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1. Abstract

In contrast to research dealing with exudate composition of *Primula* species, publications concerning their composition of tissue flavonoids are scarce. It has been hypothesized earlier, that the unusual exudate flavonoids of *Primula* must derive from an unknown biosynthetic pathway ("*Primula*-type flavones"), whereas tissue flavonoids derive from the well-known classical pathway. The aim of this survey was to assess the flavonoid composition of exudates and tissues from leaves of 30 different *Primula* collections. Therefore, comparative HPLC profiling of exudates, and of crude tissue extracts and their hydrolysates was carried out, to obtain more information on structural diversification in these compartments.

From tissue extracts of *P. auricula* subsp. *auricula*, three flavonoid glycosides were obtained as pure compounds and structurally identified as flavone-3′-*O*-β-D-glucopyranoside (**1**), 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**) and isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**3**), respectively. Of these compounds, **1** is reported herein for the first time as natural plant constituent, while glycoside **2** is new for the genus *Primula*. More information about tissue flavonoid composition was obtained from the analysis of hydrolysates, which revealed the presence of those aglycones normally incorporated in the respective tissue glycosides. Thus, it was shown that the "*Primula*-type flavones" such as 3′-hydroxyflavone, and 3′,4′-dihydroxyflavone might be more common in tissues than originally thought. This result does not support the earlier hypothesis, that *Primula* exudate flavonoids are always fundamentally different to the tissue flavonoids.

Comparative analyses of a range of species, belonging to different taxonomic groups revealed striking differences of flavonoids accumulated in exudates versus tissues. All exudates studied were characterized by the unusual substituted "*Primula*-type flavones". In the hydrolysates of these species, mostly aglycones deriving from the classical biosynthetic pathway were found. Flavonols such as quercetin, kaempferol and isorhamnetin were frequently accumulated, albeit in different combinations. The flavones luteolin and its methyl ether were present in a small group of related species only. 3′-Hydroxyflavone and 3′,4′-dihydroxyflavone, also known from exudates, were present in some species belonging to different taxonomic groups. Earlier reported infraspecific variation of exudate composition could be confirmed in the present study. A new finding is the qualitative variation of the glycoside profiles in the studied species. Accordingly, these diversifications might be of chemotaxonomic significance, which is also addressed in this study.

2. Introduction

The genus *Primula* L. comprising more than 400 species, is well known for the production of either oily or semi-crystalline (farinose) exudates produced by glandular trichomes (Fico et al., 2007; Valant-Vetschera et al., 2009). In particular, the formation of farinose exudates, being found on leaves, stems or inflorescences, is a conspicuous feature of many *Primula* species. Farina production appears to be uncorrelated to taxonomic groups, which may contain both farinose and efarinose species (Valant-Vetschera et al., 2009). Moreover, infraspecific variation concerning presence of farina was reported for e.g. *P. denticulata* (Elser et al., 2016). Occurrence of farina is a rare phenomenon in plants, and to date only *Primula* and associated genera, paralleled by some fern genera, are known for this feature (Wollenweber, 1989).

Previous studies of the composition of the either oily or farinose exudates from *Primula* species reported unsubstituted flavone together with a series unusual substituted flavones, sometimes referred to as *Primula*-type flavonoids because of their unresolved biosynthetic origin (Bhutia and Valant-Vetschera, 2012; Bhutia et al., 2012, 2013; Valant-Vetschera et al., 2009). These substitution patterns seem to be unique to the genus *Primula* and its closest allies, including the genus *Dionysia* (Valant-Vetschera et al., 2009, 2010). Erratic occurrences of some of these compounds are known for a few Thymeleaceae and for *Feijoa sellowiana* Berg. of the Myrtaceae (Hinterdobler et al., 2017). In a recent investigation, however, also the accumulation of flavonoids deriving from the regular biosynthetic pathway, was detected in exudates of some European *Primula* species, as has been earlier described for some *Dionysia* species (Elser et al., 2016; Valant-Vetschera et al., 2010). In particular, some species of *Primula* subgen. *Auriculastrum* exhibited infraspecific variation of exudate composition, accumulating either *Primula*-type flavonoids or flavonoids deriving from regular biosynthetic pathway. The observed differences correlated either with different geographic origin or with different collection dates. The authors suggested, that the shift from *Primula*-type flavones to those of the classical biosynthetic pathway may be characteristic for evolutionary new lineages within *Primula* (Elser et al., 2016).

In contrast to these unusual substituted flavones found in *Primula* exudates, the majority of reported flavonoid glycosides detected in tissues (leaves, flowers) of *Primula* species (Colombo et al., 2017) are clearly derived from the classical biosynthetic pathway (Bhutia et al., 2012). Thus, most of the identified *O*-glycosides derive from the flavonols kaempferol, quercetin and isorhamnetin, and less frequently from limocitrin, myricetin, and tamarixetin, respectively (Colombo et al., 2017). One rare exception is a glycoside based upon 7,2′-

dihydroxy flavone occurring in leaf tissues of *P. auricula* (Colombo et al., 2017; Fico et al., 2007). Interestingly, glycosides of two *Primula*-type flavones were found in leaves of *P. albenensis* and of *P. farinosa* from Italy (Colombo et al., 2014). *C*- Glycosylation in positions 6 and/or 8 of the flavones apigenin and luteolin, and of the flavonol kaempferol are less frequently reported. Available data suggest that flavones are not as frequently glycosylated as flavonols. Sugar moieties frequently consist of 2–3 units, with glucose and rhamnose being more frequent than arabinose, mannose, galactose and xylose (Colombo et al., 2017). Structure identification of glycosides is more complex compared to exudate aglycones (Fico et al., 2007; Harborne, 1968). This might be the reason, why so few glycoside data are available across the genus, and comparative studies on both exudates and tissue flavonoids of corresponding plant organs and of the same accessions are rare.

Apart from flavonoids, volatile compounds and essential oils have been described from some species, but studies in this respect are scare. These oils are source of several phenylpropanoids and sesquiterpene hydrocarbons (Colombo et al., 2017). *Primula* is exceptional as flavonoids are predominant in exudates. Most of the other plants with glandular hairs accumulate primarily volatiles together with minor amounts of flavonoids in these compartments, such as e.g. Compositae (Wollenweber and Valant-Vetschera, 1996). However, a recent report points to lack of exudate flavonoids in phylogenetically derived *Primula* species, which then were found to accumulate primin-type (benzoquinone) compounds and *ent*-kaur-16-en-19-oic acid (Elser et al., 2016).

The aim of the present study was to compare different compartments of the same accessions, across a range of species, and to evaluate the earlier impression that tissue- and exudate flavonoids are biosynthetically unrelated. As for exudate flavonoids detected here, hypothetical biosynthetic relationships based upon their structure and from literature information, were elaborated. Flavonoid diversification is discussed in view of its possible significance as chemical character in studied groups.

3. Botany

3.1 Primula L.

The genus *Primula* L. (Primulaceae, Ericales) comprises about 500 species grouped into seven subgenera and 37 sections (Elser et al., 2016). The central concentration of species diversification occurs in the Sinohimalayan mountain range, together with the adjacent ranges in central Asia, which count together for about 78% of all *Primula* species (Richards, 2002).

Away from the Asian highland center of diversity the number of species drops rapidly (Richards, 2002), with *Primula* species occupying the mountains or high latitudes of North America, Europe and Asia with a few species extending to South America, Ethiopia, Java, and Sumatra (Mast et al., 2001). The most recent, global monographic treatment of the genus was elaborated by J. Richards, who recognized seven subgenera *Sphondylia* (Duby) Rupr., *Auriculastrum* Scott, *Primula* L., *Auganthus* (Link), *Carolinella* (Helmsley) Wendelbo, *Pinnate* subgen. nov., and *Aleuritia* (Duby) Wendelbo (Richards, 2002). Further, phylogenetic investigations based on comparison of DNA sequences, indicated that genera *Dodecatheon*, *Cortusa*, and *Dionysia* have been evolved from *Primula* (Mast et al., 2001; Richards, 2002).

Primula species are perennial herbs, with rosette shoots, or rarely annuals, growing in plain meadows, the forest belt, alpine lawns, and nival and meadow tundras (Kovtonyuk and Goncharov, 2009). The large genus is morphological heterogenous. Flowers within the genus can be either solitary, or arranged in umbels, several superimposed whorls, heads or spikes, and subtending bracts are either large and leaf-like, or small, and sometimes be saccate at the base. The corolla is hypocrateriform in most instances, but some species have a campanulate corolla (Trift et al., 2002). Within *Primula* 91% of species are heterostylous, characterized by all flowers of a plant having either a long style (pin morph), with anthers lying deep in the corolla, or a short style (thrum morph), with the anthers attached near the mouth of the corolla (Richards, 2002). Further, the two morpho-types exhibit different pollen surface ornamentations and pollen sizes. Generally, it is assumed that the two different morphs promote outcrossing (Trift et al., 2002), and that viable seed is only set, when pollination occurs between the different morpho-types (Richards, 2002). Heterostyly seems to be the condition in the most recent ancestor of *Primula*, followed by several recent losses within deeply nested lineages (Vos et al., 2014).

Leaf vernation within *Primula* (arrangement of leaves within the bud) is either involute, with the margins rolled inward to the upper side, or revolute with the margins rolled backward to the upper side (Harris and Harris, 2001; Richards, 2002). The majority of *Primula* species has revolute vernation. The condition of involute leaves is only found in six sections, including sections *Sphondylia*, *Cuneifolia*, *Sufrutescens*, *Amethystina*, *Parryi*, and *Auricula*, although there is to mention, that vernation of *Sphondylia* was shown to be better interpreted as conduplicate, with leaves folded together lengthwise with the upper surface within (Richards, 2002).

The chromosome base numbers within *Primula* vary between 8 and 12 (Mast et al., 2001; Richards, 2002). Main pollen types in *Primula* are polycolpate, colporidate, or syncolpate, with the number of colpi varying, often in correlation with the ploidy level. Within *Primula* some species have multicellular, sometimes glandular hairs (trichomes), whereas other species are glabrous (Trift et al., 2002). Glandular trichomes produce exudates, which are either oily or semi-crystalline, whereby the latter is referred to as farina (Fico et al., 2007). Farinose exudates are a conspicuous feature of several *Primula* species, and may be found on leaves, stems or inflorescences. The production of farina is not correlated to taxonomic groups, which can contain farinose and non-farinose species (Valant-Vetschera et al., 2009). Further, for some *Primula* species, infraspecific variation for the presence of farina, coinciding with seasonal changes, was reported (Elser et al., 2016).

3.2 Trichomes

Trichomes are epidermal outgrowths covering most aerial parts of plants. They are composed of a single cell or of multicellular structures and are divided into two general categories, non-glandular and glandular structures (Huchelmann et al., 2017). Between different plant species, the morphological characteristics of both trichome types, glandular and non-glandular, may vary greatly. Generally, morphological and mechanical constitution of trichomes may contribute to diverse aspects of plant physiology and ecology. The functions of both trichome types, found within the plant kingdom are, diverse, and include protection against biotic and abiotic stress (Wagner, 2004). Beside the mechanical aspects, glandular trichomes are involved in synthesis, storage and/ or excretion of secondary metabolites (SMs) and can be found on approximately 30% of all vascular plant species (Huchelmann et al., 2017; Wagner, 2004). Glandular trichomes consist of a stalk and a glandular head and according to morphological characteristics two categories, peltate and capitate, can be distinguished (Huchelmann et al., 2017). Capitate trichomes, consisting of a stalk, are made up of one to a few cells, and one to a few secretory cells at the tip of the stalk. They generally synthesize non-volatile or poorly volatile compounds, which are directly excreted onto the surface of the trichome (Glas et al., 2012). As far as capitate trichomes are variable in respect to stalk cell number and length, morphology, and secretion patterns, they can be subdivided into further subgroups (Huchelmann et al., 2017). Peltate trichomes consist of a stalk made up of one cell, and a multicellular secreting head, accumulating SMs in a large subcuticular cavity (Glas et al., 2012). Generally, secondary metabolites mainly produced by glandular trichomes, were found to be terpenoids, but they are also capable to synthesize phenylpropanoids, flavonoids,

methylketones and acyl sugars (Huchelmann et al., 2017). The phytochemistry of glandular trichomes may differ between the different developmental stages of the trichomes (Bhutia et al., 2012). Many of the phytochemicals produced by glandular trichomes are involved in host defense, and in some species the amount of exudate produced by glandular trichomes, may make up to 30% of the leaf dry weight (Wagner, 2004). As far as there are no records for glandular trichomes in Bryophytes, glandular trichomes are suggested to be an invention of vascular plants, where they seem to have evolved several times independently. Fossil records of ferns indicate that multicellular glandular trichomes already existed during the late Carboniferous. Different hypotheses about the origin of glandular trichomes exist: a) migration of secretory cells from the mesophyll to the outside; b) evolvement from stomata; c) evolvement from non-glandular trichomes by differentiation of apical cells into secretory cells. However, a comprehensive study of the occurrence and types of glandular trichomes, indicating evolutionary patterns, is missing (Tissier, 2012).

