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Comparative eco-chemical studies of *Primula auricula* in the Eastern Alps with focus on flavonoid diversification

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Abstract

In continuation of previous studies, flavonoid diversification within *Primula auricula* populations was analysed, considering further locations in the Eastern Alps of Austria. *Primula auricula* is known for glandular hair derived exudates, mostly of a mealy appearance (farina). Farina consists mainly of unsubstituted flavone and small amounts of unusual, substituted flavone derivatives. These flavones are assumed to originate from a separate biosynthetic pathway as compared to the co-occurring tissue flavonoid glycosides. The study aimed to find correlations between flavonoid expression in tissue versus glandular hairs, a so far unstudied aspect. In course of this study, exudates were quantitatively and qualitatively analysed along seasonal changes, different populations, altitude gradients and leaf developmental stages using HPLC-UV. Data were evaluated using various statistical methods. Leaf tissue extracts composition of flavonoid glycosides was also analysed along different environmental variables as well as different leaf developmental stages. A clear correlation between leaf developmental stages and relative amounts of exudates was found, but no clear correlation with seasonal changes or altitudes was observed. Qualitative assessment of exudate indicates flavone (**1**) as the main component while other flavone aglycones are present in traces. Salicylic acid (**14**) was further identified as an exudate compound. Assessment of leaf tissue extracts indicated a flavonoid glycosides peak in spring towards a decline in fall and a sinus-curve progression within a year with maxima in spring and fall. No quantitative correlations were found between the relative amounts of flavonoid glycosides with the relative amounts of exudate and of flavone (**1**). From the results obtained in this study, it is suggested that *P. auricula* exudates might help regulate leaf temperature and decrease water evaporation, whereas the role of the flavonoid glycosides in the tissues remains debatable. More comparative studies working under controlled environmental conditions are needed to make final conclusions.

Zusammenfassung

In Fortsetzung früherer Studien wurde die Flavonoiddiversifizierung innerhalb von *Primula auricula*-Populationen unter Berücksichtigung weiterer Standorte in den österreichischen Ostalpen analysiert. *Primula auricula* ist für Exsudate aus Drüsenhaaren bekannt, die meist ein mehliges Aussehen haben (Farina). Farina besteht hauptsächlich aus unsubstituiertem Flavon und kleinen Mengen ungewöhnlicher substituierter Flavonderivate. Es wird angenommen, dass diese Flavone im Vergleich zu den gleichzeitig vorkommenden Flavonoidglykosiden der Gewebe aus einem anderen Biosyntheseweg stammen. Ziel der Studie war es, Korrelationen zwischen der Flavonoidexpression im Gewebe und in Drüsenhaaren zu finden, ein bislang unerforschter Aspekt. Im Zuge dieser Studie wurden Exsudate quantitativ und qualitativ anhand von saisonalen Veränderungen, unterschiedlichen Populationen, Höhengradienten und Blattentwicklungsstadien mithilfe von HPLC-UV analysiert und die Daten statistisch ausgewertet. Die Zusammensetzung der Flavonoidglykoside in Blattgewebsextrakten wurde ebenfalls anhand verschiedener Umweltvariablen sowie verschiedener Blattentwicklungsstadien analysiert. Es wurde eine klare Korrelation zwischen den Entwicklungsstadien der Blätter und den relativen Mengen an Exsudaten festgestellt, jedoch keine klare Korrelation mit jahreszeitlichen Veränderungen oder Höhenlagen. Die qualitative Bewertung des Exsudats weist auf Flavon (**1**) als Hauptbestandteil hin, während andere Flavonaglyca in Spuren vorhanden sind. Salicylsäure (**14**) wurde außerdem als bisher unbekannter Exsudatbestandteil identifiziert. Die quantitative Analyse der Blattgewebsextrakte ergab ein Maximum an Flavonoidglykosiden im Frühjahr, einen Rückgang im Herbst und einen Sinuskurvenverlauf innerhalb eines Jahres mit Maxima im Frühjahr und Herbst. Es wurden keine quantitativen Korrelationen zwischen den relativen Mengen an Flavonoidglykosiden und den relativen Mengen an Exsudat und Flavon (**1**) festgestellt. Die Ergebnisse dieser Studie legen nahe, dass *P. auricula*-Exsudate möglicherweise dabei helfen, die Blatttemperatur zu regulieren und die Wasserverdunstung zu verringern, während die Rolle der Flavonoidglykoside in den Geweben umstritten bleibt. Weitere Vergleichsstudien unter kontrollierten Umweltbedingungen sind erforderlich, um endgültige Schlussfolgerungen ziehen zu können.

1. Introduction

Primula auricula L. (*Primulaceae*) is a perennial alpine species distributed across central European mountain ranges (Zhang et al., 2004). *Primulaceae* are divided into 3 subgenera and 37 sections and encompass more than 2600 species mainly distributed on the northern hemisphere (Larson et al., 2023; *Tree of life explorer*; *World flora online plant list*). The genus *Primula* contains more than 400 species. Half of *Primula* species are endemic in the eastern Sino-Himalayan region, being the place with higher diversity (Richards, 2003). *Primula auricula* has been classified into the subsection *Euauricula* Pax of subgenus *Auriculastrum* Schott. This subsection represents the largest endemic group of *Primula* species in the Alps (Boucher et al., 2016).

Primula auricula plants grow in light exposed, steep rocky grounds made of limestone and in nutrient-poor and shallow soil (Xu & Chang, 2017; Zukal et al., 2020). Its green leaves are thick, similarly to succulent plants, and they show a green or white margin. While on bloom, flower stems are stiff. The aerial parts of the plant are often white due to the presence of a mealy exudate on its surface, produced by glandular trichomes. Oily exudates are equally produced, and the presence of a mealy exudate was considered a character for infraspecific differentiation (Holzbach et al., 2021). Their rhizomes are woody, and penetrating the limestone rocks, with their fine roots forming large networks there. Inside the fissures moisture is kept from evaporation, promoting rich soil life and a source of water, and protection from climate changes for the plant (Richards, 2003). Figure 1 shows *P. auricula* in its natural habitat in the Austrian Alps.

Figure 1. *Primula auricula* at the Austrian Alps.

Primula species have been known to produce a farinose and oily exudate on top of their aerial parts (Colombo et al., 2017). The main compound of this exudate is unsubstituted flavone (**1**), accompanied by unusually substituted flavones (flavones without substituents on their ring A), which are considered to be biosynthesised by glandular hairs. Flavone and flavonol glycosides are accumulated in the tissues and they are based mainly upon kaempferol and isorhamnetin derivatives (Holzbach et al., 2021). Unusually substituted flavones present in exudates have been named *Primula*-type flavones, due to their almost unique presence in *Primula* plants, and because of their assumed different biosynthetic pathway deviating from the classical flavonoid biosynthetic pathway (Valant-Vetschera et al., 2009). The fact that exudate and flavone (**1**) are produced in large quantities, indicates they might have a significant function on the plant and protective functions against environmental conditions such as UV-light and cold temperatures have been suggested (Colombo et al., 2014; Elser et al., 2016; Holzbach et al., 2021).

Previous research has analysed exudates compositions to determine possible correlations with environmental characteristics (Bhutia & Valant-Vetschera, 2012; Valant-Vetschera et al., 2009) and also studied *Primula* populations to determine possible significant characters that might help on the taxonomy and phylogeny distribution (Janda, 2021). In continuation of previous studies (Priemer, 2022), differentiation of chemical profiles both of exudates and tissues in selected populations of *P. auricula* are studied. Points addressed include analysis of the chemical composition of the exudate, with flavone (**1**) as main component, and the isolation of other relevant compounds. As a new aspect, the composition of flavonoid glycoside compounds in leaf tissue is analysed across populations. Based upon these results, flavonoid composition of exudate is statistically correlated with different environmental variables, seasons, altitudes and locations on the Austrian Alps. The aims of this study are detecting possible correlations between flavonoid composition and these abiotic variables, and detecting possible correlations between exudates and inner tissues.

2. Specialized Plant Metabolism

Specialised plant metabolites (SPM) are those compounds biosynthesized that contrary to primary plant metabolites are not essential for growth and reproduction but for survival on the environment (Tissier et al., 2014). Different tissues produce different specialised metabolites, and their biosynthesis can be affected by physiological and environmental conditions as well as the developmental stage of the individual. The main groups which SPM are classified into are terpenoids, the nitrogen containing metabolites called alkaloids, glucosinolates and cyanogenic glucosides which derive from amino acids and phenolic compounds. The main building blocks for secondary metabolites biosynthesis are acetyl-CoA synthesised during glycolysis from the primary carbon metabolism, aliphatic amino acids synthesised during the tricarboxylic acid cycle and isoprenoids produced by the mevalonic acid and MEP pathway (Figure 2).

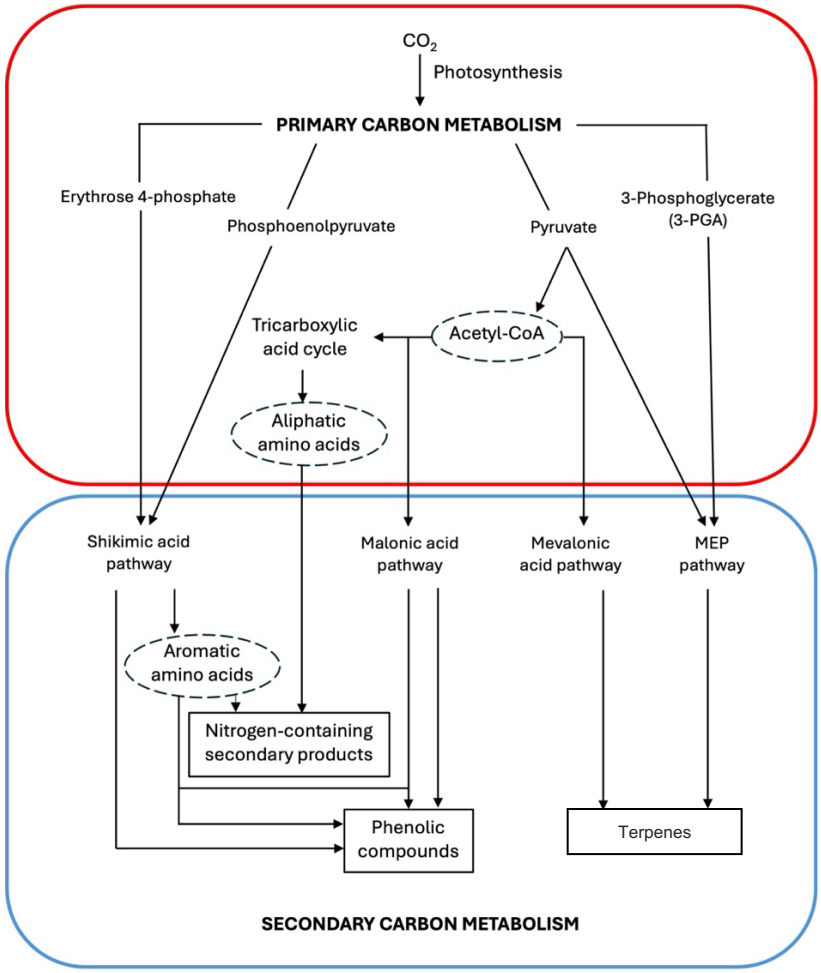

Figure 2. Biosynthesis of the main building blocks used by plants to produce secondary metabolites (Taiz & Zeiger, 2010).

3. Flavonoids. General Aspects

3.1. Flavonoid biosynthesis

Flavonoids are a group of polyphenolic secondary metabolites with a structure formed by two benzene rings (A, B) and a heterocyclic ring C (Figure 12 1). They are differentiated by the level of oxidation of the ring C and their substitution pattern, which influences the flavonoid antioxidant capacity, which is one of the major functions of these compounds. The hydroxy groups and the conjugated double bonds in flavonoids give these compounds their antioxidant activity, stabilizing free radicals by donating electrons through resonance (Cotelle et al., 2005; Kumar & Pandey, 2013; Panche et al., 2016). In plants, flavonoids are derived from two biosynthetic pathways: the phenylpropanoid pathway that produces the phenylpropanoid skeleton (C6-C3), and the polyketide pathway that produces blocks for polymeric C2 units (Meng et al., 2022).

Flavonoids are synthesized through the phenylpropanoid pathway with the conversion of phenylalanine to cinnamic acid by phenylalanine ammonia lyase (Figure 3). Then enzymes cinnamate-4-hydroxylase (C4H) adds a hydroxy group on the 4rth carbon of the phenyl and a 4-coumarate CoA ligase (4CL) binds a CoA group at the carboxy group, transforming the cinnamic acid to coumaroyl-CoA. In parallel, malonyl-CoA is produced from acetyl-CoA, and three molecules of malonyl-CoA react with coumaroyl-CoA, leading to a chalcone scaffold through the activity of chalcone synthase (CHS). Chalcones are readily isomerized by a chalcone isomerases (CHI), leading to the corresponding flavanones such as naringenin. Flavanones are the central molecules for generation of further flavonoid derivatives. Flavone synthases (FNS) react with flavanones and add one hydroxy group at the ring B and the flavones apigenin and luteolin are created (Figure 4). With the help of *O*-glycosyltransferases (FG3T), sugars can be added and transform these flavones into flavone glycosides transforming them into apigenin *7*-glycosides or luteolin *7*-glycosides. These additions will alter their solubility, reactivity and interaction with cellular targets. If for the contrary naringenin reacts with flavanone 3-hydroxylase (F3H) dihydroflavonols such as dihydrokaempferol and dihydroquercetin are formed. Flavonol synthase (FLS) will react with dihydroflavonols hydrolysing a hydrogen from ring C creating a double bond resulting in flavonols kaempferol and quercetin. Flavonol *O*-glucosyltransferase adds sugars to form flavonol glycosides, such as kaempferol 3-*O*-glycosides and quercetin 3-*O*-glycosides.

When dihydroflavonols react with dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS), the resulting structures are anthocyanidins in this case pelargonidin derived from dihydrokaempferol and cyanidin from dihydroquercetin, respectively (Figure 5) (Davies & Schwinn, 2006).

3.1.1. Factors affecting flavonoid biosynthesis

Abiotic stress

Plants are exposed to abiotic stresses including climatic and chemical changes that decrease their fitness. These stressors can be UV light exposure, temperature changes, nutrient deficiency, drought and heavy metals accumulation (Sulmon et al., 2015). Studies show that flavonoids can be related to the resistance and protection of the plant against these abiotic factors (Li et al., 1993; Xu et al., 2015). It has been observed that UV-B light treatments can affect plants by upregulating flavonoid biosynthesis key enzymes such as PAL and CHS in *Arabidopsis thaliana* L. (Li et al., 1993) and inducing differential flavonoid expression in *Primula veris* L. (El Morchid et al., 2014). The lack of nitrogen and phosphorous in the soil has also shown to enhance the transcription of the genes from the flavonoid pathway PAL, C4H, 4CL, CHS, F3H, F3'H in *A. thaliana*. (Lillo et al., 2008). When phenylpropanoid derivatives such as flavonoids are synthesised, ammonium from the phenylalanine is released. It could explain why plants would enhance flavonoid production when deficiency of nitrogen in soil exists. In this manner plants can eliminate excess of photosynthates while conserving nitrogen (Prescott et al., 2020). In maize seedlings and wheat plants, increase of flavonoid biosynthesis could be a response to drought stress to help protecting plants from drought-derivate oxidative damage (Li et al., 2021; Ma et al., 2014). A relationship between cold temperatures and an upregulation of the flavonoid pathway has been studied (Kitashova et al., 2024; Olsen et al., 2009; Zhang et al., 2022; X. Zhao et al., 2024). Yet more studies are needed to prove a relationship between cold stress and an enhanced flavonoid biosynthesis. It has also been suggested that anthocyanins can increase tolerance to moderate salinity in soils in *Brassica napus* L. and *Arabidopsis* thanks to their antioxidant activity by mitigating the effect of ROS induced by the stress (Changjiang et al., 2022; Jihye et al., 2017). Although further research about a possible increase in biosynthesis in plants on moderate saline soils and an upregulation of flavonols under lower temperatures is needed. Thanks to the neighbouring position of hydroxyl groups of flavonoids, these compounds are able to bind with metal ions and therefore have metal chelating properties in plants (Cornard & Merlin, 2003). Research also shows that flavonoids and flavonoid glycosides affected

polar auxin transport and nodulation, although specific roles are still yet to be defined (Buer et al., 2013; Franco et al., 2015; Yin et al., 2014)

