

Article

Chemical Characterization of *Fraxinus angustifolia* Vahl. ssp. *pannonica* Soó & Simon Stump-Derived Biomass: Evaluation of Valorization Potential

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Abstract

During forest exploitation, large amounts of wood biomass are left as residues after harvesting, even though this material could be valuable in reducing greenhouse gas emissions by replacing fossil fuels and serving as a source of bioactive compounds. Within the context of the European Union's circular bioeconomy strategy, enhancing the efficacy and rational application of forest logging residues constitutes a critical future objective. To evaluate the possible use of the stumps as forest residue-derived woody biomass for the first time, the chemical composition and presence of bioactive compounds in the xylem and bark of *F. angustifolia* Vahl. ssp. *pannonica* Soó & Simon stumps were analyzed, including the content of cellulose, acid-insoluble lignin, acid-soluble lignin, ash, and extractives soluble in toluene–ethanol and hot water. In hot water extracts, total phenolic content, the antioxidant activity determined by scavenging capacity toward DPPH and HPTLC, the content of elements by ICP-OES, and the identification of bioactive compounds by UHPLC-DAD-ESI MS/MS were assessed. Considering the chemical composition, the quality of the xylem of the *F. angustifolia* (cellulose: $42.46 \pm 0.45\%$; lignin: $25.05 \pm 0.02\%$; hemicelluloses: $19.74 \pm 0.46\%$) was similar to that of the stem. Hot water extracts of the bark of *F. angustifolia* stumps showed high total phenolic content (33.94 ± 1.43 mg GAE/g DW) and strong antioxidant potential (198.48 ± 3.33 μ mol TE/g DW), while toxic metals were not detected. UHPLC-DAD-ESI-MS/MS analysis indicated phenylethanoid glycosides as the dominant compounds in the extracts. The analyzed stumps of *Fraxinus angustifolia* Vahl. in the “Morović” Forest Administration represent a valuable biomass with potential for further valorization.

Keywords: *Fraxinus angustifolia* Vahl. ssp. *pannonica* Soó & Simon; stump biomass; UHPLC-DAD-ESI-MS/MS; ICP-OES; antioxidant activity; HPTLC; biomass valorization



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1. Introduction

The stem is technically the highest quality part of wood biomass and the preferred raw material for many mechanical and chemical refining processes [1]. Forest exploitation generates large amounts of wood biomass residues (branches and twigs with leaves or bristles, knots, bark, acorns, and stumps), which most often remain in the forest after cutting trees [2]. These residues are considered an important resource for obtaining antioxidant and bioactive molecules [2]. As Walmsley and Godbold (2010) stated, there is the potential for up to 9 million m³ per year of forest chips from stumps [3]. Sawmill processing generates additional residues, such as bark, which can represent 13–21 wt% of dry wood, depending on the species [4]. The stump and root possess a higher proportion of bark compared to the stem [5]. Despite the substantial annual generation of forest wood waste biomass, the utilization of products derived from this source remains limited.

The chemical properties of the xylem of stems and stumps are relatively similar, but the content of extractive substances in stumps is slightly higher [1,6,7]. Biomaterials such as forest residues and by-products from primary wood processing, which have a high extractive content, are often used for energy production [8]. For many years, stumps have been used as fuel, for tar production, and as building material for ships [3,9,10]. Aligned with the European Union's circular bioeconomy strategy, enhancing the efficiency and rational use of forest logging residues represents an important future priority [11–13]. In recent years, increased attention has been directed toward the utilization of stumps remaining in forests following tree harvesting. While the primary objective was to protect against wooden root rot, this approach also seeks to maximize the utilization of stump biomass and generate valuable commercial products, typically derived from timber [14,15]. Stumps could be a carbon reservoir, as well as hydrogen and nitrogen according to Damyanova and Dimitrova (2023) in their research on the dead wood of beech (*Fagus Sylvatica* L.) forests [16]. The heating value of stumps, which correlates with lignin, carbohydrate and extractive content, was the same or in some cases higher than in the stem, branches or root of some species of the *Pinus*, *Picea*, *Betula*, *Alnus*, and *Populus* genus as stated in the work of Nurmi (1997) [17]. Some previous research indicates that stumps and roots, especially of conifers, represent a reservoir of secondary metabolites, primarily polyphenols, tannins, lignans, resin acids, diterpenoids, sterols, and stilbenoids, with protective, antioxidant, and antimicrobial properties [8,18]. For example, Latva-Mäenpää (2017) found that the bark of stumps of *Picea abies* contains stilbenoid glucosides and also free stilbenoids (astringin, isorhapontin, piceid, piceatannol, and isorhapontigenin). Catechin and β -sitosterol were also identified. Wood samples of Scots pine stump had stilbenoids, pinosylvins and pinosylvin monomethyl ether, while the bark contained catechin [18]. In *Picea abies* stump hearts, Halmemies et al. (2023) identified lignans hydroxymatairesinol, conidendrin, lignan A, iso-hydroxymatairesinol, lariciresinol, and isolariciresinol. Stilbene glucosides were also found in stump hearts and crushed samples of stumps, where the bark and xylem were mixed together [8]. Many authors have found that the stumps of *P. abies* contain larger amounts of resin acid than stems, knotwood or roots [19]. It has also been found that *Eucalyptus globulus* stumps represent an important source for the extraction of bioactive molecules with antioxidant and antimicrobial effects, especially polyphenols, including flavonoids, which are well-known bioactive compounds [6].

Therefore, the use of stumps for other purposes, such as the extraction of bioactive molecules, is expected to gradually increase, thereby increasing the demand for stump cutting. Nevertheless, these practices raise concerns about the impacts of stump removal on forest ecosystems, particularly with respect to soil quality and forestry sustainability [7,8]. Removing stumps can significantly alter the soil structure (therefore increasing

soil erosion), cause changes in the natural carbon cycle, cause soil demineralization and acidification through leaching, and cause changes in the flora and fauna that use stumps as substrates [3,9]. Stump harvesting is mainly carried out with old technology—large excavators—often largely disturbing soil properties. This process, together with collection, transportation and conversion of stumps into a desirable product, indicates a heightened level of forest management [3,10]. On the other hand, in areas where stump harvesting is not practiced, the natural decay of stumps caused by root rot fungi can result in significant annual wood loss [14]. Insects involved in the decomposition of tree stumps can elevate the mortality risk of newly planted trees [9]. In addition, leaving a large number of stumps in the forest reduces the possibility of planting new trees and reforestation, as sufficient land space is necessary for the adequate development of new trees [10].

Given these opposing perspectives—the valorization of stumps as a raw material versus their role in ecosystem functioning and soil maintenance—the importance of understanding the chemical composition of stumps, which has not been sufficiently researched, is highlighted. However, the chemical composition of the xylem and bark of the stump of most deciduous species, such as narrow-leaved ash (*Fraxinus angustifolia* Vahl. ssp. *pannonica* Soó & Simon), has not been sufficiently investigated.

Narrow-leaved ash belongs to the *Oleaceae* family, genus *Fraxinus*, which includes 70 tree species. It is abundant in Europe (Mediterranean and Pannonia), West Asia, and North Africa [20,21]. In the Balkans and Pannonia, it is primarily located in plains, riverbanks, and floodplains near the major rivers [22]. The European program of forest genetic resources (EUFORGEN) identified narrow-leaved ash as one of 27 noble deciduous tree species [23]. Narrow-leaved ash is a medium-strong wood with average malleability, suitable for making pulp, plywood, laminated veneer lumber, and glued laminated timber [22].

According to Zhao et al. (2025), 326 compounds were isolated and identified to date from the *Fraxinus* genus [24]. Many of these compounds belong to polyphenols, which are important bioactive molecules of trees. Their chemical structure includes more than 8000 hydroxyphenyl compounds, and they range from simple molecules, such as phenolic acids, to highly polymerized structures like condensed tannins and lignins [25]. Most polyphenols are composed of fundamental building blocks derived from simpler phenolic moieties, which give them specific biochemical properties, such as the capacity to form complexes with proteins and metals and the ability to react with peroxy radicals by pulling the easily removed hydrogen from their hydroxyl group, creating a more stable radical oxidant that can help scavenge reactive oxygen species [25–27]. Nykolov et al. (1993) identified the coumarins esculin, esculetin, fraxin, and fraxetin in *F. ornus* bark [28]. From the stem bark of *F. chinensis*, 26 bioactive compounds were isolated, such as oleuropein, fraxetin, aesculetin, aesculetin dimethyl esterisoscopoletin, tyrosol, calceolarioside B, verbascoside, isoacteoside, and lugrandoside [29,30]. Jensen and Nielsen (1976) isolated derivatives of fraxidin, fraxinol, and 10-hydroxyligstroside from *F. excelsior* bark [31]. From the bark of *F. rhynchophylla* Hance, coumarins and secoiridoid glucosides such as esculetin, esculin, ligstroside, and oleuropein were isolated [32]. From the fresh fruiting leaves of *F. uhdei*, Shen et al. (1993) isolated 10-hydroxyligustroside and verbascoside, fraxuhdoside, and insularoside [33]. Kiss et al. (2020) identified 64 compounds in *F. excelsior* leaves, including phenylethanoids, flavonoids, phenolic acid derivatives, lignans, iridoids, and secoiridoids. Among these compounds, the major ones detected were ligstroside, verbascoside, oleuropein, chlorogenic acid, and quercetin-3-O-rutinoside [34]. In the leaves and bark of *F. angustifolia*, Kasmi et al. (2021) determined coumarins (esculetin, esculin, fraxin, fraxetin, fraxidin), secoiridoids (oleuropein, oleoside, ligustroside), phenylethanoids (verbascoside, isoverbascoside,

calceolariosides), phenolic acids (caffeic acid, protocatechic acid, chlorogenic acid, *p*-hydroxybenzoic acid, hydroxytyrosol), and flavonoids (rutin, nicotiflorin, quercetin-3-O-glucoside, kaempferol-O-glucoside) [35]. Many of these compounds have antimicrobial, anti-inflammatory, antioxidative, and anti-hepatotoxic activities. They inhibit enzymatic activity, while esculin aids in skin regeneration, and coumarins from narrow-leaved ash bark prevent photodynamic cell damage [36]. The bark of narrow-leaved ash has anti-inflammatory properties and is used as an antioxidant, diuretic, and astringent for digestive health [37]. Recent research has shown significant the antimutagenic and antigenotoxic activity of narrow-leaved ash bark and leaves [35,38]. After air-drying, bark is used in the pharmaceutical industry and in cosmetic preparations [23]. In the study conducted by Ahmane et al. (2019), it was noted that esculin and calceolarioside, which were isolated from the bark of narrow-leaved ash, demonstrate significant anti-nicotinamide adenine dinucleotide oxidase activity in xanthine oxidoreductase. This suggests that the bark is a valuable source of therapeutic compounds [39]. Bougelid et al. (2020) found that the ethanol extract of narrow-leaved ash bark showed significant antimutagenic and antigenotoxic properties, while chloroform/water bark extract showed selective cytotoxicity towards cancer cells [38].