Within *Primula*, non-glandular and glandular trichomes may occur (Bhutia et al., 2012). Generally, glandular trichomes cover the majority of aerial parts of *Primula* species (Wollenweber and Schnepf, 1970), but so far morphological studies on glandular trichomes of these species are scarce (Colombo et al., 2017). More detailed studies on trichome morphology in *Primula* report the occurrence of glandular trichomes of the capitate type, consisting of a one to few celled stalk with an unicellular glandular head at the tip (Colombo et al., 2014; Fico et al., 2007). Differences of glandular trichomes between species were observed in the ratios between stalks and glandular heads (Colombo et al., 2014, 2017; Fico et al., 2007; Valant-Vetschera et al., 2009; Vitalini et al., 2011). According to these characteristics capitate trichomes within *Primula* may be divided into two categories, long-stalked capitate trichomes and short-stalked capitate trichomes. Some species possess only one type of glandular trichomes on leaves, either short-stalked capitate trichomes or long-stalked capitate trichomes, where other species are characterized by both trichome types occurring side by side in the same leaf section (Bhutia et al., 2012; Colombo et al., 2014). Glandular trichomes in *Primula* can be further classified according to constitution of their secretions, which may be either oily or mealy. In both cases secretions are deposited on the tip of the glandular head (Wollenweber and Schnepf, 1970). Detailed observations on secreting trichomes of *P. obconica* Hance, carried out by light microscopy, scanning and transmission electron microscopy, indicate that the resinous secretion forms a drop at the tip of the glandular trichome. The droplet is not covered by a cutine layer, instead, it seems preserved by a thin layer of thickened material (Maleci et al., 1992). Depending on the species, major compounds in *Primula* exudates may be unusual

substituted flavones, referred to as *Primula*-type flavonoids, flavonoids deriving from regular biosynthetic pathways, primin, or primin derivatives (Elser et al., 2016).

4. Secondary metabolites (SMs)

The metabolism of plants is usually regarded to consist of two major branches: Primary and secondary metabolism (Kliebenstein and Osbourn, 2012). Primary metabolism covers all processes, that are responsible for growth and development (Hartmann, 2007), with genes required for their synthesis being largely conserved across all known plant species. Compounds deriving from secondary metabolism, referred to as secondary metabolites (SMs), cover primarily plant interactions with the environment (Kliebenstein and Osbourn, 2012).

The precursors for the biosynthesis of SMs usually derive from primary metabolism, and enzymes involved in biosynthesis are often derived from common progenitors with a function in primary metabolism (Wink, 2010). A limited number of key genes encode for the enzymes that are responsible for the synthesis of the backbone structures, resembling the different classes of SMs. Further diversification of SMs is achieved by a limited number of enzyme classes, such as glycosyl-, methyl-, and acyltransferases, decorating the single basic structures (Jørgensen et al., 2005). Although the number of corresponding pathways is restricted, the number of SMs is suggested to exceed 200 000 compounds (Wink, 2008). SMs biosynthesis is a highly coordinated process, including metabolic channeling and metabolon (multi enzyme complexes) formation, which enable a specific biosynthesis and avoid metabolic interferences. The channeled biosynthetic pathways can proceed in a channeled array in one compartment, or can involve different organelles, cell types or plant organs, including intra- and intercellular translocations of metabolites. Within the plant, some SMs were shown to be produced in all tissues, but generally SM biosynthesis was shown to be tissue- and cell specific (Wink, 2010). Further biosynthesis of SMs can be development specific (Wink, 2010), and changes of accumulation tendencies of specific SMs, correlating with seasonal changes were observed (Elser et al., 2016). SMs may be stored in vacuoles, laticifers, cell walls, cuticles, trichomes, resin ducts, oil cells, or plastid membranes. Functions of SMs within the plant kingdom include defense against herbivores, fungi, bacteria, and viruses, protection against UV light, involvement in plant-plant interactions, attraction of pollinating and seed-dispersing animals, communication between plants and symbiotic microorganisms, and selected physiological roles (Wink, 2010). Interactions with the environment impart an evolutionary pressure upon plants creating new secondary metabolites, which are lineage specific in the majority of cases (Kliebenstein and Osbourn, 2012).

4.1 Flavonoids

Flavonoids are a large and diverse group of phenolic compounds based upon a basic C_6-C_3-C_6 skeleton, comprising over 9000 different compounds (Berim and Gang, 2016). The biosynthesis of flavonoids is part of the larger phenylpropanoid pathway, which produces a range of other specialized metabolites, like phenolic acids, lignins, lignans and stilbenes (Davies and Schwinn, 2006). General flavonoid biosynthesis is well studied, both from the localization and from the biosynthetic sequence (Halbwirth, 2010). Immunolocalization experiments suggest that most of the flavonoid synthesizing enzymes are loosely bound to the endoplasmic reticulum and probably organized in multi-enzyme complexes (Falcone Ferreyra et al., 2012). Metabolic channelling by multienzyme complexes would enable synthesis without metabolic interference (Falcone Ferreyra et al., 2012), that can be caused for example by competition for substrates at the different branching points within the pathways. Moreover, intermediates may be highly reactive and potentially toxic and the overall concentrations appears to be very low (Winkel-Shirley, 2001). The end products of flavonoid biosynthesis are transported to various subcellular or extracellular locations (Davies and Schwinn, 2006).

Precursors for flavonoid biosynthesis are malonyl-CoA, and a suitable hydroxy cinamic CoA ester, which is in most cases *p*-coumaroyl-CoA (see Figure 1) (Forkmann and Heller, 1999). *P*-coumaroyl-CoA is synthesized through three enzymatic steps from phenylalanine, central to the general phenylpropanoid pathway (see Figure 1) (Davies and Schwinn, 2006). The first committed step of flavonoid biosynthesis is catalyzed by chalcone synthase (CHS) (Berim and Gang, 2016). CHS forms the parental bicyclic C_6-C_3-C_6 structure, called chalcones, by the stepwise condensation of three malonyl-CoA with *p*-coumaroyl-CoA (Berim and Gang, 2016), resulting in the formation of naringenin-chalcone (see Figure 1) (Halbwirth, 2010). Chalcones are the central intermediates from which all other flavonoids originate (Forkmann and Heller, 1999). Chalcone isomerase (CHI) gives rise to the tricyclic phenylchromane backbone skeleton typical for the majority of flavonoids (Berim and Gang, 2016). The enzyme CHI catalyzes a stereospecific cyclization of chalcones to (2S)-flavanones, which are one of the main branch points in flavonoid biosynthesis, and are direct intermediates in the formation of isoflavones, flavones, dihydroflavonols and flavan-4-ols (Forkmann and Heller, 1999). Cyclization of naringenin-chalcone by CHI leads to the formation of naringenin (see Figure 1) (Halbwirth, 2010). Further structure modifications lead to the flavonoid subclasses (see Figure 1) (Berim and Gang, 2016), which are grouped according to the oxidation level of the central heterocyclic ring C (Forkmann and Heller, 1999). As a result, the basic flavonoid structure

possesses hydroxyl groups at positions 5, and 7, deriving from malonyl-CoA, and a 4′-OH, deriving from *p*-coumaroyl-CoA (Halbwirth, 2010). This well-known pathway is named here as "classical" or "regular" biosynthetic pathway.

Another pathway leads to the formation of 5-deoxyflavonoids, as a result from a co-action of CHS and chalcone-ketide reductase (CHR) during the formation of chalcones (see Figure 1). The enzyme CHR catalyzes the reduction of the keto group in position 6′ of the coumaroyl-trione formation. The transformation from 6′-deoxychalcones to 5-deoxyflavanones requires a specific CHI. Other downstream enzymes of the flavonoid pathway seem to accept 5-deoxyflavonoids in addition to 5-hydroxyflavonoid substrates (see Figure 1). Furthermore, substitution patterns other than the basic 4′-substitution in ring B of flavonoids, may be generated in some species during the biosynthesis of chalcones by CHS accepting caffeoyl-CoA instead of *p*-coumaroyl-CoA. The generated 3,4-dihydroxylated chalcones resemble intermediates for the corresponding 3′,4′-dihydroxylated flavonoids (Halbwirth, 2010). The biosynthesis of flavonoids as occurring in exudates of *Primula* species is still unrevealed in detail, especially the formation of unsubstituted and further unusual substituted 7-deoxyflavones (Hinterdobler et al., 2017). From what is known so far about flavonoid biosynthesis, no suitable precursor could be identified (Bhutia et al., 2013). It is also not clear if the 5-deoxy pathway would contribute to the *Primula*-type flavone structures (Bhutia et al., 2012). Particularly, B-ring substitution cannot be explained by this pathway. As one possible option, endophytic fungi or bacteria were suggested to be involved in biosynthetic pathways leading to *Primula*-type flavonoids (Bhutia et al., 2013), but their presence has not yet been proven.

Outside Primulaceae, occurrence of some *Primula*-type flavonoids are reported from Thymeleaceae and Myrtaceae only. Thus, within the Thymeleaceae, flavone (E1), 2′-methoxyflavone (E8), and 3′-methoxyflavone (E19) were detected in whole plants of *Pimelea* species, and 2′-hydroxyflavone (E7) was shown to occur in the leaves of *Daphnopsis sellowina* Taub. (for structures see Figure 2 in chapter 6.1). Flavone (E1) is further reported from fruits of *Feijoa sellowiana* Taub. (Myrtaceae) (Hinterdobler et al., 2017). In these species synthesis and storage are unknown (Hinterdobler et al., 2017), and biosynthesis seems not primarily associated with glandular hairs, but so far, no biosynthetic studies have been reported from members of the genera mentioned above. Interestingly, recombinant *Streptomyces lividans* cells, carrying shuffled, bacterial biphenyl dioxygenase genes, were shown to be capable of transforming flavone to 3′-hydroxyflavone (E18) and 2′,3′-dihydroxyflavone (Chun et al.,

2003). Additionally, those strains were shown to transform unsubstituted flavanone to 2′-hydroxyflavanone, 3′-hydroxyflavanone, and 2′,3′-dihydroxyflavone (Chun et al., 2003), with flavanones generally resembling biosynthetic precursors of flavones in plants (Halbwirth, 2010). This is particularly of interest, as authors of a previous phytochemical investigation suggested "external" assistance for biosynthesis of some flavones occurring in *Primula* and species of the Thymeleaceae by microbial endophytes (Hinterdobler et al., 2017). Generally, it is assumed, that such patchy distributions of SMs may also, be the result of horizontal gene transfer from, for example, viruses or bacteria. In the case of a transfer of genes of SM synthesis, host plants most probably would contribute their own sets of pathway genes, leading to structural variation of SMs (Wink, 2008).

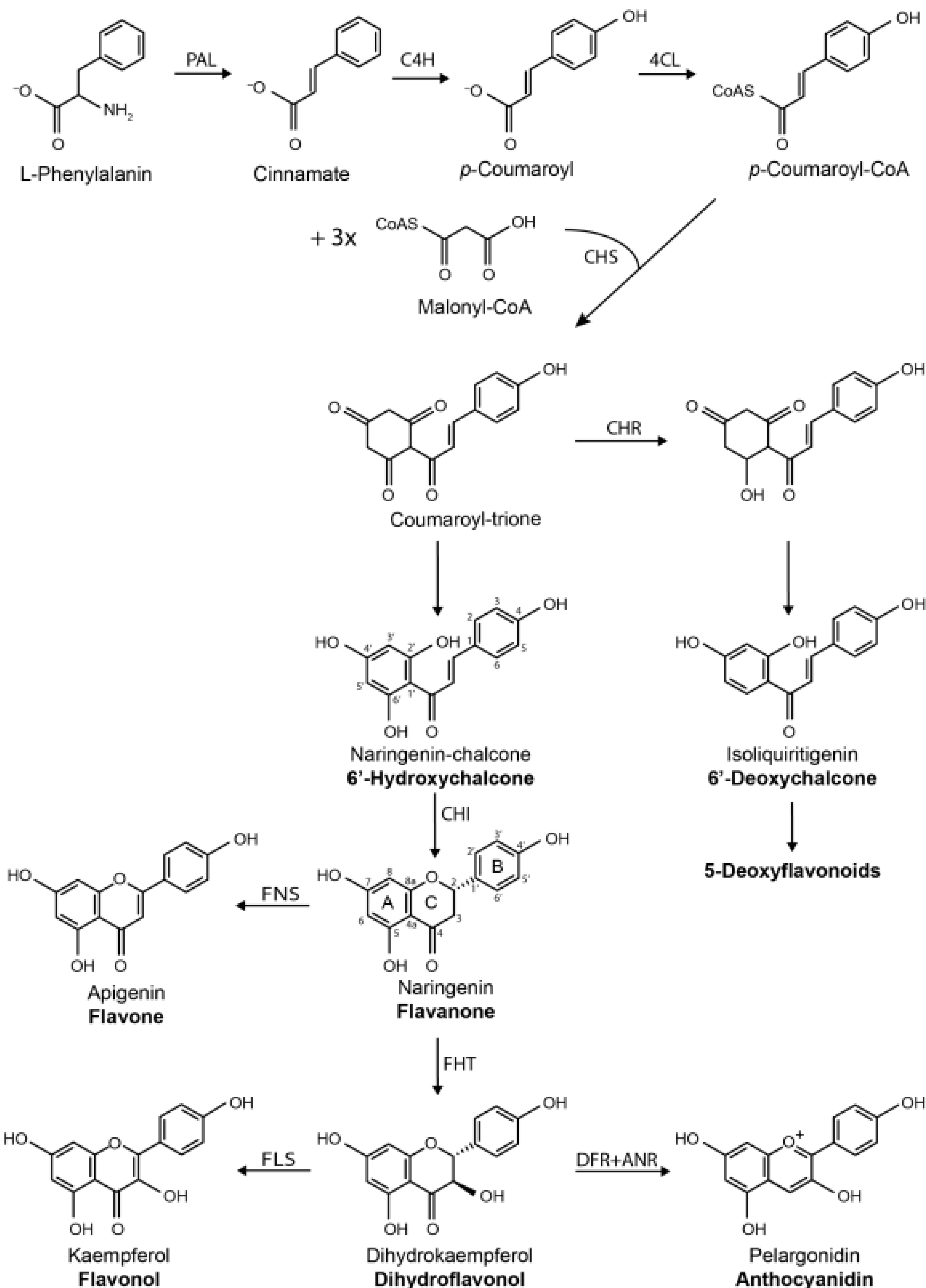


Figure 1. Simplified phenylpropanoid pathway and biosynthetic pathways leading to various classes of flavonoids. Biosynthetic pathways were taken from (Davies and Schwinn, 2006; Halbwirth, 2010). Abrev.: PAL: Phenylalanine ammonia-lyase, C4H: Cinnamate 4-hydroxlase, 4CL: 4-coumarate:CoA ligase, AUS: Aurone synthase, CHS: Chlacone synthase, CHR: Chalcone ketide reductase, CHI: Chalcone isomerase, FNS: Flavone synthase, FHT: Flavanone 3-hydroxylase, FLS: Flavonol synthase, DFR: Dihydroflavonol 4-reductase, ANR: Anthocyanidin reductase.