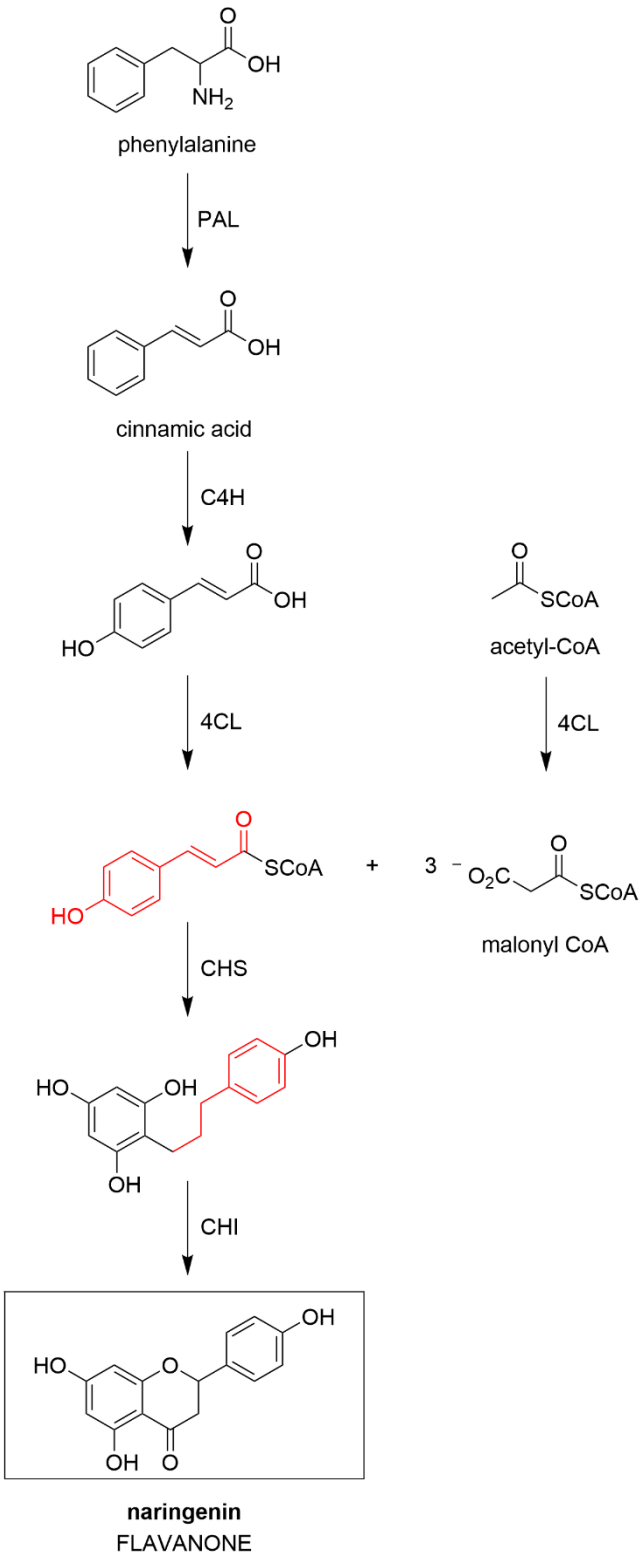

Figure 3. Phenylalanine and polyketide pathways. PAL=phenylalanine ammonia liase, C4H= cinnamate-4-hydroxylase, 4CL=4-coumarate CoA ligase, CHS=chalcone synthase, CHI=chalcone isomerase. Edited from (Davies & Schwinn, 2006).

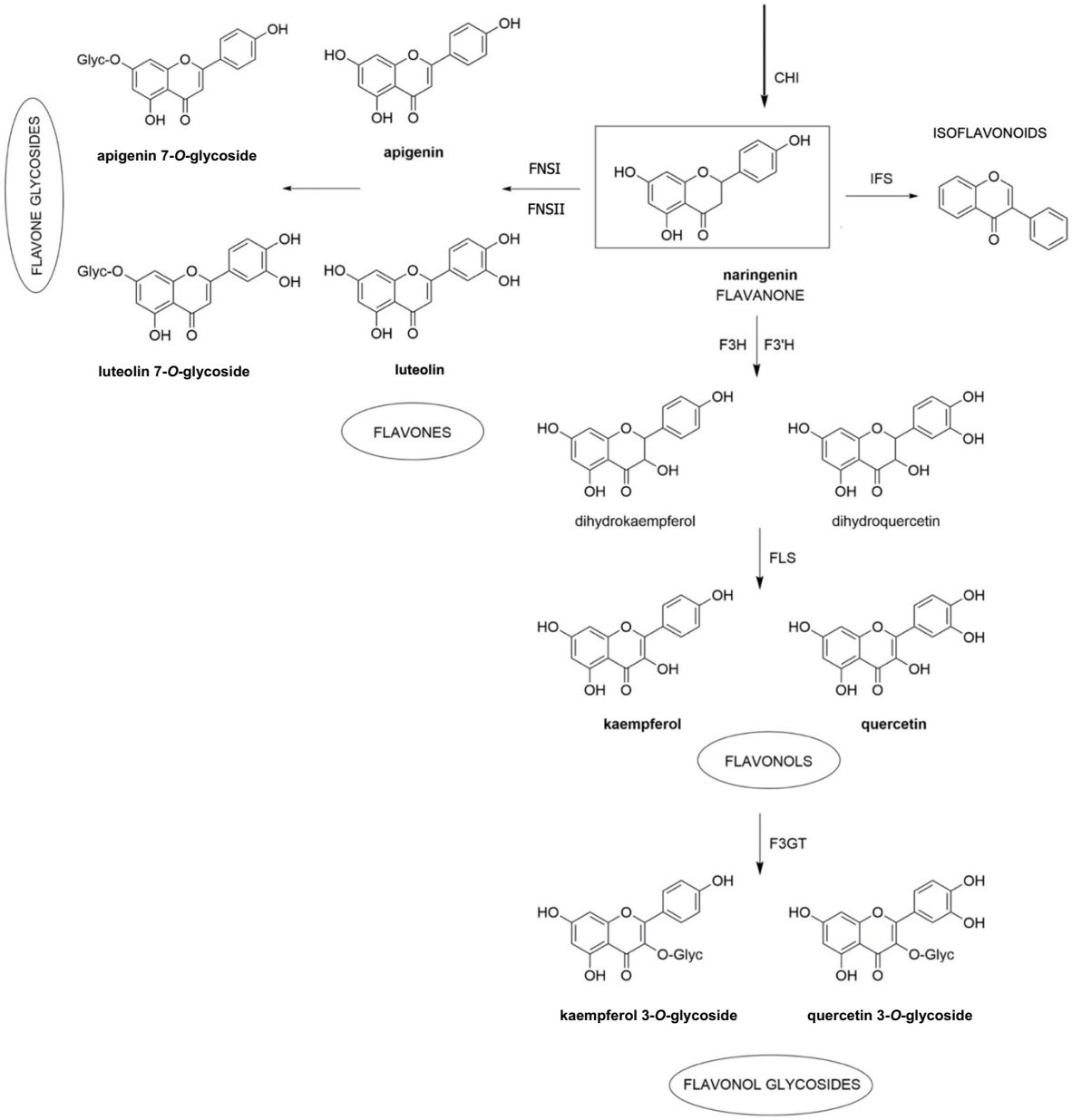

Figure 4. Continuation of the phenylalanine pathway on the flavonoid biosynthesis that results in formation of flavones and flavone glycosides, flavonols and flavonol glycosides. CHI=chalcone isomerase, FNSI=flavone synthase I, FNSII=flavone synthase II, IFS=isoflavone synthase, F3H=flavonol 3-hydroxylase, F3'H=flavonol 3'-hydroxylase, FLS=flavonol synthase, F3GT=flavonol 3-glycosyltransferase. Edited from (Davies & Schwinn, 2006).

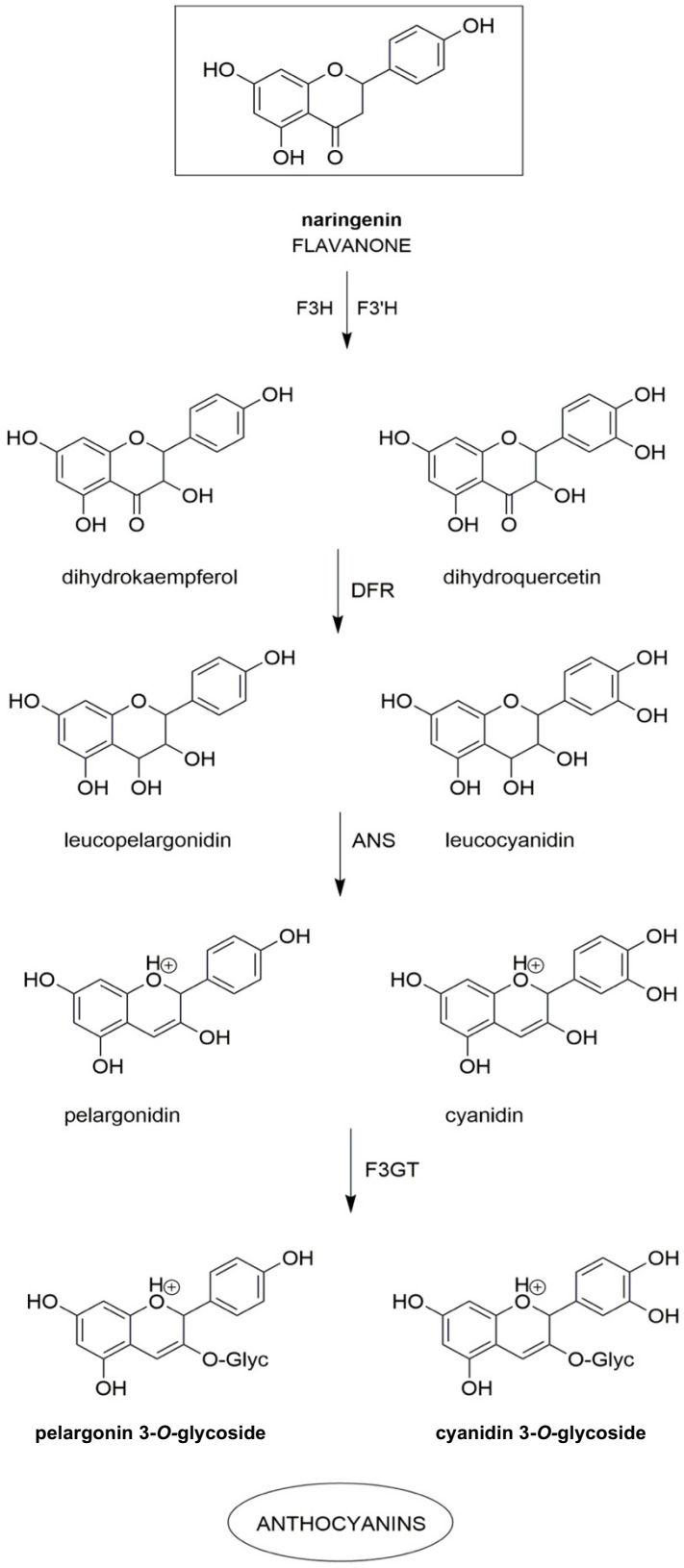

Figure 5. Continuation of the phenylalanine pathway on the flavonoid biosynthesis resulting on the formation of anthocyanins. F3H=flavonol 3-hydroxylase, F3'H=flavonol 3'-hydroxylase, DFR= dihydroflavonol 4-reductase, ANS=anthocyanidin synthase, F3GT=flavonol 3-glycosyltransferase. Edited from (Davies & Schwinn, 2006).

Biotic stress

Plants are susceptible to be negatively affected by pathogens such as viruses, bacteria, fungi and herbivores and they have developed constitutive and induced defense systems (Wilkinson et al., 2019). Constitutive protections are physical barriers like thorns, trichomes, and cuticles as well as specialised metabolites which are always produced, such as some alkaloids. Induced defense systems encompass secondary metabolites such as triterpenes and other alkaloids and flavonoid derivatives biosynthesised in case of pathogen negative effects. Studies have shown that flavonoids are related to plant resistance against different fungi and pests. Specifically, flavonoid aglycones have seen to provide higher pathogen resistance (Q. Zhao et al., 2024).

Flavonoid biosynthesis can be enhanced by the infection of fungi, endophytes or other pathogens. On the study from Janne et al. (2009) bilberry plants were infected by both an endophyte and a pathogen fungus. In both cases the production of flavonoids was increased, especially flavan-3-ols and oligomeric proanthocyanidins. The study made by Chen et al. (2023) also showed an enhanced flavonoid biosynthesis in the orchid *Dendrobium catenatum* Lindl. due to interaction with endophytic fungus *Pestalotiopsis* sp. DO14.

3.1.2. Position of flavonoids inside the plant cell

Antioxidant flavonols have been found in the vacuoles of both epidermal and mesophyll cells in leaves exposed to visible sunlight. These, together with anthocyanins, could be mediating the vacuole's role in the control of cellular ROS homeostasis. Flavonoids also have been detected at the plasmatic membrane (PM). They could be related with the capacity to regulate polar auxin transport by interacting with PM-located auxin carrier proteins. Flavonols present in PM could also have a role as ROS scavengers (Pollastri & Tattini, 2011). Emerging evidence is showing that consecutive enzymes of the phenylpropanoid and flavonoid biosynthesis are organized into complexes or metabolons that could be associated with endomembranes such as the endoplasmic reticulum (ER). These complexes would allow metabolic channelling to happen. Intermediate secondary metabolites could go from one active site to another avoiding metabolic interference. This would allow plants to synthesize specific metabolites inside the cell without needing processes to be compartmentalised (Falcone Ferreyra et al., 2012; Winkel-Shirley, 2004). Flavonols have been detected inside nuclei of cells from different trees. Applying UV-VIS spectroscopic titration it was found that there was an interaction between quercetin and rutin with

nucleosomes. The enzymes flavonol synthase I, chalcone synthase and chalcone isomerase related to the flavonoid biosynthesis have also been found in the nuclei of *Arabidopsis* cells using confocal laser scanning and wide-field deconvolution fluorescence microimaging (Polster et al., 2006). The reason for this interaction is still unknown. It is hypothesised that these enzymes together with flavonoids could be having an impact on the control of the genes related to growth and development of the plant, because they have also been detected in chloroplasts, which have been reported as capable of flavonoid biosynthesis. Anthocyanin pigments have been seen to accumulate in the vacuole or the cell wall (Pollastri & Tattini, 2011).

3.2. Flavonoids and other consituents of *Primula*

3.2.1. Exudate flavonoids and glandular trichomes

Flavones are the main components of *Primula* exudates. In previous research, exudates from different species were analysed and it was shown that the main compounds were unsubstituted flavone (**1)** and other flavone aglycones with unusual substitution patterns (Valant-Vetschera et al., 2009). Flavone (**1)** has also been found in other species, such as *Feijoa sellowiana* O. Berg from the *Myrtaceae* family, but it is otherwise rarely reported outside Primulaceae (Valant-Vetschera et al., 2024). Flavone (**1)** and the unusually substituted flavones predominant in the *Primula* exudates have been termed "*Primula* type flavones", as the structures suggest to derive from a different biosynthesis pathway other than the "classical" one (Valant-Vetschera et al., 2009).This new biosynthetic pathway, however, has not yet been elucidated. This topic is discussed at length by Vetschera et al. (2024).

For *Primula*, it has been suggested that exudate flavones are produced inside glandular hairs as indicated by detection of flavonoid biosynthetic enzymes in these compartments (Schöpker et al., 1995; Valant-Vetschera et al., 2024). This is in line with the lack of detection in leaf tissues where flavonoid glycosides accumulate (Holzbach et al., 2021). However, more in depth studies are needed. In contrast to other glandular trichomes, those of *Primula* spp. excrete both oily and farinose exudates through their cuticula (Wollenweber et al., 1971). Recently, farinose exudation was confirmed to occur at specific sites at the surface of the trichome's glandular head cell, i.e. distinct micron-size holes in the plasma membrane, as shown for the related *Dionysia tapetodes* (Bourdon et al., 2021). Endoplasmic

reticulum, mitochondria, leucoplasts, and microbodies could be involved in flavone (**1**) biosynthesis due to their abundance in the end cell of *Primula spp.* glandular hairs (Valant-Vetschera et al., 2024). It is believed as unlikely that flavone (**1**) is a product of the degradation of substituted flavones. For now, no degradation enzymes have been found. Possible precursors of flavone (**1**) could be 2′ß-dihydroxy chalcone via isomerization to the respective unsubstituted flavanone (Valant-Vetschera et al., 2024).

Glandular trichomes are specialised structures present on the aerial parts of plants, also called glandular hairs. They can be made of one or more cells, and they are the place of synthesis, storage and sometimes excretion of secondary metabolites. These structures are present in several plant families (Bhutia, 2013). In *Primula*, the excretions produced by glandular trichomes can be oily or mealy, the latter taking a thread-like or farinose structure (Bourdon et al., 2021). Figure 6 gives a survey on different types of exudates produced by *Primula* species. *Primula auricula* produces mostly a significant amount of farinose exudate, deposited on the epidermis of leaves, calyces, and flowers (Figure 6 A) by glandular trichomes (Figure 6 B). For *P. vialii* Delavay ex Franch., it was shown that the auto-fluorescent 2'OMe-flavone is accumulated as major compound only in the head and neck-cell of the glandular trichome. Bhutia et al. (2012), *Flavonoids in selected Primula spp.: bridging micromorphology with chemodiversity,* showed that glandular trichomes from the same *Primula vulgaris* Huds. leaf, producing an oily exudate, gave different colours upon excitation with blue light at 490 nm. The resulting fluorescence observed indicated that trichomes from the same leaf might have a different chemical composition (Figure 6 C). Final proof that exudate flavonoid production is exclusively located in glandular hairs, and if the synthesis of precursors occurs in underlying tissues with subsequent transport to final site of biosynthesis, awaits confirmation.

