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SALICIN CONTENT FROM *SALIX ALBA* L. AND *SALIX PURPUREA* L. EXTRACTS AND ITS ANTIBACTERIAL EFFECTS

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Abstract: The species *Salix alba L.* and *Salix purpurea L.* are medicinal herbs, frequently used in the pharmaceutical industry. This study performs phytochemical and antibacterial analysis of ethanolic extracts from the bark of these two species. The qualitative and quantitative phytochemical analyses were performed by thin-layer chromatography identifying the salicin and salicylic acid; the salicin content was assessed by high performance liquid chromatography. The bark extracts yielded 4.300 µg/mL salicin for *Salix alba* and 1.167 µg/mL salicin for *Salix purpurea*. The plant extracts were tested on Gram negative and positive bacteria (*Escherichia coli* and *Staphylococcus aureus*). Both tested strains showed a mild resistance, but *S. aureus* showed slightly higher inhibition by all extract samples.

Keywords: salicin, salicylic acid, *Salix alba* L., *Salix purpurea* L., HPLC, antibacterial activity.

Introduction

Salix alba L. and *Salix purpurea* L. belong to kingdom *Plantae*, phylum *Malpighiales*, family *Salicaceae*, genus *Salix* [22]. The distribution of *Salix alba* L. (White Willow) ranges from the Mediterranean zone to Central Russia and up to the borders with China, Iran and Anatolia [12]. On the surface of the leaves there are silky white hairs, a feature that makes it easy to recognize from a distance, hence the specific name *alba.* It has slender catkins, with leaves in the spring, and in the autumn young light-yellow shoots [12]. *Salix purpurea* L. (Purple Willow) has a distribution range from Europe to North Africa, Central Asia and Japan. It is a small tree, usually 1-2 m high [12]. One way to recognize this species is the large number of long and thin shoots formed at root level [21, 25, 26]. It adapts to drought and high soil salinity, which is why it readily grows on sandy soils. It has a bitter taste, which largely limits destruction by insects and herbivores [12].

The bark of *Salix* spp. contains numerous phenolic heterosides which confer medicinal and tinctorial properties to this family [2, 6]. The wood of *Salix alba* L. contains 54% cellulose, 26.3% lignin, and the extractive content reaches 2% [27]. The bark contains phenolic compounds such as: caffeic, ferulic, p-hydroxybenzoic, vanillic, cinnamic and p-coumaric acids and their

derivatives; and salicylic glycosides: salicin, salicortin, populin, fragilin, tremulacin and naringenin, known for their therapeutic, mainly antioxidant and anti-inflammatory, properties. The main components of *Salix* extracts are monoterpenes, such as isomers of lilac aldehydes, hotrienol, cis-/trans oxy-linalool and terpinene-4-ol [13]. Due to the high silicate concentration and rapid growth, many of them are recommended as promising sources for plant-derived medicines in the pharmaceutical industry [2, 9]. The main active ingredient of *Salix alba* L. is a derivative of salicylic acid, salicin, which is commercialized under the name acetyl-salycilate [15]. This plant is the most important natural source of salicin, an alcoholic β-glucoside. Salicin is transformed to salicylic acid within the organism. Substitutes of phenolic group in many products include groups such as methyl, acetyl and ether bonds [5].

The chemical components of *Salix purpurea* L. include a mixture of glycosides as well as phenols, salicylic acid and thiols. The active compound is salicylic acid [8].

Salicin is a white powder, with a bitter taste, which can be obtained by aqueous extraction from willow bark and leaves [10]. The other names associated with salicin are: β-D - glucopyranoside, 2-(hydroxymethyl) phenyl, 2 (hydroxymethyl) phenyl- β- D glucopyranoside, salicoside and glucoside of salicylic alcohol [16]. Many researchers claim that the storage form of salicylic acid is salicin [24].

Salicin biosynthesis is associated with the phenylpropanoid pathway (shikimic acid pathway), which begins with phenylalanine. By using marked precursors, it was proved that salicin biosynthesis takes place in five steps: deamination, ortho-hydroxylation, β-oxidation, ethyl unit removal and glycosylation. This process is considered to take place in cytoplasm [17]. The *Salix* species contain a high number of toxic phenols, including salicin. After ingestion salicin is hydrolyzed and oxidized to salicylic acid, which decouples oxidative phosphorylation in mitochondria. It is thought that the role of this compound in plant is to hinder the attacks of herbivores and pathogens [14].

