Species of the Genus *Salix* L.: Biochemical Screening and Molecular Docking Approach to Potential Acetylcholinesterase Inhibitors

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Received: 1 April 2019; Accepted: 28 April 2019; Published: 5 May 2019

Abstract: The genus *Salix* includes about 500 different, mainly woody species with potentially significant medicinal values. The aim of this study was to evaluate the chemical composition and antioxidant activity of little-studied bark and leaves extracts of seven different species of the genus *Salix*, and to examine the acetylcholinesterase (AChE) inhibitory potential of selected compounds. The extracts were characterized by High Pressure Liquid Chromatography (HPLC). Total phenolics and flavonoids content was determined spectrophotometrically and the antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and hydroxyl radical (•OH) scavenging assays. Molecular docking studies were conducted in order to elucidate the interaction and binding affinity between selected compounds of willow bark and leaves against AChE. The major components in bark and leaves of most of the species were rutin (1.26–22.09 mg/g), salicin (1.62–17.33 mg/g), chlorogenic acid (0.74–7.53 mg/g) and epicatechin (0.71–4.83 mg/g). The latter three compounds demonstrated significant inhibitory potential against AChE in docking studies. All extracts exhibited notable antioxidant activity as scavengers of both DPPH• and •OH. The obtained results indicate that willow species other than those in commercial use, and not only bark, but willow leaves as well, could be utilized as sources of valuable phytocompounds with antioxidant and neuroprotective properties.

Keywords: *Salix*; biological activity; acetylcholinesterase; molecular docking

1. Introduction

The genus *Salix* L. consists of about 500 species distributed mainly in the northern hemisphere, out of which 65 grow in Europe and about 20 are indigenous to the Balkan Peninsula [1,2]. Willow bark (*Salix* spp., Salicaceae) is a traditional herbal remedy used to treat pain, fever and inflammation [3]. Contrary to bark, leaves of many *Salix* species are mainly not studied and there is a general lack of data concerning their medicinal properties. While pharmacological effects of willow bark are mainly attributed to salicin as the major active constituent, newer studies suggest that these effects cannot be explained solely by the presence of salicin and its derivatives, but that other compounds such as flavonoids (flavan-3-ols) and simple phenols (phenolic acids) synergistically contribute to the therapeutic effects of willow bark [4–7]. Certain *Salix* species are known to exhibit strong antioxidant activity in many in vitro systems [8,9]. The anti-inflammatory effect of some phenolic acids has been associated with their antioxidant activity [10]. Antioxidants can protect biomolecules (lipids, proteins and nucleic acids) against potentially harmful effects of free radicals. Oxidative stress, resulting from the imbalance between free radical production and antioxidant defenses, is thought to promote many diseases such as cancer, cardiovascular and neurodegenerative, including Alzheimer’s disease.
(AD) [11]. One of the main causes of AD is low level of acetylcholine (ACh) in the synapses of human cerebral cortex, leading to loss of memory and impairment of multiple cerebral functions [12]. Synthetic inhibitors of acetylcholinesterase (AChE) are standard therapy of AD, preventing the hydrolysis of ACh, thus maintaining the neurotransmitter in acceptable concentrations and improving cognitive functions. However, numerous side effects limit their use and urge a search for safer and more effective therapeutic options of natural origin [13].

Studies dealing with phytochemical characterization of various species from the genus Salix have focused mainly on salicylic compounds found in bark [14]. Phenolic constituents in leaves, leaf buds, flower buds and twigs in several Finnish Salix species were investigated, but the research also included mainly the analysis of salicin derivatives [15]. Simple phenols were analyzed to a certain extent in bark of ten species of the genus Salix [10]. However, there is no comparative analysis of simple phenolics and flavonoids in bark and leaves of different Salix species, as well as of their antioxidant potential. In addition, there is no information available in literature about the chemical composition of S. amplexiaculis, S. babylonica and S. eleagnos. Therefore, the primary aim of this research was to determine the chemical composition and antioxidant potential of bark and leaves of seven species indigenous to the Balkan Peninsula, namely S. alba, S. amplexicaulis, S. babylonica, S. eleagnos, S. fragilis, S. purpurea and S. triandra. The secondary goal of this paper was to elucidate the interaction and binding affinity between selected compounds of willow extracts and AChE using molecular docking. It would be of significant interest to exploit these widely distributed and easily accessible plant crops as sources of valuable phytochemicals in the pharmaceutical industry.

2. Materials and Methods

2.1. Plant Material

Bark and leaf of seven Salix species were collected from various localities on the Balkan Peninsula: S. alba L. 1753—Pecenjevce (43° 06’ 01″ N 21° 54’ 60″ E), June 2014; S. amplexiaculis Bory et Chaub. 1838—Pecenjevce (43° 06’ 01″ N 21° 54’ 60″ E), June 2014; S. babylonica L. 1753—Bosut riverside, Morovic (44° 59’ 31″ N 19° 12’ 22″ E), September 2013; S. eleagnos Scop. 1772 subsp. eleagnos—gorge of Beli Rzav (43° 47’ 31.9″ N 19° 26’ 44.4″E), September 2013; S. fragilis L. 1753—Vrdnik (45° 07’ 13″ N 19° 47’ 16″ E), June 2013; S. purpurea L. 1753 subsp. purpurea—Mountain Deli Jovan (44° 02’ 11.7″ N 22° 12’ 49.49″ E), 95 August 2013; S. triandra L. 1753—Vlasina Lake (42° 42’ 26″ N 22° 20’ 32″ E), July 2013. Voucher specimens were identified and deposited in the Herbarium of the University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology—Herbarium BUNS (no. 2-1471–2-1482). The plant material was air-dried and stored at room temperature. Dried willow bark and leaves were ground in a mill and particle size diameter (d = 0.35 mm) was determined by sieve set (Retsch GmbH and Co KG).

