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Influence of the season on the salicylate and phenolic glycoside contents in the bark of *Salix daphnoides*, *Salix pentandra*, and *Salix purpurea*

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Summary

Due to the benefits of herbal medicine and their wide range of application for human health, the usage of natural drug products, such as willow bark extract, has increased in the last few years. The principle active compounds of the drugs comprised primarily of willow bark are phenolic glycosides like salicylates. Phenolic glycoside profiles of bark vary among species and between the seasons. To identify and preserve willow clones with high salicylate content for possible commercial usage at a later stage, we have screened three *Salix* sp. in respect to their chemical profiles. The willow species analysed were: *Salix daphnoides*, *Salix pentandra*, and *Salix purpurea*. These species had distinct phenolic glycoside profiles. The major salicylate of *S. daphnoides* and *S. purpurea* clones was salicortin, whereas the main compound of *S. pentandra* was 2'-O-acetylsalicortin. According to the chemical profiles of 140 clones, seven independent clones of *S. daphnoides* and *S. purpurea* as well as four clones of *S. pentandra* with high phenolic glycoside contents were picked to study seasonal changes in bark chemistry. Overall, the clones of *S. daphnoides* showed the highest mean salicylate and phenolic glycoside contents, followed by *S. pupurea* and *S. pentandra*. The secondary metabolite content of willow bark clones decreased during the vegetative season from March to June 2007 and further from June to July 2007. Our study revealed that for optimum yield of phenolic glycosides the species, the clone, and the time of harvest during the season have to be taken in consideration.

Introduction

Herbal medicine products are dietary supplements that people take to improve their health. Many herbs have been used for a long time because of their claimed health benefits. In recent years herbal medicine gained more and more interest, even within the scientific community. At the end of the 20th century, many medical studies published proved biotic effects of willow bark extracts. Certain phenolic glycosides of *Salix* bark, particulary salicin and its esters like tremulacin or salicortin, have been shown to relieve rheumatic disturbances, infections, and headache (Biegert et al., 2004; Chrubasik et al., 2000; Chrubasik et al., 2001; Lardos et al., 2004). These glycosides have non-inflammable, temperature-reducing, and pain-alleviating effects. Adverse effects, like a negative influence on the aggregation of the thrombocytes or local lesions of the gastric mucosa caused by the synthetic medicine product acetylsalicylic acid (Aspirin), have not been reported for salicin.

Phenolic glycosides present in bark vary among *Salix* species (Thieme, 1965; Thieme, 1971; Meier et al., 1985a). Commercial extraction of phenolic glycosides from willow bark is only economical when the content reaches a minimum of 1% total salicin, according to the „European Scientific Cooperative on Phytotherapy" (ESCOP). Furthermore, it has to be taken into consideration that the phenolic glycosides content varies with the seasons (Thieme, 1965; Thieme, 1971). To identify and preserve willow clones with high salicylate content for possible recovery of certain phenolic compounds at a later stage, we have screened clones of *Salix daphnoides*, *Salix purpurea*, and *Salix pentandra* in respect to their chemical profiles. A second object of the study was to analyse the seasonal variability of salicylate and phenolic glycoside contents in the *Salix* species to identify the optimum harvest time for secondary metabolites.

Materials and methods

Origin and selection of plant material

Osier stacks of different willow clones were collected in the north-east of Germany and the north-west of Poland in April and May 2006 and stock planted in Zepernick (Berlin). Bark samples of 140 collected clones were taken in December 2006. According to their chemical profiles seven independent clones of *S. daphnoides* (DA7, DA40, DA52, DA56, DA58, DA61, DA64) and *S. purpurea* (PU9, PU10, PU11, PU12, PU15, PU24, PU25) as well as four clones of *S. pentandra* (PE11, PE12, PE13, PE23) with high phenolic glycoside contents were picked for further studies. To determine changes within phenolic glycosides within the beginning vegetation period, willow bark for chemical analysis was collected in March, June, and July 2007.

