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Antioxidant and antiacetylcholinesterase activities of different granulometric classes of *Salix alba* (L.) bark powders

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Keywords

Salix alba (L.) bark, Grinding, Granulometric class, Antiacetylcholinesterase, Antioxidant activity, LC-ESI/MS

Abstract

This work aims at identifying and quantifying the main bioactive compounds in hydroalcoholic extracts of *Salix alba* (L.) bark powders obtained by grinding and sieving. The influence of the granulometric class on antioxidant activity and acetylcholinesterase (AChE) inhibition effect were investigated. Our study highlights that all granulometric classes revealed a high antioxidant activity and a good efficiency against the action of AChE enzyme. The 50–100 μm granulometric class led also to the highest content in phenolic compounds (circa 52.7 mg gallic acid equivalent/g of dry matter). The best results were obtained for the 50–100 μm granulometric class: the IC₅₀ value for anti-AChE activity reached 0.59 $\mu\text{g/mL}$. These results reveal the interest of the fine grinding and sieving procedure to enhance antioxidant and anti-AChE activities of *Salix alba* (L.), as significantly higher levels of bioactive compounds were found in ground and sieved fractions in comparison with unground dry *Salix alba* (L.) bark.

1. Introduction

In the last few years, the search for natural drugs increased and medicinal plants emerged as alternatives to synthetic products. They are not only used in traditional medicines but also in a great number of pharmaceutical products, due to their bioactivity. Indeed, there have been extensive efforts to investigate the preventive role of nutraceuticals against diseases [1,2]. Herbal manufacturing is receiving attention since numerous bioactive compounds present in plants serve as starting points for the development of new drugs [3]. Clinical studies have shown that herbal drugs obtained from willow bark are better tolerated by patients than synthetic derivatives [4]. Therefore, many people have begun to opt for *Salix alba* (L.) bark as an alternative to aspirin. This plant is the most important natural source of salicin, an alcoholic β -glucoside. This compound is easily assimilated in the gastrointestinal tract, releasing salicylic alcohol, which is oxidized in the liver into salicylic acid, thereby causing no damage to the gastric mucosa [5].

Several studies have concentrated on phytochemical research of *Salix* species used for the preparation of willow bark extracts. *Salix daphnoides*, *Salix pentadra*, *Salix purpurea*, *S. alba*, and *Salix fragilis* have been studied for their composition in polyphenols, known for their antioxidant activity. The latter is a fundamental property of polyphenolic compounds in plants, which have other pro-health activities, such as antimutagenic, anticarcinogenic, and anti-aging. Polyphenols have the ability to scavenge reactive oxygen species (ROS), preserving the genomic stability of cells through elimination of carcinogens and interference with DNA adduct formation [6]. Antioxidants can protect biological systems against potentially harmful effects [7]. When the production balance between ROS and defense systems is disrupted, oxidative stress sets in, promoting many diseases such as cancer, atherosclerosis, and other cardiovascular disorders, diabetes, accelerated aging [8], and Alzheimer's disease [9].

Nowadays, neurodegenerative diseases are estimated to surpass cancer as the second most common cause of death among ages by the 2040s [10]. Acetylcholinesterase is a key enzyme catalyzing the hydrolysis of acetylcholine to choline and acetic acid. In some pathologic conditions leading to low acetylcholine concentrations in the synaptic zone, it can cause loss of memory and impairment of multiple cerebral functions in the nervous system of animals [11]. Acetylcholine deficiency in the synapses of human cerebral cortex is one of the main cause of Alzheimer's disease [12]. Galantamine is a synthetic AChE inhibitor used to enhance memory for Alzheimer patients. The standard drug gal-antamine has been shown to exhibit a competitive inhibition pattern [13]. However, AChE commercial inhibitors present some restrictions, such as their short half-lives and large side effects, as well as hepatotoxicity, which is the most common and important side effect of these treatments [14]. Current pharmacology focuses on the synthesis of potent and concentrated substances with a specific therapeutic effect, even though a similar effect can be obtained using natural medicinal plant materials [5].

Ejaz Ahmed et al. [15] have evidenced an additional contribution of polyphenolic compounds to neuroprotective effects of catechin against Alzheimer's disease. Even more recently, it has been found that the presence of flavonoids in plants, mainly catechin, might be responsible for AChE inhibitory and antioxidant activities [16]. Kwon et al. [17] reported that chlorogenic acid inhibits both free radical scavenging and AChE activities. Besides, in addition to its powerful antioxidant activity, Ouattara et al. [18] reported that chlorogenic acid induces neuroprotective effects by AChE inhibition and thus presents a double benefit. The search for new AChE inhibitors derived from natural sources and having few side effects is urgently required [19]. Research on plant-derived inhibitors of AChE has accelerated in view of the benefits of these drugs to the treatment of Alzheimer's disease [20]. Nevertheless, scientific information remains very limited for various plants and compounds with notable anti-AChE activity. Gomathi and Manian [21] reported that hydroalcoholic extracts of *Scolopia crenata* bark have an inhibitory effect

against AchE as near as the common synthetic drug galantamine. Another study showed that the methanol extract of the stem barks of *Ficus carica* has a good effect against AChE [22].

A way to enhance bioactive compounds concentration in hydroalcoholic extracts from plant material could be to grind dried plants, sieve the obtained powders, and finally select the granulometric class having the best activity, before performing the hydroalcoholic extraction [23,24]. However, temperature increase during grinding is a crucial phenomenon in regard to powder quality (usually, 99% of input grinding power is converted into heat [25]), requiring a careful choice of process conditions to limit its impacts. Biochemical alterations of food materials due to temperature increase during grinding (locally up to 90 °C [26]) may result in modifications of their physicochemical properties [27] like color alteration (product surface darkening [28]), which lowers their organoleptic properties [25] and sometimes causes bioactivity losses [29]. Temperature increase is more pronounced for smaller particles, as heat comes from the breaking of the chemical bonds of the material to be ground, but also, and above all, from the friction between particles while flowing in the grinder. Besides, the grinding process has to be optimized to avoid extensive oxidation of surface compounds (indirect contact with air oxygen), especially for small particles of high specific surface area.

