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Pharmaceutical potential of willow leaves in terms of salicylic alcohol content

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ABSTRACT

Salicis cortex has analgesic, anti-inflammatory, antipyretic, and anti-rheumatic properties, primarily due to the content of salicylic alcohol derivatives (SAD) and other phenolic compounds. The Pharmacopoeia Europaea monographs willow bark and does not specify a particular species but requires a minimum content of 1.5 % SAD. This study aimed to determine whether the leaves of certain willow species could also be pharmaceutically relevant due to their SAD concentration, to identify species with high SAD levels, to figure out ideal harvest times, to investigate intraspecific variability, and to determine differences between the sexes in terms of SAD content, including less-studied species. Using a UPLC®-RP18-PDA method, 12 willow species with 42 individuals were analyzed. Concerning the average content of the entire observation period, the following species were identified as particularly SAD-rich (mean ± standard deviation): *S. purpurea* (6 ± 4 %), *S. aurita* (3 ± 4 %), *S. fragilis* (3.2 ± 2.3 %), *S. cinerea* (2.5 ± 3.0 %), and *S. lapponum* (1.7 ± 1.4 %). *S. daphnoides* (0.11 ± 0.20 %) and *S. caprea* (0.08 ± 0.21 %) are displayed as SAD-poor species. Statistical analysis revealed a slight intraspecific variation, but the interspecific variability of the SAD content was higher. The SAD values were significantly higher in mid- and late summer, except for *S. purpurea* (May) and *S. caesia* (June), as well as in 2019 compared to 2018. Moreover, no significant effect of sex could be detected. Considering the high costs of producing willow bark extracts, supplementing with willow leaves, particularly from SAD-rich species and genotypes, could be beneficial.

1. Introduction

Willow bark has analgesic, anti-inflammatory, antipyretic, and anti-rheumatic effects, and is therefore used to treat colds with fever and headaches, as well as back pain and rheumatic complaints (Chrubasik et al., 2001; Mahdi, 2010; Mills and Hutchins, 2017). These effects are similar to those of synthetic acetylsalicylic acid (ASA), one of the most widely manufactured drugs globally (Klessig, 2016; Schmid et al., 2001b). However, willow bark preparations generally have fewer side effects, such as localized gastric lesions and inhibited platelet aggregation (Krivoy et al., 2001; Vlachojannis et al., 2011). Despite these benefits and the confirmed efficacy, patients often choose synthetic alternatives due to the relatively high cost of natural extracts (Chrubasik et al., 2001). Although the active ingredients are absorbed more slowly than synthetic ASA, the effect lasts longer (Schmid et al., 2001a). The beneficial effects of willow bark and leaves are due to the synergistic action of secondary constituents such as phenolic glycosides, flavonoids, and tannins (Gligorić et al., 2023; Nahrstedt et al., 2007; Schmid et al., 2001a), with salicylic alcohol derivatives, notably salicin and salicortin, being primary contributors (Antoniadou et al., 2021; Nahrstedt et al., 2007; Schmid et al., 2001a). However, the mechanism of action and the main bioactive substances are not yet fully understood (Klessig, 2016).

The European Pharmacopoeia does not specify a particular willow species for pharmaceutical use but requires a minimum SAD concentration of 1.5 %, measured as salicin, for medical and economic viability (EDQM, 2020). Otherwise, the salicin content would be too low to be effective, or a large amount of bark would be required to achieve the necessary dose. Therefore, the production of willow bark extracts requires a plant source material that contains stable high concentrations of active constituents - above all salicylic alcohol derivatives. However, as no species is defined, different species with different genotypes can be used. Due to large intra- and interspecific differences, there can be

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considerable fluctuations in the composition and content of the phenolic constituent spectrum (Förster et al., 2008, 2009, 2010; Köhler et al., 2023; Nissinen et al., 2018; Sulima et al., 2021; Wiesneth, 2019). Therefore, morphological identification alone is insufficient due to the simple vegetative distribution, morphological similarities, as well as seasonal and environmental fluctuations (Chen et al., 2010; Förster et al., 2009; Skvortsov and Zinovjev, 1999; Wiesneth, 2019). For phytopharmaceuticals, it is necessary to characterize the phenolic profile of the individual willow species and genotypes, especially of understudied species (Köhler et al., 2023). This can contribute to the efficacy and quality of willow-based phytopharmaceuticals and increase their popularity (Sulima et al., 2017). Most research focuses on the bark, as only the bark and shoots are monographed for pharmaceutical use (Committee for Herbal Medicinal Products, 2007; EDQM, 2020), whereas leaves — typically discarded — also hold significant therapeutic potential, containing a valuable phenolic profile (Gligorić et al., 2023; Julkunen-Tiitto and Meier, 1992; Tawfeek et al., 2021; Wiesneth, 2019). Leaves are particularly rich in flavonoids, phenolic acids, and phenolic glycosides like salicylic alcohol derivatives, whereas bark is more procyanidin-rich (Tawfeek et al., 2021).

This study aimed to explore whether the leaves of specific willow species are of pharmaceutical interest based on their SAD content, to identify SAD-rich species, to determine optimal harvest times, and to assess potential interspecific and sex-based influences, including less-studied species. It was hypothesized that the SAD content varies between species, harvest years and months, sex, and within species. Moreover, it is assumed that certain species display sufficiently high SAD levels in their leaves from a pharmaceutical perspective and can therefore be used for supplementation of the bark.

2. Materials and methods

2.1. Plant material

In this study, 12 Central European *Salix* species from various related groups within the genus were analyzed, including *Salix aurita* L., *S. bicolor* EHRH. ex WILLD. (synonym: *S. phylicifolia* L.), *S. caesia* VILL., *S. caprea* L., *S. cinerea* L., *S. daphnoides* VILL., *S. fragilis* L., *S. hastata* L., *S. lapponum* L., *S. purpurea* L., *S. viminalis* L., and *S.* × *sepulcralis* SIMONK. In total, 42 individuals, which were located at the Ecological-Botanical Gardens of the University of Bayreuth (ÖBG Bayreuth), Germany. All species represented by more than eight individuals in the Ecological-Botanical Gardens were limited to eight in this study, which was the case for *S. caprea*, *S. cinerea*, *S. fragilis*, and *S. purpurea*. For all other species, all available individuals were sampled, which in most cases was a single individual, except for *S. daphnoides* (3 individuals). The sex was determined morphologically, and a balanced sex ratio was chosen for species with more than one individual, except for *S. purpurea* (3:5 in favor of females) and *S. daphnoides* (1:2 in favor of males). For the latter, a balanced ratio was logistically impossible.