Extraction procedure and analysis

The peeled bark was lyophilised in a Christ Alpha 1-4 at exerted pressure of 0.034 bars for two days up to 20°C. After milling of probes (Retsch MM 301, total samples 1-2 g) for 1.5 min at 20000 Hz the phenolic glycoside samples, 50 mg each, were extracted with 750 µl methanol. 100 µl of standard solution (resorcinol) was added to the first extraction. After conditioning the probes for 10 min in an ultrasonic bath (Bandelin SONOREX SUPER RK 513) and centrifugation for 5 min and 6000 rpm (Eppendorf Centrifuge 5416) the supernatant was collected. The pellet was used for reextraction with methanol (500 µl, 80 %) twice more. The combined supernatants were concentrated in a vacuum concentrator (Speed Vac SC110, Savant Instruments) until a volume of 150 µl was remaining. The extracts were filled up to 1 ml with ultra pure water (MilliQ quality) and filtered by using Spin-X tubes (Corning, The Netherlands). The obtained extracts were analysed by HPLC.

10 µl of willow bark extracts were injected in a Dionex P680A HPLC system equipped with an ASI-100 auto sampler and a PDA-100 photodiode array detector. Phenolic glycosides were separated on a narrow bore column (Acclaim Polar Advantage C16: 3 µm, 120 Å, 2.1 x 150 mm, Dionex). Eluents used for HPLC analysis were: A) 2% tetrahydrofuran and 0.5% phosphoric acid and B) 100% methanol. The eluent programme was: 0% B (0-5 min), 15% B (10 min), 25% B (20 min), 35% B (30 min), 50% B (35 min), 100% B (40-42 min) and 0% B (44-49 min). The flow rate was 0.35 ml/min and the eluent was detected at 270 nm. Qualitative identification of the phenolic glycosides was carried out by using standards and via specific UV-spectra (Shao, 1991). The quantitative analysis based on peak area

relative to the standard resorcinol by using respective response factors. For peak evaluation the software Chromeleon Version 6.0 was used. Data were analysed for significant differences by using analysis of variance following the mean comparison test Tukey's HSD for honest significant difference with SYSTAT 12.0.

Results and discussion

The chemical compounds identified in willow species were the salicylates salicin, salicortin, 2'-O-acetylsalicin, 2'-O-acetylsalicortin, and tremulacin and the other phenolic glycosides syrengin, picein, catechin, ampelopsin, vimalin, purpurein, and naringenin-5-glycoside. Furthermore, a not-yet identified salicin derivate (elution at circa 35 min) and an unknown phenolic glycoside (elution at about 18 min) could be detected.

Clones of *S. daphnoides* showed the highest mean salicylate and phenolic glycoside content, followed by *S. pupurea* and *S. pentandra*. Every *Salix* species had a characteristic phenolic glycoside profile. The major salicylate present in *S. daphnoides* and *S. purpurea* was found to be salicortin, whereas the main compound of *S. pentandra* was 2'-O-acetylsalicortin. Type-chromatograms for clones of the three analysed willow species are given in Fig. 1-3.

Initial total phenolic glycoside content determined in March for *S. daphnoides* was 8.24%, for *S. purpurea* 5.67%, and 3.25% for *S. pentandra*. The clone variability of secondary metabolites in willow species was found to be different with highest variability in *S. daphnoides*, followed by *S. purpurea* and *S. pentandra* (Tab. 1). The secondary metabolites generally decreased from March to June 2007 and further from June to July 2007, whereby the the total phenolic glycosides decreased significantly in all species already from March to June (Fig. 4). Furthermore, the salicylate content of all species was significantly lower in July compared to March 2007. Significant differences were found more frequently between March and June than between June and July. The salicylate and phenolic glycoside contents in clones of *S. daphnoides*, *S. pentandra*, and *S. purpurea* decreased about 30% from March to June (Tab. 1). The salicylate contents of *S. daphnoides* and *S. purpurea* decreased further from 4.71% and 3.27%, respectively around 18% from June to July. Also from June to July, the total phenolic glycoside contents decreased in *S. daphnoides* and *S. purpurea* around 22% and 14% from initially 5.14% and 4.07%. An increase of 8% from 2.15% phenolic glycosides content was detected in *S. pentandra* from June to July. But the salicylic content remained unchanged in these months in *S. pentandra*. In general it could be seen that the secondary metabolite contents decreased from March to June with more intensity than from June to July.

