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SALICYLATES AMOUNT VARIATION IN DIFERENT SPECIES OF LITHUANIAN WILLOW

Master thesis

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CONTENT

INTRODUCTION

Willow is a deciduous shrub native to Britain, central and southern Europe, Asia, and North America. (3) The material of commerce comes mainly from southeastern European countries, including Bulgaria, Hungary, Romania, the former Yugoslavia. (4)

There is 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 1 bract subtending each flower. Members of willow are difficult to conclusively identify due to high variability and much hybridization among the genus. 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. (6, 7, 9, 10)

The use of willow bark dates back to the time of Hippocrates (400 BC) when patients were advised to chew on the bark to reduce fever and inflammation. (23). Some of the modern therapeutic applications for willow bark were first reported by Dioscorides in his herbal De Materia Medica written in the first century C.E. (14). After Dioscorides, many European medical authorities, including Albertus Magnus, Dalechamp, Matthiolus, and Paracelsus, recommended willow bark for its haemostatic, antipyretic, anthelmintic, and anti-inflammatory properties. (1) During the middle Ages, willow bark was used in Europe to reduce fevers and relieve pain. (15) Willow's active chemical constituent, salicin, was identified in 1829 by the French pharmacist H. Leroux. Salicylic acid was prepared in pure form by an Italian chemist in 1838. Salicin and salicylic acid were widely used by 19 th century European physicians to treat rheumatic fever and as an antipyretic, gout remedy and analgesic, particularly for joint pain. However, the high doses used (8-10 grams daily) routinely led to vomiting and gastric irritation, and the search was on for a less noxious salicylate.(12)

Acetylsalicylic acid was first synthesized by a French chemist in 1853 and was rediscovered by Felix Hoffman at the Bayer Company in Germany who created acetylsalicylic acid from the spiric acid (spirin) found in meadowsweet (Filipendula ulmaria; syn. Spiraea ulmaria), in the 1890's. Bayer Aspirin quickly became one of the most popular drugs sold worldwide.

Nowadays, synthetic acetylsalicylic acid is used not only as an analgesic and antipyretic, but to prevent myocardial infarctions, strokes and colorectal cancer. Some herbalists recommend willow bark extract as a natural substitute for aspirin to achieve these same benefits. In Germany,

willow bark is often taken along with aspirin to enhance the therapeutic effects while minimizing side effects. The European Scientific Cooperative on Phytotherapy (ESCOP) has approved willow bark extract to treat fever, pain, and mild rheumatic complaints. (12).

Willow bark consists of the bark of the young, two to three year-old branches harvested during early spring of S. alba L., S. purpurea L., S. fragilis L., and other Salix species [Salicaceae] and their preparations in effective dosage. The bark contains at least 1% 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. (18, 19, 20) It is important to choose willow bark that contains proper amount of salicylates to achieve therapeutic effect for medical preparations.

AIM OF THE STUDY

Investigate salicylates amount variation in willow bark of different Salix species growing in Lithuania.

OBJECTIVES OF THE STUDY

- Analyze the amount of salicylates in different species of willow growing in Lithuania bark
- Compare the amounts of salicylates in willow bark, considering the age of plants and time of collection.
- Evaluate the results and availability for expected therapeutic effect.

LITERATURE REVIEW

1. CLASSIFICATION OF PLANTS:

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Malpighiales
Family:	Salicaceae
Genus:	Salix L.

2. IDENTIFICATION

2.1. Botanical Identification of Willow

Salix spp. Woody trees, many-stemmed shrubs, or creeping shrublets; 1-2 cm to 25 m; dioecious or rarely monoecious.

Leaf: Deciduous, generally petiolate and stipulate, simple, alternate to subopposite, linear to widely obovate, margins entire to serrulate, leaf underside generally hairy or glaucous, rarely glabrous.

Inflorescence: Small unisexual catkins emerging in different species either before, at the same time, or after the leaves emerge; individual flowers inconspicuous, apetalous, subtended by a single fringed or hairy bract; sepals replaced by nectaries.

Staminate Flower: Stamens 1-8, often 2;

Pistillate Flower: Ovary superior, bicarpellate, unilocular; style 1; stigmas 2, each 0-2-lobed.

Fruit: Capsule, valves 2.

Seed: Many, comose. (1)

Distribution: There are about 400 species of willow worldvide (3), including analised species, growing in Lithuania:

Salix acutifolia - *Violet Willow*

Salix alba - *White Willow*

Salix caspica – Caspic Willow

Salix daphnoides – Violet Willow

Salix dasyclados

Salix fragilis - Crack Willow

Salix integra - Dappled Willow

Salix mollissima - Silky-leaved Willow

Salix purpurea - Purple Willow

Salix rubra – Sandbar Willow, Green Osier

Salix rigida – Heart-leaved Willow,

Salix schwerinii

Salix tenuifolia

Salix triandra - Almond Willow (Black Maul)

Salix viminalis - Common Osier

Salix alba

Pic. 1. Salix alba (13)

White willow is a tall graceful tree which generally grows to between 10 and 25 m tall, though occasionally they will top 30m. The trunk is usually well developed with the principal boughs and branches ascending at a sharp angle to form a pointed or truncated crown, or a number of pointed “turrets”. The bark is deeply fissured and greyish-brown in colour. The twigs of Salix alba are at first densely pubescent with adpressed, silky hairs. They become glabrous and brown or olive in the second year. Buds are dark brown and again generally covered with adpressed pubescence; they are small, ovoid and pointed in shape.

White willow leaves are lanceolate-acuminate and between 5 and 10 cm long and about 1cm wide. Their edges are minutely serrated and the lamina at first silver/grey, this makes Salix alba easily identifiable at a distance, and become dull green with age.

Catkins appear with the leaves in late April or early May. Male catkins are about 5cm long and uniformly pale yellow, female catkins are shorter and narrower.

Salix daphnoides

Pic. 2. Salix daphnoides

Salix daphnoides is a large shrub or small tree which grows to between 6 and 8m, and occasionally to 12m. It has a rounded crown with erect or spreading branches. The bark is smooth and grey and its twigs, when young, are generally pruinose with a dense glaucous bloom becoming glabrous, lustrous and dark reddish-brown, when older.

The leaves of Violet willow are oblong or narrowly obovate, 7-12 cm long and 2-3 cm wide. At first they are thinly woolly but soon become glabrous, dark green and lustrous above and glaucous below.

Catkins appear before the leaves in February and March and are short, cylindrical and dense about 2-4 cm long and 0.8-1.8 cm wide. Female catkins are shorter and less decorative than the males.

Salix desyclados:

Pic. 3. Salix desyclados

Large schrub or small tree, vigorous 3-6 m, yelow- green new growth, leaves are lanceloate, dark green, showy large catkins on bare steams.

***Salix purpurea*:**

Pic. 4. Salix purpurea

Salix purpurea is a shrub that can sometimes grow in a low and spreading manner no more than 1.5m high and at other time more robust growing as rounded bush or small tree to 5m high.

The bark of the Purple willow is grey and smooth and yellow when the out layer is peeled off. Twigs are glabrous, slender, tough and flexible usually yellow or grey but sometimes tinged with red or purple.

Leaves of Salix purpurea are oblong, oblanceolate or narrowly obovate and variable, 2-20cm, in length and, 0.5-3cm, in width. They are a dull dark green above and paler and shinier below.

Catkins appear before the leaves in March or April, sometime as late as May in the north. They are between 1.5 and 3cm long, densely flowered and black.

Salix triandra

Pic.5. Salix triandra

Salix triandra is a small, bushy tree or robust, spreading shrub that reaches 10m in height. The bark of the Almond willow is smooth and dark grey but it flakes off in large irregular patches to expose a reddish-brown under layer. The twigs are a lustrous olive-brown, glabrous and often angled or ridged.

The leaves of Salix triandra are lanceolate, oblong-lanceolate or narrowly elliptic and between 4 and 11 cm long and 1-3 cm wide. They are regularly serrated and dark green above and green below.

Catkins appear with or a little before the leaves in April and May and sporadically throughout the summer. Male catkins are yellow, narrowly cylindrical and between 2.5-5cm long and 0.3-1.2 cm wide. Female catkins are shorter and denser than the male.

Almond willow is one of the most attractive and fragrant willow and is being used more frequently as a garden shrub.

The twigs have a pleasant flavour of rose-water when chewed.

Salix viminalis

Pic. 6. Salix viminalis

Salix viminalis is a tall shrub or small tree which usually grows to between 3 and 6 metres in height. Its erect or suberect branches usually form a rather narrow, truncate or rounded crown. The bark is greyish-brown and fissured and twigs are long and straight, very tough and flexible and at first ashy-pubescent becoming smooth and a lustrous yellowish-brown later.

Osier leaves are linear or narrowly linear-lanceolate, between 10-15cm long and 0.5-1.5cm wide, and are dull green and thinly puberulous above and silvery below.

Catkins appear before the leaves in late February, March or early April and are generally crowded towards the tips of twigs. They are yellow, between 1.5-3cm long and 0.5-1cm wide and are densely flowered. (13)

2.2. Macroscopic Identification of Willow bark

Commercial supplies are available as powder; cut flat pieces; quills; strips of whole bark of varying thicknesses, lengths, and widths; or the bark of twigs. (1).

