universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Comparative analysis of exudate production and composition in selected *Primula* species"

verfasst von / submitted by

David Elser, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien/ Vienna, 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 832
Studienrichtung lt. Studienblatt / degree programme code as it appears on the student record sheet:	Masterstudium Botanik
Betreut von / Supervisor:	Ao. Univ. Prof. Dr. Karin Vetschera

बन्धुरात्मात्मनस्तस्य येनात्मैवात्मना जितः ।
अनात्मनस्तु शत्रुत्वे वर्तेतात्मैव शत्रुवत् ॥ ६ ॥

bandhur ātmātmanas tasya

yenātmaivātmanā jitaḥ

anātmanas tu śatrutve

vartetātmaiva śatru-vat

“For him who has conquered the mind, the mind is the best of friends; but for one who has failed to do so, his mind will remain the greatest enemy.” (Bhagavad-gītā 6.6)

Acknowledgments

First of all I would like to express my sincere gratitude to my supervisor Assoc. Prof. Dr. Karin Valant-Vetschera for providing continuous support, freedom, advice and comments to lift up the quality of my work and gain new ways of thinking.

My sincere thanks goes to Dr. Johann Schinnerl for his patience in assisting during lab work and giving so much technical advice and inspirations to enhance the results of my work.

Besides I am grateful to Assoc. Prof. Dr. Lothar Brecker and Susanne Felsinger from the Institute of Organic Chemistry, University of Vienna for structure elucidation of isolated compounds.

I would like to thank Prof. Dr. Eckhard Wollenweber Institut für Botanik der TU Darmstadt, Germany for supplying his great collection of authentic flavonoid samples, which made my lab life much easier.

Additionally I would like to thank Prof. Dr. Wolfram Weckwerth, Hannes Dörfler, PhD (Department of Molecular Systems Biology, University of Vienna) and Eurofins Lebensmittelanalytik GmbH for giving access to mass spectrometry facilities.

Furthermore I am thankful to Assoc. Prof. Dr. Martina Weber for making microscopic studies possible.

Christian, Andreas, Markus, Wolfgang and Florian thank you for being always open to any questions, discussions or help.

Also I am very grateful for financial support from my family during my whole studies.

Finally, I would like to thank Shyama my partner in life for being so caring and giving so much emotional support.

Contents

1. Abstract

The mealy or oily exudates of *Primula* L. have long been of interest for many researchers. First they were considered only to consist of unsubstituted flavone, later various other flavones with unusual substitution patterns and even dihydrochalcones and orphan flavonoids were detected. Compared to the high number of species only few species (mostly Asian) have been studied for exudate composition, and research on seasonal variation is scarce. Therefore this thesis aims to get more insight into the exudate diversification especially of European alpine species, as well as possible seasonal variation of exudate profiles. Various methods for isolation of glandular trichomes were evaluated for optimal yield of exudates. Species of interest were collected from their natural habitat or were cultivated. Exudates and their components were analyzed by HPLC, MPLC, CC, TLC, MS and NMR. Results indicate that the diversity of exudates in European alpine species is not restricted to unusual flavonoids typical for *Primula*. By contrast, regular substituted flavones, flavanones and favonols are found in excretions. Additionally the diversity is enhanced by the production of diterpenes, primin- like substances and yet unknown compounds. Furthermore flavonoids in exudates could not be detected at all in some species. Qualitative and quantitative seasonal variation in exudate composition was also observed within populations, which should be investigated in more detail in further studies.

2. Introduction

The genus *Primula* has long been of interest for humans, at first mainly as ornamentals and therefore particularly for horticulture (Richards, 2002). Very early the conspicuous exudates that are produced in glandular trichomes on leaves, stems and calyces, became of interest for scientists (Brunswik, 1922; Müller, 1915). Müller (1915) first thought that the exudate consists of unsubstituted flavone only. Later researchers found chalcones (Wollenweber and Mann, 1986) and various different flavones with unusual substitution patterns as exudate constituents (Bhutia and Valant-Vetschera, 2012; Budzianowski et al., 2005; Budzianowski and Wollenweber, 2007; Valant-Vetschera et al., 2009; Wollenweber, 1974; Wollenweber et al., 1990, 1989, 1988a, 1988b; Wollenweber and Mann, 1986). Due to this unusual substitution patterns, it was assumed that they derive from a yet unknown biosynthetic pathway and thus were consequently named as "*Primula*-type" flavonoids (Bhutia and Valant-Vetschera, 2012). Further research revealed dihydrochalcones (Bhutia et al., 2013; Bhutia and Valant-Vetschera, 2012) and a new type of flavonoids which were not known from nature before (Bhutia et al., 2013). Other recent studies on *Primula* also showed that European species seem to be able to produce flavonoids of the regular biosynthetic pathway (Colombo et al., 2014; Vitalini et al., 2011). Isshiki et al., (2014) have demonstrated that unsubstituted flavone is correlated with increasing freezing tolerance in *Primula malacoides* Franch. Early in the year farinose primroses are densely covered with a farina consisting of quasi crystalline flavonoids (Isshiki et al., 2014) but during the year the meal seems to get lost probably due to mechanical action of the environment (Brunswik, 1922). The exudates may also play a role in allelopathy which is known from ferns (Cooper-Driver, 1980). Exudate flavones may also prevent the growth of various pathogens because they show cytostatic properties (Tokalov et al., 2004). Some species are and were also of interest for medicinal purposes, like *Primula veris* for its root saponins (Çalis et al., 1992) and flavonoids in and on flowers and leaves (Budzianowski et al., 2005). *Primula denticulata* Sm. also known as Indian anti-snake venom plant (Mors et al., 2000) may become of interest in cancer treatment due the effects of its exudate flavonoids (Tokalov et al., 2004).

Based upon previous work on exudate composition of *Primula* species (e.g. Bhutia et al., 2013, 2012; Bhutia and Valant-Vetschera, 2012; Valant-Vetschera et al., 2009), this thesis aims to answer the question if European alpine species exhibit similar or different biosynthetic pathways expressed in exudate composition. New species and accessions were studied in this respect and respective results are discussed in two separate chapters. An important aspect of this work is the analysis of seasonal variation of exudate production and testing of methods for deeper study of the exudates and the glandular trichomes that produce them.

3. Botany

3.1 **Primulaceae** Batsch ex. Borkh.

The Primulaceae s.l. is a family that belongs to the order Ericales (Fig. 1), it is distributed worldwide and it comprises 58 genera with about 2590 species (Stevens, 2001). It is divided in four subfamilies, the Maesoideae, Theophrastoideae, Primuloideae and the Myrsinoideae, which earlier were recognized as own families (Mast et al., 2001). Important morphological characters of the Primuloideae are articulated trichomes, leaf vernation involute or revolute, leaf margins entire to dentate or serrate, scapose inflorescences with medium sized flowers, calyx often connate, corolla salverform and the stamina often attached at or above the middle of the corolla tube (Stevens, 2001).

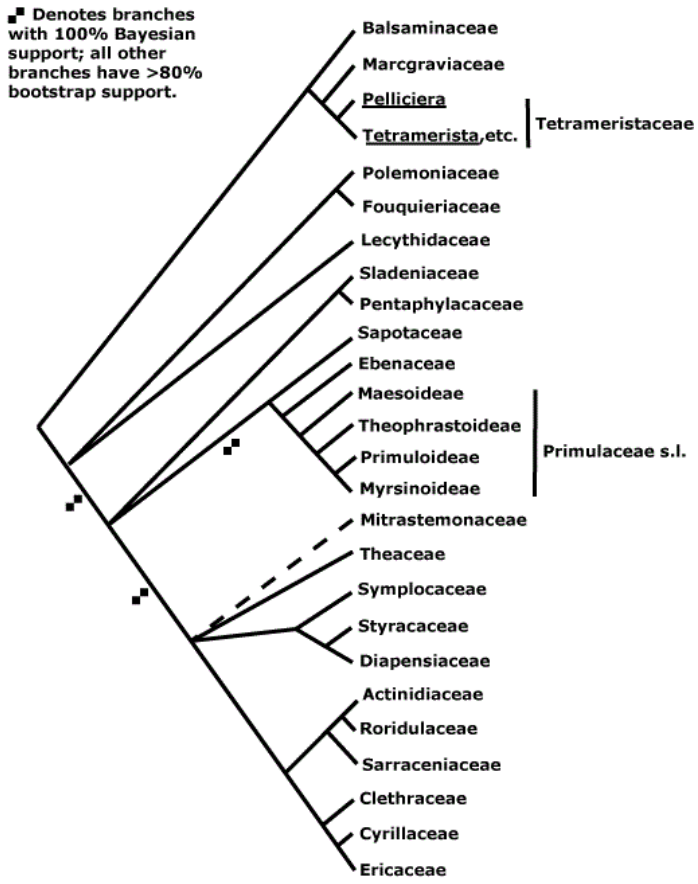

Fig. 1. Phylogenetic relationships within the Order Ericales (modified from Stevens, 2001).

3.2 Genus *Primula* L.

The genus *Primula* belongs to the subfamily Primuloideae which comprises of the genera *Androsace*, *Bryocarpum*, *Cortusa*, *Dionysia*, *Dodecatheon*. *Hottonia*, *Kaufmannia*, *Omphalogramma*, *Pomatosace*, *Soldanella* and *Vitaliana*, distributed mostly north of the Tropic of Cancer (Fig. 2) (Stevens, 2001). *Primula* has about 500 species with 7 subgenera were most of the species are found in the Sino-Himalayan region (Zhang and Kadereit, 2004). Some species also occur in South America, Africa, Arabia, Java and Sumatra. Important characters for classification are the form of styly, involution of leaves, pollen type, chromosome number, lack of or presence of meal (farina), multicellular hairs, and superimposed whorls. Heterostyly is a character that is particularly prominent in *Primula*, most of the species (91%) bear this mating type. Here the individuals have flowers with a long style (pin morph) or a short style (thrum morph) and the anthers are either deep inside the corolla tube (pin morph) or nearly reach outside of the latter (thrum morph). It is a mechanism that should avoid illegitimate crosses (selfs, plants which bear the same morph). There are also other mating systems like primary and secondary homostyly. Whereas primary homostyly is generally regarded as a primitive state in diploid species in warm temperate regions, secondary homostyly appears to be present in derived polyploid species which grow under unfavorable environmental conditions. (Richards, 2002).

Fig. 2. Geograhic distribution of the Primuloideae (Stevens, 2001).

4. Secondary metabolites

4.1 Exudates and glandular trichomes

Glandular trichomes are structures that are found on the aerial surfaces of plants such as leafs, stems or calyces. They can excrete various different secondary metabolites (e.g. terpenoids, flavonoids, phenylpropenes) and are grouped according to the nature of their exudates into trichomes excreting hydrophilic, lipophilic, proteins, poly- or monosaccharides. Additionally they can be classified according to the volatility of the material they exude (Tissier, 2012). These trichomes are built out of a basal cell in the epidermis, and one or more stalk and secretory cells, whereas the latter is responsible for the biosynthesis of the secondary metabolites.

The earliest forms of glandular trichomes were found in fossile seed ferns (*Blanzyopteris praedentata, Barthelopteris germarii*.) Earliest extant forms are present in ferns (e.g. *Pityrogramma, Cheilanthes, Notholaena*), where a farinose wax is excreted which contains primarily flavonoid aglyca and minor amounts of kaurene- type diterpenoids (Lange, 2015; Rüedi et al., 1989; Wollenweber and Schneider, 2000) There are two major hypothesis how secretory tissues have evolved. The first is that they originated from leaf mesophyll (secretory cavity) and migrated inside to build the resin ducts found in coniferous plants (Pinophyta) or that they migrated outside to form secretory tissues like in *Populus* L., or peltate and capitate glandular trichomes like in many other plant species (Fahn, 2002) .The second hypothesis is that these specialized cells evolved several times independently from stomata (Carpenter, 2006).

In *Primula* there are different types of glandular trichomes as exudate producers. The excretions may be mealy or oily, and the trichomes can be sunken, short or long stalked. There even seems to be a differentiation within one leaf, showing different types of trichomes, and a shift in developmental stages of the glandular trichomes from a pre- to secretory to a postsecretory phase is suggested (Bhutia et al., 2012). Otherwise it might be the case that different trichomes exude different compounds like known from *Helianthus* (Aschenbrenner et al., 2013). The exudates of *Primula* are mainly composed of flavonoid aglycones and related metabolites, but recent findings (Elser et al., subm.) also indicate that other compounds such as diterpenes or primin-derivatives can compose the exudate, and in somes cases, flavonoids may be totally absent.

4.2 Flavonoids

Flavonoids are a well-known class of secondary metabolites and more than 8000 different structures are known to date. The major classes of flavonoids (Fig. 4) are flavones, flavonols, favanones, dihydroflavonols and favanols (catechins). In a wider sense also anthocyanidis, chalcones, aurones, leucoanthocyanidins, procyanidins (condensed tannins) and dihydrochalcones are included in the flavonoids. They are based on a C_6-C_3-C_6 skeleton (Terahara, 2015) which is divided in an A, B and C Ring (Cao et al., 1997) (Fig. 3). The biosynthesis starts from the amino acid phenylalanine which originates from the shikimate pathway. Phenylalanine ammonia-lyase (PAL) removes ammonia from phenylalanine, then a para-hydroxyl group (p-coumaric acid) and Coenzyme A (CoA) is added which results in 4-coumaryl-CoA (p-coumaroyl CoA), a central metabolite in the phenylpropanoid pathway. In addition three units of malonyl-CoA are added by chalcone synthase (CHS) which yields chalcone. From here various different steps lead to the previously mentioned classes of flavonoids (Dixon and Steele, 1999; Falcone Ferreyra et al., 2012).

