (1H, dd, *J* = 7.9 Hz 1.7 Hz, H-6″b), 3.95 (1H, dd, *J* = 7.9 Hz 1.0 Hz, H-6″a), 4.98 (1H, d, *J* = 7.9 Hz, H-1″), 6.83 (1H, s, H-3), 7.38 (1H, d, *J* = 7.9 Hz, H-4′), 7.51 (1H, ddd, *J* = 7.9 Hz 7.0 Hz 1.0 Hz, H-6), 7.58–7.53 (2H, m, H-5′ and H-6′), 7.73 (1H, dd, *J* = 8.5 Hz 1.0 Hz, H-8), 7.83 (1H, ddd, *J* = 8.5 Hz 7.0 Hz 1.7 Hz, H-7), 8.17 (1H, dd, *J* = 7.9 Hz 1.7 Hz, H-5); ^{13}C NMR (100 MHz, CD_3OD): δ 61.69

Table 5
Primula samples.

Species[a]	Locality of harvest	Herbarium No.
P. albenensis	Monte Alben (Bergamo 1650–1750 m)	P_B-101
P. auricula	Monte Alben (Bergamo 1650–1750 m)	P_A-101
P. farinosa	Passo del Tonale (Brescia – 1850 m)	P_R-101
P. halleri	Valle dell'Alpe (Sondrio – 2400 m)	P_K-101

[a] According to Banfi and Ferlinghetti; Pignatti.

Table 6
Separation of the epicuticular flavonoids.

Species	Crude extract	Sephadex elution	Number of fractions obtained	Fraction analyzed	Flash elution Hex/EtOAc	Ref. Num.	Weight (mg)
P. albenensis	Acetone	EtOH	11	Fraction 5	–	**7**	9
				Fraction 6	6:4	**6**	14
				Fraction 7	6:4	**2**	40
P. auricula	Acetone	MeOH-Acetone (9:1)	8	Fraction 1	–	**7**	10
				Fraction 2	7:3	**6**	14
				Fraction 3	7:3	**2**	160
P. farinosa	Acetone	MeOH-Acetone (9:1)	10	Fraction 4	–	**7**	12
				Fraction 5	7:3	**2**	218
						6	15
				Fraction 6	8:2	**3**	6
				Fraction 8	8:2	**1**	5
				Fraction 9	–	**9**	6
P. halleri	Acetone	EtOH	11	Fraction 2	–	**7**	10
				Fraction 3	2:3	**2**	68.5
						6	12
				Fraction 6	2:3	**3**	10
						8	11.5
				Fraction 8	1:1	**4**	16
						5	115

(C-6″), 70.56 (C-4″), 74.05 (C-2″), 76.83 (C-3″), 77.75 (C-5″), 102.5 (C-1″), 106.1 (C-3), 114.3 (C-6′), 117.3 (C-4′), 118.7 (C-8), 119.2 (C-5′), 124.7 (C-10), 125.4 (C-5), 126.0 (C-6), 126.7 (C-1′), 134.9 (C-7), 148.0 (C-2′), 149.4 (C-3′), 156.7 (C-9), 165.2 (C-2), 179.8 (C-4); ESI-MS: ESI-MS: 439.2 [M+Na$^+$], 855.0 [2M+Na$^+$].

*Isorhamnetin 3-O-α-rhamnopyranosyl-(1→3)-O-[α-rhamnopyranosyl-(1→6)]-O-β-galattopyranoside (**13**):* yellowish solid; ^{1}H NMR (250 MHz, DMSO-d_6): δ 0.98 (3H, d, 6.1 Hz, H-6‴′), δ 1.05 (3H, d, 6.0 Hz, H-6‴), 3.13 (1H, m, H-4‴), 3.15 (1H, m, H-4‴′), 3.23 (1H, m, H-6b″), 3.31 (1H, m, H-2‴), 3.33 (1H, m, H-5‴), 3.35 (1H, m, H-6″a), 3.43 (1H, m, H-3‴), 3.52 (1H, m, H-3‴′), 3.54 (1H, m, H-5″), 3.58 (1H, m, H-4″), 3.61 (1H, m, H-2″), 3.63 (1H, m, H-5‴′), 3.79 (3H, s, -OCH$_3$), 3.81 (1H, m, H-2‴′), 3.84 (1H, m, H-3″), 4.41 (1H, brs, H-1‴), 4.72 (1H, brs, H-1‴′), 5.25 (1H, d, J = 7.1 Hz, H-1″), 6.07 (1H, d, J = 1.9 Hz, H-6), 6.34 (1H, d, J = 1.9 Hz, H-8), 7.92 (1H, brs, H-2′), 7.39 (1H, brd, J = 8.8 Hz, H-6′), 6.80 (1H, d, J = 2.0 Hz, H-5′); ^{13}C NMR (62.5 MHz, DMSO-d_6): δ 17.7 (C-6‴′), 17.8 (C-6‴), 55.8 (-OCH$_3$) 65.1 (C-6″), 67.9 (C-4″), 68.3 (C-5‴), 68.5 (C-5‴′), 70.0 (C-2‴′), 70.5 (C-3‴′ and C-2‴), 71.0 (C-3‴), 72.1 (C-4‴′ and C-4‴) 73.2 (C-2″), 73.3 (C-3″), 75.0 (C-5″), 95.6 (C-8), 99.3 (C-6), 100.1 (C-1‴), 100.8 (C-1″), 102.4.2 (C-1‴′), 103.2 (C-10), 113.2 (C-2′), 115.2 (C-5′), 120.7 (C-1′), 121.6 (C-6′), 132.4 (C-3), 138.9 (C-3′), 147.1 (C-4′), 152.1 (C-2), 153.7 (C-9), 157.4 (C-5), 160.6 (C-7), 174.6 (C-4); ESI-MS: 770.2 [M$^+$], 793.2 [M+Na$^+$].