The bark is l mm to 2mm thick and occurs in, flexible elongated, quilled or curved pieces. The outer surface is smooth or slightly wrinkled longitudinally and greenish-yellow to brownish-grey. (2) The outer surface of S. purpurea is purple. (1) The inner surface is smooth or finely striated longitudinally and white, pale yellow or reddish-brown depending on the species. The fracture is short in the outer part and coarsely fibrous in the inner region. (2) It is extremely difficult to pulverize. (1) The diameter of current year twigs is not more than 10 mm. The wood is white or pale yellow. (2)

Aroma: Freshly harvested bark has a distinct aroma reminiscent of freshly mowed grass which dissipates after drying. The aroma of S. purpurea and S. daphnoides is similar and persists after drying.

Taste: All medicinal willows are markedly bitter and most are astringent. Two of the richest sources of salicin, S. purpurea and S. daphnoides, lack a strong astringency. The bitter flavor is generally reflective of the concentration of phenolic glycosides.

Powder: Powder of older bark is beige or light reddish-brown. Powder of young inner bark is a light pinkish-beige or brown with greenish or green-yellow hues due to the presence of chlorophyll. (1)

2.3. Microscopic Identification of Medical Herbal Remedy

The powder is pale yellow, greenish-yellow or light brown. It is examined under a microscope using chloral hydrate solution R. The powder shows: bundles of narrow fibres, up to about 600 µm long, with very thick walls and surrounded by a crystal sheath containing prism crystals of calcium oxalate; parenchyma of the cortex with thick, pitted and deeply beaded walls, and containing large cluster crystals of calcium oxalate; uniseriate medullary rays; thickened and suberised cork cells. Groups of brownish collenchyma from the bud may be present. Twigs show, additionally, fragments of lignified fibres and vessels from the xylem. (2)

2.4. Chemical identification of active components:

Willow bark extract is examined by thin-layer chromatography, using a TLC silica gel plate R.

Test solution (a). To 1.0 g of the powdered drug 20 ml of methanol R are added; then heated in a water-bath at about 50 °C, with frequent shaking, for 10 min. After it is necessary to cool and filter.

Test solution (b). To 5.0 ml of test solution (a) 1.0 ml of a 50 g/1 solution of anhydrous sodium carbonate R are added and heated in a water-bath at about 60^{o} C for 10 min. Then cooled and filtered if necessary.

Reference solution: 2.0 mg of salicin R are dissolved in 1.0 ml of methanol R.

It is applied to the plate as bands 20 pm of each. Developed over a path of 15 cm using a mixture of 8 volumes of water R, 15 volumes of methanol R and 77 volumes of ethyl acetate R. The plate is dried in air. Spread with a mixture of 5 volumes of sulphuric acid R and 95 volumes of methanol R. It is heated at 100 °C to 105 °C for 5 min and examined in daylight. The chromatogram obtained with the reference solution shows in the middle third a reddish-violet zone due to salicin. In the chromatogram obtained with test solution (a), the zone due to salicin appears with only slight to moderate intensity. In the chromatogram obtained with test solution (b) the zone due to salicin is clearly more intense and there are, above the zone due to salicin, one (salicortin or 2'-0-acetylsalicortin, or possibly, two tremulacin) faint reddish-violet zones, other blue, yellow or brown zones can occur in both chromatograms. (2)

2.5. Assay of salicin

According European Pharmacopoea Willow bark extract is examined by liquid chromatography.

High Performance Liquid Chromatography (HPLC) is one mode of chromatography, the most widely used analytical technique. Chromatographic processes can be defined as separation techniques involving mass-transfer between stationary and mobile phases.

HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture is resolved into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

Liquid Chromatography was first discovered in 1903 by M.S.Tswett, who used a chalk column to separate the pigments of green leaves. Only in 1960's the more and more emphasis was placed on the development of liquid chromatography.

Types of HPLC

There are many ways to classify liquid column chromatography. If this classification is based on the nature of the stationary phase and the separation process, three modes can be specified.

In adsorption chromatography the stationary phase is an adsorbent (like silica gel or any other silica based packings) and the separation is based on repeated adsorption-desorption steps.

In ion-exchange chromatography the stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

In size exclusion chromatography the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later. Mainly for historical reasons, this technique is also called gel filtration or gel permeation chromatography although, today, the stationary phase is not restricted to a "gel".

Concerning the first type, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography.

In normal-phase chromatography, the stationary bed is strongly polar in nature (e.g., silica gel), and the mobile phase is nonpolar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

Reversed-phase chromatography is the inverse of this. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained.

Eluent polarity plays the highest role in all types of HPLC. There are two elution types: isocratic and gradient. In the first type constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run.

HPLC as compared with the classical technique is characterized by:

- small diameter (2-5 mm), reusable stainless steel columns;

- column packings with very small (3, 5 and 10 mm) particles and the continual development of new substances to be used as stationary phases;
- relatively high inlet pressures and controlled flow of the mobile phase;
- precise sample introduction without the need for large samples;
- special continuous flow detectors capable of handling small flow rates and detecting very small amounts;
- automated standardized instruments;
- rapid analysis; and
- high resolution.

Initially, pressure was selected as the principal criterion of modern liquid chromatography and thus the name was "high pressure liquid chromatography" or HPLC. This was, however, an unfortunate term because it seems to indicate that the improved performance is primarily due to the high pressure. This is, however, not true. In fact high performance is the result of many factors: very small particles of narrow distribution range and uniform pore size and distribution, high pressure column slurry packing techniques, accurate low volume sample injectors, sensitive low volume detectors and of course, good pumping systems. Naturally, pressure is needed to permit a given flow rate of the mobile phase; otherwise, pressure is a negative factor not contributing to the improvement in separation. Recognizing this, most experienced chromatographers today, refer to the technique as high performance liquid chromatography still permitting the use of the acronym HPLC.

Retention mechanism

In general, HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing bead, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process:

- Hydrophobic (non-specific) interactions are the main ones in reversed-phase separations.
- Dipole-dipole (polar) interactions are dominated in normal phase mode.
- Ionic interactions are responsible for the retention in ion-exchange chromatography.

All these interactions are competitive. Analyte molecules are compete with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface, and the weaker the eluent interaction, the longer analyte will be retained on the surface.

SEC (size-exclusion chromatography) is a special case. It is the separation of the mixture by the molecular size of its components. In this mode any positive surface interactions should be avoided (eluent molecules should have much stronger interaction with the surface than analyte molecules).

Basic principle of SEC separation is that the bigger the molecule, the less possibility for her to penetrate into the adsorbent pore space, so, the bigger the molecule the less it will be retained.

Stationary Phases (Adsorbents)

HPLC separations are based on the surface interactions, and depends on the types of the adsorption sites (surface chemistry). Modern HPLC adsorbents are the small rigid porous particles with high surface area.

Main adsorbent parameters are:

- Particle size: 3 to 10 μm
- Particle size distribution: as narrow as possible, usually within 10% of the mean;
- Pore size: 70 to 300 μm;
- Surface area: 50 to 250 m^2/g
- Bonding phase density (number of adsorption sites per surface unit): 1 to 5 per 1 nm^2

The last parameter in the list represents an adsorbent surface chemistry. Depending on the type of the ligand attached to the surface, the adsorbent could be normal phase (-OH, $-NH_2$), or reversed-phase (C8, C18, Phenyl), and even anion (NH_4^+), or cation ($-COO^-$) exchangers.

Mobile phases

In HPLC type and composition of the mobile phase (eluent) is one of the variable influencing the separation. Despite of the large variety of solvents used in HPLC, there are several common properties:

- Purity
- Detector compatibility

- Solubility of the sample
- Low viscosity
- Chemical inertness
- Reasonable price

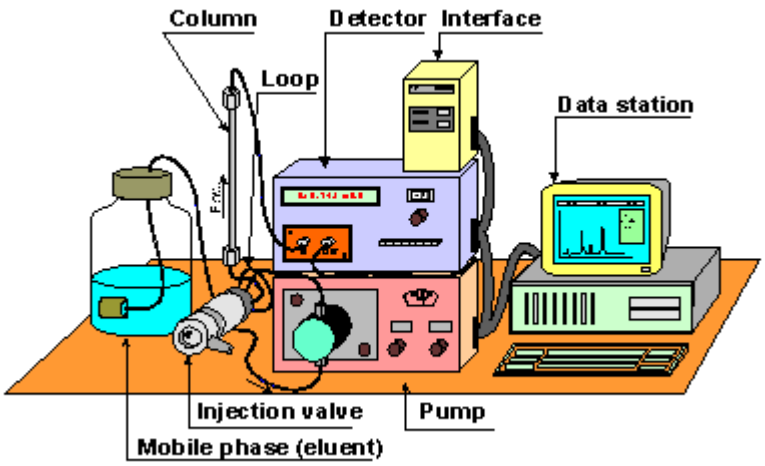
Each mode of HPLC has its own requirements. For normal phase mode solvents are mainly nonpolar, for reversed-phase eluents are usually a mixture of water with some polar organic solvent such as acetonitrile.

