IMPACT OF NATURAL DEGRADATION ON THE PHENOLIC PROFILE OF PEDUNCULATE OAK STUMP (*QUERCUS ROBUR* L.): INSIGHTS FROM HPTLC AND SPECTROPHOTOMETRIC ASSAYS

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The pedunculate oak (*Quercus robur* L.) is rich in extractives, especially in its heartwood, stumps, and bark, making it valuable to bio-based industries. Due to this reason and the increasing demand for efficient wood utilization, this study investigated the extractives of stumps remaining after freshly cut trees and *Q. robur* trees cut two years prior. During these two years, the stumps were exposed to various weather conditions (precipitation, UV radiation, and others) and the action of fungi, bacteria, and other pathogenic organisms. These factors contributed to the natural degradation of the wood and the disruption of the original chemical structure of the stumps. Water extracts from the xylem and bark of freshly cut and biodegraded *Q. robur* stumps were analyzed for total phenols, activity against H_2O_2, and free radical scavenging activity. Total phenol content was higher in degrading *Q. robur* stumps, with 71.70 and 59.17 mg gallic acid equivalents/g dry wood in bark and xylem, respectively. Degrading *Q. robur* stump bark and xylem showed higher antioxidant activity, with IC_{50} values against H_2O_2 of 682.39 µg/mL and 923.90 µg/mL, and free radical scavenging activity of 91.97% and 83.98%, respectively. High-performance thin-layer chromatography fingerprinting profile of xylem and bark extracts revealed different chemical profiles, while the HPTLC-DPPH scavenging assay evaluated antioxidant activity in extracts. This research showed that extracts from the bark and xylem of the degrading stump are stronger radical scavengers and have a richer phenolic profile than extracts from a freshly cut stump. Degrading *Q. robur* stumps may represent a valuable source of polyphenols.

Keywords: *Quercus robur* L., degrading stump, total phenol content, antioxidant potential, High-performance thin-layer chromatography (HPTLC)

Introduction

The pedunculate oak (*Quercus robur* L.) belongs to the Fagaceae family, which includes about 450 species of trees. It spreads throughout Europe, North Africa, and Asia Minor [1,2]. In the Republic of Serbia, it spreads in the valleys of large rivers (Sava, Morava, and Danube) on sandy and fertile alluvial soils [3,4]. The most significant pedunculate oak forests are around the Sava River in Srem (44°57'44"N 20°19'07"E) where some individual trees of this species are several hundred years old [4].

Genus Quercus, as well as *Acacia, Pinus, Eucalyptus, Rhizophora, Picea, Populus, Schinopsis, Castanea, Larix,* etc., is recognized for its high content of extractives (nonstructural components), especially polyphenols [5]. Extractive compounds of the genus Quercus include phenolic acids, lignans, stilbenoids, coumarins, flavonoids, tannins, as well as monoterpenes, triterpenes, steroids, fatty acids, and glycosides [6,7]. The polyphenol content is particularly high in the heartwood and the bark [8].

Polyphenols are secondary metabolites that arise in wood primarily during heartwood formation and as a response to various abiotic (climate changes, UV radiation, thermal warming, droughts, changes in soil composition, presence of heavy metals) and biotic factors (mainly fungi, but also bacteria, viruses, and various insects) [9]. The structure and amount of polyphenols can be influenced by the wood species and other factors, including the geographical origin [10] or the age of the tree [11]. High variability in polyphenolic composition even within a single species was reported by Prida and Puech (2006) [10] through the analysis of French oaks (sessile (*Q. petraea*) and pedunculate (*Q. robur*) oak) and American oak wood (*Q. alba*) species from different geographical regions.

Polyphenolic components of *Q. robur* can be classified into three large groups: volatile phenols, such as various aldehydes (vanillin, coniferylaldehyde, syringaldehyde, and sinapaldehyde), phenolic acids (gallic, ellagic, vanillic, ferulic, and syringic), and ellagitannins [12]. *Q. robur* wood extract also contains coumarins (aescule-

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tin and scopoletin) [13]. In addition, 17 triterpenoids were isolated from *Q. robur* heartwood [7]. In leaves and bark mainly, polyphenols are ellagitannins (hydrolysable tannins) and proanthocyanidins (condensed tannins) [9,11]. Phenolic compounds in *Q. robur* bark include gallic, ellagic, vanillic, protocatechuic acids, and catechin [14].

