EKSPERIMENTINIAI TYRIMAI

Application of high-performance liquid chromatography for research of salicin in bark of different varieties of *Salix*

Palmyra Kenstavičienė, Palma Nenortienė[1], Guoda Kiliuvienė[1], Andrejus Ževžikovas[1], Audronis Lukošius[2], Daiva Kazlauskienė[1]

Department of Botany, Vilnius Pedagogical University,
[1]Department of Analytical and Toxicological Chemistry, Kaunas University of Medicine,
[2]Department of Pharmacognosy, Kaunas University of Medicine, Lithuania

Key words: *willow bark; salicin; high-performance liquid chromatography.*

Summary. *Willow (Salix L.) species are widely spread in Lithuanian natural dendroflora. Willow bark contains active substances known for anti-inflammatory properties and is known as a phytotherapeutic precursor of aspirin. Bark extracts are components of analgesic and anti-rheumatic preparations. Therapeutic effectiveness is associated with salicin (2-(hydroxy-methyl)phenyl-β-D-glucopyranoside), which turns into salicylic acid. Increasing attention to natural preparations gives primary importance to research of plants. This study focused on 12 willow taxa and employed routine pharmacopoeia methods. High-performance liquid chromatography method was applied for the analysis of bark extractions. The investigation revealed that not all willow species accumulated a therapeutically sufficient amount of salicin. Bark samples were investigated after 1- and 2-year growth in autumn and spring. Salicin content ranged from 0.08 to 12.6%. Higher contents of active materials were determined in autumn and in 2-year-old willows. Certain willow taxa (Salix alba L., Salix mollissima L., Salix triandra L., Salix viminalis "Americana", Salix dasyclados L.) possessed extremely low salicin amounts.*

In the second year, analysis covered 32 willow species. Results indicated striking differences in salicin amounts (from 0.04% in Salix viminalis "Americana" to 12.06% in Salix acutifolia).

Willow species, plant age, and season should be considered when collecting medicinal plant material. The amount of salicylates in 2-year-old willow bark collected in autumn exceeded by 25% that in 1-year-old willow bark collected in spring. Bark of some analyzed willow species contained the amount of salicylates too low for using as anti-inflammatory or antipyretic remedy.

Introduction

Willow is a deciduous shrub native to Britain, central and southern Europe (1), Asia, and North America (2). The material of commerce comes mainly from southeastern European countries, including Bulgaria, Hungary, Romania, the former Yugoslavia (2, 3).

There are approximately 400 species worldwide, including 150 in the western hemisphere. *Salix* species are chiefly located in North Temperate and Arctic regions, growing primarily in moist habitats, especially along stream banks. The *Salicaceae* (includes 2 genera, *Salix* and *Populus)* are differentiated from other catkin-bearing families by the presence of only one bract subtending each flower. Members of willow species are difficult to conclusively identify due to high variability and hybridization among the genera. Important diagnostic characters include habitat; leaf position, shape, margin, and pubescence; the timing of catkin emergence; bract morphology; number of stamens per flower; shape and pubescence of the ovary (4–8).

Some of the modern therapeutic applications for willow bark originate from its early uses in ancient Greek medicine, which were first reported by Dioscorides in his herbal *De Materia Medica* written in the first century CE (9). During the Middle Ages, willow bark was used in Europe to reduce fever and relieve pain. Anti-inflammatory and fever-reducing actions of willow are attributed to salicylates, contained in the bark. These glycosidic constituents were first

Correspondence to P. Nenortienė, Department of Analytical and Toxicological Chemistry, Kaunas University of Medicine, A. Mickevičiaus 9, 44307 Kaunas, Lithuania. E-mail: npalma@takas.lt

isolated by a French chemist in 1829 and were synthesized six years later (10). Salicylic acid, first isolated from meadowsweet leaf (*Filipendula ulmaria;* syn. *Spiraea ulmaria*), is the phytotherapeutic precursor of acetylsalicylic acid, the analgesic drug commonly known as aspirin (2, 11, 12).

Willow bark comprises the bark of young two- to three-year-old branches of *S. alba* L., *S. purpurea* L., *S. fragilis* L., and other *Salix* species (*Salicaceae*) harvested during early spring and their preparations in effective dosage. The bark contains at least 1% of total salicin derivatives, calculated as salicin relative to the dried herb. As the Commission E allows for several different *Salix* species, the quantities of salicin may vary significantly depending on the species used (13–15).