2.2. Chemicals

2,2-diphenyl-1-pycrylhydrazil (DPPH) was obtained from Alfa Aesar (Germany). HPLC grade acetonitrile, methanol, ortho-phosphoric acid, tetrahydrofuran and acetic acid from J.T. Baker (Netherlands). Chlorogenic acid (≥ 95%), p-hydroxybenzoic acid (≥ 99%), syringic acid (≥ 95%), caffeic acid (≥ 98%), rutin (≥ 94%), naringenin (≥ 98%), epicatechin (≥ 98%) and trans-cinnamic acid (≥ 99%) were purchased from Sigma—Aldrich (USA); p-coumaric acid (≥ 9% 8%) from Fluka (Switzerland); quercetin (≥ 99%) from Extrasynthese (France); salicin (≥ 90%) from Carl Roth GmbH (Germany). All other reagents were of analytical grade.

2.3. Extraction Procedure

Bark and leaves of the analyzed willow species (0.5 g) were extracted with 70% aqueous ethanol (v/v) (5 mL) by maceration for 48 h at room temperature (25 °C) [16]. After maceration, the extracts
were collected, filtered and evaporated to dryness under vacuum. The weight of dried extract (d.e.) was measured and the extraction yield was calculated.

2.4. Determination of Total Phenolics and Flavonoids Content

Total phenolics and flavonoids content were determined spectrophotometrically (Agilent 8453 117 UV-Visible Spectroscopy System). The amount of total phenolic compounds in the extracts was assessed using Folin-Ciocalteu reagent at 760 nm by a method described before [17]. The concentration of total phenolics was expressed as mg of gallic acid equivalents (GAE) per g of d.e. (mg GAE/g d.e.), using a standard curve of gallic acid. Total flavonoids content in the obtained extracts was evaluated by a method based on the formation of flavonoid-aluminium complex with the absorbptivity maximum at 430 nm [18]. The flavonoids content was expressed as mg of quercetin equivalents (QE) per g of d.e. (mg QE/g d.e.), using a standard curve of quercetin. All measurements were performed in triplicate.

2.5. Chemical Characterization by High Performance Liquid Chromatography (HPLC)

Chemical characterization of the obtained extracts and quantification of the selected compounds was carried out by HPLC method [19], using Agilent HP 1100 HPLC-diode array detection (DAD) system equipped with an autosampler. The components were separated using reversed-phase Zorbax CB-C18 column (4.6 × 150 mm, 5 µm particle size) held at 25 °C. Solvent A was 0.1% aqueous acetic acid while solvent B was 0.1% acetic acid in acetonitrile. The mobile phase used was delivered in gradient mode (3.25 min 10% B; 8 min 12% B; 15 min 7 133 25% B; 15.8 min 30% B; 25 min 90% B; 25.4 min 100% B), with flow rate of 1 mL/min. UV detection was set at 280 nm. The HPLC mobile phase was prepared fresh daily and filtered through a 0.45 µm nylon filter. The injection volume was 10 µL.

Identification and quantification of gallic, chlorogenic, p-hydroxybenzoic, caffeic, syringic, p-coumaric and trans-cinnamic acid, epicatechin, rutin, quercetin and naringenin was performed. Standards of the selected compounds were dissolved in methanol and injected into the system under the same conditions in order to obtain calibration curves.

Salicin was determined by a slightly modified HPLC method described earlier [20], using Zorbax CB-C18 column (4.6 × 150 mm, 5 µm particle size) and mobile phase consisting of bidistilled water, tetrahydrofuran and ortho-phosphoric acid (97.7:1.8:0.5) (v/v/v) delivered in isocratic mode.

2.6. DPPH Radical Scavenging Assay

The DPPH-assay was performed as previously described [21]. Different volumes (10–100 µL) of samples, dissolved in methanol, were mixed with 90 µM DPPH solution (1 mL) and made up with 95% methanol to a final volume (4 mL). After 30 min at room temperature, absorbance of the resulting solutions and the control (same chemicals without sample) were measured spectrophotometrically at 515 nm. Methanol was used as a blank. For each sample, the experiment was performed in triplicate. Radical scavenging capacity (RSC), expressed as percentage, was calculated by the following equation:

\[
RSC \ (%) = 100 \times \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \tag{1}
\]

where \( A_{\text{blank}} \) represents the absorbance of the control and \( A_{\text{sample}} \) of the sample.

2.7. OH Radical Scavenging Assay

• OH scavenging assay was performed spectrophotometrically as previously described [22] with slight modifications. The reaction mixture consisted of ferrous sulfate (0.5 mL, 1.5 mM), hydrogen peroxide (0.7 mL, 6 mM), methanolic extract (1 mL) and sodium-salicylate (0.3 mL, 20 mM). After incubating the mixture at 37 °C for 1 h, the absorbance was measured at 562 nm. RSC was calculated as:

\[
RSC \ (%) = 100 \times \left( 1 - \left( \frac{A_{1} - A_{2}}{A_{0}} \right) \right) \tag{2}
\]
where $A_0$ is the absorbance of the control without extract, $A_1$ is the absorbance of the mixture with extract and $A_2$ is the absorbance without sodium-salicylate.

### 2.8. Molecular Docking

The docking calculations were performed on (-)-epicatechin ((2R,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol), chlorogenic acid ((1S, 3R, 4S, 5S)-3-[(E)-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-methyl-oxan-2-yl]oxy-methyl]oxan-3,4,5-triol-oxan-2-yl]oxy-chromen-4-one), salicin ((2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-[2-(hydroxymethyl)phenoxy]oxane-3,4,5-triol) and donepezil ((2R)-5,6-dimethoxy-2-[[1-(phenylmethyl)piperidin-4-y]methyl]-2,3-dihydroindene-1-one) (Figure 1).