Salicin (Fig. 1) is known for its antirheumatic and analgesic effects [22]. Salicin is an alcoholic β-glycoside classified as a simple phenolic compound containing D-glucose. It is obtained from different *Salix* and *Populus* species. It is a pro-drug which is gradually hydrolyzed to aligenin by intestinal bacteria and is converted to salicylic acid after absorption. Thus, it performs antipyretic action without causing gastric lesions [23]. It has anti-inflammatory effect when administered orally, and it is presumed that some salicin-containing products have anti-aging effects [10].

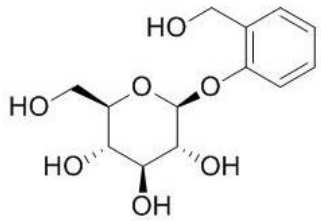

Fig. 1: The structure of salicin

The salicylic acid is present in plants as free phenolic acid or in conjugated forms, resulting from methylation, hydrolysation or glycosylation of the aromatic ring. The best-known derivative of salicylic acid is salicin, found in *Salix alba* L. and *Salix purpurea* L. In normal conditions, it is present in plant tissues in quantities of a few mg-ng per 1 g fresh mass [11]. Salicylic acid is

synthesized in plants by two different enzyme pathways, which use chorismate as primary metabolite. Salicylic acid synthesis can take place in chloroplasts. It is a two-step reaction, catalyzed by isochorismate synthase and isochorismate pyruvate synthase. It has been shown that salicylic acid synthesized by this pathway has an important role in defending plants against infection [7].

An alternative route to synthesize salicylic acid is the phenylalanine pathway, localized in cytoplasm [11]. In vascular plants it has been ascertained that salicylic acid derives from phenylalanine pathway. There are two paths of salicylic acid synthesis by this pathway, which differentiate at the point of aromatic ring hydroxylation [18]. Phenylalanine derived from chorismate is converted to trans-cinnamic acid by phenylalanine amino ligase [11]. Later, trans-cinnamic acid is oxidized to benzoic acid. The aromatic ring of benzoic acid is hydrolyzed, a reaction which leads to salicylic acid [7].

Salicylic acid is a compound used as an intermediate in synthesis of medicines and cosmetic products [9]. Obtaining salicylic acid became a necessity in order to meet the demands of industry. This is obtained by the Kolbe-Schmitt reaction (Fig. 2). Another method to obtain salicylic acid is by glycosylation. The capacity to produce glycosides is a characteristic of plant cells.

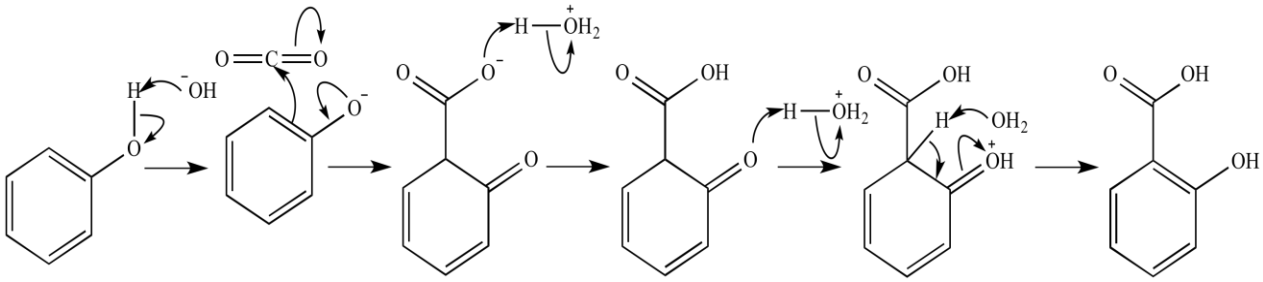

Fig. 2: Salicylic acid production by Kolbe – Schmitt reaction [35]

The aim of the present study was to perform a qualitative and quantitative analysis of the glycosides (salicin) present in the two species, as well to test the antimicrobial activity of tinctures obtained from *Salix* cortex, hypothesizing that they may act like a natural antibiotic.