The *British Herbal Compendium* indicates the use of willow bark for rheumatic and arthritic conditions and feverish conditions such as common cold or influenza (11). ESCOP indicates its application for treatment of feverish conditions, symptomatic treatment of mild rheumatic complaints, and relief of headache (16). In France, willow bark preparations are allowed for use as an analgesic to treat headache and toothache as well as painful articular conditions, tendinitis, and sprains (11, 17).

The main objective of this study was to investigate characteristics of the willow bark collected from different *Salix* species growing in Lithuania. The assay of salicin in the willow bark was done by high-performance liquid chromatography (HPLC) method according to the European Pharmacopoeia.

Materials and methods

Chemicals and reagents

Salicin, resorcinol, and picein standards were purchased from Sigma (St. Louis, MO, USA). The standards were stored in the room protected from light, at room temperature (20˚C). HPLC-grade tetrahydrofuran and phosphoric acid were obtained from Roth GmbH (Germany). Sodium hydroxide and hydrochloric acid were purchased from Fluka. Purified water was generated by a Milli-Q reagent water system (Millipore Corporation, 106 Bedford, MA).

The samples of willow bark were collected in Miroslavas region, Alytus. Internal standard solution was prepared by dissolving 50 mg of resorcinol in 10.0 mL of methanol.

Reference solution (a) was prepared by dissolving 18.5 mg of salicin in 10.0 mL of the mixture containing 20 volumes of water, 80 volumes of methanol and added 1.0 mL of the internal standard solution.

Reference solution (b) was prepared by dissolving 1.0 mg of picein R in 1.0 mL of reference solution (a).

Test solution preparation was prepared as follows: 50 mL of methanol were added to 0.5 g of the powdered bark, and this mixture was heated under a reflux condenser for 30 min, then cooled, and filtered. The residues were taken up with 50 mL of methanol and proceeded as above. The filtrates were combined and evaporated under reduced pressure. The residues were taken up with 5.0 mL of methanol; 5.0 mL of 0.1 M sodium hydroxide were added and then heated in a water-bath at 60˚C under a reflux condenser, with frequent shaking for about 1 h. After cooling, 0.5 mL of 1 M hydrochloric acid was poured. The solution was diluted to 20.0 mL with the mixture containing 50 volumes of methanol and 50 volumes of water. Then 1.0 mL of the internal standard solution was added to 10.0 mL of this solution and filtered through a membrane filter.

Instrumentation

The HPLC system consisted of separation module Alliance 2609 (Waters) and UV detector Waters 2487 set at 270 nm. A stainless steel column Hypersil H5ODS (150×4.6 mm) was used for separation of components. Mixture containing 1.8 volumes of tetrahydrofuran and 98.2 volumes of water, with 0.5% of phosphoric acid, was used as a mobile phase at a flow rate of 1.0 mL/min. Reference and test solutions (each 10 μL) were injected into the column. The percentage content of total salicylic derivatives, expressed as salicin, was calculated from the expression:

$$\% = \frac{S_1 \times S_4 \times m_2 \times p \times 2}{S_2 \times S_3 \times m_1},$$

where S_1 indicates area of the peak due to salicin in the chromatogram obtained with the test solution; S_2, area of the peak due to resorcinol in the chromatogram obtained with the test solution; S_3, area of the peak due to salicin in the chromatogram obtained with reference solution (a); S_4, area of the peak due to resorcinol in the chromatogram obtained with reference solution (a); m_1, mass of the bark in the test solution in mg; m_2, mass of salicin in reference solution (a) in mg; p, percentage content of salicin in the reference substance.

Results

Fig. 1 shows a typical chromatogram obtained from reference solutions. Salicin (retention time, tr=5.3±0.011 min; n=10), picein (tr=7.4±0.018 min; n=10), and resorcinol (tr=11.9±0.05 min; n=10)