2.2. Sample preparation

Samples were collected between May and September 2018 and 2019 on the following dates: May 07, 2018, June 11, 2018, July 02, 2018, July 30, 2018, and September 03, 2018, as well as May 13, 2019, June 03, 2019, July 01, 2019, August 05, 2019, and September 10, 2019, except for *S. hastata*. This individual perished after the collection date of May 2019. Afterwards, they were cut and dried in a vacuum over silica gel. The plant material was ground using a swing mill MM400 (Retsch GmbH) with a 25 ml grinding beaker and 10 mm ball and subsequently stored at −10 °C until further use.

2.3. Extract preparation for UPLC® analysis

The extracts were prepared following the method described by Wiesneth (2019). 50 mg of the dried plant material of each individual for each month and year was extracted in triplicate in 1.00 ml MeOH with Citropten (0.2 mg/ml) as an internal standard in a supersonic bath at room temperature for 30 min. The samples were then centrifuged at 14,000 rpm for 3 min and filtered through a 0.2 µm Perfect Flow® RC Filter (WICOM Germany GmbH, Heppenheim, Germany).

2.4. UPLC® analysis

The analysis was conducted using a Waters ACQUITY UPLC®, which included an ACQUITY H-Class QSM, FTN, and PDA detector. A reversed-phase separation was performed on a Luna® omega C18 column (1.6 µm, 100 Å, 100 × 2.1 mm, Phenomenex, Aschaffenburg, Germany). The column oven was maintained at 50 °C with an active preheater. The injection volume of the extract was 1.0 µl. Eluents used were: (A) H_2O with 1 % formic acid and (B) ACN with 1 % formic acid. The applied gradient at a flow rate of 0.5 ml/min was: 0.0–0.5 min 5 % B isocratic; 0.5–9.0 min 5–30 % B; 9.0–10.5 min 30–50 % B; 10.5-11.5 min 50–100 % B; 11.5–14.0 min 100 % B isocratic; 14.0–15.0 min 100-5 % B; 15.0–17.0 min 5 % B isocratic. UV spectra were recorded between 200 and 400 nm, with quantification at 279 nm. For calibration, a citropten standard curve was generated by measuring three stock solutions in duplicates (2–0.2 mmol/l, in MeOH). To identify the SAD content, another calibration was performed using salicin (2–0.2 mmol/l) in MeOH + 0.2 % citropten as the reference standard. This involved three stock solutions and two dilution series each. Chromatograms were integrated after blank subtraction. Peaks were then assigned using the UV maxima and a spectral database, which was developed for automated assignment. The final SAD content was calculated according to Wiesneth (2019), quantified as salicin, and referenced to the weight of dried drug material, with results expressed as % analyte per drug. Each sample was analyzed in triplicate and reported as mean values ± standard deviation (SD) unless otherwise noted.

The standard deviation of mean values of technical triplicates indicates measurement accuracy, whereas the standard deviation of mean values of the species, which were presented by more than one individual (*S. caprea, S. cinerea, S. daphnoides, S. fragilis,* and *S. purpurea*), indicates intraspecific variability. For interannual variability, the mean values shown for each species, represented by a single individual, were calculated for each month and year and were derived from the triplicate measurements. For species with multiple individuals, the mean values for each month and year included the data from all individuals of that species. In terms of interseasonal variability, for species represented by a single individual, mean values for each month were calculated by averaging across both years. For species with multiple individuals, the mean values for each month were calculated across all individuals of the species and both years. Mean values presented for intersexual variability for each month and sex were calculated by combining the data from all individuals of the respective species and sex across both years.

2.5. Statistical analysis

The statistical analysis of this study was performed using R Version 4.2.3 (Team, R.C., 2023) with the following packages: dplyr (Wickham et al., 2023), multcomp (Hothorn et al., 2008), multcompView (Gravers et al., 2024), lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), and car (Fox and Weisberg, 2019). Data were analyzed using linear mixed models with individuals nested within species as random factor unless otherwise declared. First, the mean value of each triplicate was calculated, and then the SAD concentrations were log-transformed and functioned as response variable.

To evaluate the overall influence of month, year, and sex, these variables were treated as fixed factors following a type II ANOVA. For comparisons among species, the variable species, month, and year were used as fixed factors, with individual as random factor, in order not to disregard the influence of month and year. Moreover, exclusion of the

intercept ensured that individual estimates were obtained for each species. A type II ANOVA followed by Tukey's honest significant difference (HSD) test ($p < 0.05$) was subsequently carried out. Additionally, to investigate species-specific monthly and annual effects, the fixed factors in the linear mixed model have been species in interaction with month and year. The main effects of month and year were removed here, so that only the interactions are taken into account, and the intercept was excluded. To test whether there are species-specific sex effects, species in interaction with sex as a fixed factor was used, main effects were removed, and the intercept was excluded.

Graphics were made with ggplot2 package (Wickham, 2016). P values < 0.05 were regarded as statistically significant, $p < 0.01$ as very significant, and $p < 0.001$ as highly significant.

3. Results

3.1. Interspecific variability

The following species, related to the mean value across each individual, month and year within a species (data not shown here), contained an average content of more than 1.5 % SAD, as prescribed by European Pharmacopoeia (EDQM, 2020): *S. purpurea* (6 ± 4 %), *S. aurita* (3 ± 4 %), *S. fragilis* (3.2 ± 2.3 %), *S. cinerea* (2.5 ± 3.0 %), and *S. lapponum* (1.7 ± 1.4 %). Among the 12 species analyzed, *S. daphnoides* and *S. caprea* exhibited the lowest SAD content throughout the growing season (Fig. 1).

The quantitative analysis of the salicylic alcohol derivative (SAD) content in different species revealed that *S. purpurea* had the highest average values, based on the mean value of the total data, followed by *S. aurita*, *S. fragilis*, and *S. cinerea*. The highest overall SAD content, concerning the average value of each individual within a species with the maximum detected content, was observed in *S. purpurea* (17.0 ± 0.8 %) in May 2019 (Fig. 2). Other notable concentrations were found in *S. cinerea* (14 ± 2 %) in July 2019, and in *S. fragilis* (10.20 ± 0.11 %) in September 2018 (Fig. 2). In addition, *S. purpurea* differed significantly from *S. caesia, S. caprea, S. cinerea, S. daphnoides, S. hastata, S. viminalis,* and *S. × sepulcralis*. Still, not from *S. aurita, S. bicolor, S. fragilis*, and *S. lapponum* (Table 1). Analysis of variance revealed a highly significant influence of the species variable on the SAD content (Table 2).