Materials and Methods

Materials, reagents and apparatus. Salix alba L. and *Salix purpurea* L. bark samples were collected from wild in the woods near Cluj-Napoca city, in the springtime and autumn of 2020. The fresh plant material was processed immediately after collection. A voucher specimen was sampled, retained in the herbarium, each time and the botanical identification was performed by the specialists from PlantExtrakt quality control laboratories. The extracts were obtained according to provision of European and German Homeopathic Pharmacopoeias. There were obtained on GMP (Good manufacturing practices) certified production flow mother tinctures using 90% vol. ethanol, the extraction ratio being 1:1.4 (dry part of the bark plant-solvent). The extraction was made in the cold, by maceration for 10 days, with daily mixing followed by pressing and filtering [19, 30, 33, 34].

The silica gel plate (Kiselgel F_{254}) was purchased from Merck, Germany. The HPLC (High Performance Liquid Chromatography) column, type Luna 5 µm C18 (2) 100 A of 150 x 4.6 mm

was purchased from Phenomenex, USA. The Sil-C18 SPE columns were purchased from Merck, Germany. The Teflon Millex filters were purchased from Merck-Millipore, USA. Solvents and reagents: methanol, acetic acid, ethyl acetate were supplied from Merck, Germany. The standards: salicin and salicylic acid were obtained from Phytolab, Germany.

Experimental Conditions for TLC Separation. The thin layer chromatographic analysis was performed using silica chromatographic plate with fluorescence indicator at 254 nm. As standards, methanolic solutions of salicylic acid (2.00 mg/mL) and salicin (1.00 mg/mL) [33] were used. The mobile phase was ethyl acetate (Merck) – methanol (Merck) – purified water (100:13.5:10, v/v) on 15 cm. It was applied 20 µL from each mother tincture and 25 µL from salicin and salicylic acid. After drying the plate at room temperature, the chromatograms were observed in ultraviolet radiation at 254 nm. The chromatograms were observed under a Camag Reprostar II lamp and documentation system equipped with a HP digital camera.

Experimental Conditions for HPLC Separation. The determination was carried out on a Varian Star HPLC system. A silica C18 column (Phenomenex, Luna C18, 150 x 4.6 mm, 5 mm) was used. As mobile phase, a mixture of acetic acid (Merck), methanol (Merck) and purified water (1:40:60) was used. The flow rate was 0.5 mL/min [13]. The DAD detector was operated at 270 nm and the injection volume was 10 µL for samples and standard. Each extract was diluted 1 to 10 with methanol prior injection and filtered through 0.45 µm Teflon filter. As standard was used salicin (1.00 mg/mL), in methanol. For quantitative determination different concentrations of salycin (0.05-1 mg/mL) were used. UV–visible spectrophotometric analyses were carried out with an UV–visible spectrophotometer.

Experimental procedure for bacterial susceptibility. The susceptibility of the bacteria test (Gram positive-*Staphylococcus aureus* ATCC 25923, and Gram negative-*Escherichia coli* ATCC 25922) to plant extracts was determined using a paper disc assay on Mueller-Hinton plates [1], following the diffusimetric method [4]. Diluted bacterial cultures were adjusted to 0.5 McFarland turbidity (1-2 x 10^6 cfu mL^{-1} (colony-forming units) and spread evenly over the entire surface of the Mueller-Hinton plates using a sterile cotton swab. The plates were allowed to air-dry for approximately 10 minutes before paper discs (6 mm) were placed on the agar plate. Volumes of 10 µl of *S. alba* and *S. purpurea* extracts and control (ethanol 70%) were tested. Each extract test was replicated three times. The plates were incubated at 37ºC for a 24 h period. For each microorganism tested, zones of inhibition of bacterial growth were examined, and the diameter of each zone was measured.

Results and Discussion

The TLC analysis revealed the presence of salicin and salicylic acid in *Salix alba* L. and *Salix purpurea* L. bark extracts (Fig. 3). The number of bands indicates the presence of more compounds in the composition of extracts, which were separated according to their affinity on the silica gel plate. The difference is given by the intensity of the bands, which is directly proportional to concentration. The presence of a band which migrated at the same distance in mother tinctures as the standard, indicates the presence of the compound in tinctures. On the plate could be identified the presence of the two compounds salicin (Fig. 3a) and salicylic acid (Fig. 3b) in both extracts.