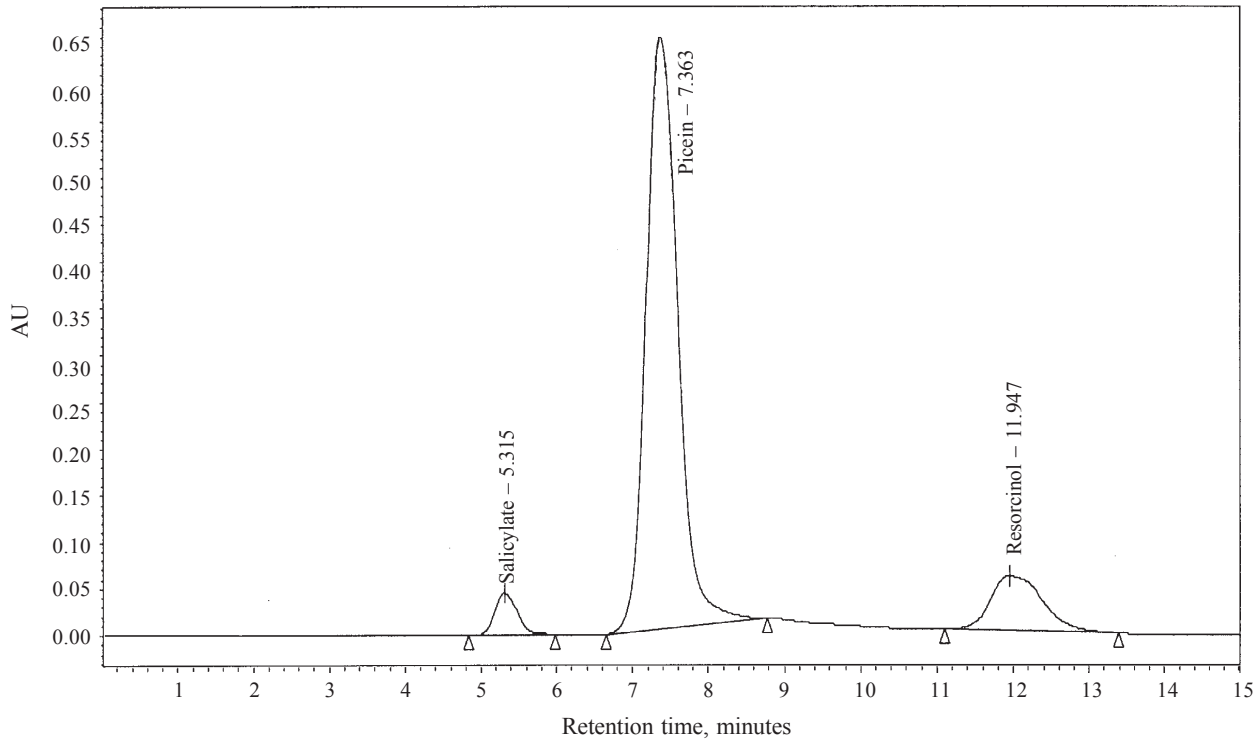

Fig. 1. **Typical chromatogram of the reference solutions**

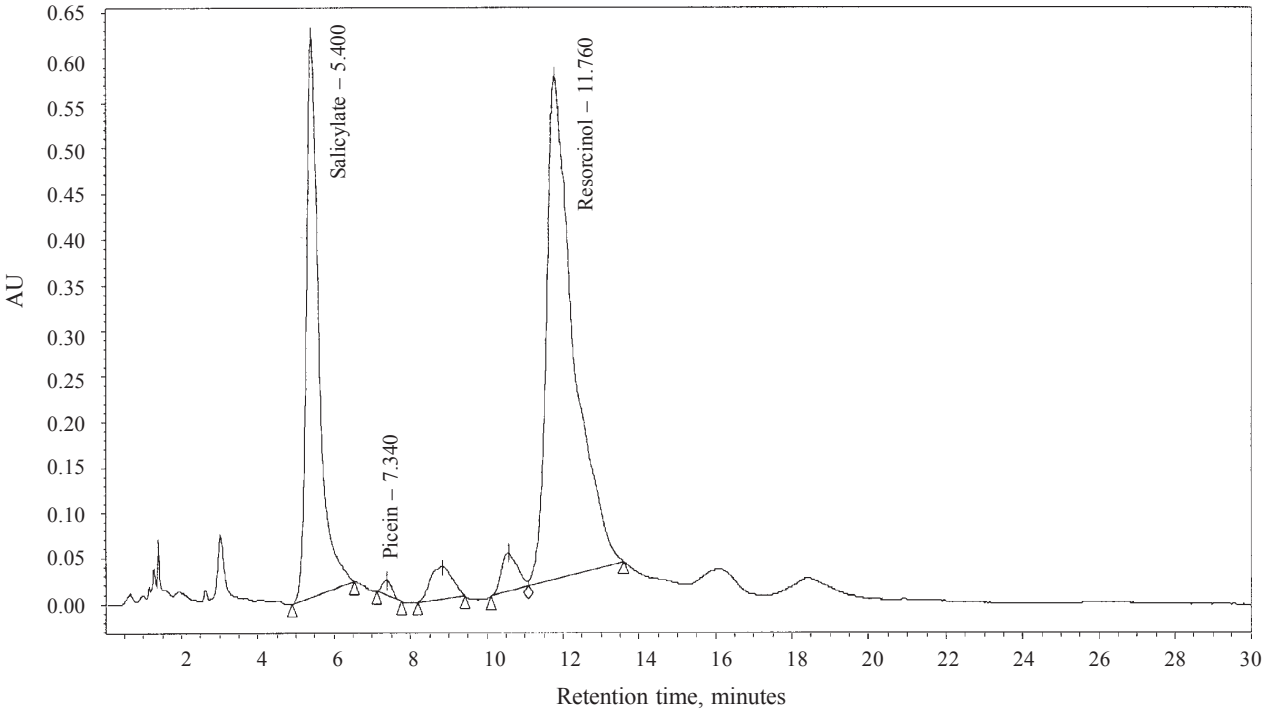

Fig. 2. **Typical chromatogram of the test solutions**

exhibited well-separated, narrow, and symmetrical peaks under the chromatographic conditions described.

Fig. 2 shows typical chromatogram obtained from test solutions.

The percentage amount of the salicylic derivatives expressed as salicin in different species of *Salix* genus is presented in Tables 1 and 2.

The amount of salicylic derivatives in the bark of willow depends on time of collection and age of the plant. The results are presented in Fig. 3–5.

Table 1. **Quantity of salicylic derivatives, calculated as salicin (%)**

Name of the plant	1-year-old growths, autumn	2-year-old growths, autumn	2-year-old growths, spring
(Salix integra × Salix kochiana)	8.09±0.4	9.48±0.47	5.92±0.17
Salix acutifolia L.	6.95±0.34	12.06±0.61	10.83±0.72
Salix daphnoides L.	10.19±0.56	11.92±0.6	9.50±0.49
Salix caspica L.	5.64±0.19	6.71±0.23	4.68±0.12
Salix rubra L.	2.11±0.06	2.88±0.07	0.95±0.04
Salix rigida L.	3.07±0.09	5.34±0.1	1.88±0.05
Salix alba L.	1.29±0.03	1.87±0.04	1.71±0.03
Salix purpurea f. "*Purpurea*"	7.16±0.35	7.77±0.41	6.36±0.27
Salix mollissima L.	0.29±0.01	0.84±0.01	0.29±0.01
Salix triandra L.	0.76±0.03	0.93±0.04	0.73±0.03
Salix viminalis "*Americana*"	0.08±0.01	0.98±0.03	0.42±0.02
Salix dasyclados L.	0.32±0.01	0.39±0.01	0.17±0.01

Table 2. **Quantity of salicylic derivatives, calculated as salicin (2-year-old growths, autumn)**