This study aims at evaluating antioxidant activity and AChE enzyme inhibition related to the particle size generated by fine grinding of *Salix alba* (L.) bark followed by sieving into seven granulometric classes. In this purpose, total polyphenol content, as well as antioxidant activity by DPPH and Ellman tests, and ant i-AChE activity were determined using UV/visible methods. Catechin, galocatechin chlorogenic acid, procyanidins, and salicin were identified and quantified by LC-ESI/MS. The contents in bioactive compounds of all granulometric fractions were compared to those of unground plant to assess the efficiency of the successive grinding/sieving process.

2. Material and Methods

2.1. Plants

Barks from 3-year-old branches of wild *Salix alba* (L.) were provided by Cailleau Herboristerie (Chemillé, France). They were manually harvested in October 2014 in Bulgaria and, for conservation purposes, they were artificially dried between 35 and 40 °C by the supplier in order to reach about 10% moisture content.

2.2. Chemicals

Chemicals Folin-Ciocalteu phenol reagent, 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid (ABTS), dihydrochloride (AAPH), gal-lic acid, galantamine, sodium chloride, hydrochloric acid, ethyl acetate, acetic acid, p-iodonitrotetrazolium chloride (INT), acetylcholinesterase (AChE), acetylcholine iodide (ATCI), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and 2-thiobarbituric acid, as well as standards of salicin, (+)catechin, (-)-galocatechin,

chlorogenic acid, procyanidin B1, procyanidin B2, procyanidin C1, and gallic acid, were acquired from Sigma-Aldrich Corporation (Saint Quentin Fallavier, France). HPLC-grade acetonitrile, water, methanol, and formic acid were purchased from Sigma Chemicals Co. (Saint Louis, MO). Sodium carbonate (Na₂CO₃), aluminum chloride (AlCl₃), sodium nitrite (NaNO₂), and sodium hydroxide (NaOH) from Acros Organics (Geel, Belgium). Stock solutions of standards were prepared in 70% methanol at a concentration of 0.5 mg/mL and diluted when needed.

2.3. Plant grinding

The fine grinding protocol was the following. Approximately 1.5 kg of dried *Salix alba* (L.) barks were ground in small batches of circa 50 g with the Ultra Centrifugal Mill ZM 200 (Retsch France), operating by impact and shearing effects. Grinding rotational speed was fixed at 12,000 rpm. Grinding was performed at ambient temperature (approximately 20 °C) using a 1 mm sieve with trapezoid holes.

2.4. Plant powder sieving

Batches of approximately 100 g of ground plants were sieved at 0.5 mm vibration amplitude for 10 min with the Analysette 3 Spartan apparatus (Fritsch, Idar-Oberstein, Germany), operating by vertical vibration. Different granulometric classes, >500 E_m, 315–500 E_m, 180–315 E_m, 100–180 E_m, 50–100 E_m, 20–50 E_m, b 20 E_m, were obtained with the appropriate 20 mm diameter sieves (Fritsch). Almost no sieved powder was found under the 20 E_m sieve, precluding the analyses of the lowest granulometric class. Powders retained on each sieve were weighted and then stored under vacuum in sealed bags at 10 °C before analysis.

2.5. Laser granulometry

Particle size distributions of *Salix alba* (L.) powders (granulometric classes and unsieved powder) were measured by laser diffraction granulometry (Mastersizer 3000, Malvern Instruments France, Orsay, France) at dry state and ambient temperature. Analyses were carried out using a 2% obscuration level, obtained by adapting the dispersion conditions to sample flowability:

– 50% air pressure, 50% feed rate, 2 mm hopper length for the whole unground plant, as well as >500 E_m, 315–500 E_m, 180–315 E_m, and 100–180 E_m granulometric classes;

– 100% air pressure, 100% feed rate, 4 mm hopper length for 50–100 E_m and 20–50 E_m granulometric classes.

The equivalent spherical diameter was chosen as particle size estimator. Particle size distributions were characterized by the particle mean diameter (D₅₀, where DX means that X% of the volume of particles has a diameter inferior to DX) and the span, a common parameter related to the width of particle size distribution, defined in Eq.(1):

$$\text{span} = \frac{D_{90} - D_{10}}{D_{50}} \quad (1)$$

2.6. Powder composition

Analyses of powder composition (moisture, lipids, proteins, minerals, carbohydrates) were performed for all granulometric classes except <20 E_m, as well as for the unground plant. Water content was measured by weight loss after drying 2 g of powder at 105 °C for 5 h [43]. Total protein content was determined by the Kjeldahl method for quantification of nitrogen, using a conversion factor of 6.25 that is suitable for plants [40]. Fat content was quantified by Folch method [30] using 3 g of sample. Mineral content was determined by putting 2 g of powder at 500 °C for 5 h until formation of white ashes [42]. Carbohydrate content was obtained by difference between 100% and the contents in other components; its standard error was deduced from the standard errors of other components by propagation of uncertainties.

2.7. Preparation of extracts

Extraction of polyphenols from *Salix alba* (L.) bark were carried out according the method of Muanda [31] with some

modifications. 2 g of powder of dry bark were macerated during 24 h under stirring at 300 rpm in 20 mL methanol–water (70–30% (v/v)). Then, the methanol–water extract was centrifuged at 6000 rpm for 20 min, the supernatant layer was filtered, brought to 15 mL by adding methanol–water 70–30% (v/v), and stored at 4 °C until analysis in a sealed dark brown vials.

2.8. UV–visible analyses

UV–visible spectrophotometric analyses were carried out with an UV–visible spectrophotometer Cary 50 Scan (Agilent, Santa Clara, California, USA).

2.8.1. Determination of the total phenolic content

Total phenolic content (TPC) were measured with the method used by F. Muanda et al. [31]. 40 E_iL of samples were added to test tubes containing 3.16 mL of distilled water followed by addition of 200 E_iL Folin-Ciocalteu reagent (1 N) and 600 E_iL sodium carbonate (Na₂CO₃, 20%). Samples and blank were thoroughly mixed and vortexed. After 40 min incubation at room temperature, the absorbance was measured at 725 nm. For calibration curves, diluted solutions of gallic acid (at 1, 1.5, 2, 2.5, and 3 mg/mL) were used and total phenolic content was expressed in terms of equivalent amounts of gallic acid per gram of dry matter (GAE/g DM). All experiments were triplicated.