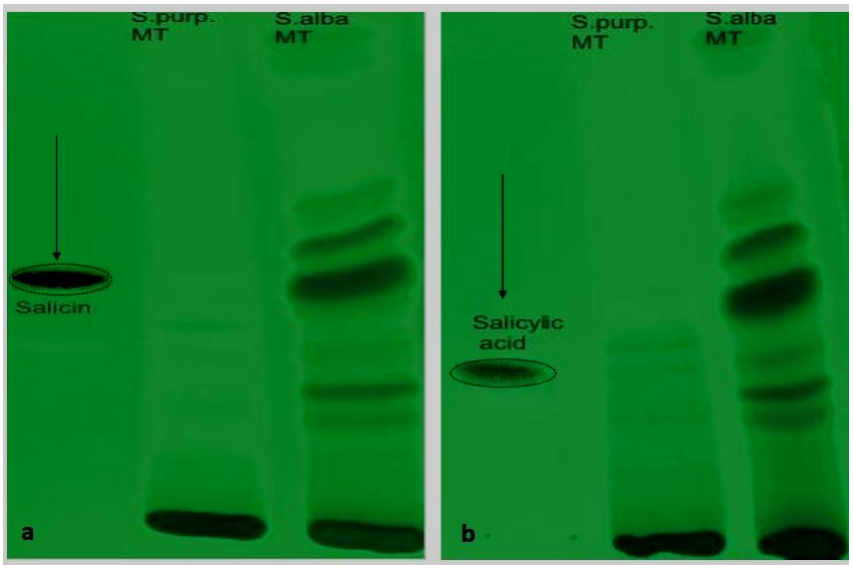

Fig. 3: The TLC chromatograms in UV 254 nm salicin (a), salicylic acid (b)

Visualizing the results of TLC does not offer us information regarding the quantity of the compounds present in tinctures. This only confirms their potential presence. A calibration curve for salicin used for determination of the content by HPLC was established.

Based on the calibration curve for salicin, the quantity of salicin in both extracts was calculated by the formula: Area = $3x10^7$ x Conc [µg/mL] + $1x10^6$; R^2 = 0.990. The salicin concentration in both extracts was assessed by HPLC and it was noticed that *S. alba* L. contains a higher concentration of salicin (4.300 µg/mL) than *S. purpurea* L. wherein only 1.167 µg/mL were detected. The HPLC analysis evidenced more peaks that were compared with the standard and the peak corresponding to salicin can be identified. Salicin standard is separated at 4.2 min. By analogy, a peak with the same shape of the absorption spectrum was identified on the chromatogram of the mother tincture of *Salix alba* at 4.4 min (Fig. 4).