Number	Name of the plant	Quantity of salicin (%)
1.	*Salix caspica*	3.87305
2.	*Salix alba* straightforward stem form	1.3731
3.	*Salix mollissima*	0.25584
4.	*Salix schwerinii × Salix dasyclados*	0.65067
5.	*Salix viminalis* "*Americana*"	0.03817
6.	*Salix viminalis* cl. 9822	0.13351
7.	*Salix viminalis* cl. 9817	0.8377
8.	*Salix viminalis* "*Americana*"	0.19693
9.	*Salix alba* ssp. *alba* cl. 04115	1.2168
10.	*Salix viminalis* cl. 04116	0.31419
11.	*Salix viminalis* "*Americana*" cl. 9976	0.10439
12.	*Salix dasyclados × S. viminalis* cl. 04120	0.26436
13.	*Salix dasyclados* cl. 9977	0.14988
14.	*Salix dasyclados* cl. 04122	0.16962
15.	*Salix dasyclados* cl. 04124	0.36989
16.	*Salix daphnoides* f. *latifolia* cl. 9904	2.56015
17.	*Salix purpurea* "Lutea" cl. 9731	6.53091
18.	*Salix purpurea × Salix triandra* ssp. *amygdalina* cl. 04131	8.35459
19.	*Salix purpurea* cl. 04132	10.0545
20.	*Salix tenuifolia (rubra)* cl. 04133	1.58693
21.	*Salix triandra × Salix purpurea* cl. 04134	1.66764
22.	*Salix dasyclados* "*Gudrun*" cl. 04135	0.54189
23.	*Salix viminalis* "*Tordis*" cl. 04136	0.05892
24.	*Salix mollissima* cl. 9868	0.07651
25.	*Salix purpurea × Salix viminalis* cl. 9714	4.61521
26.	*Salix purpurea × Salix viminalis* cl. 04141	7.26769
27.	*Salix purpurea* "*Lutea*" cl. 9727	5.95857
28.	*Salix purpurea* "*Rubra*" cl. 04130	2.58514
29.	*Salix schwerinii* "*Tora*" cl. 04137	1.109
30.	*Salix fragilis × Salix alba*	2.06709
31.	*Salix viminalis* cl. 0109	0.05716
32.	*Salix dasylados* cl. 099	0.49788