Our data collected for the seasonal variability are proof of the results reported by MEIER et al. (1985a, 1985b). They found that the salicylate and phenolic glycoside content decreased from winter to summer. Furthermore, our results show the same trend as documented by THIEME (1965, 1971). He found that the maximum content of phenolic

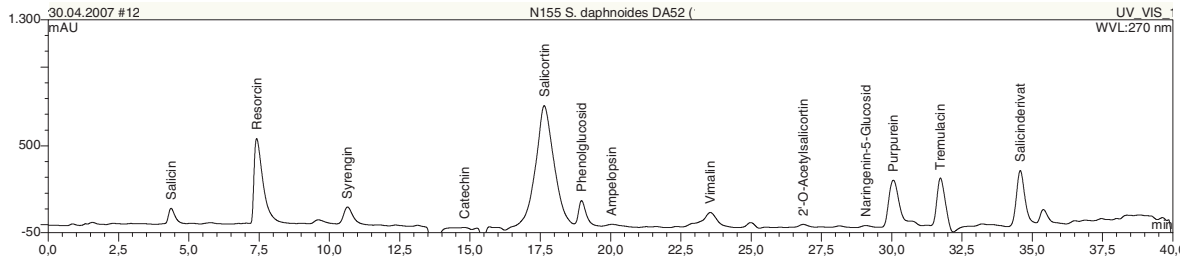

Fig. 1: Chromatogram of *Salix daphnoides* bark (clone 52, March 2007)

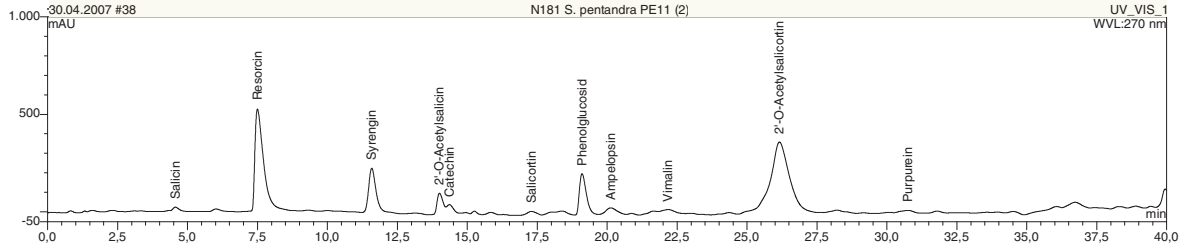

Fig. 2: Chromatogram of *Salix pentandra* bark (clone 11, March 2007)

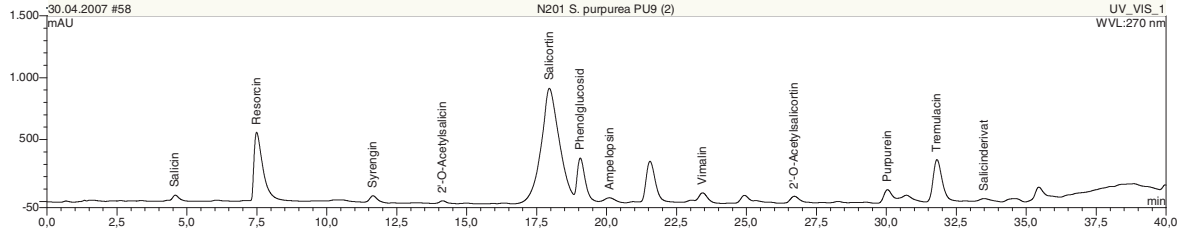

Fig. 3: Chromatogram of *Salix purpurea* bark (clone 9, March 2007)

Tab. 1: Salicylate (SC) and phenolic glycoside (PGC) contents in *Salix daphnoides*, *Salix pentandra*, and *Salix purpurea* bark in March, June, and July

	Salicylate and phenolic glycoside content [%]					
	Salix daphnoides		*Salix pentandra*		*Salix purpurea*	
	SC	PGC	SC	PGC	SC	PGC
March	6.41 ± 1.71	8.24 ± 1.96	2.41 ± 0.27	3.25 ± 0.14	4.69 ± 0.66	5.67 ± 0.68
June	4.71 ± 1.31	5.14 ± 1.48	1.84 ± 0.27	2.15 ± 0.35	3.27 ± 0.74	4.07 ± 0.77
July	3.85 ± 0.68	4.01 ± 0.84	1.80 ± 0.16	2.32 ± 0.18	2.67 ± 0.53	3.52 ± 0.49