To investigate the possible usage of stumps, the chemical composition and presence of bioactive compounds of narrow-leaved ash stumps from Morović were analyzed. The content of the basic chemical components of the xylem and bark of the stumps of narrow-leaved ash was determined, and due to the potential presence of bioactive compounds, special attention was devoted to the water extracts. The content of the total polyphenols and the antioxidant activity of the extracts were determined. In addition, individual bioactive components present in hot water extracts as well as potentially toxic elements were identified.

Comprehensive characterization of lignocellulosic biomass requires the application of complementary analytical approaches due to the complexity of its chemical composition. Spectrophotometric methods allow a quick assessment of the total content of phenolic compounds and antioxidant potential, while chromatographic techniques, such as HPTLC and UHPLC-DAD-ESI-MS/MS, provide a more detailed insight into the distribution and chemical profile of secondary metabolites. In particular, HPTLC coupled with DPPH• derivatization enables the visualization of radical-scavenging zones directly on the chromatographic plate, providing information on the distribution of zones exhibiting DPPH radical-scavenging activity within complex extracts. ICP-OES analysis additionally enables a reliable assessment of the mineral composition of the biomass. Although individual methods have certain limitations in terms of selectivity and identification of compounds, their combined application enables a more comprehensive chemical characterization and assessment of the potential of lignocellulosic residues for further valorization.

Despite the importance of *Fraxinus* species as a source of lignocellulosic biomass and phenolic compounds, data on the chemical composition and valorization potential of *Fraxinus angustifolia* stumps are very limited. To the best of our knowledge, this research represents the first integrated chemical characterization of xylem and bark of *Fraxinus angustifolia* Vahl. ssp. *pannonica* Soó & Simon stumps using standardized analysis of lignocellulosic composition, determination of total phenols and antioxidant activity, HPTLC-DPPH bioautography, UHPLC-DAD-ESI-MS/MS profiling, and ICP-OES elemental analysis. The work aimed to evaluate the chemical composition and valorization potential of this type of biomass within the sustainable use of wood biomass.

In this context, hot water extraction is increasingly considered a viable option for modifying and improving wood chip products (e.g., for the production of pellets [40,41], wood plastic composites [42,43], particleboards [44], fiberboards [45] and OSB panels [46]) while simultaneously utilizing the liquid extract phase. This approach is in line with the principles of biorefinery and complete wood biomass utilization, meaning that this manuscript can serve as a useful guideline for future researchers in this field.

2. Materials and Methods

2.1. Plant Material

The wood material was sourced from a sustainably managed forest of the Public Enterprise “Vojvodinašume” (Petrovaradin, Serbia), specifically from the Forest Estate “Sremska Mitrovica”. This enterprise holds a valid Forest Stewardship Council (FSC) certification (Certificate No. FSC-C021645, valid until August 2028 [47]). Samples of narrow-leaved ash (*Fraxinus angustifolia* Vahl. ssp. *pannonica* Soó & Simon) stumps were collected from Forest Compartment No. 9 within the “Smogva-Grabova Greda” Forest Management Unit, managed by the Forest Administration “Morović” in the Municipality of Šid (geographical coordinates 44°59'15" N 19°09'53" E). The botanical identity and strict geographic origin of the species were officially verified based on the supplier's forest management registry and further confirmed by the macroscopic features.

Sampling and sample preparation for chemical analysis were carried out in accordance with the standard procedure TAPPI T 257 [48] and ISO 14780 [49] (Figure 1). To ensure the representativeness and repeatability of the findings, samples in the form of 3 cm thick discs were taken in March 2025 from the stumps of three healthy, randomly selected, freshly felled 102-year-old narrow-leaved ash trees, immediately after regular commercial logging operations. The sampled discs were air-dried for three months under laboratory conditions and protected from light, after which the bark was manually separated from the wood. Following separation, the xylem discs were cut cross-sectionally along their diameter into four quarters. To maintain the original proportions of sapwood and heartwood, two opposite quarters of each xylem disc, as well as the entire quantity of bark, were coarsely chopped manually to obtain smaller fragments. After grinding with a hammer mill (Culatti AG micro mill DFH 48, Zürich, Switzerland), samples were pooled and thoroughly homogenized to prepare uniform samples before being sieved. Particle size fractionation of the ground material was performed via mechanical sieving using a standard set of wire cloth test sieves mounted on a laboratory sieving machine (JEL, J. Engelsmann AG, Ludwigshafen am Rhein, Germany), operating for 10 min to ensure complete and reproducible separation of the target fraction (0.5–1.0 mm) [48]. To ensure sample representativeness, the fractionated wood and bark samples were reduced to the final analytical mass using the manual coning and quartering method on a clean, antistatic substrate, following the guidelines of the ISO 14780 standard [49]. After being air-dried at room temperature to reach a stable moisture content of approximately 10–12%, the particle fraction of 0.5–1 mm was stored in sealed glass containers for subsequent analysis [48]. This yielded two prepared samples: xylem and bark.

The moisture content of the samples was measured by drying them in a laboratory dryer at 105 ± 2 °C until a constant mass was achieved [50]. The moisture content of the samples of xylem and bark was $8.07 \pm 0.02\%$ and $7.02 \pm 0.02\%$, respectively.

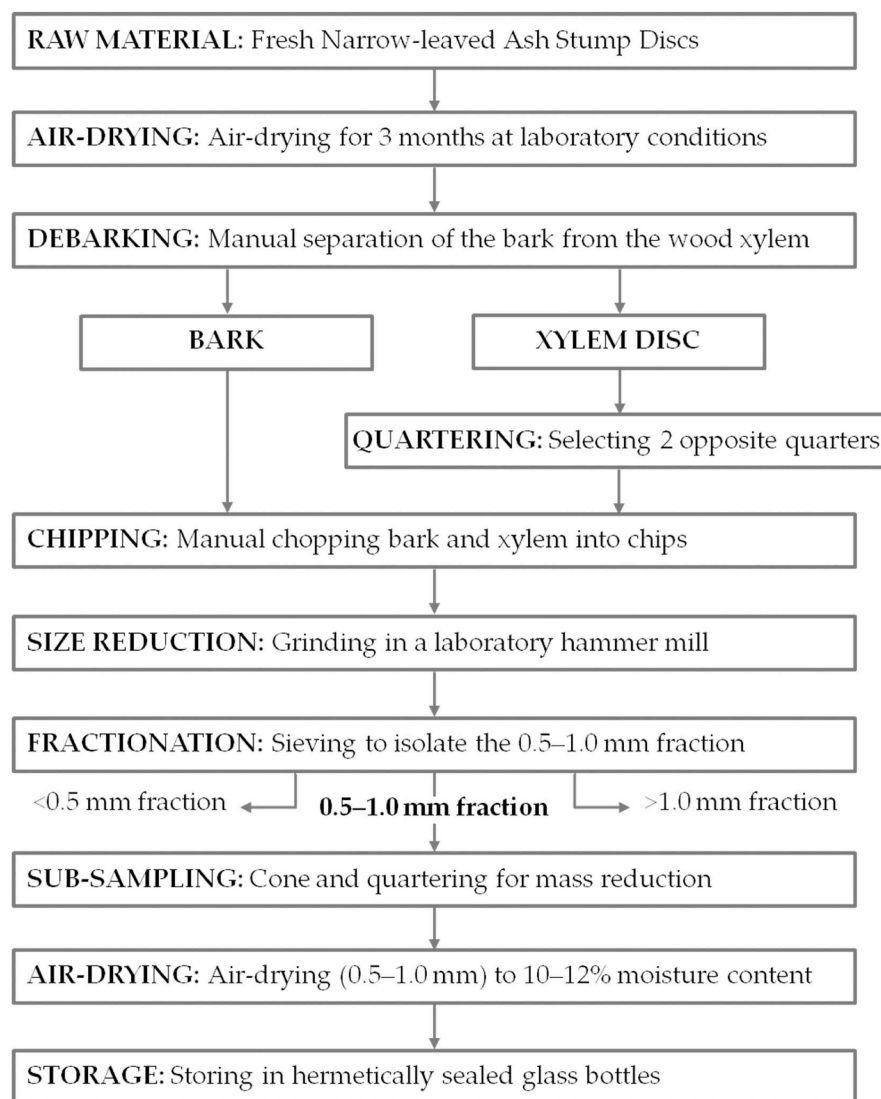


Figure 1. Schematic representation of narrow-leaved ash stump preparation for chemical analysis. The arrows indicate the sequence of the preparation steps.

