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Flavonoid diversification in different leaf compartments of *Primula auricula* (Primulaceae)

Birgit Holzbach [a], Viktor Reuter [a], Markus Bacher [b], Johann Schinnerl [a], Lothar Brecker [c], Thomas Rosenau [b,d], Karin Valant-Vetschera [a,*]

[a] Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030, Vienna, Austria
[b] Division of Chemistry of Renewable Resources, University of Natural Resources and Life Sciences Vienna (BOKU), Konrad Lorenz-Straße 24/I 3430, Tulln an der Donau, Austria
[c] Department of Organic Chemistry, University of Vienna, Währinger Strasse 38, A-1090, Vienna, Austria
[d] Johan Gadolin Process Chemistry Centre, Åbo Akademi University, Porthansgatan 3, Åbo, Turku, FI-20500, Finland

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ABSTRACT

Populations of *Primula auricula* L. subsp. *auricula* from Austrian Alps were studied for flavonoid composition of both farinose exudates and tissue of leaves. The leaf exudate yielded *Primula*-type flavones, such as unsubstituted flavone and its derivatives, while tissue flavonoids largely consisted of flavonol 3-*O*-glycosides, based upon kaempferol (**3**, **4**) and isorhamnetin (**5**–**7**). Kaempferol 3-*O*-(2″-*O*-β-xylopyranosyl-[6″-*O*-β-xylopyranosyl]-β-glucopyranoside) (**3**) and isorhamnetin 3-*O*-(2″-*O*-β-xylopyranosyl-[6″-*O*-β-xylopyranosyl]-β-glucopyranoside) (**6**) are newly reported as natural compounds. Remarkably, two *Primula* type flavones were also detected in tissues, namely 3′-hydroxyflavone 3′-*O*-β-glucoside (**1**) and 3′,4′-dihydroxyflavone 4′-*O*-β-glucoside (**2**), of which (**1**) is reported here for the first time as natural product. All structures were unambiguously identified by NMR and MS data. Earlier reports on the occurrence of 7,2′-dihydroxyflavone 7-*O*-glucoside (macrophylloside) in this species could not be confirmed. This structure was now shown to correspond to 3′,4′-dihydroxyflavone 4′-*O*-glucoside (**2**) by comparison of NMR data. Observed exudate variations might be specific for geographically separated populations. The structural diversification between tissue and exudate flavonoids is assumed to be indicative for different ecological roles *in planta*.

1. Introduction

The genus *Primula* L. is the largest genus in the family Primulaceae, and it is divided into seven subgenera and 38 sections. In general, geographic distribution of *Primula* species is concentrated in the Northern Hemisphere, with the vast majority of the 430 reported *Primula* species occurring in the mountainous Sino-Himalayan region. In Europe, on the contrary, only 34 species are distributed mostly in alpine habitats (Mast et al., 2001; Richards, 2002).

A characteristic feature of many *Primula* species is an either mealy excretion, referred to as farina, or an oily excretion, both being products of glandular hairs. Particularly the mealy excretion attracted interest, as outside *Primula* this feature is known from some ferns only (Wollenweber, 1978). In both oily and mealy excretions, flavonoids constitute the major compounds (Wollenweber and Schnepf, 1970). In addition to unsubstituted flavone, a series of unusually substituted flavone derivatives, often lacking the 5- and 7-hydroxyl groups, have been detected in the majority of species studied so far (e.g. Colombo et al., 2017; Valant-Vetschera et al., 2009). It has been postulated that these compounds originate from a yet unknown biosynthetic pathway, and hence these were termed *Primula*–type flavones (Valant-Vetschera et al., 2009). It should be mentioned that flavonoids from the "classical" biosynthetic pathway are found in exudates of more recent evolutionary lineages of the closely related genus *Dionysia*, and of some European *Primula* species (Valant-Vetschera et al., 2010; Elser et al., 2016).

A striking difference is observed between exudate flavonoid structures and tissue flavonoid glycosides even within one species (Bhutia et al., 2012; Colombo et al., 2017). In leaf tissue, flavonol 3-*O*-glycosides based on kaempferol, quercetin, and isorhamnetin predominate, while flavone-*O*-glycosides and *C*-glycosylflavonoids are less frequently reported. Structurally identical flavonol-3-*O*-glycosides may also be present in petals (Colombo et al., 2017; Harborne and Sherratt, 1961;

* Corresponding author.
E-mail address: karin.vetschera@univie.ac.at (K. Valant-Vetschera).

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Hashimoto et al., 2015). Sugars involved in *O*-glycosylation of flavonols exhibit a higher structural complexity in comparison to flavones (Colombo et al., 2017). Mostly, glycosidic flavonols derive from the classical biosynthetic pathway and constitute the majority of tissue glycosides, while some of the rare glycosidic flavones correlate to exudate flavone structures (Colombo et al., 2014; Harborne 1968).

In continuation of our studies on *Primula* flavonoids, we aimed at analysing accessions of *P. auricula* L. subsp. *auricula* (subgen. *Auriculastrum* Schott; sect. *Auricula*) more deeply, regarding flavonoid composition of different compartments. The patchy distribution pattern from western Alps to E- and S-Europe in alpine sites, along with variable characteristics, indicate *P. auricula* s.l. to be a glacial relic (Richards, 2002). Phylogenetic and evolutionary aspects of sect. *Auricula* were discussed in phylogenetic studies (Boucher et al., 2016; Zhang and Kadereit, 2004). Infraspecific taxonomy relies on the presence or absence of farina, among other characters, and thus some variation of exudate flavonoid composition is to be expected. Previous studies indicated the presence of various *Primula*-type flavones in exudates (Colombo et al., 2014; Elser et al., 2016), and the occurrence of structurally different glycosides from leaf tissue of one Italian collection (Colombo et al., 2014; Fico et al., 2007). We report on both exudate and tissue flavonoid composition of *P. auricula* accessions from Austria (Fig. 1). Chemical diversification of flavonoids of different compartments studied is discussed in view of structural and biosynthetic differences, and ecological and evolutionary significance is briefly addressed.

2. Results and discussion

Analyses of exudates produced by glandular hairs, and of flavonoid glycosides present in tissues, were done separately by comparative HPLC/UV-DAD profiling (see chapter 3, Experimental section). Different accessions from Austria were studied here for the first time. The tissue glycoside composition appeared to be rather complex, with low chromatographic resolution and inherent difficulties in separation. The compound peaks in the exudate profiles were much better separated, thus facilitating identification by HPLC-data comparison with authentic markers.