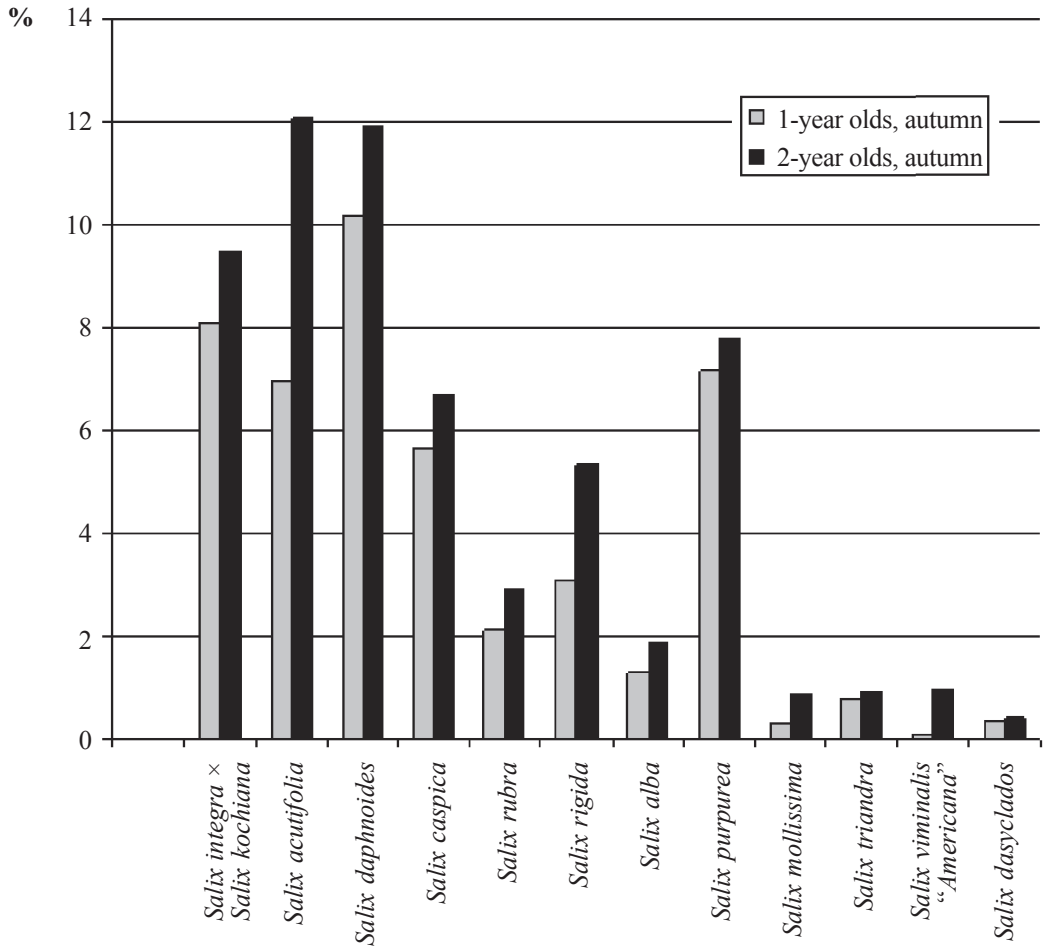

Fig. 3. **Dependence of the salicylic derivatives quantity on the age of the plant**