![Chemical structures of ligand molecules used for molecular docking](http://pubchem.ncbi.nlm.nih.gov/)

- **a**: donepezil; **b**: (-)-epicatechin; **c**: chlorogenic acid; **d**: salicin; **e**: rutin.

Chemical structures of ligand molecules were taken from PubChem database (http://pubchem.ncbi.nlm.nih.gov/). The structures of molecules were geometry optimized using the software Avogadro 2.0 following the MMFF94 method [23]. Three-dimensional crystallographic structure of human AChE enzyme (pdb code: 4ey7) complexed with donepezil [24] was retrieved from Protein Data Bank (PDB) (http://www.rcsb.org/). Before docking the ligands into the enzyme, it was prepared by removing all water molecules, heteroatoms, any co-crystallized solvent and the ligand. AutoDockTools (ADT; version 1.5.6) was used to add hydrogens and partial charges for protein and ligands using Gasteiger charges [25]. The grid box was designed to include the active and peripheral site of human AchE. The dimension of the grid box was 60 × 60 × 60 with distance of 0.375 Å between points, centered at coordinates $x = -14.108$, $y = -43.833$, $z = 27.67$. Molecular docking was conducted using AutoDock 4.2.3. program package (Molecular Graphics Laboratory, La Jolla, CA, USA). Docking simulations were performed using Lamarckian Genetic Algorithm [25] with standard docking procedure for rigid receptor and flexible ligand. A total of 25 runs along with $25 \times 10^5$ energy evaluations and 27,000 iterations were carried out. Other parameters were set to default. Conformations of docked structures with the lowest binding energy were considered as the most favorable docking pose. Discovery Studio Visualizer 4.5. (DSV; Dassault Systemes BIOVIA, San Diego, CA, USA) was used to visualize binding interactions and produce the figures. In order to validate the docking procedure, docking was performed with the co-crystallized AChE: Donepezil, which was re-prepared in DSV. The RMSD value between re-docked conformation and the original structure, which was ≤ 1.5 Å, indicated the reliability of the binding ability prediction of new ligands.
2.9. Statistical Analysis

The data were reported as mean value ± standard deviation (SD). Statistical analyses were performed by SPSS, version 22. Results of extraction yield, total phenolics and flavonoids amount, chemical characterization and antioxidant activity were subjected to one-way analysis of variance (ANOVA) using Duncan’s multiple range test to determine significant differences among samples with level of significance p < 0.05. Concentrations of extracts that inhibited 50% of DPPH/OH radicals (IC₅₀) were determined by regression analysis.

3. Results and Discussion

3.1. Preliminary Phytochemical Screening of Extracts

The content of dried extract (d.e.) obtained after 48 h of maceration with 70% (v/v) aqueous ethanol ranged from 13.48–30.03% for bark and 19.71–29.13% for leaves of seven different Salix species (Table 1). The highest extraction yield of bark was obtained for Salix purpurea, whereas Salix eleagnos and S. purpurea had the greatest yield among the leaves.

Table 1. Yield of dried extract, total phenolics and total flavonoid content in bark and leaves extracts of the investigated Salix species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Yield [%] 1</th>
<th>Total Phenolics [mg GAE/g d.e.] 1</th>
<th>Total Flavonoids [mg QE/g d.e.] 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bark</td>
<td>Leaf</td>
<td>Bark</td>
</tr>
<tr>
<td>S. alba</td>
<td>15.23 ± 0.76 b</td>
<td>21.11 ± 0.39 f</td>
<td>40.9 ± 0.46 f</td>
</tr>
<tr>
<td>S. amplexicaulis</td>
<td>19.68 ± 0.32 e</td>
<td>19.71 ± 0.29 e</td>
<td>49 ± 0.33 h</td>
</tr>
<tr>
<td>S. babylonica</td>
<td>17.41 ± 0.19 c</td>
<td>19.82 ± 0.38 e</td>
<td>20.17 ± 0.42 d</td>
</tr>
<tr>
<td>S. elegans</td>
<td>17.34 ± 0.44 c</td>
<td>28.62 ± 0.27 b</td>
<td>61.27 ± 0.73 i</td>
</tr>
<tr>
<td>S. fragilis</td>
<td>13.48 ± 0.27 a</td>
<td>21.58 ± 0.53 f</td>
<td>20.4 ± 0.40 d</td>
</tr>
<tr>
<td>S. purpurea</td>
<td>18.45 ± 0.68 d</td>
<td>29.13 ± 0.88 b</td>
<td>69.1 ± 0.25 i</td>
</tr>
<tr>
<td>S. triandra</td>
<td>30.03 ± 0.79 f</td>
<td>24.35 ± 0.36 i</td>
<td>18.41 ± 0.59 c</td>
</tr>
</tbody>
</table>

1 Results are presented as mean value of triplicate measurements ± SD. Superscript letters within the same category (yield/total phenolics/total flavonoids) indicate significant differences of means at the 0.05 level.