2.1. Tissue glycosides

Preparative separation of air-dried leaf tissue extracts of *P. auricula* yielded the flavone *O*-glycosides **1**, **2** and the flavonol 3-*O*-glycosides **3**–**7**, of which compounds **1**, **3**, and **6** are described for the first time as natural products (Figs. 2 and 3). Compounds **1**, **2**, and **5** were isolated and purified, whereas compounds in the pairs **3** and **6** as well as **4** and **7** could not be separated from each other since their chromatographic properties were quite similar. Nevertheless, it was possible to unambiguously determine the structures also of these four compounds from the respective two-component mixtures. From all samples, 1D and 2D NMR spectra as well as mass spectra were recorded. Structure elucidation showed that glycosides **4**, **5** and **7** are known already: these compounds were previously isolated from other natural sources including *Primula* species. The structures determined for these known compounds were verified additionally by comparisons with earlier original data (for references see chapter 2.2) as well as by data from ^{13}C NMR databases (CSEARCH, 2014; NMRShiftDB, 2010). The molecular structure of flavone **2**, which had been earlier described as well (Colombo et al., 2014; Peng et al., 2012; Zhang, 1993), was, however, examined more closely and compared with compounds possessing very similar ^{13}C NMR data (Ahmad et al., 1991; Fico et al., 2007). Furthermore, the newly described compounds **1**, **3** and **6** are now each identified unambiguously, including verification using ^{13}C NMR databases (CSEARCH, 2014; NMRShiftDB, 2010), and are now described in detail.

Fig. 1. *Primula auricula* L. subsp. *auricula*.

The flavone glycosides found comprise 3′-hydroxyflavone 3′-*O*-β-glucoside (**1**) and 3′,4′-dihydroxyflavone 4′-*O*-β-glucoside (**2**), respectively. Flavonol glycosides identified are based upon kaempferol and isorhamnetin, with disaccharide or trisaccharide moieties attached, thus contrasting to flavones which bear only glucose. Quercetin derivatives are also expected to be contained, based on their HPLC-UV-spectra (Fico et al., 2007), but more bulk material would be needed for successful separation. The aglycones of **1** (3′-hydroxyflavone) and of **2** (3′,4′-dihydroxyflavone) have recently also been found in exudates of several *Primula* species (Reuter, 2020).

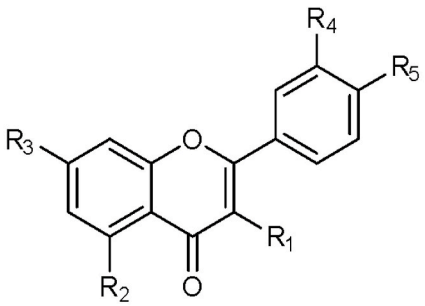
2.2. Structure elucidation

The glycoside **1** shows a molecular ion $[M+Na]^+$ of $m/z = 423.1042$, which has been determined using HR-TOF-ESI-MS. This agrees quite well with the calculated $m/z = 423.1050$ for $[M+Na]^+$ and has an absolute mass deviation of 1.89 ppm for the molecular formula $C_{21}H_{20}O_8$. A flavone skeleton can be identified as central structure. Its A-ring does not carry any substituent, which is indicated by the ^{1}H NMR signals of four protons in positions 5 to 8, which couple with each other via 3*J*H-H couplings. The annulated C-ring contains a keto function in position 4 as well as a double bond between C-2 and C-3. In position 3 there is no substituent attached and the corresponding proton H-3 shows a chemical shift of 6.95 ppm. The coupling pattern of the remaining aromatic B ring protons reveals a substituent in position 3'. H-2′ and H-6′ were identified due to long range correlations to C-2 in the HMBC spectrum. H-2′ shows only $^4J_{H\text{-}H}$ couplings whereas H-6′ showed in COSY and TOCSY spectra a $^3J_{H\text{-}H}$ coupling to H-5′ which further coupled to H-4'. The low-field shift of C-3', and a $^3J_{C\text{-}H}$ coupling of this carbon atom to an anomeric proton in HMBC show that an *O*-glycosyl group is bound at this position.

All methine protons in positions 1″ to 5″ of this glycosyl group show 3*J*H-H coupling constants in a range of 7.1–8.9 Hz. Furthermore, an additional methylene group in position 6″ of this spin system indicates the presence of an aldohexose. NOEs between H-1″, H-3″ and H-5″ as well as between H-2″ and H-4″ can been seen in the NOESY spectrum and indicate a β-*gluco*-configuration. The absolute configuration of this glucosyl, as well as of all other glycosyl residues in compounds **2** to **7** were not determined. For analogy reasons we have used the d-configuration for glucosyl and xylosyl moieties as well as the l-configuration for rhamnosyl moieties in Fig. 3.

The resulting structure was therefore identified as 3′-hydroxyflavone 3′-*O*-β-glucoside (**1**). This glycoside is new for *Primula* and has not been reported from any other plant species. The chemical structure of (**1**) is shown in Fig. 3, and NMR spectroscopic data are listed in Tables 1 and 2. NMR and MS spectra are shown in Figs. S1-S7 and S37.

The glycoside **2** shows a molecular ion $[M+Na]^+$ of $m/z = 439.1007$, which agrees well with the calculated $m/z = 439.1000$ for $[M+Na]^+$ (absolute mass deviation: 1.59 ppm) of the molecular formula $C_{21}H_{20}O_9$.



Comp.	Aglycone	R_1	R_2	R_3	R_4	R_5
1	3′-Hydroxyflavone	H	H	H	β-Glc*p*-(1→*O*	H
2	3′,4′-Dihydroxyflavone	H	H	H	OH	β-Glc*p*-(1→*O*
3	Kaempferol	β-Xyl*p*-(1→6)-[β-Xyl*p*-(1→2)]-β-Glc*p*-(1→*O*	OH	OH	H	OH
4	Kaempferol	β-Xyl*p*-(1→6)-[α-Rha*p*-(1→2)]-β-Glc*p*-(1→*O*	OH	OH	H	OH
5	Isorhamnetin	β-Xyl*p*-(1→6)-β-Glc*p*-(1→*O*	OH	OH	OMe	OH
6	Isorhamnetin	β-Xyl*p*-(1→6)-[β-Xyl*p*-(1→2)]-β-Glc*p*-(1→*O*	OH	OH	OMe	OH
7	Isorhamnetin	β-Xyl*p*-(1→6)-[α-Rha*p*-(1→2)]-β-Glc*p*-(1→*O*	OH	OH	OMe	OH

Fig. 2. Flavone and flavonol glycosides isolated from rinsed leaf tissue of *P. auricula* accessions.