Wood extracts are mixtures of various compounds, including polyphenols, terpenoids, saponins, and alkaloids. These components contain bioactive molecules that enhance the natural durability of wood [8]. Bioactive molecules (synthetic or natural) can interact with living organisms and cause changes in them [8]. Most of the biological capacity of plant extracts is attributed to the antioxidant and antimicrobial potential of polyphenols [6,8]. For example, polyphenols protect wood by being toxic to microorganisms, inhibiting their growth and interfering with their metabolism through mechanisms such as blocking transport through membrane cells and inactivating microbial enzymes [2]. During periods of oxidative stress in plants, polyphenols can neutralize hydroxyl and superoxide radicals through reduction reactions. This is possible because polyphenols can donate an electron or a hydrogen atom from their hydroxyl group to the radical [15].

Bioactive molecules originating from plant extracts have a high antibacterial, antioxidant, and anticancer potential. They are commonly used in various extractive industries, including pharmaceuticals, cosmetics, and food [8]. In addition to their antioxidant properties, polyphenols exhibit various beneficial characteristics, such as anti-inflammatory, antimicrobial, analgesic, anti-allergic, and anti-proliferative effects [15]. These properties make polyphenols valuable in the treatment of several conditions, including cardiovascular and neurodegenerative diseases, type 2 diabetes, osteoporosis, and asthma [15]. They are also utilized in the food industry to enhance quality and extend the shelf life of food and beverages. Furthermore, in cosmetics, polyphenols have proven effective in formulations aimed at combating skin aging and offering protection from UV radiation [15].

Due to concerns about the potential health risks associated with synthetic phenolic antioxidants, research is being conducted to find new antioxidants from natural sources, including agricultural, forestry, and industrial residues [16]. Many studies have estimated that wood and forest residues (wood chips, sawdust, branches, and knots) can be utilized for the production of bioactive molecules [8].

Due to this high polyphenol content, the genus Quercus is used for the extraction of tannins and other bioactive compounds [14]. The heartwood, bark, and lower parts of trees, such as stumps, are particularly rich in extractives [14], making them valuable raw materials for bio-based industries.

There has been a rapid development of petroleum-based materials recently. Due to fluctuating oil prices and strict environmental regulations, there is a growing demand for bio-based materials, such as plant polyphenols. These plant polyphenols can be copolymerized to create new polymers or reactive intermediates with existing polymeric materials, thereby enhancing their properties. This makes them excellent candidates for producing materials that are resistant to fire, suitable for water treatment, or usable in sensing and stimuli-responsive applications [17,18].

For example, Gogoi and Karak (2014) found that tannic acid can be used as a substitute for fossil-based polyols in the production of waterborne hyper-branched bio-based polyurethane. This substitution enhances the characteristics of the resulting material, making it biodegradable, antibacterial, and improving its thermal stability and mechanical properties [19].

Given the increasing demand for wood products, fully utilizing all parts of the tree, including stumps, is an important consideration. For example, pine stumps are used for resin extraction [20]. Also, Luís *et al*. (2014) [21] established that the extracts from the stumps of Eucalyptus globulus have a high content of total phenolic compounds and flavonoids, highlighting their significance as a source of bioactive molecules with antioxidant and antimicrobial effects, especially polyphenols.

However, stumps frequently remain in the forest after tree logging because removing stumps from the soil can disrupt soil heterogeneity, as it may result in the loss of a significant amount of entrapped soil from the site. This disruption can lead to soil erosion and the leaching of carbon and nutrients as water drains away [22]. Due to their biodegradation, stumps can be valuable sources of carbon and nutrients for future generations of forests [23].

During the natural biodegradation of wood, its structural integrity is disturbed by the action of fungi and enzymes from other microorganisms, which break down cellulose, hemicellulose, and lignin polymers [23, 24]. Insects attracted to decayed wood break down cellulose and hemicellulose using their digestive enzymes, feeding on the monosaccharides produced from this process. To get to cellulose and hemicellulose, they first break down the complex polyphenol network of lignin through the oxidative action of peroxidase, laccase, and the formation of free radicals [25].

Tannins have toxic effects on various organisms, but certain microorganisms produce enzymes (tannase and others) that can degrade them. Condensed and complex tannins are harder to degrade than ellagitannins, while gallotannins are easily broken down by these enzymes. Through the catalytic action of different enzymes produced by microorganisms, tannins are degraded into oligomeric tannins and other derivatives, such as gallic acid, ellagic acid, pyrogallol, and so on [26]. The increased moisture content and temperature of the soil lead to faster decay of wood [27]. According to Onuchin *et al.* (2018), during the natural degradation of wood, the levels of starch and pentosans decrease, while the amounts of substances soluble in water and benzene increase [28].