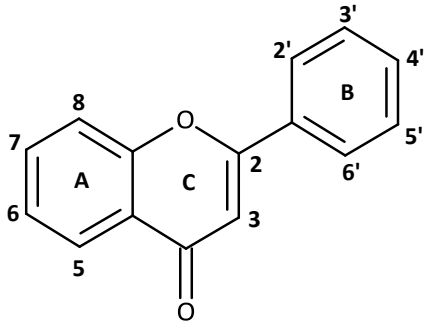

Fig. 3. Structural formula of flavone.

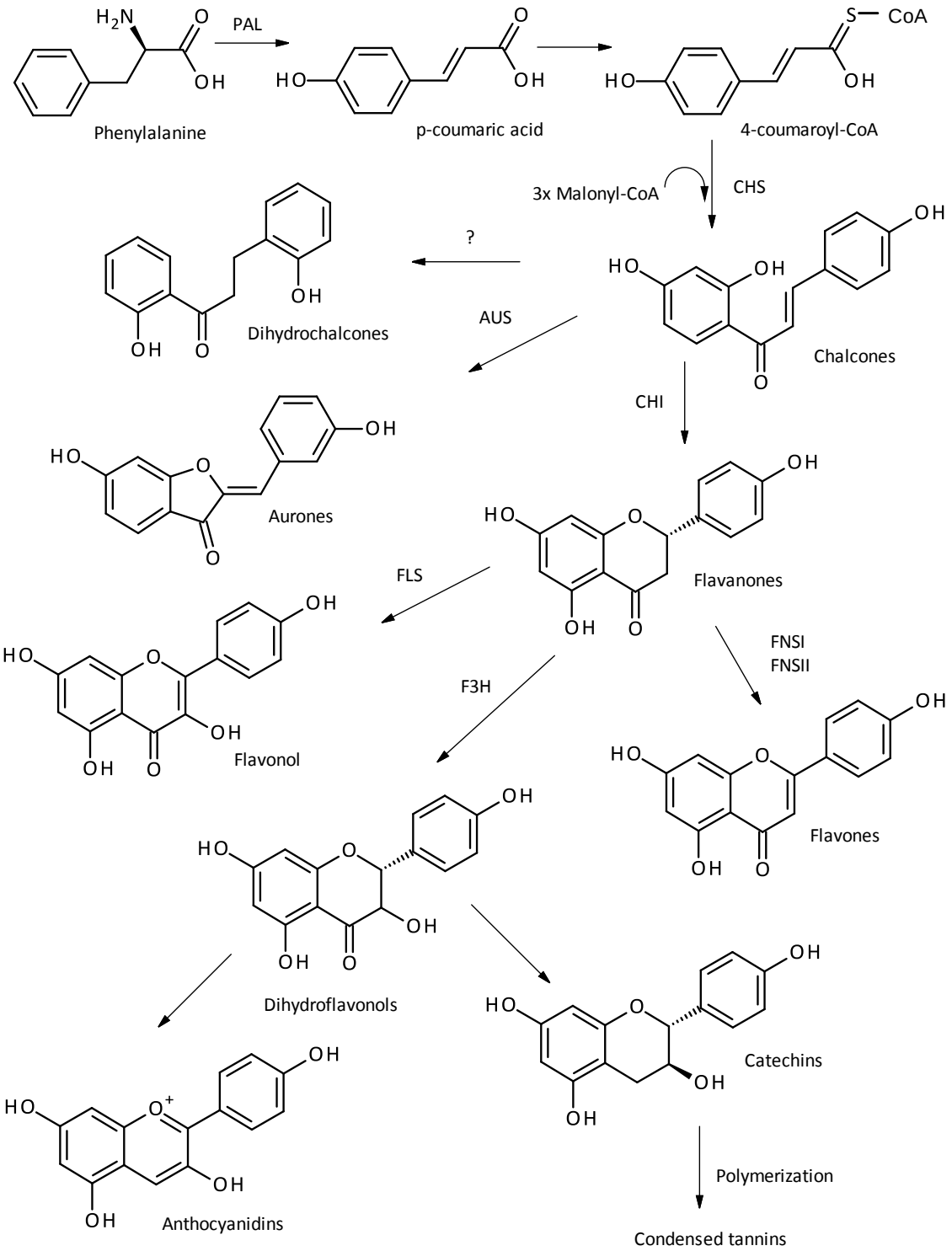

Fig. 4.: Biosynthesis of the major classes of flavonoids. PAL: Phenylalanine ammonia-lyase; CHS: Chalcone synthase; CHI: Chalcone Isomerase; AUS: Aureusidin synthase; FLS: Flavonol synthase; F3H: Flavanone 3β –hydroxylase; FNSI, FNSII: Flavone synthase 1 & 2.

Flavonoids are usually present as glycosides in vacuoles of leaves, stems or roots cells. However as lipophilic aglyca they are usually excreted. Generally flavonoids are produced across the whole kingdom of Plantae, but also some fungi and corals can produce them (Iwashina, 2000), since 1962 they are used as taxonomic markers (Harborne et al., 1975). Due to their great structural diversity many authors have used them for different taxonomic problems e.g. in Asteraceae (Emerenciano et al., 2001; Valant-Vetschera and Wollenweber, 2001), Primulaceae (Harborne, 1968; Valant-Vetschera et al., 2010, 2009) or Aspleniaceae (Iwashina et al., 1995).

In or on the plant these compounds may have different functions, and it is generally assumed that they serve as UV protectants (Ryan et al., 2002). They also play a role in plant microorganism interaction e.g. nodulation or as signal molecules in pathogen interaction, or may exhibit anti-microbial activity (Dixon and Steele, 1999).

Due to the various different bioactivities they are of interest for many research fields. In nutrition they are of interest for their antioxidative and anti-inflammatory properties (Kris-Etherton et al., 2004). In medicine they are now of special interest in treating cancer, here on the example of Chrysin (Kasala et al., 2015) which may have an enormous potential in future therapies. This flavone has also recently been isolated from the leaf exudate of *Primula halleri* Honck. (Colombo et al., 2014).

Primula-type flavones are flavones with unusual substitution patterns. They usually lack the classical 5, 7 ring A , 3'and/or 4' ring B hydroxyl groups (Valant-Vetschera et al., 2010). The biosynthesis of these flavonoids takes place in the glandular trichomes, probably in the head cells, as has been demonstrated by the presence of the biosynthetic enzymes PAL, CHS (Schöpker et al., 1995) and fluorescence microscopy of end products (Bhutia et al., 2012).

4.3 Terpenoids

Terpenoids are the most diverse and largest class of secondary metabolites which comprises an estimated number of 55000 different structures. They are based on the molecule isoprene, a C_5 hydrocarbon which is present as isopentenyl pyrophosphat (IPP) or dimethylallyl-pyrophosphate (DAMPP). These can originate either from the mevalonic acid (MVA) or the 2C-methyl-D-erythritol 4-phosphate (MEP) pathyway. The MVA pathway takes place in the cytosol, the endoplasmic reticulum and the MEP pathway in the plastids. One isoprene unit represents the hemiterpenes, these molecular building blocks are combined, while two units build the monoterpenes (C_{10}). Three isoprene units form the sesquiterpenes (C_{15}), two terpene units the diterpenes (C_{20}) and three terpene units triterpenes (C_{30}). Then there are also the sesterpenes (C_{35}) tetraterpenes (C_{40}) and polyterpenes (C_n, more than 8 isoprene units) (Fig. 5). Terpenoid production in plants is usually associated with secretory structures, where they play an important role in plant-herbivore and plant pathogen interaction (Christianson, 2008; Lange, 2015; Zwenger and Basu, 2008). One example is the induced defence, where plants can produce different volatile compounds upon an attack from an herbivore which includes terpenoids (e.g. monoterpenes, sequiterpenes), these volatiles then attracts the predator of the herbivore (Arimura et al., 2005). For the lima bean (*Phaseolus lunatus* L.*)* this has been shown clearly. After an attack form spider-mites the leaves show production of *trans*-β-ocimene and *trans*-4,8-dimethyl-1,3,7-nonatriene which is not or only produced in traces by unifested leaves (Takabayashi et al., 1991).

Essential oils are complex mixtures of volatile, lipophilic compounds with low molecular weight. Usually they are composed of 20–60 components, of which two to three compounds represent the main constituents. They are extracted from various aromatic plants via different methods e.g. distillation, use of liquid carbon dioxide, microwaves or extraction with lipophilic solvent. To date about 3000 different essential oils are known from which 300 are commercially used. Mono- and sesquiterpenes are the main constituents of most essential oils, but also may contain large amounts of aromatic or aliphatic compounds (Bakkali et al., 2008). The production takes place in different specialized cells, as shown for glandular trichomes of *Humulus lupulus* L. or *Mentha piperita* L. (Wang et al., 2008). The ecological function of these oils in plants ranges from antibacterials, antivirals, anitfungals, insecticides to deterrents. Human applications are also diverse such as in perfumes, make up products, sanitary products, dentistry, agriculture, food-additives and preservers, natural remedies or as in repellants (Bakkali et al., 2008; Nerio et al., 2010).

The diterpenoids will be mentioned here as separate short chapter as they were found by Elser et al., (subm.) to occur in the exudates of *Primula*. Diterpenoids have also been detected in exudates of *Salvia divinorum* Epling & Játiva (Siebert, 2004), *Blakiella bartsiifolia* (S.F.Blake) Cuatrec. (Rodriguez-Hernandez et al., 2014) or different Ferns (e.g. *Notholaena peninsularis* Maxon & Weath., *N. pallens* Weath.) species (Wollenweber et al., 1983).

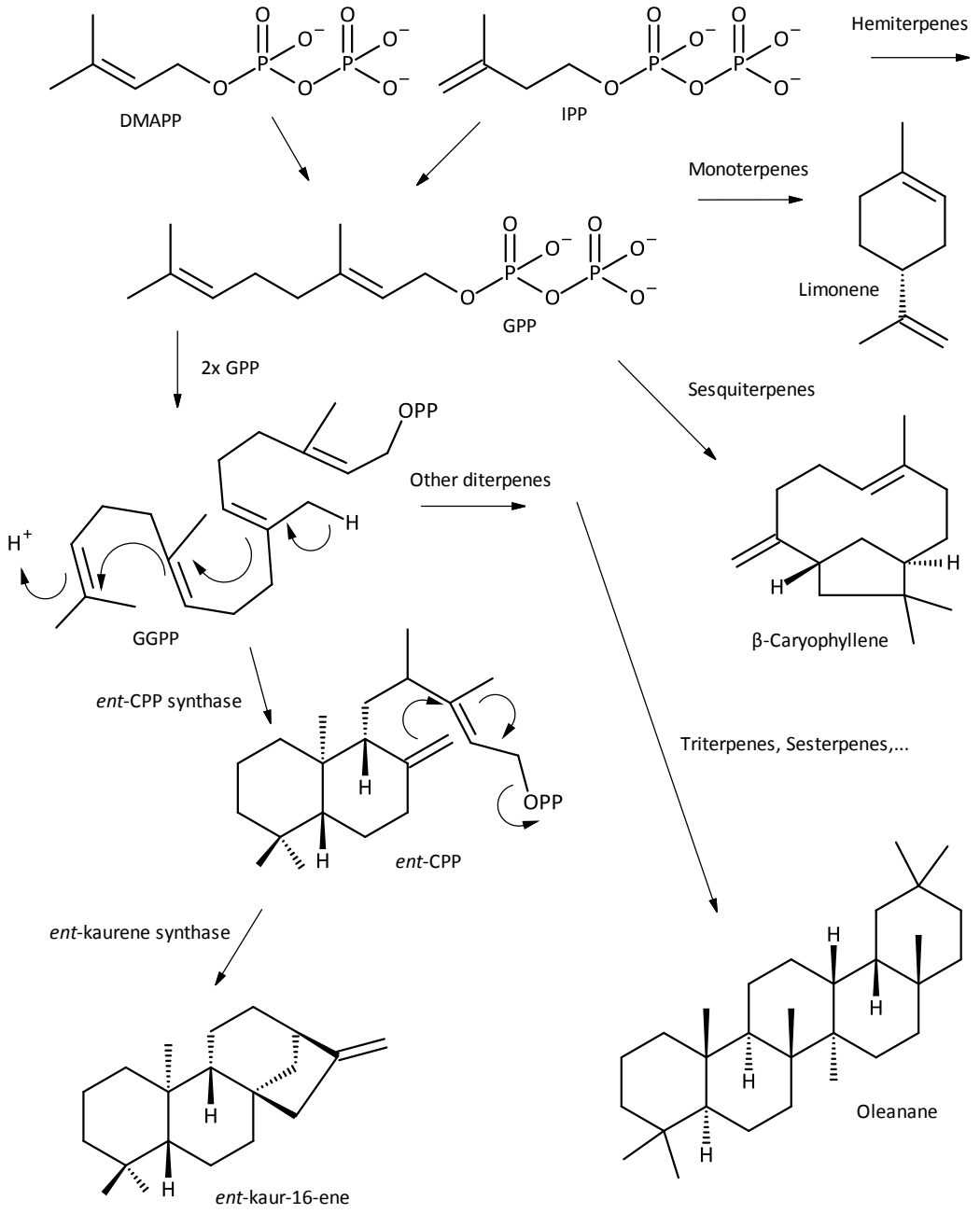

Fig. 5.: Biosynthesis of different classes of terpenoids. DMAPP: Dimethylallyl pyrophosphate; IPP: Isopentenyl pyrophosphate; GPP: Geranyl pyrophosphate; GGPP: Geranylgeranyl pyrophosphate; *ent*-CPP: copalyl pyrophosphate.