Compound **2** comprises the same basic flavone structure as found for compound **1**. On the one hand this is again clearly indicated by the four aromatic protons of ring A at positions 5 to 8, which possess a series of 3*J*H-H coupling with each other. Their 2JC-H and 3JC-H couplings to the quaternary carbon atoms in positions 4a and 8a complete the assignment of the aromatic ring A. 13C NMR chemical shifts of these two nuclei indicate an oxygen to be bound to C-8a and a keto function bound to C-4a. A further 3JC-H coupling from H-5 to the keto function in position 4 shows the C-ring to be annulated here. The ^{1}H NMR signal of proton H-3 in this ring C indicates position 3 to be unsubstituted. This proton H-3 shows in HMBC further 2JC-H couplings to C-4 and C-2, respectively. The low field shifts of C-2 and C-3 (165.5 ppm and 108.0 ppm) denote a polarized C=C double bond between these two nuclei. It carries on C-2 the oxygen (pos. 1) which closes ring C to C-8a.

Proton H-3 has another 3JC-H coupling to C-1′ in ring B, also connected to C-2. In contrast to compound **1**, however, ring B carries two substituents. Two $^{3}J_{C\text{-}H}$ couplings (HMBC) from C-2 to H-2′ and H-6′ as well as a further $^{3}J_{H\text{-}H}$ coupling (COSY) from H-6′ show the protons to be located in positions 2′, 5′and 6′. The two low-field shifted 13C NMR resonances of C-3‘, and C-4‘, indicate oxygen atoms to be bound in these positions. No further $^{3}J_{C\text{-}H}$ coupling of C-3’ indicates a hydroxy group to be present in position 3’. However, C-4′ shows a $^{3}J_{C\text{-}H}$ coupling to the anomeric proton of a glycosyl moiety which again was identified as β-glucosyl moiety by the 3*J*H-H coupling constants extracted from the 1H NMR spectrum ranging from 7.1 to 8.9 Hz as well as by NOEs between H-1″, H-3″ and H-5″ as well as between H-2″ and H-4‘’. The resulting structure was hence identified as 3′,4′-dihydroxyflavone 4′-*O*-β-glucoside (**2**). The chemical structure is shown in Fig. 3, and the NMR spectroscopic data are listed in Tables 1 and 2 NMR and MS spectra are displayed in Figs. S8–S14 and S38.

Previously, compound **2** was reported from leaves of *P. albenensis* Banfi & Ferl. (Colombo et al., 2014), a species being distantly related to *P. auricula,* from the more remote *P. faberi* Oliver (subgen. *Auriculastrum,* sect. *Amethystina;* Zhang, 1993), and from the unrelated *P. sikkimensis* Hook. (subgen. *Aleuritia*, sect. *Sikkimensis*; Peng et al., 2012). The analytical data reported for these compounds are in good agreement with the data reported here for **2**. Thus, this compound is probably more widespread in different taxonomic groups of *Primula* as already suggested by Harborne (1968), who detected the corresponding aglycone in several hydrolyzed leaf extracts. These findings are confirmed by current analyses (Reuter, 2020).

We also checked our samples for the presence 7,2′-dihydroxyflavone-7-*O*-β-glucopyranoside (macrophylloside), reported from *P. auricula* leaves collected at an Italian alpine site (Fico et al., 2007). This compound had been identified by comparison of NMR-data with an earlier publication on *P. macrophylla* D. Don (Ahmad et al., 1991). We were unable to detect this glycoside, neither directly in extracts, nor its aglycone in hydrolysates, by comparison with a commercial sample of 7, 2′-dihydroxyflavone.

However, there is a striking similarity between the NMR spectroscopic data that Ahmad et al. (1991) assigned to 7,2′-dihydroxyflavone-7-*O*-β-glucopyranoside (macrophylloside) with those we obtained for 3′,4′-dihydroxyflavone 4′-*O*-β-glucoside (**2**). A prediction of ^{13}C NMR spectroscopic data performed by a^{13}C NMR database (NMRShiftDB, 2010) for the structure of compound **2** fits quite well with the data measured for both compounds. However, an analogous prediction for the structure of macrophylloside differs significantly from the data reported for macrophylloside (Fig. S15). It is therefore most likely that the earlier structural assignment of macrophylloside needs to be revised and changed to compound **2**. This is further supported by the fact that 7,2′-substitution appears somewhat odd in view of substitution patterns observed in *Primula* flavones so far. Also, no comparable structure (7,2′-dihydroxyflavone-7-*O*-β-glucopyranoside or its aglycone) has been observed as a natural product, to our knowledge. The compound isolated by Ahmad et al. (1991) is thus very likely 3′,4′-dihydroxyflavone 4′-*O*-β-glucoside (**2**), in agreement with the previous and current spectroscopic data.

Glycoside **3**, isolated as a mixture with **6**, shows in HR-TOF-ESI-MS a molecular ion $[M+Na]^+$ of m/z = 735.1739 corresponding to the calculated m/z 735.1741 for $[M+Na]^+$ (absolute mass deviation: 0.27 ppm) of the molecular formula $C_{31}H_{36}O_{19}$. Compound **3** has a flavone structure carrying hydroxyl groups in the A-ring in positions 5 and 7,