Abiotic factors can also accelerate wood decomposition. For instance, UV rays from sunlight can initiate the

formation of free radicals within the aromatic structure of lignin, leading to its degradation [29].

This study aimed to respond to the growing need for efficient wood utilization while simultaneously applying the principles of green chemistry. Accordingly, the antioxidant potential of water extracts of wood and bark from freshly cut and *Q. robur* stumps, after partial natural degradation over two years, was examined. Water was used for extraction, which, as a polar solvent, dissolves polyphenols well [26,27]. According to data from the literature, most of the heartwood and bark polyphenols are water-soluble [13]. For example, from 77.7 mg/g of wood dry extracts extracted with methanol/water (1:1), the water-soluble fraction was 72.5 mg/g of wood [13]. Research has shown that more total polyphenols are extracted with an ethanol-water mixture [11] or 75% methanol [30] than with water. However, due to the potential application of *Q. robur* extracts in medicine, pharmaceutical, food, and cosmetic industries, water is more suitable as a solvent due to its non-toxicity [31]. In addition to the spectrophotometric assays, total phenol content and scavenging capacities, this study aimed to apply High-performance thin-layer chromatography (HPTLC) coupled with various detection methods, natural product (NP) reagent, and DPPH• reagent, as a powerful technique for the screening of phenolic profiles.

Materials and methods

Chemicals and Materials

All chemicals and reagents for this study were sourced from commercial suppliers. Folin-Ciocalteu (FC) reagent (for microscopy) was from CARLO ERBA reagent (Cornaredo, Italy), and the standard of gallic acid (99.97%) was from BLD PHARMATECH GmbH (Reinbek, Germany). Ethanol, sulfuric acid, acetic acid (glacial), sodium carbonate, phosphate-buffered saline (PBS), polyethylene glycol (PEG) 4000, and silica gel 60 (Art. 105461) HPTLC glass plates were supplied by Merck (Darmstadt, Germany). 2,2-di(4-tert-octylphenyl)-1 picrylhydrazyl (DPPH) free radical, *p*-anisaldehyde, standard of ascorbic acid (99%), (2-aminoethyl diphenylborinate, and 30% H_2O_2 p.a. were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Standard of 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid (Trolox, ≥98%) was supplied by Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

Plant Material and Extraction

The samples of *Quercus robur* L. were collected in the forest administration “Morović” (geographical coordinates 44°59'15"N 19°09'53"E) managed by the Public Enterprise “Vojvodinašume”. The IUCN (The International Union for Conservation of Nature). Red List (2025) categorizes Quercus robur as a species of Least Concern [32]. The samples were taken from two stumps of *Q. robur* tree of the same age (110 years) - one freshly cut and one cut two years earlier. From each stump, two discs (each 3 cm thick) were sampled: one from the surface and one from 10 cm below. Due to the potential presence of impurities, a thin surface layer was previously removed from the oak stump cut two years ago. The samples were air-dried for 3 months. After separation of the bark, the xylem discs were cross-cut along the diameter into four parts. To preserve the original proportions of sapwood and heartwood, two opposite quarters of each xylem disc were coarsely chopped, as well as the entire amount of bark. After grinding with a hammer mill (Culatti AG micro mill DFH 48, Schweiz, Switzerland) and sieving, particles smaller than 0.25 mm were stored in sealed containers and used for analysis [33]. This yielded four prepared samples: xylem and bark from a tree stump of freshly cut *Q. robur* (Q and BQ), and xylem and bark from a tree stump of *Q. robur* cut two years ago (Q2Y and BQ2Y).

Before extraction, the moisture content of the samples was determined by drying them in a laboratory dryer to a constant mass at 105±2 °C [34]. Moisture content of samples of xylem and bark was 7.77±0.11% (Q); 8.21±0.06% (BQ); 8.08±0.06% (BQ2Y); 7.60±0.10% (Q2Y).

Extraction of the prepared xylem and bark samples was performed according to the modified standard method TAPPI T 207, as well as ASTM D1110-21 [35,36]. 2 g of each sample was extracted with 100 mL of distilled water under reflux in a boiling water bath for 1 h. The extracts were filtered through a Gooch crucible (porosity grade 3) with a vacuum pump. The resulting aqueous extracts were collected and stored at 4 °C until further analysis.