4.2 Functions of flavonoids in plants

Flavonoids are widely distributed in the plant kingdom, and occur in different amounts, according to species, organ, developmental stage, and growth condition (Petrussa et al., 2013). The function of flavonoids depends on number and nature of substituents. Water solubility of flavonoids increases with the number of hydroxyl groups or of sugars, whereas it decreases with the number of methylations (Halbwirth, 2010). The majority of flavonoids occurs as glycosides (Iwashina, 2000). Generally, the most reactive hydroxyl groups of flavonoids are glycosylated, in particular at position 7 of flavones, and position 3 of flavonols. Glycosylation prevents autooxidation of the reactive hydroxyl group and increases the solubility in aqueous cellular compartments (Kumar and Pandey, 2013). Dihydroxy B-ring substituted flavonoid glycosides (e.g. luteolin (H5) or quercetin (H2) (for structures see Figure 3 in chapter 6.1)) were detected in different compartments including chloroplasts, nucleus, and vacuoles. They may inhibit the generation of reactive oxygen species (ROS), and it is assumed that they prevent photo-oxidative damage. Flavonoid glycosides were also detected in cell walls of some species. Assumed functions of cell wall flavonoids are protection against UV radiation, and pathogens (Agati et al., 2012). Another noteworthy function of flavonoid glycosides is the colouring of flowers, fruits and seeds (Wollenweber, 1989) for attracting pollinators and seed dispersers (Winkel-Shirley, 2001).

Beside flavonoid glycosides, also aglycones possess functions in plants. Quercetin (H2), apigenin, and kaempferol (H1), for example, can outcompete auxines for binding sites on plasma membranes, thus influencing auxin transport (Gould and Lister, 2006). It must be mentioned, though, that aglycones rarely occur in plants, except those produced by glandular structures and accumulated in exudates externally (Wollenweber, 1989). The majority of exudate flavonoids are lipophilic and methylated aglycones (Onyilagha and Grotewold, 2004). Generally, the methylation of hydroxyl groups decreases the antioxidant potential of flavonoids and increases their stability and ability for permeating membranes (Berim and Gang, 2016). Exudate flavonoids usually differ in structure from vacuolar flavonoids of the same plant (Onyilagha and Grotewold, 2004). Associated functions, according to their external storage, include UV protection, antimicrobial activity or herbivore feeding deterrent effects (Berim and Gang, 2016). Further, allelopathic effects from flavonoids of *Cistus ladanifer* L. (Cistaceae; Malvales), hindering the development of *Rumex crispus* (Polygonaceae; Caryophyllales) seedlings, were demonstrated. Thus, it was assumed, that flavonoids enter the soil either via root exudation or decaying leaves (Onyilagha and Grotewold, 2004). For unsubstituted flavone

(E1) (for structure see Figure 2, in chapter 6.1), a common exudate constituent of *Primula*, it has been demonstrated that it is involved in freezing tolerance (Elser et al., 2016). Results of bioactivity tests further indicated, that the generalist *Spodoptera frugiperda* avoids plants containing flavone (Onyilagha and Grotewold, 2004). For the flavones of *P. denticulata* Sm. (P2) cytostatic properties were demonstrated leading to the suggestion, that they might limit the growth of pathogens (Elser et al., 2016). So far exudate flavonoids gained less attention compared to vacuolar flavonoids, and still many questions concerning ecological role of exudate flavonoids and their distribution within the plant kingdom are unresolved (Onyilagha and Grotewold, 2004).

5. Experimental

5.1 Plant material

Studied species comprise collections from nature as well as cultivated material, and older exudate samples. For list of samples see Table 1. Voucher specimens, including names of collectors, are deposited at the Herbarium of the University of Vienna, Austria (WU).

5.2 Analytical methods

5.2.1 TLC

TLC analyses were carried out on silica gel 60 UV_{254} aluminium plates (Marchery-Nagel), thickness 0.2 mm, developed in ethyl acetate/ formic acid/ glacial acetic acid/ water (100:11:11:26) (Marston and Hostettmann, 2006). Compounds with chromophore were detected under UV light at 254 and 366 nm, and also by derivatization with Naturstoffreagenz A (1% in MeOH). For purity checks TLC plates were sprayed with anisaldehyde.

5.2.2 HPLC

HPLC analyses of exudates and extracts were performed on Agilent 1100 series with UV diode array detector (detection WL 230 nm), using a Hypersil column BDS-C18, 250 x 4.6 mm, 5 µm particle size, eluted with methanol (MeOH) (B) in aq. buffer containing 15 mM H_3PO_4 and 1.5 mM Bu_4NOH (A). For analyses of exudates, and the aglycone part of flavonoid glycosides the applied gradient started with 55% B in A to 90% B in A at 17 min and to 100% B at 20 min kept for 8 min. The flow rate was 1 mL/min, and the injection volume was 10 µL. For analyzing the extracts, following gradient was applied: 0–15 min 10% B in A, from 15–20 min 70% B in A, from 20–22 min 80% B in A, from 22–28 min 100% B, at a flow rate of 1 mL/min and an injection volume of 10 µL.

5.3 Preparative methods

5.3.1 Medium pressure liquid chromatography (MPLC)

This method was used for isolation of flavonoid glycosides. The separation was achieved either via a silica gel 60 column (40–63 µm particle size), and/or a reversed phase column (RP-18, 40–63 µm) were used. The system further consists of a Büchi Pump Module C-601, Büchi Pump Controller C-610, and a Teledyne Isco UA-6 UV/VIS Detector.

5.4 Extraction and isolation

5.4.1 Exudate

Dried leaves of selected *Primula* species (compare Table 1) were used to analyze epicuticular flavonoids. The leaf material was briefly rinsed with acetone and the obtained solution was filtered and concentrated under reduced pressure for further TLC and HPLC analyses (Elser et al., 2016).

5.4.2 Extraction

For analysis of the tissue flavonoids, rinsed leaf material (50 or 100 mg) was weighed into Eppendorf tubes. Additionally, 3 glass beads were added, and the plant material was disrupted by using a tissue lyser. After adding 1 mL MeOH and sonication for 20 minutes, samples were centrifuged at 13.500 rpm, and the supernatant was decanted. Analyses of extracts were performed by HPLC. For isolation of flavonoid glycosides, 42.3 g of rinsed leaf material of *P. auricula* subsp. *auricula* (P16, P17) (for collection data see Table 1) was powdered and extracted three times over one week using MeOH at room temperature. The gained extract was filtered and evaporated to dryness under reduced pressure at 35°C. The dry weight for the crude methanolic extract was 3.25 g.

5.4.3 Hydrolysis

For the analysis of the aglycone part of flavonoid glycosides, extracts were concentrated to a minimum of MeOH, and 1 mL HCl (2 M) was added. The solution was heated in a water bath for one hour, and resulting hydrolysates were extracted with EtOAc to gain the flavonoid aglycones. After evaporation of EtOAc, dry hydrolysates were dissolved in MeOH and analyzed by HPLC. Additionally, isolated flavone-3′-*O*-β-D-glucopyranoside (**1**) (compare chapters 5.4.4, and 6.2) was hydrolysed according to the above described procedure, to gain 3′-hydroxyflavone (E18), which was used as standard for comparative HPLC profiling.

5.4.4 Isolation

The obtained extract was separated in 24 fractions by MPLC on normal-phase column (NP-MPLC) using a silica gel 60 column (40–63 µm particle size) eluted with mixtures of PE/ EtOAc (40% EtOAc–60% EtOAc, 400 mL), EtOAc (100 mL), and a mixture of EtOAc/ MeOH (60% EtOAc–20% EtOAc, 400 mL). The obtained fractions were analyzed by TLC using Naturstoffreagenz A as detection reagent, and flavonoid containing fractions were further analyzed by HPLC. Fractions 12, 13, 15 and 16 were further chromatographed separately by reversed-phase MPLC (RP-MPLC1–RP-MPLC4), using a RP-18 column (40–63 µm), eluted with mixtures of H_2O/ acetonitrile (10% ACN–25% ACN). The obtained flavonoid containing fractions were further analyzed by HPLC. The pooled fraction 6 of RP-MPLC1 (NP-MPLC fraction 12) and fraction 24 of RP-MPLC2 afforded 1.6 mg flavone-3′-*O*-β-D-glucopyranoside (**1**). Fraction 17 of RP-MPLC2, fractions 11 and 13 of RP-MPLC3 and fractions 12, 13 and 16 of RP-MPLC4 were combined to gain 5.3 mg 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**). Fractions 7 and 8 of RP-MPLC4 gained 2 mg isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**3**).

Table 1. List of studied *Primula* species.

	Collection date	Location/ source	E	H
Subgenus *Sphondylia* (Duby) Rupr.				
Section *Sphondylia*				
Primula verticillata Forskk. (P01)	23.11.2018	Bot. Garden, Univ. Vienna	x	x
Subgenus *Aleuritia* (Duby) Wendelbo				
Section *Denticulata*				
P. denticulata Sm. (P02)	04.04.2016	Bot. Garden, Univ. Vienna	x	x
Section *Aleuritia* Duby				
P. frondosa Janka (P03)	23.11.2018	Bot. Garden, Univ. Vienna	x	x
Section *Armerina*				
P. involucrata subsp. *yargongensis* Sm. & For. (P04) (Sub *P. munroi* subsp. *yargongensis*)	31.05.2017	Bot. Garden, Univ. Vienna, (KG),	x	x
Section *Petiolares* Pax				
P. boothii subsp. *repens* A.J. Richards (P05)	23.11.2018	Bot. Garden, Univ. Vienna; (KG),	x	x
Section *Proliferae* Pax				
P. aurantiaca W.W. Sm. & Forrest (P06)	31.05.2017	Bot. Garden, Univ. Vienna; (KG),	x	x
P. bulleyana subsp. *beesiana* Forrest (P07) (Sub *P. beesiana* Forrest.)	31.05.2017	Bot. Garden, Univ. Vienna; (KG) ,	x	x
P. secundiflora Franch. (P08)	31.05.2017	Bot. Garden, Univ. Vienna; (KG),	x	x
P. serratifolia Franch. (P09) (Sub *P. serratifolia* Franch.)	31.05.2017	Bot. Garden, Univ. Vienna; (KG),	x	x
P. poissonii Franch. (P10)	31.05.2017	Bot. Garden, Univ. Vienna; (KG),	x	x
P. wilsonii Dunn (P11)	31.05.2017	Bot. Garden, Univ. Vienna; (KG)	x	x
Subgenus *Auganthus* (Link) Wendelbo				
Section *Bullatae* Pax				
P. bullata var. bracteata (Franch.) P. Eveleigh, J. Nielsen & D.W.H. Rankin (P12) *	23.11.2018	Bot. Garden, Univ. Vienna	x	x
P. bullata var. forrestii (Balf. f.) P. Eveleigh, J. Nielsen & D.W.H. Rankin (P13) *	23.11.2018	Bot. Garden, Univ. Vienna	x	x
Section *Cortusoides* Balf. f				
P. cortusoides L.(P14)		Exudates of E. Wollenweber	x	
Section *Monocarpicae* Franchet ex Pax				
P. malacoides Franch. (P15)		Exudates of E. Wollenweber	x	
Subgenus *Auriculastrum* Schott				
Section *Auricula* Duby				
P. auricula L. subsp. *auricula* (P16) **	12.07.2014	Austria, Carinthia, Nockberge (WU0082545) ***	x	
P. auricula L. subsp. auricula (P17) **	01.08.2017	Austria, Styria, Gaiswinkelkar (WU0107690) ***	x	
P. hirsuta L. (P18)	01.07.2013	Switzerland, Graubünden/Uri, Oberalppass (WU0083088) ***		x
P. hirsuta L. (P19)		Austria, Tyrol, Stubaier Alpen, Schrankogel (WU0083093) ***		x
P. villosa Wulfen (P20)	12.07.2014	Austria, Carinthia, Nockberge (WU0082549) ***		x
P. minima L. (P21)	07.07.2014	Austria, Carinthia, Großfragant (WU0082541) ***	x	x
P. glutinosa Wulfen (P22)	06.07.2014	Austria, Carinthia, Großfragant (WU s.n.)	x	x
P. spectabilis Tratt (P23)	23.11.2018	Bot. Garden, Univ. Vienna	x	x
Subgenus *Primula*				
Section *Primula*				
P. elatior (L) Hill (P24)	19.05.2016	Bot. Garden, Univ. Vienna,	x	x
P. veris (Bunge) L. (P25)	23.11.2018	Bot. Garden, Univ. Vienna;	x	x
P. veris subsp. *macrocalyx* (Bunge) L. (P26)	23.11.2018	Bot. Garden, Univ. Vienna;	x	x
P. veris subsp. *suaveolens* Gutt. & Ehrend. (P27)	05.07.2017	Italy, Liguria Monte Beigua (WU s.n.)***	x	x
P. veris subsp. *suaveolens* Gutt. & Ehrend. (P28)	07.07.2017	Italy, Liguria, Monte Toraggio (WU s.n.)***	x	x
P. vulgaris cf. x *elatior* (P29) ****	04.04.2016	Roggendorf;	x	x
Section *Sredinskya* Stein				
P. grandis Trautv. (P30)	31.05.2017	Bot. Garden, Univ. Vienna; (KG)	x	x

Nomenclature according to (Richards, 2002), except of * : according to (Eveleigh et al., 2014), ****: according to (Fischer et al., 2008); KG: ordered from Kevock Garden, Scotland; x= exsudate (E) and hydrolysis of tissue extracts (H) performed; existing** extracts were combined and used for isolation of flavonoid glycosides. In column "Location/source": Numbers of vouchers are indicated in brackets.