2.2. Chemicals and Materials

All chemicals and reagents for this study were sourced from commercial suppliers. Folin-Ciocalteu reagent (for microscopy) was from CARLO ERBA Reagents (Cornaredo, Italy), and the standard of gallic acid (99.97%) was from BLD PHARMATECH GmbH (Reinbek, Germany). Sulfuric acid ($\geq 97\%$), nitric acid (65%), acetic acid (glacial), sodium carbonate (anhydrous, $\geq 99.5\%$), polyethylene glycol (PEG) 4000, and silica gel 60 (Art. 105461) HPTLC glass plates were supplied by Merck (Darmstadt, Germany). Toluene (p.a., $>99\%$) and ethanol (p.a., 96%) were obtained from Centrohem d.o.o. (Stara Pazova, Serbia). 2,2-diphenyl-1-picrylhydrazyl (DPPH, $\geq 95\%$) free radical, *p*-anisaldehyde, and 2-aminoethyl diphenylborinate (NP reagent) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Sodium acetate trihydrate G.R. ($\geq 99\%$) was purchased from Lach-Ner, S.R.O. (Neratovice, Czech Republic). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid (Trolox, $\geq 98\%$) was supplied by Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Methanol (LC-MS grade, $\geq 99.9\%$) and water (LC-MS purity) were purchased from Fisher Scientific Co. (Ward Hill, MA, USA). Formic acid (HPLC grade, $\geq 98\%$) was purchased from CARLO ERBA Reagents (Val-de-Reuil, France).

2.3. Chemical Composition Determination

Cellulose content was determined by the Kürschner–Hoffer method. The sample was treated with a mixture of 65% nitric acid and 96% ethanol in a 1:4 (*v/v*) ratio in a water bath (WKP-14, ViMS-elektrik d.o.o., Tršić, Serbia) maintained at the boiling temperature of the reaction mixture. This reflux treatment was performed in successive 1 h cycles (two for wood and three for bark) to dissolve lignin and hemicelluloses. The remaining cellulose residue was filtered, washed to neutrality with distilled water, dried at 105 ± 2 °C to a constant mass, and quantified gravimetrically [51]. Lignin content was quantified by combining the gravimetric method for acid-insoluble lignin and the spectrophotometric method for acid-soluble lignin. Acid-insoluble (Klason) lignin was isolated via two-stage hydrolysis of extractive-free samples using 72% sulfuric acid at 20 ± 1 °C for 2 h in the first step, followed by dilution to a 3% acid concentration and secondary hydrolysis under reflux in a sand bath (Sutjeska, Belgrade, Serbia) at the boiling temperature of the mixture for 4 h. The insoluble precipitate was filtered, washed to neutrality with hot distilled water, dried to a constant mass at 105 ± 2 °C, and quantified gravimetrically [52]. Meanwhile, the acid-soluble lignin fraction was quantified by measuring the absorbance of the supernatant obtained prior to filtration, at a wavelength of 205 nm with an Evolution 300 UV–Vis spectrophotometer (Thermo Electron Corporation, Altrincham, UK) [53]. Organic-soluble extractives were quantified using a 150 mL toluene:ethanol (2:1, *v/v*) mixture. After 8 h of extraction in a Soxhlet apparatus in a sand bath, the toluene–ethanol extractive content was determined by drying and weighing the residue after solvent evaporation; concurrently, this phase yielded the extractive-free samples required for subsequent lignin analysis [54]. The hot-water-soluble extractives were determined via extraction in a boiling water bath. After 3 h of extraction, the resulting water extracts were collected and stored at 4 °C until further analysis. The remaining wood and bark residues were separated by filtration, washed, and dried to a constant mass at 105 ± 2 °C. The water-soluble extractive yield was calculated gravimetrically based on the mass loss of the extracted residue relative to the oven-dry mass of the initial sample [55,56]. Finally, the inorganic fraction (ash content) was determined gravimetrically by weighing the residue after complete combustion at 900 ± 25 °C in a muffle furnace (ViMS-elektrik d.o.o., Tršić, Serbia) until a constant mass was reached [57]. All analyses were conducted in triplicate (three independent analytical replicates), and the results are expressed on an oven-dry weight (DW) basis and presented as mean values with standard deviations. The content of hemicelluloses, including other unquantified components, was determined approximately by subtracting the sum of all quantified components (cellulose, lignin, total extractives, and ash) from the total sample mass (100%). The results are shown as histograms created using Microsoft Excel 2010, version 14.0.4760.1000.

2.4. Total Phenolic Content (TPC)

TPC was determined for extractives soluble in hot water using the method of Aksić et al. (2023) [58]. In the 0.5 mL of diluted water extracts (1000 µg/mL), 2 mL of 10% (*v/v*) Folin-Ciocalteu reagent was added. After waiting for 3 min, 2.5 mL of 10% Na₂CO₃ was added and left in the dark for 30 min. Absorbance was measured in quartz cuvettes at 765 nm with an Evolution 300 UV–Vis spectrophotometer. TPC was read from the calibration curve obtained with different concentrations of gallic acid (10, 25, 50, 75, and 100 µg/mL) and expressed as gallic acid equivalents per g dry weight (mg GAE/g DW). The obtained calibration curve had the correlation coefficient $R^2 = 0.999$. Sample replicates were tested to maintain a relative standard deviation below 10%. Analysis was performed in triplicate, and the results are presented as mean values with standard deviations.

2.5. Antioxidant Potential of Water Extracts Determined Spectrophotometrically

Water is a good solvent for polyphenols, which are often characterized by antioxidant activity. Thus, the antioxidant activity of narrow-leaved ash stump water extracts was determined by the scavenging capacity of the DPPH free radical using the method of Smailagić (2019) [59]. DPPH• solution was prepared by mixing 186 μM of freshly prepared ethanol solution of DPPH• and acetate buffer (pH 5) in a ratio of 2:1 *v/v* in a volumetric flask, which was protected from light with aluminum foil. In 0.2 mL of diluted water extract (1000 $\mu\text{g/mL}$), 2.8 mL of DPPH• solution was added, and after incubation for 90 min in the dark, the decrease of absorbance was measured at 525 nm with an Evolution 300 UV-Vis spectrophotometer. The calibration curve was established using a Trolox solution in ethanol with different concentrations in a range of 50–450 μM . DPPH radical scavenging capacity of samples was expressed as μmol of Trolox Equivalent (TE) per g of dry weight ($\mu\text{mol TE/g DW}$). The obtained calibration curve had the correlation coefficient $R^2 = 0.995$. Sample replicates were tested to maintain a relative standard deviation below 10%. Analysis was performed in triplicate, and the results are presented as mean values with standard deviations.

2.6. Antioxidant Potential of Water Extracts Determined by HPTLC

The DPPH method using high-performance thin-layer chromatography (HPTLC) was used to investigate the antioxidant capacity of water extracts of narrow-leaved ash stumps. HPTLC is a quick and cost-effective analytical method that enables the simultaneous analysis of several samples with minimal solvent use [60]. A particular advantage of this method is the possibility of direct visualization and assessment of the biological activity of the separated components on the plate, which makes it very suitable for the screening and comparative analysis of complex plant extracts, including the assessment of their antioxidant potential.

A 30 μL aliquot of extract (20 mg/mL) was applied to HPTLC glass plates with silica gel 60 F254, as 6 mm wide bands using a CAMAG Linomat 5 (Muttens, Switzerland). The band was placed 8 mm from the bottom edge of the plate, with a minimum distance of 12 mm from both sides. Chromatographic development was carried out using the mobile phase ethyl-acetate:water:formic acid (80:10:10; *v/v/v*) [61] in a saturated Twin Trough chamber. The chamber was saturated with the mobile phase for 20 min prior to development, with a solvent migration distance of 70 mm. The resulting HPTLC chromatogram was documented under white light conditions, and images were recorded in TIFF format.

Antioxidant activity was determined by the DPPH method, by immersing the plate in a 0.05% (*w/v*) methanolic solution of DPPH• for 1–2 s to completely coat the plate and then incubating it for 30 min in the dark. Antioxidant activity was visible as bright zones on a purple background under white light [62].

2.7. UHPLC-DAD-ESI-MS/MS Analysis

Ultra-high performance liquid chromatography coupled with diode array detection and electrospray ionization tandem mass spectrometry (UHPLC-DAD-ESI-MS/MS) analysis was performed using a Dionex Ultimate 3000 UHPLC+ system equipped with a diode array (DAD) detector and an LCQ Fleet ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA). The chromatographic column was Hypersil Gold C18 (50 $\times$ 2.1 mm, 1.9 μm) from the same producer, set at 25 $^{\circ}\text{C}$. The mobile phase at flow rate 0.25 mL/min consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol with the following eluting gradient program: first two minutes, from 10% to 30% (B); 2–4 min, 30% to 35% (B); 4–7 min, from 35% to 40% (B); 7–8 min, 40–60% (B); 8–11 min,

from 60% to 90% (B); an isocratic run at 90% (B) from 11 to 15 min; for 15–15.01 min, from 90% to 10% (B); and an isocratic run with 10% (B) to the 20th min. For the analysis, dry extracts were dissolved in a water: methanol (9:1, *v/v*) mixture to a concentration of 10 µg/mL, and the injection volume of the samples was 4 µL.