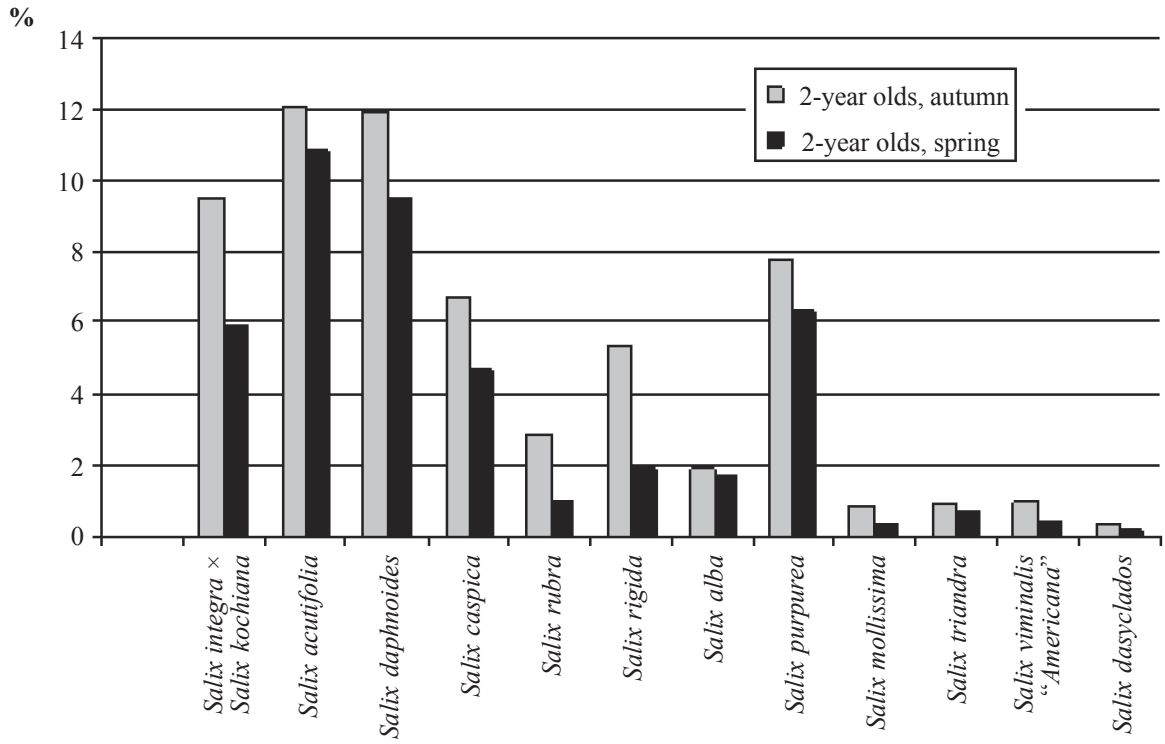

Fig. 4. **Dependence of the salicylic derivatives quantity on the time of collection**