6. Results and Discussion

The aim of this study was to obtain information about accumulation trends within different *Primula* species. Additionally, the flavonoid composition of the leaf surface and tissues was comparatively analyzed by HPLC. The methods applied were already employed in earlier studies (Bhutia et al., 2012; Elser, 2016; Elser et al., 2016; Harborne, 1968, Hinterdobler et al., 2017; Mabry et al., 1970; Marston and Hostettmann, 2006). The obtained chromatograms from the leaf tissue flavonoid profiles proved to be quite complex, with resolution of HPLC chromatograms being low due to the accumulation of structurally related glycosides. For identification of the flavonoid aglyca, leaf tissue extracts were hydrolysed and subsequently analyzed by HPLC. The structural relationship of identified flavonoid aglyca is discussed in chapter 6.1, for structures see Figures 2, and 3. The structures of the isolated flavonoid glycosides is depicted in Figure 6 and discussed in chapter 6.2. Results for flavonoid profiles of single species are summarized in Table 2 and discussed in chapter 6.3. Further, observed accumulation tendencies and the significance of flavonoid distribution patterns within the studied taxonomic groups of *Primula* are discussed in chapter 6.4.

6.1 Diversification of aglycones in exudates and tissues

The majority of exudate flavonoids identified during this study belong to the so-called *Primula*-type flavones, assumed to originate from a yet unknown biosynthetic pathway (Elser et al., 2016). Altogether, 25 flavones were identified by HPLC profiling and structures confirmed by retention times and UV spectra corresponding to those of authentic samples of the HPLC database. In Figure 2 exudate flavones are grouped according to similarities of their substitution patterns, in increasing order of substituents, and under consideration of possible biosynthetic sequences. This resulted in groups based upon 5-hydroxyflavone (E2), 2′-hydroxyflavone (E7), 5,2′-dihydroxyflavone (E12) and 3′-hydroxyflavone (E18). As for substitution patterns, 5,6-disubstitution (E4) and 3′,4′,5′-trisubstitution (E22, E23) are the only patterns that are not confined to *Primula*-type flavones (Wollenweber and Dietz, 1981). Correlation of unusual B-ring substitution patterns in flavone (E1) and 5-hydroxyflavone (E2) are remarkable, as are 2′,5′-analogues between flavone (E1) and 5-hydroxyflavone (E2). As the biosynthetic steps are still in the dark (Elser et al., 2016), it may be speculated that flavone (E1) is a potent substrate for further enzymatic decoration (Figure 2).

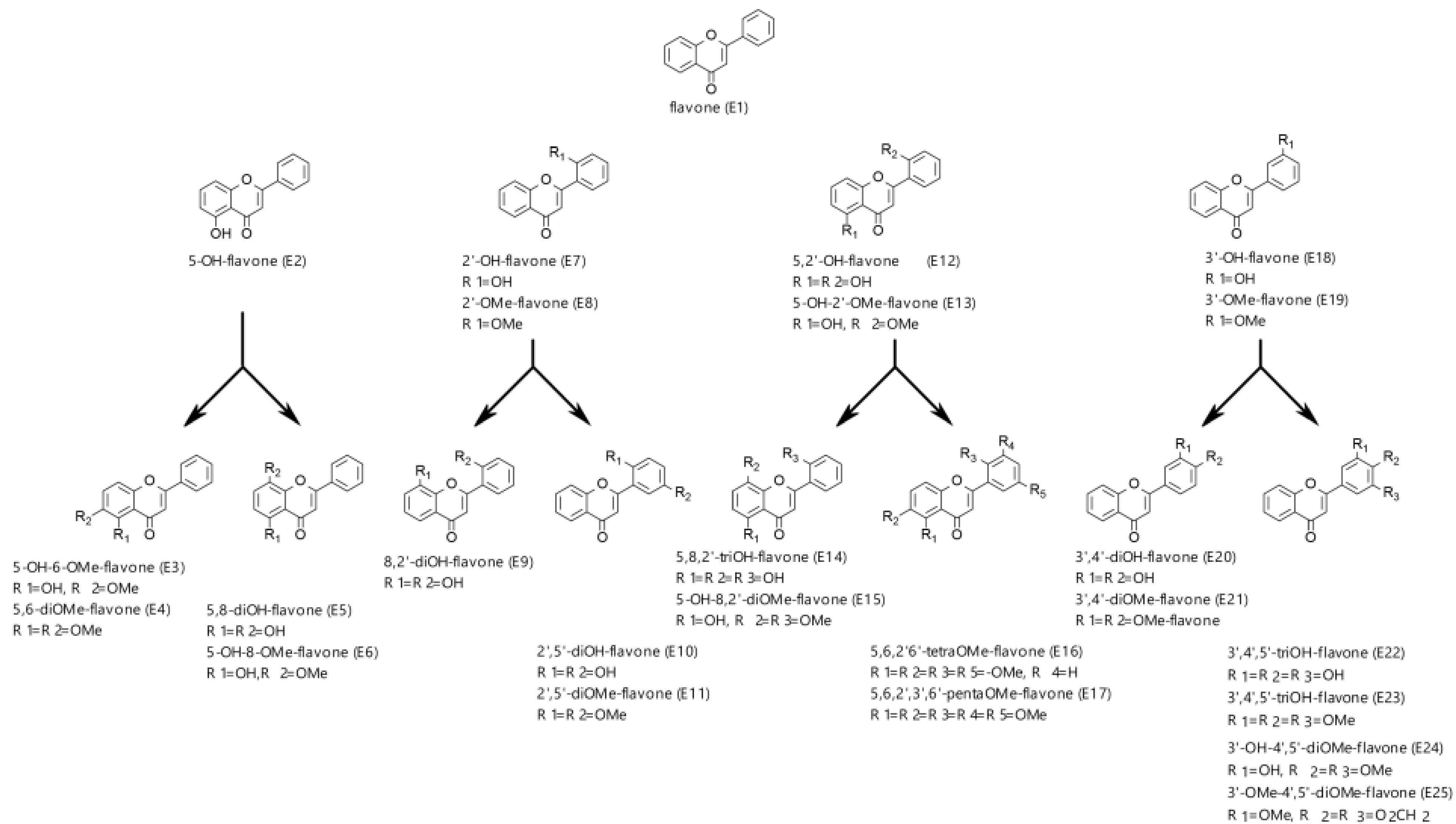
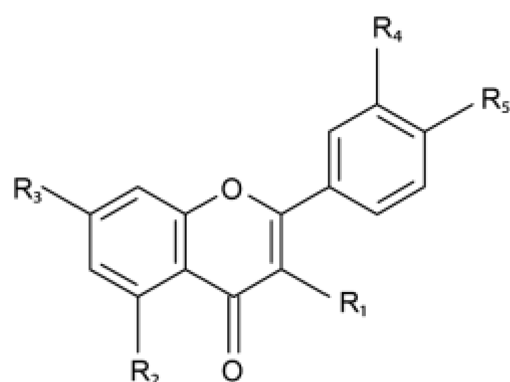


Figure 2. Structures of flavones identified in exudates of studied *Primula* species. Codes for individual structures are written in parentheses.

Diversification of glycosides is limited on the aglycone level, but apparently more diverse concerning sugar components (Harborne, 1968), with partly complex structural combinations (Colombo et al., 2014, 2017). In contrast to the large number of exudate flavones, only seven aglycones were obtained all together from tissue hydrolysates. Major compounds found are the flavonols kaempferol (H1) and quercetin (H2), and the flavone luteolin (H5) (see Figure 3). Two further flavonoids (UK1, UK2 in Table 2, and Figure 3) could not be fully identified solely on basis of retention times and UV spectra, with both being almost identical (see Figures 4, and 5). The basic structure of UK1 is quercetin (H2) with a methoxylation at either position 3′ (H3) or 4′ (H4) of ring B (see Figure 3). Relevant literature reports both glycosides of the 3′-substituted isorhamnetin (H3) and its 4′-isomer tamarixetin (H4) in *Primula* (Colombo et al., 2017), albeit with higher frequency of the 3′-substituted flavonol (H3) and only one report of its 4′-isomer (H4). This makes it more likely that isorhamnetin (H3) might rather be present in the studied sample. Similar difficulties are experienced with structurally corresponding flavones present in *Primula*. HPLC data do not allow unambiguous identification of the presence of either chrysoeriol (H6) or diosmetin (H7) (see Figure 5). Anyway, both compounds have not been reported for *Primula* so far.

Two of the *Primula*-type flavones (E18, E20) were detected in both, the exudates and hydrolysates from tissue extracts, however, not always in parallel in the same accession (compare Table 2, and chapter 6.3). The accumulation of 3’-hydroxyflavone (E18) in exudates was described before only for P. *veris* subsp. *macrocalyx* (P26) (compare Table 2) (Li et al., 2019). Furthermore, its accumulation in tissues of *Primula* species is reported here for the first time. It is excluded that these results are an artefact, as only carefully rinsed tissue, thus being largely devoid of exudate components, was analyzed for tissue composition. Recent studies revealed the presence of regular substituted flavonoids in exudates of some European alpine species of *Primula* sect. *Auricula*, albeit only as exudate and not as tissue components. Flavonoids found included the flavanones naringenin and naringenin-7,4′-dimethylether, the flavonols kaempferol (H1) and kaempferol-3-methylether, and the flavones apigenin and apigenin-7-methylether. It was hypothesized that this diversification might be a feature of phylogenetic younger lineages (Elser et al., 2016).



	R_1	R_2	R_3	R_4	R_5
Kaempferol (H1)	OH	OH	OH	H	OH
Quercetin (H2)	OH	OH	OH	OH	OH
Isorhamnetin (H3)	OH	OH	OH	OMe	OH
Tamarixetin (H4)	OH	OH	OH	OH	OMe
Luteolin (H5)	H	OH	OH	OH	OH
Chrysoeriol (H6)	H	OH	OH	OMe	OH
Diosmetin (H7)	H	OH	OH	OH	OMe

Figure 3. Structures of common flavones and flavonols from hydrolysates of *Primula*. (H1), (H2), and (H5): identified HPLC data; (H3) or (H4) = UK1 in Figure 4; (H6), or (H7): UK2 in Figure 5.

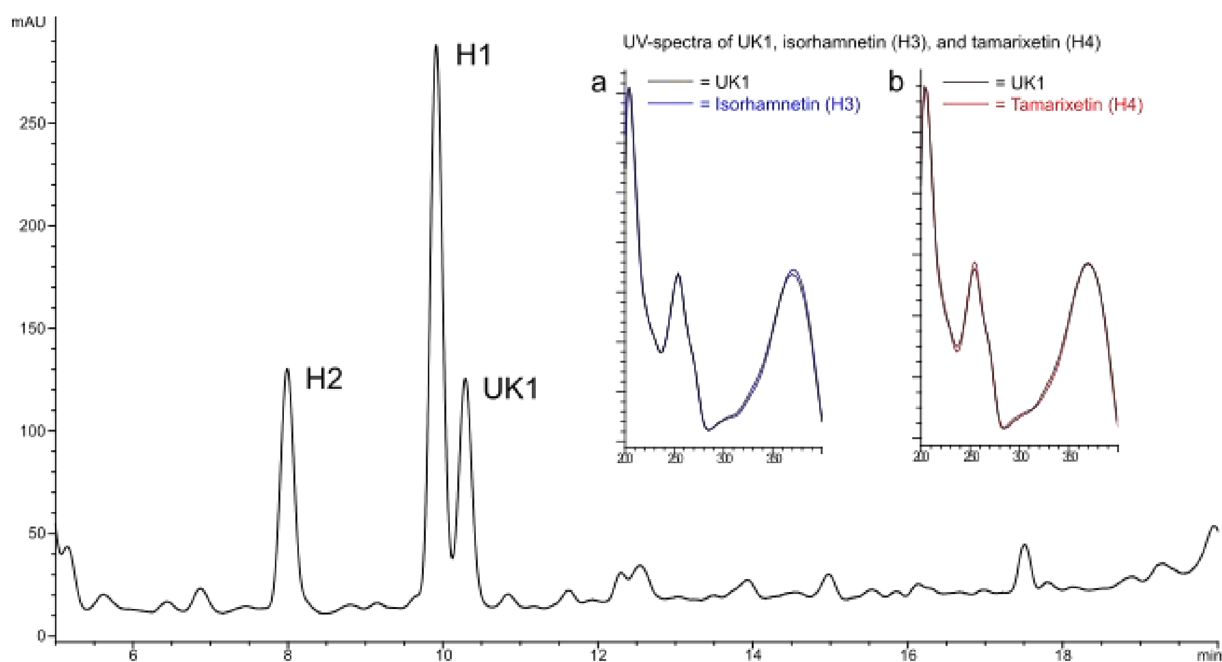


Figure 4. Chromatogram of the hydrolysate of *P. hirsuta* (P19). Quercetin (H2) and kaempferol (H1) are identified by UV spectra and retention time by comparison with authentic samples. UK1 could correspond either to 4a) isorhamnetin (H3) or to 4b) the isomeric tamarixetin (H4), with identical UV spectra and retention time.