The amount of total phenolics in the analyzed species of the genus Salix varied widely and ranged from 18.41–69.1 mg gallic acid equivalents (GAE)/g d.e. and 10.26–87.06 mg GAE/g d.e., for bark and leaves respectively (Table 1). The content of total flavonoids was in range from 2.88–31 mg quercetin equivalents (QE)/g d.e. for bark and 11.4–32.82 mg QE/g d.e. for leaves (Table 1). Generally, the amounts of phenolics were significantly higher in bark than in leaves extracts of different Salix species, except for S. triandra. In addition, bark of the analyzed Salix species contained larger amounts of phenolics than flavonoids. On the other hand, the amount of flavonoids was higher in leaves than in bark, except for S. purpurea and S. triandra. In contrast to the total phenolics content in glycolic bark extract of Salix alba (6.2 mg GAE/g) [26], the amounts of phenolics in ethanolic willow bark extracts from our study were 3–11 times higher, and in leaves extracts 1.75 to even 14 fold higher. This could be explained by differences in the polarity of solvents used for extraction, but genetic and environmental factors as well. Compared to another previously published study that reported very high concentrations of total phenolics in ethanolic bark extracts of S. alba and S. purpurea, our results are 9.5 and 6 times lower in bark for these species, respectively [27]. Since the extractant and sample to solvent ratio was the same, differences could possibly be attributed to extraction length (2 days vs. 2 weeks). Comparing the obtained results with those reported earlier for S. alba it can be noted that the analyzed extracts contain greater amounts of flavonoids [28]. Comparison of the obtained results with those previously published for other Salix species indicate that beside salicin and its derivatives, flavonoids were one of the major fractions of phenolic compounds present in ethanolic bark extracts of Salix species [27].
3.2. Chemical Composition of Bark and Leaves Extracts of Various Species from the Genus Salix

Even though many research articles studied the chemical composition of bark of various Salix species to a certain extent, these studies mainly focused on salicylic and salicin derivatives and only few dealt with simple phenolics in willows [10,14,15]. Moreover, leaves of Salix species are usually considered as waste product after bark collection and are mainly not studied. It is known that waste products from plant processing represent promising sources of valuable bioactive compounds [29]. In addition, many species of willows widely distributed in nature are until present poorly studied or lack any kind of data related to their biological activities and chemical characterization. Therefore, chemical composition of bark and leaves of seven Salix species was determined by HPLC and is shown in Tables 2 and 3. A representative chromatogram of S. amplexicaulis bark extract is presented in Figure 2.

### Table 2. Phytochemical characterization of bark extracts.

<table>
<thead>
<tr>
<th>Compound</th>
<th>S. alba</th>
<th>S. amplexicaulis</th>
<th>S. babylonica</th>
<th>S. elaeagnos</th>
<th>S. fragilis</th>
<th>S. purpurea</th>
<th>S. triandra</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>0.17 ± 0.00 a</td>
<td>n.d.</td>
<td>0.17 ± 0.01 a</td>
<td>n.d.</td>
<td>0.19 ± 0.01 b</td>
<td>n.d.</td>
<td>0.26 ± 0.03 c</td>
</tr>
<tr>
<td>CHLA</td>
<td>1.65 ± 0.17 b</td>
<td>1.51 ± 0.14 b</td>
<td>1.92 ± 0.02 bc</td>
<td>2.08 ± 0.14 c</td>
<td>1.54 ± 0.01 b</td>
<td>1.14 ± 0.01 a</td>
<td>1.63 ± 0.01 b</td>
</tr>
<tr>
<td>PHB</td>
<td>0.32 ± 0.01 a</td>
<td>0.92 ± 0.01 b</td>
<td>1.21 ± 0.02 c</td>
<td>1.24 ± 0.01 c</td>
<td>0.39 ± 0.04 a</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>VA</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.24 ± 0.04</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>SA</td>
<td>0.22 ± 0.01 b</td>
<td>n.d.</td>
<td>0.34 ± 0.01 4 c</td>
<td>0.20 ± 0.04 ab</td>
<td>0.16 ± 0.01 a</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CA</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.53 ± 0.01 a</td>
<td>n.d.</td>
<td>1.05 ± 0.01 b</td>
<td>n.d.</td>
</tr>
<tr>
<td>EPC</td>
<td>1.17 ± 0.05 a</td>
<td>1.30 ± 0.00 a</td>
<td>2.68 ± 0.06 d</td>
<td>1.95 ± 0.15 bc</td>
<td>2.57 ± 0.09 d</td>
<td>2.08 ± 0.02 c</td>
<td>1.77 ± 0.25 b</td>
</tr>
<tr>
<td>PCA</td>
<td>0.15 ± 0.01 ab</td>
<td>1.55 ± 0.04 c</td>
<td>0.15 ± 0.01 ab</td>
<td>0.14 ± 0.00 ab</td>
<td>0.08 ± 0.00 a</td>
<td>1.53 ± 0.05 c</td>
<td>0.22 ± 0.01 b</td>
</tr>
<tr>
<td>R</td>
<td>1.75 ± 0.03 a</td>
<td>22.09 ± 0.04 d</td>
<td>1.36 ± 0.15 a</td>
<td>5.20 ± 0.34 c</td>
<td>1.26 ± 0.04 a</td>
<td>4.30 ± 0.03 b</td>
<td>1.73 ± 0.06 a</td>
</tr>
<tr>
<td>Q</td>
<td>0.38 ± 0.02 a</td>
<td>0.61 ± 0.01 b</td>
<td>0.52 ± 0.00 ab</td>
<td>1.28 ± 0.04 c</td>
<td>0.41 ± 0.01 a</td>
<td>1.13 ± 0.01 c</td>
<td>0.67 ± 0.01 b</td>
</tr>
<tr>
<td>TCA</td>
<td>0.15 ± 0.05 a</td>
<td>0.65 ± 0.14 b</td>
<td>0.57 ± 0.01 a</td>
<td>0.52 ± 0.03 b</td>
<td>0.49 ± 0.00 b</td>
<td>0.13 ± 0.01 a</td>
<td>0.53 ± 0.03 b</td>
</tr>
<tr>
<td>N</td>
<td>0.20 ± 0.01 a</td>
<td>0.48 ± 0.01 d</td>
<td>0.27 ± 0.09 b</td>
<td>0.38 ± 0.06 c</td>
<td>0.26 ± 0.00 b</td>
<td>0.26 ± 0.01 b</td>
<td>0.33 ± 0.00 c</td>
</tr>
<tr>
<td>S</td>
<td>3.99 ± 0.18 a</td>
<td>8.29 ± 0.09 b</td>
<td>3.11 ± 2.50 a</td>
<td>2.60 ± 0.06 a</td>
<td>3.47 ± 0.04 a</td>
<td>7.53 ± 0.30 b</td>
<td>2.87 ± 0.03 a</td>
</tr>
</tbody>
</table>