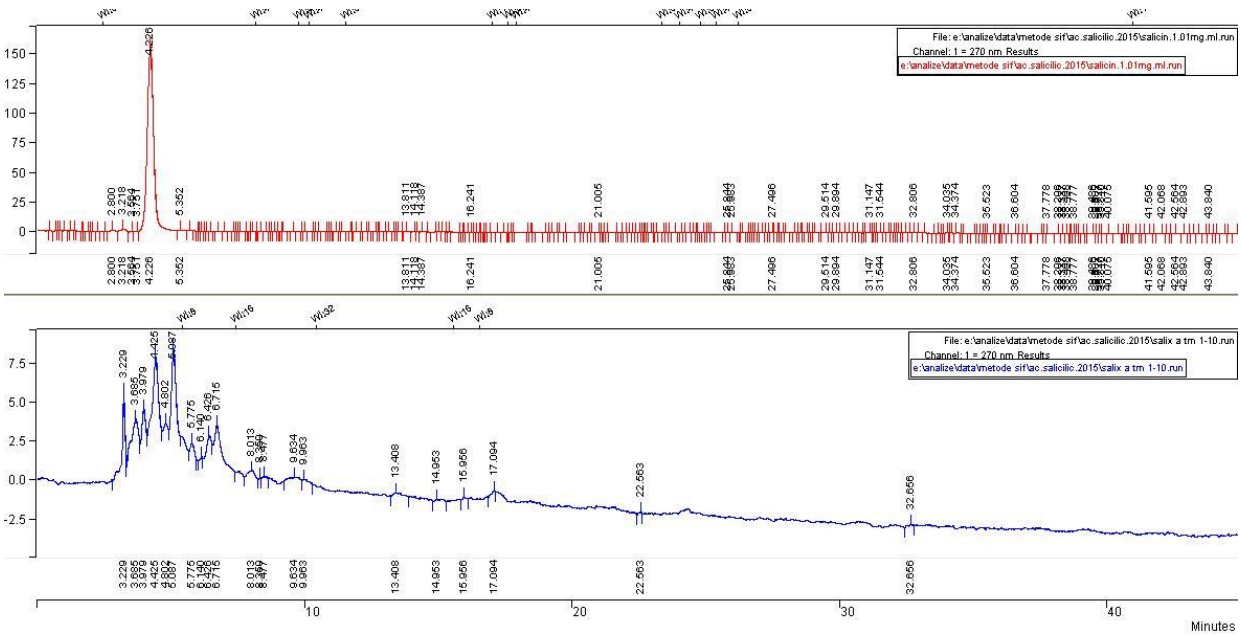

Fig. 4: Chromatogram of the standard salicin (red) and chromatogram of *Salix alba* mother tincture (blue)

In the case of *Salix purpurea* tincture, the peak whose absorption spectrum has the same shape as standard was marked out at 4.6 min (Fig. 5).

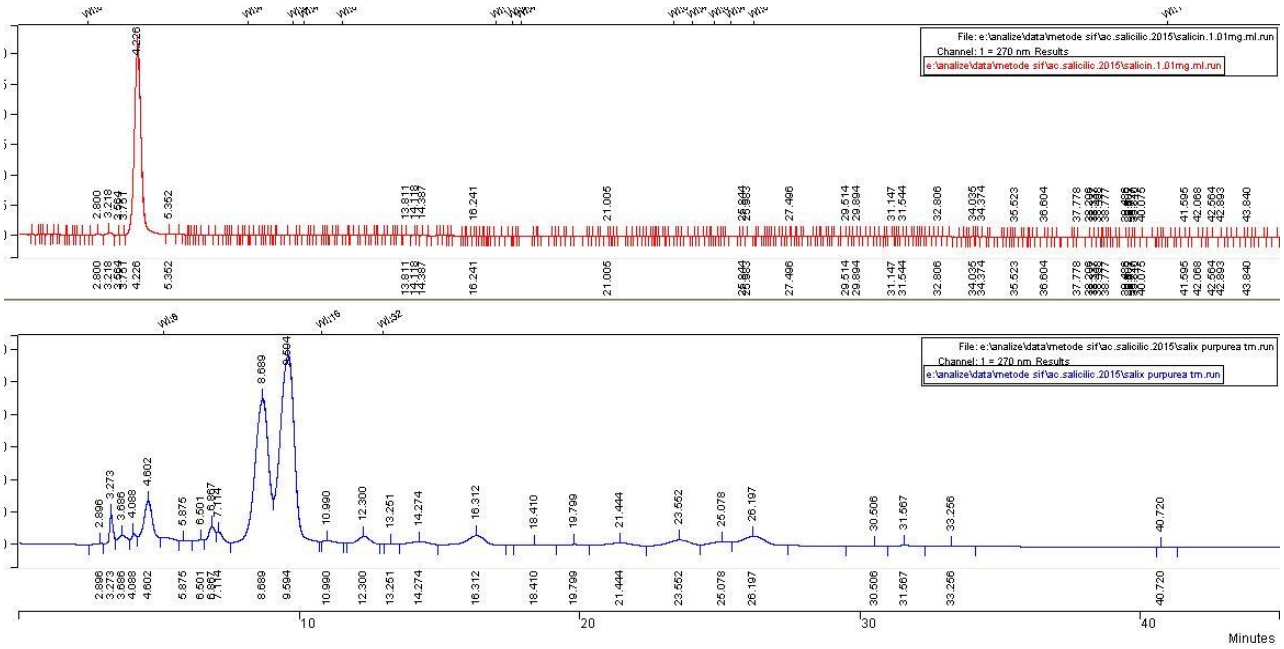

Fig. 5: Chromatogram of standard salicin (red) and chromatogram of *Salix purpurea* mother tincture (blue)