2.8.2. Antioxidant activity

2.8.2.1. ABTS tests

The method described by F. Muanda et al. [32] was used in this study. The ABTS radical anion solution was prepared by dissolving AAPH and ABTS at 1 and 2.5 mM, respectively, in phosphate buffer saline (PBS) solution (100 mM, pH 7.4) containing 150 mM NaCl. After incubation in a water bath at 68 °C for 20 min, the resulting ABTS solution was diluted with methanol to obtain a blue-green coloration with an absorbance of 0.70 ± 0.02 at 734 nm, then 2.94 mL of ABTS radical anion solution (buffer) was added. The decrease in absorbance was measured at 734 nm. The radical stock solution was daily prepared and all tests were triplicated. The ABTS radical scavenging activity was expressed in terms of half maximal inhibitory concentration (IC₅₀), which refers to the smallest concentration of antioxidants required to scavenge 50% of the ABTS radical.

2.8.2.2. DPPH tests

The DPPH radical scavenging activity was evaluated according to the method described by Shariffar, Dehghn-Nudeh, and Mirtajaldini [33]. 1 mL of 100 E_iM DPPH solution in methanol was mixed with 1 mL plant extract. The reaction mixture was incubated in the dark for 20 min and the optical density was recorded at 517 nm against the blank. For the control, 1 mL DPPH solution in methanol was mixed with 1 mL methanol and optical density of solution was recorded after 20 min. The DPPH radical scavenging activity was expressed in terms of IC₅₀.

2.8.3. In vitro AchE activity assay

Inhibitory effect of hydroalcoholic extracts of *Salix alba* (L.) bark was studied in vitro by incubating various concentrations of plant extract with AChE and assessing their activities by Ellman's colorimetric method. AChE inhibitory assays, with kinetics analysis, was conducted according to the protocol described by Kiendrebeogo [34] with some modifications. 650 E μ L Tris-HCl buffer (50 mM, pH 8) were added to 50 E μ L extract and 5 E μ L AChE (0.26 U/mL). The mixture was incubated at room temperature for 15 min before the addition of 250 E μ L DTNB (3 mM) and 75 E μ L substrate (ATCI at 15 mM). The developing yellow color was measured after a new incubation period of 10 min at 405 nm with a spectrophotometer. Galantamine, a reference compound employed in Alzheimer's disease cure, was used as positive control at final concentrations of 5, 10, 20, 30, and 40 E μ g/mL in the assay mixture. The activity of AChE inhibition was expressed as inhibition percentage of AChE, calculated as $(1 - B / A) \times 100$, where A is the change in absorbance of the assay without the plant extract and B is the change in absorbance of the assay in the presence of the plant extract. The sample concentration providing 50% inhibition (IC₅₀) was calculated from the plot representing the evolution of the inhibition percentage with sample concentration.

2.9. LC-ESI/MS analyses

LC-ESI/MS analyses were performed on a LC-MS 2020 system (Shimadzu, Tokyo, Japan) coupled with an electrospray ionization source (ESI). Separation was performed on a Gemini 3 E μ m C18 130 Å reversed phase column (Phenomenex, Torrance, CA, USA) of 150 mm length and 4.6 mm i.d.

2.9.1. Analytical conditions

The column oven was fixed at 30 °C. The mobile phase consisted in a mixture of (A) 0.5% formic acid in water and (B) acetonitrile. The injection volume was 20 E μ L and the flow rate 0.6 mL/min. The employed 25 min gradient was as follows: 0–

15.5 min from 95:05 to 45:55 (A:B); 15.5–16 min from 45:55 to 10:90 (linear elution); 16–20.5 min held at 10:90; 20.5–21 min from 10:90 to 95:05 (linear elution); 21–25 min held at 95:05 (re-equilibration step). Compounds were quantified with the external calibration method. Before analysis, extracts were filtrated and diluted ten times. Data were expressed in milligrams of standard per gram of dry matter. The ESI source was operated in negative mode. The nebulization gas flow was set at 1.5 L/min, drying gas flow at 20 L/min, the heat block temperature was fixed at 350 °C, and the desolvation line (DL) temperature at 250 °C. The probe voltage was set at –4500 V.

2.9.2. Calibration method

For the determination of calibration curves, standard compounds of salicin, chlorogenic acid, gallic catechin, procyanidin B1, procyanidin B2, procyanidin C1, and catechin were prepared in 70/30 (v/v) methanol/ water. Quantification of standards was performed using the Selected Ion Monitoring (SIM) mode. The method sensitivity and linearity were assessed by determining the limits of detection (LOD) and quantification (LOQ), defined as the concentrations leading to a signal-to-noise ratio (S/N) of 3 and 10, respectively. The determination of LOD and LOQ values was achieved with standards in the following concentration ranges: salicin (0.03–1 mg/mL); catechin (0.05–1 mg/mL); chlorogenic acid (0.01–0.1 mg/mL); gallic catechin (0.01–1 mg/mL); procyanidin B1 (0.01–1 mg/mL); procyanidin B2 (0.01–1 mg/mL); procyanidin B3 (0.01–1 mg/mL). The SIM mode was used in order to improve detection sensitivity in comparison with the full scan mode.

2.10. Statistical analyses

For all experiments, the average value and standard deviation from three replicates were calculated. Statistical analysis (ANOVA) was conducted using SAS statistical software (SAS Institute, NC) with $p < 0.05$ as significance level.

3. Results and Discussion

3.1. Granulometry and particle size distributions

Laser granulometry was carried out on ground and sieved *Salix alba* (L.) bark powders in order to check if the employed grinding/sieving procedure was efficient in producing powders fraction having well-different particle size (Table 1). It can be seen in Table 1 that enough powder for further analyses were obtained in all granulometric classes, except for the smallest one (<20 Nm). All studied powder samples presented monomodal particle size distributions, with their mean particle size comprised in (lower granulometric classes) or slightly above (upper granulometric classes) the particle size range, as well as span values around 2, which confirmed the correct running of the sieving process. The fact that the mean particle size was slightly outside the granulometric class ranges for 315–500 Nm and >500 Nm fractions can be explained by the expected non-sphericity of large particles, as ground fibers generally lead to large rod-shaped particles.

