BIOACTIVE PHENOLIC COMPOUNDS FROM WHITE WILLOW (*SALIX ALBA*) BARK, LEAVES AND BRANCHES

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This study aims to investigate the composition of different parts of white willow (Salix alba) which is commonly used in the treatment of painful mobility disorders, such as back pain and arthritis, due to the content of salicylic derivatives, tannins and other phenols. Total polyphenols, total flavones, total tannins and the antioxidant activity were determined by UV-Vis spectrophotometric methods. The salicylic derivatives were determined by a HPLC method. The results show a higher concentration of salicin in the mature bark sample of 20 mg/g. Polyphenols have similar values in the bark and leaves with higher results for the mature willow.

Keywords: white willow, phenols, tannins, HPLC, salicylic derivatives

1. Introduction

White willow (*Salix alba*) is a deciduous tree that grows on the waterfront, in meadows, wetlands, in hilly and plain areas. The stem, which can often grow crooked, reaches heights of 25-30 m. The bark is smooth at first, gray-green, and then cracks appear in length. The leaves are lanceolate, up to 10 cm long, with irregular edges and greenish-silver color. For therapeutic purposes, the bark of young branches, leaves and flowers of the male type (catkins) are harvested.

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White willow is the main source of salicin and other salicylic derivatives - salicortin, 2'-O-acetylsalicortin and tremulacin - similar compounds in terms of structure with aspirin (acetylsalicylic acid), often white willow being called "vegetable aspirin". Salicin is cleaved into glucose and saligenin (o-oxybenzyl alcohol or saligenol) by enzymatic hydrolysis induced by emulsin and diastase. Saligenin in turn produces, by oxidation, salicylic acid, with notable analgesic, antipyretic and antirheumatic properties, thus achieving a gradual, prolonged effect. Also, the tannins present in willow bark have a tonic, astringent, coagulant and slightly hemostatic action[1].

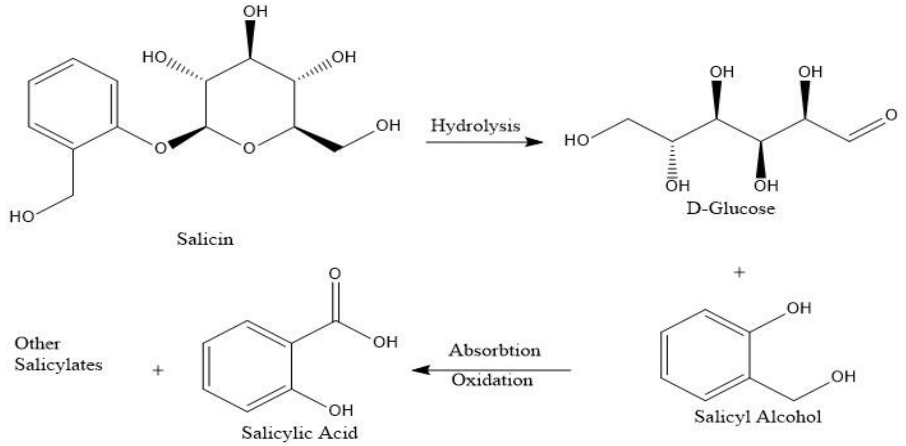

Fig. 1. Conversion of Salicin→Saligenin→Salicylic Acid

As peripheral analgesics, salicylic acid and aspirin belong to the group of anionic drugs that inhibit the generation of pain in nociceptors; at the same time it develops an obvious anti-inflammatory action which is based on the ability to concentrate the active substance in the tissue environment which has become acidic as a result of the inflammatory process. Pain is triggered in nociceptors by a series of chemical mediators released with tissue alteration, and sensitivity to pain is exacerbated by the presence of PGE1 and PGE2 in the outbreak. Salicylic acid resulting from the metabolism of salicoside and related glycosides intervenes in the metabolism of arachidonic acid by inhibiting COX and, thus, the synthesis of PG, PC and TX. As the concentration of PG in the inflammatory focus decreases, the pain decreases in intensity or even disappears[2].