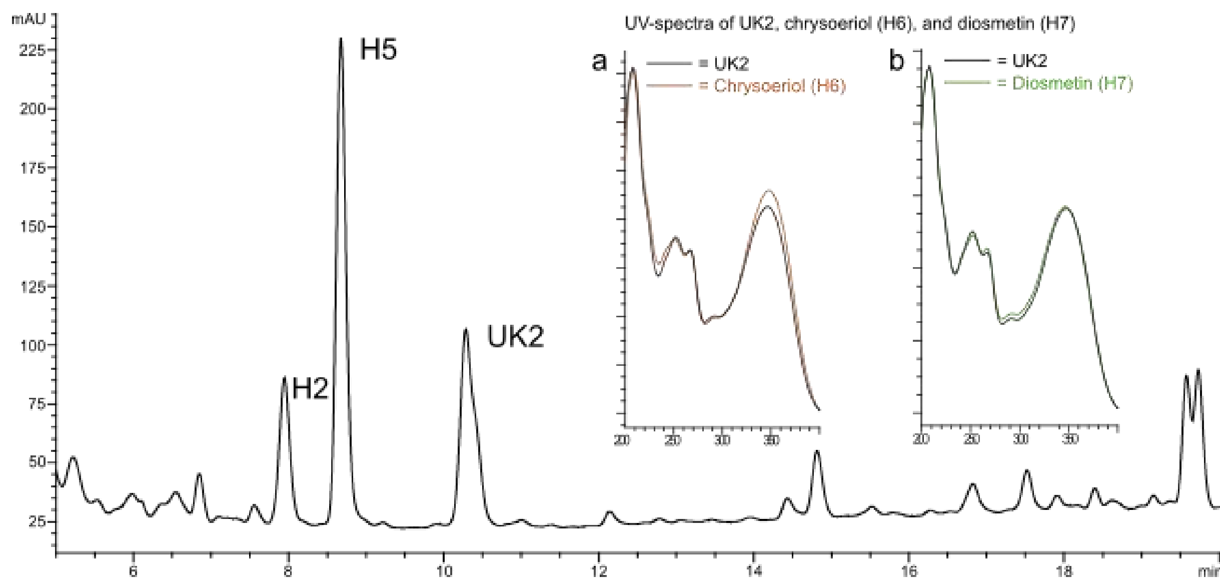


Figure 5. Chromatogram of the hydrolysate of *P. spectabilis* (P23). Quercetin (H2) and luteolin (H5) were unambiguously identified by UV spectra and retention time by comparison with authentic samples. UK2 could correspond either to 5a) chrysoeriol (H6) or to 5b) the isomeric diosmetin (H7), with identical UV spectra and retention time.

6.2 Chemical structures of isolated compounds

To obtain additional structural information about flavonoid glycosides accumulated in leaf tissues of *Primula* species, a methanolic extract of *P. auricula* subsp. *auricula* leaves (P16, P17), that had been rinsed before for separating exudate from tissue compounds, was used for isolation of flavonoid glycosides. Structure elucidation of purified compounds was performed by NMR and MS, respectively, at the Institute of Chemistry and Renewable Resources at the University of Natural Resources and Life Sciences (BOKU). Three glycosides were thus unambiguously identified as flavone-3′-*O*-β-D-glucopyranoside (**1**), 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**) and isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**3**), respectively (for structures see Figure 6; for UV spectra see Figure 7).

Flavone-3′-*O*-β-D-glucopyranoside (**1**) has not been described from the plant kingdom yet and hence it can be considered as a new report. This compound is based upon 3′-hydroxyflavone (E18), which is, together with its methyl ether (E19), a common exudate constituent of investigated *Primula* species (compare Figure 2, and chapter 6.2). Outside of Primulaceae the occurrence of its methyl ether, 3′-methoxyflavone (E19) was reported for whole plant extracts of *Pimelea decora* Domin (Thymeleaceae) (Freeman et al., 1981), although storage sites of this lipophilic flavonoid within the plant are unknown (Hinterdobler et al., 2017). Reports of flavonoids with B-ring glycosylation are scarce for *Primula* species

and are so far limited to glycosides based upon flavones glycosylated at positions 3′ or 4′ (Colombo et al., 2014). Glycosylation of position 3′ is so far, only reported from 2′-hydroxyflavone-3′-*O*-β-galactopyranosyl occurring in leaf tissues of *P. farinosa* (Colombo et al., 2017). Its aglycone, 2′,3′-dihydroxyflavone, is so far not known as exudate component from *Primula*. Earlier results indicate the presence of 2′-hydroxy-3′-methoxyflavone and 2′,3′-dimethoxyflavone as exudate constituents in some species of the related genus *Dionysia* (Hinterdobler et al., 2017).

The aglycone of glycoside **2** is 3′,4′-dihydroxyflavone and the sugar moiety consists of glucose linked to the 4′-position of the flavone. Early phytochemical analyses revealed the occurrence of this flavone in hydrolysates of aerial parts in several sections of the genus *Primula*, and in the closely related genera *Dionysia*, *Dodecatheon*, and *Cortusa* (Harborne, 1968). Harborne (1968) isolated and suspected the presence of 4′-*O*-glucoside of 3′,4′-dihydroxyflavone in extracts of *P. pulverulenta* Duthie. 3′-Hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**) is further known to occur in *P. albenensis* Banfi. & Ferl. of subsect. *Euauricula* of sect. *Auricula*, and *P. faberi* Oliv. of sect. *Amethystina*, with the latter sections being members of subgen. *Auriculastrum* (Colombo et al., 2014). The aglycone of glycoside **2** and its dimethyl ether, were identified during this survey in several species, regardless of their subgeneric alignment (compare Figure 2, and chapter 6.3). A recent comparison of published NMR data to those of 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**) revealed that the earlier reported macrophylloside (2′-hydroxyflavone-7-*O*-β-D-glucopyranoside (Ahmad et al., 1991; Colombo et al., 2017)) corresponds structurally to **2** (L. Brecker, pers. comm.) The corresponding aglycone could also not be detected in any hydrolysate when compared to an authentic commercial sample, thus being in line with NMR data analysis. Moreover, this compound has not been reported from any other natural source so far.

Glycoside **3** is based upon isorhamnetin, with the disaccharide D-xylopyranosyl-(1→6)-β-D-glucopyranoside linked at position 3 of the flavonol aglycone and is described here for the first time for *Primula*. Isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**3**) has been isolated before from *Psittacanthus calyculatus* G. Don (Loranthaceae, Santalales) (Moustapha et al., 2011), and pollen of *Cistus ladanifer* L. (Cistaceae; Malvales) (Tomás-Lorente et al., 1992). Isorhamnetin (H3) is rarely reported to occur as free aglycone (Valant-Vetschera and Wollenweber, 2006), mostly rather in glycosylated form in several plant families (Williams, 2006). During this survey, isorhamnetin (H3) was detected only in hydrolysates of species assigned to subgenera *Primula* and *Auriculastrum* (compare Table 2, and chapter 6.3).

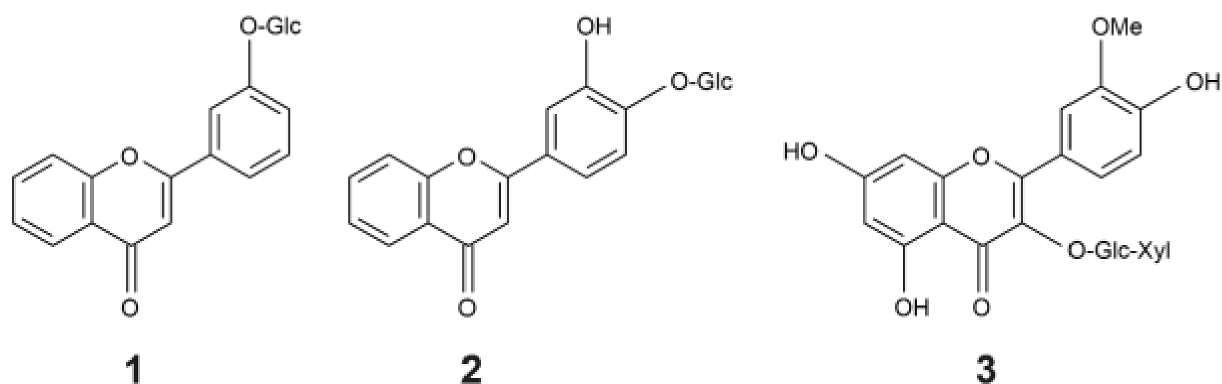


Figure 6. Structural formulae of isolated flavonoid glycosides. Flavone-3′-*O*-β-D-glucopyranoside (**1**), 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**) and isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**3**).

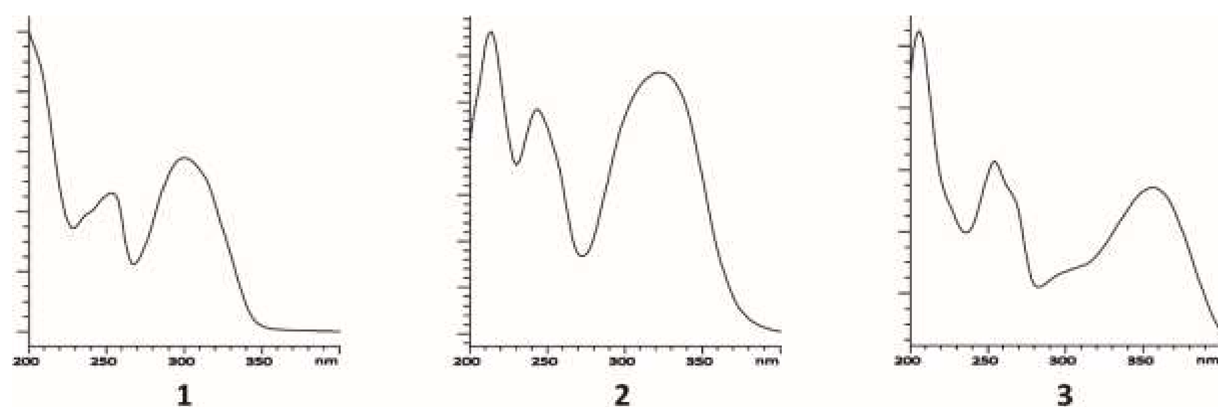


Figure 7. UV spectra of isolated flavonoid glycosides **1–3**. Glycoside **1** (UV_{max}: 254 nm, 300 nm), glycoside **2** (UVmax: 244 nm, 322 nm), and glycoside **3** (UVmax: 254 nm, 356 nm).

6.3 Flavonoid composition of studied species

Composition and accumulation tendencies of flavonoids of investigated *Primula* species were elaborated by listing flavonoids identified in exudates and hydrolysates for single species (see Table 2). Additionally, further occurrences of isolated flavonoid glycosides **1–3** in crude extracts of investigated species were included. The results were compared to published data of flavonoid composition in *Primula*. The grouping of species in subgenera and sections, generally follows Richards (2002), with exception of sectional alignment for species of subgen. *Auriculastrum,* following results of a molecular phylogenetic study (Zhang and Kadereit, 2004). The results for flavonoid composition of 30 collections, belonging to subgenera *Sphondylia* (Duby) Rupr., *Aleuritia* (Duby) Wendelbo, *Primula* L., *Auriculastrum* Schott., and *Auganthus* (Link) Wendelbo, are shown in Table2.

Comparison of results of the present study to previous phytochemical surveys partly indicates infraspecific variation of the flavonoid composition in tissues and exudates. Infraspecific variation of exudate composition as observed is rather common in *Primula,* as has been reported earlier. The authors of a recent phytochemical publication suggested that the diversification of exudate profiles in *Primula* may be the result of both, the function of flavonoids in response to the environment and/or the result of the phylogenetic history of species. Adaptions to the environment may include responses to seasonal changes, as was shown, for example, for the production of flavone, suggesting its involvement in freezing tolerance (Elser et al., 2016). Further, also changes in the composition of flavonoid glycosides in leaves of *Primula* species in response to UV were reported (El Morchid et al., 2014). Similar to inconsistencies of exudate composition (Elser et al., 2016), also variation in accumulation trends in hydrolysates was observed for both populations of *P. hirsuta* (P18, P19) (Table 2). The absence of *Primula*-type flavonoids in exudates of *P. hirsuta* (P19) coincides with the hydrolysates, as neither 3′-hydroxyflavone (E18) nor 3′,4′-dihydroxyflavone (E20) were detectable. In contrast, *P. hirsuta* (P18) accumulates typical *Primula* flavonoids in both compartments, but its composition differs between compartments, with 3′-hydroxyflavone (E18) and 3′,4′-dihydroxyflavone (E20) being present in hydrolysates only. This observation indicates infraspecific variation of flavonoid composition in both compartments. According to present results, causes for variation of flavonoid-*O*-glycoside composition in *P. hirsuta* (P18, P19) are only speculative. It may be assumed, however, that variation is driven by similar factors, such as different collection date and/or geographic origin, as suggested earlier for exudate composition (Elser et al., 2016). Following, results are discussed according to the subgeneric alignment of the investigated species.

Table 2. Flavonoids detected in exudates, and hydrolysates of *Primula* species.