Size-exclusion HPLC has a special reqirements, SEC eluents has to dissolve polymers, but the most important is that SEC eluent has to supress all possible interactions of the sample molecule with the surface of the packing material.

Instrumentation : HPLC systems

HPLC instrumentation includes a pump, injector, column, detector and recorder or data system, connected as shown in the Picture below (Pic.7). The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometer size porous particles, a high pressure pump is required to move the mobile phase through the column.

The chromatographic process begins by injecting the solute onto the top of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or peak) on the recorder. Detection of the eluting components is important, and this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyze the chromatographic data, computers, integrators, and other data processing equipment are frequently used.



Pic. 7. Functional schematic of HPLC instrument. (26)

Acording to European Pharmacopoea and to American Herbal Pharmacopoea chromatographic conditions for willow bark extract are:

Column:

Stainless steel column 0.10 m long and 3-4 mm in internal diameter packed with octadecylsilyl silica gel for chromatography (3 µm).

Mobile Phase:

Tetrahydrofuran:water (with 0.5% V/V phosphoric acid) (l,8:98,2).

Flow Rate:

1.0 mL/minute.

Detection:

270 nm

Injection Volume:

10 µl of reference solution B.

Run Time:

Chromatograph is continued for 4 times of the elution time of the peak corresponding to salicin (approximately 70 minutes).

Elution Order:

Salicin (16.68 minutes), L-picein (20.48 minutes), resorcin (28.46 minutes) (1).

3. TESTS OF MEDICAL HERBAL REMEDY

3.1. Foreign matter. Not more than 3 per cent of twigs with a diameter greater than 10 mm, and not more than 2 per cent of other foreign matter.

3.2. Loss on drying. Not more than 11 per cent, determined on 1.000 g of the powdered drug, by drying in an oven at 100 °C to 105 °C for 2 h.

3.3. Total ash. Not more than 10 per cent. (2).

4. HANDLING OF MEDICAL HERBAL REMEDY

4.1. Collection

The twigs or bark of twigs of young branches of 2-to 3-year- old trees should he gathered in the early spring when glycoside content is highest and the bark is easily peeled off. If bark of older trees is used; the outer bark should be removed. (1)

4.2. Drying

Tree bark: For maximum preservation, extractability, and solubility of the phenolics, the fresh bark should be dried quickly in a well-ventilated oven at 48 °C for 8-16 hours. Drying bark slowly at room temperature results in a considerable decrease of phenolics. In one study of the drying conditions on leaves of willow, vacuum-drying was reported to preserve high amounts of both condensed tannins and phenolics. Twigs: Before drying, the twigs should be cut into small pieces 3-4 mm long and then dried in a system, that maintains a 10% relative humidity at temperatures of 5 °C to 20 °C for a period of 40 hours. The smaller pieces allow for a greater efficiency of evaporation and significantly reduce decomposition of the phenolics. Freeze-drying and drying above 22 °C is reported to cause a serious decomposition of the phenolics. (1)

4.3. Storage

Willow bark is stored in a well-closed container protected from light and moisture. (1)

4.4. Adulterants

Wood, leaf, and outer bark material may be present in commercial powders, With the exception of their presence with in acceptable limits and the use of twigs within the specified range of not greater than 10 mm in diameter of current growth these plant parts should be considered as adulterants. Powder that is dark brown indicates the presence of a significant amount of outer bark. (1)

4.5. Preparations

A variety of willow bark preparations are in commerce with various manufacturers preparing their products according to their own specifications utilizing fresh and/or dried material. (1)

Infusion: 32 g of fresh or dried bark fragments are infused in 500 mL of water for 10-15 minutes (1).

Decoction: 32 g of fresh or dried bark fragments are simmered in, 500 mL of water for 10 minutes (1).

Liquid Extract (1:5): 20.0 g of the powdered bark is extracted in 1000 mL of a 25% ethanol water menstruum (wt:V). Some sources recommend heating the menstruum.

5. CONSTITUENTS OF WILLOW BARK

5.1. Phenolic Glycosides

a) Salicin, rarely more than 1% when the botanical is dried adequately, and the esters of salicin:

- salicortin (0.03% to 4%) or 2'-0-acetylsalicortin (0.5% to 10%), 2'-0-acetylsalicin (0.03% to 4%) (described as fragilin in most references),
- tremulacin (0.12% to 2%).

Total salicin content (after hydrolysis) varies according to species. Species rich in total salicin include S. daphnoides (2% to 10%) (primary compound salicortin), S. purpurea (3% to 8.5%) (primary compound salicortm), S. fragilis (2% to 10%) (primary compound 2'-acetylsalicortin) (1) and S. alba (0.5-1%). (12)

There were a lot of investigations of willow bark constituents made by different scientists. The minimal and maximal amounts of salicylates are shown in table 5.1.

	Salicin		Salicortin		2'-O-acetyl-salicin (fragilin)		2'-0-acetyI-salicortin		Tremulacin		Total Salicin After Hydrolysis		References
Species	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	
S. alba	0	0.37	0	2.82	0	0	0	0	0	0.16	0.53	0.98	Shao 1991
S. daphnoides	0.31	1.02	3.5	8.85	0	0.04	0	0	0.33	1.2	1.98	10.2	Meier and others 1985a Shao1991
S. elaeagnos	0.18	0.3	1.12	2.58	0	0	0	0	0.72	1.06	mv	mv	Shao 1991
S. elaeagnos angustifolia	mv	0.5	mv	4.93	0	0	0	0	mv	2.25	3.87	5.36	Shao 1991
S. fragilis	0.25	1,18	0.33	2.96	0	1.06	0.5	7.71	0	0.74	1.90	10.24	Meier and others 1988
S.hastata	0.13	0.97.	1.64	9.2	0	0	0	0	0	0	1.29	6.34	Meier 1988; Meier and others 1988 Shao 1991
S. lanata	0.3	0.4	6.65	8.15	0	0	0	0	0	0	mv	7.38	Meier and others 1988
S. myrsinifolia	0.19	0 55	1.71	4.84	0.03	0.93	0.06	2.67	0	0	mv	mv	Julkunen-Tiitto and Tahvanainen 1989 Meier and others 1988
S. myrsinites	1,02	1,23	6.59	7.6	0	0	0	0	0	0	mv	7.66	Meier and others 1988
S.pentandra	0	0,59	0	3.22	0	2.59	4.82	10.1	0	0	1.14	7.60	Julkunen-Tiitto and Tahvanainen 1989
S. phylicifolia	01	0.4	1.6	5.30	0	3.90	0	0.42	0	2.00	mv	mv	Julkunen-Tiitto and Tahvanainen 1989
S. purpurea	0.11	0.8	3.04	11,1	0	0.04	0	0	0.12	1.97	3.34	8.47	Julkunen-Tiitto and Tahvanainen 1989 Meier and others 1985a; Shao 1991
S. repens	0.07	0.54	3.39	9.04	0	0	0	0	0	0	2.25	5.69	Shao 1991
S. rosmarinifolia	0.12	0.64	3.52	10.9	0	0	0	0	0.12	0.28	2.76	6.04	Meier and others 1988

mv = missing values

Table 5.1 Salicin and salicin derivatives in selected Salix species by wt% (bark and current growth twigs) (1)

Salicylates are found in a number of plants including many Salix species, wintergreen (Gaultheria procumbens), black cohosh root (Cimicifuga racemosa), poplar tree bark (Populus spp.) and sweet birch tree bark (Betula lenta). Salicylate content appears to be highest in S. fragilis bark (1-10%), S. purpurea bark (3-9%), S. repens leaves (5-12%), and S. rosmaninifolia leaves (7-10%) and lowest in white willow bark (1.5-11%). The concentration and availability of salicylates also varies within species according to growing conditions, processing and preparation (12).

b) Phenolic glucosides:

- picein (S. bicolor, S. Cinema, S. nigricans),
- purpurein (S:-purpurea),
- syringin (S. purpurea, S. daphnoides),
- triandrin (S.triandra, S. viminalis),
- Salireposid (S. purpurea, S. repens),
- salidrosid (S. triandra),
- vimalin (S.viminalis)

5.2. Flavonoids

a) Flavonoid glycosides:

- (+)- and (-)-naringenin-5-glucoside (0.3% to 1% in S. daphnoides and S. purpurea),
- naringenin-7-glucoside (0.3% to 1.5% in S. daphnoides),
- eriodictyol-7-glucoside (S. purpurea),
- polyphenols of the flavan-3-ol type

b) Chalcone glycosides: isosalipurposid (S. daphnoides and S. purpurea)

c) Aglyca: biflavones

d) Flavanones:.(+)catechin, (-) epicatechin (traces).

e) Condensed flavanones: dimeric and trimeric procyanidins. (1)

Tannins are phenolic compounds found in many herbs and common foods such as sorrel and rhubarb. Tannins are astringent and are traditionally used topically to treat wet or oozing skin conditions such as poison ivy and eczema. Tannins have also been used internally to treat diarrhea and inflamed mucus membranes. Plants that contain more than 10% tannins have potential adverse effects including upset stomach and nausea and vomiting acutely, and renal damage, hepatic necrosis, and increased risk of esophageal and nasal cancer with long term use.