This paper presents studies done to investigate the composition of different parts of young and mature white willow – bark, branches and leaves – by HPLC: total salicylic derivatives expressed as salicin equivalents and UV-Vis spectrophotometric methods: total polyphenols expressed as gallic acid equivalents (Folin-Ciocalteu method), total flavones expressed as rutin equivalents and total tannins expressed as pyrogallol equivalents. We also aimed to correlate the results obtained for polyphenols, flavones and tannins with

antioxidant activity using the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) method.

The specialty literature contains only a small amount of data regarding the characterization of both young and mature white willow, so this article could represent a starting point for new detailed research resulting in huge benefits for the natural supplements field. Moreover, this article presents a detailed analysis of all the components of white willow – bark, branches and leaves – which represents a novelty itself based on the fact that, in spite of the long and compelling history of traditional use of willow bark, its medical properties are still not fully understood.

2. Materials and methods

Materials:

Tetrahydrofuran (Merck), methanol (Merck), ethanol 96% (Merck), phosphoric acid (Merck), chlorhydric acid 97% (Merck), sodium hydroxide pellets (Merck), D (-) salicin (Sigma-Aldrich), sodium acetate (VWR), aluminium chloride (VWR), rutin (Sigma-Aldrich), gallic acid monohydrate (Sigma-Aldrich), Folin-Ciocalteu reagent (Merck), sodium carbonate anhydrous (Merck), copper sulfate pentahydrate (Merck), neocuproine (Sigma-Aldrich), ammonium acetate (Merck), trolox (Sigma-Aldrich), hide powder (Sigma-Aldrich), pyrogallol (Sigma-Aldrich) were of analytical grade and used with no further purification. Ultrapure water (18.2 MΩ) was used for all experiments. Filtering of the HPLC samples was performed using PA filters with diameter pores of 0.2 μm.

Bark, tree branch and leaves from young and mature trees of white willow were collected from Hofigal Export-Import Company in June 2020. The samples were hand-separated, dried and ground.

Methods:

HPLC was performed using an Agilent EZChrom *Elite* system equipped with a DAD detector and a HiCHROM LiChrosorb 100 RP-18 column, 10 μm particle (4.6 x 250 mm).

Extraction of salicylic derivatives: for the analysis of the samples represented by bark, branches and willow leaves, the sample (1 g) was extracted with 8 mL of a solution consisting of a part of 4.2 g/L NaOH solution and a part of methanol. The mixture was stirred at 60°C at reflux for 60 min. After cooling, 0.4 mL of 10 g/L HCl solution was added and the mixture was centrifuged at 9000 rpm for 5 minutes. The supernatant was diluted to 10 mL with a mixture of equal volumes of methanol and high purity water. Before HPLC analysis, the samples were filtered with a PA filters.

Determination of total salicylic derivatives content expressed as salicin equivalents: qualitative and quantitative determinations of salicin derivatives have

been performed according to the adapted method of the 9th edition of the European Pharmacopoeia[3]. The analysis was performed in duplicate at a flow rate of 1 mL/min using tetrahydrofuran as phase A and 0.005 M phosphoric acid as phase B in a gradient program as detailed in Table 1. The volume of the sample injected was 10 µL. Analytes were detected at 270 nm.