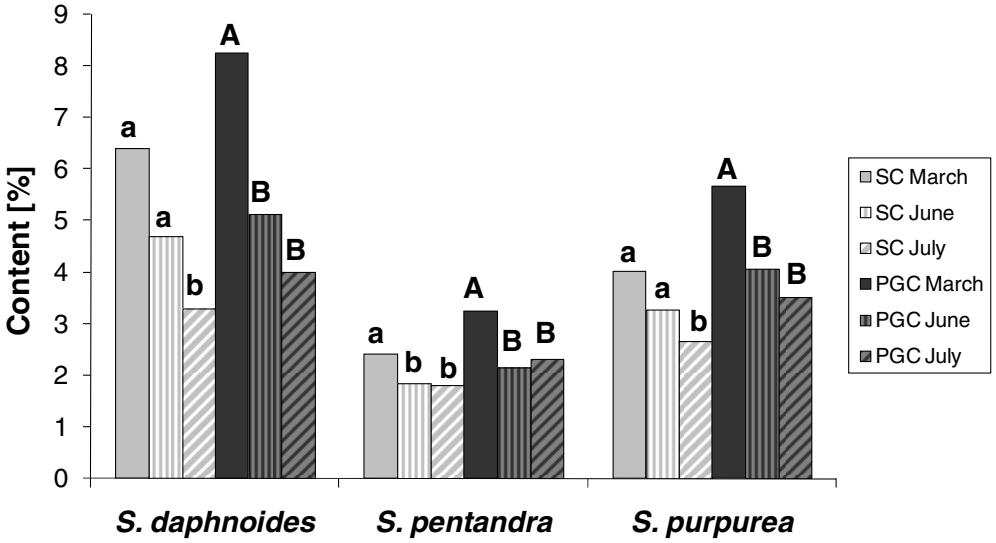

Fig. 4: Seasonal variability of salicylates and phenolic glycosides in the bark of willow species (Different small letters indicate seasonal significant differences of salicylate contents within the species and large letters indicate differences within levels of phenolic glycosides, Tukey's HSD test, $p < 0.05$; salicylate: SC and phenolic glycoside: PGC).

glycosides could be detected during spring, in March. The content of phenolic glycosides decreased from spring over summer to come to a minimum in September/October. In autumn and winter, an accumulation of these compounds was found by THIEME (1965, 1971). Further research in our institute about the seasonality of phenolic glycoside contents of willow bark in autumn and winter is ongoing.

Several compounds in the three willow species restrained differently in their seasonal development. From March to June most of the compounds in *S. daphnoides* decreased, especially purpurein, tremulacin, ampelopsin, catechin, syringin, salicin, 2'-O-acetylsalicortin. In some clones, a few compounds like tremulacin, catechin or purpurein could not be detected anymore. Naringenin-5-glycoside showed an increase from March to June but a decrease from June to July. Another decreasing compound was salicortin. Syrengin, ampelopsin, purpurein, tremulacin, and 2'-O-acetylsalicortin increased from March to July. In *S. pentandra* from March to June decreasing concentrations of purpurein, syrengin, 2'-O-acetylsalicin, 2'-O-acetylsalicortin, as well as vimalin and increasing concentrations of salicin, picein, and salicortin could be detected. From June to July some compounds like 2'-O-acetylsalicortin, syrengin, vimalin, purpurein increased, and some compounds like picein, salicortin, and ampelopsin decreased. In *S. purpurea* the content of syrengin, picein, 2'-O-acetylsalicin, ampelopsin, vimalin, purpurein, and tremulacin declined from March to June, whereas the content of catechin and 2'-O-acetylsalicortin increased. From June to July decreasing concentrations of 2'-O-acetylsalicortin, purpurein, and salicortin and increasing concentrations of vimalin and tremulacin could be found. A general, clear trend could not be found. Only some compounds like syrengin, 2'-O-acetylsalicin, ampelopsin, vimalin, purpurein, and tremulacin decreased in all three willow species from March to June. From June to July an increasing content of syrengin, vimalin, and tremulacin and a decreasing content of salicortin could be detected.

The production of willow bark extracts is very expensive. Therefore, the use of willow species and clones with highest amounts of salicylates is obligatory. Furthermore, we had to select individual clones from which maximum harvests per hectare can be gained, e. g. with fast growth performance (RIIPI et al., 2002). Besides the bark, other plant organs of willow, like the leaves contain phenolic glycosides (JULKUNEN-TIITTO, 1992; THIEME 1965). Usage of these parts for phenolic glycoside production could be also considered. There are other factors which influence secondary metabolite content in willow bark, such as the species, the clone (JULKUNEN-TIITTO, 1992), the climate (MEIER et al., 1985b; JULKUNEN-TIITTO, 1985), the nutrient supply of the plant (Hakulinen, 1998; Julkunen-Tiitto et al., 1993), the physiological age (JULKUNEN-TIITTO, 1992; THIEME, 1965;

THIEME, 1971; SULIMA et al., 2006), the time of day (Thieme, 1971), and the gender (Thieme, 1965). All parameters need to be considered for effective commercial recovery of phenolic glycosides.