3.2. Chemical composition

Table 2 displays the analytical results for moisture, lipid, protein, ash, and carbohydrate contents for all granulometric classes except the <20 Nm one, as well as for the whole unground plant. It can be seen in Table 2 that the successive grinding/sieving procedure had a great influence on the chemical composition of studied samples. On one hand, the chemical composition significantly differed between whole unground plant and powder samples: the moisture contents were lower for powder samples than for the unground plant, owing to the temperature increase induced by the grinding process. This mostly resulted in greater dry extracts in powder samples, i.e. higher contents in proteins, lipids, ashes, and carbohydrates, which may contribute to increase the content in bioactive molecules in powder samples. On the other hand, the chemical composition depended also on powder granulometry. As fibers are presumably more difficult to grind, their concentration in higher size fractions by the successive grinding/sieving procedure was expected. This leads to higher contents in proteins and lipids in the lowest granulometric classes and may be an indication of higher contents in

bioactive molecules, as the latter are often associated with proteins or lipids in plant tissues.

3.3. Total phenolic content

The Folin-Ciocalteu test was used to evaluate TPC of the different granulometric classes of *Salix alba* (L.) bark powders (Fig. 1). The evolution of TPC with powder granulometry was bell-shaped, with maximal values found in the 50–100 Nm and 100–180 Nm fractions (respectively, 53.6 and 45.3 mg GAE/g DM). These values were two times higher than that of the >500 Nm granulometric class (22.3 mg GAE/g DM). This can be explained by the higher content in carbohydrates (fibers) of larger particles, decreasing the bioactive molecule content of powders of higher granulometry, but also by the temperature increase induced by grinding, which is more pronounced for the smaller particles, leading to a small decrease in TPC for <20 Nm and 20–50 Nm samples. Last, Fig. 1 confirmed the efficiency of the successive grinding/sieving procedure in enhancing the polyphenol content in powder samples, as the unground plant exhibited one of the lowest TPC, excepted for the >500 Nm fraction.

3.4. In vitro antioxidant activity

DPPH is an extensively used method for screening antioxidants. Gomathi and Manian [21] reported that hydroalcoholic extracts from stems, barks, and leaves of *Scolopia crenata* contain high amounts of bioactive compounds and effectively in vitro scavenged DPPH and ABTS. As shown in Table 3, all extracts of *Salix alba* (L.) bark were able to scavenge DPPH radicals, and IC50 decreased with the particle size until the 20–50 Nm fraction (DPPH: 58.54 Ng/mL; ABTS: 46.91 Ng/mL). The lowest antioxidant activity was recorded for the >500 Nm fraction (DPPH: 65.92 Ng/mL; ABTS: 53.36 Ng/mL). This confirms the hypothesis of Bau-delaire [23] regarding the suitability of a successive grinding/sieving procedure to separate bioactive compounds initially located indifferent plant parts. Materials which are difficult to grind (i.e. hard or fibrous materials) are expected to be concentrated in large particles, and bioactive molecules, often bound to proteins or lipids, may preferentially be found in small particles. Besides, ABTS and DPPH tests showed similar variations, as confirmed by Pearson's correlation coefficients: 0.87 for IC50 values, indicating positive correlations of strong intensity.

3.5. AChE inhibition activity of *Salix alba* (L.) extracts

AChE inhibitory acti Powder fractions of *Salix alba* (L.) bark extracts exhibited antioxidant and AChE inhibition abilities. These effects could derive from a synergy between salicin and other active compounds like polyphenols. Results revealed that the AChE inhibition of *Salix alba* (L.) extracts was close to the galantamine action. Moreover, the combination of grinding and vity of extracts of granulometric classes of *Salix alba* (L.) bark, expressed as IC50 values, are presented in Fig. 2. All samples presented significant inhibitory activities towards AChE. IC50 values were found to depend on powder granulometry: AChE inhibition activity increases when particle size decreases until 100–180 Nm and then reaches a maximum for lower granulometric classes, consistently with the above-mentioned decrease in carbohydrates, especially

fibers, and increases in proteins and lipids for smaller particles. The maximal AChE inhibitory activity of *Salix alba* (L.) extracts was obtained for the 20–50 Nm granulometric class (IC50 = 0.59 Ng/mL), while unground plant parts gave the lowest anti-AChE activity (IC50 = 1.52 Ng/mL). Even if the reference control, galantamine, showed a strongest inhibition of AChE (IC50 = 0.34 Ng/mL), it remains a synthetic drug as opposed to *Salix alba* (L.) extracts that are obtained from natural plant powders. In this paper, the maximal IC50 value of hydroalcoholic extracts is very significant, in comparison with the best values obtained for *Scolopia crenata* bark, IC50 = 9.20 Ng/mL [21] and *Viburnum lantana* extract, IC50 value = 50 Ng/mL [35]. Showing the potential of *Salix alba* (L.) bark powder extracts in constituting a possible natural medicine for the treatment of neurological effects of Alzheimer's disease.

3.6. LC-ESI/MS analyses

3.6.1. Identification of bioactive compounds in *Salix alba* (L.) bark powders

The main bioactive compounds in *Salix alba* (L.) bark powders were identified and quantified by LC-ESI/MS. LC-ESI/MS analyses of standard compounds allow determining the retention times and m/z ratios of compounds. Extracted-ion chromatograms of identified compounds are displayed in Fig. 3. Table 4 presents retention times and m/z ratios of these molecules. Two catechins were detected in extracts, namely (+)-catechin (m/z 289) and (–)-gallocatechin (m/z 305) identified at 9.18 and 7.26 min retention times, respectively. The presence of three condensed tannins was also confirmed. Procyanidin B1 ((–)-epicatechin-(4 β → 8)-(+)-catechin), procyanidin B2 ((–)-epicatechin-(4 β → 8)-epicatechin) and procyanidin C1 ((–)-epicatechin-(4 β -8)-epicatechin-(4 β -8)-epicatechin) were identified at 8.76 and 8.20, and 8.92 min retention times, respectively. Another signal recorded at m/z 865 (tR = 9.97 min) close to this of procyanidin C1 could be attributed as an isomer. This behavior is similar as this observed for procyanidin B1 and procyanidin B2. However, the lack of standard compound of other procyanidin C isomers commercially available could not allow us to confirm this assumption. Salicin was detected in *Salix alba* (L.) extract as for other *Salix* varieties [36]. However, this compound was not detected in the deprotonated form (m/z 285) but in a formate adduct form (m/z 331), similarly to Blazics, Papp, and Kéry [37], who operated in close experimental conditions (water acidified with 0.5% formic acid in water/acetonitrile solution).