*Quercetin 3-O-α-rhamnopyranosyl-(1→3)-O-[α-rhamnopyranosyl-(1→6)]-O-β-galattopyranoside (**14**):* yellowish solid; ^{1}H-NMR (250 MHz, DMSO-d_6): δ 0.98 (3H, d, 5.8 Hz, H-6‴′), δ 1.07 (3H, d, 6.1 Hz, H-6‴), 3.12 (1H, m, H-4‴), 3.15 (1H, m, H-4‴′), 3.21 (1H, m, H-6b″), 3.32 (1H, m, H-2‴), 3.34 (1H, m, H-5‴), 3.35 (1H, m, H-6″a), 3.43 (1H, m, H-3‴), 3.51 (1H, m, H-3‴′), 3.56 (1H, m, H-5″), 3.58 (1H, m, H-4″), 3.62 (1H, m, H-2″), 3.63 (1H, m, H-5‴′), 3.80 (1H, m, H-2‴′), 3.83 (1H, m, H-3″), 4.40 (1H, brs, H-1‴), 4.70 (1H, brs, H-1‴′), 5.16 (1H, d, J = 7.2 Hz, H-1″), 6.05 (1H, d, J = 1.9 Hz, H-6), 6.36 (1H, d, J = 1.9 Hz, H-8), 6.71 (1H, d, J = 8.5 Hz, H-5′), 7.46 (1H, d, J = 2.0 Hz, H-2′), 7.59 (1H, d, J = 8.5 Hz 2.0 Hz, H-6′); ^{13}C NMR (62.5 MHz, DMSO-d_6): δ 17.7 (C-6‴′), 17.9 (C-6‴), 65.3 (C-6″), 68.1 (C-4″), 68.3 (C-5‴), 68.6 (C-5‴′), 70.0 (C-2‴′), 70.5 (C-3‴′ and C-2‴), 71.0 (C-3‴), 72.2 (C-4‴′, C-4‴ and C-2″), 73.3 (C-3″), 74.9 (C-5″), 94.6 (C-8), 99.3 (C-6), 100.1 (C-1‴), 100.6 (C-1″), 102.4 (C-1‴′), 103.2 (C-10), 115.1 (C-5′), 115.3 (C-2′), 120.3 (C-1′), 121.9 (C-6′), 133.1 (C-3), 145.2 (C-3′), 149.9 (C-4′), 150.0 (C-2), 155.4 (C-9), 156.9 (C-5), 160.8 (C-7), 175.9 (C-4); ESI-MS: 756.2 [M$^+$], 779.2 [M+Na$^+$].

Volatile compounds

The solid phase microextraction (SPME) was carried out by Supelco SPME devices coated with polydimethylsiloxane (PDMS, 100 μm), used for sampling the headspace of *Primula* aerial parts placed into a 100 ml glass septum vial and allowed to equilibrate for 30 min. After the equilibration time, the fiber was exposed to the headspace for 30 min at room temperature. At the end of sampling, the fiber was withdrawn into the needle and transferred to the injection port of the GC or GC–MS system, operating as follows.

The GC analyses were accomplished with a HP-5890 Series II instrument equipped with HP-WAX and HP-5 capillary columns (30 m × 0.25 mm, 0.25 μm film thickness), and with the following conditions: temperature program 60 °C for 10 min, followed by an increase of 5 °C/min to 220 °C; injector and detector temperatures at 250 °C; carrier gas helium (2 ml/min); splitless injection; detector dual FID.

For both the columns, identification of the chemicals was performed by comparison of their retention times with those of pure authentic samples and by means of their Linear Retention Indices (LRI) relative to the series of *n*-hydrocarbons.

GC-EIMS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a HP-5 ms capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. The analytical conditions were the following: injector and transfer line temperatures 250 and 240 °C respectively; oven temperature from 60 to 240 °C at 3 °C/min; carrier gas helium at 1 ml/min; splitless injection. Identification of the constituents was based on comparison of their retention times with those of authentic samples, comparing their Linear Retention Indices relative to the series of *n*-hydrocarbons, and on computer matching against commercial mass spectra (NIST 98 and ADAMS 95) and those of our library, built up from pure substances and components of known essential oils and MS literature data [346 (Stenhagen et al., 1974); 347 (Massada, 1976); 348 (Jennings and Shibamoto, 1980); 364 (Swigar and Silverstein, 1981); 349 (Davies, 1990); 350 (Adams, 1995)]. Moreover, the molecular weights of all the identified substances were confirmed by GC–CIMS, using MeOH as CI ionizing gas.

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