Table 1

Mobile phase gradient of the HPLC method

Time, minutes	Mobile Phase A, %	Mobile Phase B, %
0 - 8	5	95
8 - 9	5→10	95→90
9 - 30	10	90
30 - 31	10→5	90→95
31 - 41	5	95

Salicin was used as the standard in order to perform the quantitative determinations. Retention time for salicin is at about 7.8 minutes. Results were quantified as salicin equivalents using a standard curve of 0.1 - 0.5 mg/mL salicin. The correlation coefficient was 0.9992 which shows a strong linear relationship between the two variables – concentration and area. (Fig. 2)

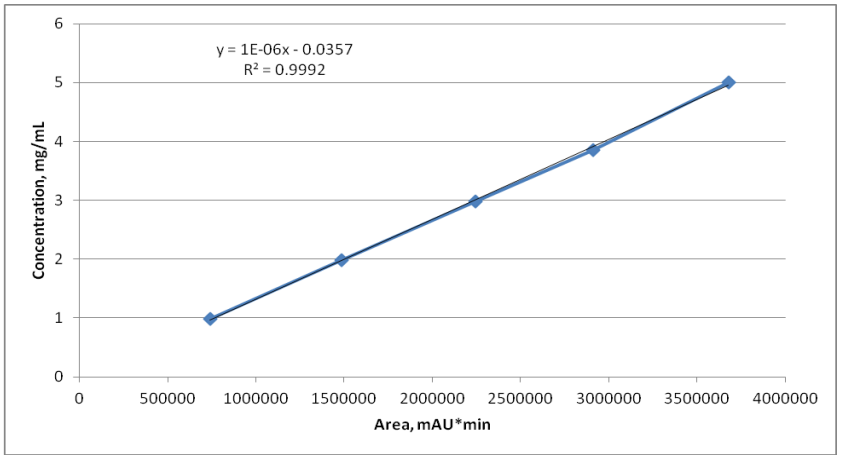

Fig. 2. The calibration curve of salicin

UV-VIS Spectrophotometry was performed on a Jasco V-530 UV-Visible Spectrophotometer, with 2.0 nm Resolution and Double-beam Configuration.

Extraction of phenolic compounds: the samples were extracted using the following method: an extract was prepared in a 1:10 of plant:solvent ratio in an ethanol solution in water with a concentration of 50% vol. by refluxing for 30 minutes. The obtained extract was filtered hot.

Determination of total polyphenolic content expressed as gallic acid equivalents: the polyphenols in the extracts are determined colorimetrically using Folin-Ciocalteu phenol reagent. The reagent contains phospho-tungstic acids as

oxidants, which on reduction by readily oxidized phenolic hydroxy groups yield a blue colour with a broad maximum absorption at 765 nm. This is due to the formation of so-called tungsten and molybdenum blues. The Folin-Ciocalteu phenol reagent reacts with a wide range of polyphenol compounds and, although the response can vary with the individual components, selection of gallic acid as a calibration standard enables useful total polyphenol data to be obtained[4].

Determination of total flavones content expressed as rutin equivalents: the flavone derivatives content was determined by the reaction with aluminum chloride and total content was expressed in rutin.

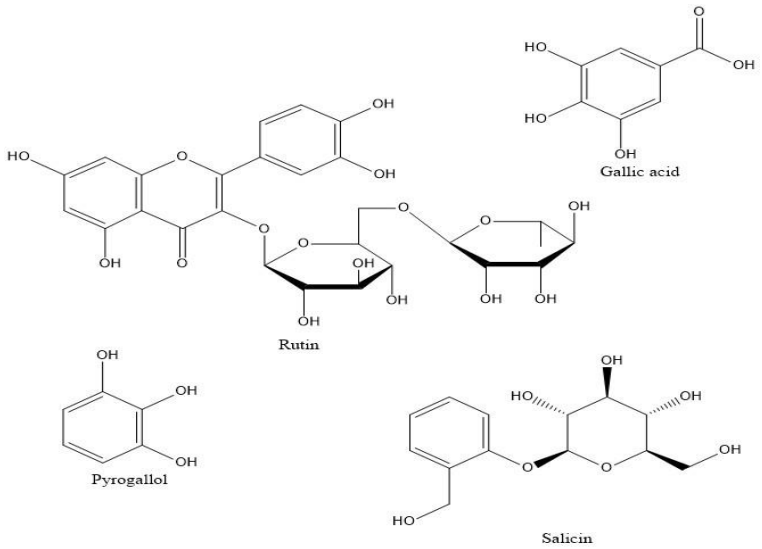

Fig. 3. Bioactive compounds of white willow

Extraction of tannins: 2.5 g of the sample were extracted in 150 mL of ultrapure water at reflux for 30 minutes. The contents were then transferred to a 250 mL flask, rinsed with water. The extract was decanted and then filtered; the first 50 mL of filtrate were removed and will be hereinafter referred to as stock solution.