3.6.2. Quantification of active compounds

Coefficients of determination (R2) for the calibration curves obtained with the external method are displayed in Table 4. These calibration curves were used to quantify catechin, gallocatechin, chlorogenic acid, procyanidin B1, procyanidin B2, procyanidin C1, and salicin in bark powder extracts. Fig. 4 exhibits their contents calculated for all granulometric classes, as well as for unground plant parts. The highest content was determined for procyanidin B1 with 5.9 mg/g DM for the 50–100 Em particle sizes. Procyanidin B2 and procyanidin C1 contents were also noteworthy with 3.4 and 1.8 mg/g DM, respectively, for the 50–100 Em fraction. Catechin content in powder extracts were higher (about three times) than

galocatechin content. Chlorogenic acid exhibited the lowest content of all the compounds quantified. The influence of the granulometric class on catechin, galocatechin, procyanidins and chlorogenic acid contents was similar: maximum contents were found for granulometric fractions between 20 and 180 μm and the lowest content for the N 500 μm fraction, similarly with TPC results. Contents in catechin, galocatechin, procyanidins, and chlorogenic acid were consistent with the trends observed for the antioxidant activity (Table 3). Indeed, significant positive correlations between these six compound contents and antioxidant activity were evidenced by Pearson's coefficients, calculated for DPPH IC₅₀ values and bioactive compound contents: -0.68 for catechin, -0.62 for galocatechin, -0.62 for procyanidin B1, -0.60 for procyanidin B2, -0.64 for procyanidin C1, and -0.59 for chlorogenic acid. A slight different trend was evidenced for salicin which is not a polyphenol: its highest content was obtained for the smallest granulometry (1.85 mg/g DM for the b 20 μm fraction), nevertheless its content was also well correlated to antioxidant activity (Pearson's coefficient of -0.65 with DPPH IC₅₀ values). Contents in bioactive compounds of unground *Salix alba* (L.) bark were lower than the maximal value obtained for a specific granulometric class, in accordance with previous results. For all investigated bioactive compounds, it was confirmed that the successive grinding/sieving procedure allowed to concentrate bioactive compounds in powders of a particular size range, improving the extraction efficiency in comparison with unground plant parts. The total ion chromatogram (TIC) values showed the same trend as for quantified compounds (see Fig. 4). The highest TICs were recorded between 20 and 180 μm with a maximum reached for the 50– 100 μm fraction (899,226,463 a.u.), whereas the minimum TIC was recorded for particle size N500 μm (537,216,287 a.u.). This behavior suggests that contents in the different constituents are similarly affected by granulometric class size. This induces

that the extraction process may not significantly affect content in minor constituents.

3.7. Bioactivity correlation

A marked correlation was observed between antioxidant activity and polyphenol content (Pearson's coefficients of -0.73 between TPC and DPPH IC₅₀ values, and -0.72 between TPC and ABTS IC₅₀ values), meaning that antioxidant activity could mainly be attributed to poly-phenols as described by Wafa et al. [38]. Moreover, a strong correlation has also been noticed between anti-AChE activity and TPC (Pearson's coefficient of -0.77 between TPC and anti-AChE IC₅₀ values, showing a strong negative correlation: the more polyphenols in the powder, the lower is the anti-AChE IC₅₀, and the higher the anti-AChE activity). The latter information allows to link the presence of salicin and poly-phenols to anti-AChE activity. This was confirmed by the good correlations obtained between anti-AChE IC₅₀ values and bioactive compound contents (Pearson's coefficients of -0.74 for salicin, -0.64 for catechin, -0.76 galocatechin, -0.68 for procyanidin B1, -0.65 for procyanidin B2, -0.72 for procyanidin C1, and -0.60 for chlorogenic acid), confirming the role of the identified bioactive compounds in anti-AChE activity of investigated powders. Erdogan-Orhan et al. [35] reported that salicin and *Viburnum lantana* methanol extracts (containing salicin) inhibited the AChE. A salicin derivative, the salicylate, irreversibly inhibits the enzymatic activities via its catalytic site by interaction with serine [39]. It is known that the catalytic site of AChE has also a serine and it could be salicin inhibiting enzyme action by a link with serine. Finally, these correlations show that there could be a synergistic effect between salicin and polyphenols for the inhibition of AChE.

4. Conclusion

Powder fractions of *Salix alba* (L.) bark extracts exhibited antioxidant and AChE inhibition abilities. These effects could derive from a synergy between salicin and other active compounds like polyphenols. Results revealed that the AChE inhibition of *Salix alba* (L.) extracts was close to the galantamine action. Moreover, the combination of grinding and sieving processes was successful to generate granulometric classes significantly differing in bioactive compound contents,

and thus in antioxidant and anti-AChE activities. Indeed, it was shown that these biological activities were correlated to the presence of bioactive compounds, mainly catechin, galocatechin, procyanidins, chlorogenic acid, and salicin. This reveals that these powder extracts could constitute an alternative way to treat Alzheimer's disease. Finally, this study also that bioactive compounds were concentrated in *Salix alba* (L.) bark powders by grinding, sieving, and selection of the adequate granulometric class.

Table 1. Sieved masses, mass fractions, mean particle size, and span of particle size distribution of granulometric classes and unsieved powder of *Salix alba* (L.) bark.