Willow bark contains between 8-20% tannins, a higher concentration than in almost any other plant. Tannins may interfere with the absorption of alkaloid compounds, salicylic acid, iodine, zinc, iron and copper. Drug-tannin precipitates may form in alkaline environments, interfering with proper dosing. (12)

6. PHARMACOCINETICS OF SALICYLATES

Salicin and salicortin are the primary salicylates found in willow bark. Salicortin is hydrolyzed in the small intestines into salicin and its esters which are further hydrolyzed into saligenin and glucose. Saligenin is absorbed and oxidized to salicylic acid in the blood and liver. The rate of absorption depends on many factors, including solubility of the preparation, dosage, gastric and intestinal pH, gastric emptying time, and the presence of food in the gastrointestinal (GI) tract. More than 80% of salicin is absorbed after oral administration. Because of the time required to metabolize the willow's salicylates, the time to achieve therapeutic effects and the duration of effects are longer than with synthetic salicylates. (1, 12).

7. PHARMACODYNAMICS OF SALICYLATES

Much research has focused on the ability of salicylates to suppress the synthesis of prostaglandins, thought to play an integral role in pain, inflammation, and fever. Two specific enzymes are presently considered to be predominantly involved in this process, cyclooxygenase-l and -2 (COX-1 and -2). COX-1 is mainly present in platelets, blood vessels, and other organs, whereas COX-2 occurs in inflamed tissues. Aspirin (acetylsalicylic-1 acid) blocks the synthesis of prostaglandins through the acetylation of the enzyme cyclooxygenase (PCH-syhthase), especially in platelets (COX-1), by an irreversible transfer of the acetyl group into the enzyme. Salicylic acid and salicylates, which lack an acetyl group, are only effective in inhibiting platelet aggregation at concentrations that are so high (> 300 µg/mL) as to be clinically irrelevant. Salicylates have been shown to reduce prostaglandin levels in body tissues through an inhibition of PCH-synthase in inflamed tissues (COX-2) and are predominantly used for their analgesic properties. There may be an advantage to using COX-2 over COX-1 inhibitors since most of the side effects associated with currently avail able nonsteroidal anti-inflammatories (NSAIDs) may be due to a blockade of COX-1.

It has been proposed that the anti-inflammatory actions of salicylates may be due to mechanisms independent of prostaglandins. Sodium salicylate has been shown to inhibit the functioning of neutrophils, the most abundant cells associated with acute inflammation. (1)

8. POTENTIAL CLINICAL USES OF WILLOW BARK

With the advent of synthetic aspirin, the use of willow bark fell by the wayside both clinically and scientifically. Very few studies have addressed the physiologic effects of willow bark as opposed to aspirin. Modern herbalists have tended to extrapolate the data from studies on aspirin and apply those findings to willow bark. Studies cited refer to willow bark and/or salicin.

8.1. Cardiovascular: none

8.2. Pulmonary: none

8.3. Renal and electrolyte balance: none

8.4. Gastrointestinal/hepatic: none

8.5. Neuro-psychiatric: Analgesic. Salicin does have analgesic properties. However, due to the low concentration of salicin in willow bark, the amount extracted in typical preparations and the metabolic transformation required to convert salicin and salicortin to salicylic acid, achieving therapeutic effects would require approximately 35 grams of white willow bark three times daily. At typical doses, willow bark is not expected to exert significant analgesic effects. However, recent unpublished data suggests that willow bark extracts may provide significant therapeutic benefit even with low salicylate levels.

a) In vitro data: none

b) Animal data: none

c) Human data: In a randomized, double-blind placebo-controlled trial of 54 migraine sufferers, half were given a topical medicament containing salicin (dose unknown) and half were given a placebo. All were advised that when a headache began, they should lie down, apply the medicament to their forehead (amount unknown), cover the face with a photo-occlusive mask and take their regular headache medications. Those treated with salicin reported significantly more improvement than those assigned to placebo treatment. (12)

A randomized, placebo-controlled double-blind clinical trial investigated the analgesic effects of a standardized willow bark extract. Seventy-eight patients with osteoarthritis of the knee and/or hip were given either 4 tablets per day (intake of 1360 mg extract containing 240 mg total salicin per day) or placebo for 2 weeks. The pain subscale of the WOMAC osteoarthritis index was used as the primary out come measure. Secondary outcome measures included total WOMAC scale

(including pain, stiffness, and function sub scales), daily visual analog scales for pain and function, and final assessments by the physicians and patients. As determined by a change in the WOMAC pain index, a significant analgesic effect was observed by day 14 of administration of the preparation ($P < 0.05$). Using visual analog scales, a significant difference was seen in the verum ($n = 78$) versus the per-protocol evaluation ($n = 68$). A tendency toward improvement in stiffness and function was observed although this was not statistically significant. Final assessments by the physicians and patients reported highly significant differences between verum acid placebo (physicians: $P = 0.0073$; patients P = 0.00p2). According to the researchers, the analgesic effect was reported to be comparable to the effect of tenoxicam (20 mg/day) and approximately 40% lower than the effect of diclofenac (150 mg/day). In this study, adverse events were reported to be less frequent in the treatment group than the placebo group. (1)

In a study of nearly 200 people with low back pain, participants who received willow bark experienced a significant improvement in pain compared to those who received placebo. Moreover, participants who received higher doses of willow bark (240 mg salicin) had more significant pain relief than those who received low doses (120 mg salicin). (23)

8.6. Endocrine: none

8.7. Hematologic: Anticoagulant and antithrombotic: Some herbalists recommend willow bark as a natural alternative to aspirin to prevent myocardial infarction and strokes. However, willow's salicylates do not appear to interfere with coagulation.

a) In vitro data: Unlike aspirin, sodium salicylate had no effect on platelet adhesion or platelet release of ADP 27 .

b) Animal data: none

c) Human data: Unlike aspirin, sodium salicylate did not prolong bleeding time in adults; nor did it inhibit platelet aggregation.

8.8. Rheumatologic: Arthritis (See Immune modulation: Anti-inflammatory)

8.9. Reproductive: none

8.10. Immune modulation: Anti-inflammatory

a) In vitro data: Unlike aspirin, salicin did not inhibit PHA-induced lymphocyte transformation.

b) Animal data: none

c) Human data: Although salicin does have anti-inflammatory properties, the concentration in willow bark is so low that 25-35 liters of willow bark tea would be required to achieve dosages comparable to 4 grams of aspirin daily. However, a recent unpublished case series suggests that standardized willow bark extracts may provide significant therapeutic benefit for patients with

rheumatic pain. (12) Researchers Meier and Liebi explain the long history of use of willow preparations and clinical effectiveness by suggesting the metabolism process of the phenolic glycosidic complex in willow results in maximum plasma levels that are sustained for hours after ingestion. They further suggest constituents besides salicylates may contribute to the activity. (1) A detailed pharmacological screening of the aqueous willow bark extract STW 33-I addressed the question of the identification of fractions contributing to the overall effect. All in vivo and in vitro models studied pointed to relevant contributions of the fraction of polyphenols and flavonoids. The single compounds or their combinations responsible for the effect remain to be elucidated. (25).

8.11. Antimicrobial: Antifungal

a) In vitro data: Aqueous extracts of dried white willow bark displayed antifungal activity against

Botrytis cincerea and Penicillium digitatum, but were inactive against Aspergillus fumigatus, Aspergillus niger, Rhizopus nigricans, Trichophyton mentagrophytes and Candida albicans and a preparation of S. fragilis L. inhibited growth of Aspergillus fumigatus, Aspergillus niger, and Aspergillus flaws (12, 1).

a) Animal data: none

b) Human data: none (12)

8.12. Antineoplastic: Prevention of colorectal cancer.(12) Recently, there have been extensive efforts to evaluate the chemopreventive role of substances present in natural products. The aim of the study was to examine the effects of the main groups of compounds (salicylalcohol derivates, flavonoids, proanthocyanidins), and salicin isolated from willow bark extract BNO 1455 on proliferation and apoptosis in human colon and cancer cells.

Human colon cyclooxygenase-2 (COX-2)-positive HT 29 and (COX-2)-negative HCT 116 or lung COX-2 proficient A 549 and low COX-2 expressing SW2 cells were used. After treatment for 72 h with various concentrations of single substances and acetylsalicylic acid (ASA) as control, inhibition of cell growth and cytotoxicity were measured by colorimetric WST-1 assay and propidium iodide uptake by flow cytometry, respectively. Apoptotic cells were identified by annexin V adhesion using flow cytometry.

Studies on dose-dependent effects of BNO 1455 and its fractions showed anti-proliferative activity of all compounds with 50% maximal growth inhibitory concentrations (GI(50)) between 33.3 and 103.3 microg/ml for flavonoids and proanthocyanidins fractions and 50.0-243.0 microg/ml for salicylalcohol derivates and extract. Apoptosis induction was confirmed by annexin V adherence and analysis of cell morphology based on light scattering characteristics using flow cytometry in all cell lines at GI(50).

The research showed that willow bark extract BNO 1455 an its fractions inhibit the cell growth and promote apoptosis in human colon and lung cancer cell lines irrespective of their COX-selectivity. (24)

8.13. Antioxidant: none

8.14. Skin and mucus membranes: none

8.15. Other/miscellaneous: Antipyretic; wart remedy

In central Italy dried willow bark is applied topically to treat warts; a modern home remedy is to tape a plain adult aspirin over a wart. There have been no controlled trials reported on willow bark's use for this purpose, but it is likely to be as effective as other home remedies.