Determination of total tannins content expressed as pyrogallol equivalents: the tannins were determined by Folin-Ciocalteu method. The reaction product from Folin-Ciocalteu is result of unspecific oxidation with polyphenols. Thus, the coloration and intensity observed after reaction does not correspond exclusively with tannins. Therefore, the complexation of tannin is indispensable to attribute specificity to the method. Then, the tannins are removed by filtration or centrifugation after formation of insoluble complexes with macromolecules. After this, the content of tannin is established by the difference between the reaction product of total polyphenols and the reaction product of the fraction of non-adsorbed polyphenols.

Determination of antioxidant activity

The CUPRAC - cupric ion reducing antioxidant capacity - method is a simple and versatile antioxidant capacity assay useful for a wide variety of polyphenols, including phenolic acids, hydroxycinnamic acids, flavonoids, carotenoids, anthocyanins, as well as for thiols, synthetic antioxidants, and vitamins C and E. This method is based on electron transfer – it detects the ability of a potential antioxidant substance to transfer an electron to reduce any compound. The calibration curve was performed with a Trolox solution of known concentrations, 10-60 μg/mL.

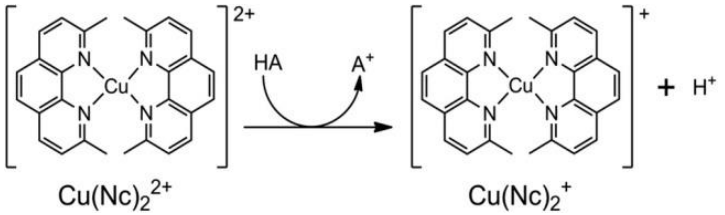

Fig. 4. Reaction mechanism of copper reducing antioxidant capacity (CUPRAC)

3. Results and discussions

Salicin derivatives are the main constituents of willow bark and can be quantified as salicin equivalents. According to the Monograph of the European Pharmacopoeia[3] and the Evaluation Report of the European Medicines Agency [2], the content of salicin derivatives in the bark of different species of willow varies from 0.5 to 10%. However, quality standards involve at least 1.5%. Results in Fig. 5 show a higher content of salicylic derivatives in the willow bark than in the tree branch and in the leaves salicylic derivatives were undeterminable, for both young and mature willow.

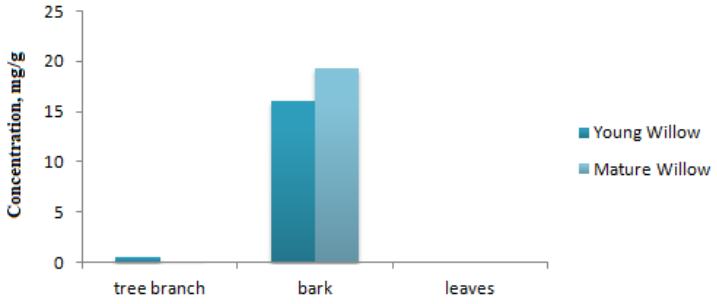

Fig. 5. Total salicylic derivatives expressed as salicin, mg/g

Figs. 6-8 depict the HPLC chromatograms of tree branch/bark/leaves *Salix alba* extracts. Salicin was identified by the external standard method and by comparing the retention times (RT) with the one of the standard, in the same

chromatographic conditions and quantified by the external standard method. The retention time for both external standard and samples was 7.8 minutes.