	Primula	2,2'-diOH-chalcone	Flavone (E1)	2'-OH-flavone (E7)	2'-OMe-flavone (E8)	2',5'-diOH-flavone (E10)	2',5'-diOMe-flavone (E11)	8,2'-diOH-flavone (E9)	5-OH-flavone (E2)	5,8-diOH-flavone (E5)	3'-OH-flavone (E18)	3'-OMe-flavone (E19)	3',4'-diOH-flavone (E20)	3',4'-diOMe-flavone (E21)	5,2'-diOH-flavone (E12)	5-OH-2'-OMe-flavone (E13)	5,6,2',6'-OMe-flavone (E16)	5,8,2'-triOH-flavone (E14)	3',4',5'-triOH-flavone (E22)	3',4',5'-triOMe-flavone (E23)	3'-OH-4',5'-diOMe-flavone (E24)	3'-OMe-4',5'-O2CH2-flavone (E25)	5,6-diOMe-flavone (E4)	5-OH-8-OMe-flavone (E6)	5-OH-8,2'-diOMe-flavone (E15)	3'-OH-flavone (E18)	3',4'-diOH-flavone (E20)	Kaempferol (H1)	Quercetin (H2)	UK 1 (H3 or H4)	Luteolin (H5)	UK2 (H6 or H7)	Refs.
SS	*verticillata* (P1)		●	○		○			○	○			○		○	○	○	○								●	●	●	●				
ALAL	*frondosa* (P3)		●	●		○			●	○	○		🔴		○												●		●				1, 2
ALD	*denticulata* (P2)	●	●	🔴	●	🔴			🔴	🔴	●	●					●	🔴								●	●	●	●				1, 3
ALA	*involucrata* subsp. *yargongensis* (P4)		●	○					●						○													●	●				
ALPE	*boothii* subsp. *repens* (P5) **																											●	●				
ALP	*secundiflora* (P8)	●	●	○	●				○		●	●		○		○										●		●	●				
ALP	*poissonii* (P10) **																											●	○				
ALP	*wilsonii* (P11) **																											●	●				
ALP	*bulleyana* subsp. *beesiana* (P7)		●	○	●	🔴		🔴	○		○	○	🔴			○											●	●	●				1
ALP	*aurantiaca* (P6)		●						○		●	●	○														○	●	●				
ALP	*serratifolia* (P9)	●	●	○					○			○		○	○											●							
AUB	*bullata var. bracteata* (P12)	●	○																										●				
AUB	*bullata var. forrestii* (P13)	●	●	🔴					●	●					●											●			●				1
AUCO	*cortusoides* (P14) *		●	●	○		●		○		●			○	○																		
AUMO	*malacoides* (P15) *		●	○	○	○			○	🔴	○	○	🔴	○	○		○	🔴	○	🔴													1, 4
AUAEU	*auricula* subsp. *auricula* (P16)		●	●				○	○	●	●		○		○			○															
AUAEU	*auricula* subsp. *auricula* (P17)		●	●		○		○	○	●	○		○		○																		
AUAEU	*hirsuta* (P18)		🔴																							●	●	●	●	●			5
AUAEU	*hirsuta* (P19)																											●	●	●			
AUAEU	*villosa* (P20)																											●	●	●			
AUACY	*minima* (P21)		🔴						🔴	🔴																			●		●	●	5
AUACY	*glutinosa* (P22)	●							●						●														●	●	●	●	
AUACY	*spectabilis* (P23)																												●		●	●	
PP	*elatior* (P24)	●			🔴		🔴					●		●						●	●		●					●	●	●			1, 6
PP	*veris* (P25)		○	🔴	●		🔴				●	●		○			●		○	●	🔴	🔴		○				●	●	○			1, 6
PP	*veris* subsp. *macrocalyx* (P26) **		🔴	🔴	🔴		🔴				🔴	🔴					🔴				🔴						●	●	●				7
PP	*veris* subsp. *suaveolens* (P27)				●									●					○	●	●			○	○			●	●	●			
PP	*veris* subsp. *suaveolens* (P28) *					○					○	○		○					○	●	●	●											
PP	*vulgaris* cf. x *elatior* (P29)		○				●					●		●					○	●	●	●					○	●	●				
PS	*grandis* (P30)		●						○	○	○																●	●	●	●			

Sectional alignment according to (Richards, 2002; Zhang and Kadereit, 2004). Abrev.: SS (*Sphondylia* sect. *Sphondylia*), ALAL (*Aleuritia* sect. *Aleuritia*), ALD (*Aleuritia* sect. *Denticulata*), ALA (*Aleuritia* sect. *Armerina*), ALPE (*Aleuritia* sect. *Petiolares*), ALP (*Aleuritia* sect. *Proliferae*), AUB (*Auganthus* sect. *Bullatae*), AUCO (*Auganthus* sect. *Cortusoides*), AUMO (*Auganthus* sect. *Monocarpicae*), AUAEU (*Auricula* sect. *Auricula*; subsect. *Euauricula*), AUACY (*Auricula* sect. *Auricula*; subsect. *Cyanopsis*), PP (*Primula* sect. *Primula*), PS (*Primula* sect. *Sredinskya*). Coding: blue = exudates; green = hydrolysates. Compound numbers as in Figures 2, and 3; H = hydrolysates; E = exudates; ● = major compound, ○ = trace amounts, ● = cited literature data; * no results for hydrolysates; ** = no results for exudates; Cited literature: 1 = (Valant-Vetschera et al., 2009); 2 = (Colombo et al., 2017); 3 = (Elser, 2016); 4 = (Wollenweber, 1974); 5 = (Elser et al., 2016); 6 = (Bhutia and Valant-Vetschera, 2012); 7 = (Li et al., 2019).

6.3.1 Subgenus Sphondylia (Duby) Rupr.

Subgenus *Sphondylia* comprises only sect. *Sphondylia,* with eight species (Bhutia and Valant-Vetschera, 2012), and it is distributed from South-East Turkey, Sinai, and Ethiopia to Northern-India (Richards, 2002). Its members have toothed leaves with winged stalks, and vernation is interpreted as conduplicate. Further they are characterized by superimposed umbels of yellow flowers. DNA studies indicate a close relationship to members of *Dionysia* (Richards, 2002).

The only investigated species of this subgenus, *P. verticillata* (P1)*,* exhibited a quite complex exudate profile with 10 detected flavonoids, including flavone (E1), 3′,4′-dihydroxyflavone (E20), and flavonoids based upon 5-hydroxyflavone (E2), 2′-hydroxyflavone (E7), and 5,2′-dihydroxyflavone (E12) (see Table 2). Comparison with published data indicated that 5,6,2′,6′-tetramethoxyflavone (E16), 5-hydroxy-2′-methoxyflavone (E13), and 5,8,2′-trihydroxyflavone (E14) are new lists for *P. verticillata* (P1). Results of previous phytochemical investigation of exudate composition in further *Sphondylia* species show relatively low diversification of flavonoids (Bhutia and Valant-Vetschera, 2012; Valant-Vetschera et al., 2009). Flavones based upon 5,2′-dihydroxyflavone (E12) are so far not reported from this subgenus.

Results for hydrolysates of *P. verticillata* (P1) indicate the presence of flavonoid glycosides based upon 3′-hydroxyflavone (E18), 3′,4′-dihydroxyflavone (E20), kaempferol (H1), and quercetin (H2). Interestingly, neither 3′-hydroxyflavone (E18) nor 3′-methoxyflavone (E19) were detected in exudates of *P. verticillata* (P1), although the presence of tissue flavonoids based upon 3′-hydroxyflavone (E18) is indicated by accumulation of 3′-hydroxyflavone (E18) in hydrolysates. Additionally, neither flavone-3′-*O*-β-D-glucopyranoside (**1**), nor 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**) could be detected in crude extracts. Similarly, isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**3**) was not detected in crude extracts.

6.3.2 Subgenus Aleuritia (Duby) Wendelbo

Subgenus *Aleuritia* is the largest subgenus within *Primula* (Richards, 2002), comprising 18 sections (Bhutia and Valant-Vetschera, 2012). Results of a phylogenetic study indicate that subgen. *Aleuritia* is polyphyletic (Mast et al., 2001). Investigated species of subgen. *Aleuritia*, were *P. frondosa* Janka (P3) of sect. *Aleuritia* Duby, *P. denticulata* Sm. (P2) of sect. *Denticulata* Watt, *P. involucrata* subsp. *yargongensis* (Petitm.) Sm. & Forrest (P4) of sect. *Armerina* Lindley, *P. boothii* subsp. *repens* (P5) of sect. *Petiolares*, and *P. secundiflora* Franch.

(P8), *P. poissonii* Franch. (P10) and *P. wilsonii* Dunn (P11), *P. bulleyana* subsp. *beesiana* Forrest (P7), *P. aurantiaca* W.W. Sm. & Forrest (P6), and *P. serratifolia* Franch. (P9), all of sect. *Proliferae*. With exception of *P. frondosa* (P3), which occurs in Europe, all investigated species have Asiatic occurrence (Richards, 2002). HPLC chromatograms and UV spectra for *P. involucrata* subsp. *yargongensis* (P4), had too low signals for analysis. *Primula secundiflora* (P8), *P. aurantiaca* (P6), and *P. serratifolia* (P9) were investigated here for the first time with respect to their flavonoid composition (compare Table 2).

Exudate flavonoids detected in species of subgen. *Aleuritia*, were 2′-hydroxyflavone (E7), 3′-hydroxyflavone (E18), 5-hydroxyflavone (E2), 5,2′-dihydroxyflavone (E12), and their derivatives (see Table 2). In contrast to the present results, previous phytochemical research reported 5,8-dihydroxyflavone (E5), 8,2′-dihydroxyflavone (E9), 5,8,2′-trihydroxyflavone (E14), 5-hydroxy-8,2′-dimethoxyflavone, and 2′,5′-dihydroxyflavone (E10) as common exudate components for species of subgen. *Aleuritia* (Bhutia and Valant-Vetschera, 2012; Valant-Vetschera et al., 2009), but they were hardly detected during this survey (compare Table 2). Further, comparison to published data indicates infraspecific variations for some of the species. The detection of 2′,5′-dihydroxyflavone (E10), 5,8-dihydroxyflavone (E5), and 3′-hydroxyflavone (E18) in exudates of *P. frondosa* (P3) of sect. *Aleuritia*, are new reports, as are 2′-methoxyflavone (E8), 3′-hydroxyflavone (E18), 3′-methoxyflavone (E19), and 5-hydroxy-2′-methoxyflavone (E13) for *P. bulleyana* subsp. *beesiana* (P7) of sect. *Proliferae*.

Major flavonoids in investigated hydrolysates were kaempferol (H1) and quercetin (H2). In the hydrolysate of *P. frondosa* (P3) only quercetin (H2), and in the hydrolysate of *P. serratifolia* (P9) neither quercetin (H2) nor kaempferol (H1) were detected. Additionally, in hydrolysates of *P. denticulata* (P2), *P. frondosa* (P3), and *P. aurantiaca* (P6) 3′,4′-dihydroxyflavone (E20) was detected, indicating the presence of flavonoid-*O*-glycosides based upon 3′,4′-dihydroxyflavone (E20). However, 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**), was not detected in crude extracts of these species. Although 3′,4′-dihydroxyflavone (E20) was detected in hydrolysates of *P. denticulata* (P2), and *P. frondosa* (P3), it was not detected in exudates of these species during this survey. Hydrolysates of species *P. secundiflora* (P8), and *P. serratifolia* (P9) yielded 3′-hydroxyflavone (E18). Furthermore, analysis of these species indicated the presence of flavone-3′-*O*-β-D-glucopyranoside (**1**) in crude extracts, and of 3′-hydroxyflavone (E18) and 3′-methoxyflavone (E19) in exudates. In contrast to own results Colombo et al. (2017) indicate the presence of a glycoside based upon isorhamnetin, namely isorhamnetin-3-*O*-α-rhamnopyranosyl-(1→3)-*O*-[α-rhamnopyranosyl-(1→6)]-*O*-β-

galactopyranoside, that was isolated from leaf tissues of *P. halleri* J.F. Gmel.. This appears to be the only record of a glycoside based upon isorhamnetin in leaf tissues of subgen. *Aleuritia* (Colombo et al., 2017).

6.3.3. Subgenus Auganthus (Link) Wendelbo

Subgenus *Auganthus* contains nine sections, mainly distributed in Eastern Asia. Recent molecular phylogenetic research suggests subgen. *Aughanthus* only as monophyletic if subgen. *Carolinella* would be included (Liu et al., 2015). Investigated species of subgen. *Auganthus* were *P. bullata var. forrestii* (Balf. f.) P. Eveleigh, J. Nielsen & D.W.H. Rankin (P13), *P. bullata var. bracteata* (Franch.) P. Eveleigh, J. Nielsen & D.W.H. Rankin (P12), both species of section *Bullatae Pax*, *P. cortusoides* L.(P14) of section *Cortusoides Balf*, and *P. malacoides* Franch. (P15) of section *Monocarpicae* Franchet ex Pax (Richards, 2002). *Primula bullata* subsp. *bracteata* (P12) exhibited a quite undiversified chromatogram, where only 2,2′-dihydroxychalcone and flavone (E1) were detected. *Primula bullata* subsp. *forrestii* (P13) revealed a more complex exudate profile, consisting of 2,2′-dihydroxychalcone, 5-hydroxyflavone (E2), 5,2′-dihydroxyflavone (E12), and 5,8-dihydroxyflavone (E5). During the present survey no flavonoids based upon 2′-hydroxyflavone (E7) or 3′-hydroxyflavone (E18) were detected. In contrast, for *P. forrestii* (Balf. f.), which is now named *P. bullata var. forrestii* (P13) (Eveleigh et al., 2014), an earlier phytochemical survey is indicating the presence of 2′-hydroxyflavone (E7) (Valant-Vetschera et al., 2009). The substitution patterns of flavonoids detected in exudates of *P. cortusoides* (P14) and *P. malacoides* (P15) were similar compared to those of subgen. *Aleuritia,* including 5-, 2′-, 3′-, 2′,5′-di, 5,2′-di-, and 3′,4′-disubstituted flavones. The detection of 5-hydroxy-6-methoxyflavone (E3) in exudates of both species are new lists for these species. The number of flavonoids in exudates of *P. malacoides* (P15) found during this survey together with the number of flavonoids reported from prior surveys is quite amazing, as 16 exudate flavonoids out of 25 listed in Table 2 are co-occurring.