In modem herbal therapy, willow is predominantly used as an anti-inflammatory for symptomatic relief of gouty arthritis and as an analgesic for mild neuralgic pains, toothaches, and headaches. It is not widely used for its antipyretic activity, having been replaced by diaphoretics and other febrifuges. These uses have also been cited in numerous authoritative sources. (1). Some professional herbalists may recommend willow bark for the following conditions, although at present, no scientific studies have been conducted to support these uses:

- Sexual dysfunction
- Mild diarrhea with cramps
- Fever
- Flu
- Tendonitis
- Bursitis
- Ankylosing spondylitis (a form of rheumatoid arthritis affecting the lower spine) (23)

9 TYPICAL DOSAGES OF WILLOW BARK PREPARATIONS

Plants containing salicylates have a very bitter taste, so willow bark tea may be unpalatable for most patients, particularly for children.

A typical 500 mg dose of aspirin is equivalent to 794 mg of salicin. If extraction was 100% effective, this amount of salicin could be derived from 80-150 grams of dried willow bark. The usual dose of willow bark tea is typically 1-3 grams of bark per cup of water. Thus, the usual herbal products contain minute amounts of salicylates compared with aspirin. Tea is made by steeping ½-2 tsp. (1-3 grams) of the dried bark in 1 cup of boiling water for 10- 20 minutes (infusion) or bringing this amount of cold water and dried bark to a boil for 5 minutes (decoction). Some herbalists

suggest drinking one cup a day to help prevent myocardial infarction and stroke. To treat fever and pain, 3-5 cups a day are recommended (containing a total of 60-120 mg of salicin).

Larger amounts are likely to lead to gastrointestinal upset due to the high tannin content of the bark.

Tincture or glycerite: ½ tsp. three times daily.

Liquid extract (1:1 in 25% alcohol): 1-3 ml three times daily

Capsules: Two to three 379 milligram capsules taken every 3-6 hours 8 (12)

Decoction: 30-60 mL 3 times daily or as indicated

Infusion: 180-240 mL 3 times daily or as indicated (1)

Topically: Infused in vegetable oil and rubbed over painful joints or muscles as needed (12)

Pediatric dosages: 4-10 years old: equivalent to 30-60 mg of total salicin daily.

10-l6 years old equivalent to 60-120mg of total salicin daily according to body weight and stature. (1)

10. TOXICITY AND CONTRAINDICATIONS OF WILLOW BARK

All herbal products carry the potential for contamination with other herbal products, pesticides, herbicides, heavy metals and pharmaceuticals.

Allergic reactions can occur to any natural product in sensitive persons.Allergic reactions to willow bark have been reported. Persons known to be allergic to aspirin should avoid herbal remedies containing willow bark.

Potentially toxic compounds in willow bark: Salicin, salicortin, tannins.

Acute toxicity: Toxicity is far less with willow bark than with aspirin due to the low levels of salicylates in the plant products. High doses can be associated with gastric and renal irritation, nausea, vomiting, and gastrointestinal bleeding, but side effects are far more likely from the high tannin levels than the salicylates.

Chronic toxicity: None reported with willow bark, but theoretically possible due to salicylates.

Limitations during other illnesses or in patients with specific organ dysfunction: Patients with tinnitus should avoid using herbal remedies such as willow bark that contain salicylates.

Although side effects have not been reported, the salicylate content warrants cautious use by patients with allergies to aspirin, asthma, active peptic ulcer disease, diabetes, gout, hemophilia and other bleeding disorders, or hepatic or renal disease.

Interactions with other herbs or pharmaceuticals: None reported. There is a potential for interaction with salicylate-containing medications and other non-steroidal anti-inflammatory medications (NSAIDs). Tannins may interfere with absorption of other medications.

Safety during pregnancy and/or childhood: Due to the potential of developing Reye's syndrome from salicylates, children with influenza or varicella should avoid willow bark. Safety has not been established for pregnancy and lactation. Salicylates in breast milk were reported to cause a rash in one breast-fed baby. (1, 12).

11. PROBLEMS

According to the reviewed literature, willow bark has a lot of indications. The most of them are based on the effect of salicylates. Howewer the amounts of salicylates varies among different species of willow. As the differences may also depend on growing conditions, the sourses of information about investigations made in other geographical zones may be not reliable.

This study was made in order to investigate species of willow, appropriate for medical usage as the source of salicylates in Lithuania dendroflora.

The assay of salicine in the willow bark was made by HPLC method according European Pharmacopoea.

EXPERIMENTAL

1. SAMPLE

The samples of Willow bark were collected from Botanical Institute of Vilnius colection. 12 samples of twigs and young branches of 1 year old trees and 12 samples from the same species of 2 year old trees were gathered in autumn in 2005. 12 samples of the same species were collected in spring in 2006 from 2 years old plants. These 3 samples of each of 12 species were used in the first part of the study. For the second part of the study, 32 samples of twigs and young branches were collected from 2 year old trees in autumn in 2006.

The bark was peeled off, dried at room temperature, cut into small pieces and sifted.

2. CHEMICALS AND REAGENTS

Salicin, resorcinol and picein standards were purchased from Sigma (St. Louis, MO, USA). The standards were stored protected from light at routine room temperature (20°C). HPLC-grade tetrahydrofuran and phosphoric acid were obtained from Roth GmbH (Germany). The 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).

Internal standard solution was prepared dissolving 50 mg of resorcinol in 10 ml of methanol.

Reference solution (a) was prepared dissolving 18.5 mg of salicin in 10.0 ml of a mixture of 20 volumes of water and 80 volumes of methanol and adding 1.0 ml of the internal standard solution.

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

Test solution preparation:

To 0.5 g of the powdered bark 50 ml of methanol were added and heated under a reflux condenser for 30 min. Cooled and filtered. The residues was taken up with 50 ml of methanol and proceeded as above. The filtrates were combined and evaporated under reduced pressure. The residues was taken up with 5.0 ml of methanol, add 5.0 ml of 0.1 M sodium hydroxide and heated in a water-bath at about 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 a

mixture of 50 volumes of methanol and 50 volumes of water. 1.0 ml of the internal standard solution was added to 10.0 ml of this solution and filtered through a membrane filter.

2. INSTRUMENTATION

The HPLC system consisted from separation module Alliance 2609 (Waters) and UV detector waters 2487 set at 270 nm. A stainless steel column Hypersil H50DS (150x4.6 mm) was used for separation of components. As mobile phase at a flow rate 1.0 ml/min a mixture of 1.8 volumes of tetrahydrofuran and 98.2 volumes of water, containing 0.5% of phosphoric acid was used. 10µl of reference and test solutions were injected onto column.

The percentage content of total salicylic derivatives, expressed as salicin, was calculated from the expression:

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

S_1 = 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.

3. RESULTS

Fig. 1 shows typical chromatogram obtained from reference solutions. Salicine (retention time tr = 5.511 ± 0.011 min, n = 10), picein (tr = 6.421 ± 0.018 min, n = 10) and resorcinol (tr = 8,067 ± 0.05 min, n = 10) exhibited well separated, narrow and symmetrical peaks under the chromatographic conditions described.