4.3.1 Diterpenoids

Various classes of diterpenoids exist, such as acyclic, bicyclic, labdanes, halimanes and clerodanes as well as tricyclic (abietanes, pimaranes) tetracyclic, macrocyclic and miscellaneous ones (Fig. 6). They may have various properties e.g. anti-microbial, anti-cancer, psychoactive or sweet tasting (Hanson, 2013; Jennewein and Croteau, 2001; Siebert, 2004). Prominent anti-microbial compounds are pleuromutilin and its semisynthetic derivative tiamulin, which are used in veterinary medicine (Giguère et al., 2013). Taxol is another well-studied drug which is active against various types of cancer (e.g. ovary, breast, lung, head carcinomas). It was isolated for the first time from the bark of *Taxus brevifolia* Nutt. (Jennewein and Croteau, 2001). These three latter mentioned compounds belong to the macrocyclic diterpenes. Psychoactive activity is known from salvinorin A, a clerodane diterpenoid isolated from *Salvia divinorum*, used by the mazatec shamans to induce visionary states (Siebert, 2004). Steviol glycosides from *Stevia rebaudiana* Bertoni, are used for their sweet taste. Its aglycone steviol is a tetracyclic diterpenoid with similar structure to the gibberelins (Carakostas et al., 2008; Ruddat et al., 1963).

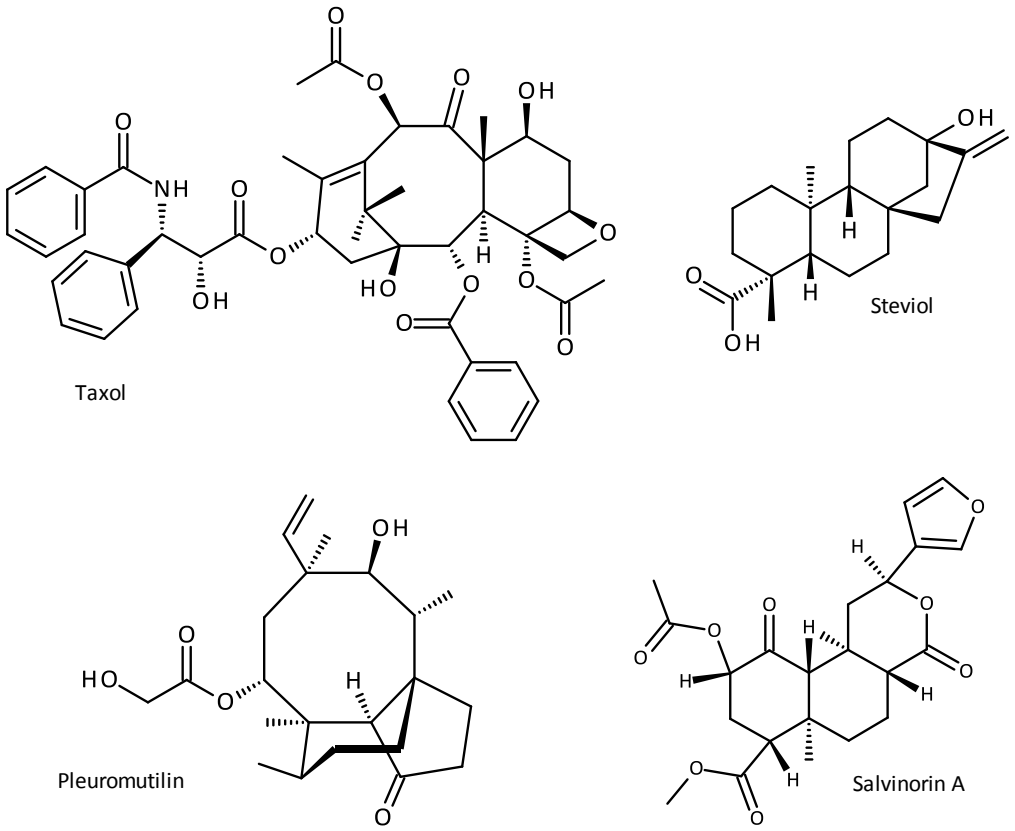

Fig. 6.: Diversity of different diterpene structures. Taxol and pleuromutilin are macrocyclic diterpenes. Steviol is the aglycone of Rebaudiosde A (tetracyclic diterpene). Salvinorin A belongs to the group of the clerodane diterpenes.

5. Experimental

Plant material

Leaf material of *P. denticulata* was collected throughout the year from the Botanical Garden of the University of Vienna (HBV) in order to reveal seasonal changes in exudate composition. For collection dates see Tab. 1, for other plant material used see Tab. 3 in Elser et al. (subm.).

5.1 Extraction & isolation

Exudates

Air-dried or fresh leaves were used to gather exudates, respectively. Various methods were tested and compared to obtain best results inspired by earlier studies (Gershenzon et al., 1992, 1987; Marks et al., 2008). Usually leaf material was briefly rinsed with acetone and the obtained exudate was filtered, concentrated under reduced pressure and then dissolved in methanol (MeOH) for further analyses. When the leaf material was too small for rinsing, a teabag was filled with leaves and shortly dipped into acetone. For comparative purposes the exudates were standardized to a concentration of 0.5 mg/mL.

Methods used to gather glandular trichomes:

1. Shake dry leaf with glass beads (0.25-0.5 mm) in shaking device
2. Shake dry leaf with glass beads (0.25-0.5 mm) on Vortex
3. Shake dry leaf on Vortex in graduated flask filled with glass beads (0.25-0.5 mm), refill 3(4) times with the same beads
4. Abrasion of dry leaf in Eppendorf tube filled half full with silicagel 60 (0.2-0.5 mm) for 15 min in Mixer Mill Retsch MM200 at 30/s

The abrasion material (beads or silica gel) was extracted with different solvents (MeOH, acetone, petroleum ether (PE)/acetone (50:50) and subjected to HPLC.

Extraction

Rinsed leaves were ground and used for extraction. Therefore, 50 mg of dried leaves were ground with the help of 3 glass beads in an Eppendorf tube using Mixer Mill Retsch MM200 at 30/s for 3 minutes. Then 600 µL MeOH was added and the material was extracted for 20 minutes in an ultrasonic bath. This extract was centrifuged (13.500 rpm) for 15 minutes and then the supernatant was subjected to HPLC and TLC analyses respectively.

Isolation

The exudate of *P. villosa* Wulfen (Fig. 17, 248 mg) was first separated by column chromatography (CC) over Sephadex LH-20 column eluted with acetone. The volume of each fraction was approx. 10 mL. The obtained fractions were analyzed by TLC and HPLC, respectively, and further pooled according to the results of these analyses. The pooled fractions 3 (Fig. 18a) and 4 (153.7 mg) were subjected to CC with ~27g silica gel 60 (0.2-0.5 mm), eluted with mixtures of 100 mL PE and ethylacetate (EtOAc) (95:5), 100 mL PE/ EtOAc (80:20), 200 mL PE/ EtOAc (60:40), 100 mL MeOH/ acetone (50:50). Fractions 2 to 5 were combined and further separated via MPLC (Lobar 240-10 LiChroprep Si60, 40-63 µm) column) eluted with 100 mL PE, 200 mL in cyclic elution PE/ PE + 3% isopropanol (50:50), 100 mL MeOH and yielded impure fractions. Fractions 7–16 were combined (9.4 mg, Fig. 18b) and HPLC analyses revealed the occurrence of naringenin-7,4'-diMe.

5.2 Analytical methods

Thin layer chromatography (TLC)

Silica gel 60 $_{F254}$ TLC plates, 0.2 mm or 0.25 mm layer thickness (Machery-Nagel or Sigma-Aldrich) were used to study the characteristics of the exudates, to find ways for separation and to control fractions after each separation. Compounds with chromophoric moieties could be detected using UV_{254} light due to the extinction of fluorescence of the indicator or with UV_{366} light when substances showi auto-fluorescence. For the eluents, please see Elser et al. (subm.). Plates were sprayed with Anisaldehyde reagent in order to detect substances without chromophore or with Naturstoffreagenz A to show differences in putative flavonoids.

Naturstoffreagenz A (Wagner and Bladt, 1996)
A 1% methanolic solution of diphenylboric acid 2-aminoethyl ester. Detection of compounds was done immediately after spraying or 10 minutes after at UV_{365}.

Anisaldehyde-sulfuric acid reagent (Wagner and Bladt, 1996)
This spraying reagent contains 0.5 mL of anisaldehyde, 10 mL acetic acid, 85 mL MeOH and 5 mL conc. sulfuric acid. The plate was heated after spraying to 100 °C until coloration appears.

High performance liquid chromatography (HPLC)

High performance liquid chromatography was used for phytochemical profiling, checking purities of fractions and substance identification (UV spectra and retention times) with authentic standards from Eckhart Wollenweber (and UV-Spectra Database). Exudates were standardized to a concentration of 0.5 mg/mL to enable comparison of chromatograms. HPLC analyses were performed on Agilent 1100 Series equipped with a UV diode array detector (for details see Elser et al., subm.). Furthermore, the HPLC was used for purification of samples which were further subjected to mass spectrometry (MS) analyses. Therefore, samples were concentrated to about 2.5 mg/mL and HPLC Agilent 1100 Series eluted with MeOH in aq. buffer containing 0.05% formic acid and 10 mM ammonium formate at a flow rate of 0.8 mL/min and an injection volume of 10 µL. The applied gradient for preparative purposes, was the same as in chapter Elser et al.,(subm.) six runs were performed in order to acquire enough material of the selected peaks for further analysis via MS.

Mass spectrometry (MS)

Mass spectrometry determines the mass to charge ratio (m/z) of a molecule. It was used to figure out the molecular mass of unknown compounds and get structural information of molecules of interest. The concentrated substances were dissolved and diluted in 0,1% formic acid in MeOH and then directly infused to Thermo Scientific Orbitrap LTQ XL or ABSciex QTRAP 3200 mass spectrometer. Positive or negative ionization mode was used and collision energies were adjusted to optimize fragmentation. A full scan was performed and ms2 spectra of the peaks with highest intensity were recorded. For Data analysis Mass ++ (Tanaka et al., 2014), Massbank (Horai et al., 2010) and HR2 (Kind and Fiehn, 2007) were used.

Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy is a technique that uses strong magnetic fields in order to record shifts in atoms of a molecule. These analyses were performed at the Institute of Organic Chemistry, University of Vienna. For parameters and equipment used see Elser et al. (subm.).

5.3 Preparative methods

Column chromatography (CC)

Column chromatography with different bead sizes of silica gel 60 (0.2-0.5 mm or 40-63 µm, respectively) was used for separation of compounds according to their polarity. A slurry out of silica gel and the starting eluent was for packinging the column. Silica gel is amorphous silicon dioxide, it is a hygroscopic colorless substance which has a high surface area. Due to its hydroxyl groups it is used in normal phase chromatography, where polar substances are retained longer than apolar ones. By contrast in reversed phase chromatography, hydroxyl groups are usually modified with C_{18}-chains.

Sephadex LH20 was used as stationary phase for size exclusion chromatography. Sephadex consists of hydroxylated, crosslinked dextrane with a bead size of 25-100 µm. As acetone was used as eluent, a slurry of Sephadex in acetone was prepared to pack the column.

Middle pressure chromatography (MPLC)

This method was used to achieve further separation, when the compounds were difficult to separate by CC. The more densly packed MPLC column provides a better separation performance. The MPLC consists of a Büchi Pump Module C-601, Büchi Pump Controller C-610, a Teledyn Isco, UA-6 UV/VIS Detector set at 254 or 280 nm detection wavelength. A Lobar 240-10 LiChroprep Si60 (40-63µm) (Merck) was employed. The flow rate was 5 mL/min.

Preparative TLC

Additionally, preparative thin layer chromatography (TLC) was used for separating small amounts of compounds (Merck silica gel 60 F_{254} glass plates 0,25mm layer thickness). Eluents and gradients used for preparative purposes are either mentioned in chapter 5.1 (Extraction & Isolation) or the following chapter. Elser et al. (subm.).

6. Results and discussion

Results are presented and discussed in two separate chapters. The first part included here (chapter 6.1) is a submitted manuscript to the journal *Natural Product Communications*, currently under review (Elser et al., subm.). The second part deals with isolation techniques developed to isolate glandular hairs, and with their influence on exudate composition. The second chapter is complemented by analysis of exudate profile variation (seasonal, populational) in selected *Primula* species.

6.1 Natural Product Communications

2016
Vol. 11
No. 0
1 - 2

Striking Diversification of Exudate Profiles in Selected *Primula* Lineages

David Elser[a], Christian Gilli[a], Lothar Brecker[b] and Karin M. Valant-Vetschera[a]

[a]*Chemodiversity Research Group, Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030 Vienna, Austria*

[b]*Institute of Organic Chemistry, University of Vienna, Währinger Strasse 38, A-1090 Vienna, Austria*

karin.vetschera@univie.ac.at

Received: January XX, 2016; Accepted: XX, 2016

In continuation of previous studies on glandular exudates of *Primula,* we analyzed eleven so far unstudied species and several populations for exudate composition. Unsubstituted flavone and unusual substituted flavones, normally predominant in *Primula* exudates, were not detected in all of the analyzed samples. Instead, some species exhibited regular substituted flavonoids, and in some cases, no flavonoids could be detected at all. The detection of a diterpene (**1**) in *P. minima* exudates is new to *Primula.* On basis of MS and NMR, **1** was structurally characterized as *ent*-kaur-16-en-19-oic acid. Comparative profiling of exudates as performed by HPLC and TLC against authentic markers indicated further the presence of primin and derivatives in some exudates. Thus, exudates of newly studied species contrast markedly to those analyzed so far. The significance of observed exudate diversification is discussed in view of the phylogeny of derived lineages in European alpine regions.

Keywords: *Primula* subgenera *Auriculastrum, Aleuritia, Primula*; flavonoids; primin derivatives; diterpene acid.