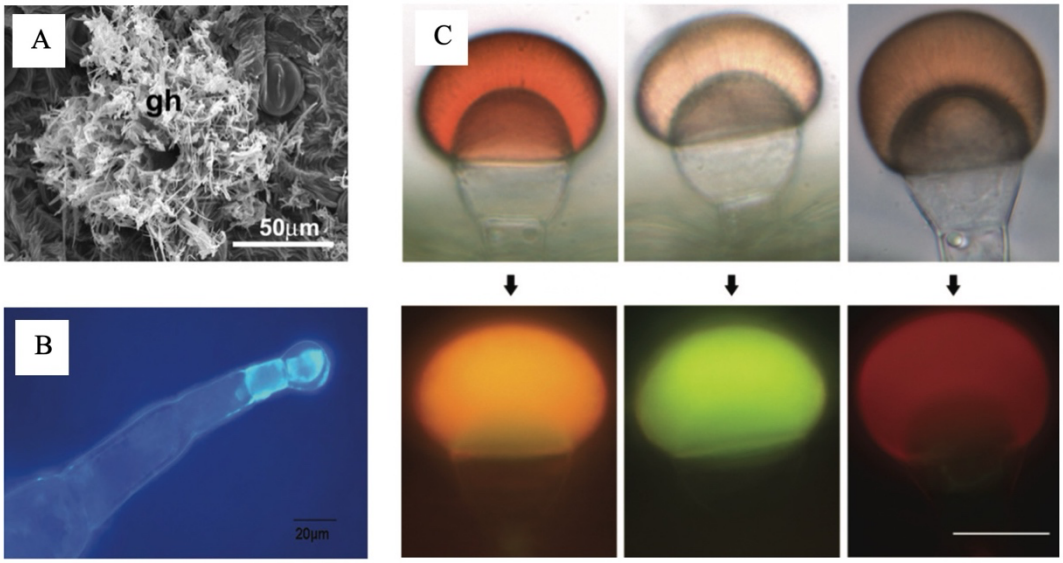

Figure 6. (A) Farinose exudate from *P. auricula* (Colombo et al., 2014). (B) Glandular trichome producer of oily exudate from *Primula vialii* Delavay ex Franch leaves (Valant-Vetschera et al., 2009). (C) Oily exudate from *Primula vulgaris* Huds. plants belonging to the same population (Bhutia & Valant-Vetschera, 2012)

3.2.2. Tissue flavonoids

In *Primula* leaf tissue flavonoid *O*-glycosides predominate. The most frequent flavonoid glycosides found in this plant species tissue are flavonol 3-*O*-glycosides based upon the aglycones kaempferol, quercetin, and isorhamnetin, but also the flavones apigenin, luteolin and, less frequently, the flavonols limocitrin, myricetin and tamarixin have been reported. *Primula*-type flavones have also been identified in the tissues, 3′-hydroxyflavone 3′-O-β-glucoside and 3′,4′-dihydroxyflavone 4′-O-β-glucoside (Holzbach et al., 2021). Flavonoid glycosides found in leaf tissue have two to three sugars, usually being glucose, rhamnose, gentiobiose, neohesperidose and sophorose. However, arabinose, mannose, galactose and xylose have also been identified (Colombo et al., 2017).

3.2.3. Further constituents of *Primula*

Saponins are another group of secondary metabolites identified in *Primula* spp. as compounds of root tissue. Those described so far are protoprimulagenin A (PSap1), primulasaponin I (PSap2), priverosaponin-B-22 acetate (PSap4), primulasaponin II (PSap5) (Włodarczyk Maciej et al., 2020). It has been shown that saponins are of limited distribution within the genus but are predominant in subgenera *Primula*.

The composition of volatiles present in oily exudates could change due to climatic and environmental pressures and herbivore or parasite attacks. In *Primula* spp., volatiles are mainly synthetized from the terpenoid and phenylpropene pathways. In *P. vulgaris* it has been seen that altitude also causes variations to the essential oils and fatty acid methyl esters present in oily exudates (Yaylı et al., 2016). Salicylic acid derivatives have also been found in *P. auricula* exudates although their specific structure has not been yet identified (Priemer, 2022).

4. Material and Methods

4.1. Plant material

Whole individuals of *Primula auricula* have been collected, around midday, from five different locations of Austria: Schneeberg (S), Mödling (M), Rax (R), Rote Wand (RW) and Leopoldsteinersee (L, Figure 7). In these sampling sites, plant samples were collected at different altitudes. The samples have been collected in different years and in different seasons (AX I). Due to the different availability of individuals, the number of samples and altitudes vary along the sampling sites from which they were collected.

Figure 7. *Primula auricula* collection sites.

Once individuals were collected, we separated each leave and rinsed each one with acetone in Eppendorf tubes to obtain the exudate. After the leaves were rinsed, they were fixed on a white paper sheet in order, from youngest to oldest, and dried them at 30°C for at least two weeks. Leaves were categorized in inner leaves, for those being part of the bud, and outer leaves, for those not forming part of the bud (Figure 8). After the leaves have been dried, each sheet of paper containing *Primula auricula* individuals was scanned to afterwards perform leaf area determination.

Figure 8. *Primula auricula* individual CPS-PA-OH-1.13. leaves arranged from youngest (leaf number one) to oldest (leaf number fifteen). Leaves forming part of the bud were classified as inner leaves and the those that were not part of the bud were classified as outer leaves.

4.2. High-performance liquid chromatography

Exudates and extracts were analysed by high-performance liquid chromatography (HPLC) using the Agilent 1100 series with an UV diode array detector and a LC column (Hypersil BDS-C18 column with dimensions of 100 × 4.0 mm, particle size of 3 µm, Agilent). The detection settings were set according to Elser et al, (2016). The wavelength used was 300 nm for exudate analysis and 254 nm for leaf extract analysis, respectively. The flow rate was set at 0.5 mL/min and the solvent gradient of methanol (A) in aqueous buffer (B) started with 10 % A in B for 0–5 min, 55 % A in B from 5–9 min and 78 % A in B from 9–15 min. The buffer composition was 15 mM ortho-phosphoric acid (H_3PO_4) and 1.5 mM tetra-butyl ammonium hydroxide (Bu_4NOH) in gradient grade water. The column oven temperature was set at a temperature of 30 °C and a volume of 5 µL of sample was injected each time. The software Agilent ChemStation (Agilent Technologies) was used to process and analyse the chromatograms and the UV-spectra. The identification of compounds was made comparing UV-spectra and retention time with the in-house flavonoid database. The integration parameters set for peak area were slope sensitivity 5, peak width 0.02, area rejected 5, height rejected 1, shoulders off.

4.3. Calibration

Calibration parameters were assessed and used to transform HPLC peak areas into concentrations, to determine amounts of flavone and flavonol glycosides (Table 1). The compounds used for calibration for flavone glycosides and flavonol glycosides were 3′,4′-dihydroxyflavone 4′-*O*-β-glucoside and kaempferol 3-*O*-beta-glucosylgalactoside. Initially, 1.0 mg/mL of stock solutions of 3′,4′-dihydroxyflavone 4′-*O*-β-glucoside and kaempferol 3-O-beta-D-glucosylgalactoside (Panasenoside) were prepared using methanol as solvent. From these stock solutions dilution series with concentrations of xx µg/mL etc were prepared. For this purpose, 150 µL from the initial dilution were transferred into Eppendorf tubes and 150 µL of methanol were added in each of them. These two steps were repeated four times more to obtain a dilutions series of 1.0 mg/mL, 0.5 mg/mL, 0.5 mg/mL, 0.125 mg/mL, 0.0625 mg/mL and 0.03125 mg/mL. From each dilution 50 µL were taken and transferred HPLC glass tubes. These dilutions were then subjected to HPLC measurements. From the obtained chromatograms the peak areas at 230 nm were assessed and the following parameters calculated: y= 8068.5x – 6.9624 with an accuracy of R2. = 0.9994 for 3′,4′-dihydroxyflavone 4′-*O*-β-glucoside and y= 9955.6x – 45.124 with an accuracy of R^2 = 0,9991 for panasenoside. For quantification of flavone (**1)** and flavone aglycones calibration parameters given in Priemer (2022) were used.

Table 1. Classification of type of compounds found in exudates and leaf extracts with related calibration parameters.

Substitution type	Compound	Calibration regression
Unsubstituted*	Flavone (**1)**	$y = 50634\,x + 321.89$
A-Ring*	5-hydroxyflavone (**2**)	$y = 28665\,x + 6.3389$
B-Ring*	2'-hydroxyflavone (**5**)	$y = 38853\,x + 9.1222$
AB-Rings*		
Chalcone*	2,2'-dihydroxychalcone (**13**)	$y = 26549\,x + 2.5764$
Flavonol glycoside	3′,4′-dihydroxyflavone 4′-*O*-β-glucoside	$y = 26549\,x + 2.5764$
Flavone glycoside	Panasenoside	$y = 9955{,}6x - 45{,}124$

* Calibration parameters taken from Priemer (2022)

4.4. Exudate preparation

To obtain the exudate, young *P. auricula* leaves were submerged in Eppendorf tubes with acetone (ACS Reagent ≥ 99.5 %, Sigma Aldrich) as solvent, dipping the leaf five times. Older leaves that did not fit inside the tubes were rinsed with the solvent in small beakers three times. The tubes and beakers were set under a hood for an hour to evaporate the solvent. New tubes for each exudate were weighted and labelled. Once tubes containing the exudate solution were almost dried, they were rinsed three times with solvent and the solution was filtered using glass pipettes containing cotton filter. This step has been important to purify the sample and eliminate soil and other solids that might obstruct the HPLC column used for future measurements. Filtered solutions were transferred into previously weighed tubes. For the solvent to evaporate, all Eppendorf tubes were set again under a hood and afterwards inside a vacuum centrifuge until fully dry. The tubes were weighed again to obtain the amounts of exudate before adding solvent to dissolve the exudate at a concentration of 2 mg/mL. From each of these exudate solutions 50 µL were placed into 96-well plate (VWR Tissue Culture Plates, VWR) to afterwards let the solvent evaporate under a hood. In each well 50 µL of DMSO was added and analysed by HPLC.

4.5. Extract preparation

Inner leaves and outer leaves were selected from each *Primula* individual. From each leaf, a piece was cut out and ground inside 2 mL Eppendorf tubes. The same amounts of material (from 2 mg to 25 mg) was not used in all leaves for two main reasons. The first one being that not all leaves were dried for the same amounts of time, therefore the water content inside the leaf was varying consequently making vary the amounts of extract obtained for the same amounts of leaf. The second one was because the most inner leaves were very small, and thus the amounts of extract obtained from them was many times zero. Therefore, using the same amounts of leaf for more than three hundred samples resulted into a number of samples that could not be used for comparison. The results obtained were going to be related to the amounts of extract obtained instead of the amounts of leaf.

Before grinding the samples three glass weights were added inside each tube and samples were set inside tissue lyser for 10 minutes at a frequency of 30 Hz. Then 500 µL of methanol (HiPerSolv Chromanorm, VWR) were added in each tube and they were placed into an ultrasonic bath for 10 minutes. Eppendorf tubes were centrifuged for 10 minutes at

3500 rpm and supernatants were transferred into new pre-weighed tubes. Methanol was evaporated under a hood and afterwards inside vacuum evaporator to obtain the weight of extract in each tube.

Once the amounts of extract was assessed, these were resuspended at a concentration of 2 mg/mL with methanol. From each of these solutions 50 µL were placed into 96-well plate to afterwards let the solvent evaporate under a hood. In each well 50 µL of DMSO was added and the samples were ready to be analysed on the high-performance liquid chromatography (HPLC). In addition, a dilution series of a flavonoid (Table 2) was made to be able to compare the HPLC results and obtain the concentration of compounds present in the samples.

4.6. Leaf area determination

The leaf area has been determined using Adobe Photoshop Version 23 by selecting each leaf to automatically calculate the number of pixels of one side of the leaf, then doubling the number of pixels and finally multiplying the area of a pixel per number of pixels that each leaf occupies. In some leaves, the area was needed to be selected manually, although the pixel calculation of such area was also done automatically.

4.7. Compound isolation

4.7.1. Liquid-liquid extraction

Chromatograms of exudates obtained from samples collected at Mödling measured in HPLC were compared to select the compound for isolation. We proceed to isolate the compound that eluted in HPLC at minute 4.000-4.008 min. This compound was mentioned in Priemer (2022), proposing to be a possible salicylic acid derivative. Exudates samples from Mödling collected during winter were pooled and (670.8 mg) were used for separation steps liquid-liquid extraction using water/chloroform ($CHCl_3$).

4.7.2. Medium pressure liquid chromatography

The apolar phase of the liquid-liquid separation was dried and together with some methanol was placed in 3 g of silica gel 0.2–0.5 mm diameter. Twenty grams of silica gel 60 (40–63 µm) was used as stationary phase. Once it dries, a medium pressure liquid chromatography (MPLC) connected to a UV-Vis detector was set to measure absorbance 254 nm and 300

nm (AX 2). With a flow rate of 5 mL/min the sample elutes with mobile phase of petrol ether (PE) and ethyl acetate (EtOAc) with ratios of 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 0:100. We collected a total of 38 fractions none of the samples contains a pure compound.

4.7.3. Thin layer chromatography

Selected fractions obtained from the size exclusion chromatography were pooled (Table in appendix), and together with the apolar phase of the liquid-liquid separation two TLC plates were prepared for further purification and isolation of the desired (Figure 9).

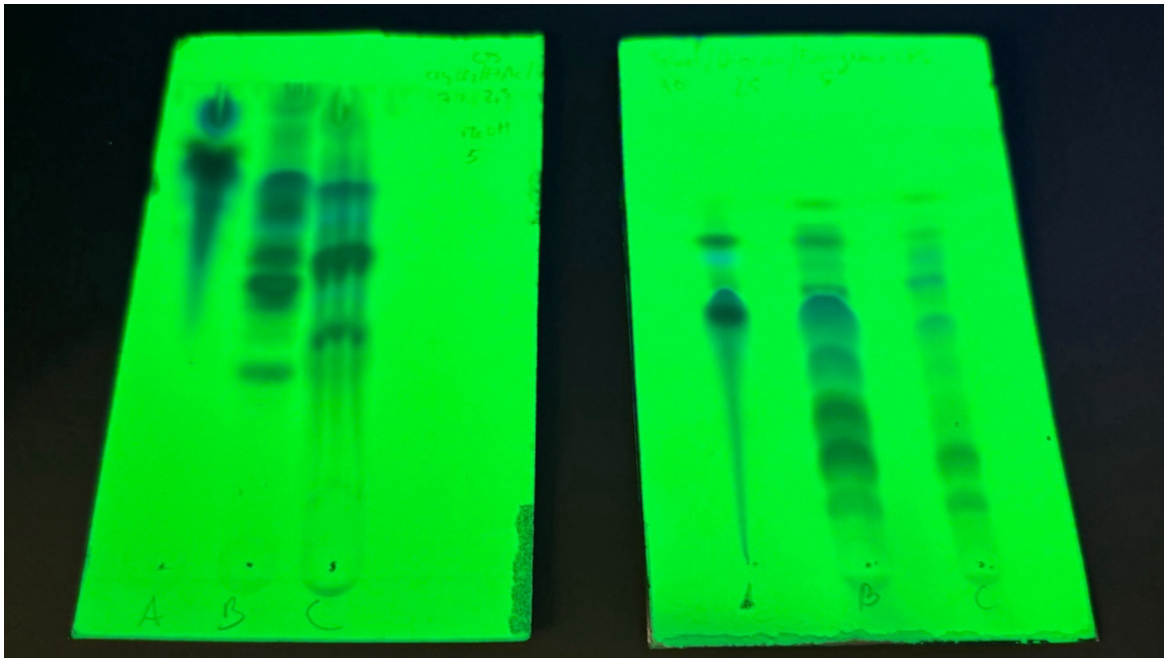

Figure 9. TLC plates eluted with a mobile phase of 70% chloroform, 2.5% ethyl acetat and 5% methanol (left) and 90% toluene, 25% dioxan and 5% acetic acid (right) at 254 nm. The samples eluted were A = pool of silica gel MPLC fraction 38, B = pool of Sephadex LH20 column chromatography fractions 30–33, 36, 37 and 39, C = pool of Sephadex LH20 column chromatography fractions 34, 35 and 36 alaysed by NMR. Visualised under green light at 254 nm.