Granulometric classes	Sieved mass (g)	Sieved mass fractions (%)	Mean particle size d_{50} (E μ m)	Span (-)
<20 E μ m	4	0.30%	n.d.	n.d.
20–50 E μ m	112	7.20%	29 \pm 0	2.0 \pm 0.0
50–100 E μ m	150	9.70%	81 \pm 1	2.3 \pm 0.0
100–180 E μ m	164	10.70%	187 \pm 3	1.8 \pm 0.0
180–315 E μ m	100	6.50%	323 \pm 2	1.6 \pm 0.0
315–500 E μ m	70	4.50%	523 \pm 5	2.0 \pm 0.1
N 500 E μ m	940	61.00%	714 \pm 15	1.9 \pm 0.0
Unsieved powder (total)	1540	100%	451 \pm 1	3.7 \pm 0.1

n.d.: not determined.

Table 2. Chemical composition (moisture, lipids, proteins, ashes, carbohydrates) on wet basis of all granulometric classes (except the <20 Nm one) of *Salix alba* (L.) bark and the whole ungroundplant.

Sample	Unground plant	20–50 Nm	50–100 Nm	100–180 Nm	180–315 Nm	315–500 Nm	>500 Nm
Moisture (% (w/w))	9.83 ± 0.08 ^a	8.86 ± 0.43 ^c	9.46 ± 0.19 ^b	8.71 ± 0.36 ^c	8.13 ± 0.25 ^d	8.25 ± 0.37 ^d	7.80 ± 0.27 ^d
Lipids (% (w/w))	4.27 ± 0.54 ^d	1286 ± 1.20 ^a	6.52 ± 0.83 ^b	7.56 ± 0.65 ^b	7.70 ± 0.76 ^b	5.44 ± 0.49 ^c	5.52 ± 0.80 ^c
Proteins (% (w/w))	6.90 ± 0.21 ^e	1399 ± 1.42 ^c	1551 ± 0.74 ^b	1752 ± 0.94 ^a	1133 ± 0.25 ^d	1235 ± 0.72 ^c	5.80 ± 1.01 ^f
Ashes (% (w/w))	7.19 ± 0.06 ^c	1357 ± 0.11 ^a	6.51 ± 0.15 ^d	6.99 ± 0.29 ^c	7.05 ± 0.24 ^c	7.59 ± 0.29 ^b	7.14 ± 0.15 ^c
Carbohydrates (% (w/w))	7181 ± 0.89 ^b	5072 ± 3.16 ^e	6200 ± 1.91 ^d	5922 ± 2.24 ^d	6579 ± 1.50 ^c	6554 ± 1.87 ^c	7647 ± 2.23 ^a

Presented data are mean ± standard deviation of triplicate measures; mean values in the same line followed by different letters were statistically different ($p < 0.05$).

Table 3. Radical scavenging assays (IC₅₀) for the different granulometric classes and unground plant parts of *Salix alba* (L.) bark

Granulometric class	DPPH test	ABTS test
	IC ₅₀ (Ng/mL)	IC ₅₀ (Ng/mL)
<20 nm	59.47± 0.03 ^a	47.37± 0.02 ^a
20–50 nm	58.54± 0.02 ^b	46.91± 0.06 ^b
50–100 nm	60.12± 0.04 ^c	48.44± 0.06 ^c
100–180 nm	61.10± 0.05 ^d	49.24± 0.04 ^d
180–315 nm	61.36± 0.02 ^e	49.85± 0.07 ^e
315–500 nm	61.76± 0.08 ^f	50.17± 0.08 ^f
>500 nm	65.92± 0.02 ^g	53.36± 0.04 ^g
U.B.	61.43± 0.08 ^e	49.14± 0.07 ^d

DM: dry matter; U.B.: unground *Salix alba* (L.) bark. Means ± standard errors in the same column followed by different letters corresponded to statistically different results ($p < 0.05$). Presented data are mean ± standard deviation of triplicate measures; mean values in the same column followed by different letters were statistically different ($p < 0.05$).

Table 4. Retention times, m/z ratios, coefficients of determination (R²), LOD and LOQ values.

Compound	m/z	Retention time (min)	LOD (ppm)	LOQ (ppm)	Coefficient of determination (R ₂)
(+)-Catechin	289	9.18	3.4	11.2	0.998
(-)-	305	7.26	3.9	12.1	0.995
Salicin ^a	331	7.53	0.3	1.1	0.999
Chlorogenic acid	353	8.91	2.2	7.3	0.989
Procyanidin B2	577	8.20	3.5	10.6	0.991
Procyanidin B1	577	8.76	3.4	10.5	0.990
Procyanidin C1	865	8.92	4.1	12.4	0.982

^a Detected under the form of formate adduct.