In preceding studies on different subgenera of *Primula* L.*,* exudates were investigated for their chemodiversity [1–3] and also for the micromorphology of glands as their production site [4]. These exudates may be found on leaves stems, calyces or inflorescences and are either of mealy (farina) or oily appearance [2]. Earlier, unsubstituted flavone and flavones with unusual substitution patterns, named as *Primula*-type flavonoids because of their yet unknown biosynthesis, were found as major exudate constituents [1,2]. The same compounds are also found in exudates of the closely related genus *Dionysia* Fenzl, but some *Dionysia* species also accumulate regular flavonoids in their exudates [5]. However recent publications indicate that some European *Primula* species also are able to produce flavonoids of the regular biosynthetic pathway [6,7]. Exudates are produced by glandular trichomes that can vary in size, shape and color [4,6–8]. The production of farina is correlated with seasonally low temperatures and it is produced from winter to spring in large quantities. It has been demonstrated that unsubstituted flavone of the exudate increases the freezing tolerance [9]. *Primula* is a genus of about 500 species which is mostly distributed in the Sino-Himalayan region, comprising 7 subgenera and 37 sections [10,11]. Biogeographically, the center of origin is assumed to be in the Sino-Himalayan region, with an estimated age of about 30 million years and the majority of species found there. The lineages occurring in Europe and N-America consist of a smaller number of species that are considered to be phylogenetically derived [12,13].

The present study focuses on species of subgen. *Auriculastrum* Schott sect. *Auricula* Duby, a group that is endemic to Central and S-European mountain ranges with no apparent relationships to other European lineages. Relationships are expected rather with sect. *Parryi* W.W. Smith ex Wendelbo (North America) and sect. *Cuneifolia* Balf (East Asia-North America) [10]. In this study seven species from sect. *Auricula* were analyzed for the first time for exudate diversification, considering infraspecific variation whenever possible. Results are compared to those of four Turkish taxa from other taxonomic groups (subgen. *Aleuritia* (Duby) Wendelbo and subgen. *Primula* L.), not studied so far. Observed flavonoid profile diversification is discussed in relation to literature data and compared to that of the closely related genus *Dionysia*, particularly in view of the production of non-*Primula*-type flavonoids. In addition ecological significance of pattern variation is briefly addressed.

Representatives and new accessions from subgen. *Auriculastrum*, subgen. *Primula* and subgen. *Aleuritia* were analyzed for exudate profile composition. Major compounds detected comprise a series of well-known *Primula*-type flavonoids, ranging from unsubstituted flavone to unusually substituted flavones described before [1]. Surprisingly, regular substituted flavones and flavanones as well as derivatives of the flavonol kaempferol were detected for the first time in exudates of the studied species, which are new reports for the genus *Primula*. So far, regular substituted flavonoids do not co-occur with *Primula*-type flavones and thus appear to be mutually exclusive. Some exudate profiles revealed predominance of primin and primin-like compounds corresponding in their UV-spectra to that of primin, a constituent well known for *Primula*.[14] The diterpene acid (**1**) is found in exudates of *Primula* species for the first time. Variation of profile composition within single species is observed in some cases thus questioning the usability of exudate compounds as chemical characters. The results are compiled in Table 2 and are discussed against literature data and the phylogenetic background of the studied species.

The occurrence of *ent*-kaur-16-en-19-oic acid (**1**) (Fig.1) in exudates of *Primula* species is a strikingly new feature, as only sesquiterpenoids have been described from leaf oils of some *Primula* taxa so far [6,7]. Earlier, **1** was reported as exudate constituent in farinose ferns such as *Notholaena peninsularis* and *N. pallens* [15]. Compound **1** was further reported from resinous exudates of some *Pseudognaphalium* spp. (Asteraceae), where its presence had been correlated with antimicrobial activity [16]. Occurrence in both farinose (ferns) and hardly farinose (*P. minima*) excretions is remarkable and may point to a specific function.

Further sources of **1** are fruits of *Anonna glabra* (Annonaceae) [17] or roots of different *Smallanthus* species (Asteraceae) [18]. In addition Padla et al. demonstrated antifungal activity for this compound [19]. Notably, **1** serves as biosynthetic precursor of the plant hormones gibberelins [20].

ent-Kaur-16-en-19-oic acid (**1**) was structurally characterized on basis of MS and NMR. The deprotonated molecular ion of compound **1**, $[M-H]^-$ was detected by HR-TOF ESI MS in negative ionization mode with m/z of 301.2197. This corresponds well to the calculated m/z of 301.2173 for a $[M-H]^-$ of $C_{20}H_{29}O_2$. Consequently $C_{20}H_{30}O_2$ is the summed formula of the isolated natural product. An attached proton test (APT) ^{13}C NMR spectrum, a HMBC and a ^1H NMR indicated compound **1** to consist of two methyl groups, ten methylene groups, three methine groups and five quaternary carbon atoms. The ^1H and ^{13}C chemical shifts as well as the 2D NMR spectra indicate an annulated tetracyclic system carrying two methyl groups (C-18, C-20), a carboxyl group (C-19) and an *exo*-methylen group (C-17). 2D TOCSY and COSY show the presence of four separated ^1H spin systems, namely between C-1, C-2 and C-3; between C-5, C-6 and C-7; between C-9, C-11, C-12, C-13 and C-14 as well as the separated C-15. $^2J_{C-H}$ and $^3J_{C-H}$ long range couplings determined from HMBC present the interaction from H-20 to C-1, C-5, C-9 and C-10 as well as the interaction from H-18 to C-3, C-4, C-5 and C-19. These indicate two annulated six-membered rings carrying the methyl groups in positions C-4 and C-10, respectively and the carboxyl group on C-4. The protons of the *exo*-methylen group C-17 have long range couplings to C-13, C-15 and C-16, which indicate its position on C-16. Further, C-8 has amongst others couplings to H-7, H-9, H-14 and H-15.

These spectroscopic data allow the conclusion that the investigated compound is known *ent*-kaur-16-en-19-oic acid (**1**). Its presence was confirmed by the use of combined CSEARCH and SPECINFO database system for spectral similarity searches by application of the SAHO-protocol [21]. All recorded spectroscopic data are in good agreement with earlier reported values [22]. The absolute configuration of the isolated compound **1** has hence not be proven, but was supposed to congruent with *ent*-kaur-16-en-19-oic acid (**1**) isolated earlier from other sources. NMR spectroscopic data are summed in Table 1 and the structure is shown in Figure 1a.

Table 1: NMR spectroscopic data of compound **1**, which was solved in $CDCl_3$. Chemical shifts [ppm] and multiplicities are given. Indication of the positions is made in accordance to Figure 1a.

Position	^{13}C [ppm, mult.]	^1H [ppm, mult.]	Integral
1	41.2, t	1.88, m & 0.81, m	2
2	19.4, t	1.87, m & 1.45, m	2
3	38.0, t	2.16, m & 1.01, m	2
4	n.d.	-	-
5	56.8, d	1.06, m	1
6	21.9, t	1.83, m	2
7	41.5, t	1.52, m & 1.44, m	2
8	44, s*	-	-
9	55.2, d	1.06, m	1
10	40, s*	-	-
11	18.4, t	1.56, m	2
12	33.1, t	1.61, m &1.47, m	2
13	43.8, d	2.63	1
14	39.7, t	1.99, m & 1.14, m	2
15	49.4, t	2.06, m & 2.04, m	2
16	155.8, s	-	-
17	103.0, t	4.80, m & 4.74, m	2
18	29.7, q	1.25, s	3
19	182, s*	-	-
20	14.8, q	0.95, s	3

* determined via HMBC

Morphological characters such as involute leaf vernation, a reduced number of stomata and colporate pollen grains characterize members of **subgen. *Auriculastrum* Schott sect. *Auricula* Duby**.

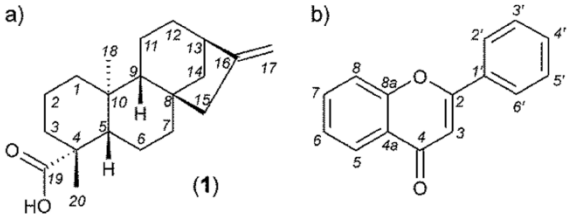

Figure 1: a) *ent*-Kaur-16-en-19-oic acid (**1**) isolated from *Primula minima* leaf exudate. b) Basic structure of flavone.

Species of its subsect. *Cyanopsis* (Schott) Pax, comprising ten species, and subsect. *Euauricula* Pax with fifteen species, show highest species diversity in the Alps, and are all hexaploid or hypohexaploid. Chromosome numbers based upon x=11 can be quite high, amounting to 2n= 198 as e.g. for *P. clusiana* Tausch, and they may vary within species. Members of subsect. *Euauricula* have papillose seed surfaces and bracteose bracts, differing from those of subsect. *Cyanopsis* with smooth seed surfaces and foliose bracts [10].

The exudate profiles of eight studied species of subsect. *Euauricula* (EUA in Tab. 2) are quite diversified. Thus, *Primula*-type flavones, flavonoids derived from the regular biosynthetic pathway, as well as primin and derivatives are the main accumulation tendencies observed here. The occurrence of *Primula*-type flavones and of flavonoids derived from the regular biosynthetic pathway appears to be mutually exclusive, while primin and its derivatives may co-occur with regular flavonoids. Two collections of *P. daonensis* Leyb. yielded primin-like compounds that appear as a cluster of peaks between approx. 20–27 min in the HPLC chromatogram showing UV-spectra similar to the known compound primin, as illustrated for *P. pedemontana* Thom. ex. Gaudin (see Fig. 2). Primin and its derivatives are known to appear with different alkyl side chains [23], this would explain the high number of peaks with same UV spectra but different retention times. Earlier, this species

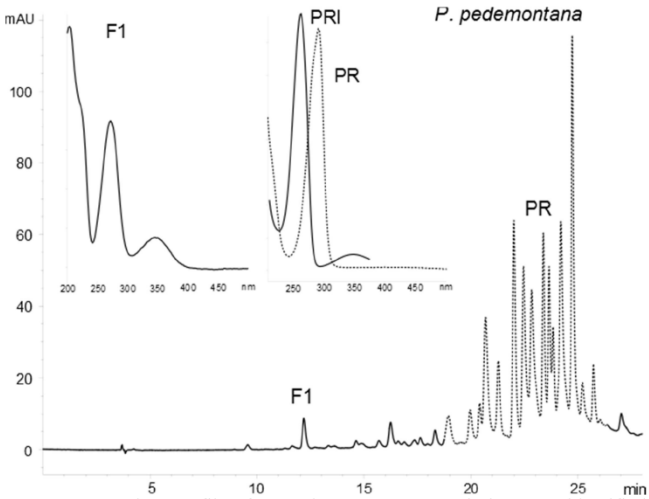

Figure 2: Exudate profile of *P. pedemontana.* PRI Primin, F1 unidentified flavonoid. PR: Predominant compounds showing primin like UV-spectra (dotted lines; UV_{max}: PR 292 nm; Primin 270 nm). Different retention times but identical UV-spectra possibly due to different alkyl side chains. Reversible color reactions on TLC from violet to orange-yellow indicative of a quinoid structure.

was studied for flavonoid glycosides and exudates [8], but the exudate composition was not specified. The depicted UV-spectrum points to a flavonol derivative that was not detected in the present samples. A predominance of the primin derivatives is obvious in

exudates of *P. pedemontana*, *P. villosa* Wulf. and *P. hirsuta* All. which are all characterized by redish-brownish glands that appear to be correlated to primin production [24]. Interestingly, these taxa were formerly grouped in sect. *Erythrodrosum* Schott, a taxonomic entity currently not recognized [10]. In addition to these compounds, an unidentified flavonoid (F1 in Tab. 2) was present in the exudate of *P. pedemontana*, while accessions of *P. hirsuta* showed conspicuous variation in profile composition. Thus, the exudate of accession 7 from July is dominated by unsubstituted flavone, followed by lower amounts of 2'-OH-5'OAc-flavone in addition to the primin mixture. A switch from the typical *Primula*-type flavonoids to those from the classical biosynthetic pathway is observed for the *P. hirsuta* collections 6 and 7 from August, with primin substances and apigenin as main compounds, accompanied by lower amounts of kaempferol, kaempferol-3-Me, apigenin-7-Me and the flavanone naringenin.

Earlier studies did not indicate the presence of primin or derivatives in a cultivated sample (*P. hirsuta*[a], Tab. 2) [2]. Naringenin 7,4'-diMe was finally detected in *P. villosa* exudate after prep. chromatography in addition to dominating primin derivatives. These regular substituted flavonoids are reported here for the first time as exudate constituents of *Primula* species. The significance of profile variation within *P. hirsuta* could either be related to geographic origin or to different collection dates. For the production of flavone, it is known that seasonal variation may exist as is shown for the unrelated *P. denticulata* Sm. (D. Elser, in prep.), and which would favor the assumed function of flavone being involved in cold tolerance [9]. However, this does not explain the shift from *Primula*-type flavones to those derived from the regular biosynthetic pathway.

Presence of the typical *Primula*-type flavonoids only (unsubstituted flavone, 2'OH-flavone and 5,8-diOH-flavone) is shown for *P. latifolia* Lapeyr., which coincides with literature data on other species of subsect. *Euauricula* (*P. albenensis* E. Banfi & R. Ferlinghetti, *P. marginata* Curt., *P. auricula* L. (Tab. 2. The profiles of *P. auricula* and *P. albenensis* are reported to be identical in their composition [7], which is somewhat surprising. Both species, however, differ in the composition of leaf essential oil, with the acetophenone paeonal as predominant constituent in *P. albenensis* oil and sesquiterpenes in that of *P. auricula*. Earlier studies on *P. auricula* describe a different exudate composition [1] thus indicating infraspecific variability. Taken together, this subsection is chemically quite diversified and consistent profile composition appears to be not the rule even within single species. In earlier studies on *P. palinuri* Petagn. calyces [1], a rich exudate profile based upon *Primula*-type flavones was indicated. This species is rather distant to *P. auricula* in the molecular phylogenetic tree [10] which might correspond to observed chemical differences.