4.7.4. Size exclusion chromatography

To further purify the compound of interest we use a size exclusion chromatography connected to a UV-Vis detector on the MPLC fraction number 38 (27.9 mg). The detector was set at 302 nm. The sample was eluted with methanol through the stationary phase of Sephadex LH20. The fractions and their absorbance measurements can be consulted in AX 3 The fractions 34, 35 and 36 were pooled and sent to be analysed by nuclear magnetic resonance (NMR). These measurements and spectra interpretation was done by Professor Lothar Brecker at the Department of Organic Chemistry, University of Vienna.

4.8. Statistical analysis

From the collected plants, exudate and leaf extract samples obtained. The amounts of exudate from individual leaves and their leaf areas were measured to afterwards calculate ratios of amounts of exudate per leaf area in mg/cm^2. Selected exudate and extract samples were submitted to the HPLC. Using the Agilent Chemstation Rev. B04.02 software, resulting chromatograms for each exudate and extract sample were obtained. Chromatogram peaks were associated with a type of flavonoid and their area values were obtained. To determine the type of compounds UV spectra diagrams were assessed. With these area values, amounts of type of flavonoids were calculated. The handling of data was performed using python (version 3.11.2) in the text editor Visual Studio Code (version 1.92.2). The library plotly was used to visualise the data and the module scipy.stats was used to apply the statistical tests. Shapiro-Wilk tests was used to determine the data distribution. Kruskal-Wallis tests were performed to determine the presence of significant differences between medians of compounds in leaf exudates along inner and outer leaves, seasons, altitudes and mountains where sample collection was made. And two-way ANOVA tests determined significant mean differences between amounts of flavonoid glycosides from extracts and the different independent variables. To further identify between what groups from a variable the significant differences lay, post hoc tests Dunn's and Tukey's were done on exudate and extract data respectively. To finalise the statistical analysis, correlation tests Spearman's and Pearson's were made.

5. Results

5.1. Analysed exudates

During this study, a total of 87 *P. auricula* plants were collected in summer, fall and winter in 2023/2024 along all locations and altitudes, but no samples were collected during spring. The leaves of the plants were carefully separated and processed for further investigations. From these plants, 724 exudate samples were prepared and further investigated taking into account their developmental stage. From all these samples, total amounts of exudate were determined and the relative amounts in mg/cm² calculated. The mean total amounts in summer were 0.02 mg/cm² (min <0.01/max 0.97/std 0.11) in fall 0.01 (<0.01/ 0.20/ 0.02) and in winter 0.01 (<0.01/0.16/0.02). Total amounts of exudate in different leaf developmental stages were 0.02 mg/cm² (min <0.01/max 0.97/std 0.07) in inner leaves and <0.01 (0/ 0.03/ 0.01) in outer leaves (Tables 3 and 4). The detailed results are given in the following sections.

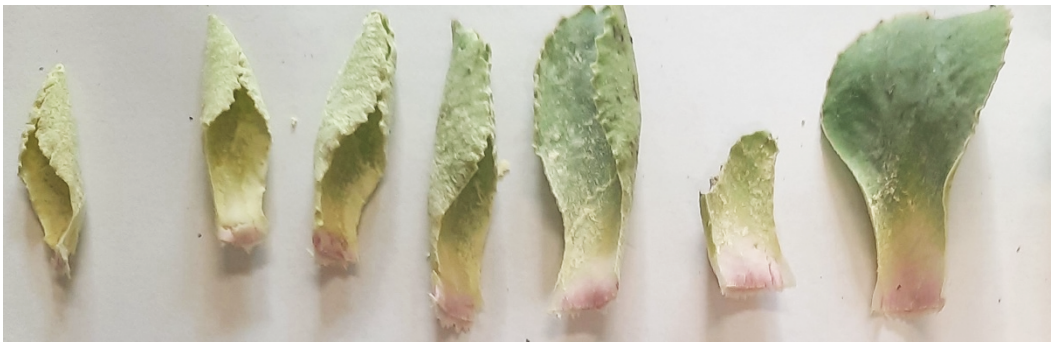

Figure 10. *Primula auricula* leaves covered with exudate, ordered from youngest (left) to oldest (right).

5.1.1. HPLC analysis

From this pool of samples 293 of them were selected and analysed by HPLC and their composition of flavonoid aglycones determined. The chromatograms obtained showed that flavone (**1**) was the most abundant compound, while other flavonoid aglycones e.g. 5-hydroxyflavone (**2**), 2'-hydroxyflavone (**4**), 3'-hydroxyflavone (**5**), 2'-hydroxy-5-acetoxyflavone (**13**) were present in traces (Figure 11 and Figure 12). To determine the present type of aglycones detectable in the chromatograms, UV-spectra and retention times were compared with those of previously identified flavonoids. These flavonoid aglycones were previously identified were available in reference database used. A total of 15

compounds could be identified in the chromatograms. Their structural formulae are given in Figure 12.

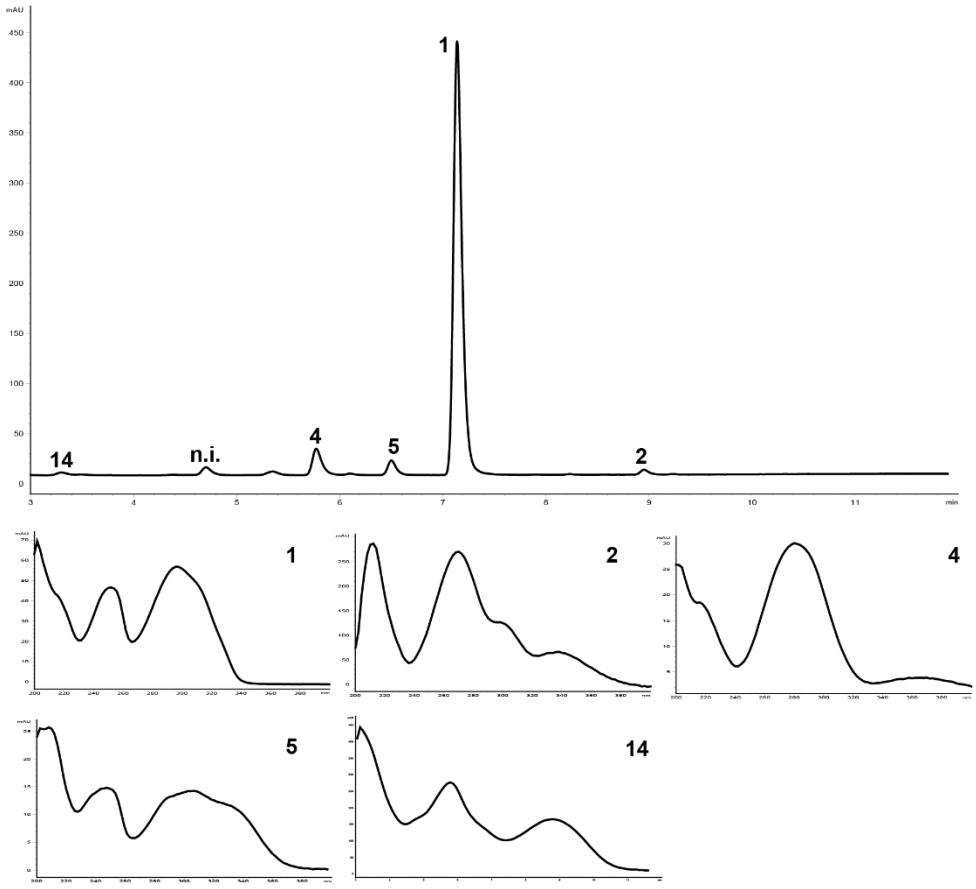

Figure 11. Exemplary HPLC chromatogram obtained of *P. auricula* leaf exudate samples at 300 nm. **1**= flavone, **2**=5-hydroxyflavone, **4**=2'-hydroxyflavone, **5**=3'-hydroxyflavone, **14**=salicylic acid, n.i.=not identified.

For quantitative evaluation, peak areas were assessed and the amounts calculated using the regression parameters given in section 5.3. Identified flavone aglycones were separated into the three groups: those with functional groups on ring A, those with functional groups on ring B and those with functional groups on the A- and B-ring (Figure 12). From the latter group only peak areas were assessed and compared. Additionally to the identified flavonoid aglycones, the benzoic acid derivative salicylic acid (**14**) could be isolated and its identity determined by NMR spectroscopy. The contents of tables 2–4 give an overview about all the analysed samples. Detailed results are provided in sections 6.1.2–6.1.4. All samples showed a huge concentration of flavone (**1**). Its concentration was sometimes ten times higher than that of the other derivatives. The concentration of flavone (**1**) increased

from summer to fall but decreased in winter. The concentrations of A- and B-ring substituted flavone aglycones per leaf area were almost equal in the samples collected in summer (1.03 to 1.06 µg/cm²) and winter (0.24 to 0.21 µg/cm²). By contrast, the samples collected in fall showed a more than the two-times higher concentration of B-ring substituted flavonoid aglycones. The concentration of chalcones was highest in summer (0.72 µg/cm²) over fall (0.49) and winter (0.28).

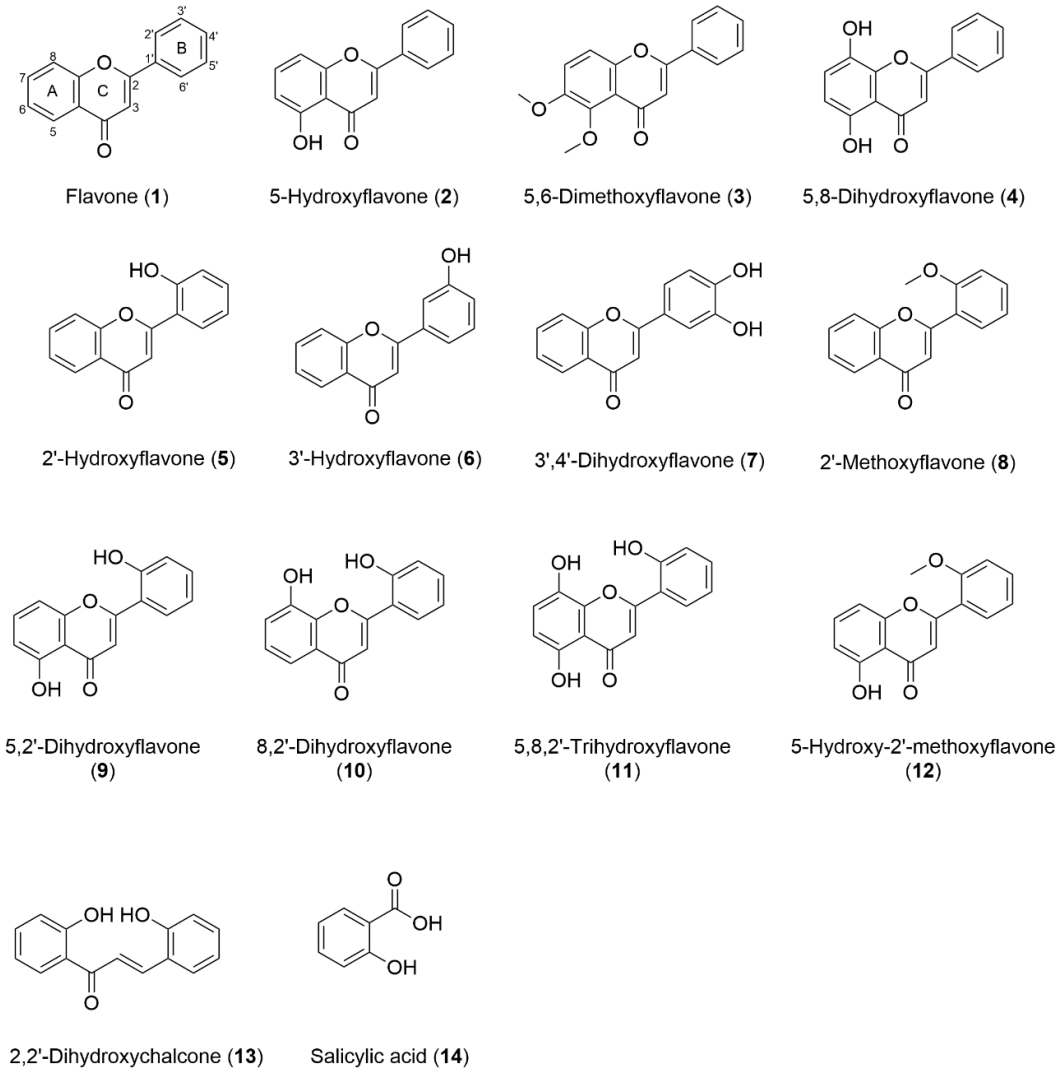

Figure 12. Chemical structures of the identified compounds in *P. auricula* exudates.

The values of exudate are given in mg/cm^2 leaf area, and the values of constituents in µg/mg exudate. Ring A indicates flavonoid aglycones with substituents present on the A-ring and Ring B indicates flavonoid aglycones with substituents present on the B-ring. n.a.= not analysed.

T**able 2.** Relative amounts of exudate and constituents present in exudates along seasons.

	SEASONS															
	Spring				**Summer**				**Fall**				**Winter**			
	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std
Exudate	n.a.	n.a.	n.a.	n.a.	0,02	0,00	0,97	0,11	0,01	0,00	0,20	0,02	0,01	0,00	0,16	0,02
Flavone (**1**)	n.a.	n.a.	n.a.	n.a.	10.58	0.22	76.27	11.45	13.68	0.07	82.35	15.89	7.20	0.02	102.50	14.90
Ring A	n.a.	n.a.	n.a.	n.a.	1.03	<0.01	22.27	2.42	0.61	<0.01	2.76	0.61	0.24	<0.01	4.84	0.68
Ring B	n.a.	n.a.	n.a.	n.a.	1.06	0.01	12.20	1.46	1.42	<0.01	7.76	1.72	0.21	0.01	4.35	0.61
Chalcones	n.a.	n.a.	n.a.	n.a.	0.72	<0.01	6.66	1.35	0.49	<0.01	3.09	0.73	0.28	<0.01	3.07	0.64
Salicylic acid (**14**)	n.a.	n.a.	n.a.	n.a.	0.38	0.01	3.33	0.56	0.25	0.01	1.12	0.25	0.27	0.01	1.91	0.40

Table 3. Relative amounts of exudate and constituents present in exudates along populations.

	POPULATIONS											
	Schneeberg				**Rax**				**Rote Wand**			
	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std
Exudate	0.02	<0.01	0.97	0.07	0.01	<0.01	0.16	0.02	0.01	<0.01	0.20	0.02
Flavone (**1**)	10.99	0.02	82.35	15.01	9.73	0.12	102.50	18.56	9.35	0.08	30.00	9.02
Ring A	0.43	<0.01	1.97	0.50	0.33	<0.01	4.84	0.91	0.54	0.01	2.24	0.52
Ring B	0.86	<0.01	7.13	1.31	0.32	<0.01	4.35	0.81	1.02	<0.01	3.03	0.83
Chalcones	0.90	<0.01	6.66	1.43	0.29	<0.01	3.07	0.65	0.19	<0.01	1.18	0.36
Salicylic acid (**14**)	0.38	0.01	1.88	0.49	0.41	0.05	1.91	0.51	0.17	0.01	0.90	0.18
	Leopoldsteinersee				**Mödling**							
	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std				
Exudate	<0.01	<0.01	0.02	0.01	0.01	<0.01	0.03	0.01				
Flavone (**1**)	11.66	0.29	76.27	12.79	0.46	0.04	1.05	0.31				
Ring A	1.37	<0.01	22.27	2.95	0.01	0.01	0.01	<0.01				
Ring B	1.36	0.03	12.20	2.02	0.01	0.01	0.01	<0.01				
Chalcones	0.44	<0.01	3.09	0.80	n.a.	n.a.	n.a.	n.a.				
Salicylic acid (**14**)	0.34	0.02	3.33	0.51	n.a.	n.a.	n.a.	n.a.				

Table 4. Relative amounts of exudate and constituents present in exudates along leaf developmental stages.

LEAF DEVELOPMENTAL STAGES								
	Inner Leaves				**Outer Leaves**			
	Mean.	Min.	Max.	± std	Mean.	Min.	Max.	± std
Exudate	0.02	<0.01	0.97	0.07	<0.01	0	0.03	0.01
Flavone (**1**)	11.28	0.06	102.50	13.98	9.60	0.02	82.35	14.23
Ring A	0.57	<0.01	4.84	0.75	0.81	<0.01	22.27	2.23
Ring B	0.78	<0.01	5.82	1.10	1.12	<0.01	12.20	1.73
Chalcones	0.81	<0.01	6.60	1.28	0.87	<0.01	0.38	0.87
Salicylic acid (**14**)	0.34	0.01	1.91	0.39	0.29	0.01	3.33	0.49

5.1.2. Exudate amounts along seasons, leaf developmental stages and populations

The amounts of exudate from samples of plants collected along the seasons of summer, fall and winter were compared. An increase on the exudates median value was observed in fall and winter (Figure 13 a), while during summer we observe more extreme outlier values (Figure 13 A). *Primula auricula* plant leaves were categorised by their developmental stage: inner leaves (younger leaves forming a bud) and the outer leaves (older leaves). Amounts of exudate were present in maximum quantities in inner leaves and decreased drastically in outer leaves (Figure 13 B). This particular result was in some cases seen at plain eyesight Figure 10). Younger leaves forming a bud were thickly covered by the white mealy coat of exudate while the amounts of exudate visibly decreased in quantity along the older leaves.