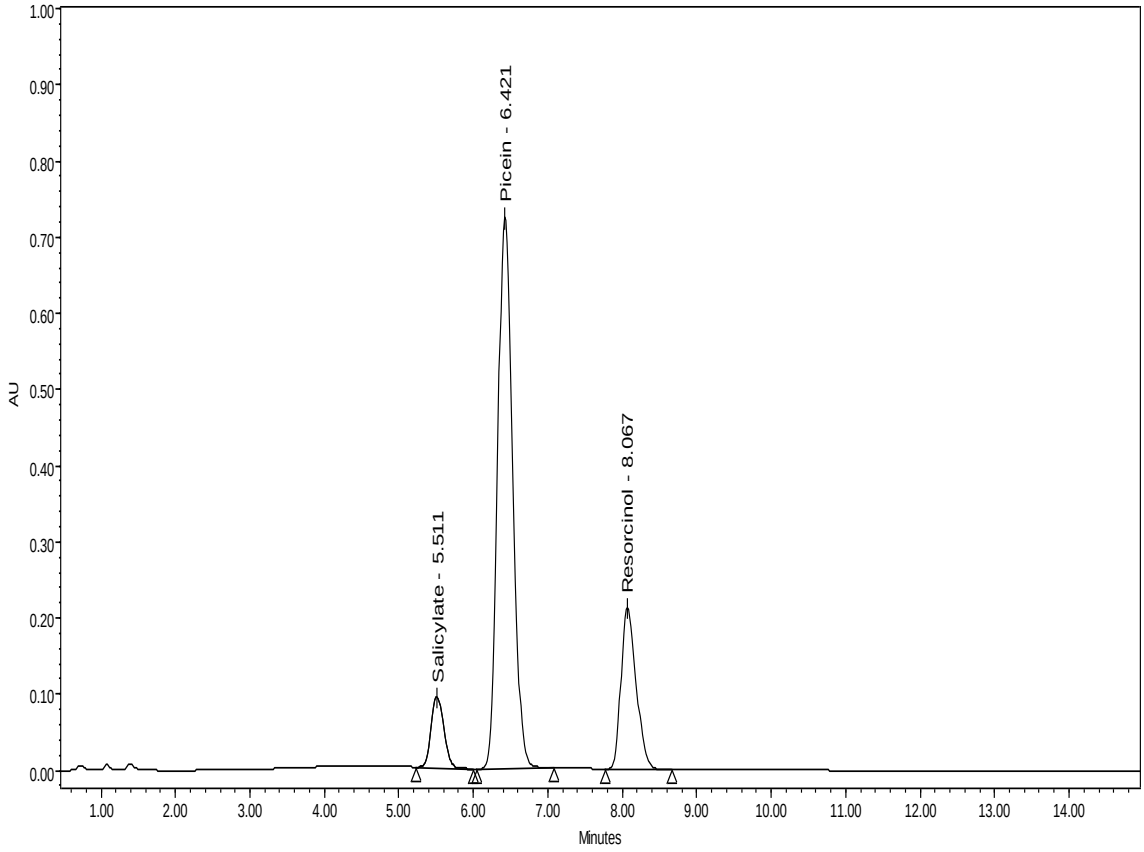


Fig. 1. Typical chromatogram obtained from reference solution

Fig.2 and 3 shows typical chromatograms obtained from test solutions:

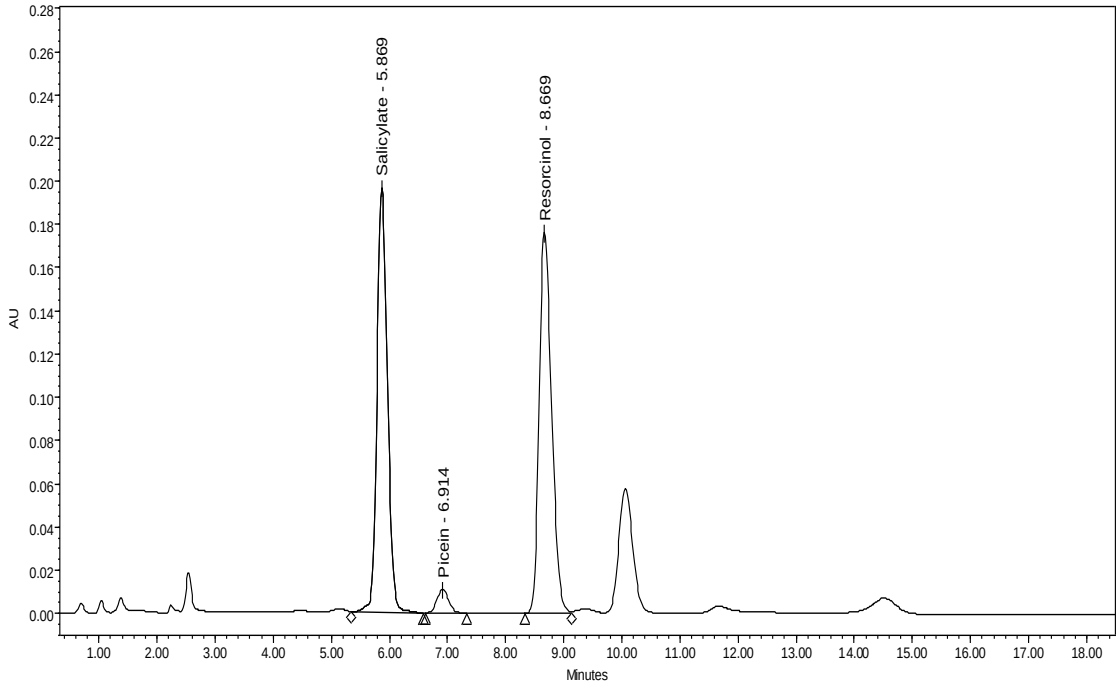


Fig. 2. Chromatogram obtained from test solution of Salix purpurea cl. 04132

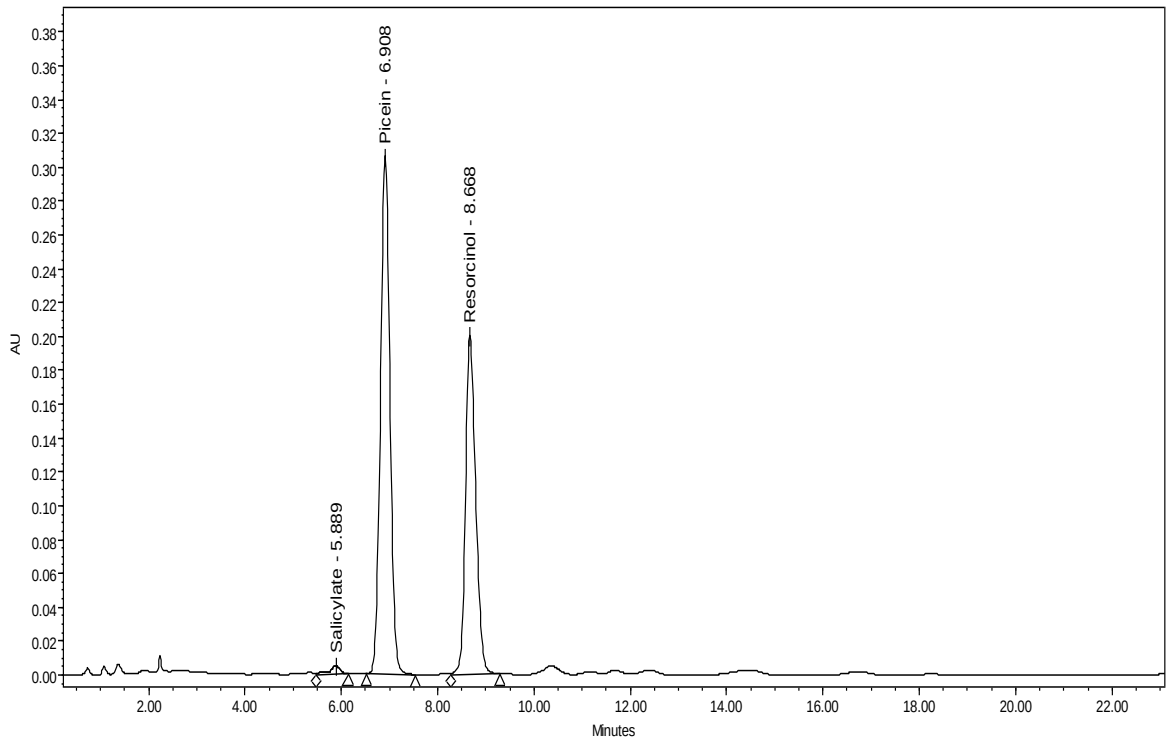


Fig. 3. Chromatogram obtained from test solution of Salix dasyclados x S. viminalis cl. 04120

At first 12 species of willow were analysed. There were 3 samples of each species bark: the first were colected in autumn of 1 year old plants, the second – 2 years autumn and the third – 2 years spring. The results are shown in tabble 2:

Name of the plant	**1 year autumn %**	**2 years autumn %**	**2 years spring %**
Salix integra x salix kochiana	8,09	9,48	5,92
Salix acutifolia	6,95	12,06	10,83
Salix daphnoides	10,19	11,92	9,50
Salix caspica	5,64	6,71	4,68
Salix rubra	2,11	2,88	0,95
Salix rigida	3,07	5,34	1,88
Salix alba	1,29	1,87	1,71
Salix purpurea f. "Purpurea"	7,16	7,77	6,36
Salix mollissima	0,29	0,84	0,29
Salix triandra	0,76	0,93	0,73
Salix viminalis "Americana"	0,08	0,98	0,42
Salix dasyclados	0,32	0,39	0,17

Tab. 2. Quantity of salicylates, calculated as salicin

The amount of salicylic derivatives in the bark of willow depends on time of collection and age of the plant (figure 4 and 5).