The DAD-detector was set at a total spectral range between 190 and 700 nm. The mass spectrometry (MS) analysis was performed using an LCQ 3D-ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA) with electrospray ionization (ESI) in the negative and positive ion mode using both full MS between m/z 100 and m/z 1000 and a data-dependent scan method for the precursor ions with a collision-induced dissociation (CID) set at 30 eV in a stream of He. The ESI-source parameters were set as follows: sheath and auxiliary gas were nitrogen, set at 32 and 8 flow arbitrary units; capillary temperature 350 °C for both modes; source voltage 4.5 kV and 5.0 V, capillary voltage −41 V and 49 V, and tube lens voltage −95 V and 115 V for negative and positive ESI-MS modes, respectively.

Instrument control, data acquisition, and data analysis were conducted with Xcalibur software (version 2.1, Thermo Fisher Scientific, USA). The compounds were tentatively identified based on their retention times and elution order, available UV–Vis spectra from the DAD detector, MS spectra with the corresponding molecular ion peaks ($[M-H]^-$, $[M+H]^+$) and their corresponding adduct ions ($[M-H+HCOOH]^-$, $[M+Na]^+$, $[M+NH_4]^+$), and the characteristic ion fragmentation of selected peaks (MS/MS spectra) from UHPLC-DAD-ESI-MS chromatograms (Figures S1–S4). The corresponding chromatography and spectral data were compared with the available literature. Compound identification was considered tentative due to the absence of authentic standards for all detected compounds and was based on the combined interpretation of chromatographic behavior, UV–Vis spectral characteristics, and MS/MS fragmentation patterns.

2.8. ICP-OES Analysis

The content of macro- and microelements in water extracts of xylem and bark stumps was determined by Inductively Coupled Plasma—Optical Emission Spectrometry (ICP-OES) (ARCOS FHE12, SPECTRO Analytical Instruments, Kleve, Germany). The operating conditions of the ICP-OES instrument and the parameters for determining the concentrations of selected elements are given in Table S1.

The water extracts of narrow-leaved ash stumps were diluted with HPLC-grade distilled water, filtered through 0.45 µm membrane filters, and directly analyzed by ICP-OES. No additional dilution or digestion procedure was applied since the analysis was performed on water extracts. HPLC-grade water used for sample preparation was analyzed as a method blank, and all monitored elements in the blank were below the corresponding limits of detection. The carrier gas was Argon with a grade of 5.0 (99.999% purity). Calibration solutions were prepared by diluting standard solutions, ensuring that the concentrations of the standards used for the calibration curves covered the expected concentration range of the analyzed elements. The selected analytical wavelengths, the correlation coefficient (*R*), the linearity ranges (LR), and limits of detection (LOD) for the analyzed elements are summarized in Table S2.

To evaluate the safety of the extracts, the concentrations of the analyzed elements (µg/mL) were compared with the Permitted Concentration Limits (PCLs, µg/mL) for both oral and cutaneous routes [63]. In accordance with the International Council for Harmonisation Guideline for Elemental Impurities (ICH Q3D) guideline, the PCL values (oral and cutaneous) were calculated based on the Permitted Daily Exposures (PDEs, µg/day) for elemental impurities, assuming a conservative maximum daily product intake of 10 g [64]. Since the samples were water extracts, a density of approximately 1.0 g/mL was

considered to convert the daily mass intake of 10 g into an equivalent volume of 10 mL/day, enabling a direct comparison of the regulatory limits (PCLs) with the experimental values expressed in $\mu\text{g/mL}$. For elements that were not detected in the extracts, the instrumental limits of detection (LODs) of the ICP-OES method (Table S2) were used as the upper concentration estimates for comparison with the corresponding permitted concentration limits (PCLs).

3. Results and Discussion

3.1. Chemical Composition

Analysis of the chemical composition of narrow-leaved ash stumps from Morović showed that there are differences in the content of structural biopolymers between xylem and bark (Figure 2). The stump xylem contains a high proportion of cellulose and lignin, while bark, on the other hand, contains less cellulose and hemicelluloses but more lignin, which is in line with general trends in hardwoods. These variations in chemical composition between xylem and bark are driven by their specific biological and physiological functions within the living tree. While the xylem provides mechanical support and water conductivity, which accounts for the predominance of structural carbohydrates, primarily cellulose, the bark serves a predominantly protective function against environmental factors. This protective role is reflected in its higher proportions of lignin, suberin and non-structural compounds [5,63,65,66].

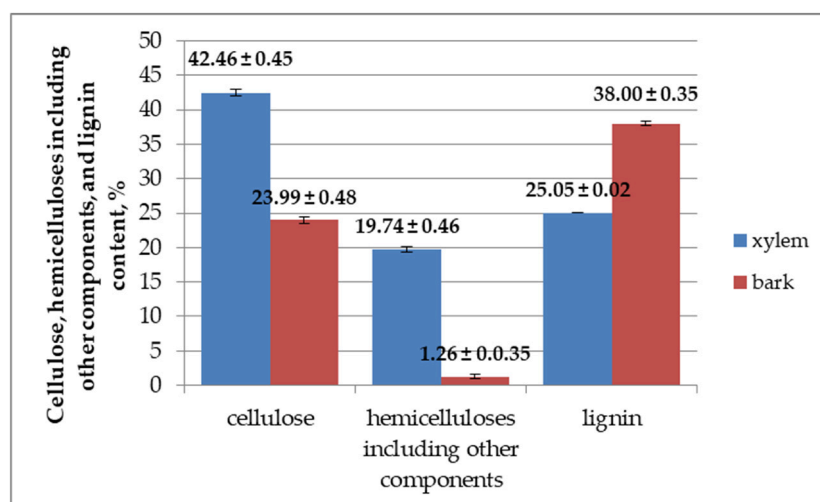


Figure 2. Cellulose, hemicelluloses with other components, and lignin content of xylem and bark of narrow-leaved ash stumps, expressed as % DW (mean value $\pm$ standard deviation).

In addition to the clear differences in chemical composition between wood and bark, it is well established that the chemical profile of the xylem itself is not uniform; rather, it is subject to variations in both axial (along the stem height) and radial (from the pith to the periphery) directions. These variations are related to tree age, the transition from juvenile to mature wood, and heartwood formation, which is accompanied by the deposition of secondary metabolites (extractives, particularly polyphenols). Furthermore, wood formation is strongly influenced by both genetic factors and environmental conditions (such as photoperiod, nutrient availability, precipitation, and hydrological conditions, as well as mechanical stresses that induce reaction wood formation), which further contributes to the variability in chemical composition between trees from different localities [67,68].

In accordance with the evident variability in chemical composition, previous studies have noted that the cellulose content in wood changes depending on the position in both axial and radial directions, as well as with the age of the tree. Popović et al. (2016)

established that at 1.3 m, the cellulose content in the 10-year-old juvenile wood of narrow-leaved ash from Morović (45.46%) was lower than in the mature wood of the same species aged 70–73 years (46.83%) [69]. Conversely, Yalcin and Sahin (2015) reported higher cellulose content (53.46% at a height of 1.5 m) for 22-year-old narrow-leaved ash from Turkey [70]. This higher value could be a consequence of specific growth conditions or the presence of tension wood, which is characterized by a high cellulose content and is frequently present in young trees [71]. Bodirlau et al. (2007) also observed that the cellulose content in oak stemwood is related to its axial and radial position [72]. These variations in cellulose, as well as in other wood constituents along both axial and radial directions, result from the varying proportions of juvenile and mature wood, alongside the presence of the extractive-rich heartwood that occupies the central parts of the stem. At a height of 1.3 m, the sample showed a trend of increasing cellulose content from the pith toward the cambium. In the axial direction, within the inner (0–40 annual rings) and outer (70–80 annual rings) sections of the stem, the cellulose content increases up to a certain height (from 1.3 m to 8 m) before eventually decreasing (from 8 m to 15 m). In contrast, the central growth rings (40–70 annual rings) display a steady decreasing trend in the axial direction from 1.3 m to 15 m [72]. Popović et al. (2016) also established a decreasing trend in cellulose content along the axial direction, dropping from 45.46% (1.3 m) to 44.61% (1/2 tree height) and 43.99% (3/4 tree height) in juvenile narrow-leaved ash wood. A similar trend was observed in the mature wood of the same species, decreasing from 46.83% (at 1.3 m) to 46.14% (1/2 tree height) and 44.85% (3/4 tree height) [69]. Nevertheless, when comparing the cellulose content in the xylem of narrow-leaved ash stumps presented in Figure 2 to the cellulose content in the wood of a 70–73-year-old mature tree from the same locality reported by Popović et al. (2016) (46.83% at 1.3 m) (Table 1), it can be seen that the cellulose content in the investigated xylem stumps is slightly lower. This lower cellulose content within the stump zone can be attributed to the accumulation of extractive compounds, which naturally concentrate over time in the oldest basal parts of the tree and mathematically reduce the relative ratio of structural carbohydrates. Nevertheless, the cellulose content in the xylem of narrow-leaved ash stumps is within the limits of the values stated in the literature for the genus *Fraxinus* (Table 1), which range from 38.22 to 49.24% for *F. americana* L. Marsh and from 40.86 to 46.77% for *F. excelsior* L. *Oleaceae* [73], as well as the value of 43.81% that Bodirlău et al. (2007) reported for 102-year-old *F. excelsior* L. at a height of 1.3 m. In this context, it can be concluded that the cellulose content of analyzed narrow-leaved ash stumps does not differ from the commercial parts of trees of the genus *Fraxinus*.

Bark is less important than xylem and is usually treated as waste, so it is less studied, and it is difficult to find data on its chemical composition for most wood species. Cellulose content in the bark of narrow-leaved ash stumps, presented in Figure 2, is much lower than in the xylem, which is in accordance with the cellulose content ratio of bark and xylem stated in the work of Pásztor et al. (2016), who noted that wood contains 40–50%, phloem 18–25%, and outer bark 3–17% cellulose [66].