The performed statistical analysis made on data obtained from exudates revealed that the data did not follow a normal distribution (Shapiro-Wilk test p value>0.05). According to the results from Kruskal Wallis tests, the amounts of exudate per leaf area (mg/cm^2) were significantly different between seasons (p value <0.05) and leaf developmental stages (p value <0.05). The post-hoc Dunn's test confirmed significant differences in relative amounts of exudate being present between summer and fall, and summer and winter. Although no significant differences were detected between fall and winter. No correlation was confirmed between relative amounts of exudate and seasons (Spearman's test p value >0.05). In the case of relative amounts of exudate and leaf developmental stages, a negative correlation was found (Spearman's coefficient=-0.43, p value <0.05), which confirms the results observed.

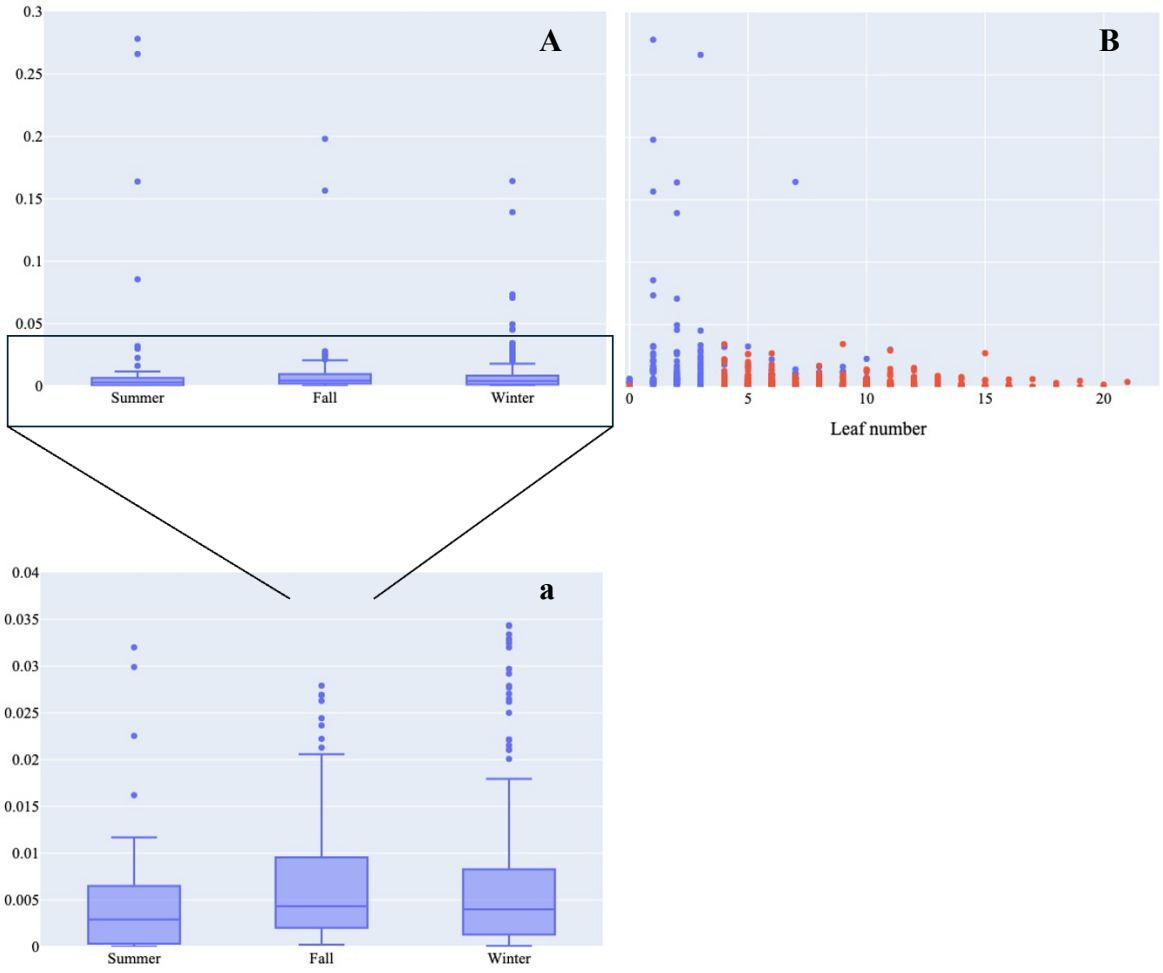

Figure 13. (A) Relative amounts of exudate (mg/cm2 leaf area) along the seasons. (B) Relative amounts of exudate in inner leaves (blue) and outer leaves (red). (a) Zoom in from figure A. All outliers were considered during the statistical analysis.

For this investigation plants were collected from five different populations at Schneeberg, Rax, Rote Wand, Leopoldsteinersee and Mödling. The collection of these populations followed an altitudinal transect of the mountain sites (Figure 14). Schneeberg and Rax were the locations at higher altitudes, followed by Rote Wand, Leopoldsteinersee and the lowest altitude location being Mödling. Relative exudate amounts along different populations show that plants at higher altitudes produced higher amounts of exudate than those situated in lower altitudes. As it has been seen in the overall observation of exudate samples, inner leaves were covered with higher amounts of exudate than outer leaves. This pattern was conserved in all sampled populations (Figure 15 A and B). Significant differences on the amounts of exudate between plants from different populations (Kruskal Wallis test p value <0.05) and plants collected along the different altitudes (Kruskal Wallis

test p value <0.05) were visible in the median values. These significant differences were present between Leopoldsteinersee and Schneeberg, Leopoldsteinersee and Rax and Leopoldsteinersee and Rote Wand. A positive correlation between amounts of exudate and altitude has also been corroborated (Spearman's coefficient=0.3197, p value<0.05).

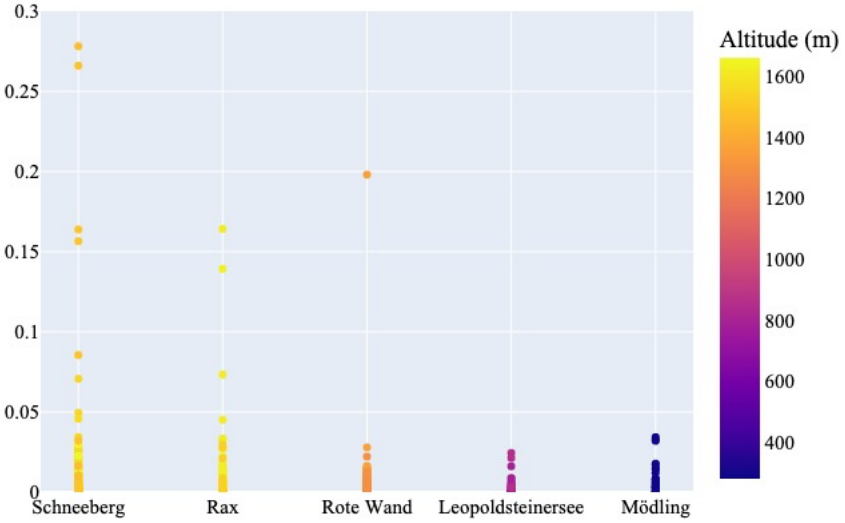

Figure 14. Relative amounts of exudate (mg/cm2 leaf area) along the different populations and altitudes. All data is shown and all outliers were considered in the statistical analysis.

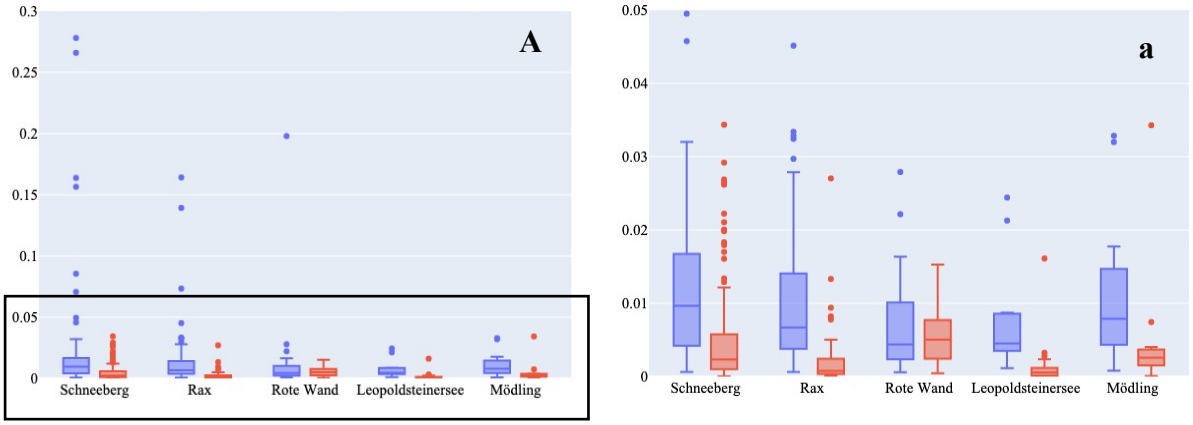

Figure 15. (A) Relative amounts of exudate (mg/cm2 leaf area) in inner (blue) and outer (red) leaves. (a) Zoom in from figure A. All outliers were considered in the statistical analysis.

5.1.3. Amounts of flavone (1) and other aglycones

The main component of *P. auricula* exudates was flavone (**1**) (Figure 11). Median relative amounts of flavone (**1**) were higher during summer and fall and decreases during winter (Figure 16 A). Comparing the relative amounts of flavone (**1**) along locations, no trend was

observed (Figure 16 B). Inner leaves showed higher relative amounts of flavone (**1**) compared to outer leaves (Figure 16 C), but no specific pattern was observable regarding the relative amounts of flavone (**1**) along altitudes (Figure 16 D). The values of relative amounts of flavone (**1**) along seasons were significantly different between summer and winter, and fall and winter (Kruskal Wallis test p value <0.05; Dunn's test p value <0.05). With the Spearman's correlation test, a negative correlation between the relative amounts of flavone (**1**) and seasons was found (Spearman's coefficient=-0.27, p value <0.05). This supports the visible variations mentioned previously. By contrast, no significant differences on flavone (**1**) between populations, altitudes nor leaf developmental stages could be observed (Kruskal Wallis test p value >0.05).

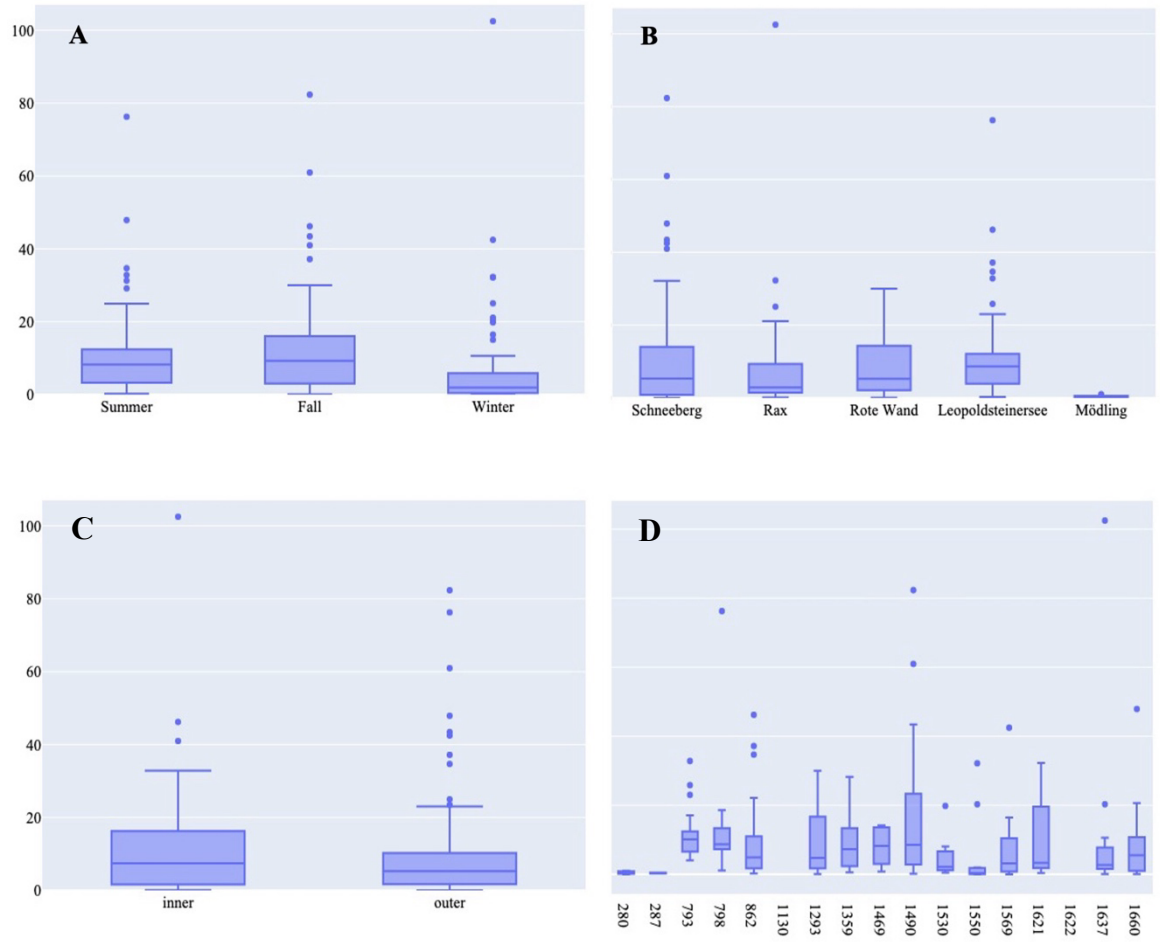

Figure 16. (A) Relative amounts of flavone (**1**) (µg/mg exudate) along seasons, (B) populations, (C) leaf developmental stages and (D) altitudes. All outliers were considered in the statistical analysis.

Other flavone aglycones also present in *P. auricula* exudates have been classified as flavones with functional groups on ring A, flavones with functional groups on ring B and

chalcones. To simplify the writing, from now on flavones with functional groups on ring A are going to be referred as group-A, flavones with functional groups on ring B are going to be referred as group-B and chalcones are going to be referred as group-C. The results indicated that group-B aglycones were overall more abundant than aglycones group-A and group-C (Figure 17 A). The accumulation of aglycones experienced a significant decline from summer and fall to winter (Figure 17 B; Kruskal Wallis test p_A value= <0.05, p_B value <0.05, p_{Chal} value <0.05; Dunn's test p value <0.05). Comparing the amounts of aglycones per amounts of exudate along the populations, a pattern cannot be observed (Figure 17 C).

Focusing on leaf developmental stages, from inner leaves higher relative amounts of group-A and group-C were measured than from outer leaves (Figure 17 D). On the contrary, outer leaves showed higher relative amounts of group-B than inner leaves. The relative amounts of aglycons along altitudes increases in batches of altitudes, but no steady increase can be seen (Figure 17 E). The statistical analysis indicated significant differences between the median values of the ratios of relative amounts of group-A and relative amounts of group-B and the relative amounts of group-C along seasons (Kruskal Wallis test: p_A value <0.05. p_B value <0.05. p_{Chal} value <0.05), populations (Kruskal Wallis test: p_A value <0.05. p_B value <0.05. p_{Chal} value <0.05) and altitudes (Kruskal Wallis test: p_A value <0.05. p_B value <0.05. p_{Chal} value <0.05). The differences of the relative amounts of group-A and group-C along seasons were significant between summer and winter and also between fall and winter (Dunn's test p value <0.05). However the differences of relative amounts of group-B were significant along all the three seasons summer, fall, winter (Dunn's test p value <0.05). Possible correlations were determined after applying the Spearman's correlation test (Table 5). A positive correlation between relative amounts of group-A, group-B and group-C and relative amounts of flavone (**1**) was also observed (Spearman's correlation test p value <0.05).