n.d.—not detected; GA—gallic acid; CHLA—chlorogenic acid; PHB—p-hydroxybenzoic acid; VA—vanillic acid; SA—syringic acid; CA—caffeic acid; EPC—epicatechin; PCA—p-coumaric acid; R—rutin; Q—quercetin; TCA—trans-cinnamic acid; N—naringenin; S—salicin. Data are presented as mean of triplicate measurements ± SD. Superscript letters within the same line indicate significant differences of means at the 0.05 level.

### Table 3. Phytochemical characterization of leaves extracts.

<table>
<thead>
<tr>
<th>Compound</th>
<th>S. alba</th>
<th>S. amplexicaulis</th>
<th>S. babylonica</th>
<th>S. elaeagnos</th>
<th>S. fragilis</th>
<th>S. purpurea</th>
<th>S. triandra</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.20 ± 0.01</td>
<td>n.d.</td>
<td>5.82 ± 0.09 a</td>
<td>1.04 ± 0.27 b</td>
<td>7.53 ± 0.07 f</td>
</tr>
<tr>
<td>CHLA</td>
<td>2.26 ± 0.04 d</td>
<td>0.74 ± 0.35 a</td>
<td>1.62 ± 0.02 c</td>
<td>2.22 ± 0.21 d</td>
<td>2.57 ± 0.09 d</td>
<td>2.08 ± 0.02 c</td>
<td>1.77 ± 0.25 b</td>
</tr>
<tr>
<td>PHB</td>
<td>0.36 ± 0.11 ab</td>
<td>0.27±0.13 a</td>
<td>0.46±0.15 b</td>
<td>0.39±0.05 b</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>VA</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.35±0.35</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>SA</td>
<td>0.11 ± 0.01 a</td>
<td>n.d.</td>
<td>0.20 ± 0.01 a</td>
<td>0.19±0.00 a</td>
<td>0.16±0.05 b</td>
<td>n.d.</td>
<td>0.18±0.08 b</td>
</tr>
<tr>
<td>CA</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.46±0.04</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>EPC</td>
<td>0.96 ± 0.06 b</td>
<td>0.71 ± 0.01 a</td>
<td>1.55±0.04 d</td>
<td>1.19±0.04 bc</td>
<td>2.09±0.19 a</td>
<td>4.83±0.22 f</td>
<td>1.30±0.11 c</td>
</tr>
<tr>
<td>PCA</td>
<td>0.19 ± 0.04 ab</td>
<td>0.68 ± 0.06 c</td>
<td>0.13±0.01 a</td>
<td>0.19±0.00 ab</td>
<td>0.24±0.01 b</td>
<td>1.92±0.11 d</td>
<td>0.17±0.02 ab</td>
</tr>
<tr>
<td>R</td>
<td>1.74 ± 0.03 a</td>
<td>11.4 ± 1.13 c</td>
<td>2.05 ± 0.05 a</td>
<td>18.56 ± 0.03 d</td>
<td>1.57 ± 0.78 a</td>
<td>9.44 ± 0.05 b</td>
<td>1.69 ± 0.99 a</td>
</tr>
<tr>
<td>Q</td>
<td>0.52 ± 0.02 ab</td>
<td>1.46±0.09 d</td>
<td>0.38±0.01 a</td>
<td>0.68±0.00 b</td>
<td>0.46±0.07 a</td>
<td>0.76±0.23 bc</td>
<td>0.47±0.01 a</td>
</tr>
<tr>
<td>TCA</td>
<td>0.33 ± 0.10 ab</td>
<td>1.45 ± 0.11 d</td>
<td>0.25±0.02 a</td>
<td>0.45±0.06 b</td>
<td>0.45±0.06 b</td>
<td>1.59±0.15 e</td>
<td>0.78±0.04 e</td>
</tr>
<tr>
<td>N</td>
<td>0.19 ± 0.00 a</td>
<td>0.88±0.01 d</td>
<td>0.22±0.00 a</td>
<td>0.91±0.00 d</td>
<td>0.55±0.01 b</td>
<td>0.69±0.02 c</td>
<td>0.24±0.02 a</td>
</tr>
<tr>
<td>S</td>
<td>n.d.</td>
<td>6.01±0.11 b</td>
<td>1.63±0.10 a</td>
<td>5.87±0.74 b</td>
<td>2.73±0.19 a</td>
<td>17.33±1.54 e</td>
<td>1.92±0.08 a</td>
</tr>
</tbody>
</table>

n.d.—not detected; GA—gallic acid; CHLA—chlorogenic acid; PHB—p-hydroxybenzoic acid; VA—vanillic acid; SA—syringic acid; CA—caffeic acid; EPC—epicatechin; PCA—p-coumaric acid; R—rutin; Q—quercetin; TCA—trans-cinnamic acid; N—naringenin; S—salicin. Data are presented as mean of triplicate measurements ± SD. Superscript letters within the same line indicate significant differences of means at the 0.05 level.
was determined in methanolic bark and leaves extracts of several within the same willow species is particularly evident. It is notable that the highest concentration higher in bark and leaves extracts of all seven analyzed species [32]. These variations could be ascribed to different part of the plant, polarity of solvent used for extraction, as well as environmental and growth conditions of the species. Moreover, secondary metabolite content in willows varies with season and time of the day, but also gender and age of the plant [33].