Results of hydrolysates were obtained for *P. bullata* var. *bracteata* (P12) and *P. bullata* var. *forrestii* (P13) only*,* as no leaf material for *P. malacoides* (P15) and *P. cortusoides* (P14) was available. A characteristic feature of their hydrolysates is the accumulation of quercetin (H2) and the absence of kaempferol (H1). In addition, 3′-hydroxyflavone (E18) was detected in hydrolysates of *P. bullata* var. *forrestii* (P13). Interestingly, 3′-hydroxyflavone (E18), and flavonoids based upon it, were not detected in exudates of the latter species. Comparison of crude extracts did not indicate the presence of flavone-3′-*O*-β-D-glucopyranoside (**1**), 3′-

hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**), or isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**3**).

6.3.4 Subgenus Auriculastrum Schott.

Subgenus *Auriculastrum* consists of sections *Auricula* Duby, *Suffrutescens* A.J. Richards, *Amethystina* Balf. f, J. Roy, *Parryi* W.W. Smith ex. Wendelbo, and *Cuneifolia* Balf. F., with all of them characterized by involute vernation, which is a unique feature within *Primula* (Richards, 2002). All investigated species of subgen. *Auriculastrum* belong to sect. *Auricula*. Its members are polyploid and endemic to Europe, where they most probably have migrated from Asia. Results of a phylogenetic study confirm a closer relationship of European sect. *Auricula* to East Asian/ North American sect. *Cuneifolia*, and Western North American sect. *Parryi*, rather than to any other section occurring in Europe. Analysis of ITS and AFLP sequences resulted in two major clades within sect. *Auricula*, designated as subsect. *Euauricula*, and subsect. *Cyanopsis*. Accordingly, investigated species *P. glutinosa* Wulfen (P22), *P. minima* L. (P21), and *P. spectabilis* Tratt. (P23), are members of subsect. *Cyanopsis* whereas *P. villosa* Wulfen (P20), *P. hirsuta* L. (P18, P19), and *P. auricula* L. subsp. *auricula* (P16, P17) belong to subsect. *Euauricula* (Zhang and Kadereit, 2004). Results for exudate composition of *P. hirsuta* (P18, P19), *P. villosa* (P20), and *P. minima* (P21) in Table 2 refer to a recent phytochemical survey (Elser et al., 2016). Leaf material of this study was used for further analysis.

Collections of *P. auricula* subsp. *auricul*a (P16, P17) were the only samples of subsect. *Euauricula* studied for their exudate composition during this survey. The samples exhibited a quiet complex exudate profile based upon flavonoids typical for *Primula* species (see Table 2). Comparison of literature data revealed that 5,8,2′-trihydroxyflavone (E14), 8,2′-dihydroxyflavone (E9), and 2′,5′-dihydroxyflavone (E10) are new reports for the *P. auricula* species complex, and they were detected within subsect. *Euauricula* before only in exudates of *P. palinuri* Petagna (Colombo et al., 2017). The two collections of *P. auricula* subsp. *auricula* (P16, P17) differed in their exudate composition. 5,8,2′-Trihydroxyflavone (E14) was detected only in collection P16 but it could not be detected in population P17. Vice versa, 2′,5′-dihydroxyflavone (E10) was detected only in exudates of collection P17. Both samples were of different geographic origin and time of collection differed (see Table 1). The other species of this subsection had been analyzed before (Elser et al., 2016), and were found to be deviating in their exudate composition. They mainly accumulated flavonoids from the regular biosynthetic pathway, and as for *P. hirsuta* (P18, P19), *Primula*-type flavones and those of the regular

biosynthetic pathway were accumulated in a mutually exclusive way. In exudates of population P18 only flavone (E1), common in the genus *Primula*, was detected (compare Table 2), but population P19 yielded flavonoids from the regular biosynthetic routes, such as the flavones apigenin and apigenin-7-Me, the flavonols kaempferol (H1), and kaempferol-3-methyl ether, and the flavanone naringenin (data not shown in Table 2). The authors proposed, that observed variation between both populations may be either the result of different collection dates or of different geographical origin. Further, during the latter survey the only detected flavonoid, within exudates of *P. villosa* (P20) was the flavanone naringenin-7,4′-dimethyl ether (Elser et al., 2016). Another phytochemical study indicates the presence of quercetin-7,3,4′-trimethyl ether, in *P. spectabilis* (P23) (Vitalini et al., 2011). Investigated species of subsect. *Cyanopsis* exhibited low diversification of *Primula*-type flavonoids in exudates (compare Table 2). This finding is supported by earlier phytochemical research (Colombo et al., 2017, Elser et al., 2016), and so far, flavonoids based upon 3’-hydroxyflavone are not reported from this subsection.

According to results of hydrolysates, the presence of flavonoid glycosides based upon kaempferol (H1), quercetin (H2), and the unknown flavonoid UK1 is indicated in leaf tissues of all investigated species from subsect. *Euauricula* (see Table 2). UK1 is a methylated quercetin derivative, corresponding either to tamarixetin (H4) or isorhamnetin (H3). In hydrolysates of population P18 of *P. hirsuta*, additionally 3′-hydroxyflavone (E18) and 3′,4′-dihydroxyflavone (E20) were detected. Further, flavone-3′-*O*-β-D-glucopyranoside (**1**), and 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**), both occurring in leaf tissues of *P. auricula* subsp. *auricul*a (P16, P17) (compare chapter 6.2), were also detected in leaf tissues of population P18 of *P. hirsuta*. Interestingly, neither *Primula*-type flavonoids, nor glycosides (**1**) and (**2**) were detected in population P19 of *P. hirsuta*. The results for the tissue flavonoid composition of investigated species from subsect. *Euauricula* were compared to published data, confirming the presence of glycosides based upon kaempferol (H1), quercetin (H2), isorhamnetin (H3), and tamarixetin (H4) (Colombo et al., 2017).

Within subsect. *Cyanopsis*, major flavonoids were shown to be luteolin (H5), quercetin (H2) and UK2 (see Table 2). Comparison of rt and UV spectra of UK2 with the HPLC data base and authentic standards, suggested the presence of a monomethyl ether of luteolin (UK2). During this study, luteolin (H5) and UK2 were not detected in any other subgenus. Comparison to published data indicates that *O*-glycosides based upon luteolin (H5) are not reported for subgen. *Auriculastrum*. Further, *O*-glycosides based upon chrysoeriol (H6) or diosmetin (H7) are so far unknown for *Primula* species. In the hydrolysate of *P. glutinosa* (P22), additionally

UK1 was detected. The only records for flavonoid-*O*-glycosides within subsect. *Cyanopsis* that were found in literature, concern glycosylated derivatives of quercetin (H2) (Colombo et al., 2017).

6.3.5 Subgenus Primula

Subgenus *Primula* consists of sect. *Primula*, distributed in Western Eurasia, Siberia and North Africa in the Atlas region, and of sect. *Sredinskya* Stein distributed in the western end of the main Caucasian chain, and in parts of Georgia. Section *Sredinskya* was former treated as own genus but results of comparison of DNA sequences indicate that its only species *P. grandis* Trautv (P30) is related to species of sect. *Primula*. *Primula grandis* (P30) is the only species within the genus possessing tube shaped flowers lacking a limb (Richards, 2002). Investigated species of sect *Primula* are *P. elatior* (L.) Hill (P24), *P. veris* L. (P25), *P. veris* subsp. *macrocalyx* (Bunge) L. (P26), *P. veris* subsp. *suaveolens* Gutterman & Ehrend., (P27, P28), *P. vulgaris* cf. x *elatior* (P29). The UV signals of single peaks in HPLC chromatograms of exudates from species *P. veris* subsp. *macrocalyx* (P26) were too weak for identification of flavonoids.

A characteristic tendency within subgen. *Primula* is the accumulation of a series of 3′,4′,5′-trisubstituted flavones lacking A-ring substitution, shared by all analyzed exudates of sect. *Primula*. 3′-Hydroxy-4′,5′-dimethoxyflavone (E24) was detected during this survey only in species of sect. *Primula*, excluding *P. veris* (P25). Results of previous phytochemical survey differ in respect to the occurrence of 3′-hydroxy-4′,5′-dimethoxyflavone (E24) in the latter species (Bhutia et al., 2012), indicating infraspecific variation in exudate composition for this species. In the present study 3′-methoxy-4′,5′-O_2CH_2-flavone (E25) is restricted to species of sect. *Primula*, including *P. veris* subsp. *suaveolens* (P28), and *P. vulgaris* x *elatior* (P29). Further occurrence of 3′-methoxy 4′,5′-O_2CH_2-flavone (E25) was reported earlier in *P. veris* (P25) (Bhutia et al., 2012; Valant-Vetschera et al., 2009). In contrast to sect. *Primula,* the tendency of accumulating 3′,4′,5′-trisubstituted flavones, was not shared by *P. grandis* (P30) of sect. *Sredinskiya*. Exudates of *P. veris* subsp. *suaveolens* (P27, P28) were investigated for the first time with respect to its flavonoid composition. The two different populations of the latter species (P27, P28) exhibit a rather diverse exudate profile, but they share the accumulation of 3′,4′-dimethoxyflavone (E21), and 3′,4′,5′-trisubstituted flavones (E22, E23, E24) (compare Table 2).

Major flavonols detected in hydrolysates of subgen. *Primula* were kaempferol (H1) and quercetin (H2). Additionally, the unidentified compound UK1 was detected in hydrolysates of

P. elatior (P24), *P. veris* (P25), *P. veris* subsp. *suaveolens* (P27), and *P. grandis* (P30), indicating the presence of flavonoid glycosides based upon either isorhamnetin (H3) or tamarixetin (H4) deduced from its UV spectrum. Published data indicated the presence of flavonoid-*O*-glycosides based upon isorhamnetin (H3) in flowers of *P. elatior* (P24) and *P. veris* (P25). Identified flavonoids were isorhamnetin-3-*O*-β-glucopyranoside, isorhamnetin-3-*O*-rutinoside, and isorhamnetin-3-*O*-(rhamnopyranosyl)-robinobioside, isorhamnetin-3-*O*-robinobioside (Colombo et al., 2017). Another phytochemical survey reported the presence of several flavonoid glycosides based upon methylated quercetin derivatives, and a flavonoid glycoside based upon methylated myricetin in leaves and flowers of *P. veris* (P25), but without information of the positions of methylations and glycosylations (Apel et al., 2017). Flavone-3′-*O*-β-D-glucopyranoside (**1**), 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**), and isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**3**) could not be detected in crude extracts of species of subgen. *Primula.*

6.4 Observed accumulation trends within Primula L.

Results of present study indicate a striking difference between the composition of flavonoids occurring in exudates and tissues. Exudates are characterized by flavone (E1) and a series of unusual substituted flavones based upon 5-hydroxyflavone (E2), 2′-hydroxyflavone (E7), 5,2′-dihydroxyflavone (E12) and 3′-hydroxyflavone (E18). The high diversification of unusual substituted flavones in *Primula* is remarkable, as much as 25 different substituted flavones were detected during this survey (Figure 2). Comparison to published data indicates the occurrence of even further flavonoid structures (Bhutia et al., 2012; Bhutia and Valant-Vetschera, 2012; Colombo et al., 2017; Elser et al., 2016; Valant-Vetschera et al., 2009). *Primula*-type flavones are further reported from genera *Cortusa* and *Dionysia* (Valant-Vetschera et al., 2009), that both are suggested to have evolved from within *Primula* (Richards, 2002). Besides the characteristic accumulation of *Primula*-type flavonoids, recent research indicates the presence of flavonoids deriving from regular biosynthetic pathways in exudates. This observation is so far restricted to evolutionary younger lineages occurring in Europe, including mainly species of subgen. *Auriculastrum*. Thus, the earlier hypothesis that only *Primula*-type flavonoids occur in exudates is not fully supported (Elser et al., 2016).

Despite the high structural diversity of exudate flavonoids, a characteristic tendency, unambiguously identifying one taxonomic group within genus *Primula*, is not obvious from the present results. For example, the accumulation of 3′-hydroxy-4′,5′-dimethoxyflavone (E24), and 3′,4′,5′-trihydroxyflavone (E22), or its trimethyl ether (E23), was observed in all

investigated species of sect. *Primula*. Although the accumulation of 3′,4′,5′-trisubstituted flavones is a rare condition within *Primula*, presence of 3′,4′,5′-trihydroxyflavone (E22) in *P. malacoides* (P15) of subgen. *Auganthus* merits attention (compare Table 2). Further, previous phytochemical research reports that 3′,4′,5′-trisubstituted flavones appear to be absent in Turkish relatives of *P. elatior*, namely *P. elatior* subsp. *pallasii* and *P. elatior* subsp. *meyeri*, which are both members of sect. *Primula*. The significance of such diversification in exudate patterns is still unclear. Generally, it is assumed that evolutionary younger lineages are characterized by a higher degree of diversification in exudates, and that exudates serve functions, that are adapted to the environment (Elser et al., 2016).