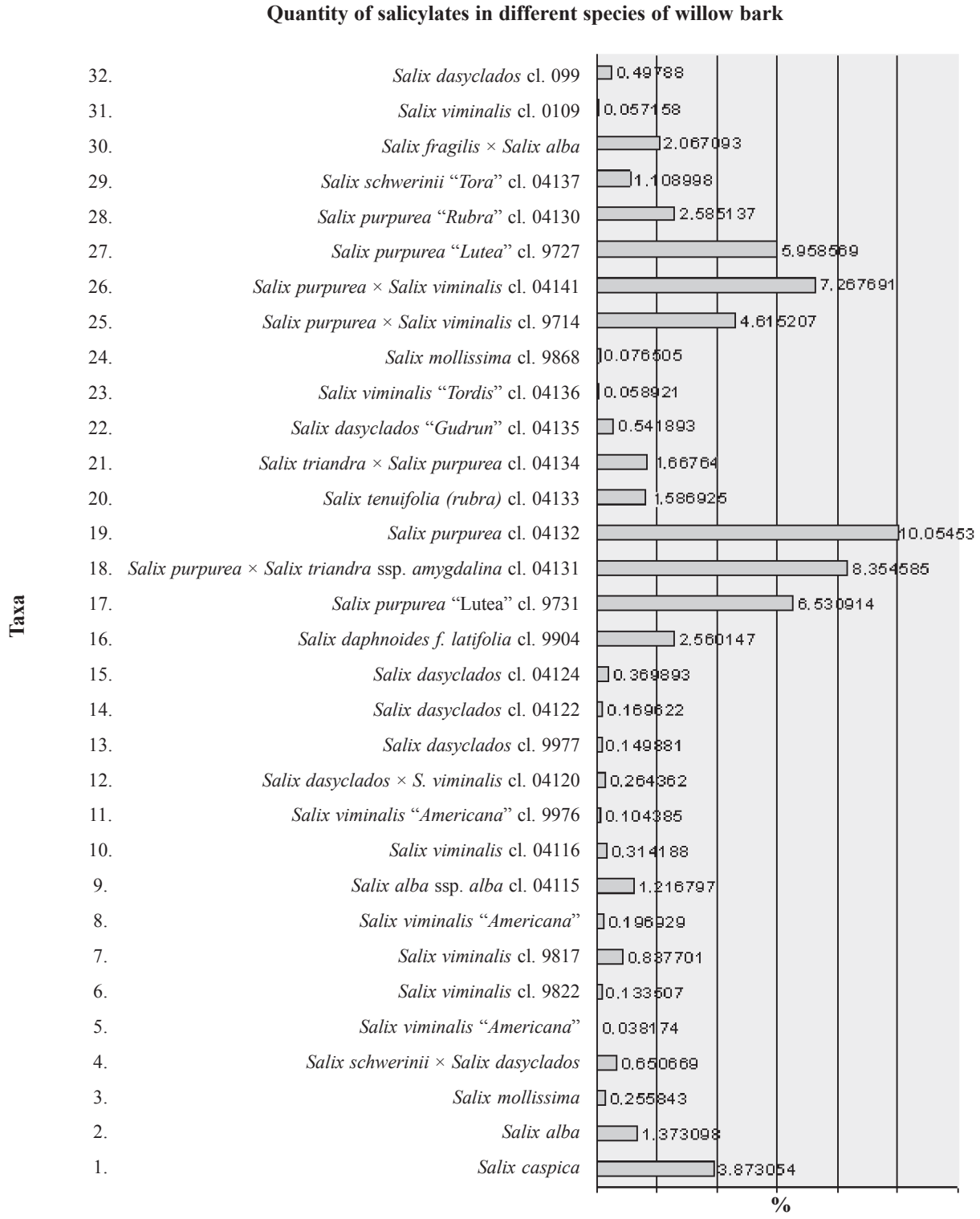

Fig. 5. **Variation of the amount of salicylates among different *Salix* species**

Discussion

Willow bark is a perspective herbal remedy, which possesses a lot of therapical effects, such as antipyretic, anti-inflammatory, analgesic, antineoplastic, and antifungal. As the effect is based on salicin content, this investigation was conducted in order to compare the amounts of salicin in different species of *Salix*. The results of this study show striking differences in amounts of salicylic derivatives among different *Salix* species (Fig. 3–5). According to the European Pharmacopoeia, minimal amount of 1.5% of salicin is

required in willow bark. Considering this requirement, it is possible to divide species analyzed in this study to the ones that can be used in preparation of qualitative herbal remedies and to the ones, inappropriate for medical usage. *Salix integra* × *Salix kochiana* (5.92%–9.48%), *Salix acutifolia* (6.95%–12.06%), *Salix daphnoides* (9.50%–11.92%), *Salix caspica* (3.87%–6.71 %), *Salix rigida* (1.88%–5.34%), *Salix tenuifolia* (10.05%), all analyzed clones of *Salix purpurea* and its hybrids with other species (2.56%–10.05%) contain high amounts of salicin (Tables 1 and 2). According to the results of this study, medical usage of the species mentioned above is recommended. However, many species were noticed to possess very low amounts of salicin: *Salix dasyclados* (0.15%–0.39%), *Salix mollisima* (0.25%–0.84%), *Salix triandra* (0.73%–0.98%), and all clones of *Salix viminalis* (0.038%–0.98%). Hybrids of *Salix viminalis* (the lowest amount of salicin) and *Salix purpurea* (the highest amount of salicin) possess the required amount of salicin (4.61%–7.26%).

The first step of the study focused on three samples of each plant, collected at different growing period (first-year autumn, second-year autumn, and second-year spring). Comparison of the quantities of the salicylic derivatives, depending on age of the plant (Fig. 4) and time of collection (Fig. 5), showed that two-year-old plants and the ones collected in the autumn accumulated the greatest amounts of active substances (approximately a 25.0% increase) when compared with one-year-old plants and the material collected in spring season. These findings agree with data reported by other authors (13–15).

Salix alba is a common species in Lithuania, and it is described in foreign literature as herbal remedy as well. According to this study, it possessed only 1.29% of salicin in the spring of the first year, but in autumn of the second year, it accumulated an appropriate amount of salicin (1.87%). Therefore, time of collection of willow bark is important to consider. The amount of 1.87% is not very high, and according to literature, it would be necessary to use 35 g of white willow bark to achieve the effect of one tablet of aspirin. Such high amount of bark can be toxic due to effect of tannins. The usual dosage is 1–3 g of willow bark. In this case, the effect of herbal remedies cannot be based on the effect of salicin only. It is proposed to evaluate other components (tannins, flavonoids), which can contribute to overall effect.