Spectrophotometric Assays

Total phenolic content (TPC) was determined using the method of Aksić *et al.* (2023) [37]. 2 mL of 10% *v/v* Folin-Ciocalteu reagent was added to 0.5 mL of diluted extracts (1000 µg/mL) or gallic acid standard (25, 50, 75, 100, and 125 µg/mL). After 3 min, 2.5 mL of 10% Na_2CO_3 was added, and after 30 min of incubation in the dark, the absorbance was measured at 765 nm. The blank solution contained 0.5 mL of water instead of extracts/standards. TPC was calculated from the calibration curve equation and expressed in gallic acid equivalents (GAE) per g dry wood (DW) as mg GAE/g DW.

H_2O_2 scavenging activity was determined by the method of Ghenima *et al.* (2015) [38] with slight modification. 3.4 mL of extracts with different dilutions (500-1250 µg/mL) were added to 0.6 mL of a 40 mM solution of H_2O_2 (prepared in phosphate-buffered saline, pH 7.4). After incubating for 10 minutes in the dark, the absorbance value was measured at 230 nm against a blank solution containing water and phosphate-buffered saline without H_2O_2. Absorbance was also measured for the control solution, which consisted of water mixed with phosphate-buffered saline and H_2O_2. Ascorbic acid was used as a positive control (with concentrations of 75-175 µg/mL). The % of the H_2O_2 radical scavenging activity

was calculated from the absorption value using the following equation:

$$\%\ scavenging\ activity = \frac{A\ control - A\ sample}{A\ control} * 100 \quad \text{.....(1)}$$

The concentration at which the sample scavenging activity is 50% (IC_{50}) for each sample was determined by an online tool [39] using a two-parameter logistic regression model.

DPPH scavenging activity was determined by the method of Smailagić (2019) [40]. DPPH• solution was prepared by mixing a 186 µM solution of DPPH• in ethanol and acetate buffer (pH 5.03) in a ratio of 2:1, *v/v* In 0.2 mL of extracts diluted in ethanol (1000 µg/mL) was added 2.8 mL of DPPH• and after incubation for 90 min in the dark absorbance value was measured at 525 nm against ethanol as a blank solution. Control absorbance was measured for the DPPH• using 0.2 mL of ethanol instead of the extract, and the calibration curve was established with different concentrations of Trolox in ethanol (200-800 µg/mL). DPPH scavenging activity was expressed as mg of Trolox Equivalent (TE) per g of dry wood (mg TE/g DW). The % of the DPPH radical scavenging activity was calculated from the absorption value using equation (1).

Total phenol content and scavenging capacities were determined with an Evolution 300 UV-Vis spectrophotometer (Thermo Electron Corporation, England, UK). All analyses were performed in triplicate and expressed as mean values.

HPTLC and chemical derivatization

30 µL of extracts were applied as 6 mm bands onto 20 cm × 10 cm HPTLC glass silica gel plates using a CAMAG Linomat 5 (Muttenz, Switzerland). The bands were placed 8 mm from the lower edge of the plate, with a minimal separation of 13 mm from each side. Chromatographic development was carried out using the mobile phase ethyl acetate: *n*-hexane: formic acid: water (11:2:1:0.5 *v/v/v/v*) in a saturated Twin Trough Chamber (20 cm × 10 cm), allowing the mobile phase to travel a distance of 70 mm. The images of the obtained HPTLC chromatograms were captured in TIFF format and subsequently processed using VideoScan (version 1.02, CAMAG).

Antioxidant activity was determined using the DPPH assay. The plate was sprayed with a 0.05% (*w/v*) methanol solution of DPPH• and incubated for 30 minutes in the dark. Antioxidant activity was indicated by bright zones against a purple background under white light [41].

A NP reagent was prepared by dissolving 500 mg of 2-aminoethyl diphenylborinate in 100 mL of methanol, and the polyethylene glycol 4000 (PEG) solution contained 5% (*w/v*) polyethylene glycol 4000 in methanol. Plates were sprayed sequentially with the NP reagent, dried with hot air, and then sprayed with the PEG reagent before being observed under UV 366 nm for visualization of flavonoids and other phenolic compounds [42].

Terpenoids were detected through derivatization using a *p*-anisaldehyde/sulfuric acid (ASA) reagent, followed by heating on a plate at 110 °C for 10 minutes or until the zones became visible. The ASA reagent was freshly prepared by mixing 10 mL of glacial acetic acid and 85 mL of methanol, and in a refrigerated solution, 5 mL of concentrated sulfuric acid and 0.5 mL of *p*-anisaldehyde were added [43].