Figure 2. Chromatograms of *S. amplexicaulis* bark extract: (a) a-chlorogenic acid; b-p-hydroxybenzoic acid; c-epicatechin; d-p-coumaric acid; e-rutin; f-quercetin; g-trans-cinnamic acid; h-naringenin; (b) s-salicin.

In the analyzed extracts, a total of 13 compounds were identified and quantified: Gallic, chlorogenic, p-hydroxybenzoic, vanillic, syringic, caffeic, p-coumaric and trans-cinnamic acids, flavonoids epicatechin, rutin, quercetin and naringenin, and the salicylic glycoside salicin. It is notable that chlorogenic, p-coumaric and trans-cinnamic acids and the analyzed flavonoids were present in bark and leaves extracts of all willow species, but their amounts vary between samples.

In bark of five out of seven willow species, the most abundant components were salicin, rutin, epicatechin and chlorogenic acid. Bark extracts of *S. amplexicaulis* and *S. purpurea* showed a slightly different pattern. Beside salicin and rutin, dominant compounds in bark of *S. amplexicaulis* were p-coumaric and chlorogenic acid, whereas in bark of *S. purpurea* those were epicatechin and p-coumaric acid. A distribution of components similar to that in bark was observed in leaves extracts of the respective species as well. Presence of variability in concentrations of salicin, rutin, chlorogenic acid and epicatechin between leaves samples of different species, as well as between bark and leaves within the same willow species is particularly evident. It is notable that the highest concentration of salicin was found in leaves of *S. purpurea*, but it was not detected in leaves of *S. alba*. Among all the extracts, the highest amount of salicin was found in leaves of *S. purpurea*, which is about twice as high as the amount in bark of the same species. Bark of *S. purpurea* is considered as one of the richest sources of salicin among the willows [16]. Knowing that leaves of this species contain even higher concentrations of salicin opens up new possibilities for the exploitation of leaves as valuable and easily accessible sources of salicin.

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Presence of rutin in the analyzed extracts is in accordance with the results obtained for *S. aegyptiaca*, where rutin was also one of the major flavonols in the ethanolic bark extract, whereas its amount was higher in bark and leaves extracts of all seven analyzed *Salix* species [34]. High levels of rutin are
noteworthy in *S. amplexicaulis*, *S. eleagnos* and *S. purpurea*, with emphasis on bark of *S. amplexicaulis* and leaves of *S. eleagnos* being the highest. Our findings are in accordance with other previously published reports which indicate that epicatechin is also one of the most dominant flavonoids in willow bark extracted in polar solvents [35]. Vanillic acid was present in low amounts in bark and leaf of *S. fragilis* only. Vanillic acid was reported in bark of some *Salix* species, including *S. alba* clone 1100, *S. purpurea* and *S. triandra*, which was not the case in our study [10]. Caffeic acid was found in barks of *S. eleagnos* and *S. purpurea*, as well as in leaves of *S. fragilis*. The presence of caffeic acid in bark of *S. purpurea* was previously confirmed, but its amount was not quantified [10]. The caffeic acid content of *S. purpurea* and *S. eleagnos* bark extracts was found to be 17.5 and 8.5 times higher, respectively, than that in bark of *S. aegyptiaca* (0.06 mg/g) [34]. Moreover, the level of caffeic acid in *S. fragilis* leaves extract was more than 2.5 fold higher than that in leaves of *S. aegyptiaca* (0.15 mg/g) [34]. P-hydroxybenzoic acid was determined in a majority of species, except for *S. purpurea* and *S. triandra*. In addition, it was not found in leaves of *S. fragilis*. P-hydroxybenzoic acid was previously reported in the wood of *S. nigra*, *S. babylonica* and *S. eriocephala*, but the content of these compounds was not presented [10]. To the best of our knowledge this is the first report on detailed chemical characterization and comparative analysis of simple phenolics and flavonoids in bark and leaves of seven different *Salix* species indigenous to the Balkan Peninsula.

### 3.3. Antioxidant Activity

Natural products with antioxidant properties have become a hot topic nowadays as they represent safe alternatives to potentially toxic and carcinogenic synthetic antioxidants (such as butylated hydroxytoluene and butylated hydroxyanisole), which are widely used in food, pharmaceutical and cosmetic industry. The ability of bark and leaves extracts of various *Salix* species to scavenge DPPH• and •OH was assessed. DPPH• is a stable, nitrogen-containing free radical, violet/purple colored in ethanol solution, which fades to shades of yellow color in the presence of antioxidants. DPPH assay is one of the most widely used methods for screening antioxidant activities of plant extracts. It allows testing of lipophilic as well as hydrophilic compounds unlike other methods that are restricted in the nature of antioxidants [36]. •OH is known as the most potent oxidant and one of the most reactive natural free radicals. It is directly involved in the irreversible damage caused by oxidative stress, which is one of the fundamental mechanisms underlying many human diseases such as malignant, cardiovascular and neurodegenerative [36,37].

Concentrations of extracts that inhibited 50% (IC50) of DPPH• ranged from 1.83–7.79 µg/mL in bark and 1.95–8.07 µg/mL in leaves extracts of different species of the genus *Salix* (Table 4).