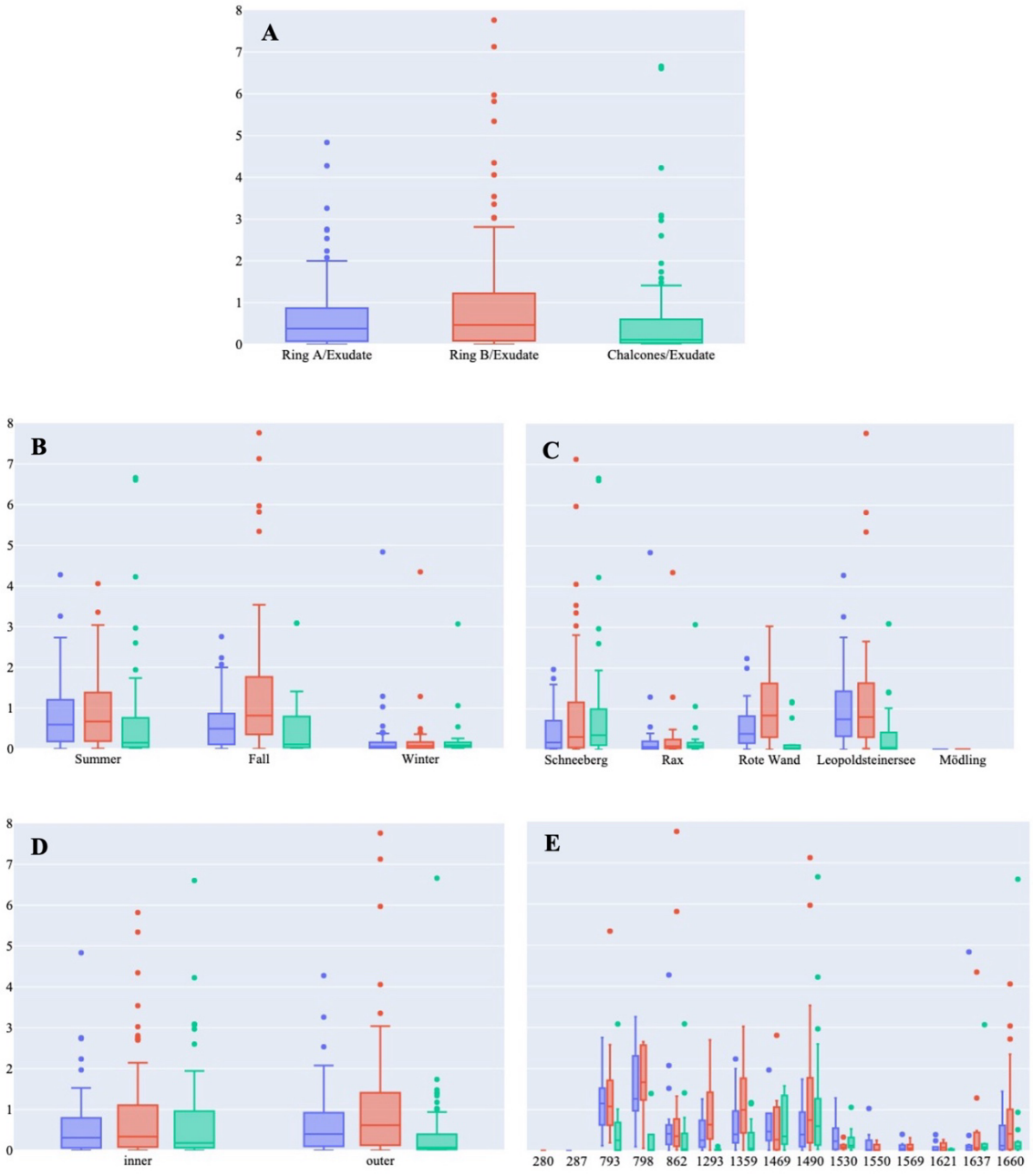

Figure 17. (A) Overall relative amounts of flavonoid aglycones (µg/mg exudate) along all measured samples, (B) seasons, (C) populations, (D) leaf developmental stage and (E) altitudes. Blue=group-A aglycones, red= group-B aglycones, green= group-C aglycones. All outliers considered in the statistical analysis.

5.1.4. Correlation between variables

Correlations between variables found with significant differences were calculated using Spearman's correlation test (Table 5). Between summer and fall, an increase of relative amounts of flavone (**1**) was observed while there was a decrease between fall and winter. This decrease was more pronounced than the increase between summer and fall. For this reason, the overall correlation between relative amounts of flavone (**1**) and seasons was

negative (Figure 17 A). In contrast positive correlations were found in relative amounts of group-A and group-B aglycones and a negative correlation along group-C aglycones. These three correlations can be observed on the visualisation of data (Figures 17). On values along leaf developmental stages no correlations were found on flavonoids from exudates except on type-C aglycones. And positive correlations were present between relative amounts of flavone (**1**) and relative amounts of aglycones group-A, group-B and group-C.

Table 5. Correlations calculated by the application of Spearman's correlation test considering all data values. n.c.= no correlation.

	Spearman's coefficient				
	Season	**Population**	**Altitude**	**Developmental stages**	**Flavone (1)**
Exudate	n.c.	n.c.	0.09	-0.43	n.c.
Flavone (**1**)	-0.27	n.c.	n.c.	n.c.	-
Ring A	-0.44	0.33	-0.46	n.c.	0.74
Ring B	-0.38	0.23	-0.35	n.c.	0.72
Chalcones	n.c.	-0.35	n.c.	-0.24	0.73
Salicylic acid (**14**)	n.c.	n.c.	n.c.	n.c.	0.46

5.2. Analysed leaf tissue extracts

A total of 382 samples of *P. auricula* leaf extracts were prepared from plants collected on the years 2021-2024 and their composition was analysed by HPLC. Resulting chromatograms show that the main components present were flavone and flavonol glycosides (Figure 18). The differentiation between flavonol and flavone glycosides was based on their UV spectra which show conspicuous differences (Figure 18, a and b). Flavonol glycosides occur in a higher number of derivatives with lower amounts whilst the number of flavone glycosides was low but with higher quantities. Since both types of flavonoid glycosides often differ in their sugar moieties (Holzbach et al., 2021) the assignment to distinct derivatives was impossible with analytical method used. For quantitative evaluation, the respective peak areas obtained from the HPLC chromatograms were summed up as flavonol and flavone glycosides and these data further was used for statistical analyses.

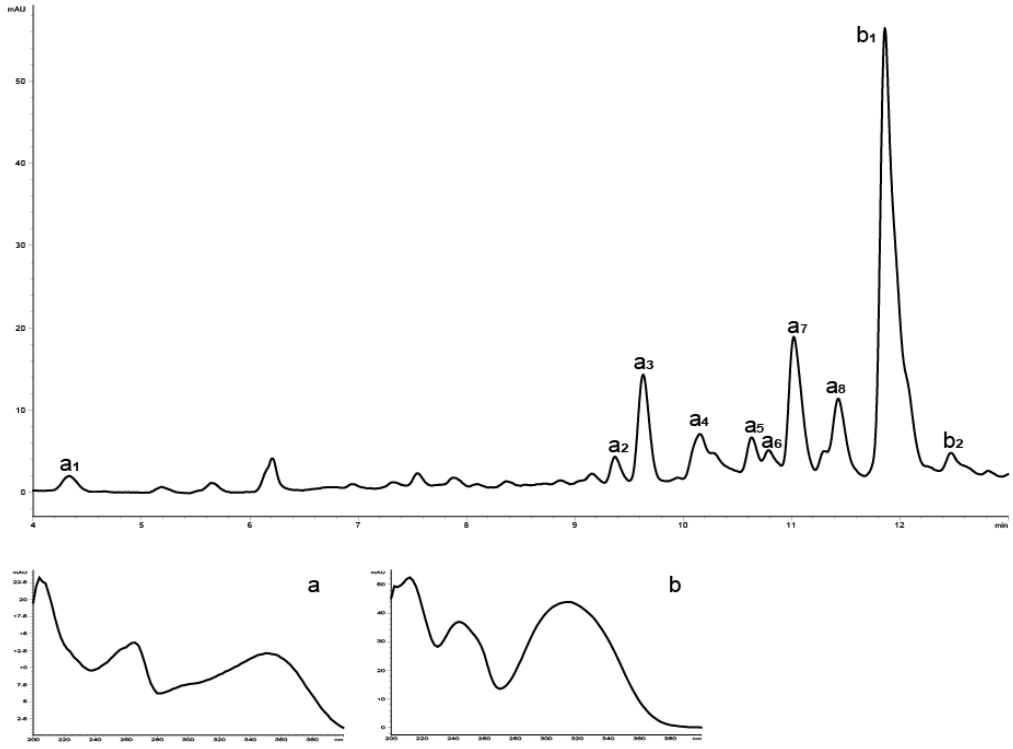

Figure 18. Exemplary HPLC chromatogram obtained of *P. auricula* leaf tissue extract samples at 254 nm. a=flavonol glycosides, b=flavone glycosides.

5.2.1. Amounts of flavonoid glycosides in leaf tissue along seasons

Quantitative evaluation revealed that flavone glycosides were generally present in higher concentrations (Figure 19 A). These flavonoid glycosides also tend to decrease from spring until winter (Figure 19 B) and the relative amounts of flavonoid glycosides were slightly higher in inner leaves (Figure 19 C).

5.2.2. Amounts of flavonoid glycosides in leaf tissue along locations

The calculated means amounts of flavonoid glycosides along the different locations did not show a clear trend (Figure 20). Only a few samples exhibited high amounts of flavone and flavonol glycosides, but these samples did not lead to an increase of the means. Throughout the seasons the quantities of flavone glycosides show a scattered distribution which was not clearly reflecting the calculated mean (Figure 20). Nevertheless, the seasonal concentration of these compounds was highest in spring (1.93 µg/mg extract), decreased clearly in autumn (0.53 µg/mg extract) and increased again over winter (1.66 µg/mg extract Table 6). The seasonal trend of the flavonol concentration decreased from spring (1.86 µg/mg extract) to summer (0.69 µg/mg extract).

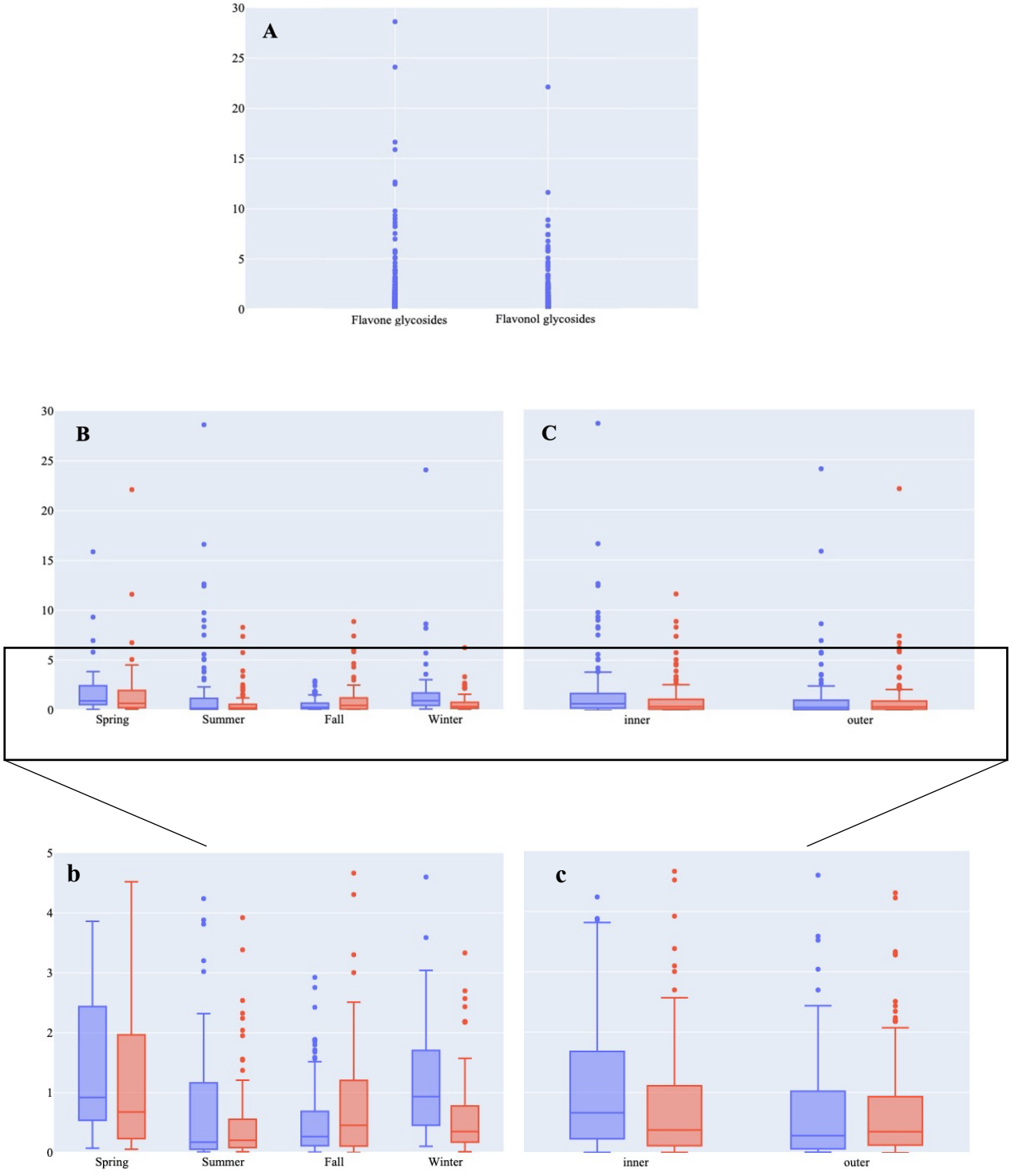

Figure 19. (A) Overall relative amounts of flavone and flavonol glycosides (µg/mg extract). (B) Relative amounts of flavone and flavonol glycosides along the seasons. (C) Relative amounts of flavone and flavonol glycosides and along leaf developmental stages. (b) Zoom in from figure B. (c) Zoom in from figure C. Blue=flavone glycosides, red=flavonol glycosides. All outliers were considered in the statistical analysis.

The samples collected in autumn showed a higher concentration than the samples in summer and winter (Table 6, Figure 19). Comparing both, the flavone and the flavonol glycosides, the ratios were almost 1 in spring whilst the concentration of flavone glycosides was around 2.5 times higher in summer. Remarkably, this ratio was inverted during autumn with 0.53 µg/mg extract of flavone glycosides and 0.99 µg/mg extract flavonol glycosides. In winter, was the ratio of flavone glycosides to flavonol glycosides similar to summer (1.66 to 0.70 µg/mg extract) (Table 6). A detailed description of the assessed ratios was given in chapter 6.3.1.

The concentrations of flavonoid glycosides was highest in Schneeberg (1.94 µg/mg extract) the population at highest altitude, and decreased along Rax (1.88 µg/mg extract), Rote Wand (0.84 µg/mg extract) and Leopoldsteinersee (0.62 µg/mg extract) populations in a decreasing order of maximum altitude (Table 7). Interestingly, the samples from Mödling did not follow this trend (1.02 µg/mg extract). Comparing both, the flavone and the flavonol glycosides, the ratios were 3 in Schneeberg, 1 in Rax a, Rote Wand and Mödling. In Leopoldsteinersee the ratio was inverted to a 0.6, with 0.62 µg/mg of flavone glycosides and 0.93 µg/mg of flavonol glycosides (Table 7).

Regarding the concentrations of flavone and flavonol glycosides in leaf developmental stages, the inner leaves exhibited a higher concentration of flavone glycosides with 1.77 µg/mg extract. This concentration decreased in the outer leaves to 0.99 µg/mg extract. The concentrations of flavonol glycosides were almost identical in both developmental stages (Table 8).

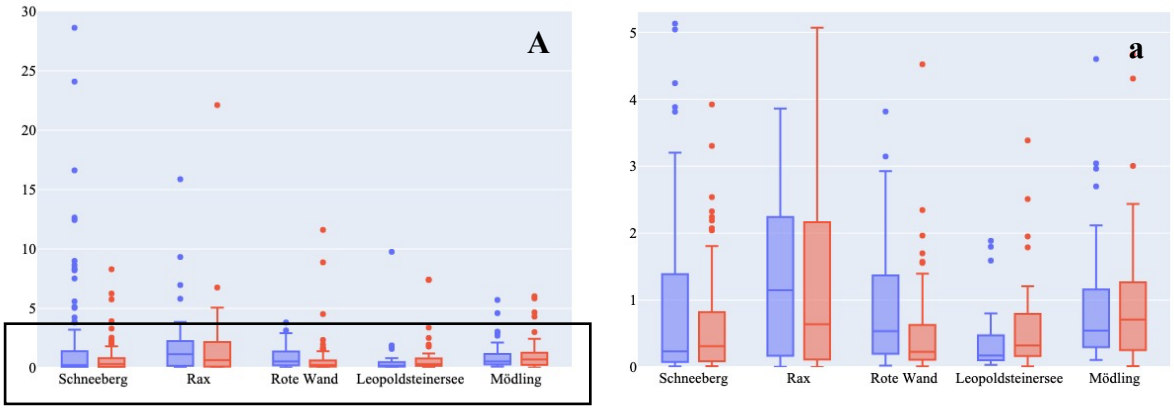

Figure 20. (A) Relative amounts of flavone glycosides (blue) and flavonol glycosides (red, µg/mg extract) along populations. (a) Zoom in from figure A. All outliers were considered during the statistical analysis.