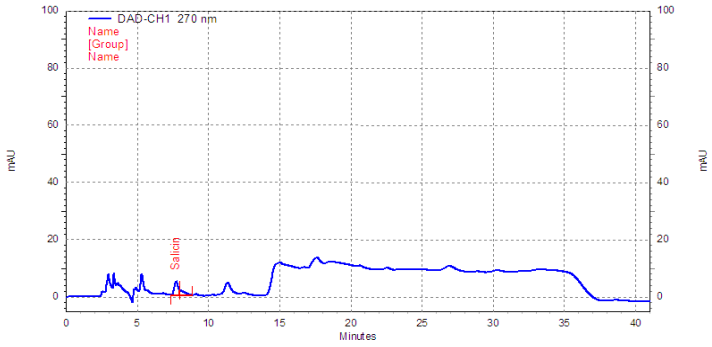

Fig. 6. HPLC chromatogram for willow tree branch

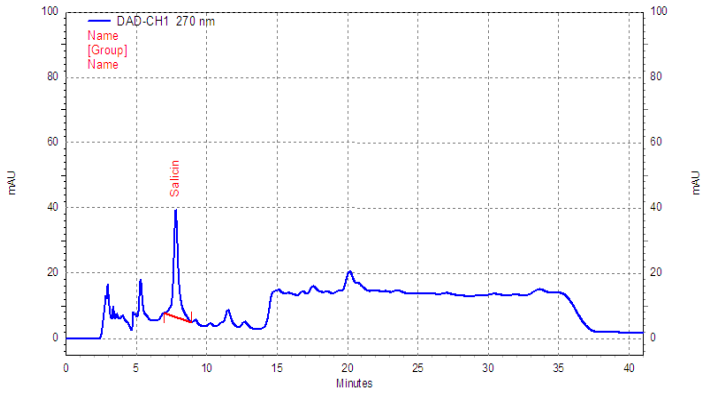

Fig. 7. HPLC chromatogram for willow bark

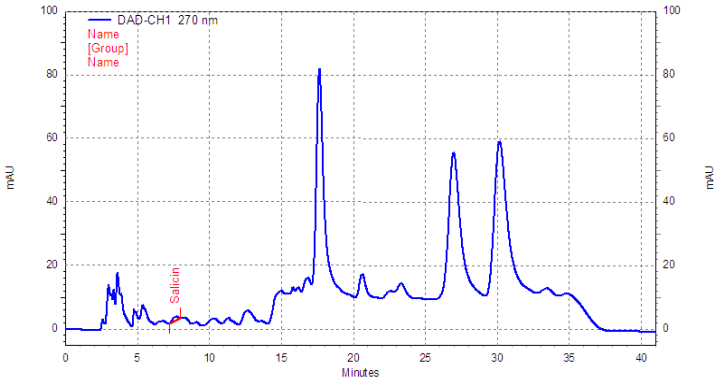

Fig. 8. HPLC chromatogram for willow leaves

In the present study, the phytochemical profile of hydroethanolic extracts of *Salix alba* branch, leaves and bark was analyzed using UV-Vis

Spectrophotometry. The results of the qualitative and quantitative analyses are summarized in Figs. 9–12.

According to reported studies the total polyphenolic content in the *Salix alba* bark is between 20 and 50 mg/g, so the values obtained in our study, 53,2 mg/g and 63,1 m/g for young, respectively mature willow bark are above this range.[5, 6, 7] Polyphenols have similar values in the bark and in the leaves, but much lower values in the tree branch, also the results are higher for mature willow.