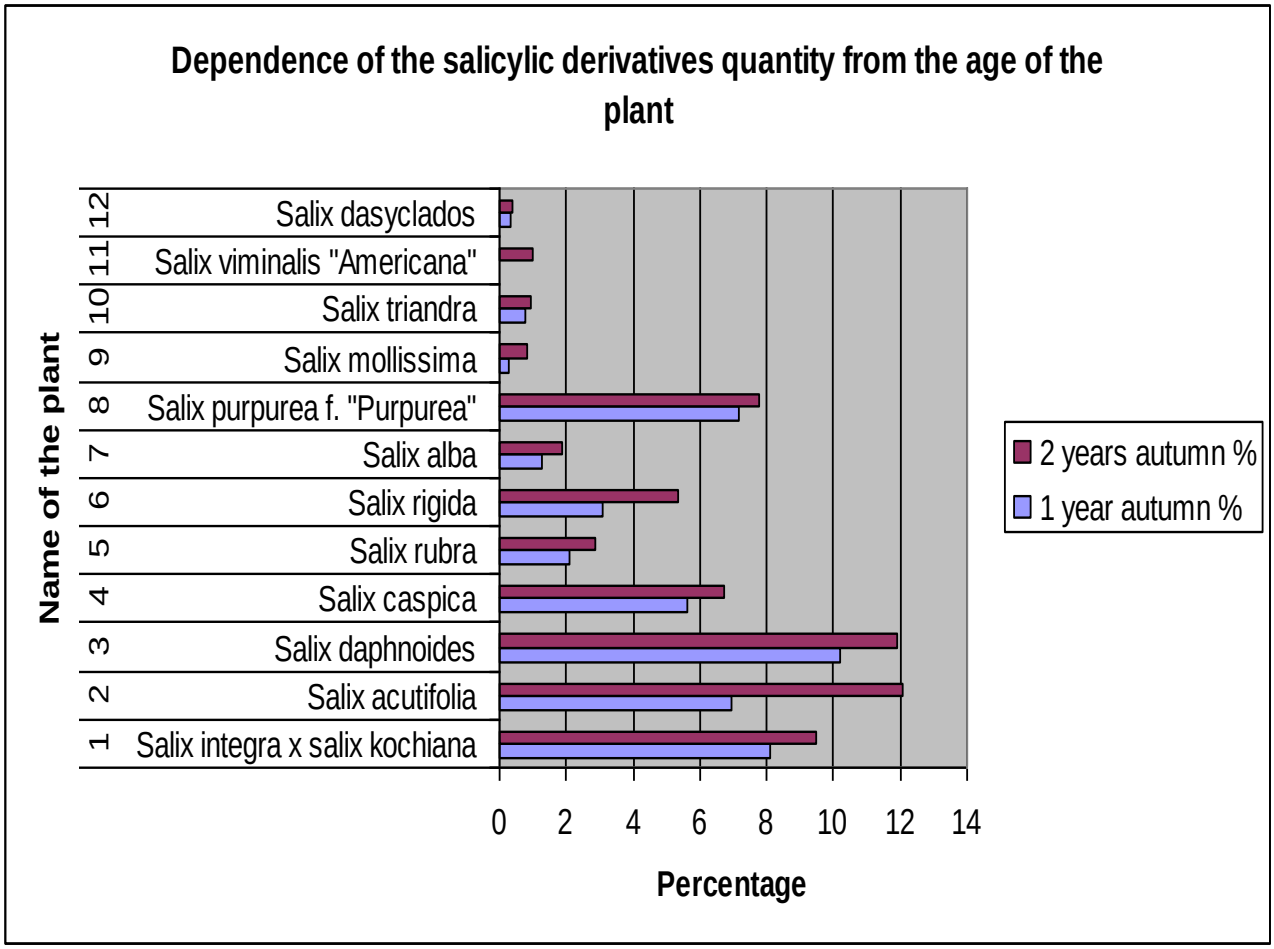


Fig. 4. Dependence of the salicylic derivatives quantity from the age of the plant

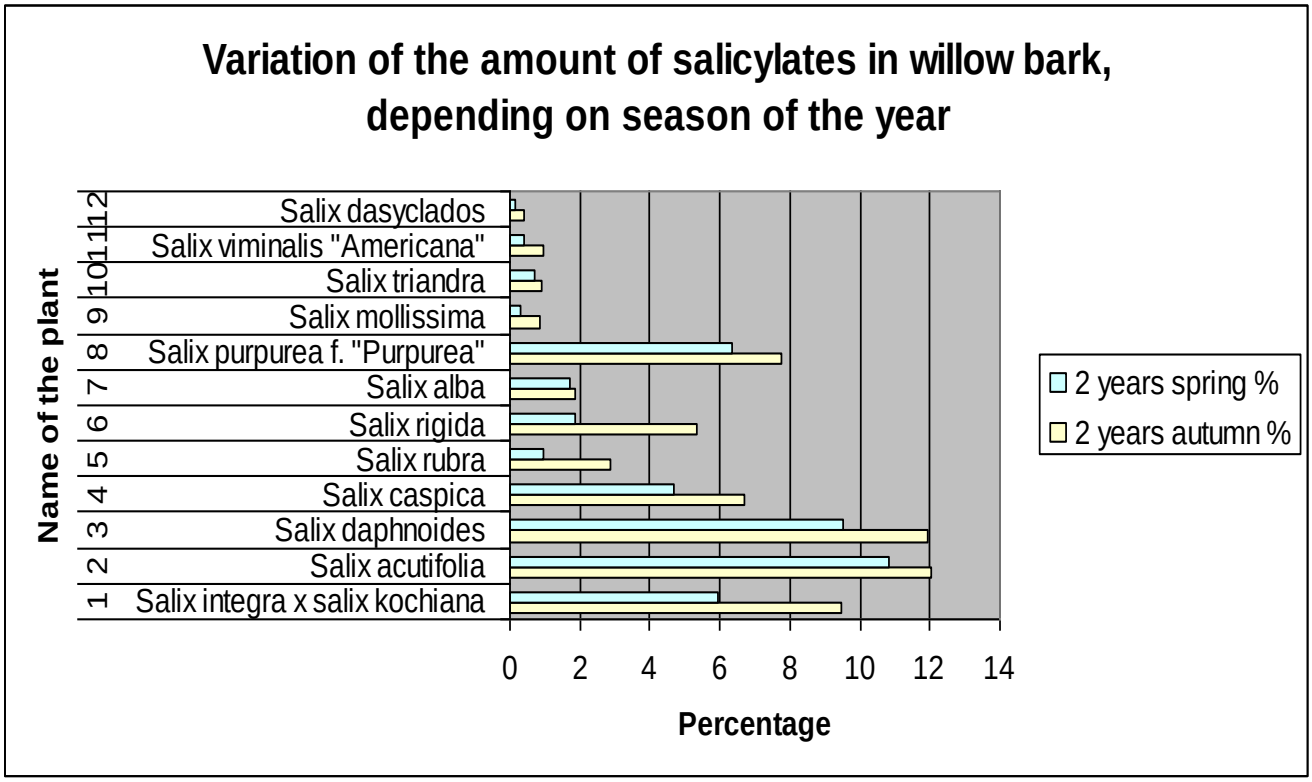


Fig.5. Variation of the amount of salicylates in willow bark, depending on season of the year

The investigation demonstrated that salicylates are not accumulated in spring. The biggest amounts of salicylates were found in 2 years old willow bark collected in autumn, for that reason the second experimental part was made among 32 species of willow collected in autumn of 2 years old plants.

The percentage amount of the salicylic derivatives expressed as salicin in the different species of Salix genus is presented in the table 3.

Number	Name of the plant	Quantity of salicin (%)
1	Salix caspica	3,87305
2	Salix alba tiesiakamienė forma	1,3731
3	Salix mollissima	0,25584
4	Salix schwerinii x Salix dasyclados	0,65067
5	Salix viminalis ‚Americana‘ žaliažievė forma	0,03817

6	Salix viminalis kl. 9822	0,13351
7	Salix viminalis kl. 9817	0,8377
8	Salix viminalis ‚Americana‘	0,19693
9	Salix alba ssp. alba kl. 04115	1,2168
10	Salix viminalis kl. 04116	0,31419
11	Salix viminalis ‚Americana“ kl. 9976	0,10439
12	Salix dasyclados x S. viminalis kl. 04120	0,26436
13	Salix dasyclados kl. 9977	0,14988
14	Salix dasyclados kl. 04122	0,16962
15	Salix dasyclados kl. 04124	0,36989
16	Salix daphnoides f. latifolia kl. 9904	2,56015
17	Salix purpurea ‚Lutea‘ kl. 9731	6,53091
18	Salix purpurea x Salix triandra ssp. amygdalina kl. 04131	8,35459
19	Salix purpurea kl. 04132	10,0545
20	Salix tenuifolia (rubra) kl. 04133	1,58693
21	Salix triandra x Salix purpurea kl. 04134	1,66764
22	Salix dasyclados ‚Gudrun‘ kl. 04135	0,54189
23	Salix viminalis ‚Tordis‘ kl. 04136	0,05892
24	Salix mollissima kl. 9868	0,07651
25	Salix purpurea x Salix viminalis kl. 9714	4,61521
26	Salix purpurea x Salix viminalis kl. 04141	7,26769
27	Salix purpurea ‚Lutea‘ kl. 9727	5,95857
28	Salix purpurea ‚Rubra‘ kl. 04130	2,58514
29	Salix schwerinii ‚Tora‘ kl. 04137	1,109
30	Salix fragilis x Salix alba	2,06709
31	Salix viminalis kl. 0109	0,05716
32	Salix desylados kl. 099	0,49788

Table 3 Quantity of salicylic derivatives calculated as salicin

The variation of amount of salicylates is expresed in figure 6.