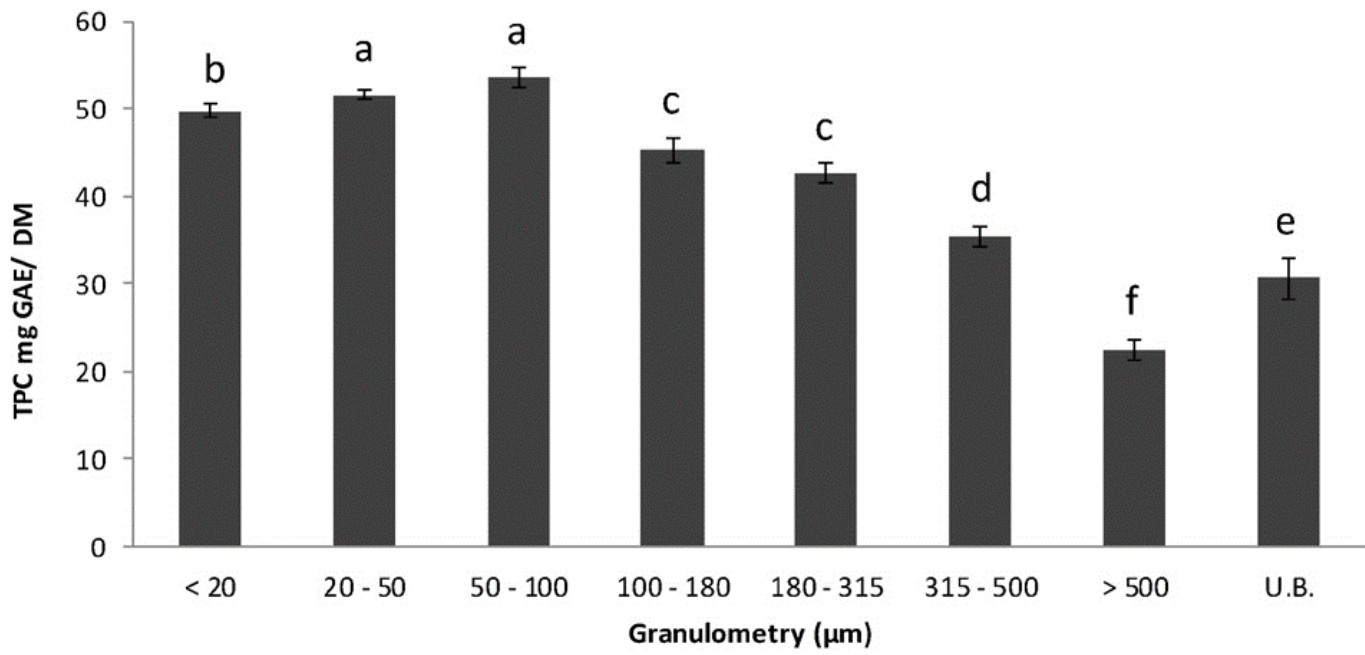


Figure 1. Total phenolic content of *Salix alba* (L.) bark powders

DM: dry matter; GAE: gallic acid equivalent; U.B.: unground *Salix alba* (L.) bark. Bars topped by different letters were statistically different ($p < 0.05$)

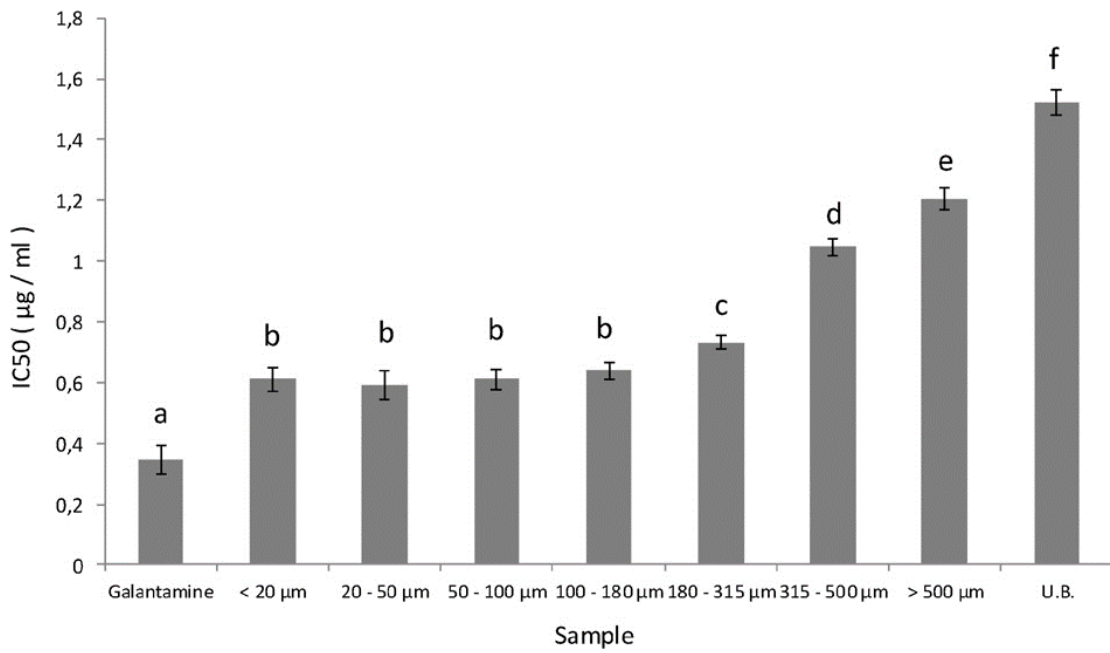


Figure 2. Anti-AChE assays (IC50) of the different granulometric classes and unground sample of *Salix alba* (L.) bark and galantamine

U.B.: unground *Salix alba* (L.) bark. Bars topped by different letters were statistically different ($p < 0.05$).