Conclusions

1. The results of this study show striking differences in amount of salicylic derivatives among different *Salix* species from 0.04% (*Salix viminalis* "*Americana*") to 12.06% (*Salix acutifolia*).

2. The amount of salicin in the bark of some analyzed species is lower than it is required according to the European Pharmacopoeia (1.5%). These species are not recommended to be used for treatment due to very low amount of salicin.

3. It is important to consider season of the year and age of the plant during collection of willow bark. The comparison of the quantities of the salicylic derivatives depending on age of the plant showed that two-year-old plants and the ones collected in the autumn accumulated the greatest amounts of active substances (approximately a 25.0% increase) when compared with one-year-old plants and the material collected in the spring.

4. Further studies are required to evaluate amounts of other compounds (flavonoids, tannins, or salicin esters), which may contribute to the overall effect while salicylate levels are too low to explain therapeutic activity.

Acknowledgments

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Efektyviosios skysčių chromatografijos metodo taikymas salicinui tirti įvairių rūšių gluosnių žievėje

Palmyra Kenstavičienė, Palma Nenortienė[1], Guoda Kiliuvienė[1], Andrejus Ževžikovas[1], Audronis Lukošius[2], Daiva Kazlauskienė[1]

Vilniaus pedagoginio universiteto Botanikos katedra,

Kauno medicinos universiteto [1]Analizinės ir toksikologinės chemijos katedra, [2]Farmakognozijos katedra

Raktažodžiai: gluosnio žievė, salicinas, efektyvioji skysčių chromatografija.

Santrauka. Natūralioje Lietuvos floroje gluosnių (*Salix* L.) rūšys yra plačiai paplitusios. Jų žievėje yra aktyviųjų medžiagų, kurių priešuždegiminis veikimas žinomas nuo senų laikų. Gluosnių žievė yra fitoterapinis

aspirino pirmtakas. Gluosnių žievės ekstraktai įeina į priešreumatinių ir skausmą malšinamųjų preparatų sudėtį. Šis gydomasis poveikis yra susijęs su salicinu (2-(hidroksimetil)fenil-β-D-gliukopiranozidu), kuris organizme virsta salicilo rūgštimi. 21 a. vis didesnis dėmesys skiriamas natūraliems, ne sintetiniams preparatams. Taigi, augalų, pasižyminčių gydomosiomis savybėmis, tyrimai įgyja didelę reikšmę. Tyrimo metu išanalizuota 12 gluosnių taksonų. Tyrimai atlikti naudojant farmakopėjinį efektyviosios skysčių chromatografijos analizės metodą. Žievės ekstraktai buvo tiriami efektyviosios skysčių chromatografijos metodu. Tyrimų duomenimis, ne visi gluosnių taksonai sukaupia pakankamą salicino kiekį. Ištirti vienerių ir dvejų metų pavasarį ir rudenį rinkti žievės pavyzdžiai. Salicino kiekis pavyzdžiuose kito nuo 0,08 iki 12,06 proc. Didesni aktyviųjų medžiagų kiekiai rasti rudenį dvejų metų gluosnių žievės pavyzdžiuose. Kai kurių gluosnių taksonų pavyzdžiuose rastas labai mažas salicino kiekis nepriklausomai nuo augalų amžiaus.

Antraisiais metais buvo analizuojami 32 gluosnių taksonai. Visi pavyzdžiai buvo rinkti rudenį, nuo dvejų metų gluosnių žievės. Tyrimo duomenimis, tarp skirtingų gluosnių taksonų yra didžiuliai salicino kiekio skirtumai – nuo 0,04 iki 12,06 proc.

Renkant vaistinę augalinę žaliavą, būtina atsižvelgti į gluosnio taksoną, augalo amžių ir metų laiką. Rudenį rinktų dvimečių augalų žievėje salicilatų kiekis yra apie 25 proc. didesnis nei vienerių metų gluosnių žievėje, rinktoje pavasarį. Kai kurių gluosnių taksonų žievėje salicilatų kiekis toks mažas, kad ji netinkama vartoti gydymui kaip priešuždegiminė ir antipiretinė priemonė.

Adresas susirašinėti: P. Nenortienė, KMU Analizinės ir toksikologinės chemijos katedra, A. Mickevičiaus 9, 44307 Kaunas. El. paštas: npalma@takas.lt

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