3.2. Interannual variability

The comparison between the harvest years 2018 and 2019 in terms of SAD content is presented in Fig. 1, Table 1, and Table 2. Data for

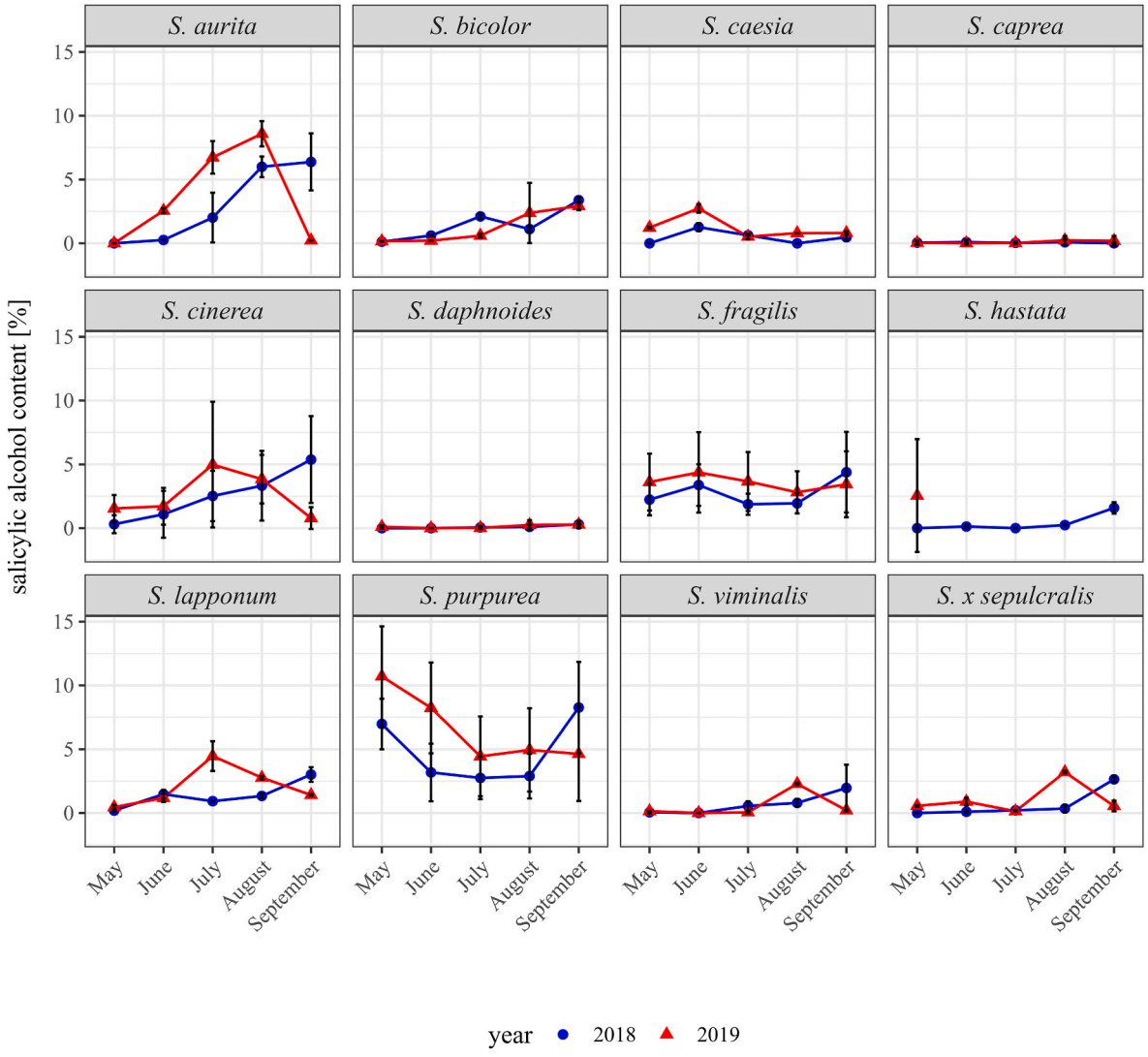

Fig. 1. Comparison of the annual SAD trend of two subsequent years (interannual variability). Data are presented as mean ± SD, calculated as salicin. For species represented by a single individual, the mean for each month and year is calculated based on the corresponding triplicate. For species represented by more than one individual, the mean for each month and year includes the data of each individual measured for the species.

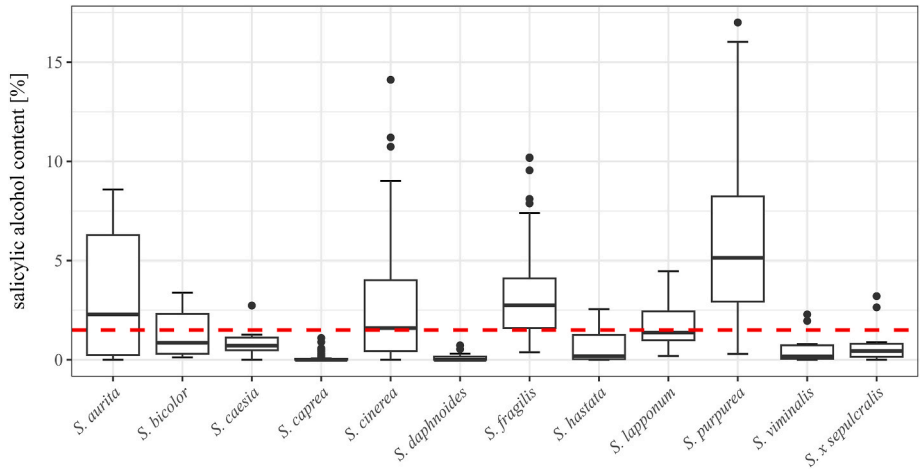

Fig. 2. Overview of the salicylic alcohol derivative content for each species and thus of the interspecific variability. The red line marks the minimum of 1.5 % total SAD content prescribed by the European Pharmacopoeia, calculated as salicin. Each data point represents the mean of three technical replicates of each particular sample. This results in one data point per individual, month, and year. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Results of linear mixed models. Comparison of the species in terms of SAD content with Tukey's HSD test, as well as species-specific effects of year, month, and sex. R^2 in general are log-transformed concentration estimates of SAD in mg/g DW[a]. R^2 of species-specific effects represents the average log-transformed content without reference to the other species. R^2 of year-specific effects refers to 2018 as the reference year, R^2 of month-specific effects relates to May as the reference month, and R^2 of sex-specific effects refers to females as the sex reference.