Comparing the means of amounts of flavone and flavonol glycosides present in *P. auricula* leaf extracts along the different locations, no pattern can be observed. The relative amounts of glycosides range from 0 to approximately 4 µg/mg with a 1:1 ratio (Figure 20). When having the outlier values into account, a decreasing tendency of relative amounts of flavonoid glycosides can be observed from populations at higher altitudes to populations at lower altitudes.

The data obtained comprising amounts of flavonoid glycosides per amounts of exudate did present normal distribution once converted to logarithmic data (Shapiro-Wilk test p value<0.05). The two-way ANOVA Tests show that the means of flavonoid glycosides for each plant sample of the logarithmic data of flavone glycosides (a) and flavonol glycosides (b) per amounts of exudate along the independent variables such as populations (p $value_a$ <0.05, p $value_b$ <0.05), seasons (p $value_a$ <0.05, p $value_b$ <0.05) and altitudes (p $value_a$ <0.05. p $value_b$ <0.05) were significantly different, while the difference between means of the type of leaf were significant just for flavone glycosides (p $value_a$ <0.05) and not for flavonol glycosides (p $value_b$ >0.05) The post hoc Tukey's HSD test confirmed (p value <0.05) the significant differences between spring-summer, spring-fall for both flavonoid glycosides. Summer-winter, fall-winter means of flavone glycoside showed to have significant different means as well as spring-fall, spring-winter means of flavonol glycosides (Figure 19 B). The post hoc ANOVA test did not detect significant differences between the means of flavonoid glycosides along the different altitudes nor populations (p value >0.05), but it did detect significant differences between leaf developmental stages and flavone glycosides. The Pearson's correlation test confirmed a potential correlation between leaf developmental stages and flavone glycosides with a coefficient of -0.24 and p value <0.05.

Table 6. Relative amounts of flavonoid glycosides present in *P. auricula* extracts along seasons. The values are given in µg/mg extract.

	SEASONS															
	Spring				**Summer**				**Fall**				**Winter**			
	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std
Flavone glycosides	1.93	0.07	15.87	2.68	1.70	0.01	28.62	4.07	0.53	0.01	2.93	0.64	1.66	0.10	24.09	3.04
Flavonol glycosides	1.86	0.06	22.11	3.54	0.69	0.01	8.30	1.38	0.99	<0.01	8.88	1.55	0.70	0.02	6.25	0.96

Table 7. Relative amounts of flavonoid glycosides present in *P. auricula* extracts along populations. The values are given in µg/mg extract.

	POPULATIONS											
	Schneeberg				**Rax**				**Rote Wand**			
	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std
Flavone glycosides	1.94	0.01	28.62	4.44	1.88	0.01	15.87	2.75	0.84	0.02	3.82	0.83
Flavonol glycosides	0.76	0.02	8.30	1.27	1.66	<0.01	22.11	3.36	0.76	0.01	11.61	1.69
	Leopoldsteinersee				**Mödling**							
	Mean	Min.	Max.	± std	Mean	Min.	Max.	± std				
Flavone glycosides	0.62	0.03	9.76	1.55	1.02	0.11	5.71	1.15				
Flavonol glycosides	0.93	0.01	7.43	1.66	1.15	0.02	6.02	1.42				

Table 8. Relative amounts of flavonoid glycosides present in *P. auricula* extracts along leaf developmental stages. The values are given in µg/mg extract.

	LEAF DEVELOPMENTAL STAGES							
	Inner Leaves				**Outer Leaves**			
	Mean.	Min.	Max.	± std	Mean.	Min.	Max.	± std
Flavone glycosides	1.77	0.01	0.01	3.35	0.99	0.01	0.01	2.46
Flavonol glycosides	1.01	0.01	0.01	1.72	0.94	<0.01	<0.01	2.07

5.3. Evaluation of ratios

5.3.1. Ratios of flavone glycosides to flavonol glycosides

Depiction of the amounts of flavone glycosides on x-axis against flavonol glycosides on the y-axis showed a weak positive correlation between both subclasses of compounds (Figure 21). Most of the samples exhibited almost identical concentrations of both types of glycosides. In only a few samples from Schneeberg, Rax and Leopoldsteinersee was a much higher flavone glycoside concentrations (15–30 µg/mg extract) than flavonol glycoside concentration. In these cases, flavonol glycosides concentration exceeded 10 µg/mg.

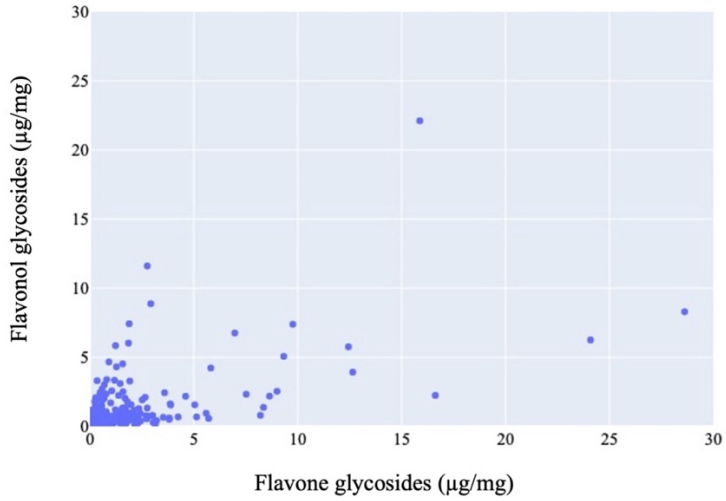

Figure 21. Overall relative amounts of flavone glycosides (x-axis, µg/mg extract) per overall relative amounts of flavonol glycosides (y-axis, µg/mg extract) in *P. auricula* leaf extracts.

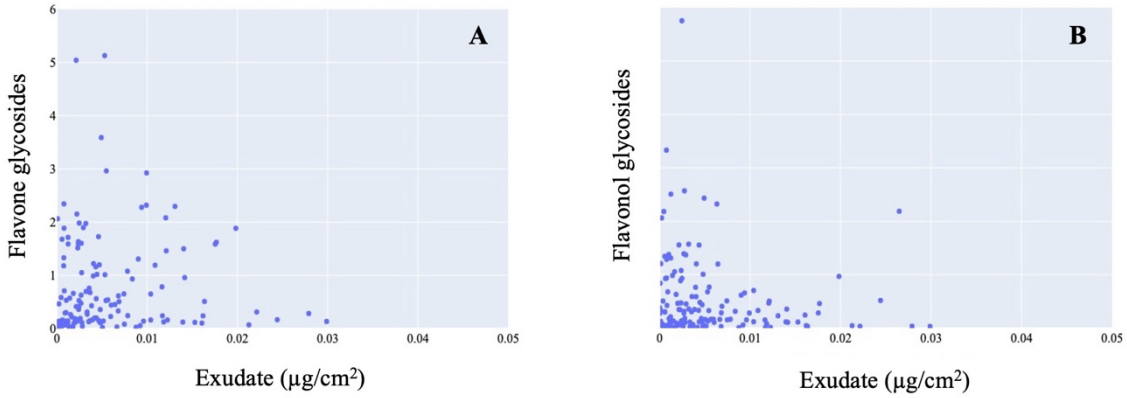

Figure 22. (A) Relative amounts of flavone glycosides (µg/mg extract) per relative amounts of exudate (mg/cm2 leaf area). (B) Relative amounts of flavonol glycosides (µg/mg extract) per relative amounts of exudate (mg/cm2 leaf area).

5.3.2. Ratios of flavonoid glycosides to flavone (1)

No quantitative correlation between flavone glycosides and flavone (**1**) was observed (Figure 23 A). This was also valid for flavonol glycosides (Figure 23 B). An increase of flavone (**1**) does not reflect into an increase of relative amounts of flavonoid glycosides.

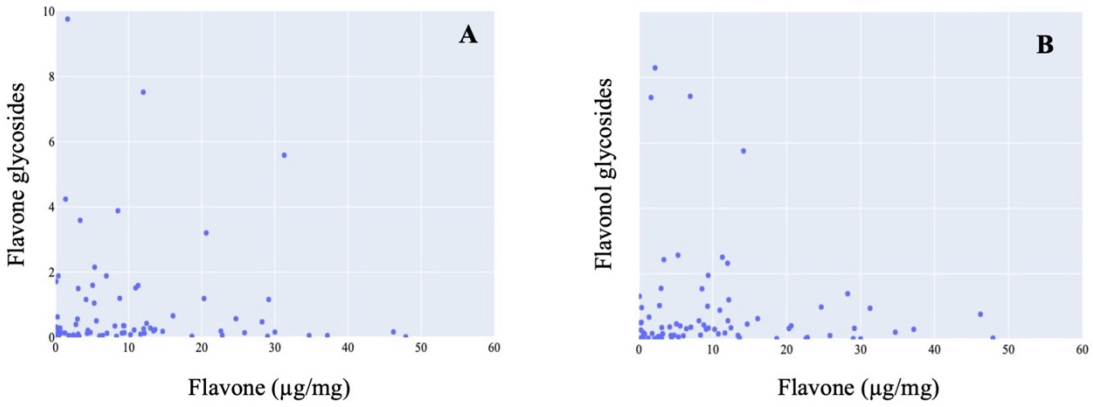

Figure 23. (A) Relative amounts of flavone glycosides (µg/mg extract) per amounts of flavone (**1**) (µg/mg exudate). (B) Relative amounts of flavonol glycosides (µg/mg extract) per amounts of flavone (**1**) (µg/mg exudate). Some extreme outliers have not been portrayed in the plots, even though they have all been considered for statistical analyses.

5.4. Identification of salicylic acid (**14**)

During this work a compound was purified from *P. auricula* exudates using preparative chromatographic methods. This compound was tentatively determined by Priemer (2022) as a salicylic acid derivative due to the similarity of its UV spectrum to salicylic acid (**14**). A posterior determination of the compound was done by nuclear magnetic resonance spectroscopy (NMR) combined with mass spectrometry, which confirmed its identity as salicylic acid (**14**). The relative amounts of salicylic acid (**14**) in *P. auricula* exudates tend to decline from summer to winter (Figure 24 A) following the pattern of flavone (**1**). In the different populations, exudate samples showed lower salicylic acid (**14**) concentrations in samples collected at Leopoldsteinersee and Rote Wand (Figure 24 B). Regarding the different leaf development stages, a slight decrease in relative concentrations of this compound was observed in outer leaves (Figure 24 C) and a positive increase of means along the altitudes can be observed in intervals (Figure 24 D). Comparing the UV spectra of

all significant peaks in the chromatograms obtained from extracts did not lead to the identification of salicylic acid (**14**).

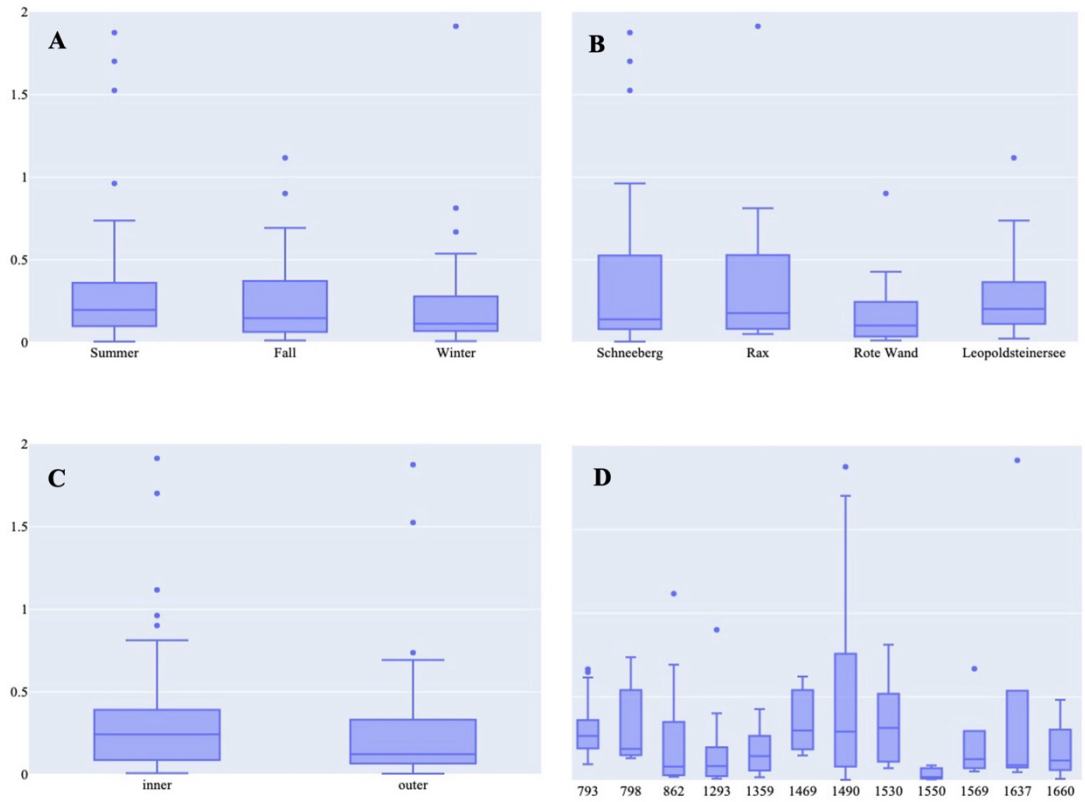

Figure 24. (A) Relative amounts of salicylic acid (**14,** µg/mg exudate) along the seasons, (B) populations, (C) leaf developmental stages and (D) altitudes. All outliers were considered during the statistical analysis.

No significant differences in the relative concentrations of salicylic acid (**14**) in exudate samples collected along the seasons, populations and leaf development (Kruskal Wallis test *p* value >0.05) were observed. Although there were significant differences between altitudes (Kruskal Wallis test *p* value <0.05), no correlation was found. A positive correlation between the amounts of salicylic acid (**14**) and amounts of flavone (**1**) was determined (Table 5).

6. Discussion

Continuous excretion of specialized metabolites in a farinose form is a rare feature in the plant kingdom, and it appears to be confined to species of the genus *Primula* inclusive *Dionysia*. Farinose exudates are also known of some fern taxa, but the structure of the glandular hairs and the secretion mechanism are different (Rodriguez et al., 2018). In *Primula* and *Dionysia*, both oily and farinose excretions are present on the cutinised surface of glandular hairs (Bourdon et al., 2021; Wollenweber et al., 1971), whereas in farinose ferns the substances are stored in a subcuticular space (Rodriguez et al., 2018)., as is common for glandular hairs also in flowering plants (Muravnik, 2020). Cuticle micropores have been described both from farinose ferns (Rodriguez et al., 2018), and from *Dionysia tapetodes* (Bourdon et al., 2021). Functional aspects of glandular hairs and their products have been discussed *inter alia* by (Wollenweber, 1989) and (Muravnik, 2020).

So far, hardly any studies on the impact of abiotic factors on the flavonoid composition exist for *Primula*, except one dealing with UV-B impact on flavonoid production in leaves and flowers of *P. veris* in a greenhouse experiment (El Morchid et al., 2014), and the study of Priemer (2022) on *P. auricula*. In continuation of the latter study, extended analyses on the evaluation of farina, exudate and, as a new aspect, tissue flavonoid production in different locations in East Austria were carried out along altitudinal gradients, considering also seasonal variation. As far as the exudate composition is concerned, the current results complement previous studies on the exudate composition from this species (Elser et al., 2016; Holzbach et al., 2021; Priemer, 2022; Reuter, 2020). The qualitative assessment indicates flavone (**1**) as major constituent in the exudate. The other flavonoid aglycones, e.g. 5-hydroxyflavone (**2**) 2'-hydroxy flavone (**5**), 2'-methoxyflavone (**8**), (Figure 11) were detectable only in very small quantities. This composition was observed in all the samples analysed, indicating a conservative pattern. In some samples collected at Rote Wand, Leopoldsteinersee (Styria), Schneeberg, Rax and Mödling (Lower Austria) salicylic acid (**14**) was clearly detectable. Compound **14** has not been identified before from *P. auricula*, and it corresponds to the assumed salicylate derivative described by Priemer (2022).

Quantitative evaluation of both exudate and tissue flavonoid composition was performed to test for the significance under changing environmental factors. In particular, results were tested for correlation of flavonoid production in the respective compartments (glandular hairs versus tissue), as it is assumed that there should be different functions in an

ecological context. Similarly, seasonal variation as earlier described by Priemer (2022), was detected in the quantities produced, both of farinose exudates and of flavone (**1**). However, no consistent pattern is obvious when statistical analyses are compared. Discrepancies may be rooted in various ecological parameters and reflect a high phenotypic plasticity concerning exudate production. Since the current work is the second study regarding the seasonal progression, a deeper discussion on this subject is not possible at this stage of research. Greenhouse or common garden experiments would be needed to confirm effects of temperature and UV-light on the production of flavonoids under comparable culture conditions.