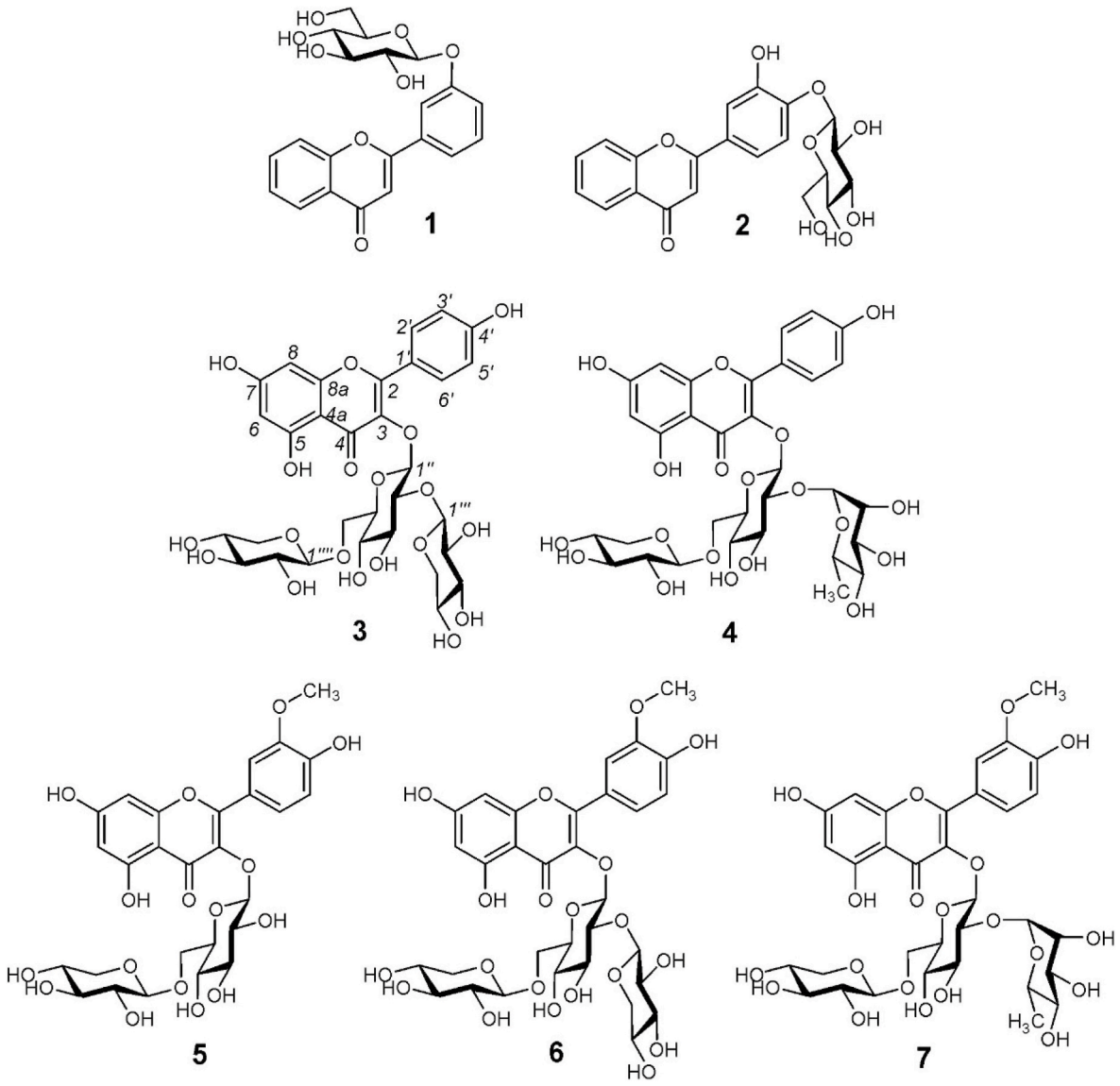


Fig. 3. Structures of flavone glycosides and flavonol glycosides isolated from rinsed leaf tissue of *P. auricula* accessions. For compound **3** the numbering used in Tables 1 and 2 is shown exemplarily.

which are indicated by a low-field shift of the 13C NMR signals to 158.5 ppm and 166.3 ppm respectively. Furthermore, the two 1H NMR resonances of H-6 and H-8, showing a typical 4*J*H-H coupling (2.1 Hz), support this substitution pattern. On the B-ring, only one hydroxyl group is present in position 4′, which is supported by the corresponding NMR shifts for protons and carbons in positions 3′ and 5′ as well as in positions 4′ and 6', respectively. In the C-ring, position 4 is oxidized to a ketone which forms a hydrogen bond to the proton of the hydroxyl group in position 5. Additionally, there is a double bond between C-2 and the quaternary C-3, where the carbohydrate moiety is bound.

The TOCSY spectrum of this carbohydrate unit shows a strong coupling between all seven carbon bound protons, which have shifts in the spectral region between 5.63 ppm (H-1″) and 3.34 ppm (H-4″). The spectrum indicates all 3*J*H-H couplings from H-1″ to H-5″ to be axial/axial, ranging from 7.2 to 8.9 Hz, and a CH2-O-R group in position 6 to be present. Furthermore, comparable to glucosyl units in **1** and **2**, signals of protons H-1″, H-3″ and H-5″ as well as the protons H-2″ and H-4″ in the NOESY spectrum indicate 1,3-diaxial couplings. All these data are strong evidence of a β-glucopyranosyl moiety. Its proton and carbon NMR chemical shifts and the observed HMBC long-range correlations indicate that additional glycosyl moieties are bound at positions 2″ and 6''.

The 3JC-H couplings in HMBC as well the NOEs from these two positions allow identification of the anomeric carbons and protons of these terminal glycosyl residues. Both glycosyls show sets of very similar NMR spectroscopic shifts, which indicate them to be the same pentapyranosyl moieties. Each of these moieties show strong 3*J*H-H coupling between protons of three methine and one methylene group in the TOCSY spectrum. Additionally, all carbon and proton chemical shifts of these spin systems indicate each carbon to be bound to an oxygen atom. NOEs can be detected between H-1, H-3 and H-5ax as well as between H-2 and H-4 of these moieties. All these findings indicate both pentapyranosyl groups to be β-xylopyranosyl moieties.

The resulting structure can hence be identified as kaempferol 3-*O*-(2″-*O*-β-xylopyranosyl-[6″-*O*-β-xylopyranosyl]-β-glucopyranoside) (**3**). The compound has not yet been reported; its chemical structure is shown in Fig. 3. All NMR spectroscopic data are listed in Tables 1 and 2, and the corresponding NMR and MS spectra are shown in Figs. S16-S22 and S39.

The glycoside **4**, isolated in mixture with **7**, has a structure very similar to that of compound **3**. The molecular ion $[M+Na]^+$ of m/z = 749.1912 correlates with the calculated m/z 749.1898 for $[M+Na]^+$ (absolute mass deviation: 1.86 ppm) of the molecular formula

Table 1
^{1}H NMR spectroscopic data of compounds **1**–**7**.