In contrast to exudates, hydrolysates were generally dominated by flavonoids mainly derived from the regular biosynthetic pathway, including kaempferol (H1), quercetin (H2), luteolin (H5), UK1, which is assumed to be either isorhamnetin (H3) or tamarixetin (H4) (compare Figures 3, and 4), and UK2, assumed to be either chrysoeriol (H6) or diosmetin (H7) (compare Figures 3, and 5). In hydrolysates, major compounds kaempferol (H1) and quercetin (H2) were present in all subgenera. Differences between species and subgenera were observed in the accumulation of UK1 (H3 or H4), luteolin (H5) or UK2 (H6 or H7). Compound UK1 was identified during the present survey in all investigated members of subsect. *Euauricula,* part of species of subsect. *Cyanopsis*, and part of species of subgen. *Primula*. Although *P. grandis* (P30) is not sharing the accumulation of 3′,4′,5′-trisubstituted flavones in exudates with other investigated species of subgen. *Primula*, it is sharing the tendency to accumulate UK1 in hydrolysates with some members of sect. *Primula* (compare Table 2). Structure elucidation of glycoside **1**, and results of previous phytochemical research indicate the presence of flavonoid-*O*-glycosides based upon both, isorhamnetin (H3) and tamarixetin (H4), in species of subgen. *Auriculastrum*. So far, the only report for the occurrence of a flavonoid-*O*-glycoside based upon tamarixetin (H4) concerns a species of subsect. *Euauricula*, namely *P. daonensis* Leyb.. Occurrence of flavonoid-*O*-glycosides based upon isorhamnetin (H3) within the genus is reported beside members of subgen. *Auriculastrum* from flowers of *P. veris* (P25), and *P. elatior* (P24) (both subgen. *Primula*), and from *P. halleri* J. F. Gmel. of (subgen. *Aleuritia*). So far, flavonoid-*O*-glycosides based upon isorhamnetin (H3) or tamarixetin (H4) are reported only from European distributed species (Colombo et al., 2017), and from *P. grandis* (P30), occurring in the Caucasian mountains (Richards, 2002). Concerning the latter observation, it is worth mentioning that the majority of literature for tissue flavonoids used for comparison deals with European distributed species, thus leading to an incomplete picture.

Another difference in accumulation tendencies in hydrolysates is the presence of luteolin (H5) and UK2 (H6 or H7), which appears to be restricted to species of subsect. *Cyanopsis* (compare Table 2). In contrast to own results, literature data indicate further occurrence of *O*-glycosides based upon luteolin (H5) outside subsect. *Cyanopsis*. Luteolin-7-*O*-β-D-glucopyranoside was recently reported to occur in herbal extracts of *P. veris* of sect. *Primula* (Latypova et al., 2019), but without specification, which plant parts were used for extraction. Concerning UK2, its detection in all species of subsect. *Cyanopsis* (subgen. *Auriculastrum*) merits attention. *O*-Glycosides based upon either diosmetin (H7) or chrysoeriol (H6) would be new reports for *Primula*. Isolation and structure elucidation of the respective flavone-*O*-glycoside has to be performed for structural identification. Additionally, comparison of either hydrolysates or crude extracts of a larger number of species with reference compounds are recommended to assess specific accumulation of UK2 in subsect. *Cyanopsis*.

According to previous published data (Colombo et al., 2017), and the results of this survey, all, so far known, *Primula*-type flavones being involved in the formation of *O*-glycosides, are 3′-hydroxyflavone (E18), 3′,4′-dihydroxyflavone (E20), and 2′,3′-dihydroxyflavone. During the present survey, a scattered distribution of flavone-*O*-glycosides based upon 3′-hydroxyflavone (E18), and 3′,4′-dihydroxyflavone (E20) was observed within the genus. Structure elucidation of flavone-3′-*O*-β-D-glucopyranoside (**1**), and 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**) confirmed the occurrence of flavonoid-*O*-glycosides based upon these flavonoids in leaf tissues of *P. auricula* subsp. *auricula* (P16, P17). Additionally, previous research indicates the presence of glycoside **2** in *P. albenensis,* Banfi. & Ferl. (sect. *Auricula*), and *P. faberi* Oliv. (sect. *Amethystina*), both being members of subgen. *Auriculastrum* (Colombo et al., 2014). Additionally, HPLC profiling, performed in the present survey, suggests further occurrence of glycoside **1** in *P. secundiflora*, (P08) and *P. serratifolia* (P09) of subgen. *Aleuritia*, and accumulation of glycosides **1** and **2** in population P18 of *P. hirsuta* (subgen. *Auriculastrum*). Presence of these compounds in other crude extracts may not be excluded, as the applied HPLC method gave partly insufficient separation of glycosides, thus hindering analysis of crude extracts. According to this observation, an improvement of the HPLC method for future investigations is suggested for efficient separation of flavonoid glycosides.

Interestingly, results of the present survey indicate differences for the accumulation of 3′-hydroxyflavone (E18) and 3′,4′-dihydroxyflavone (E20) between tissues and trichomes in some species. Beside the accumulation or absence of 3′-hydroxyflavone (E18) and 3′,4′-

dihydroxyflavone (E20) in both compartments, in some species, the accumulation was detected only in one of both compartments. In *P. grandis* (P30), and population P18 of *P. hirsuta,* accumulating either 3′-hydroxyflavone (E18) or 3′,4′-dihydroxyflavone (E20) in hydrolysates, none of the aglycones was detected in exudates. Vice versa, 3′-, or 3′,4′-substitution patterns are present in exudates of *P. veris* (P25), and *P. elatior* (P24), but absent from hydrolysates of the same species (compare Table 2). So far, no literature dealing with this aspect was found. According to own results it is not yet clear if transport of 3′-hydroxyflavone (E18) and 3′,4′-dihydroxyflavone (E20) occurs between different compartments, or if biosynthetic pathways may occur in both compartments in parallel. Besides glycosides based upon 3′-hydroxyflavone (E18) and 3′,4′-dihydroxyflavone (E20), another flavone apart from regular 5,7,4′-substitution, 2′,3′-dihydroxyflavone, is reported to occur as glycoside in leaf tissue of *P. farinosa* (Colombo et al., 2017). 2′,3′-Dihydroxyflavone is another flavonoid so far undetected in exudates of *Primula* species, but its mono- and dimethyl ether are known to occur in the closely related genus *Dionysia* (Hinterdobler et al., 2017).

Concerning the sugar moiety, all so far known *Primula* flavonol-*O*-glycosides, deriving from regular biosynthetic pathways, share glycosylation at position 3. They possess partly complex sugar combinations, consisting of up to three sugar units, including glucose, rhamnose, arabinose, mannose, galactose and xylose. The so far reported *Primula* flavone-*O*-glycosides are based upon *Primula*-type flavones, and upon flavones deriving from regular biosynthetic pathways. They have in common that they are all monosaccharides, involving either glucose or galactose. Contrary to flavonol-*O*-glycosides, the sugar moiety of *Primula* flavone-*O*-glycosides may be attached at different positions of the flavone core. So far, in all flavone-*O*-glycosides, possessing hydroxyl groups at ring A and B, glycosylation seems to occur always on position 7 of the flavone core (Colombo et al., 2017). This is especially confirmed for luteolin-7-*O*-β-D-glucopyranoside (Latypova et al., 2019). The so far reported *Primula*-type flavones, known to be involved in the formation of *O*-glycosides, possess no A ring substitution, and glycosylation for this type occurs at positions 3′ or 4′ (Colombo et al., 2017).

Chemotaxonomic value of flavonoid glycosides can so far not be assessed on basis of present results and published data. Generally, fewer literature dealing with flavonoid glycosides compared to literature dealing with exudate flavonoids is found, and the present study concerns only a small part of *Primula* species. Furthermore, results of existing literature seem to be incomplete. A review of phytochemical investigations on European *Primula* species, for example, reports only three flavonoid glycosides occurring in leaf tissues of *P. auricula*

(Colombo et al., 2017). Flavone-3′-*O*-β-D-glucopyranoside (**1**), 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**), and isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (**3**) are added now as new tissue glycosides of this species, and two of these are new records for the genus *Primula*.

7. Conclusion

Results of the current study indicate a striking difference between the composition of flavonoids accumulating in exudates and aerial tissues of *Primula* species. Glandular exudates exhibited a high structural diversity of flavone aglycones of unknown biosynthetic origin (*Primula*-type flavones). In contrast, corresponding leaf tissue was characterized by a high number of unknown flavonoid glycosides. Major flavonoids in hydrolysates mainly follow regular substitution patterns, including kaempferol, quercetin, luteolin, UK1 (isorhamnetin or tamarixetin), and UK2 (chrysoeriol or diosmetin). Of the latter, UK2 was detected exclusively in species of subsect. *Cyanopsis*. Further research is recommended, including isolation and structure elucidation of UK2, to confirm the presence of either chrysoeriol or diosmetin, both unknown for *Primula*. The low number of detected aglycones in hydrolysates of leaf extracts, vice a large number, of glycoside peaks in the HPLC chromatogram suggests that structural diversity of *O*-glycosides is caused rather by diversity and complexity of attached sugars.

The isolation of flavone-3′-*O*-β-D-glucopyranoside (**1**), and 3′-hydroxyflavone-4′-*O*-β-D-glucopyranoside (**2**) from leaf tissue extract of *P. auricula* subsp. *auricula*, gave two rare examples of *O*-glycosides in leaf tissues based upon *Primula*-type flavonoids. Further, the scattered distribution of, 3′-hydroxyflavone, and 3′,4′-dihydroxyflavone, in hydrolysates of investigated species, suggests a more widespread occurrences of the corresponding *O*-glycosides. So far, the accumulation of 3’-hydroxyflavone in leaf tissues is a newly reported feature for *Primula*, and the detection of glycoside **1** is the first report for its occurrence in the plant kingdom. Interestingly, 3′-hydroxyflavone, and 3′,4′-dihydroxyflavone were not always accumulated in both compartments (glandular hairs, leaf tissue) in parallel. This observation requires further research to evaluate, if either, sites of synthesis are active in both compartments, or if transport of compounds may occur between different compartments.

According to the high number of unknown flavonoid glycosides detected in investigated species, their chemotaxonomic value cannot be evaluated at present. Notable, infraspecific exudate variation of *P. hirsuta* as reported earlier, was now also shown for tissue flavonoids by analysis of hydrolysates of the same species. Thus, infra-taxon variation of flavonoid profiles

is an important feature that needs further investigation, primarily in respect to seasonal changes and responses to stress. Checking for variation is important before chemicals are used as additional systematic characters. Finally, critical remarks should be made on the HPLC methods applied for separation of flavonoid glycosides. Generally, there is no method applicable today that shows good resolution of structurally related flavonoid glycosides as is the case in *Primula*, so improvements are strongly warranted in the future.

8. References

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9. Zusammenfassung

Diese Arbeit beschäftigt sich mit der Verbreitung von Flavonoiden in Geweben und Exsudaten innerhalb der Gattung der Primeln. Bisherige Arbeiten beschäftigten sich hauptsächlich mit der Zusammensetzung der Flavonoid-Aglyka in den Exsudaten der Primelarten, die in Drüsenhaaren gebildet werden. Die Mehrzahl dieser Exsudat-Flavonoide sind Flavone, die durch ungewöhnliche Substitutionsmuster charakterisiert sind. Die Biosynthese dieser primeltypischen Flavonoide ist bis heute ungeklärt. Im Gegensatz dazu, ist Literatur zum Vorkommen einzelner Flavonoid-Glykoside innerhalb der Gattung nur spärlich vorhanden. Die Mehrzahl der bekannten Flavonoid-Glykoside innerhalb der Gattung der Primeln, basiert auf Flavonoiden, die aus bekannten Biosynthesewegen stammen.

Für die Untersuchung der Zusammensetzung der Flavonoide in den unterschiedlichen Kompartimenten wurden die Exsudate, Rohextrakte und deren Hydrolysate, von 30 verschiedenen Primelproben, mit der HPLC analysiert und die Ergebnisse miteinander verglichen. Im Rahmen dieser Untersuchung wurden drei Flavonoid-Glykoside, Flavon-3′-*O*-β-D-glucopyranosid (**1**), 3′-Hydroxyflavon-4′-*O*-β-D-glucopyranosid (**2**), und Isorhamnetin-3-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranosid (**3**), isoliert. Dies ist der erste Bericht über das Vorkommen von Glykosid **1** innerhalb des Pflanzenreiches.

Die Zusammensetzung der einzelnen Flavonoide, zeigt einen deutlichen Unterschied zwischen den beiden Kompartimenten. Die Exsudat-Flavonoide weisen eine hohe strukturelle Vielfalt und die für die Primeln typischen Substitutionsmuster auf. Im Gegensatz dazu akkumulieren in den Geweben eine Vielzahl unbekannter Flavonoid-Glykoside, die auf nur wenigen Flavonoid-Aglyka basieren. Zu den häufig detektierten Aglyka in den Hydrolysaten der untersuchten Arten zählen die Flavonole Kämpferol und Quercetin, welche aus bekannten Biosynthesewegen stammen. Vereinzelt wurden auch Luteolin und zwei unbekannte Flavonoide, bei denen es sich vermutlich um die 3′-, oder 4′-Methylether des Luteolins und des Quercetins handelt, nachgewiesen. Das Vorkommen von Luteolin und seinem Methylether war auf Arten der Untersektion *Cyanopsis* beschränkt. Zusätzlich zu diesen Flavonoiden wurden vereinzelt, zwei aus den Exsudaten bekannte Verbindungen, 3′-Hydroxyflavon und 3´,4′-Dihydroxyflavon, nachgewiesen. Die Akkumulation von 3′-Hydroxyflavon in Hydrolysaten von Primeln ist hier zum ersten Mal nachgewiesen. Die Ergebnisse deuten darauf hin, dass *O*-Glykoside, basierend auf den primeltypischen Exsudat-Flavonoiden, häufiger vorkommen als ursprünglich angenommen. Die während dieser Arbeit festgestellte Diversität der Flavonoid-Glykoside, kann eine Grundlagen für weitere phytochemische Untersuchungen darstellen.