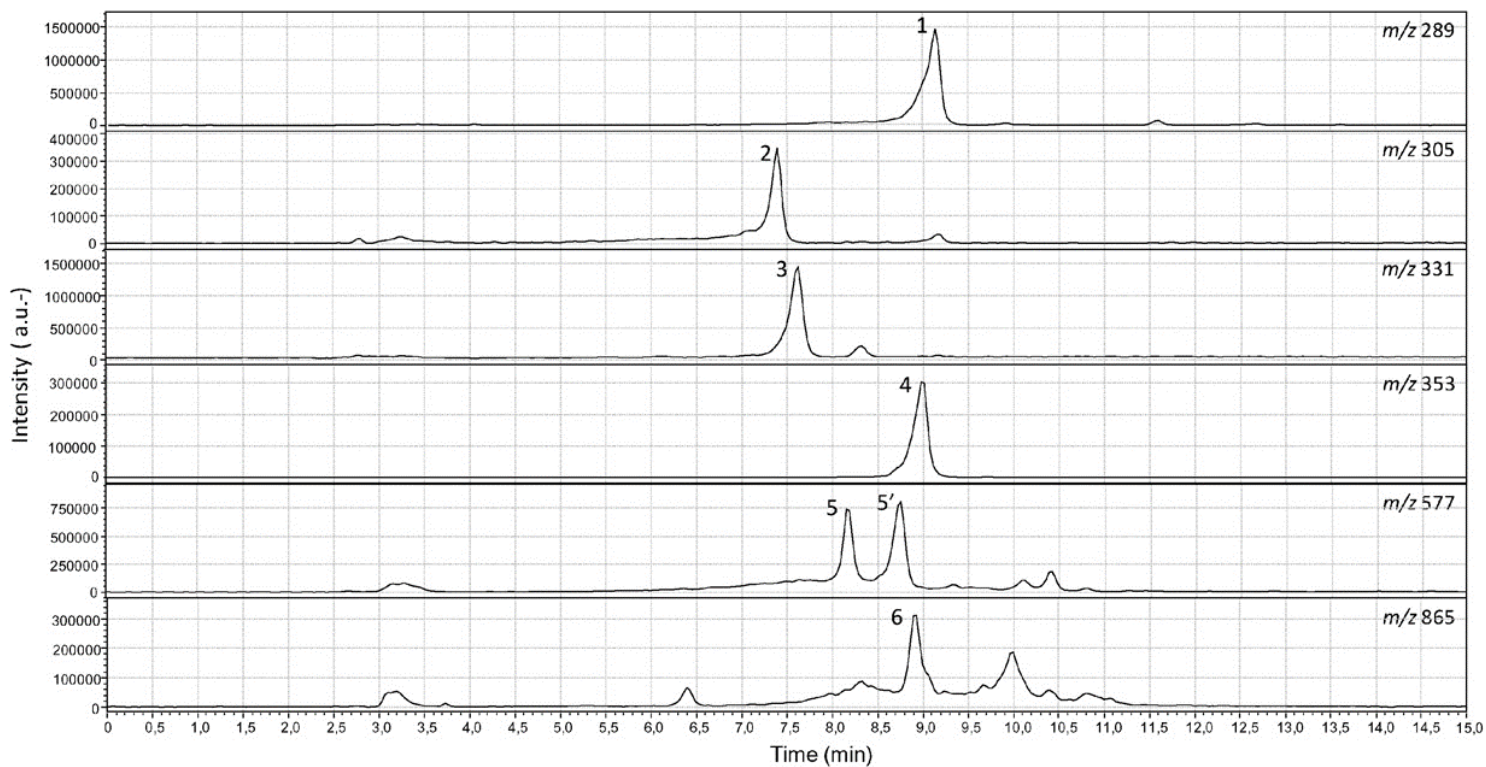


Figure 3. Extracted-ion chromatograms from LC-ESI/MS analysis of hydroalcoholic extracts from *Salix alba* (L.) bark powders

Peak assignments were confirmed by retention times and m/z ratios, as deduced from standard compounds, (+)-catechin (1); (-)-gallocatechin (2); salicin (3); chlorogenic acid (4); procyanidin B2 (5); procyanidin B1 (5'); procyanidin C1 (6).

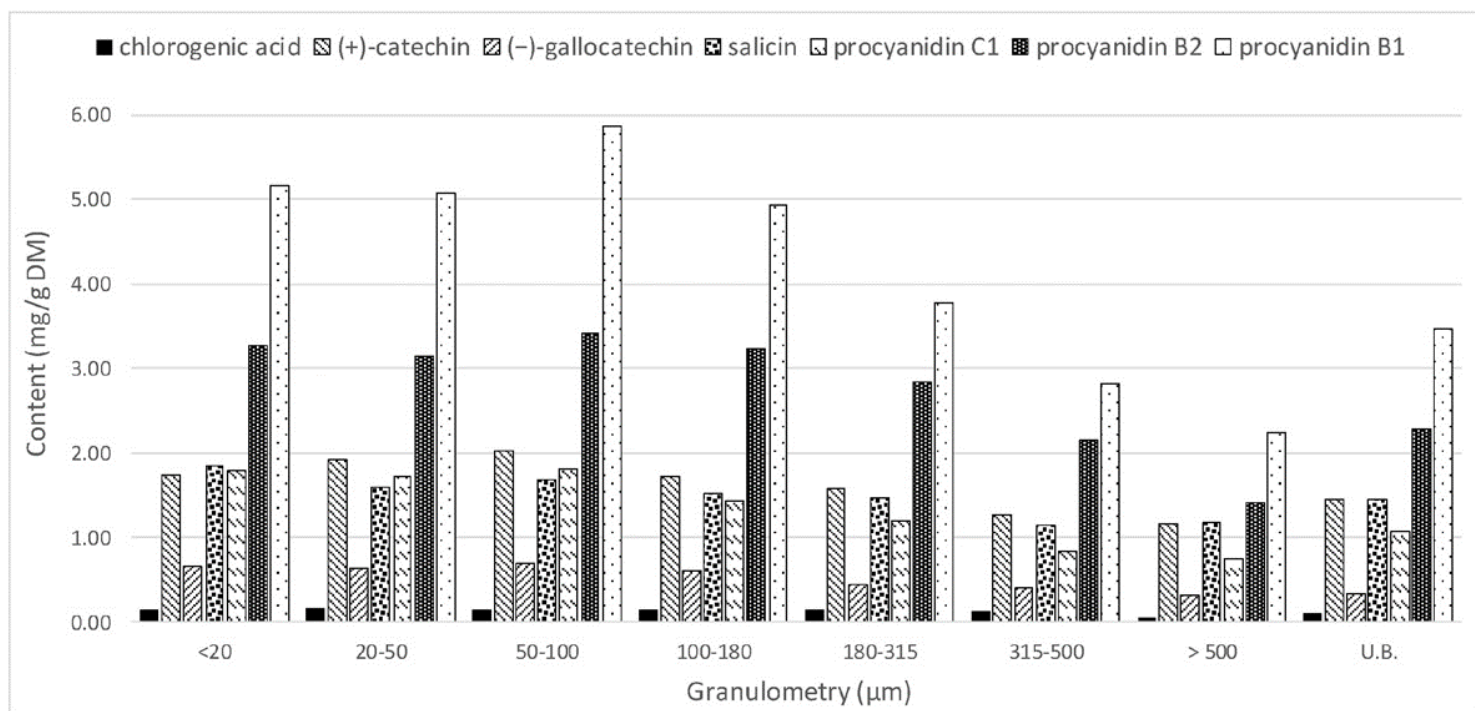


Figure 4. LC-ESI/MS quantification of chlorogenic acid (m/z 353), (+)-catechin (m/z 289), (-)-gallocatechin (m/z 305), salicin (m/z 331), procyanidin C1 (m/z 577), procyanidin B2 (m/z 577), and procyanidin B1 (m/z 577) in *Salix alba* (L.) bark powders. U.B.: unground *Salix alba* (L.) bark.

5. References

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