Position	1	2	3	4	5	6	7
Aglycone							
3	6.95; s; 1H	6.82; s; 1H					
5	8.17; dd; 7.9, 1.5; 1H	8.15; dd; 8.0, 1.6; 1H					
6	7.51; m; 1H	7.50; ddd; 8.0, 7.1, 1.0; 1H	6.40; d; 2.1; 1H	6.40; d; 2.1; 1H	6.41; s; 1H	6.41; d; 2.1; 1H	6.41; d; 2.1; 1H
7	7.84; ddd; 8.5, 7.5, 1.6; 1H	7.82; ddd; 8.2, 7.1, 1.6; 1H					
8	7.78; d; 8.5; 1H	7.72; d; 8.2; 1H	6.18; d; 2.1; 1H	6.19; d; 2.1; 1H	6.19; s; 1H	6.19; d; 2.1; 1H	6.19; d; 2.1; 1H
2′	7.82; br.s; 1H	7.53; d; 2.3; 1H	8.10; d; 9.0; 1H	8.09; d; 8.9; 1H	8.02; d; 1.5; 1H	7.93; d; 2.1; 1H	8.03; d; 2.1; 1H
3′			6.89; d; 9.0; 1H	6.89; d; 8.9; 1H			
4′	7.35; dd; 8.1, 2.0; 1H						
5′	7.51; m; 1H	7.35; d; 8.5; 1H	6.89; d; 9.0; 1H	6.89; d; 8.9; 1H	6.91; d; 8.2; 1H	6.92; d; 8.5; 1H	6.92; d; 8.4; 1H
6′	7.74; d; 8.0; 1H	7.54; dd; 8.5, 2.3; 1H	8.10; d; 9.0; 1H	8.09; d; 8.9; 1H	7.64; dd; 8.2, 1.5; 1H	7.68; dd; 8.5; 2.1; 1H	7.59; d; 8.4, 2.1; 1H
O-CH_3					3.96; s; 3H	3.97; s; 3H	3.98; s; 3H
	3′-Glc	4′-Glc	3-Glc	3-Glc	3-Glc	3-Glc	3-Glc
1″	5.01; d; 7.2; 1H	4.95; d; 7.6; 1H	5,42; d; 7.6; 1H	5.63; d; 7.7; 1H	5.34; d; 7.5; 1H	5.53; d; 7.7; 1H	5.78; d; 7.6; 1H
2″	3.52; m; 1H	3.56; dd; 7.6, 9,3; 1H	3.70; dd; 9.0; 7.6; 1H	3.62; m; 1H	3.49; m; 1H	3.74; dd; 9.0; 7.7; 1H	3.65; m; 1H
3″	3.51; m; 1H	3.51; dd; 9.3; 8.8, 1H	3.64; m; 1H	3,56; m; 1H	3.46; m; 1H	3.64; m; 1H	3,59; m; 1H
4″	3.39; t; 9.0; 1H	3.43; dd; 9.5; 8.8; 1H	3.36; m; 1H	3.34; m, 1H	3.37; m; 1H	3.35; m; 1H	3.34; m, 1H
5″	3.54; m; 1H	3.50; m; 1H	3.43; m; 1H	3.44; m; 1H	3.40; m; 1H	3.42; m; 1H	3.44; m; 1H
6a''	3.96; dd; 12.0, 2.1; 1H	3.93; dd; 12.2; 2.3; 1H	3.89; dd; 11.9; 1.8; 1H	3.91; dd; 11.9; 1.9; 1H	3.94; br.d; 11.9; 1H	3.93; dd; 2.0; 11.9; 1H	3.59; dd; 11.8; 1.7; 1H
6b''	3.72; dd; 12.0, 6.2; 1H	3.74; dd; 12.2; 5.7; 1H	3.56; m; 1H	3.59; m; 1H	3.63; dd; 11.9, 5.5; 1H	3.59; m; 1H	3.34; m; 1H
			2″-Xyl	2″-Rha		2″-Xyl	2″-Rha
1‴			4.80; d; 1H	5.22; d; 1.5; 1H		4.85; m; 1H	5.18; d; 1.3; 1H
2‴			3.39; m; 1H	4.01; m; 1H		3.39; m; 1H	4.01; m; 1H
3‴			3.40; m; 1H	3.78; m; 1H		3.38; m; 1H	3.78; m; 1H
4‴			3.51; m; 1H	3.34; m; 1H		3.49, m; 1H	3.34; m; 1H
5a'‴			3.97; m; 1H	4.07; m; 1H		3.97; dd; 11.6; 2.8; 1H	4.05; dq; 9.1, 6.3; 1H
5b'‴			3.25; dd; 9.9; 7.6; 1H			3.23; dd; 11.6; 8.8; 1H	
6‴				0.99; d; 6.3; 3H			0.91; d; 6.3; 3H
			6″-Xyl	6″-Xyl	6″-Xyl	6″-Xyl	6″-Xyl
1⁗			4.07; d; 7.4; 1H	4.08; d; 7.3; 1H	4.09; d; 7.4; 1H	4.08; d; 7.4; 1H	4.10; d; 7.3; 1H
2⁗			3.01; dd; 8.9; 7.4; 1H	3.00; dd; 8.8, 7.3; 1H	3.04; dd; 8.7, 7.4; 1H	3.01; dd; 8.9; 7.4; 1H	3.01; dd; 8.9, 7.3; 1H
3⁗			3.09; dd; 8.9; 8.9; 1H	3.10; dd; 8.8, 8.8; 1H	3.11; dd; 8.7, 8.7; 1H	3.09; dd; 8.9; 8:9; 1H	3.10; dd; 8.9, 8.9; 1H
4⁗			3.36; m; 1H	3.34, m; 1H	3.37; m; 1H	3.35; m; 1H	3.34, m; 1H
5a'⁗			3.67; m; 1H	3.65; m; 1H	3.65; dd; 11.5, 5.5; 1H	3.64; m; 1H	3.61; m; 1H
5b'⁗			2.93; dd; 11.4, 10.0; 1H	2.86; dd; 11.5, 10.1; 1H	2.89; t; 10.9; 1H	2.88; dd; 11.5, 10.1; 1H	2.86; dd; 11.4, 10.3, 1H

For numbering of positions see Fig. 3, compound 3. Spectra of compounds 1 and 3–7 were measured at 600 MHz; spectra of compound 2 were measured at 700 MHz.

$C_{32}H_{38}O_{19}$. The kaempferol moiety as well as the β-glucopyranosyl and the β-xylopyranosyl moieties bound at position 6“, as described for **3**, are also present in compound **4**. The difference to **3** is located in the sugar unit located at position 2″ of the β-glucosyl group. This sugar unit is a hexopyranose, which does not carry a hydroxyl group in its position 6‴. Furthermore, the H-1‴ shows a $^3J_{H\text{-}H}$ coupling constant of 1.5 Hz. The chemical shifts of all its ^{1}H and ^{13}C nuclei indicate that this sugar unit is a terminal α-rhamnopyranosyl moiety, particularly supported by the detection of the 6‴ methyl group. This fits well with the molecular formula of **4** comprising an additional CH_2 unit compared to that of **3**.

The resulting structure is kaempferol 3-*O*-(2″-*O*-α-rhamnopyranosyl-[6″-*O*-β-xylopyranosyl]-β-glucopyranoside) (Fig. 3), which has earlier been described from *P. hirsuta* All. leaves by Fico et al. (2007). It was furthermore reported to be a natural product in *P. maximowiczii* Regel (Qu et al., 2011), *Lysimachia stenosepala* var. *flavescens* F.H. Chen & C.M. Hu (Wang et al., 2012), *Camellia oleifera* Abel (Li and Luo, 2003) and in *Rostellularia* (Qin et al., 2015). The spectroscopic data, listed in Tables 1 and 2, are in good accordance with those reported by Fico et al. (2007). The NMR and MS spectra are shown in Figs. S23–29 and S40.