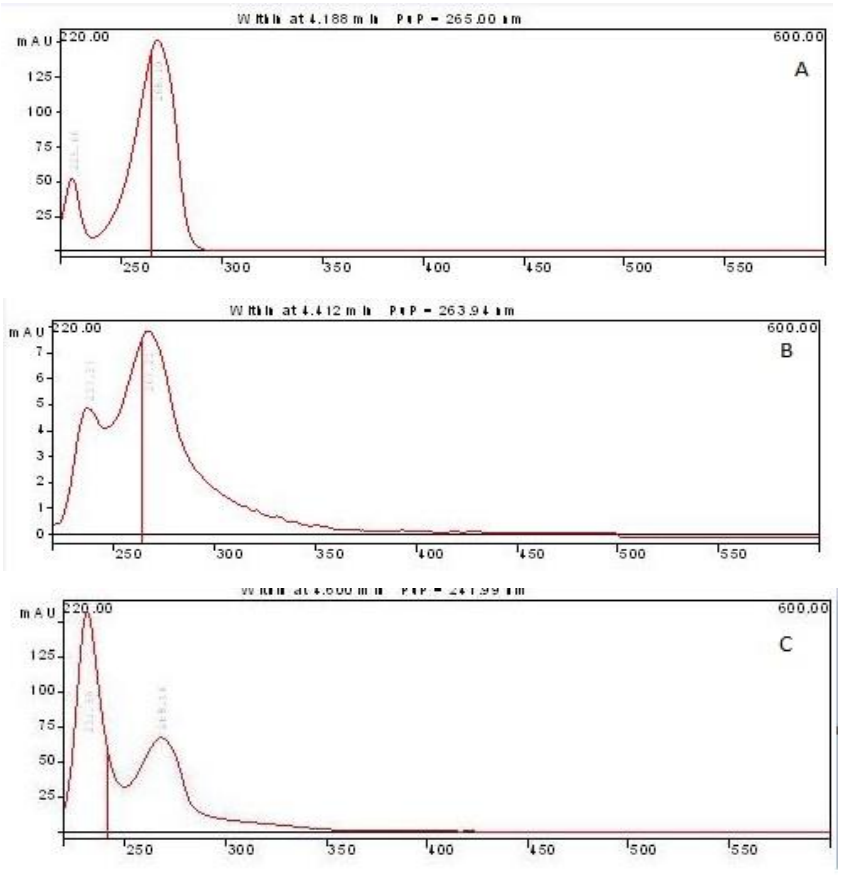

Fig. 6: UV-Vis spectra of salicin (A), *Salix alba* mother tincture (B), *Salix purpurea* mother tincture (C)

In figure 6 are presented the UV-Vis spectra of salicin but also UV-Vis spectra for *Salix alba* mother tincture and *Salix purpurea* mother tincture. These analyses were carried out with an UV–visible spectrophotometer.

For testing the bacterial sensitivity to *Salix* spp. extracts, two plates were inoculated with bacterial suspension of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. On each plate with Mueller-Hinton culture medium, 5 disk papers impregnated with plant extracts and control (ethanol 70% vol.) were applied and were incubated for 24 h. In Fig. 7A are presented the plates with extract samples and control, respectively 70% vol. ethanol at the beginning of testing.

After 24 h of incubation, the inhibition zones for the tested strains were observed (Fig. 7B). According to the diameter of the clear zone the intensity of the antimicrobial effect is assessed [20]. It can be observed that for the Gram negative strain (*E. coli*) the sensitivity is lower than in the case of *S. aureus*, reaching an average inhibition zone of maximum 7.5 mm for *Salix alba* extract.