Exudates from five of the studied species of subsect. *Cyanopsis* revealed the presence of not only the *Primula*-type flavones, but also of compounds not known from *Primula* so far. Earlier studies could not detect any flavonoid compound in the exudates of *P. minima* L., *P. wulfeniana* Schott, and *P. clusiana*, respectively, but indicated the presence of unknown structures [2]. From exudates of *P. minima* (collection 14), the diterpene *ent*-kaur-16-en-19-oic acid (**1**) (Fig.1) could now be isolated and structurally determined. In addition, five yet unidentified compounds were detected and are assumed to be related structures, as suggested by UV-spectra (unknown compds. in Tab. 2). Low amounts of exudate, however, prevented isolation and further purification. Interestingly, no flavonoids originating from the regular biosynthetic pathway were detected in this group in our study. This is contrary to the report on endemic *P. spectabilis* Tratt. accumulating the flavonol quercetin 7,3',4'-trimethyl ether only [6], a species clustering together with the chemically different *P. glaucescens* Moretti in Giorn in the phylogenetic tree (Tab. 2) [10]. Exudates of *P. glutinosa* Wulf. yielded three different dihydrochalcones in previous studies[3], of which 2,2'-dihydrochalcone could be detected together with 5-OH-flavone in a recollection from the same locality (No. 9 in Tab. 2,3), thus providing a further example of profile variation.

Collections of *P. minima*, a species of disjunct distribution occurring on the Eastern Alps and on various Eastern mountain ranges such as the Carpathians, Tatras, and Rila mountains, showed inconsistent exudate composition in collections from various locations in Austria. Exudate composition of a herbarium collection from Salzburg (No. 10 in Tab. 2, 3) proved to correspond largely to that of *P. glaucescens*, accumulating unsubstituted flavone and some other *Primula*-type flavones. Herbarium collection 11 from Styria, on the other hand, showed flavone as the only exudate constituent. All of the other studied populations come from Carinthia, and they deviated by producing the unknown compounds possibly derived from **1** (Tab. 2). Populations from Carinthia were found to be separated in a phylogenetic tree from those of Eastern Europe [10], so the observed exudate diversification may reflect this alignment. It must be mentioned that differences may also be related to the source of collection, originating either from natural habitats (12-15 in Tabs. 2, 3) or from herbarium specimens (collections 10 and 11) sampled at different times of the growing season. Preceding studies in other species, however, did not indicate striking chemical differences between natural collections and old herbarium material. Generally, exudate flavonoids are quite stable and farina is normally well preserved [2]. Extended studies, both on additional plant material from different locations, considering equally seasonal variation, are required to assess the significance of pattern variation in *P. minima*. *Primula integrifolia* L. exudate contained only unidentified compounds and no flavonoids, which was similarly observed for *P. clusiana,* both species part of the same clade [10].

Some congruence is apparent between our results and those of the phylogenetic study of Zhang and Kadereit [10]. Thus, primin and derivatives occur in members of two of the more derived clades of subsect. *Euauricula*, while *Primula*-type flavonoids are typical for the more basal clades in which the type species *P. auricula* is placed. Lack of flavonoids and presence of diterpenes is found in some members of subsect. *Cyanopsis*. The accumulation of regular substituted flavones and flavonols appears to be more common in subsect. *Euauricula* (Tab. 2). Earlier, *P. rusbyi* Greene from the closely related sect. *Parryi,* was studied and found to accumulate *Primula*-type flavonoids only [1]. Further studies are needed to analyze profile diversification for a better insight into the diversification patterns in geographically separated sections and lineages within subgen. *Auriculastrum*.

The other species studied here (Tab. 2) were collected in different locations in Turkey and belong to other and distant taxonomic groups of *Primula*. The exudate flavonoid profiles observed for members of subgen. *Aleuritia* fall well in the range of expected composition, with unsubstituted flavone as main compound and the typical 5-OH-flavone derivatives in addition (Tab. 2). Thus, profile composition of *P. longipes* Freyn & Sint. exudate fits well to those of the earlier studied *P. megalocarpa* Hara and *P. macrophylla* D. Don (sect. *Crystallophlomis* (Rupr.) Federov) and *P. specuicola* Rydb. and *P. darialica* Rupr. (sect. *Aleuritia*) [2].

Table 2: Phytochemical diversification of exudate profiles in selected *Primula* species.

Primula		Flavone	2'-OH-Flavone	2'-OMe-Flavone	2'-OH-5'-OAc-Flavone	3'-OMe-Flavone	3',4'-diOMe Flavone	4'-OH-Flavone	5-OH-Flavone	5,2'-diOH -Flavone	5,8-diOH-Flavone	Apigenin	Apigenin-7-Me	Kaempferol	Kaempferol -3-Me	Naringenin	Naringenin 7,4'-diMe	Flavonoid derivatives	Primin	Primin derivatives	*ent*-kaur-16-en-19-oic acid (**1**)	Unknown compds.
daonensis[1]	EUA																			●		
daonensis[2]	EUA																			●		
pedemontana[3]	EUA																	F1		●		
villosa[4]	EUA																x		x	●		
hirsuta[5]	EUA											●	x	x	x	x				●		
hirsuta[6]	EUA											●	x	x	x	x				●		
hirsuta[7]	EUA	●			x															x		
hirsuta[a]	EUA	●	x		x						x											
albenensis[b]	EUA	●	x			x	x															
albenensis[c]	EUA	x	x					x														
latifolia[8]	EUA	●		x	x	x					x											
marginata[b]	EUA	●	●						●		●											
auricula[b]	EUA	●	●				x		●	x	●											
auricula[c]	EUA	x	x					x														
glutinosa[9]	CYA								●									F2				
glaucescens[a]	CYA	●			x				x		●											
minima[10]	CYA	●			x				x		x											
minima[11]	CYA	●																				
minima[12]	CYA																					●
minima[13]	CYA																					●
minima[14]	CYA																				x	●
minima[15]	CYA																					●
clusiana[16]	CYA																					●
integrifolia[17]	CYA																					●
longipes[18]	CRY	●			x				x		●											
algida[19]	AL	●			x				x		x											
elatior subsp. *pallasii*[20]	PR	●			x				x		●											
elatior subsp. *meyeri*[21]	PR	●							x													

Legend to Table 2.

● = Major compound **x** minor compound Flavonoid derivatives: F1= unknown; F2= 2,2'-dihydrochalcone, primin-derivatives: a mixture of compounds that have similar UV-spectra to primin; Numbers in column 1 indicate sources (see Tab. 4); letters indicate references: a [2] b [1], c [7] Taxonomic alignment: EUA: subgen. *Auriculastrum* sect. *Auricula* subsect. *Euauricula*; CYA: subgen. *Auriculastrum* sect. *Auricula* subsect. *Cyanopsis;* CRY: subgen. *Aleuritia* sect. *Crystallophlomis* subsect. *Crystallophlomis*; AL: subgen. *Aleuritia* sect. *Aleuritia* subsect. *Algida*; PR: subgen. *Primula* sect. *Primula*.

The exudate profiles of two members of subgen. *Primula* are hardly distinguishable from those of the other members of subgen. *Aleuritia* studied here (Tab. 2). However, striking diversifications between subspecies of *P. elatior* Hill of Central Europe and their Turkish relatives were detected in exudate composition. While European *P. elatior* exudates contain a series of 3´,4´,5´-trisubstituted flavones [1], compounds of this type could not be detected in the present study. Recent phylogenetic studies indicate that *P. elatior* is genetically heterogenous and non-monophyletic [25]. It would be interesting to study the phylogenetically analyzed samples in parallel for exudate diversification, to test for the hypothesis of younger lineages diversifying from the otherwise quite monotonous profiles of their Asian ancestors.

The significance of exudate diversification within *Primula* is still somewhat obscure, and reasons may be both at the functional level and/or the phylogenetic history of single groups. Infraspecific changes in exudate composition may be the result of seasonal responses as mentioned by Isshiki et al. [9] on the example of unsubstituted flavone. *Primula hirsuta* clearly shows this pattern, with the collection 7 from July dominated by flavone and collections 5 and 6 (later in the season), showing a switch to the production of regular flavonoids (Tab. 2). Similarly, accessions of *P. minima* differed in the presence/absence of flavone. Preliminary results indicate seasonal variation in flavone production in exudates of cultivated *P. denticulata*, coinciding with a loss of the densely cover of farina in spring (D. Elser, pers. comm.). A similar observation was made for farina production of *P. malacoides* Franch. [9], and much earlier, the impact of abiotic factors such as wind, snow, or rain was suggested as cause for disappearance of farina in *P. auricula* [26]. This could serve as one explanation of the inconsistency in accumulation of specific compounds and of the absence of flavone in some of the investigated species. It would be interesting to see if this dynamics in accumulation is typical for derived lineages of *Primula*.

The exudates of *Primula* seem to serve multiple functions according to the needs of adaptation to the environment. One proven function is the already mentioned freezing tolerance. Another function seems to be the protection against UV irradiation as generally known from flavonoids [27]. Some of the compounds such as dihydrochalcones or diterpenes might protect against fungi or other pathogens [3,16,19]. The flavones of *Primula denticulata* have also been shown to have cytostatic properties [28] which might limit the growth of pathogens. Although the studied species occur on different soils, so far no correlation can be observed between soil chemistry and exudate composition, as an explanation for primin or terpenoid predominance in exudates, or a shift to flavonoids from the regular biosynthetic pathway.

As suggested previously from studies on the closely related genus *Dionysia* [5], a larger degree of diversification, in terms of complexity of exudates may represent a more derived character. This is also indicated by our present analyses, as most of the species

studied now differ clearly from the ones previously studied e.g. [1,2]. Similarly, both genera have members that obviously do not produce *Primula*-type flavonoids or any other flavonoids. Molecular and cytogeographical data suggest that subgen. *Auriculastrum* sect. *Auricula* represents a derived lineage which originated about 3.6 million years ago [10], thus representing a young lineage when compared to the estimated 30 million years of origin of the genus [13]. Parallels become evident when exudate composition of *P. hirsuta* is compared to that of *Dionysia diapensiifolia* Boiss., both sharing a similar derived status within the respective genera. Similar tendencies towards production of apigenin, kaempferol, kaempferol-3-Me, apigenin-7-Me and naringenin are observed, with *D. diapensiifolia* accumulating naringenin-7-Me and kaempferol-7-Me in addition [5]. Therefore, our results are in line with the hypothesis of diversification of exudates (increasing complexity in derived species) proposed earlier for *Dionysia* [5]. To get more support for this hypothesis, we suggest more species to be analyzed from North America, Europe, as well as the isolated taxon (*P. magellanica*) from South America, from sect. *Parryi* subsect. *Aleuritia*, and the remaining species from sect. *Auricula*. In any case, seasonal variability should be considered, especially when exudate profiles should be applied as chemical characters in this phylogenetically most interesting genus.

Experimental

NMR spectroscopy: Compounds were dissolved in 99.8% CD_3OD or 99.9% $CDCl_3$ (~5 mg in 0.7 mL) and transferred into 5 mm NMR sample tubes (Promochem, Wesel, Germany). Spectra were measured with Topspin 3.2 software on Bruker AV-III-600 or AV-III-700 spectrometer (Bruker, Rheinstetten, Germany) at 298.1 K +/-0.1 K. For 1D spectra 32k data points were recorded and Fourier transformed to spectra with a range of 7200 Hz (^{1}H) / 30,000 Hz (^{13}C) or 8400 Hz (^{1}H) / 35,000 Hz (^{13}C), respectively. COSY, TOCSY, NOESY, HMQC, and HMBC spectra were measured by 128 experiments with 1024 data points each. Linear forward prediction, sinusoidal multiplication and Fourier transformation led to 2D-spectra with a range of 10 ppm and up to 220 ppm for ^{1}H and ^{13}C, respectively. CH_3OH or $CDCl_3$ were used as respective internal standard for ^{1}H (δH 3.34 or 7.24) and ^{13}C (δC 49.86 or 77.02) spectra.

Mass spectrometry: Mass spectra were measured on a high resolution time-of-flight (HR-TOF) mass spectrometer (maXis, Bruker Daltonics) by direct infusion electrospray ionization (ESI) in positive ionization mode (mass accurancy +/- 5 ppm). TOF MS measurements have been performed within the selected mass range of m/z 100-2500. ESI was made by capillary voltage of 4 kV to maintain a (capillary) current between 30-50 nA. Nitrogen temperature was maintained at 180°C using a flow rate of 4.0 L min^{-1} and the N_2 nebulizer gas pressure at 0.3 bar.

HPLC: HPLC analyses were performed on Agilent 1100 Series equipped with a UV diode array detector (detection WL 230 nm), 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), at a flow rate of 0.8 mL/min and an injection volume of 10 µl. The applied gradient was 0–17 min 55% B in A from 17–20 min 90% B in A, from 20–28 min 100% B in A.

TLC: TLC was carried out on silica gel 60 F_{254} aluminum plates (Machery-Nagel or Sigma-Aldrich) thickness 0.2 mm, developed in petrol ether (PE)/ ethyl acetate (EtOAc)/ MeOH (70:30:1) (for terpenoids), toluol/ ethylmethylketone (90:10) (for primin derivatives), and toluol/ dioxan/ acetic acid (90:25:5) for flavonoids. Compounds with chromophores were detected under UV light at 254 or 366 nm, respectively. TLC plates were sprayed either with Naturstoffreagenz A (1% in MeOH, w/v) for detection of flavonoids, or with anisaldehyde for detection of other compounds.
Isolation of compounds was performed using CC with silicagel 60 (40-63 µm) or Sephadex LH-20, and MPLC with a Lobar 240-10 LiChroprep silicagel 60 (40–63 µm) column. In addition preparative TLC (Merck glass plates, thickness 0.25 mm silica gel 60 F_{254}) was used, if required.