Compound **5** comprises a flavone structure as central moiety, which again bears two hydroxy groups in positions 5 and 7 of the A-ring. The B-ring of glycoside **5** shows a 3′,4′-dioxygenation, indicated in the ^{13}C NMR spectrum by two signals of quaternary carbons. The proton pattern, with protons in positions 2′, 5′ and 6′, was verified by the typical coupling pattern of $^4J_{H2'\text{-}H6'}$ (1.5 Hz) and $^3J_{H5'\text{-}H6'}$ (8.3 Hz) as well as by the $^3J_{H\text{-}C}$ couplings between H-2′ and H-6′ with C-2. In position 3′, a methoxy group is attached, proved by the $^3J_{C\text{-}H}$ couplings between the methoxy protons and C-3’. A NOE between this methoxy group and H-2′ also sustains this substitution pattern. In position 4′, a hydroxy group is located. Position 4 of the C-ring is a carbonyl moiety, comparable to those of the structure in compounds **3** and **4**. Additionally, there is again a double bond between C-2 and C-3, with the carbohydrate moiety being attached at the quaternary C-3. This carbohydrate moiety was identified as β-glucopyranosyl group with a β-xylopyranosyl moiety on its 6″-position, comparable to the glycosyl moieties in compounds **3** and **4**. However, position 2″ is unsubstituted. The compound has a molecular formula of $C_{27}H_{30}O_{16}$, and its structure is isorhamnetin 3-*O*-β-xylopyranosyl (1 → 6)-β-glucopyranoside (Fig. 3). This glycoside is a new result for *Primula,* but it was reported to occur in pollen of *Cistus ladanifer* L. (Cistaceae) (Tomás-Lorente et al., 1992) and in whole plants of Psittacanthus calyculatus (DC.) G. Don (Loranthaceae) (Bah et al., 2011), respectively. Furthermore, it was reported to occur in bee pollen extracts

Table 2
^{13}C NMR spectroscopic data of compounds **1**–**7**.

Position	1	2	3	4	5	6	7
Aglycone							
2	165.5, s	165.9, s	158.3, s	158.5, s	158.4, s	158.3, s	158.2, s
3	108.0, d	106.8, d	134.9, s	134.5, s	135.4, s	135.0, s	134.5, s
4	180.7, s	180.6, s	179.5, s	179.3, s	179.3, s	179.3, s	179.2, s
4a	124.6, s	124.5, s	105.8, s	106.1, s	105.6, s	105.5, s	106.1, s
5	126.2, d	126.1, d	158.5, s	158.6, s	158.5, s	158.5, s	158.4, s
6	126.8, d	126.7, d	94.9, d	94.7, d	95.0, d	94.9, d	94.7, d
7	135.8, d	135.6, d	166.3, s	165.6, s	166.8, s	166.3, s	165.7, s
8	119.7, d	119.4, d	100.0, d	99.7, d	100.2, d	100.0, d	99.7, d
8a	157.9, s	157.8, s	163.1, s	163.1, s	163.0, s	163.1, s	163.2, s
1′	134.1, s	127.4, s	122.8, s	123.1, s	123.0, s	123.2, s	123.3, s
2′	115.9, d	115.1, d	132.3, d	132.2, d	114.5, d	114.3, d	114.6, d
3′	159.8, s	149.0, s	116.2, d	116.0, d	148.4, s	148.5, s	148.4, s
4′	121.61*, d	150.2, s	161.1, s	161.3, s	150.9, s	150.9, s	150.6, s
5′	131.4, d	118.0, d	116.2, d	116.0, d	116.1, d	116.1, d	116.1, d
6′	121.63*, d	119.8, d	132.3, d	132.2, d	123.8, d	124.9, d	123.5, d
O-CH_3						56.9, q	57.1, q
	3′-Glc	4′-Glc	3-Glc	3-Glc	3-Glc	3-Glc	3-Glc
1″	102.6, d	103.2, d	100.7, d	100.4, d	103.9, d	100.8, d	100.4, d
2″	74.9, d	74.8, d	81.9, d	79.8, d	76.0, d	81.3, d	80.2, d
3″	78.0, d	77.5, d	78.3, d	78.7, d	78.0, d	78.2, d	78.6, d
4″	71.5, d	71.3, d	71.0, d	71.9, d	71.0, d	71.0, d	71.8, d
5″	78.5, d	78.5, d	77.5, d	77.5, d	77.8, d	77.6, d	77.6, d
6″	62.6, t	62.4, t	69.4, t	69.5, t	69.3, t	69.3, t	69.3, t
			2″-Xyl	2″-Rha		2″-Xyl	2″-Rha
1‴			105.2, d	102.6, d		104.6, d	102.8, d
2‴			76.8, d	72.3, d		74.4, d	72.4, d
3‴			74.4, d	72.4, d		76.4, d	72.3, d
4‴			71.4, d	74.0, d		71.0. d	73.9, d
5‴			66.1, t	69.9, d		66.2, t	69.9, d
6‴				17.5, q			17.4, q
			6″-Xyl	6″-Xyl	6″-Xyl	6″-Xyl	6″-Xyl
1⁗			105.2, d	105.1, d	105.1, d	105.1, d	105.0, d
2⁗			74.7, d	74.7, d	74.8, d	74.7, d	74.7, d
3⁗			77.3, d	77.5, d	77.6, d	77.5, d	77.6, d
4⁗			71.3, d	71.0, d	71.2, d	71.3, d	71.0, d
5⁗			66.5, t	66.5, t	66.6, t	66.5, t	66.6, t

For numbering of positions see Fig. 3, compound 3. ^{13}C NMR spectra are measured as "distorsionless enhancement by polarization transfer including the detection of quaternary nuclei" (DEPTQ) (Burger and Bigler, 1998). Spectra of compounds 1 and 3–7 were measured at 150 MHz (600 MHz NMR); spectra of compound **2** were measured at 176 MHz (700 MHz NMR).

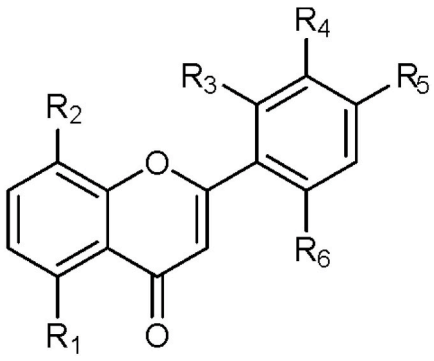
(Li et al., 2019). All NMR spectroscopic data of **5**, listed in Tables 1 and 2, are in good agreement with previous data. NMR spectra are shown in Figs. S31–S37.

HR-TOF-ESI-MS of glycoside **6**, isolated in a mixture with **3**, indicates a molecular ion $[M+Na]^+$ of m/z = 765.1853, which correlates quite well with the calculated m/z 765.1847 for $[M+Na]^+$ (absolute mass deviation: 0.78 ppm) of the molecular formula $C_{32}H_{38}O_{20}$. Compound (**6**) has an isorhamnetin core structure as has compound **5**. The carbohydrate moiety is bound at position 3 of the C-ring and consists of the same trisaccharide moiety as that in compound **3**. The resulting structure was hence identified as isorhamnetin 3-*O*-(2″-*O*-β-xylopyranosyl-[6″-*O*-β-xylopyranosyl]-β-glucopyranoside) (**6**), a hitherto not yet reported structure (Fig. 3). All NMR spectroscopic data are listed in Tables 1 and 2, and the corresponding NMR and MS spectra in Figs. S16–S22 and S39.