	Species-specific		Year-specific		Month-specific		Sex-specific	
Species	R^2	SE	R^2	SE	R^2	SE	R^2	SE
S. aurita	2.6 ab	0.5	0.1	0.5	0.74 ***	0.18	NA	NA
S. bicolor	2.3 ab	0.5	−0.3	0.5	0.65 ***	0.18	NA	NA
S. caesia	1.8 bcd	0.5	1.2 *	0.5	−0.05	0.18	NA	NA
S. caprea	0.31 d	0.17	0.09	0.18	0.06	0.07	0.0	0.4
S. cinerea	2.60 bc	0.17	0.31	0.18	0.34 ***	0.07	−0.4	0.4
S. daphnoides	0.48 d	0.28	0.15	0.29	0.26 *	0.11	0.0	0.7
S. fragilis	3.28 ac	0.17	0.22	0.18	0.00	0.07	−0.2	0.4
S. hastata	1.5 bd	0.6	NA	NA	0.61 *	0.25	NA	NA
S. lapponum	2.6 ab	0.5	0.4	0.5	0.37 *	0.18	NA	NA
S. purpurea	3.78 a	0.17	0.33	0.18	−0.13 *	0.07	−0.1	0.4
S. viminalis	1.3 bd	0.5	−0.4	0.5	0.55 **	0.18	NA	NA
S. × sepulcralis	1.7 bcd	0.5	0.8	0.5	0.43 *	0.18	NA	NA

R^2 - regression coefficient.
SE - standard error of regression coefficient.
NA – not detectable.
*p < 0.05, **p < 0.01, *** <0.001.
Different letters indicate interspecific significant differences in SAD content (p < 0.05).
[a] DW: dry weight.

Table 2
Summary of the overall effects from linear mixed models and type II ANOVA of the variables species, year, month, and sex on SAD content. R^2 in general are log-transformed concentration estimates of SAD in mg/g DW[1]. R^2 of years relates to 2018 as reference, R^2 of month refers to May as reference, and R^2 of sex refers to females as reference.

	R^2	SE	X^2	d.f.
Species	NA	NA	1299.67 ***	12
Year	0.26 **	0.09	9.60 **	1
Month	0.137 ***	0.030	21.53 ***	1
Sex	−0.18	0.16	1.42	2

R^2 - regression coefficient.
SE - standard error of regression coefficient.
X^2 – chi-squared value.
d.f. – degree of freedom.
NA – not detectable.
*p < 0.05, **p < 0.01, ***p < 0.001.

S. hastata are not shown here because of missing comparable values. Linear mixed model analysis displayed a significant overall annual effect on the SAD content in terms of higher values in 2019 (Table 2). Consequently, for most species, the SAD content was higher in 2019, but for *S. caesia* this effect was even significant (Table 1). On the other hand, for *S. bicolor* and *S. viminalis* the negative regression coefficient showed that they exhibited higher SAD values in 2018, but this effect was not significant.

3.3. Interseasonal variability

Figs. 3 and 4 illustrate the seasonal variation in salicylic alcohol derivatives. Maximum values were found for almost all species in mid- and late summer (July–September), except for *S. purpurea* (May) and *S. caesia* (June). Concerning the minimum contents, the trend was less distinct but generally pointed towards early and midsummer. None of the species displayed the lowest levels in September. Regression analysis confirmed that the content increases with month significantly (Table 2).

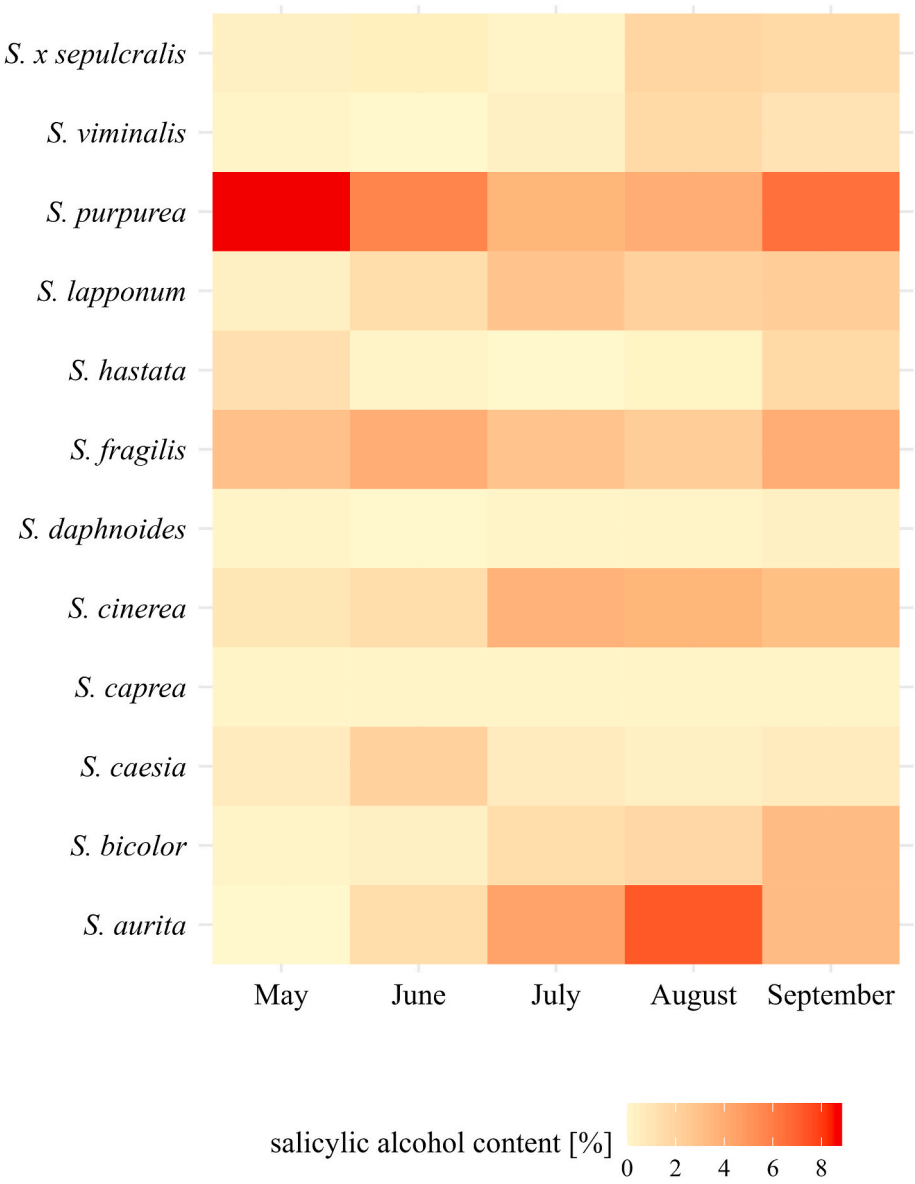

Fig. 3. Salicylic alcohol content based on harvest time represents the interseasonal variability. The redder a field is, the higher the salicylic alcohol derivative content, and the lighter yellow a field is, the lower. Data are shown as the mean of each species and month across both investigated years, calculated as salicin. For species represented by a single individual, the mean for each month is calculated across each replicate and both years. For species represented by more than one individual, the mean includes the data of every individual investigated for this species for each month across both years. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

A closer look at the different species showed that this effect was significant in almost all species, except for *S. caprea* and *S. fragilis*. Furthermore, the opposite trend was observed in *S. caesia* and *S. purpurea,* whereby this was even significant for the latter (Table 1). Sufficiently high concentrations above 1.5 % were found in *S. aurita* (July–September), *S. bicolor* (August–September), *S. caesia* (June), *S. cinerea* (July–September), *S. fragilis* (May–September), *S. hastata* (September), *S. lapponum* (July–September), *S. purpurea* (May–September), *S. viminalis* (August), and *S.* × *sepulcralis* (August–September), whereas the highest SAD content was detected for *S. purpurea* (9 % ± 4) in May.