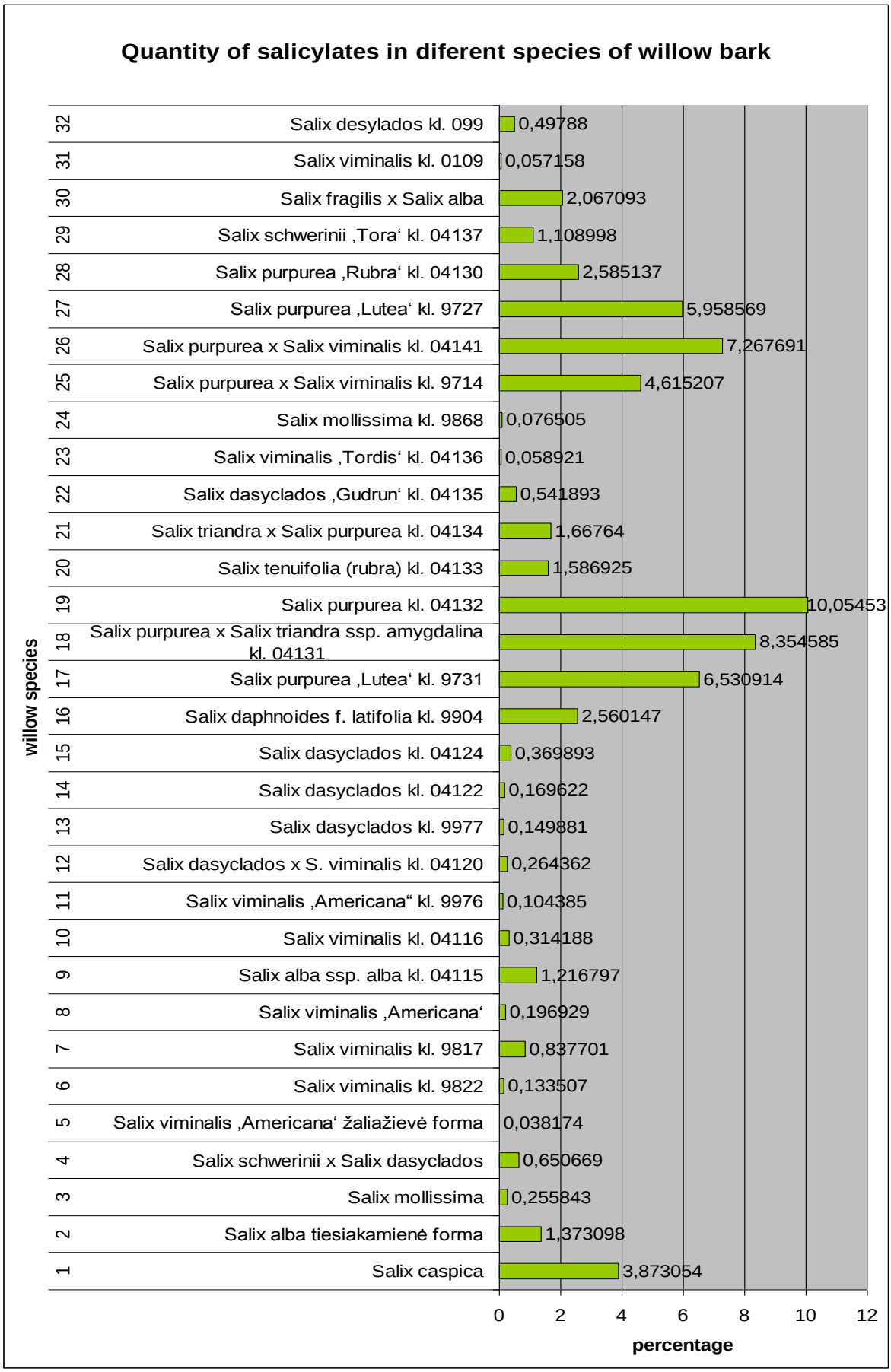


Fig. 6 The variation of amount of salicylates

4. DISCUSSION

Willow bark is a perspective herbal remedy, which possess a lot of therapical effects, such as antipyretic, antiinflamatory, analgesic, antineoplastic and antifungal. As the effect is based on salicin this investigation was made to compare the amounts of it in different species of Willow. The results of this study shows, that there is striking differences in salicylic derivatives amount among different Salix species. (Fig. 4, Fig. 5, Fig. 6). Acording to the European Pharmacopoea, minimal amount of 1,5% of salicin is required in Willow bark. Considering this requirement, it is possible to divide species analised in this study to species that can be used to prepare qalitative herbal remedies and to species, inapropriate for medical usage. Salix integra x 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 hybrides with other species (2,56% - 10,05%) contains high amounts of salicin (Tab. 2, Tab.3). Medical usage of species mentioned above is recomended acording to this study. However a lot of 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%) , all clones of Salix viminalis(0,038% – 0,98%) and hybrides with it. Hybrides of Salix viminalis (lowest amount of salicin) and Salix purpurea (highest amount of salicin) possess apropriate required amount of salicin (4,61% – 7,26%).

The first step of study was made among 3 samples of each plant, colected at different growing period (1-st year autumn, 2-nd year autumn and 2-nd year spring). Comparison of the quantities of the salicylic derivatives depending upon age of the plant (Fig. 4) and time of collection (Fig. 5) shows, that plants at two years of growth and collected in the autumn accumulate greatest amounts of active substances (approximately 25.0% increase) when comparing with the 1 year old plants and material collected at spring season. These findings agree with data reported by (Meier [18] Meier [19]; Thieme [20]). However they doesn’t agree with American Herbal Pharmacopoeia, which suggests that the twigs or bark of twigs of young branches of 2-to 3-year- old trees should he gathered in the early spring when glycoside content is highest and the bark is easily peeled off. It is possible to make a conclusion, that the accumulation of highest amounts of salicin during different seasons depends on geographical zones and growing conditions.

Salix alba is a common species in Lithuania, it is described in foreign literature as herbal remedy as well. According to this study it possess only 1,29% of salicin in 1-st year spring, but on the 2-nd year autumn it accumulates appropriate amount of salicin (1,87%). So it is important to consider time of collection of Willow bark. The amount of 1,87% is not very high, and according to

literature (12) it would be necessary to use 35 g. of White Willow bark to achieve the effect of 1 tablet of aspirin. Such high amount can be toxic due to effect of tannins. Usual dosage is 1-3 g of Willow bark. In this case, the effect of herbal remedies can not be only based on the effect of salicin. It is proposed to consider other components (tannins, flavonoids) which can contribute to overall effect.

FINDINGS

1. The results of this study shows, that there is striking differences in salicylic derivatives amount among different Salix species (from 0,04% (Salix viminalis “Americana”) to 12,06% (Salix acutifolia).

2. It is important to consider season of the year and age of the plant during collection of Willow bark. Plants at two years of growth and collected in the autumn accumulate greatest amounts of active substances.

3. The amount of salicin was lower than it is required according to European Pharmacopoeia (1,5%) in the bark of some analyzed species. These species are not recommended to be used for treatment.

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

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SANTRAUKA

Natūralioje Lietuvos floroje gluosnių (*Salix* L.) rūšys yra plačiai paplitusios. Gluosnio žievė – nuo seno žinoma kaip priešuždegiminiu poveikiu pasižyminti vaistinė augalinė žaliava. Jos ekstraktai įeina į priešreumatinių ir analgezinių preparatų sudėtį. Terapinis veikimas yra susijęs su salicinu, kuris organizme virsta salicilo rūgštimi.Gluosnio žievė yra fitoterapinis aspirino pirmtakas. XXI amžiuje vis didesnis dėmesys krypsta į natūralius, o ne sintetinius preparatus. Tokiu būdu, augalų, pasižyminčių terapinėmis savybėmis tyrimai įgyja didelę reikšmę. Skirtingų rūšių gluosnių žievėje, kaupiamas salicino kiekis skiriasi. Europos farmakopėjoje reglamentuojamas minimalus salicino kiekis yra 1,5%. Šio tyrimo tikslas buvo Ištirti Lietuvoje augančių gluosnio rūšių salicilatų kiekio įvairavimą vaistinėje augalinėje žaliavoje (žievėje). Darbo uždaviniai- atlikti įvairių Lietuvoje augančių gluosnio rūšių salicilatų kiekio analizę, palyginti salicilatų kiekį gluosnio žievėje, atsižvelgiant į augalo amžių bei rinkimo laiką bei įvertinti gautus rezultatus, numatomą terapinį veikimą. Tyrimai atlikti naudojant farmakopėjinius analizės metodus. Žievės ekstraktai buvo tiriami efektyviosios skysčių chromatografijos metodu.Šio tyrimo pirmo etapo metu išanalizuota 12 gluosnių taksonų. Buvo ištirti vienerių ir dviejų metų, taip pat pavasarį ir rudenį rinkti žievės pavyzdžiai. Didesni kiekiai aktyvių medžiagų rasta rudenį ir 2 metų amžiaus gluosnių žievės pavyzdžiuose. Antrajame tyrimo etape buvo tiriami 32 rudenį rinkti 2 metų amžiaus gluosnių žievės pavyzdžiai. Tyrimo rezultatai parodė, kad tarp skirtingų gluosnio rūšių yra didžiuliai salicino darinių kiekio skirtumai (nuo 0,04% (Salix viminalis “Americana”) iki 12,06% (Salix acutifolia). Kai kurių rūšių gluosnių žievėje nustatytas salicilatų kiekis buvo toks mažas, kad galima teigti, jog ji yra netinkama vartoti gydymui kaip priešuždegiminė ir antipiretinė priemonė.Taigi renkant vaistinę augalinę žaliavą būtina atsižvelgti į gluosnio rūšį, augalo amžių ir metų laiką.