Plant material: Different *Primula* species were collected by Christian Gilli, Andreas Berger and David Elser. Voucher specimens were deposited at the Herbarium of the University of Vienna, Austria (WU). Additionally herbarium specimens of *P. minima* were used for comparison (10; 11 in Tab. 3). Collection details of the plant material are given in Tab. 3.

Table 3: Plant material

No.	*Primula*	Altitude [m]	Location	Voucher	Collection Date
1 [C]	*daonensis*	2460	Switzerland, Graubünden	WU 0083091	30.06.2013
2 [C]	*daonensis*	2090	Italy, Lombardia	WU 0067589	24.07.2011
3 [C]	*pedemontana*	2115	Italy, Valle d'Aosta	WU 0083092	03.07.2013
4	*villosa*	1997	Austria, Carinthia	WU 0082549	12.07.2014
5 [C]	*hirsuta*	2780	Austria, Tyrol	WU 0083089	09.08.2014
6 [C]	*hirsuta*	2900	Austria, Tyrol	WU 0083093	19.08.2014
7 [C]	*hirsuta*	2230	Switzerland, Uri	WU 0083088	01.07.2013
8 [C]	*latifolia*	2220	Italy, Lombardia	WU 0067592	18.07.2011
9	*glutinosa*	2486	Austria, Carinthia	WU 0082547	05.07.2014
10	*minima*	2300-2687	Austria, Salzburg	WU 0082539	23.06.2005
11	*minima*	1880-2275	Austria, Styria	WU 0082538	05.07.1999
12	*minima*	2350	Austria, Carinthia	WU 0082541	07.07.2014
13	*minima*	2453	Austria, Carinthia	WU 0082548	05.07.2014
14	*minima*	2308	Austria, Carinthia	WU 0082542	05.07.2014
15	*minima*	2095	Austria, Carinthia	WU 0082540	05.07.2014
16 [A]	*clusiana*	1320	Austria, Salzburg	WU 0083086	21.08.2015
17 [C]	*integrifolia*	2580	Switzerland, Graubünden	WU 0083105	14.07.2015
18 [C]	*longipes*	3040	Turkey, Erzurum	WU 0083100	25.06.2014
19 [C]	*algida*	2960	Turkey, Erzurum	WU 0083096	25.06.2014
20 [C]	*elatior* subsp. *pallasii*	2300-2400	Turkey, Gümüşhane	WU 0083099	01.07.2014
21 [C]	*elatior* subsp. *meyeri*	2960	Turkey, Erzurum	WU 0083097	25.06.2014

C = collected by C. Gilli, A = collected by A. Berger, others collected by D. Elser

Extraction and isolation: Air dried leaf material was briefly rinsed with acetone. From the herbarium specimens 10 and 11 plants were cautiously unmounted and the leaves were briefly rinsed with acetone, and subsequently remounted. The resulting exudate was filtered, concentrated under reduced pressure at 35°C and then suspended in MeOH for further analysis. When leaf material was too small for rinsing, a small teabag was filled with leaves and shortly dipped into acetone. Phytochemical profiling of exudates (standardized at a concentration of 0.5 mg/mL) and isolation of compounds was performed using HPLC and TLC. Isolated compounds were identified by MS and NMR. UV spectra and retention times obtained from HPLC analysis were compared with those authentic standards obtained from E. Wollenweber (Darmstadt, Germany).

The exudate of *P. minima* population 12 was first separated in 10 mL fractions via a Sephadex LH-20 column in acetone. The fractions 6, 7 and 8 were combined (14.9 mg) and further separated via MPLC eluted with 350 mL PE/ EtOAc (90:10), 140 mL (PE/:EtOAc (70:30) and 100 mL PE/ EtOAc (60:40) which yielded samples that were not pure enough for structure elucidation. After comparison of profiles of the populations (Nos. 12–15), exudates were combined (~100 mg) and subjected to separation on silica gel, 202 mL PE/ EtOAc/ MeOH/ (70:30:1), 100 mL PE/ EtOAc (60:40) and 100 mL MeOH, to yield 3.2 mg *ent*-kaur-16-en-19-oic acid (**1**).

Acknowledgements - We would like to thank Dr. Eckhard Wollenweber (Institut für Botanik der TU Darmstadt, Germany) for providing authentic flavonoid samples. We are also very grateful to Andreas Berger for providing plant material. Assistance in field work by Gerald Schneeweiss and Michaela Sonnleitner (Plant Biogeography Research Group, Department of Botany and Biodiversity Research, University of Vienna) is gratefully acknowledged. We are particularly thankful to Susanne Felsinger and Alexander Ronacher (Faculty of Chemistry, University of Vienna) for recording NMR and mass spectra, respectively.

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6.2 Specific examples of exudate variation

This chapter describes the results of various isolation techniques to separate glandular hairs of leaf tissue and their possible influence on exudate composition. *Primula denticulata*, growing in abundance in the Botanical Garden (University of Vienna), lent itself as a suiting research object. In addition, seasonal variation in exudate composition was studied in this species as well. Additional data not covered in the publication (6.1) are presented for *P. villosa*, and *P. minima*, respectively.

6.2.1 *Primula denticulata* Sm.: Evaluation of methods for isolation of glands

Four different methods were applied to gather glandular trichomes as the production site of exudates, which were subsequently analysed for composition by HPLC profiling. As reference the traditional method of briefly rinsing the leaf material with acetone was used (Fig. 7c and d). These isolation methods are described in chapter 5.1. Isolated glands were further microscopically checked for physical intactness which is required for analysis of their chemical composition. It could be shown that method 4 (abrasion of dried leaves) gives the highest yield in exudate and largest quantity of its compounds. For microscopic studies see Fig. 7, showing a leaf of *P. denticulata* that is in full production of exudates. Rinsing the leaf with acetone nearly removed all exudates completely and made the glandular trichomes visible as shown in Fig. 7c and d. Method 4 has been shown to be able to remove the glandular trichomes (Fig. 8b–e) from the leaf surface including the intact exudates. Preliminary results also indicate that this method should work for fresh leaf material too, if an isotonic solution is added during abrasion. These are important features for metabolomic and proteomic studies of these structures.

Glandular trichomes obtained by method 4 revealed the presence of a putative dihydrochalcone (Fig. 10) when silica gel was extracted with MeOH. Its UV spectrum (Fig. 9) resembles that of 2,2'-dihydrochalcone, a structure not detectable in exudates obtained from rinsing with acetone (Fig. 10). Apart from this compound, method 4 led to similar results as did rinsing with acetone, as far as profile composition is concerned (Fig. 10). Leaf material was also briefly rinsed with MeOH, but yielded the same exudate composition, as did rinsing fresh leaf material with acetone or MeOH. One remaining task is to implement a good technique to separate trichomes from silica gel, which could be achieved via a percoll gradient as applied in previous studys on *Mentha* L. (Champagne and Boutry, 2013).

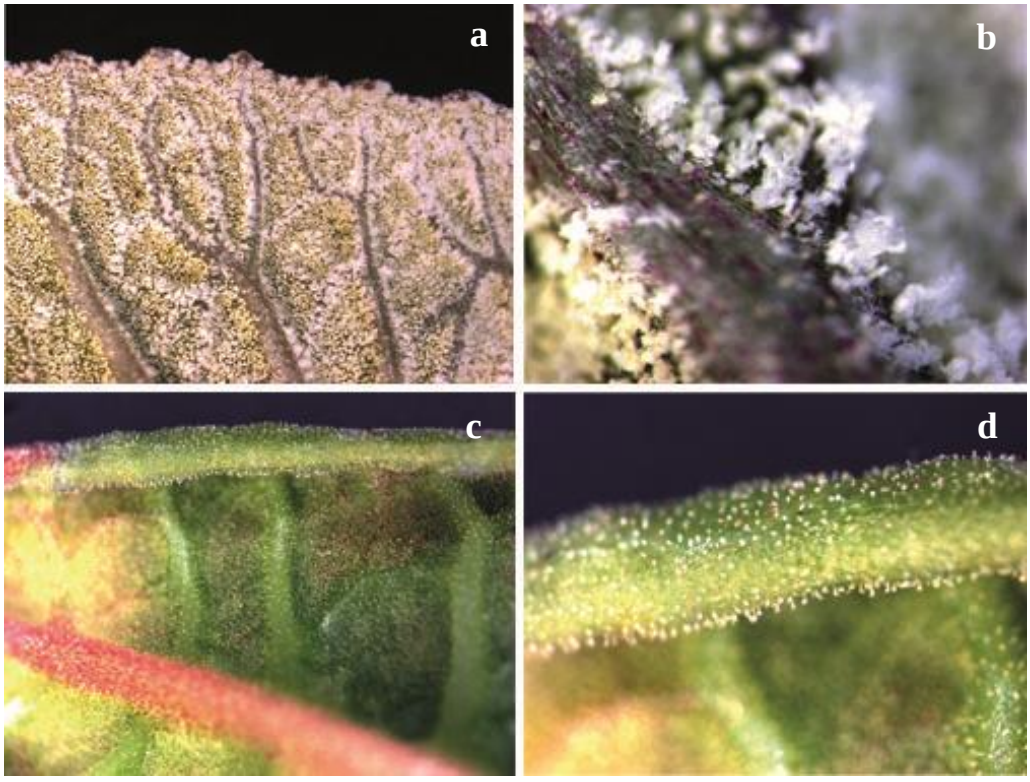

Fig. 7.: Fresh leaf material of *P. denticulata*. **a**: Leaf densly covered with farina; **b**: Glandular trichomes with nearly crystalline flavonoid exudates. **c**: Leaf after rinsing with acetone; **d**: Glandular trichomes after rinsing with acetone.

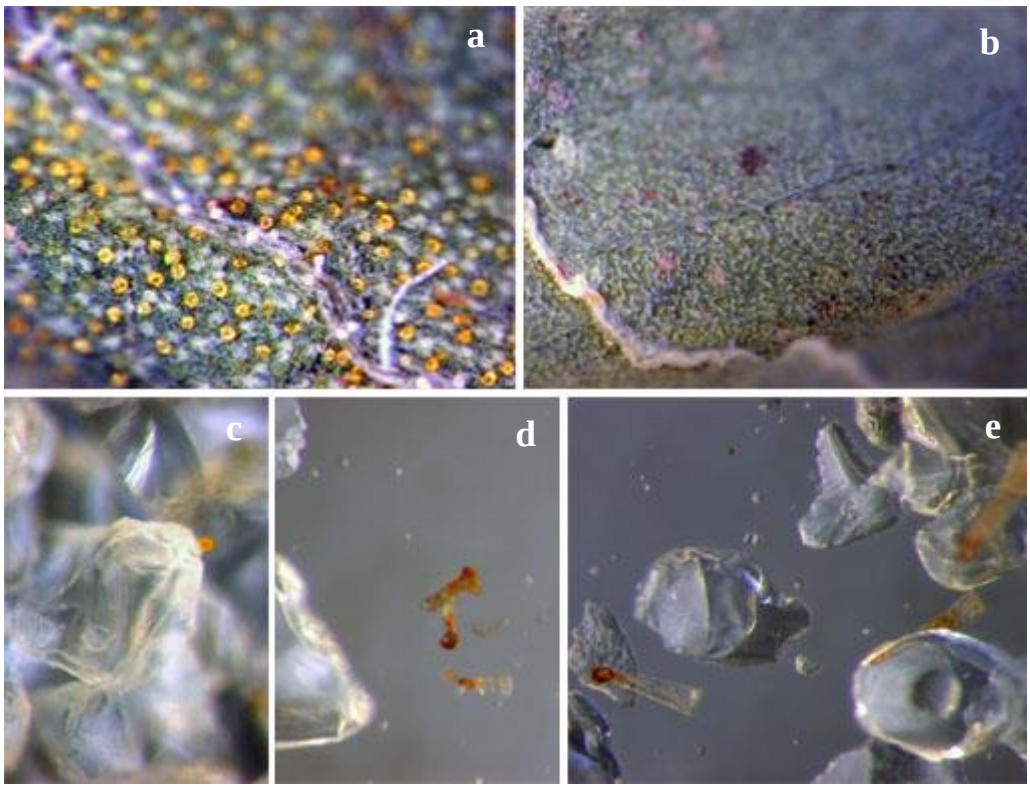

Fig. 8.: Abrasion of dry leaf material. **a**: Leaf surface with glandular trichomes before abrasion with silica gel; **b**: Leaf surface after abrasion with silica gel. **c**: Silica gel after abrasion (*P. denticulata*); **d**, **e**: Silica gel with glandular trichomes of *P. villosa*, after abrasion of leaf surface.

6.2.2 *Primula denticulata*: Seasonal variation of exudate profiles

Comparative HPLC analysis was carried out to check on the seasonal exudate composition over a period ranging from October to July. The results are summarized in Table 1. A clear distinction between the different seasons is apparent, particularly as the formation of flavone and 2,2'-diOH-chalcone as major compounds is concerned. The full range of 5-OH-flavone derivatives do not occur constantly over the seasons. Thus, 2'-OH-5'-OAc-flavone was detected in collections from March to April (6d-10d), but the structurally corresponding 5-OH-2'-OMe-flavone occurred in those from fall (1d-4d) and late spring (12d). A similar pattern was observed for 3-OMe-flavone. Most of the collections from early spring (6d-9d) and 11d (leaf holding farina residues) showed presence of lower amounts of 5,8-diOH-flavone in their profile. Noteworthy 11d differed completely from 12d although collected at the same time. Some compounds appear primarily in fall. Apart from 5,6,2',6'OMe-flavone (1d-4d), a so far unidentified chalcone derivative was detected in collections 1d and 4d, respectively. The UV-spectrum (UV_{max} 215 nm, 254 nm, 322 nm) of this chalcone resembles that of 2,2' dihydrochalcone (Fig. 9), but retention times are different (Fig. 10). The compound 2-OMe-2'OH-chalcone could only be detected in 12d and 14d. The latter collection showed presence of compound x1 (*ent*-kaurenoic acid derivative) in addition, which was also detected in *P. minima* and *P. clusiana*.