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References

BIEGERT, C., WAGNER, I., LÜDTKE, R., KÖTTER, I., LOHMÜLLER, C., GÜNAYDIN, I., TAXIS, K., HEIDE, L., 2004: Efficacy and Safety of Willow Bark Extract in the Treatment of Osteoarthritis and Rheumatoid Arthritis: Results of 2 Randomized Double-Blind Controlled Trials. The Journal of Rheumatology 31, 2121-2130.

CHRUBASIK, S., EISENBERG, E., BALAN, E., WEINBERGER, T., LUZZATI, R., CONRADT, C., 2000: Treatment of Low Back Pain Exacerbations with Willow Bark Extract: A Randomized Double- Blind Study. The American Journal of Medicine 109, 9-14.

CHRUBASIK, S., KÜNZEL, O., BLACK, A., CONRADT, C., KERSCHBAUMER, F., 2001: Potential economic impact of using a proprietary willow bark extract in outpatient treatment of low back pain: An open non-randomized study. Phytomedicine 8, 241-251.

HAKULINEN, J., 1998: Nitrogen-induced reduction in leaf phenolic level is not accompanied by increased rust frequency in a compatible willow (*Salix myrsinifolia*)- *Melampsora* rust interaction. Physilogia Plantarium 102, 101-110.

JULKUNEN-TIITTO, R., 1992: Analyse und Verteilung phenolischer Glycoside (Salicinderivate) in Weiden (*Salicaceae*). Die Holzzucht 46, 2-5.

JULKUNEN-TIITTO, R., 1985: Chemotaxonomical screening of phenolic glycosides in northern willow twigs by capillary gas chromatography. Journal of Chromatography 324, 129-139.

JULKUNEN-TIITTO, R., TAHVANAINEN J., SILVOLA J., 1993: Increased CO_2 and nutrient status changes affect phytomass and the production of plant defensive secondary chemicals in *Salix myrsinifolia* (Salisb.). Oecologia 95, 495-498.

LARDOS, A., SCHMIDLIN, C.B., FISCHER, M., FERLAS-CHLODNY, E., LONIEWSKI, I., SAMOCHOWIEC, L., MUSIAL, H.D., 2004: Wirksamkeit und Verträglichkeit eines wässrig ausgezogenen Weidenrindenextraktes bei Patienten mit Hüft- und Kniearthrose. Zeitschrift für Phytotherapie 25, 275-281.

MEIER, B., LEHMANN, D., STICHER, O., BETTSCHART, A., 1985a: Identifikation und Bestimmung von je acht Phenolglykosiden in *Salix purpurea* und *Salix daphnoides* mit moderner HPLC. Pharmaceutica Acta Helvetiae 60, 269-275.

MEIER, B., STICHER, O., BETTSCHART, A., 1985b: Weidenrinden-Qualität. Gesamtsalicinbestimmung in Weidenrinden und Weidenpräparaten mit HPLC. Deutsche Apotheker Zeitung 125, 341-347.

RIIPI, M., OSSIPOV, V., LEMPA, K., HAUKIOJA, E., KORICHEVA, J., OSSIPOVA, S., PIHLAJA, K., 2002: Seasonal changes in birch leaf chemistry: Are there Trade-offs between leaf growth and accumulation of phenolics? Oecologia 130, 380-390.

SHAO, Y., 1991: Phytochemischer Atlas der Schweizer Weiden. Zürich 1991, DISS. ETH NR. 9532.

SULIMA, P., PRZYBOROWSKI, J.A., WIWART, M., 2006: Willow bark – herbal raw material harvested from plants cultivated on arable lands. Herba Polonica 52 18-25.

THIEME, H., 1965: Die Phenolgykoside der Salicaceen 5. Mitteilung: Untersuchungen über die Gykosidspektren und den Glykosidgehalt der mitteldeutschen Salixarten. Pharmazie 20, 570-574.

THIEME, H., 1971: Vorkommen und Verbreitung von Phenolglucosiden in der Familie der Salicaceen. Herba Polonica 17, 248-257.

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