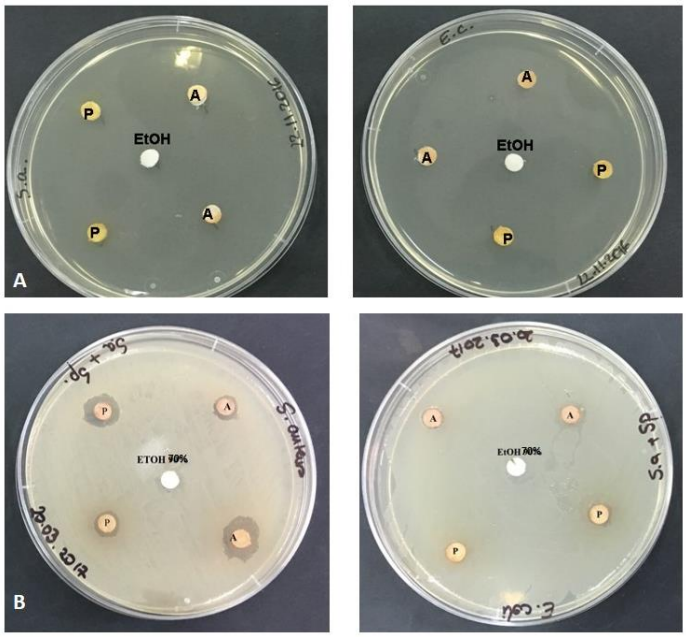

Fig. 7: Disk papers on Mueller-Hinton culture media inoculated with *S. aureus* (S.a.), *E. coli* (E.c.) (control = ethanol 70% vol.; A= TM *Salix alba*; P= TM *Salix purpurea*). Line A=at the 0 moment; line B=after 24 hours of incubation

The average diameters of the inhibition zones for mother tinctures of *Salix alba* and *Salix purpurea*, measured on plates inoculated with test strains of *Staphylococcus aureus* and *Escherichia coli* are presented in Table 1.

Various metabolites which have been studied for their bioactivity are known to be contained in the willow bark and, among these, phenolic compounds have the highest concentration [28]. Bioactive phenolics are known to represent a range of compounds, which comprises phenolic acids, phenolic glucosides, lignans, flavonoids, stilbenes and tannins [31]. Salicin is one of the most important bioactive compounds present in willow bark, which acts within the metabolic

pathway, responding to pathogens and environmental stress in order to limit their negative effects [29].

Table 1: Inhibition zone diameters (mm) for paper disks impregnated with tinctures

Samples	Average diameters for inhibition zone (mm)	
	Staphylococcus aureus ATCC 25923	*Escherichia coli* ATCC 25922
Control (EtOH 70%)	0 mm	0 mm
Salix alba	9.5 mm	7.5 mm
Salix purpurea	9 mm	7 mm

More studies investigated the anti-inflammatory properties of willow bark extract by studying salicin and other compounds. Not only salicylic acid and salicin but also other phenolic glucosides occurring in willow biomass have antipyretic, analgesic, anti-inflammatory, antirheumatic, antimicrobial and anticoagulant effects in humans, animals and bacterial cells [3, 31, 32].

Conclusions

This study highlights the differences and the similarities between the phytochemical profiles of two species of *Salix* (willow). In it was presented an analysis of *Salix alba* and *S. purpurea* extracts, used in diverse therapies, that proved to have salicin and salycilic acid, but at different concentrations.

The quantity of salicin in the tested extracts was much higher in the *Salix alba* extract compared to the *Salix purpurea* extract. This result was also observed when testing antimicrobial activity: *Salix alba* extract showed a more intense inhibition on the bacterial growth, mostly for *Staphylococcus aureus*. This led us to conclude that possibly the salicin and salicylic acid have a main contribution to the antimicrobial effect of the extracts.

Not only the identified compounds (salicin and salicylic acid) are responsible for the antimicrobial activity, but also the whole amount of secondary metabolites present in the two extracts. In any case these results lead us to conclude that the *Salix* spp. extracts, with their phytocomplex, can be therapeutically useful also as antibiotics in *Staphylococcus aureus* infections.

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CONȚINUTUL SALICINEI DIN EXTRACTE VEGETALE DE *SALIX ALBA* L ȘI *SALIX PURPUREA* L. EFECTELE ANTIBACTERIENE ALE ACESTEIA

(Rezumat)

Speciile *Salix alba L.* și *Salix purpurea L.* sunt plante medicinale folosite frecvent în industria farmaceutică. Acest studiu include analize fitochimice și biologice asupra extractelor etanolice din scoarța celor două specii. Analizele fitochimice calitative s-au realizat prin cromatografie în strat subțire indentificându-se salicina și acidul salicilic în cele două extracte. Prin cromatografie de lichide de înaltă performanță s-a înregistrat conținutul de salicină pentru cele două extracte: 4.300 µg/mL pentru *Salix alba* respectiv 1.167 µg/mL pentru *Salix purpurea*. Extractele vegetale au fost testate pe tulpini bacteriene Gram negative și pozitive (*Escherichia coli* și *Staphylococcus aureus*). Ambele tulpini au arătat rezistență medie, dar la *S. aureus* inhibiția a fost putin mai mare pentru ambele extracte vegetale.

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