Tab. 1 *P. denticulata* leaf exudates, collected from HBV.

No.	*P. denticulata* Collections	2,2'-diOH-Chalcone	2-OMe-2'-OH-Chalcone	Unidentified Dihydrochalcone	Flavone	2',5'-diOH-Flavone	2'OMe-Flavone	2'-OH-5'-OAc-Flavone	3'OMe-Flavone	5-OH-Flavone	5-OH-2'-OMe-Flavone	5,8,2'-triOH-Flavone	5,8-diOH-Flavone	5,6,2',6'OMe-Flavone	x1: Rt 24.338
1d	20.10.2014	●		●	x				x		x			x	
2d	20.10.2014	●							x		x			x	
3d	20.10.2014	●							x		x			x	
4d	20.10.2014	●		●					x		x			x	
5d	17.11.2014				●					x					
6d	10.03.2015				●			x		x		x	x		
7d	18.03.2015				●			x					x		
8d	19.03.2015				●			x					x		
9d	20.03.2015				●			x		x			x		
10d	08.04.2015				●			x		x		x			
11d*	07.05.2015				●							x	x		
12d	07.05.2015	●	x		x	x	x		x	x	x				
13d	02.06.2015	●			x		●			x					
14d	27.07.2015	●	x		x		x			x					x

● major compound **x** minor compound *leaf with farina residues

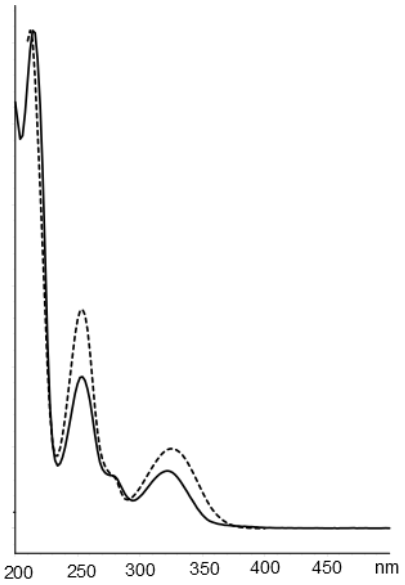

Fig. 9.: UV-spectra of the unknown dihydrochalcone substance (UV_{max} 215 nm, 254 nm, 322 nm) from *P. denticulata* (1d, 4d) and 2,2' dihydrochalcone (dotted line).

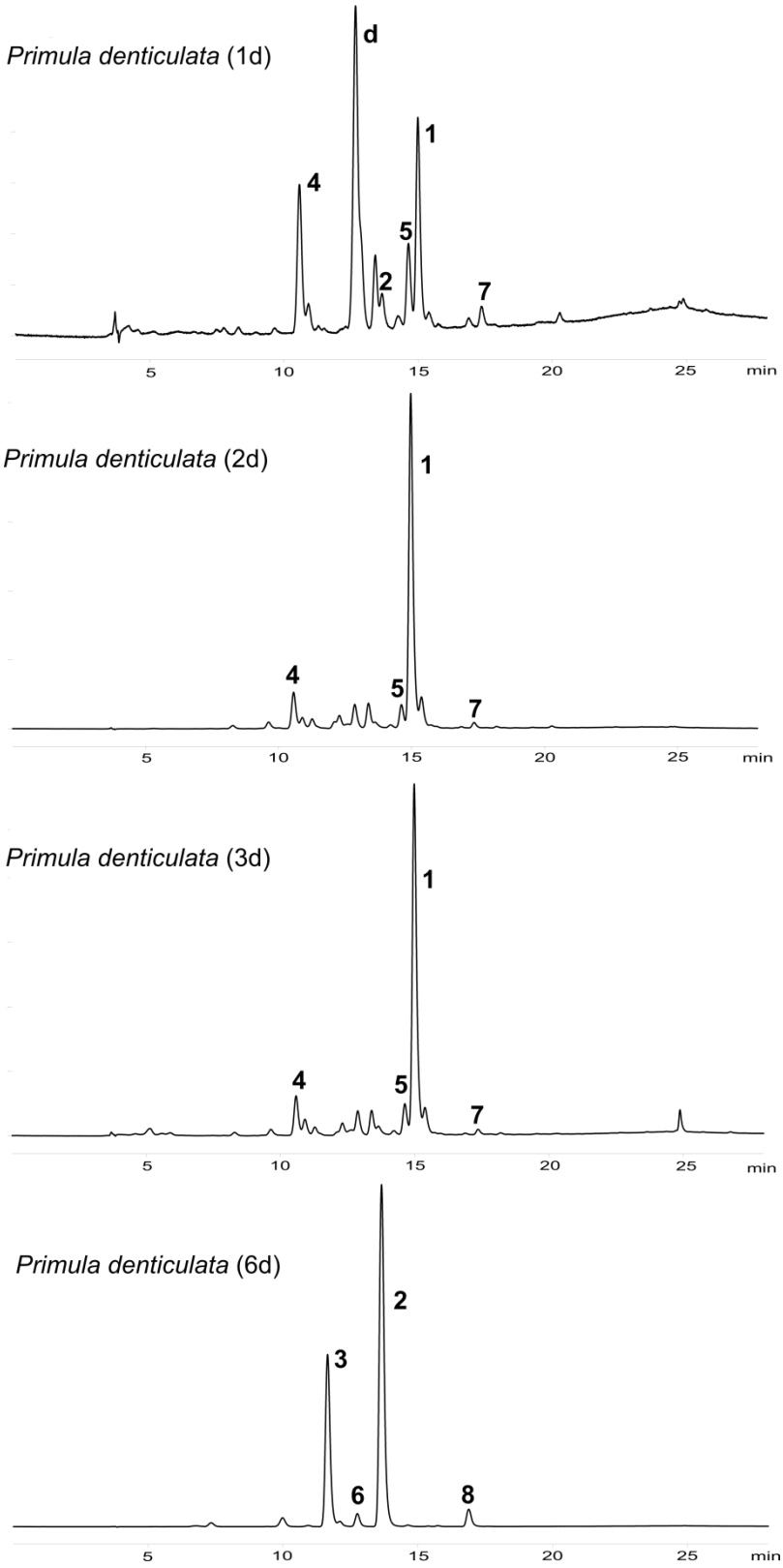

Fig. 10.: HPLC-chromatograms of *P. denticulata* leaf exudate collected at 20.10.2014 (1d), 20.10.2014 (2d, 3d), 10.03.2015 (6d). Exudate 1d and 3d were gathered via method 4, the other two via rinsing with acetone. Numbers and letters indicate compounds: **1**: 2,2'-diOH-chalcone, **2**: flavone, **3**: 5,8-diOH-flavone, **4**: 5,6,2',6'-OMe-flavone, **5**: 3'-OMe-flavone, **6**: 2'-OH-5'-OAc-flavone, **7**: 5-OH-2'-OMe-flavone, **8**: 5-OH-flavone, **d**: unidentified dihydrochalcone (UV-spectra see Fig. 9.).

As shortly addressed in chapter 6.1 (Elser et al., subm.) a seasonal shift in exudate compostion of *P. denticulata* collections could be demonstrated, this correlates to morphological observations performed when collecting the material. During winter *P. denticulata* was densely covered with white farina (Fig. 7a) and at the end of spring the farina was nearly completely absent. This is in line with the studies from Isshiki et al., (2014), attributing farina and flavone to increasing freezing tolerance. Collection 11d (see Tab. 1, leaf with farina residues) still had some farina left on the leaf surface whereas on 12d, collected at the same date, no farina could be observed. This clearly shows that the farina production is correlated to the production of flavone and the related "winter" exudate pattern (Fig. 10-6d). The summer exudate pattern is dominated by 2,2'diOH-chalcone or the unknown dihydrochalcone observed in exudates obtained by method 4 (Fig. 10-1d-3d). This dihydrochalcone seems to be a byproduct of the method as the silicagel in MeOH is able to act as acidic catalysis (J. Schinnerl, pers. comm.). Our HPLC measurements support this hypothesis. Two vials with pure MeOH and 2,2'diOH-chalcone at 1 mg/mL, one with and one without silica gel was monitored and showed the reaction to the unknown dihydrochalcone (Fig. 9). The peak area of the unknown dihydrochalcone increased about seven times (from 320,5 to 2334,5) within a month (18.11–15.12.2015) with added silica gel (Fig. 11b) whereas without added silica gel (Fig. 11a) there was only a doubling of the peak area (217 to 450) of the unknown dihydrochalcone within a month.

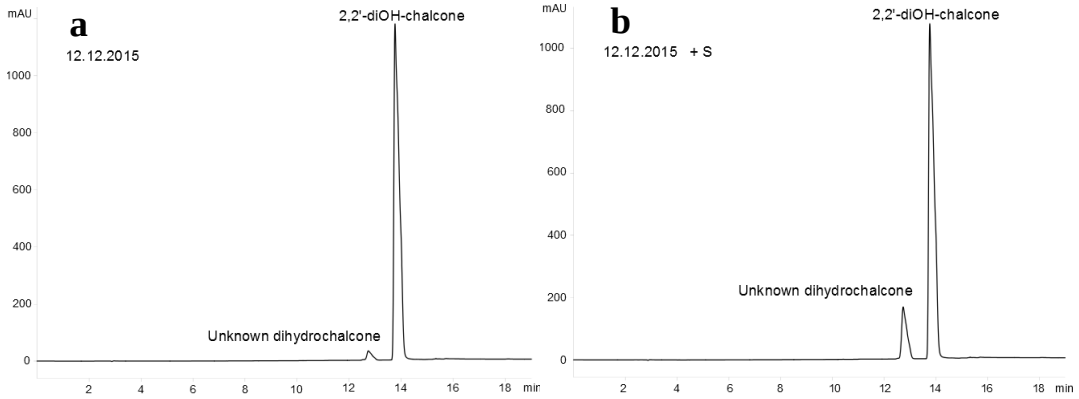

Fig. 11. Conversion of 2,2-diOH chalcone to a unknown dihydrochalcone in MeOH by the addition of silica gel within a month (18.11–15.12.2015). **a**: Without added silica gel. **b**: With silica gel added.

6.2.3 *Primula minima* L.

The exudate of *P. minima* exhibited four unknown compounds (Fig. 12). The recorded mass (Fig. 13–15) spectra of compound **x2** showed no matches in the online database Massbank (Horai et al., 2010). From these spectra it was deduced that compound **x2** consists of at least two compounds (**x2_1**, **x2_2**) or a loss of H_2O appears. Compound **x2_1** showed a calculated molecular mass of 426.2763 and a calculated molecular formula of $C_{27}H_{38}O_4$ which appears as adduct ions with of $[M+Na]^+$ (positive mode) with m/z of 449.2655 (Fig. 13 and Fig. 15a) and $[M+Cl]^-$ (negative mode) with m/z of 461.2 (calc. 461.2457) (Fig. 15b and Fig. 16). In negative ionization mode it mainly appears as molecular ion of $[M-H]^-$ with m/z of 425.3 (calc. 425.2690) (Fig. 15b and Fig. 16). Compound **x2_2** showed a calculated molecular mass of 408.2658 with a molecular ion of m/z of 409.2731($[M+H]^+$) (Fig. 13 and Fig. 14) which corresponds well to a calculated water loss from **x2_1** of 409.2724. The observed isotopic pattern of the two compounds is visible, looking at the given ratios it is likely that these molecules are mainly built up from $C_{27}H_{38}O_4$ (Fig. 14a and Fig. 15b), the calculated (M+1) peak of $C_{27}H_{38}O_4$ should have about 29.89 % height of the main peak which is in line with the observed isotopic pattern. The calculated isotopic pattern for the molecular ion $[M+H]^+$ $C_{27}H_{37}O_3$ of compound **x2_2** is shown in Fig. 14b. It corresponds well to the observed pattern.

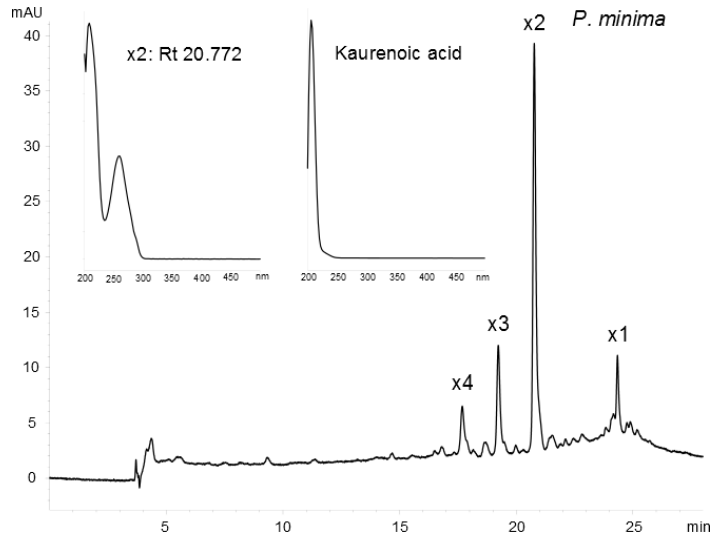

Fig. 12. HPLC-chromatogram of *P. minima* (population 12) leaf exudate. Letters x1-4 indicate unknown compounds, probably derived from kaurenoic acid.