The content of hemicelluloses, including other unquantified components of the xylem of narrow-leaved ash stumps as presented in Figure 2, was lower than the hemicellulose content reported in the work of Mazri et al. (2022), where they found 28.38% in sapwood and 27.60% in heartwood (Table 1) [74]. It is also lower than the hemicellulose content of 24.93% in stemwood reported in the work of Yalcin and Sahin (2015) (Table 1) [70]. The content of hemicelluloses, including other unquantified components, is still within the limits stated for hardwood hemicelluloses by Pettersen (1984) and Sjöström (1993), which range from 15% to 30% for glucuronoxylans and 2% to 5% of dry wood for glucomanans [64,75]. However, it should be noted that the content of hemicelluloses, including

other unquantified components, is calculated approximately, by subtracting the sum of cellulose, lignin, total extractives, and ash content from the total mass (100%), where a portion of the soluble hemicellulose components may be present in the water extract, along with some water-soluble minerals. Pásztor et al. (2016) state that the polysaccharide content is much higher in wood (62.8 to 70.6%) than in bark (44.1 to 47.6%) [66]. However, as we can see from Figure 2, there is an extremely low content of hemicelluloses, including other components, in the bark of the ash stumps. Concurrently, the hot-water extractive yield is remarkably high (Figure 2). This strongly indicates that a substantial portion of polysaccharides, such as easily water-soluble hemicelluloses and pectins, along with certain water-soluble mineral matter, likely dissolved during the hot-water extraction process and was consequently quantified within the hot-water extractive fraction. This mass transfer to the extract fraction may explain the underestimated and unexpectedly low calculated value of hemicellulose (including other components) content in the bark. To analyze the differences in hemicellulose content between stump and stemwood, it is crucial to further investigate the monosaccharide contents of narrow-leaved ash stumps, specifically xylose, mannose, glucose, and glucuronic acid, which are the primary constituents of hardwood hemicelluloses [74,76].

The lignin content of narrow-leaved ash stump xylem, presented in Figure 2, is within the limits for the stemwood, from 21.40% for acid-insoluble (Klason) lignin [70] to 25.53% for acid-insoluble (Runkel) lignin [77], and is close to the total lignin content value of 24.70% (at 1.3 m) reported by Popović et al. (2016) for this species from the same locality (Table 1). Mazri et al. (2021) report a somewhat lower total lignin content of 21.9% (sapwood) and 23.4% (heartwood) (Table 1) [74].

Table 1. Chemical composition of certain *Fraxinus* species, represented in %.

Age (Years)	Sampling Height on the Tree (m)	Cellulose (%)	Hemicelluloses (%)	Lignin (%)	Extractives (Organic Solution) (%)	Extractives (Hot Water) (%)	Ash (%)	Reference
<i>F. angustifolia</i>								
70–73	1.3	46.83		24.70	4.39	4.44–5.59	0.36	[69]
22	1.5	53.46 (sapwood)	24.93	21.40 (acid-insoluble, Klason)	4.18	5.14	0.83	[70]
62–64				25.53 (acid-insoluble, Runkel)	4.90–8.08	11.23	0.44–0.77	[77]
27	2.5		28.38 (sapwood) 27.60 (heartwood)	21.9 (sapwood) 23.4 (heartwood)			0.83	[74]
stem bark					5			[39]
					5.85			[38]
<i>F. americana</i> , L. Marsh.								
stem wood		38.22–49.24						[73]
<i>F. excelsior</i> L.								
stem wood		40.86–46.77						[73]
102	1.3	43.81						[72]

As shown in Figure 2, the lignin content in the bark of analyzed narrow-leaved ash stumps is equal to or greater than that found in the stem bark of other hardwood species (willow 37.3%; oak 32.67–38%; beech 24.63–34.04%; poplar 28.17–36.04%) [78–83]. The high content of lignin increases the heating value of lignocellulose and makes it suitable for energy production. In the investigation of Dibdiakova et al. (2014), it was stated that the bark of some hardwood trees has higher effective calorific values than other tree

biomass, such as stems, branches, stump xylem, roots, and foliage [84]. Nosek et al. (2016) reported that birch bark has a significantly higher calorific value than xylem, by roughly 4.28 MJ/kg [85].

The extractive contents of analyzed narrow-leaved ash stump xylem, presented in Figure 3, were higher than the extractive content in the upper parts of the wood, as stated in some studies. Yalcin and Sahin (2015) found that the hot water extractive content of narrow-leaved ash xylem sampled at 1.5 m height was 5.14%, while Popović et al. (2016) reported a hot water extractive content of 5.59%, 4.44%, and 5.08% in xylem samples taken at 1.3 m above the ground and 1/2 and 3/4 of total tree height, respectively [69,70] (Table 1). However, Elmas (2011) states a value of 11.23% for hot water extractives, which may be due to external factors (Table 1). According to Popović et al. (2016), this high content of hot water extractives in the lower part of a tree is because water, in addition to primary metabolites, also dissolves secondary metabolites, such as polyphenols, hydrolyzable tannins, and pectins [69], whose concentration is higher in the lower parts of a tree [86]. The toluene-ethanol extractive content of narrow-leaved ash stump xylem is higher than the content of these substances, at 4.39% (at 1.3 m) as reported by Popović et al. (2016), and is within the limits of 4.18–8.08% reported by Yalcin and Sahin (2015) and Elmas (2011) for the ethanol-benzene extract of this species in Turkey (Table 1). Considering that organic solvents dissolve primarily secondary substances, and hot water, as stated before, also dissolves some primary substances, we can conclude that the analyzed narrow-leaved ash stumps are much richer in extractives than stemwood, which is in accordance with the existing literature [86]. Comparing the extractive content of the bark and xylem in our study (shown in Figure 3), we can see that the extractive content of the bark is higher than that of the xylem of the narrow-leaved ash stump. The bark has a protective role and is known for its high content of extractives [87], so this result is expected. The extractive content of narrow-leaved ash stumps' bark was also much higher than the extraction yield of the ethanolic extracts obtained in some studies for narrow-leaved ash tree bark, which ranges from 5% [39] to 5.85% [38] (Table 1). In their research, Bougellid et al. (2020) and Ahmane et al. (2019) found that the bark of narrow-leaved ash is rich in bioactive phenolic compounds, including tannins and flavonoids. Therefore, the high content of hot water extractives in the analyzed stumps' bark deserves further research.

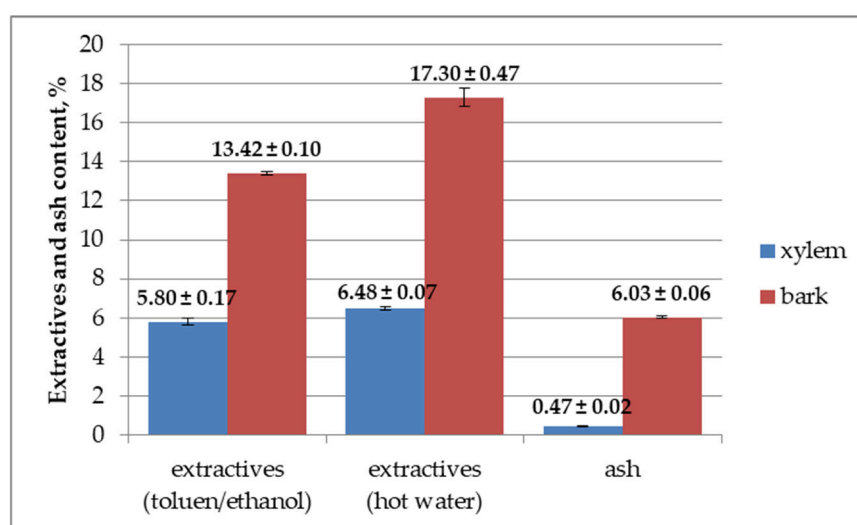


Figure 3. Extractives and ash content of xylem and bark of narrow-leaved ash stumps, expressed as % DW (mean value ± standard deviation).

The mineral material content of the narrow-leaved ash stumps is represented as ash content in Figure 3. When compared to the studies by Elmas (2011), Yalcin and Sahin (2015), and Mazri (2022) which reported ash contents of 0.77% (62 to 64-year-old trees), 0.44% (33 to 36-year-old trees) [77], and 0.83% [70,74] in stemwood, we observe that the ash content in the xylem of the narrow-leaved ash stumps is similar or lower (Table 1). However, the ash content of stumps is slightly higher than in the tree (0.36%) of this species from the same locality, which is reported by Popović et al. (2016) (Table 1). However, the ash content is higher in stump bark than in stump xylem, consistent with findings reported in previous studies and the existing literature [66,86].

3.2. TPC and Antioxidant Capacity Determined Spectrophotometrically

Considering the high content of water extractives of the analyzed narrow-leaved ash bark and xylem stumps of 6.48% and 17.30%, respectively, and the fact that polyphenols, which are often linked with antioxidative capacity, are water-soluble, the TPC and antioxidant potential of water extracts of the bark and xylem of narrow-leaved ash stumps were investigated.