<table>
<thead>
<tr>
<th>Species</th>
<th>Bark DPPH• IC50 (µg/mL)</th>
<th>Leaf DPPH• IC50 (µg/mL)</th>
<th>Bark •OH 1</th>
<th>Leaf •OH 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. alba</em></td>
<td>1.83 ± 0.09 a</td>
<td>2.43 ± 0.11 bc</td>
<td>34.76 ± 0.24 b</td>
<td>42.18 ± 0.32 i</td>
</tr>
<tr>
<td><em>S. amplexicaulis</em></td>
<td>2.12 ± 0.12 ab</td>
<td>7.05 ± 0.15 b</td>
<td>51.69 ± 0.31 j</td>
<td>31.38 ± 0.14 f</td>
</tr>
<tr>
<td><em>S. babylonica</em></td>
<td>2.59 ± 0.16 c</td>
<td>4.43 ± 0.17 de</td>
<td>40.44 ± 0.56 h</td>
<td>44.61 ± 0.39 c</td>
</tr>
<tr>
<td><em>S. eleagnos</em></td>
<td>2.34 ± 0.28 bc</td>
<td>5.51 ± 0.19 f</td>
<td>29.71 ± 0.29 e</td>
<td>51.96 ± 0.14 j</td>
</tr>
<tr>
<td><em>S. fragilis</em></td>
<td>4.11 ± 0.13 d</td>
<td>8.07 ± 0.43 h</td>
<td>22.20 ± 0.36 a</td>
<td>25.15 ± 0.46 b</td>
</tr>
<tr>
<td><em>S. purpurea</em></td>
<td>4.73 ± 0.26 c</td>
<td>7.34 ± 0.16 g</td>
<td>26.31 ± 0.63 c</td>
<td>63.51 ± 0.17 k</td>
</tr>
<tr>
<td><em>S. triandra</em></td>
<td>7.79 ± 0.21 h</td>
<td>1.95 ± 0.08 a</td>
<td>28.01 ± 0.29 d</td>
<td>36.10 ± 0.18 h</td>
</tr>
</tbody>
</table>

1 Results are presented as mean value of triplicate measurements ± SD. Superscript letters within the same category (DPPH•, •OH) indicate significant differences of means at the 0.05 level.

Significant difference between the radical scavenging potential of bark and leaves extracts within the same species, as well as of different species, was found. The highest radical scavenging activity...
against DPPH• was observed in bark of *S. alba*, and the lowest in bark of *S. triandra*. Contrary to bark, leaves extract of *S. triandra* exhibited the strongest DPPH• quenching ability among the seven analyzed extracts. This could be explained by the fact that leaves extract of *S. triandra* contains significantly higher amounts of total phenolics and flavonoids compared to bark of this species. The ability of ethanolic bark and leaves extracts of all seven analyzed species to scavenge DPPH• was significantly stronger than the one of methanolic bark extract of *S. alba* (15.5 µg/mL) [28] indicating higher selectivity of ethanol for the extraction of antioxidant compounds.

The analyzed bark and leaves extracts of willows also showed notable potential to inhibit *•*OH generated in Fenton reaction. IC\textsubscript{50} values ranged from 22.20–51.63 µg/mL and 25.15–63.51 µg/mL for bark and leaves, respectively (Table 4). Generally, bark extracts had stronger *•*OH scavenging potential than leaves, except for *S. amplexicaulis*, which showed the opposite. Extracts of all of the analyzed willow species exhibited significantly higher *•*OH quenching ability than bark extract of *S. purpurea* (ca. 15 mg/mL) and *S. myrsinifolia* (ca. 5 mg/mL) from an earlier study [38]. Comparing the IC\textsubscript{50} values of extracts against DPPH• and *•*OH it can be noted that the radical scavenging capacity was stronger against DPPH• than *•*OH in all of the tested extracts. The results are in agreement with other previously tested extracts where a similar pattern was observed [39]. This is the first report on comparative analysis of *•*OH scavenging activity of bark and leaves of seven different Salix species, to our knowledge.

### 3.4. Molecular Docking

As one of the important tools to investigate the active site of a protein and to elucidate the interactions between ligands and the biological molecule, molecular docking was conducted. The most abundant compounds of willow bark and leaves extracts were selected to study their binding affinity and interactions to AChE enzyme. While docking results have shown a number of possible ligand conformations, only the lowest binding energy was chosen. Results of docking analysis are shown in Table 5. The more negative the binding energy value, the better affinity the ligand has for the receptor. The inhibition constant (Ki) was also predicted for the selected ligands, which brings about additional information along with energy values. A very good linear correlation between pKi, where pKi = −logKi, and docked binding energies was observed (R\textsuperscript{2} = 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Energy (Kcal/mol)</th>
<th>Inhibition Constant (nM)</th>
<th>Interaction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epicatechin</td>
<td>−9.33</td>
<td>145.64</td>
<td>Tyr 72, Trp 86, Tyr 124, Trp 286, Phe 295, Phe 297,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tyr 337, Phe 338, Tyr 341</td>
</tr>
<tr>
<td>Salicin</td>
<td>−7.38</td>
<td>3890</td>
<td>Tyr 72, Asp 74, Tyr 124, Trp 286, Phe 295, Phe 297,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phe 338, Tyr 341</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>−7.87</td>
<td>1700</td>
<td>Tyr 72, Trp 86, Gly 121, Tyr 124, Ser 203, Trp 286,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phe 295, Phe 297, Tyr 337, Phe 338, Tyr 341, His 447</td>
</tr>
<tr>
<td>Rutin</td>
<td>−0.87</td>
<td>232030000</td>
<td>Gly 345, Ser 347</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Trp 86, Gly 121, Tyr 124, Ser 203, Trp 286, Phe 295,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phe 297, Tyr 337, Phe 338, Tyr 341, His 447</td>
</tr>
<tr>
<td>Donepezil</td>
<td>−11.43</td>
<td>4.2</td>
<td></td>
</tr>
</tbody>
</table>

The standard, donepezil, showed the strongest binding energy toward AChE and the largest number of residues (Table 5). The nature of the interactions is presented in Figure 3.
Rutin demonstrated the weakest binding affinity toward AChE among the studied ligands. Previous reports also indicate that rutin was not inhibiting AChE and are consistent with our data [40,41]. Epicatechin, salicin and chlorogenic acid showed good affinity toward AChE in comparison with the standard inhibitor donepezil. Indeed, epicatechin showed the lowest binding energy among the ligands. In addition, these three ligands interact with important amino acid residues at the active site of AChE [42]. In order to get insight into the interactions of these ligands with the active site of AChE, 3D and 2D structures of AChE-ligand complexes are shown in Figures 4–6.