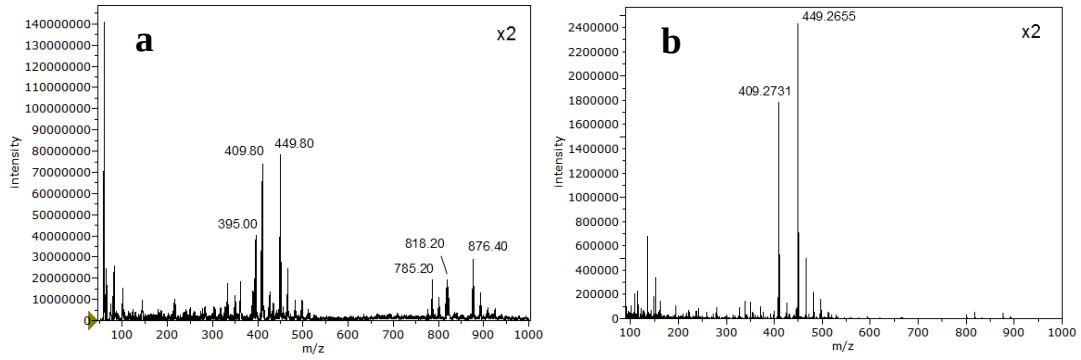

Fig. 13. a: Full scan mass spectrum of compound **x2** in positive ionization mode from *P. minima*. **b**: Full scan HRMS of compound **x2** in positive ionization mode from *P. minima*.

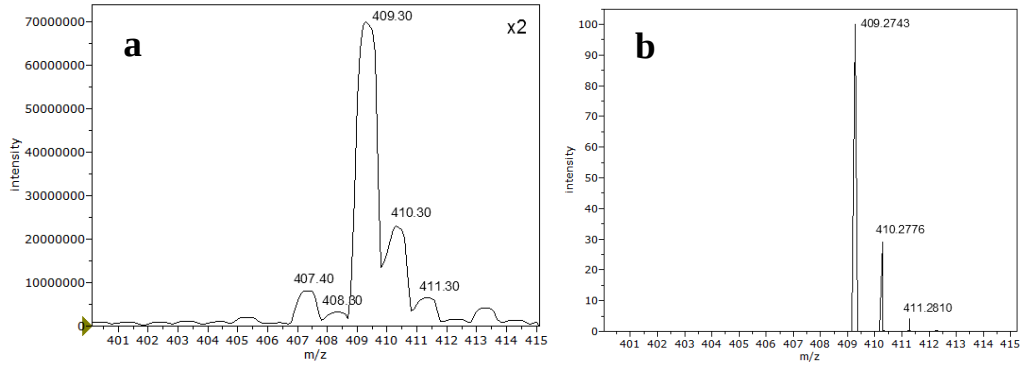

Fig. 14. a: Mass spectrum of compound **x2_2**, scanrange m/z 400-415 in positive ionization mode from *P. minima*. **b**: Calculated isotopic pattern of $C_{27}H_{37}O_3$ the molecular ion $[M+H]^+$ of compound **x2_2**.

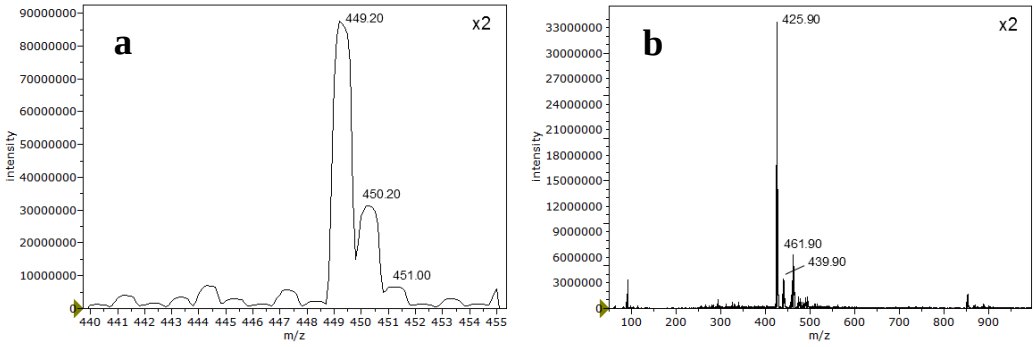

Fig. 15. a: Mass spectrum of compound **x2_1**, scanrange m/z 440-455 in positive ionization mode from *P. minima*. **b**: Full scan mass spectrum of compound **x2** in negative ionization mode from *P. minima.*

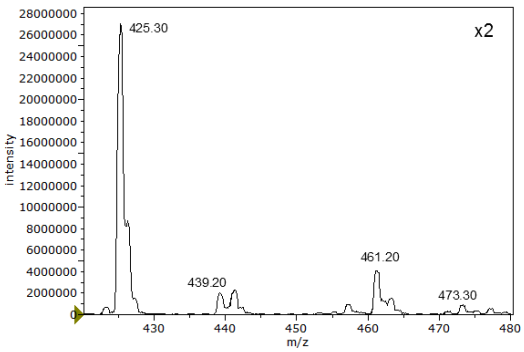

Fig. 16. Mass spectrum of compound **x2**, scanrange m/z 420-480 in negative ionization mode from *P. minima*.

The exudate composition of *P. minina* is still unknown, but the identification of the diterpene *ent*-kaurenoic acid gave some indication as to the types of compounds present. The results of mass spectrometry, the UV-spectra and the color reaction on TLC indicate that compound x2 seems to be a diterpenoid. The occurrence of diterpenoids in exudates is not new. Thus clerodane ditpernes are found in *Salvia divinorum* (Siebert, 2004) or in *Blakiella bartsiifolia* (Rodriguez-Hernandez et al., 2014). However, they are so far undescribed from the genus *Primula* and are hence a new result. The exudates of ferns (Rüedi et al., 1989; Wollenweber et al., 1983) exhibited kaurenoic acid derivatives, which however did no match with the observed mass from **x2**.

6.2.4 *Primula villosa* Wulfen

The leaf exudate of *P. villosa* at frist exhibited primarily primin-like compounds as main constituents (Fig. 17). Separation via Sephadex LH-20 (Fig. 18a) allowed for confirmation of primin as exudate compound. After further separation steps the flavanone naringenin 7,4'-diMe was detected. (Fig. 18b). Both depicted fractions (Fig. 18) contained a series of compounds with a primin like UV-spectrum.

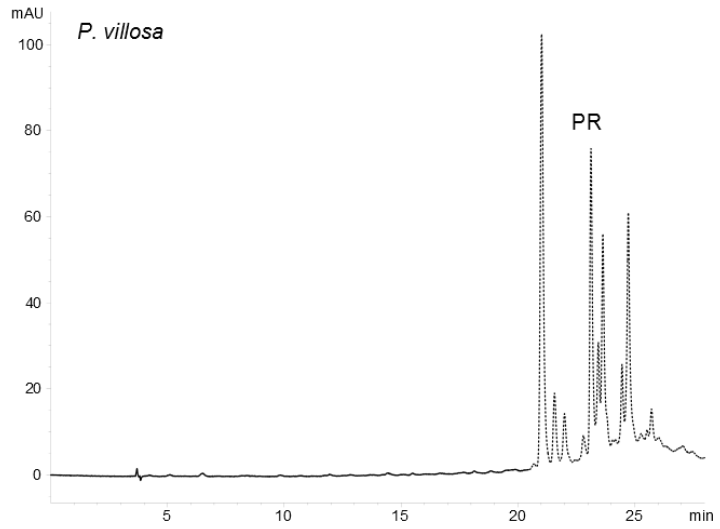

Fig. 17. HPLC-chromatogram of *P. villosa* leaf exudate. PR: Compounds showing primin like UV-spectra (dotted lines).

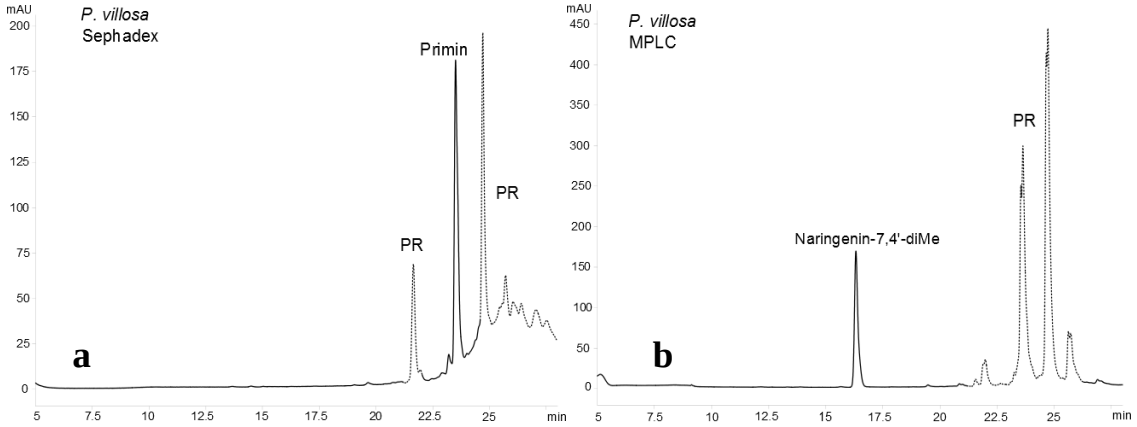

Fig. 18. HPLC-chromatogram of *P. villosa* leaf exudate. PR: Compounds showing primin like UV-spectra (dotted lines). **a**: Leaf exudate fraction 3 after separation via CC on Sephadex LH-20. **b**: Leaf exudate fraction 7-16 after separation via CC on Sephadex LH-20, Silicagel 60, and MPLC.

Separation of individual exudate compounds presented some difficulties. Even after three separation procedures, we were not able to obtain any pure compound. The primin-like compounds seem to mask the major composition of the exudate. They also seem to be quite reactive as reversible colour reactions show. In addition, the occurrence of primin and its lack after further separation procedures might indicate that a structural conversions occurred. The problems in isolation could be due to the use of solvents mixtures of inadequate lipophilicity. Horper and Marner, (1995) mentioned that all of their isolated compounds (primin and its derivatives) eluted best with PE and diethylether (Et_2O) on CC with silicagel. They achieved further separation with preparative TLC (toluene/MeOH) or a modified gradient of PE and Et_2O on CC with silicagel. Previously primin and its derivatives were isolated from *P. obconica* Hance leaf exudate. In addition various primin derivatives could be isolated from seeds of different *Iris* species and they appeared as phenols, resorcinols, quinones or their methyl ethers with different alkyl or alkenyl side chains (Marner and Horper, 1992). Such compounds could be present in the exudates where primin like substances could be detected (Fig. 17 and Fig. 2 in 6.1, Elser et al., subm.) and would explain the high number of different peaks with nearly identical UV-spectra.

7. Conclusion

The data obtained in the present study suggest that:

1. Methods to isolate glandular hairs may affect exudate composition. Critical review of obtained exudate composition patterns is recommended. An optimized method is suggested for *Primula*.

2. Seasonal variation is to be expected, particularly as far as the production of unsubstituted flavone is concerned. This might bear relevance in view of climatic conditions and should be studied further both in natural populations and under different climatic regimes. Studies under controlled climatic conditions (climatic chambers), including biotic and abiotic stress factors may reveal specific exudate compositions as reactions to modified environmental conditions.

3. Evolutionary younger lineages within *Primula*, such as European alpine taxa, may deviate from the widely distributed exudate composition that is based upon unsubstituted flavone and irregular substituted derivatives ("*Primula*-type" flavones). They tend to accumulate flavonoid compounds from the regular biosynthetic pathway, non flavonoid compounds such as primin and derivatives, or rare ditepernes in their exudates. Investigation of further species especially form North America, South America (*Primula magellanica*) and the remaining European ones is recommended.

4. Exudate composition may be an additional character when seasonal and populational variation is taken into account. This is specifically addressed in the manuscript submitted for publication and may lead to new aspects in the chemotaxonomy of *Primula*.

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9. Zusammenfassung

Die mehligen oder öligen Exsudate der Gattung *Primula* L. sind seit langem von Interesse für viele Wissenschaftler. Zuerst wurde angenommen das diese nur aus unsubstituiertem Flavon bestehen, später aber konnten sowohl unterschiedlichste Flavone mit unüblichen Substitutionsmustern, Dihydrochalkone und sogenannte Orphan Flavonoide isoliert warden. Verglichen mit der großen Anzahl an Arten wurden bisher nur wenige Arten (hauptsächlich asiatischer Herkunft) auf die Zusammsetzung ihrer Exsudate hin untersucht. Ebenso sind Arbeiten, die sich mit der jahrzeitlichen Variabilität befassen, selten. Das Ziel diser Arbeit ist es daher mehr Einblick in die Diversifikation der Exsudate zu gewinnen, im speziellen über die europäisch-alpiner Arten. Zusätzlich wurden unterschiedliche Methoden zur Isolierung von Drüsenhaaren erprobt, um die Exsudatgewinnung zu optimieren. Ebenso werden mögliche Jahreszeitliche Veränderungen miteinbezogen. Die Arten von Interesse wurden am natürlichen Standort gesammelt oder kultiviert. Die Exsudate und deren Inhaltsstoffe wurden mit Hilfe von HPLC, MPLC, Säulenchromatographie, Dünnschichtchromatographie, MS und NMR analysiert. Die Ergebnisse weisen darauf hin, dass sich die Zusammensetzung der Exsudate europäisch-alpiner Arten nicht auf das Vorkommen von Flavon und Flavonen mit unüblichen Substitutionsmustern welche sich als typisch für die Gattung *Primula* erwiesen haben, beschränkt. Demnach konnten hier regulär substituierte Flavone, Flavanone und Flavonole in den Exkreten nachgewiesen werden. Außerdem wird die Stoffvielfalt erweitert durch die Produktion von Diterpenen sowie von Primin- ähnlichen Substanzen und bis jetzt noch unbekannten Verbindungen. Interessanterweise konnten in einigen Arten keinerlei Flavonoide nachgewiesen werden. Bemerkenswert ist die beobachtete jahrzeitliche qualitative und quantitative Variabilität der Exsudateprofile in Populationen, welche jedoch noch intensiver zu erforschen wäre.