Glycoside **7**, isolated in a mixture with **4**, shows a molecular ion $[M+Na]^+$ of m/z = 779.2019, agreeing with the calculated m/z 779.2003 for $[M+Na]^+$ (absolute mass deviation: 2.05 ppm) of the molecular formula $C_{33}H_{40}O_{20}$. This compound also possesses an isorhamnetin core structure, as do compounds **5** and **6**. The carbohydrate moiety, bound to position 3 in the C-ring is the same trisaccharide moiety as detected in compound **4**. The structure is therefore isorhamnetin 3-*O*-(2″-*O*-α-rhamnopyranosyl-[6″-*O*-β-xylopyranosyl]-β-glucopyranoside) (Fig. 3). This compound was earlier described by Oueslati et al. (2014), isolated from *Suaeda fruticosa* (Forssk. ex J.F. Gmel.), but it has not been reported in *Primula* so far. NMR spectroscopic data are listed in Tables 1 and 2 and the corresponding NMR and MS spectra in Figs. S23–29 and S40.

2.3. Exudate flavonoids

Exudate flavonoids produced by glandular hairs were obtained by careful short rinsing with acetone which does not disturb surface cells, contrary to more aggressive solvents, such as chloroform. Earlier, we showed that extracts from isolated glandular hairs had the same composition as leaves washed with acetone (Elser, 2016). The exudate composition was analyzed by HPLC profiling, and individual compounds were identified against authentic substances, isolated previously by E. Wollenweber from various *Primula* species (Wollenweber and Mann, 1986; Wollenweber et al., 1988 a,b). All compounds detected belong to *Primula*-type flavones (Fig. 4; Fig. S 41). Earlier reports have indicated the presence of flavone (**8**), 2′-hydroxyflavone (**9**) and the corresponding 5-hydroxyflavone derivatives, along with 5,8-dihydroxyflavone (**15**) in a cultivar (Valant-Vetschera et al., 2009). These compounds were also detected in our samples, which, however, exhibited a more complicated aglycone profile. Particularly interesting is the accumulation of 3′-hydroxyflavone (**10**) in populations a, and b (Fig. 4). 3′,4′-Dihydroxyflavone (**13**) was detected only in collections from the central and southern Austrian alps (a,b in Fig. 4). Recent studies on populations from East Austrian Alps indicated presence of **8**, **9**, **10** and **15**, with some quantitative variations both of flavones and farina observed in populations from different altitudes (C. Priemer, pers. comm.). For assessing links between geographic patterns and exudate diversification, more populations of the *P. auricula*-complex in Austria should be studied deeper in the future. In addition to **8** and **9**,



Comp.	Aglycone	R_1	R_2	R_3	R_4	R_5	R_6	a	b
8	flavone	H	H	H	H	H	H	●	●
9	2′-hydroxyflavone	H	H	OH	H	H	H	●	●
10	3′-hydroxyflavone	H	H	H	OH	H	H	●	○
11	5-hydroxyflavone	OH	H	H	H	H	H	○	○
12	2′,5′-dihydroxyflavone	H	H	OH	H	H	OH	n.d.	○
13	3′,4′-dihydroxyflavone	H	H	H	OH	OH	H	○	○
14	5,2′-dihydroxyflavone	OH	H	OH	H	H	H	○	○
15	5,8-dihydroxyflavone	OH	OH	H	H	H	H	●	●
16	8,2′-dihydroxyflavone	H	OH	OH	H	H	H	○	○
17	5,8,2′-trihydroxyflavone	OH	OH	OH	H	H	H	○	n.d.

Fig. 4. Exudate profile of *P. auricula* accessions ● = major amount, ○ = trace amounts, n.d. = not detected. Locations: a = Nockberge in Carinthia (see Fig. S41); b = Northern Limestone Alps in Styria. Identification of these aglycones by comparison of retention times and UV-spectra with previously isolated compounds (Wollenweber and Mann, 1986; Wollenweber et al., 1988a;b). For UV-maxima of 8–17 see Table S1.

4′-hydroxyflavone was reported for exudates of an accession from the Italian Alps, Bergamo region. In the same study, the latter compound was also detected in some other *Primula* species from this region (Colombo et al., 2014), but not in collections of the present study. Thus, exudate diversification might be correlated also with geographic diversification in this species, expectable for a glacial relic.

2.4. *Significance of flavonoid structural diversification and compartmentalization*

Structural diversification between glycosidic and exudate aglycones is quite remarkable in the studied species, in view of different biosynthetic processes. So far, there is no evidence for common biosynthesis and transport of glandular and tissue flavonoids. Glandular hairs are capable of flavonoid biosynthesis, as confirmed by the presence of phenylalanine-ammonia lyase and chalcone synthase in glands of *P.* × *kewensis* W.Watson, in association with specific organelles. In mutants, incapable to produce farina, the respective enzymes could not be localized (Schöpker et al., 1995). Recently, fluorescence microscopic analyses revealed the presence of *Primula* type flavones exclusively in glandular hairs of *P. vialii* Delavay ex Franch., and of *P. vulgaris* Huds., respectively (Bhutia et al., 2012). Also, transport of lipophilic flavonoids from tissue to glandular hairs does not occur normally as demonstrated for e.g. *Empetrum nigrum* L. (Wollenweber et al., 1992). Thus, it is too early to hypothesize why some of the exudate flavonoid structures, such as **10**, are accumulated as glycoside **1** in *P. auricula* (Figs. 2 and 4). In comparison to other *Primula* type flavones, the substitution patterns at positions 3′ and 4′ of Ring B would suggest a regular biosynthetic origin, but the sole 3′-substitution cannot be explained by this pathway (Halbwirth, 2010). So far, nothing is known about the quality of flavone as substrate for respective hydroxylase activity, accounting for the sequence of observed hydroxylation patterns.

From the functional point of view, different roles are suggestive for the glandular versus the tissue flavonoids. Classical substitution patterns of tissue flavonols are in line with an anti-oxidative role (Halbwirth, 2010). Flavonol glycosides of *Primula* species tend to be quite complex at the glycosyl part, with several sugars involved, as reviewed by Colombo et al. (2017). Thus, gentiobiose, robinobiose and neohesperidose are common, as are several combinations with rhamnose and xylose. There are hardly any studies on the ecological significance of specific sugar moieties of flavonol glycosides (e.g. Neugart et al., 2012). One would suppose that tissue flavonoids have functions for plants in harsh alpine habitats with challenging climatic conditions and high UV-radiance. Interestingly, several of these sugars are also present in other glycosides, such as saponins or simple phenol glycosides (Colombo et al., 2017; Włodarczyk et al., 2020), indicating a common pool of available sugars for glycosylation.