When examining each species individually, *S. aurita* showed a clear seasonal fluctuation, with levels ranging from 0 % to 7 %. The highest concentration was observed in August, while the lowest occurred in May. There was an increase in SAD content from May to August, followed by a decline in September. Of the 12 species analyzed, the SAD content of *S. aurita* varied the most, namely by more than 7-fold. The SAD content of *S. bicolor* also changed during the growth period. The highest content was found in September, up to 3.2 ± 0.4 %, followed by August and July, whereas the lowest was measured in May (0.142 ± 0.020 %). An increase in content can be seen throughout the entire growth period. During the sampling period, the SAD content of *S. caesia* was relatively low, ranging from 0.4 to 2.0 %. The highest concentration was recorded in June, while the lowest occurred in August. An increase was observed from May to June, followed by a decline after the June peak. Subsequently, the content remained consistently low, staying below 0.64 %. For *S. caprea,* the contents were also very low (0.03–0.16 %). The highest content was detected in August and the lowest in July. Therefore, a small increase can be seen from July to August. A seasonal concentration curve in the range of 1–4 % can be recognized for *S. cinerea*. The highest content of SAD was measured in July, followed by August and September, while the lowest was monitored in May. There was an incline until July, and thereafter, the content remained constant. *S. daphnoides*, on the other hand, again showed a generally low content

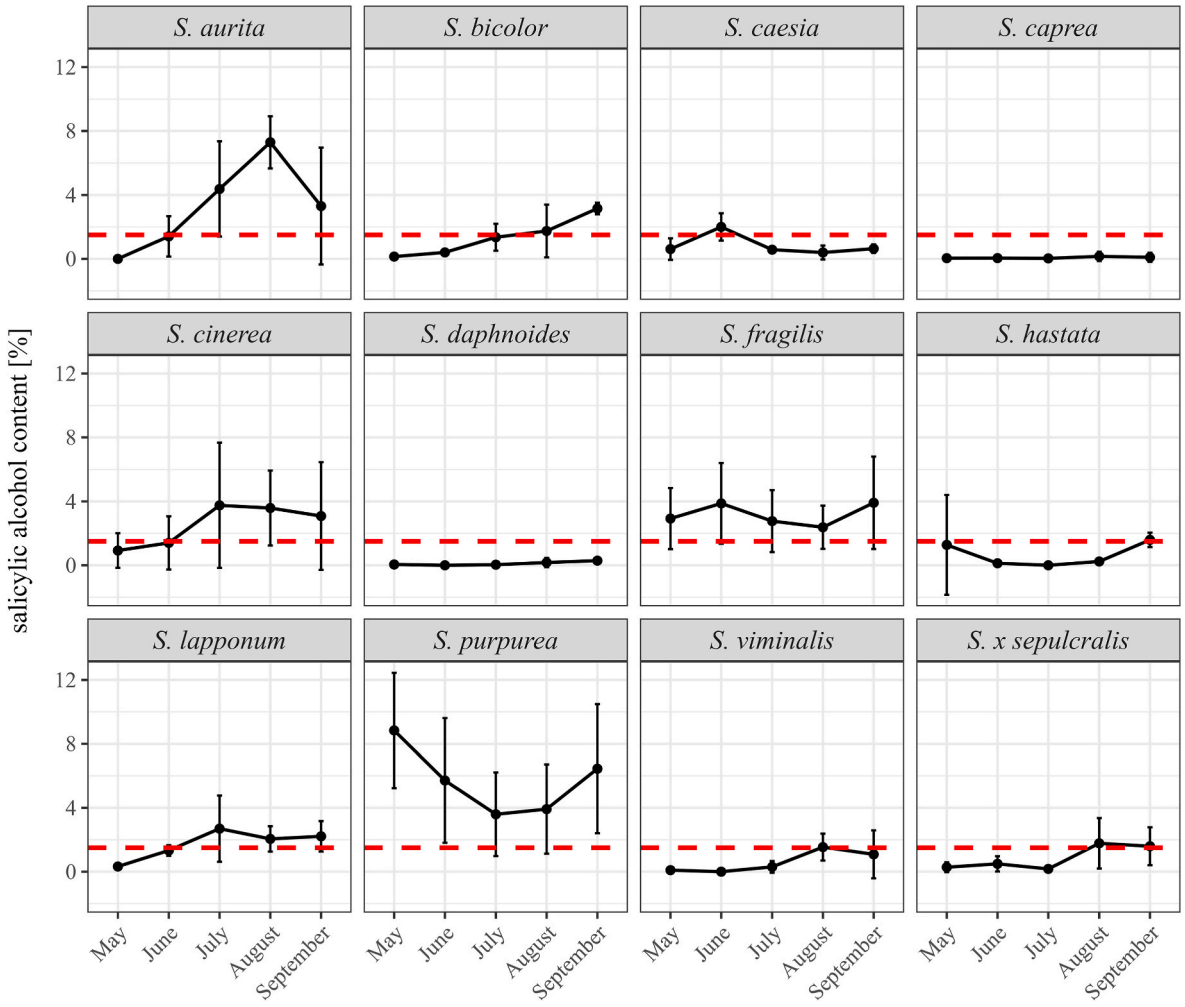

Fig. 4. Overview of the variation in SAD levels during the growing period across both investigated years (interseasonal variability). The red line marks the minimum content of 1.5 % total SAD content prescribed by the European Pharmacopoeia, calculated as salicin. Data are shown as mean ± SD. For species represented by a single individual, the mean for each month includes the data across both years for the concerning individual. For species represented by more than one individual, the mean for each month includes the data of each individual across both years. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in the range of 0.00–0.29 %. Nevertheless, the SAD content observed in September was higher than the lowest level measured in June. The SAD content in *S. fragilis* showed some variation throughout the growth period, though it remained fairly stable if SD is also considered, fluctuating between 2 % and 4 %. The highest level was observed in September, followed by June, whereas the lowest occurred in August. Notably, there was a decline from June to August, followed by an increase from August to September. For *S. hastata* the SAD levels ranged from 0.0 % in July to 1.6 ± 0.5 % in September. However, it should be noted that values from June–September 2019 were missing. The highest content of salicylic alcohol derivatives in *S. lapponum* was found in July, followed by September, while the lowest was measured in May, ranging from 0.32 to 3 %. An increase can be seen from May to July. For *S. purpurea,* the content changed considerably during the sampling period. The highest SAD contents were monitored in May (9 % ± 4), followed by September (6 % ± 5) and June (6 % ± 4), while the lowest were found in July and August (4 % ± 3). There was a drop in content from May to July, and an increase from July to September. The content of *S. viminalis* was at a low level until late summer. The highest content was then achieved in August (1.5 % ± 0.9), and the lowest was in May (0.10 % ± 0.08) and June (0.00 %). The situation was similar to *S.* × *sepulcralis.* Here as well, the level remained very low until late summer. An increase was observed from July to August, with a content ranging from 0.17 to 2.00 %.