TPC in the water extracts of the bark of narrow-leaved ash stumps, presented in Table 2, is higher than TPC in the water extracts of stemwood bark (20.53 mg GAE/g DW and 21.86 mg GAE/g DW) reported in the work of Touhami et al. (2017) [88]. This higher content of polyphenols in lower parts of a tree is in accordance with some previous studies and the existing literature [6,86,89]. TPC in hot water extracts of *F. angustifolia* stump bark is even higher than TPC in ethanolic extracts of stemwood bark (22.85 mg GAE/g DW and 24.84 mg GAE/g DW) reported in the investigation of Touhami et al. (2017) and ethanolic extracts of *F. angustifolia* bark (25.15 ± 0.70 mg GAE/g DW) reported in the work of Azib et al. (2020) [88,90]. On the other hand, the TPC of stump bark in this investigation is similar to the TPC of 80% aqueous methanol extracts (33.89 mg GAE/g DW) of *F. angustifolia* bark found by Tahirović and Bašić (2016) [21]. These comparisons add importance to the water extracts of stump bark as a source of polyphenols, knowing that plant extraction with ethanol, methanol, acetone, and ethyl acetate results in a higher yield of polyphenols [91–94]. To date, there has been no study on TPC in the xylem of narrow-leaved ash, likely due to the low content of polyphenols.

Table 2. TPC and antioxidant capacity of water extracts of narrow-leaved ash stumps, expressed as mean value $\pm$ standard deviation.

Narrow-Leaved Ash Stump	TPC mg GAE/g DW	DPPH μ mol TE/g DW
xylem	6.26 ± 0.21	47.79 ± 3.71
bark	33.94 ± 1.43	198.48 ± 3.33

In our study, we observed that the antioxidant capacity of stump bark water extracts is much higher than that of stump xylem water extracts (Table 2). Unfortunately, we could not find any studies on the antioxidant capacity of *F. angustifolia* xylem or bark toward DPPH, expressed as the amount of Trolox Equivalent per sample, to compare with our results. This finding requires further investigation to identify components of water extracts from the xylem of narrow-leaved ash stumps that contribute to its scavenging capacity.

3.3. Antioxidant Potential of Water Extracts by HPTLC–DPPH• Scavenging Assay

When the DPPH radical-scavenging activity of water extracts from the xylem and bark of narrow-leaved ash stumps was examined using the HPTLC–DPPH• free radical scavenging assay, several zones of activity were observed. On the HPTLC plate, zones with DPPH radical-scavenging activity are expressed as yellow zones on a purple background.

The water extract of bark showed higher antioxidant activity, while significantly weaker activity was recorded in the water extract of xylem (Figure 4). These findings are in agreement with the results obtained by the spectrophotometric DPPH assay.

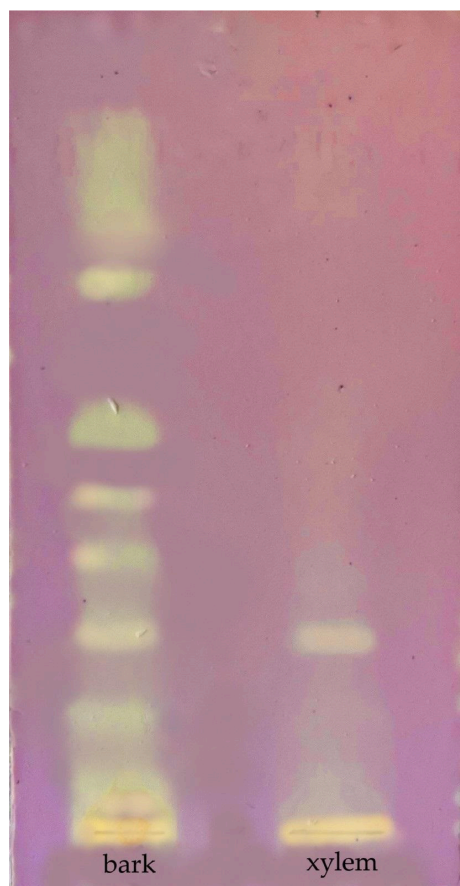


Figure 4. HPTLC-DPPH antioxidant profile of the xylem and bark water extracts of narrow-leaved ash stumps under visible light.

The water extract of the bark shows more pronounced DPPH radical-scavenging activity, while significantly weaker activity was observed in the water extract of xylem (Figure 4). A complex and rich chromatographic profile containing zones with DPPH radical-scavenging activity and different chromatographic characteristics was observed [95]. At lower R_F values (~ 0.08 – 0.10), very polar compounds with DPPH radical-scavenging activity are noticed, which may include highly polar phenolic constituents based on their chromatographic behavior [96]. In the R_F region of 0.15 – 0.20 , zones indicating moderately polar constituents with DPPH radical-scavenging activity are observed, which may be associated with phenolic acid derivatives based on their chromatographic characteristics, and require further confirmation with reference standards [96]. In the middle R_F range, zones at ~ 0.29 – 0.30 , ~ 0.40 , and ~ 0.47 – 0.48 are observed, indicating the presence of an average to lower amount of polar constituent compounds. The lowest zone may correspond to more polar constituents, while the higher zones may correspond to fewer polar derivatives and related compounds. The presence of prominent zones with DPPH radical-scavenging activity in this intermediate polarity region is highly consistent with phenolic compounds, including phenolic glycosides, which may include phenylethanoid glycoside-type compounds such as verbascoside and forsythioside derivatives. These compounds are known for their exceptional antioxidant activity due to the presence of caffeoyl and hydroxytyrosol moieties in their structures [97]. A particularly pronounced zone at $R_F \approx 0.55$ – 0.60 indicates the presence of fewer polar constituents exhibiting DPPH radical-scavenging activity.

Based on their chromatographic behavior, these zones may be associated with fewer polar phenolic compounds; however, their chemical identity cannot be confirmed solely based on R_F values. In the higher R_F region ($R_F \geq 0.75$), zones corresponding to fewer polar compounds were observed.

In contrast to the bark, the water extract of xylem shows a significantly weaker DPPH radical-scavenging activity, as observed in the HPTLC–DPPH assay (Figure 4). In the region of $R_F \approx 0.08$ – 0.10 , a weak zone with DPPH radical-scavenging activity corresponding to very polar constituents was detected. The most pronounced zone with DPPH radical-scavenging activity was located at $R_F \approx 0.25$ – 0.30 , corresponding to moderately polar constituents.

3.4. UHPLC-DAD-ESI-MS/MS Analysis

UHPLC-DAD-ESI-MS/MS analysis of the water extracts of xylem and bark of stumps of narrow-leaved ash enabled the identification of a wide range of secondary metabolites. Several classes of compounds were tentatively identified, with phenylethanoid glycosides (such as verbascoside, β -hydroxyverbascoside, forsythoside, caffeoylverbascoside), as well as phenolic acids (such as malic acid, quinic acid, caffeic acid derivatives), flavonoids, secoiridoids, and lignans (Table 3). These results provide insight into the chemical profile of the extracts and the possible potential value of *F. angustifolia* stump biomass in biotechnological and phytotherapeutic applications.

Table 3. List of detected compounds by UHPLC-DAD-ESI MS/MS analysis in negative and positive ionization modes of water extracts of xylem and bark of stumps of narrow-leaved ash.

t_r , min	λ_{max} , nm	Molecular Ion Peak [M-H] [−] * [M-H + HCOOH] [−] ** [M + H] ⁺ *** [M + Na] ⁺ **** [M + NH ₄] ⁺ , m/z	ESI MS/MS Fragment Ions, m/z	Tentative Annotation (Reference)	Sample	
					Xylem	Bark
0.67	-	181	163 (100%), 131, 119, 101, 89, 71, 59	n.i. Derivative of p-coumaric acid	+	+
0.75	-	* 387	* 341 (100%), 179	Caffeoyl hexose (PubChem CID:6124135)	+	+
0.77	-	133	115 (100%), 87	Mallic acid (standard)	-	+
0.96	-	191	173, 111 (100%)	Quinic acid (PubChem CID: 1064)	+	+
1.15	286	393	375, 363 (100%), 331, 213, 183, 151, 139	n.i.	+	+
1.25	280	* 377	* 331 (100%), 169	Galloyl hexoside (PubChem CID:124021)	-	+
1.35	289 320	* 479 *** 457 **** 452	* 433 (100%), 293 *** 302, 295, 267 (100%), 237, 207	n.i. Flavanone hexoside	+	+
1.53	-	393	375, 363 (100%), 331, 213, 183, 151, 139	n.i.	+	+
1.80	-	*377	* 331 (100%), 169	Galloyl hexoside (PubChem CID:124021)	-	+
2.38	233 281 306	315	179, 153, 135 (100%)	Hydroxytyrosol hexoside [65]	+	+
2.62	320	375	357, 331 (100%), 191, 179, 161, 151	n.i. Phenolic acid	+	+
3.17	279 313	407	389, 375/377, 357 (100%), 345, 313	n.i.	+	+

Table 3. Cont.