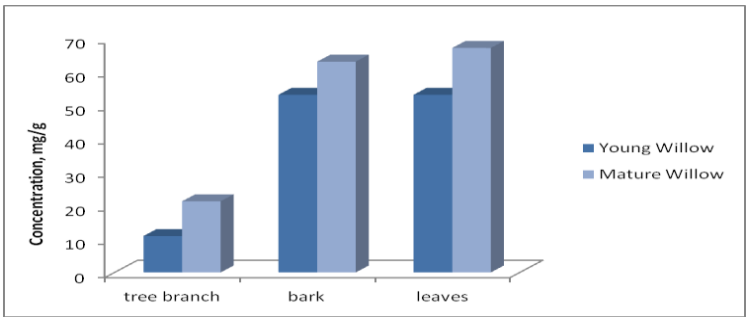

Fig. 9. Total polyphenols expressed as gallic acid, mg/g

In addition to total polyphenolic content, spectrophotometric methods are also used in the determination of total contents of individual groups of phenolic compounds, such as tannins and total flavones. Flavones determination show a higher content in the young willow, the highest value was obtained for the leaves.

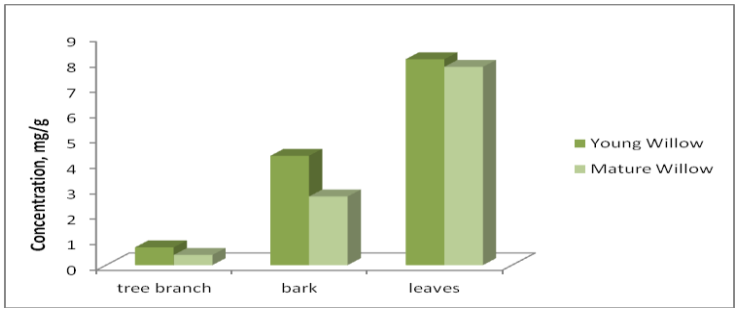

Fig. 10. Total flavones expressed as rutin, mg/g

The lowest tannins content was obtained for the tree branch – an equal value for young and mature willow – higher values were registered, as expected, for willow bark samples.

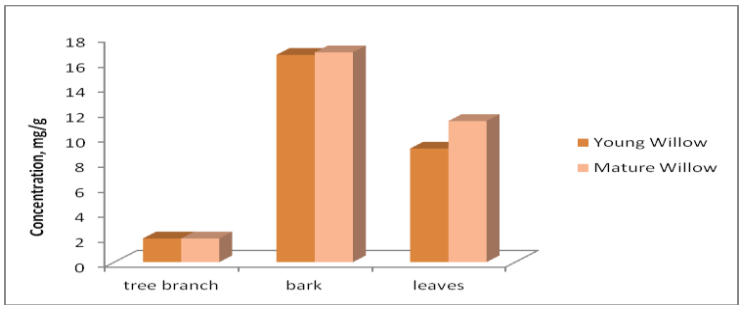

Fig. 11. Total tannins expressed as pyrogallol, mg/g

The results for the antioxidant activity of the willow extracts indicate the influence of the polyphenols and flavones present in each extract. For both young and mature willow tree branch the antioxidant activity is very low in comparison with the other samples, also the highest values are registered for willow bark samples[8].

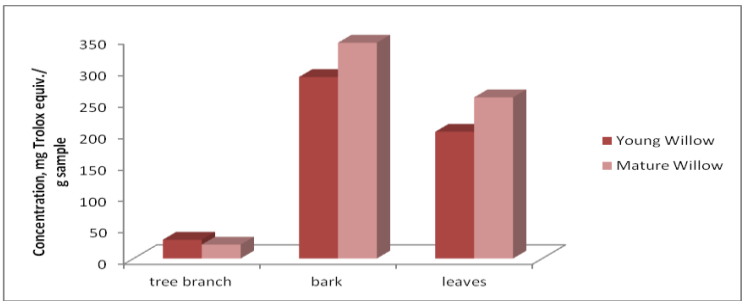

Fig. 12. Antioxidant activity, mg Trolox equiv./g sample

4. Conclusions

We have investigated the composition of different parts of *Salix alba*, tree branch, bark and leaves in order to determine the content in biological active substances such as: salicin, polyphenols, flavones, tannins. Although willow bark remains the main source of salicin, willow leaves proved to be an important source of polyphenols (flavones, tannins) with antioxidant activity comparable to willow bark.

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