3.4. Intraspecific variability

The result of the linear mixed model showed that the content of salicylic alcohol derivatives varied slightly among individuals within species (Variance = 0.1, SE = 0.4). This is also indicated by the standard deviation of *S. caprea, S. cinerea, S. daphnoides, S. fragilis,* and *S. purpurea* (Fig. 1). Nevertheless, the variation between species was higher (Variance = 1.2, SE = 1.1).

3.5. Intersexual variability

Fig. 5 illustrates the seasonal variation in salicylic alcohol levels, categorized by sex. The negative regression coefficient indicates a slight trend towards lower levels in male individuals, although there was no significant effect of sex on the SAD content (Table 2). The same was detected within species for *S. cinerea, S. fragilis,* and *S. purpurea*, which were, among others, selectively analyzed for this purpose (Table 1).

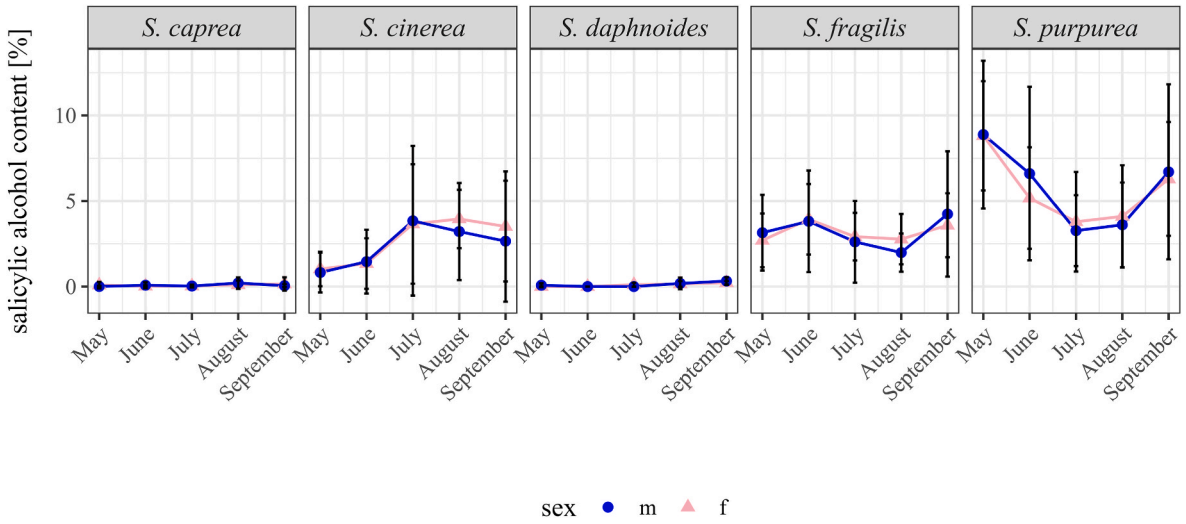

Fig. 5. Comparison of sexes among the selected species concerning SAD content, displaying intersexual variability. Data are presented as mean ± SD, calculated as salicin. The mean for each month and sex includes the data of each individual and sex across both years.

4. Discussion

4.1. Interspecific variability

To investigate interspecific variability, 12 willow species were examined for this purpose and compared to previous studies. The observed values are largely consistent with existing literature, although in some cases exceeding earlier findings. *S. purpurea* had previously been described as a species with a high phenolic glycoside content, which can be confirmed by this study (Gligorić et al., 2023; Meier et al., 1988; Thieme, 1965a; Wiesneth, 2019). The SAD content found in *S. fragilis* showed consistent values with Thieme (1965a) and Meier et al. (1988), although the May data exceeded these levels (Fig. 4). Besides, *S. aurita, S. caprea, S. cinerea*, and *S. viminalis* in general contained less than 0.1 % SAD in May, as previously reported (Thieme, 1965a, 1965b), except for the leaves of *S. cinerea.* Literature reports on *S. hastata* are conflicting, with Julkunen-Tiitto (1989) and Meier et al. (1988) describing high levels in the leaves from August–September on the one hand and negligible amounts on the other hand, which were not confirmed here. For *S. caprea* and *S. daphnoides*, the results align with those of Meier et al. (1988), Julkunen-Tiitto (1989), Gligorić et al. (2019), and Wiesneth (2019), reporting consistently low SAD levels in the leaves. Compared to the findings of Wiesneth, the data for the leaves of *S. purpurea* are similar, but there were higher SAD levels in *S. cinerea*, *S. fragilis*, *S. caprea*, and *S. daphnoides* recorded. While Wiesneth showed that these species, except for *S. purpurea*, tend to accumulate SAD in their shoots, the obtained results indicated higher SAD levels in the leaves, apart from *S. caprea* and *S. daphnoides* (Wiesneth, 2019).

Several studies have shown that the bark of *S. alba* (Sulima et al., 2021; Thieme, 1965a), *S. caprea* and *S. cinerea* (Thieme, 1965a; Wiesneth, 2019), *S. daphnoides* (Förster et al., 2009; Köhler et al., 2023; Sulima et al., 2021; Wiesneth, 2019), *S.* pentandra (Thieme, 1965a; Wiesneth, 2019), *S. purpurea* (Förster et al., 2009; Gligorić et al., 2023; Julkunen-Tiitto, 1989; Köhler et al., 2023; Sulima et al., 2021; Thieme, 1965a; Wiesneth, 2019), *S.* repens (Julkunen-Tiitto, 1989; Wiesneth, 2019) and *S. viminalis* (Thieme, 1965a) are SAD-rich. However, some authors (Gligorić et al., 2023; Wiesneth, 2019) presented a higher content in leaves of *S. purpurea* compared to the bark. The obtained research results suggest that the leaves of additional species, including *S. aurita, S. bicolor, S. caesia, S. cinerea, S. fragilis, S. hastata, S. lapponum, S. purpurea, S. viminalis,* and *S.* × *sepulcralis,* depending on harvest time, may also be suitable for pharmaceutical use.