t_r , min	λ_{max} , nm	Molecular Ion Peak [M-H] ⁻ * [M-H + HCOOH] ⁻ ** [M + H] ⁺ *** [M + Na] ⁺ **** [M + NH ₄] ⁺ , m/z	ESI MS/MS Fragment Ions, m/z	Tentative Annotation (Reference)	Sample	
					Xylem	Bark
3.40	279 313	407	389, 375/377, 357 (100%), 345, 331, 313	n.i.	+	+
3.40	-	461	315 (100%), 297, 281, 161, 135	Decaffeoylverbascoside, verbascoside [67]	+	+
3.87– 4.00	-	345	299 (100%), 179	n.i.	+	+
4.86		* 387	* 341 (100%), 179	Caffeic acid hexoside (PubChem CID: 6124135)	-	+
5.35	270	431	413, 299 (100%)/300, 251, 191, 179, 161, 149, 131	pentoside	+	+
5.53	266	611	565 (100%), 403	n.i.	+	+
5.73	266	611	565 (100%), 403	n.i.	+	+
5.83	342	369	207 (100%), 192	n.i.	+	+
6.16	329	429	383, 369, 221 (100%), 206, 191, 177, 163	n.i.	+	+
6.34	361	583	537 (100%), 375	n.i.	+	+
6.86	269 300	477	431, 341, 315, 179, 161 (100%), 135	Hydroxytyrosol dihexoside (PubChem CID: 177827552)	+	+
7.01	329 300 sh	639	621 (100%), 529, 459	β -hydroxyverbascoside [66]	+	+
7.28	328 300 sh	581 565	535, 337 (100%), 193, 175 519 (100%), 501	n.i. Coumaroylquinic acid derivative	+	+
7.28	341	525	481, 363 (100%), 345, 301, 283, 207, 181	Demethyloleuropein [68]	-	+
7.51	327 300	787	741 (100%), 579	n.i.	+	+
7.75	324 300 sh	639	621 (100%), 529, 459	β - hydroxyisoverbascoside [66]	+	+
8.00	281	477	431, 341, 315, 179, 161 (100%), 135	Hydroxytyrosol dihexoside (PubChem CID: 177827552)	+	+
8.36	333 300 sh	623 ** 625 *** 647 **** 642	461 (100%), 315 ** - *** - **** 625 (100%), 479, 471, 325, 309	Phenylethanoid glycoside isomer (forsythoside/acetoside- related compound) [63,65]	+	+
8.56	281	477	431, 341, 315, 181, 161 (100%), 135	Hydroxytyrosol dihexoside (PubChem CID: 177827552)	+	+
8.58	333 300 sh	623	461 (100%), 315	Phenylethanoid glycoside isomer (verbascoside-related compound) [63,65]	+	+
9.05	330 290 sh	623	461 (100%), 315	Phenylethanoid glycoside isomer (verbascoside-related compound) [63,65]	+	+

Table 3. Cont.

t_r , min	$\lambda_{\max}$, nm	Molecular Ion Peak [M-H] [−] * [M-H + HCOOH] [−] ** [M + H] ⁺ *** [M + Na] ⁺ **** [M + NH ₄] ⁺ , m/z	ESI MS/MS Fragment Ions, m/z	Tentative Annotation (Reference)	Sample	
					Xylem	Bark
9.29	198 224 242	731	685 (100%), 523, 345	Oleoside/secologanoside derivative [68]	+	+
9.54	314 300	539	377 (100%), 359, 291, 275	Secoiridoid glycoside isomer (oleuropein-related compound) [68]	-	+
10.00	202 231 280	539 ** 541	377, 345, 327, 307 (100%), 275 ** 511, 331 (100%), 286, 233	Secoiridoid glycoside isomer (oleuropein-related compound) [68]	+	+
10.21	283 335	637	619, 491, 475, 461 (100%), 443, 297, 235, 193	Eukovoside [69]	+	+
10.80	228 240 sh	523 ** 525 *** 547	361 (100%), 291, 259 ** - *** 385 (100%), 369/367, 353, 311, 233	Secoiridoid glycoside (ligstroside-related compound) [68]	+	+
11.23	-	895 * 569 523	793, 663, 509 (100%), 347 * 523 (100%) 403, 385, 361 (100%), 291, 347	Lignan hexoside derivative (secoisolaricresinol-related compound) [65]	+	+
11.76	-	* 569 523	* 523 (100%) 453, 385, 361 (100%), 291, 259	Lignan hexoside derivative (secoisolaricresinol-related compound) [65]	+	+
12.14– 12.20	240	* 955 909 ** 929	935, 909 (100%) - ** 731/732, 551 (100%)/552, 519, 477	n.i.	+	+

t_r —retention time, detected from ESI-MS signal. $\lambda_{\max}$ —absorption maximum wavelength in the UV-Vis spectrum, from DAD signal. The most intense fragment ions in the ESI MS/MS spectrum are marked as 100%. The second-most intense ions in the ESI MS/MS spectrum are the underlined fragment ion(s). PubChem CID—database at <https://pubchem.ncbi.nlm.nih.gov> (accessed on 6 August 2026). n.i.—not identified.

Based on Table 3, it can be seen that phenylethanoid glycosides represented a major class of tentatively identified compounds in the water extract of xylem and bark from narrow-leaved ash stumps in both negative and positive ionization modes. Verbascoside and related phenylethanoid glycosides were tentatively identified among the most prominent chromatographic signals, with peaks detected at retention times of 8.58 and 9.05 min and the molecular ion [M-H][−] at m/z 623. Their MS/MS spectra showed dominant fragment ions at m/z 461 (100%) and 315, which are formed by the successive loss of a caffeoyl residue and a sugar unit from the molecule, which represents a typical fragmentation pattern for phenylethanoid glycosides of this type [97,98]. In addition to verbascoside, β -hydroxyverbascoside ([M-H][−] m/z 639, at retention times 7.01 and 7.75) was also identified, whose molecular mass is 16 Da higher than verbascoside, which indicates an additional hydroxyl group in the molecular structure. Its MS/MS spectrum has characteristic fragments at m/z 621 (100%), 529, and 459, which are consistent with previously described fragmentation pathways for hydroxylated verbascoside derivatives [99]. Other phenylethanoid glycosides with molecular ion m/z 623 were also detected in this fraction, including *forsythio-*

side A, H, and I, i.e., *acetoside*, which in positive ionization mode were registered as $[M + H]^+$, $[M + Na]^+$, and $[M + NH_4]^+$ adducts. Their MS/MS spectra showed characteristic fragment ions at m/z 461 (100%) and 315, which further confirms their belonging to this class of compounds [97,98]. *Decaffeoylverbascoside* (m/z 461, 3.40 min) was also detected, whose MS/MS spectrum contains a dominant fragment at m/z 315 (100%), which corresponds to the hydroxytyrosol-glucoside part of the molecule. Due to the great structural similarity and identical fragmentation patterns, distinguishing individual isomers of phenylethanoid glycosides was possible only based on retention time and data from the literature, and identifications were made tentatively [100]. *Hydroxytyrosol* derivatives were also detected in the analyzed water extracts of xylem and bark. The compound with molecular ion m/z 315 $[M-H]^-$ and fragment ions m/z 179, 153, and 135 was tentatively identified as *hydroxytyrosol-hexoside* at 2.38 min [98]. In addition, several compounds with molecular ion m/z 477 $[M-H]^-$ were detected in the chromatogram at retention times of 6.86, 8.00, and 8.56 min, tentatively identified as *hydroxytyrosol dihexosides*. In addition to the most intense peak at m/z 161 (100%), fragments at m/z 431, 341, and 315 were also observed on the chromatogram, which are formed by the loss of sugar units, while fragments at m/z 179, 161, and 135 originate from the fragmentation of the hydroxytyrosol aglycone (Table 3, Figures S1–S4).

As shown in Table 3, some compounds from the group of *iridoid glycosides*, predominantly *secoiridoids*, were found in the analyzed extracts. This group of compounds is characterized by a glycosidic unit linked to the iridoid structure, which is recognized in MS/MS spectra by the characteristic loss of a sugar residue. *Oleuropein (isomers)* was detected at 9.54 and 10.00 min, in both negative and positive ionization modes with $[M-H]^-$ m/z 539 and $[M + H]^+$ m/z 541. Its MS/MS fragmentation in negative mode is characterized by the appearance of an ion at m/z 377 (100%), resulting from the loss of the hexose unit (162 Da), as well as further fragments at m/z 345, 307 (100%), and 275, which originate from the degradation of the aglycone moiety and are typical of the secoiridoid structure of oleuropein (Table 3, Figures S1–S4) [101]. *Ligstroside* was detected at 10.80 min, with ion m/z 523 ($[M-H]^-$) and dominant fragment m/z 361 (100%), typical for this class of compounds [101]. *Oleoside/secologanoside derivative* was detected at 9.29 min, with molecular ion m/z 731 and fragments m/z 685, 523, and 345, indicating a more complex structure with more functional groups [101]. *Eukovoside* was detected at 10.21 min, with an ion of m/z 637 ($[M-H]^-$) and a characteristic fragment of m/z 461 (100%), which is formed by the loss of a sugar moiety [102].

In the region of retention times 11.23–11.76 min, two compounds from the lignan class were detected, with characteristic fragmentation patterns indicating the presence of *secoisolariciresinol derivatives* and their structural isomers, confirming the diversity of the lignan fraction in the analyzed water extracts [98].

The analysis of water extracts identified mainly two sub-groups of phenolic acids, hydroxycinnamic and hydroxybenzoic acids (Table 3). A *p*-coumaric acid derivative at 0.67 min with characteristic fragment m/z 163 is present, as well as caffeic acid derivatives (caffeoyl and caffeic acid hexoside; 0.75 min and 4.86 min) with fragment m/z 179 (Table 3, Figures S1–S4). Gallic acid derivatives were confirmed by the presence of galloyl hexoside at 1.25 min and 1.80 min with m/z 169 (Table 3, Figures S1–S4). A derivative of coumaroyl-quinic acid at 7.28 min was also detected, as well as free quinic acid at 0.96, which indicates the presence of ester forms (Table 3, Figures S1–S4).

A particularly significant finding of the UHPLC-DAD-ESI-MS/MS analysis is the detection of phenylethanoid glycosides, including verbascoside and related derivatives, which represented a prominent class of tentatively identified secondary metabolites in the water extracts of *F. angustifolia* stump biomass. This group of compounds represents the

characteristic secondary metabolites of the *Fraxinus* species, and their structural properties, including the presence of caffeoyl and hydroxytyrosol units, may contribute to the antioxidant potential of the extracts, which is consistent with the antioxidant activity observed in the HPTLC–DPPH assay. However, the contribution of individual compounds remains preliminary because quantitative determination was not performed in the present study.