![Figure 3](image3.png)

**Figure 3.** Active site of the AChE enzyme with donepezil: (a) 3D view; (b) 2D view.

![Figure 4](image4.png)

**Figure 4.** Active site of the AChE enzyme with epicatechin: (a) 3D view; (b) 2D view.
AChE has a narrow 20 Å gorge with two binding sites: the catalytic active site at the bottom of the structure and the peripheral anionic site (PAS) near the entrance of the gorge. Consequently, ligands that bind to either one or two of the sites represent promising inhibitors of AChE in treatment of AD [43].

Epicatechin showed several hydrogen bonds with prominent amino acid residues of the PAS anionic subsite of AChE, namely Asp 74, Tyr 124 and Tyr 341, while van der Waals interaction with Trp 286. Hydrogen bond interactions of –OH groups of phenyl moiety with Asp 74 carboxylate group are established. In addition, a hydrogen bond between the Tyr 124-OH and the –OH group (in position 3) of chromene moiety is formed. Moreover, Tyr 341 makes pi-pi stacking with the phenyl ring of epicatechin. Interactions of epicatechin with Trp 86, Tyr 337 and Phe 338, all relevant amino acid residues of the PAS. The interaction between salicin and Phe 286, which are significant amino acid residues of the PAS. The interaction between salicin and Phe 286, which are significant amino acid residues of the PAS.

The lowest energy conformer of salicin exhibited hydrogen bond interactions with Tyr 124 and Tyr 341, van der Waals interactions with Tyr 72 and Asp 74, and pi-pi stacking interaction with Trp 286. Hydrogen bond interactions of –OH groups of phenyl moiety with Asp 74 carboxylate group are established. In addition, a hydrogen bond between the Tyr 124-OH and the –OH group (in position 3) of chromene moiety is formed. Moreover, Tyr 341 makes pi-pi stacking with the phenyl ring of epicatechin. Interactions of epicatechin with Trp 86, Tyr 337 and Phe 338, all relevant amino acid residues of the PAS. The interaction between salicin and Phe 286, which are significant amino acid residues of the PAS. The interaction between salicin and Phe 286, which are significant amino acid residues of the PAS.
286, which are significant amino acid residues of the PAS. The interaction between salicin and Phe 338, important amino acid residue of AS, is hydrophobic. Interactions of salicin with amino acid residues of the ABP, Phe 295 and Phe 297, are hydrogen bonding and van der Waals, respectively (Figure 5).

Chlorogenic acid was the only ligand, beside donepezil, that showed interactions with prominent residues of the catalytic site of AchE, Ser 203 and His 447, the former being hydrogen bonding and the latter pi-cation interaction. Chlorogenic acid also showed several van der Waals interactions with the important amino acid residues of AS of AchE, namely Trp 86, Tyr 337 and Phe 338, as well as of ABP, Phe 295 and Phe 297. Most of interactions between chlorogenic acid and significant amino acid residues of PAS peripheral anionic site, Tyr 72, Trp 286 and Tyr 341, are hydrophobic, while Tyr 124 forms a hydrogen bond. In addition, a hydrogen bond is formed between chlorogenic acid and the amino acid residue of the oxyanionic hole—Gly 121 (Figure 6). Zaiter et al. (2016) [12] reported significant in vitro inhibitory activity of S. alba bark extracts toward AchE and good correlations between anti-AchE IC\textsubscript{50} values and bioactive compounds—salicin, catechin, gallocatechin, procyanidins B1, B2, C1 and chlorogenic acid, confirming the role of these compounds in anticholinesterase activity, which is in accordance with our data.

4. Conclusions

Phytochemical analysis of various species from the genus Salix revealed that species other than those considered as biological sources of salicis cortex contain health beneficial bioactive compounds and could be utilized in the pharmaceutical industry. Moreover, not only bark of the investigated Salix species, but also leaves present rich natural sources of phenolic compounds, mainly flavonoids, and possess significant antioxidant activity. Therefore, willow leaves and bark have potential to be utilized as antioxidant agents to preserve food and pharmaceutical products, as well as in prevention of diseases associated with oxidative stress. In addition, our molecular docking studies demonstrated the inhibitory activity of compounds present in willow bark and leaves toward AchE. Epicatechin, salicin and chlorogenic acid exhibited a number of strong hydrogen bonds and hydrophobic interactions with significant amino acid residues of active sites of AchE, supporting the inhibitory potential of these compounds. This research set the foundation for further in vitro and in vivo studies that could lead to development of new therapeutics with neuroprotective effects against AD.

Author Contributions: Data curation, E.G.; Formal analysis, E.G.; Investigation, E.G.; Project administration, N.G.-L.; Supervision, R.I. and N.G.-L.; Visualization, L.S.; Writing—original draft, E.G.; Writing—review & editing, R.I., L.S. and N.G.-L.

Funding: This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, projects no. 172021 and III 4609.

Conflicts of Interest: The authors declare no conflict of interest.

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