A direct comparison with bark data from the literature remains difficult due to many influencing factors, such as location, detection and quantification method, harvest time, or climatic conditions. Unequal sample sizes can lead to certain species disproportionately influencing the overall patterns. In the statistical analysis, species were therefore treated as a fixed factor, while individuals were considered as random factors nested within species. While avoiding overrepresentation of more frequently sampled species, this method ensured that interspecific differences were explicitly modelled.

In summary, these results demonstrate that the SAD content in willow leaves varies considerably depending on the species, making interspecific variability a crucial factor for their pharmaceutical use.

4.2. Interannual variability

In order to assess the influence of harvest year on the SAD content, samples were collected during the growing season in two consecutive years. The overall trend of the obtained data showed that the content of salicylic alcohol derivatives in almost all species was higher in 2019 than in 2018, with significant annual influence towards increasing years (Tables 1 and 2). These findings, however, contradict previous literature, which documented a decrease in phenolic glycoside content as ontogeny or tissue age progressed (Bingaman and Hart, 1993; Boeckler et al., 2011; Donaldson et al., 2006; Julkunen-Tiitto, 1985a, 1985b; Nissinen et al., 2018; Thieme, 1965b). It has been suggested that salicylic alcohol derivatives are replaced by other phenolic compounds in later ontogenetic stages (Donaldson et al., 2006; Julkunen-Tiitto and Virjamo, 2016; Nissinen et al., 2018). A notable observation is also that larger species, which are less susceptible to mammalian herbivores, may exhibit more stable or lower salicylic alcohol derivative levels (Nissinen et al., 2018; Tahvanainen et al., 1985). Interestingly, an opposite trend was found in the seedlings of *S. sericea*, where the phenolic glycoside content increased (Fritz et al., 2001; Orians et al., 2010). This supports the hypothesis that phenolic glycosides function as a defense mechanism, providing younger plants with enhanced protection against herbivores (Tahvanainen et al., 1985). But in contrast, Nissinen et al. (2018) found no significant influence of age on phenolic concentrations in the leaves and bark of *S. myrsinifolia* over seven years, suggesting that defense levels remain constant. Direct comparison with earlier measurements from the same location in 2016 (Wiesneth, 2019), revealed a decline in content for *S. fragilis* and *S. purpurea,* but for *S. cinerea,* the content remained nearly unchanged.

The impact of harvest years is difficult to separate from climatic fluctuations. Furthermore, the short duration of the study (two years)

limits the significance. To prevent this problem, the harvest year was treated as a fixed factor in the linear mixed effects model, both as a main effect (in the overall analysis) and in interactions with species. This made it possible to test whether the SAD content of individual species changes over the years. This approach prevented incorrect interpretation of individual differences or random fluctuations in individual species as a harvest year effect.

The study reveals that the content of salicylic alcohol derivatives in willow leaves can vary between harvest years, with annual environmental conditions and plant ontogeny influencing these fluctuations.

4.3. Interseasonal variability

The salicylic alcohol derivative content in willow bark and leaves is known to fluctuate seasonally (Förster et al., 2008, 2009; Thieme, 1965a; Wiesneth, 2019). To identify an optimal harvest time for pharmaceutical use, seasonal studies on the SAD content were conducted between May and September during the growth period in 2018 and 2019.

Contrary to Thieme (Thieme, 1965a, 1965b), except for *S. purpurea* and *S. caesia*, the data did not align with the conclusion that maximum leaf content is reached by late May after a sharp rise 4–6 weeks post-sprouting, followed by a continuous decline until leaf fall. Wiesneth (2019), on the other hand, described peak levels mainly in September and minimum levels in June, which is consistent with the obtained results (Fig. 4). Although, *S. purpurea* was also out of line and, unlike most other species, presented its maximum in early summer and minimum in late summer. In addition, in this study, *S. purpurea* displayed a rise in content after July, continuing until the end of the growth period, discrepant with Thieme (Thieme, 1965a, 1965b) and Wiesneth (2019). *S. daphnoides* and *S. caprea* exhibited low SAD levels, consistent with Wiesneth (2019). For *S. fragilis*, there was a similar content curve compared to Wiesneth (2019). Furthermore, the seasonal patterns of *S. aurita* and *S. viminalis* did not align with Thieme (Thieme, 1965a, 1965b). The pattern observed in *S. cinerea* aligns more closely with Wiesneth (2019), where an increase was observed during the growth period, and the data presented in this study showed an increase in SAD content until July.

Seasonal fluctuations are influenced by environmental factors and cannot be fully represented by monthly samples, which were collected on a single day of the month. Differences between species can also be masked if seasonal development is not considered on a species-specific basis. For this purpose, the harvest month was considered as a fixed factor in this model, and additionally, it was modelled in interaction with species and year. This allowed species-specific seasonal patterns to be identified without distorting the results. By excluding the main effects of the variables month and year in this interaction model, general trends were prevented from being incorrectly interpreted as species-specific variability.

The results demonstrate seasonal fluctuations in the SAD content of willow leaves, with species-specific maxima occurring at different times, but mainly from July to September.

4.4. Intraspecific variability

The variation in quantitative SAD content was observed not only at the interspecific level but also at the intraspecific level. Statistical analysis revealed a minor overall effect considering the SAD content between different individuals within the species. Despite this, all individuals of *S. fragilis,* and *S. purpurea* consistently exceeded the 1.5 % threshold, related to the mean of each individual within these species, not separated by month and year (data not shown here), as already described by Sulima et al. for the bark of *S. purpurea* (Sulima et al., 2021).

The intraspecific variability has been described by several studies (Förster et al., 2008, 2009, 2010; Heiska et al., 2007; Köhler et al., 2023; Nichols-Orians et al., 1993; Nissinen et al., 2018; Nybakken et al., 2012; Nyman and Julkunen-Tiitto, 2005). The genus *Salix* is known for the tendency to hybridize without displaying significant morphological differences, which complicates the identification of the pure species (Julkunen-Tiitto, 1985a). Genotypic variations within *Salix* enable these plants to adapt and thrive under diverse and changing conditions (Liao et al., 2016). This is particularly evident in species like *S. purpurea* and *S. fragilis*, which can colonize distant areas via broken shoots dispersed by rivers (Sulima et al., 2017). Furthermore, related genera within a family tend to evolve distinct defensive compounds to counteract herbivore pressure (Becerra, 2007; Kursar et al., 2009; Volf et al., 2018). Salicylic alcohol derivatives and their degradation products generally act as deterrents to generalist herbivores (Osier and Lindroth, 2001; Pentzold et al., 2014; Volf et al., 2015), but interestingly, some specialized herbivores are attracted to high concentrations of SAD (Tahvanainen et al., 1985).