The altitudinal progression assessed by analysing samples from the populations at Schneeberg, Rax, Rote Wand, Leopoldsteinersee and Mödling, did also not exhibit a clear trend, although a weak positive correlation was found. A clear correlation regarding the amounts of exudates and the developmental stages of leaves was found. Young leaves were mostly covered thickly with farina while older leaves were almost lacking any farina. Since the old leaves were lost during the next vegetation period the coverage of leaves forming the bud may have an ecological meaning for the plants, such as keeping an optimal temperature for leaf growth and development (Raza et al., 2023). Due to the fact, that farina consists mainly of flavone (**1**) the flavone-related bioactivities (Valant-Vetschera et al., 2024) may account for the bioactivities of the exudate. These bioactivities might be complemented by the irritant properties of salicylic acid (**14**). This compound is known as an important plant hormone (Koo et al., 2020) but the amounts detected in the exudate suggests a role as potent defense chemical.

For investigation of tissue flavonoids, HPLC analyses were performed with MeOH extracts of rinsed leaves. Comparison of UV-spectra indicated the presence of flavone glycosides as previously described by Holzbach et al. (2021) in the extracts. In this work also flavonol glycosides based on kaempferol and isorhamnetin as aglycone moiety were described. Comparison of UV-spectra using the in-house UV-spectral library also indicated the presence of quercetin glycosides in the leaf tissue. For the quantitative assessment, the peaks present in the chromatograms were assigned either to flavone glycosides or to flavonol glycosides, not distinguishing single derivatives. In the chromatograms, the flavonol glycosides were present in a larger number of derivatives, but in lower concentrations as compared to the co-occurring flavone glycosides. The relative amounts of both glycoside

types were almost identical and a slight increase of both was observed over the altitudes. Seasonally, the highest relative amounts of flavone glycosides were detected in spring with a decline towards fall. By contrast, flavonol glycosides showed a sinus-curve progression within a year with maxima in spring and fall. Assessing correlations to the exudate amounts and flavone (**1**), no correlations were found. This indicates a clear separation of the biosynthesis of glandular flavone (**1**) vice the detected tissue glycosides of flavones and flavonols, confirming the assumption of a deviating pathway leading to the production of flavone (**1**) in glandular hairs (Valant-Vetschera et al., 2024). Thus, the data obtained from the conducted quantitative assessment in this work suggests, that the biosynthesis of flavone (**1**) is not accomplished via the "classical" biosynthetic route which starts from *ortho*-coumaric acid but, that other precursors or enzymes may be involved. In this context, the accumulation of salicylic acid (**14**) may be significant, as some hypotheses suggest salicylic acid (Gondor et al., 2016) and acetophenone (Sashidhara et al., 2012) as possible precursors (Valant-Vetschera et al., 2024).

7. Conclusion

This study has shown that the relative amounts of exudate and flavone (**1**) along seasons and altitudes are more flexible. While Priemer (2022) observed a positive correlation between relative amounts of exudate and flavone (**1**) along seasons, in study a negative correlation was observed. In most of the studies samples, the ratios of flavone glycosides to flavonol glycosides were 1:1 throughout the year with non-significant variations. Between relative amounts of exudate and flavonoid glycosides as well as flavone (**1**) and flavonoid glycosides no correlations have been found, suggesting that flavonoids present in exudates and flavonoids present in tissues follow different biosynthetic routes. From the results obtained in this study we create the hypothesis that *P. auricula* exudates might help regulate leaf temperature and decrease water evaporation. More studies working in controlled environmental conditions are needed to make final conclusions.

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9. Appendix

AX 1. Collection data of *P. auricula* individuals.

Collection date	Season	Mountain	Altitude (m)	Plant
10.09.22	Fall	Schneeberg	1840	CPS-PA-OH1-1.1
10.09.22	Fall	Schneeberg	1469	CPS-PA-OH1-1.2
10.09.22	Fall	Schneeberg	1490	CPS-PA-OH2-1.3
10.09.22	Fall	Schneeberg	1490	CPS-PA-OH2-1.4
10.09.22	Fall	Schneeberg	1490	CPS-PA-OH2-1.5
10.09.22	Fall	Schneeberg	1490	CPS-PA-OH2-1.6
10.09.22	Fall	Schneeberg	1660	CPS-PA-OH3-1.7
10.09.22	Fall	Schneeberg	1660	CPS-PA-OH3-1.8
10.09.22	Fall	Schneeberg	1660	CPS-PA-OH3-1.9
10.09.22	Fall	Schneeberg	1660	CPS-PA-OH3-1.10
10.09.22	Fall	Schneeberg	1660	CPS-PA-OH4-1.11
10.09.22	Fall	Schneeberg	1660	CPS-PA-OH4-1.12
10.09.22	Fall	Schneeberg	1660	CPS-PA-OH4-1.13
10.09.22	Fall	Schneeberg	1660	CPS-PA-OH4-1.14
10.09.22	Fall	Schneeberg	1660	CPS-PA-OH4-1.15
10.11.22	Winter	Schneeberg	1569	CPS-PA-ST1-2.1
10.11.22	Winter	Schneeberg	1569	CPS-PA-ST1-2.2
10.11.22	Winter	Schneeberg	1569	CPS-PA-ST1-2.3
10.11.22	Winter	Schneeberg	1569	CPS-PA-ST1-2.4
10.11.22	Winter	Schneeberg	1569	CPS-PA-ST1-2.5
10.11.22	Winter	Schneeberg	1569	CPS-PA-ST2-2.6
10.11.22	Winter	Schneeberg	1550	CPS-PA-ST2-2.7
10.11.22	Winter	Schneeberg	1550	CPS-PA-ST2-2.8
10.11.22	Winter	Schneeberg	1550	CPS-PA-ST2-2.9
10.11.22	Winter	Schneeberg	1550	CPS-PA-ST2-2.10
26.08.23	Summer	Schneeberg	1469	CPS-PA-OH1-6.1
26.08.23	Summer	Schneeberg	1469	CPS-PA-OH1-6.2
26.08.23	Summer	Schneeberg	1490	CPS-PA-OH2-6.3
26.08.23	Summer	Schneeberg	1490	CPS-PA-OH2-6.4
26.08.23	Summer	Schneeberg	1490	CPS-PA-OH2-6.5
26.08.23	Summer	Schneeberg	1490	CPS-PA-OH3-6.6
26.08.23	Summer	Schneeberg	1490	CPS-PA-OH3-6.7
26.08.23	Summer	Schneeberg	1490	CPS-PA-OH3-6.8
26.08.23	Summer	Schneeberg	1660	CPS-PA-OH4-6.9
26.08.23	Summer	Schneeberg	1660	CPS-PA-OH4-6.10
26.08.23	Summer	Schneeberg	1660	CPS-PA-OH4-6.11
29.09.20	Fall	Mödling	280	M1-11

29.09.20	Fall	Mödling	280	M1-12
29.09.20	Fall	Mödling	287	M2-11
29.09.20	Fall	Mödling	287	M2-15
03.12.22	Winter	Mödling	280	CPS-PA-M1-4.1
03.12.22	Winter	Mödling	280	CPS-PA-M1-4.2
03.12.22	Winter	Mödling	280	CPS-PA-M1-4.3
03.12.22	Winter	Mödling	287	CPS-PA-M2-4.4
03.12.22	Winter	Mödling	287	CPS-PA-M2-4.5
03.12.22	Winter	Mödling	287	CPS-PA-M2-4.6
April	Spring	Mödling	280	M1-19
April	Spring	Mödling	287	M2-23
April	Spring	Mödling	287	M2-25
	Fall	Rote Wand	1359	CPS-PA-RW1-3.5
	Fall	Rote Wand	1359	CPS-PA-RW1-3.6
	Fall	Rote Wand	1293	CPS-PA-RW2-3.7
	Fall	Rote Wand	1293	CPS-PA-RW2-3.8
26.11.22	Winter	Rote Wand	1359	CPS-PA-RW1-3.1
26.11.22	Winter	Rote Wand	1359	CPS-PA-RW1-3.2
26.11.22	Winter	Rote Wand	1293	CPS-PA-RW2-3.3
26.11.22	Winter	Rote Wand	1293	CPS-PA-RW2-3.4
16.04.22	Spring	Rote Wand	1359	T1-1
17.04.22	Spring	Rote Wand	1359	T1-2
18.04.22	Spring	Rote Wand	1293	T2-1
19.04.22	Spring	Rote Wand	1293	T2-2
September	Fall	Rax	1130	R01-01
September	Fall	Rax	1130	R01-04
September	Fall	Rax	1550	R03-05
September	Fall	Rax	1550	R03-06
14.01.23	Winter	Rax	1621	CPS-PA-R1-5.1
14.01.23	Winter	Rax	1621	CPS-PA-R1-5.2
14.01.23	Winter	Rax	1621	CPS-PA-R1-5.3
14.01.23	Winter	Rax	1637	CPS-PA-R2-5.4
14.01.23	Winter	Rax	1637	CPS-PA-R2-5.5
14.01.23	Winter	Rax	1637	CPS-PA-R2-5.6
14.01.23	Winter	Rax	1530	CPS-PA-R3-5.7
14.01.23	Winter	Rax	1530	CPS-PA-R3-5.8
14.01.23	Winter	Rax	1530	CPS-PA-R3-5.9
May	Spring	Rax	1130	R1-39
May	Spring	Rax	1130	R1-40
May	Spring	Rax	1550	R3-35
May	Spring	Rax	1550	R3-36
	Fall	Leopoldsteinersee	793	CPS-PA-L-6
	Fall	Leopoldsteinersee	862	CPS-PA-L-7
03.06.23	Summer	Leopoldsteinersee	793	CPS-PA-L-1
03.06.23	Summer	Leopoldsteinersee	862	CPS-PA-L-2
03.06.23	Summer	Leopoldsteinersee	862	CPS-PA-L-3

03.06.23	Summer	Leopoldsteinersee	798	CPS-PA-L-4
03.06.23	Summer	Leopoldsteinersee	798	CPS-PA-L-5
17.12.20	Winter	Leopoldsteinersee	793	CPS-PA-L-8
17.12.20	Winter	Leopoldsteinersee	862	CPS-PA-L-9

AX 2. MPLC fractions and absorbance measurements.

Fraction	Detection wavelength	
	254 nm	300 nm
1	1060	822
2	982	770
3	822	652
4	777	607
5	613	471
6	327	240
7	213	157
8	139	105
9	165	127
10	250	198
11	423	338
12	550	441
13	618	563
14	761	611
15	1504	1224
16	1580	1282
17	1565	1270
18	1462	1184
19	1019	885

Fraction	Detection wavelength	
	254 nm	300 nm
20	788	640
21	475	382
22	371	299
23	237	189
24	253	204
25	182	146
26	107	83
27	77	61
28	110	94
29	123	100
30	136	108
31	147	149
32	120	91
33	110	85
34	120	92
35	92	61
36	363	220
37	-	-
38	-	-

AX 3. Size exclusion chromatography fractions and absorbance measurements. Pink: fractions pooled into sample C; Green: fractions pooled into sample B.

Fraction	Detection wavelength	
	236 nm	302 nm
1	8.12	5.10
2	10.15	5.88
3	12.28	6.96
4	16.2	9.00
5	26.74	11.30
6	24.5	13.30
7	31.3	15.90
8	33.57	17.50
9	37.18	19.27
10	38.57	19.79
11	39.46	20.08
12	39.86	2<0.01
13	40.2	20.59
14	38.78	19.87
15	37.75	19.56
16	35.09	18.22
17	33.6	17.69
18	31.11	16.48
19	30.79	16.39
20	26.5	15.79
21	28.97	17.29
22	-	-

Fraction	Detection wavelength	
	236 nm	302 nm
23	27.67	19.09
24	32.57	29.90
25	45.5	50.42
26	50.85	54.81
27	48.36	44.91
28	48.45	44.50
29	49.31	34.38
30	42.24	25.34
31	39.26	23.10
32	42.33	25.35
33	58.78	36.12
34	77.4	47.50
35	85.3	52.40
36	96.94	57.04
37	94.5	53.20
38	72.13	40.39
39	39.51	33.32
40	19.23	12.63
41	7.54	5.96
42	5.25	4.23

AX 4. Compounds eluted from *P. auricula* exudates HPLC. Written in elution order.

flavone
3-OH-flavone
5-OH-flavone
5.8-diOH-flavone/primetin
2'-OH-flavone
3'-OH-flavone
3'.4'-diOH-flavone
5.2'-diOH-flavone
8.2'-diOH-flavone
5.8.4'-triOH-flavone
5.8.2'-triOH-flavone
2'-*O*Me-flavone
5.6-di*O*Me-flavone
5.7-di*O*Me-flavone
5-OH-2'-*O*Me-flavone
2.2'diOH-chalcone
salicylic acid

AX 5. Compounds eluted from *P. auricula* leaf extracts HPLC. Written in elution order.

3′.4′- dihydroxyflavone 4′-*O*-β-glucoside
3'.4'-dihydroxyflavone 4'-*O*-ß-D-glucoside
isorhamnetin-3-*O*-glucoside
flavone-3-O-ß-D-glucopyranoside
4'-OH-flavone-3'-beta-D-glucopyranoside
Isorhamnetin 3-O-β-glucopyranoside
vanillic acid
4'-Hydroxyacetophenone/piceol
3-*O*-(2′′-*O*-α-l-rhamnopyranosyl-[6′′-*O*-β-D-xylopyranosyl]-β-D-glucopyranoside & kaempferol 3-*O*-(2′′-*O*-α-l-rhamnopyranosyl-[6′′-*O*-β-D-xylopyranosyl]-β-D-glucopyranoside
isorhamnetin 3-*O*-(2′′-*O*-β-D-xylopyranosyl-[6′′-*O*-β-D-xylopyranosyl]-β-D-glucopyranoside kaempferol 3-*O*-(2′′-*O*-β-D-xylopyranosyl-[6′′-*O*-β-D-xylopyranosyl]-β-D-glucopyranoside
4-hydroxy-3-methoxybenzoic acid
4'-hydroxyacetophenone

Zusammenfassung

In Fortsetzung früherer Studien wurde die Flavonoiddiversifizierung innerhalb von *Primula auricula*-Populationen unter Berücksichtigung weiterer Standorte in den österreichischen Ostalpen analysiert. *Primula auricula* ist für Exsudate aus Drüsenhaaren bekannt, die meist ein mehliges Aussehen haben (Farina). Farina besteht hauptsächlich aus unsubstituiertem Flavon und kleinen Mengen ungewöhnlicher substituierter Flavonderivate. Es wird angenommen, dass diese Flavone im Vergleich zu den gleichzeitig vorkommenden Flavonoidglykosiden der Gewebe aus einem anderen Biosyntheseweg stammen. Ziel der Studie war es, Korrelationen zwischen der Flavonoidexpression im Gewebe und in Drüsenhaaren zu finden, ein bislang unerforschter Aspekt. Im Zuge dieser Studie wurden Exsudate quantitativ und qualitativ anhand von saisonalen Veränderungen, unterschiedlichen Populationen, Höhengradienten und Blattentwicklungsstadien mithilfe von HPLC-UV analysiert und die Daten statistisch ausgewertet. Die Zusammensetzung der Flavonoidglykoside in Blattgewebsextrakten wurde ebenfalls anhand verschiedener Umweltvariablen sowie verschiedener Blattentwicklungsstadien analysiert. Es wurde eine klare Korrelation zwischen den Entwicklungsstadien der Blätter und den relativen Mengen an Exsudaten festgestellt, jedoch keine klare Korrelation mit jahreszeitlichen Veränderungen oder Höhenlagen. Die qualitative Bewertung des Exsudats weist auf Flavon (**1**) als Hauptbestandteil hin, während andere Flavonaglyca in Spuren vorhanden sind. Salicylsäure (**14**) wurde außerdem als bisher unbekannter Exsudatbestandteil identifiziert. Die quantitative Analyse der Blattgewebsextrakte ergab ein Maximum an Flavonoidglykosiden im Frühjahr, einen Rückgang im Herbst und einen Sinuskurvenverlauf innerhalb eines Jahres mit Maxima im Frühjahr und Herbst. Es wurden keine quantitativen Korrelationen zwischen den relativen Mengen an Flavonoidglykosiden und den relativen Mengen an Exsudat und Flavon (**1**) festgestellt. Die Ergebnisse dieser Studie legen nahe, dass *P. auricula*-Exsudate möglicherweise dabei helfen, die Blatttemperatur zu regulieren und die Wasserverdunstung zu verringern, während die Rolle der Flavonoidglykoside in den Geweben umstritten bleibt. Weitere Vergleichsstudien unter kontrollierten Umweltbedingungen sind erforderlich, um endgültige Schlussfolgerungen ziehen zu können.