In addition to phenylethanoid glycosides, the detection of secoiridoids, including oleuropein and related derivatives, as well as phenolic acids and lignans, additionally confirms the complexity of the secondary metabolism of the analyzed biomass. Based on the UHPLC–DAD–ESI–MS/MS analysis, the biomass of analyzed narrow-leaved ash stumps from “Morović” contains diverse classes of tentatively identified secondary metabolites, including phenylethanoid glycosides (such as verbascoside and related derivatives), secoiridoids (including oleuropein, ligstroside, and related derivatives), lignans, and phenolic acids. The obtained results indicate that *F. angustifolia* stump biomass, in addition to its major structural components, is also a potential source of secondary metabolites of interest for further chemical characterization and valorization of forest residues within the concept of biorefining.

3.5. ICP-OES Analysis of Water Extracts

During hot water extraction, a part of the inorganic content of the wood tissue is simultaneously dissolved. These elements are also present in aqueous extracts, depending on their content in the wood tissue and solubility. Bearing in mind the biological activity of water extracts of xylem and stump bark and their potential application in pharmacy and biomedicine, the content of macro- and microelements in the extracts was examined for the assessment of elemental composition and safety profile. Results of the elemental analysis of the water extracts of analyzed narrow-leaved ash stumps performed by ICP-OES as well as the permitted concentration limits (PCL) for the purpose of safety assessment are represented in Table 4.

Table 4. Macro- and microelement composition of water extracts from xylem and bark of narrow-leaved ash stumps, with PCLs based on ICH Q3D guidelines for oral and cutaneous routes.

Concentrations of Macro- and Microelements and PCLs (µg/mL)																	
	As	Ag	B	Ba	Bi	Ca	Cd	Co	Cr	Cu	Fe	In	K	Li	Mg	Mn	Na
xylem	ND	ND	ND	0.04	ND	11.12	ND	ND	0.05	0.22	0.01	ND	44.43	ND	2.55	ND	1.00
bark	ND	ND	0.18	0.12	ND	26.00	ND	ND	0.07	0.14	0.02	ND	92.63	ND	6.73	ND	1.10
PCL oral	1.5	15	-	140	-	-	0.5	5	1100	300	-	-	-	55	-	-	-
PCL cutaneous	3	15	-	700	-	-	2	5	1100	300	-	-	-	250	-	-	-
	Ni	Pb	Sr	Ti	Zn	Hg	Si	P	S	Ce	Sn	Ta	Ti	V	W	Nb	Ge
xylem	ND	ND	ND	ND	0.20	ND	0.99	3.72	13.27	ND	ND	ND	ND	ND	ND	ND	ND
bark	ND	ND	ND	ND	0.29	ND	0.90	7.56	12.97	ND	ND	ND	ND	ND	ND	ND	ND
PCL oral	20	0.5	-	0.8	-	3.00	-	-	-	-	600	-	-	10	-	-	-
PCL cutaneous	20	5	-	-	-	3.00	-	-	-	-	600	-	-	-	0.8	10	-

ND—not detected (below the limit of detection). PCL—permitted concentration limits.

The concentration of macroelements is higher in bark water extracts, except for S (Table 4), which is slightly higher in the xylem water extracts of narrow-leaved ash stumps, which is consistent with the higher ash content found in the bark [66] (Figure 3). Among the microelements, only Si and Cu exhibit higher concentrations in xylem water extracts (Table 4). The macro- and micro-element composition of water extracts corresponds to elements typically found in wood, according to available research. About 80% of wood’s inorganic substances consist of Ca, K, and Mg, which are found in wood in the form of salts (carbonates, oxalates and, sulfates) or are bound to carboxyl groups of pectic materials and other components of the cell wall, while Na is somewhat less abundant [103]. The rest of

the inorganic components of wood are mostly Fe, Cu, Zn, Ba, B, and Cr, and other elements may also be present [103]. The content of inorganic components varies greatly depending on the species and part of the tree, as well as the environmental conditions under which the tree grew [103]. Bark contains more mineral substances than xylem, and Ca and K are dominant [66].

In both types of water extracts, K has the highest concentration among macroelements, while Si is the most abundant among the detected elements present in lower concentrations. Although Ca is the most abundant inorganic element in wood, it is found in tissue primarily in the insoluble forms—calcium oxalate and pectate [104,105]. This could be related to the relatively low concentration of Ca in the water extracts of analyzed narrow-leaved ash stumps compared to minerals that are less abundant in wood but more soluble, such as K. Among the macroelements, Na shows the lowest concentration in both extracts. According to the ICH Q3D guideline, Permitted Daily Exposures (PDEs) have not been established for the most abundant elements found in the water extracts of the analyzed stumps (K, Ca, Mg, and Na) due to their low inherent toxicity [64]. Within this group of elements, Zn and Fe were present in smaller quantities, with Fe showing the lowest concentrations among microelements in both types of water extracts. B is not detected in xylem water extracts, but it is present in bark extract, while Si, P, and S were found in both types of extracts. The remaining analyzed elements from the group without established PDEs (Mn, W, Bi, In, Sr, Ti, Ce, Ta, Nb, and Ge) were not detected in the analyzed xylem and bark extracts of the narrow-leaved ash. Highly toxic elements (Class 1), such as As, Cd, Pb, and Hg, were below the limit of detection (LOD) in the analyzed water extracts of narrow-leaved ash stumps (Table 4). The instrumental LODs of the ICP-OES method used in this study for these elements were well below the regulatory thresholds for both oral and cutaneous Permitted Concentration Limits (PCLs). This analytically confirms that the analyzed extracts satisfy the strictest pharmaceutical purity criteria regarding the presence of heavy metals. Ni, Co, and V, which are considered potentially toxic elements at elevated concentrations and require a risk assessment (Class 2A according to ICH Q3D), were also below the limit of detection, with LOD values safely below the PCLs for both oral and cutaneous exposure routes (Table 4). Among the elements with low natural abundance (Class 2B), only Ag and Tl were analyzed. These elements were not detected in the water extracts from the xylem and bark of the narrow-leaved ash stumps from “Morović”, with their LODs remaining below the oral and dermal PCLs. Furthermore, the concentrations of relatively low-toxicity elements with high PDE limits (Class 3), such as Ba, Cr, Cu, Mo, and Sb, were far below the PCLs for both oral and cutaneous routes (Table 4). A similar trend was observed for the LODs of the remaining elements from this group (Sn and Li), which were not detected by the ICP-OES analysis. Finally, it should be mentioned that previous studies have shown that certain metals dissolved from wood, such as Zn, Cu [106–109], Fe [108–110], Mg, Ca [109], Na [107], Cr [108], and others, can bind with flavonoids and other polyphenols by chelation and other mechanisms, forming stable complexes. In this way, these metals may potentially influence the antioxidant potential of polyphenols. However, these interactions were not investigated in the current study and would require extensive additional analyses.

4. Conclusions

Based on the analyses of the chemical composition and bioactive compounds, it was determined that the stumps of *Fraxinus angustifolia* Vahl. ssp. *pannonica* Soó & Simon at the Forest Administration “Morović” site represent a valuable and, so far, underutilized source of biomass, with the potential for further valorization. The xylem of stumps shows a chemical composition similar to that of stemwood, particularly in cellulose and lignin content, suggesting its potential for use in industries that rely on lignocellulosic raw

materials. At the same time, water extracts of bark are characterized by a high content of total phenolic compounds and pronounced antioxidant activity, with the absence of toxic metals, which makes them a suitable raw material for further research in terms of potential use in bio-based industries. Based on the UHPLC-DAD-ESI-MS/MS analysis, it can be concluded that the biomass of analyzed narrow-leaved ash stumps from “Morović” is rich in different classes of secondary metabolites, dominated by phenylethanoid glycosides and secoiridoids, with the presence of lignans and phenolic acids. Comparison of the samples suggested that the bark sample is chemically more diverse, especially in terms of exhibiting a broader diversity of tentatively identified iridoid and phenolic constituent profiles, indicating its greater potential for further valorization, while the xylem also contains key bioactive compounds, but with somewhat lower diversity. The obtained results show that stumps of *F. angustifolia* from the “Morović” Forest Administration site, considering their chemical composition, have potential for further investigation regarding cellulose and lignin content, as well as bioactive components. To confirm this, it would be necessary, for further investigation, to examine a larger number of stumps from the same site individually.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/analytica7030056/s1>, Figure S1, Representative UHPLC chromatograms of the xylem: (-) and (+) ESI-MS detection by the base ion peak in the mass range m/z 100–1000; Figure S2, Representative UHPLC-DAD chromatograms of the xylem: detection at reference wavelength 280 nm and 300 nm; Figure S3, Representative UHPLC chromatograms of the bark: (-) and (+) ESI-MS detection by the base ion peak in the mass range m/z 100–1000; Figure S4, Representative UHPLC-DAD chromatograms of the bark: detection at reference wavelength 280 nm and 300 nm; Table S1: Operating conditions of the instrument; Table S2: Parameters of the calibration curve for the measured elements.

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Abbreviations

The following abbreviations are used in this manuscript:

CID	Collision-induced dissociation
DW	Dry weight
DAD	Diode array detector
DPPH	2,2-diphenyl-1-picrylhydrazyl

GAE	Gallic acid equivalent
HPTLC	High-performance thin-layer chromatography
ICH Q3D	International Council for Harmonisation Guideline for Elemental Impurities
ICP-OES	Inductively coupled plasma—optical emission spectrometry
LODs	Limits of detection
LR	Linearity ranges
ND	Not detected (below the limit of detection)
PDE	Permitted daily exposure
PCL	Permitted concentration limit
R	Correlation coefficient
TE	Trolox equivalent
TPC	Total phenolic content
UHPLC-DAD-ESI-MS/MS	Ultra-high-performance liquid chromatography coupled to diode array detection and electrospray ionization mass spectrometry
UV-Vis	Ultraviolet-visible

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