A critical limitation of this study is the unequal number of sampled individuals per species. While multiple individuals allow a more reliable estimation of mean values and intraspecific variability, data derived from a single individual cannot be regarded as fully representative of a species, as they are more susceptible to random effects of genotype or fluctuating environmental conditions. Furthermore, the genotype of individuals within a species was not determined, which means that a species could be represented by several individuals of the same genotype or also by different genotypes in this study. As a result, intraspecific variability may be underrated, and species-specific effects could partly reflect an individual plant rather than the species. In regard to this imbalance, the data were analyzed using linear mixed models, in which individuals were nested within species as random factors. This statistical approach mitigates the bias arising from unequal replication and enables comparisons at the species level, considering intraspecific variation. Nevertheless, results from species represented by a single individual should be approached cautiously due to the unknown extent of intraspecific variability.

This study suggests that the content of salicylic alcohol derivatives varies between individuals within a species, although intraspecific differences are likely to be smaller than the interspecific variability.

4.5. Intersexual variability

To investigate the influence of sex on the SAD content, the species *S. caprea*, *S. cinerea*, *S. daphnoides, S. fragilis*, and *S. purpurea* were examined more precisely.

No significant differences were found between the sexes regarding the SAD content, consistent with previous reports (Julkunen-Tiitto, 1989; Nichols-Orians et al., 1993; Nissinen et al., 2018). However, in the genus *Salix*, the sex ratio tends to be skewed towards females (Lloyd, 1974; Myers-Smith and Hik, 2012). These usually evolve, as males typically have lower viability (Crawford and Balfour, 1990; Yang et al., 2020) and are more exposed to herbivores (Cornelissen and Stiling, 2005; Fritz, 1995). Females, on the other hand, generally exhibit higher biomass (Hou et al., 2017; Jiang et al., 2016), higher levels of defensive compounds (Nybakken and Julkunen-Tiitto, 2013; Palo et al., 1992), and are less susceptible to insects (Cepeda-Cornejo and Dirzo, 2010; Cornelissen and Stiling, 2005). Yang et al. (2020) confirmed this trend but argued that the higher phenolic content in females is not primarily for defense purposes. Instead, it may relate to the higher nitrogen content in female leaves, which could deter insects.

Although linear mixed models with individuals nested within species were conducted to account for the unbalanced number of individuals, the uneven sex distribution across species limits the conclusion that can be drawn about sex-specific effects. This unequal sampling reduces statistical power to detect small to moderate sex-specific differences. It also increases the risk that species-specific or site-specific effects will distort sex-associated patterns. Therefore, the absence of significant intersexual differences should be interpreted with caution. Confirmation

requires a larger sample size and a more balanced sex distribution per species.

It was hypothesized that SAD levels differ between the sexes, with female individuals expected to have higher levels of SAD. However, no significant sex-specific differences were found in this study.

5. Conclusion

Comparisons with the literature should generally be treated with caution, as some sources only determine the phenolic glycosides, which contain salicylic alcohol derivatives, but also other substances, depending on the definition, even if only in small quantities (Julkunen-Tiitto, 1989; Meier et al., 1988; Thieme, 1965a) or in some cases, only salicin (Gligorić et al., 2019, 2023) is determined. Different results are also obtained mainly due to different detection and quantification methods, but also to different harvest times, locations, climatic conditions, and intraspecific variability. Considering the high cost of producing pharmaceutical willow bark extracts (Förster et al., 2008), many factors need to be considered. Above all, selecting species rich in salicylic alcohol derivatives (Förster et al., 2008, 2009; Köhler et al., 2023) and focusing on SAD-rich genotypes within those species (Förster et al., 2009, 2021; Heiska et al., 2005; Köhler et al., 2023), is essential. Additionally, it is important to consider both, the overall phenolic profile (Förster et al., 2021; Köhler et al., 2023; Nahrstedt et al., 2007), and the qualitative composition of the salicylic alcohol derivatives (Köhler et al., 2023), as variations in these could lead to inconsistent and unpredictable therapeutic outcomes (Antoniadou et al., 2021; Sulima and Przyborowski, 2019). Therefore, the extract should be characterized to ensure a consistent composition of the relevant constituents across all batches, thereby guaranteeing comparability of the clinical effects between products and over time. In addition, willow leaves should also be considered due to their substantial SAD content in some species (Gligorić et al., 2023; Heiska et al., 2005; Julkunen-Tiitto and Meier, 1992; Meier et al., 1988). They are particularly suitable for supplementation during the summer months, as the SAD maxima in the bark tend to occur in spring (Förster et al., 2009; Thieme, 1965b). As well as selecting the appropriate harvest time (Förster et al., 2008, 2009; Thieme, 1965a; Wiesneth, 2019), though this can be complicated by fluctuating climatic conditions (Wiesneth, 2019). To optimize SAD production, breeding SAD-rich clones (Heiska et al., 2005; Julkunen-Tiitto and Meier, 1992) or controlled hybridization (Sulima et al., 2021) of high-SAD species with high-biomass species is a viable strategy (Bubner et al., 2018; Förster et al., 2021). Furthermore, factors like climate (Gligorić et al., 2023; Sulima et al., 2017), nutrient supply (Hale et al., 2005; Lower et al., 2003), soil management (Heiska et al., 2005), ontogenetic age (Julkunen-Tiitto, 1985a; Nissinen et al., 2018), time of the day (Thieme, 1965a), biotic and abiotic influences (Hale et al., 2005), as well as sample processing, extraction and the selected methods for quantification (Gligorić et al., 2019) are important.

Overall, the results of this study confirm several hypotheses regarding the SAD content in willow leaves. It varies considerably between species, highlighting interspecific differences as an important factor for pharmaceutical potential. Furthermore, seasonal fluctuations lead to species-specific maxima during the growing season. In addition, annual fluctuations reflect the influence of environmental conditions and ontogenetic development. Moreover, there is also variability between individuals within species, but this is generally weaker than the interspecific differences. Finally, no significant differences were observed between male and female individuals.

Identifying SAD-rich willow species is crucial to ensure the efficacy and cost-effectiveness of willow extracts, as high concentrations of salicylic alcohol derivatives reduce raw material requirements and contribute positively to the effectiveness. Furthermore, willow leaves are a valuable additional source, as some species can reach high SAD levels during the summer months, thus supplementing the bark. These parameters can significantly increase the efficiency and economy of willow extract production and thus also its popularity.

CRediT authorship contribution statement

Leonie Kayser: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Thomas Olaf Gruber:** Investigation, Formal analysis. **Gregor Aas:** Methodology, Conceptualization. **Guido Jürgenliemk:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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