The production of high amount of farina and concomitantly, of flavone, indicates a functional role, as observed for *P. auricula*

populations from higher altitudes and exposed habitats (C. Priemer, pers. comm.). This is in line with the suggested role of flavone in cold resistance (Isshiki et al., 2014). Furthermore, exudate flavonoids showed partly antifungal activity (Holzbach, pers. comm.), which might help the plant to fend off pathogens during the wet season. Thus, structural diversification in different compartments of the leaves would enable the plants to react to different biotic and abiotic stresses. Apparently, studying such complex interactions involves a lot of field research and lab experiments. The *Primula auricula* complex could evolve as a suitable model plant for future integrative studies in the fields of evolution and ecology.

3. Experimental

3.1. General experimental procedures

For NMR spectroscopic measurements, each sample was dissolved in CD_3OD (~3.0 mg in 0.7 mL) and transferred into 5 mm high precision NMR sample tubes. Spectra have been measured on a Bruker Avance III 600 at 600.25 MHz (^1H) or 150.94 MHz (^{13}C) or on a Bruker Avance III 700 at 700.40 MHz (^1H) or 176.13 MHz (^{13}C), respectively, and processed using the Topspin 3.5 software. 1D spectra were recorded by acquisition of 32k data points and after zero filling to 64k data points, and Fourier transformation spectra were performed with a range of 7200 Hz (^1H) and 32,000 Hz (^{13}C), respectively. To determine the 2D COSY, TOCSY, NOESY, HSQC, and HMBC spectra, 128 experiments with 2048 data points each were recorded and Fourier-transformed to 2D-spectra with a range of 6000 Hz and 32,000 Hz for ^1H and ^{13}C NMR, respectively. Measurement temperature was 298 K ± 0.05 K. Incompletely deuterated methanol (CHD_2OD) was used as the internal standard for ^1H (δH 3.34) and CD_3OD for ^{13}C (δC 49.0) measurements.

Mass spectra were recorded on a high-resolution time-of-flight (HR-TOF) mass spectrometer (maXis, Bruker Daltonics) by direct infusion electrospray ionization (ESI) in positive ionization mode (mass accuracy ± 5 ppm) as well as in negative mode (mass accuracy ± 10 ppm). HR-TOF MS measurements have been performed within the selected mass range of *m/z* 100–2500. ESI used a capillary voltage of 4 kV to maintain a (capillary) current between 30 and 50 nA. Nitrogen temperature was maintained at 180 °C using a flow rate of 4.0 L/min and a N_2 nebulizer gas pressure of 300 hPa.

HPLC analyses were performed on an Agilent 1100 Series with UV-DAD detector (detection at 230 nm), and a Hypersil BDS-C18 column (250 × 4.6 mm, 5 μm particle size). The flow rate was 1 mL/min and the injection volume was set at 10 μL. The compounds were eluted with MeOH (B) in aq. buffer (15 mM H_3PO_4 and 1.5 mM Bu_4NOH) (A) with two different solvent gradients. For analyses of tissue flavonoids, the solvent gradient was set as following: From 0 to 15 min from 10% to 70% B, 15–20 min from 70 to 80% B, 20–22 min isocratic with 80% B and from 22 to 28 min isocratic with 100% B. The solvent gradient for exudates was: From 0 to 17 min 55–90% B, from 17 to 20 min 90–100% B, 100% B was kept for 8 min. The concentration of the samples was set at 20 mg/mL in methanol.

TLC analyses were performed on Macherey–Nagel pre-coated TLC plates SIL G/UV_{254} (0.20 mm). For the plant extracts a mixture of $CHCl_3$/MeOH (80:20) was used and analyses of the exudates were performed in a mixture of toluene/dioxane/acetic acid (90:25:5). The compounds were detected by Naturstoffreagenz A (1% in MeOH) or anisaldehyde reagent. For normal- and reversed-phase MPLC, columns with dimensions of 300 × 25 mm were used. The size of the collected fractions varied from 10 to 100 mL.

3.2. Plant material

Two accessions of Primula *auricula* L. subsp. *auricula* (Primulaceae) from different locations were analyzed: Accession a was collected at Nockberge, Carinthia (Austria) on July 12, 2014, (WU0082545), and accession b at Gaiswinkelkar, Northern Limestone Alps, Styria (Austria) on August 1, 2017 (WU0107690), respectively. Species were determined by Mag. Ch. Gilli, University of Vienna, and respective voucher specimens were deposited at the Herbarium of the University of Vienna, Austria (WU).

3.3. Extraction and isolation

First, exudates were obtained by rinsing leaf material shortly with acetone. Each leaf was held in tweezers and each side of the leaf was rinsed 5 times with ca. 1 mL acetone. Small leaves were dipped in acetone five times. The obtained exudates were pooled, filtered, the solvent removed by using a rotary evaporator and subsequently analyzed. The rinsed leaves (42.3 g) were ground for extraction with MeOH to obtain the tissue components, as described e.g. in Bhutia et al. (2012). The extraction was achieved by soaking the powdered leaves in 200 mL methanol. After two days, the solvent was replaced. The obtained extracts were pooled, filtered, and concentrated under reduced pressure at 35 °C. The extract (3.25 g) was adsorbed on silica gel (40–63 μm) and subsequently chromatographed (silica gel, Si 60, 40–63 μm), eluted with mixtures of ethyl acetate (EtOAc) and methanol (MeOH) starting from 60% EtOAc to 20% EtOAc. The fraction size was 50 mL. Fractions were concentrated and analyzed by TLC Macherey–Nagel pre-coated TLC plates SIL G/UV_{254} (0.20 mm thickness) with Naturstoffreagenz A and also by HPLC. Four flavonoid-containing samples were chromatographed separately by reversed phase MPLC using a RP C18 column (40–63 μm) eluted with H_2O/acetonitrile (10%–25% MeCN). This step yielded the pure compounds **1** (1.6 mg), **2** (5.3 mg) and **5** (2 mg). The remaining fractions were combined and separated by column chromatography using silica gel 60 (40–63 μm) eluted with mixtures of $CHCl_3$ and MeOH, starting from 85% $CHCl_3$ to 55% $CHCl_3$. The gained fractions were further chromatographed separately by size exclusion chromatography over a Sephadex LH–20 column eluted with MeOH, to yield a mixture of compounds **4** and **7** in a 1:2 ratio. A mixture of compounds **3** and **6** (ratio 1:2.5) was obtained by RP-MPLC eluted isocratically with H_2O/MeOH (55:45).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bse.2021.104310.

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