Statistical analysis

The results were analyzed using regression analysis, correlation analysis, and single-factor ANOVA with Microsoft Excel's built-in "Data Analysis ToolPak", followed by the Tukey-Kramer test at a confidence level of 95% with the "Real Statistics Resource Pack", a Microsoft Excel add-in for Microsoft Excel from 2010 until the present [40,44–46].

Results and discussion

Spectrophotometric assays and statistical analysis

The analyzed extracts (Figure 1) indicated a higher TPC in bark (BQ) compared to xylem (Q), as well as in the stump of *Q. robur* cut two years ago (Q2Y and BQ2Y) versus the stump of freshly cut *Q. robur* (Q and BQ), which is consistent with existing literature [5,28,47]. Onuchin *et al*. (2018) [28] stated that during wood decay, there is an increase in the content of water-soluble substances due to changes in its chemical composition.

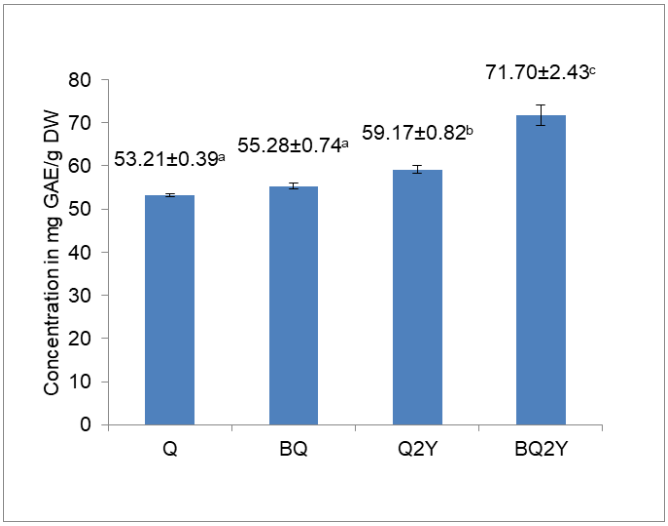

Figure 1. Total phenol content of stump water extracts as mean ± standard deviation: xylem of freshly cut *Q. robur* (Q), bark of a freshly cut *Q. robur* (BQ), xylem of degraded *Q. robur* (Q2Y), bark of degraded *Q. robur* (BQ2Y). The measurements were performed in triplicate; a, b, c indicate significant differences at $p < 0.05$; post-hoc Tukey-Kramer test at a confidence level of 95%.

TPC values of 56.6 mg GAE/g reported by Fernández De Simón *et al.* (1996) [13] for a methanol/water (1:1) extract and 55.51 and 54.21 mg GAE/g reported by [40] for an ethanol (60%, *v/v*) extract of *Q. robur* heartwood

are slightly higher than the TPC of Q (53.21 mg GAE/g). However, it should be borne in mind that methanol/water or ethanol/water extraction results in a higher yield of polyphenols, as well as the fact that the sapwood contains fewer polyphenols than the heartwood [47], so its presence affects the reduction of TPC of the tested *Q. robur* stumps. In addition, the geographical origin (growing conditions) also affects the content of polyphenols [11], which is confirmed by the TPC values that range from 37.91-55.51 g GAE/kg reported by Smailagić *et al*. (2019) [40] for heartwood ethanol (60%, *v/v*) extract of *Q. robur* from different localities.

The TPC of BQ of 55.28 mg GAE/g is consistent with the content reported for the aqueous extract of *Q. robur* by Dedrie *et al*. (2015) [11] (54.3-54.9 mg GAE/g) and Dróżdż and Pyrzynska (2018) [31] (55.4-56.4 mg GAE/g). However, the TPC of BQ2Y extract of 71.70 mg GAE/g is higher than the value of 60.8-62.9 mg GAE/g reported by Dedrie *et al*. (2015) [11] for bark from sawmill storage, while Dróżdż and Pyrzynska (2018) [31] reported a similar TPC value of 60.4 mg GAE/g for commercially available *Q. robur* bark. These differences were probably influenced by environmental conditions and/or duration of exposure.

Statistical analysis using ANOVA revealed a significant difference between the mean TPC values across the samples (F (3.8) =113.13, p < 0.001). The results of the Tukey HSD/Kramer test, illustrated in Figure 2, indicate a significant difference among all extracts, except for Q and BQ, regarding their polyphenol content.

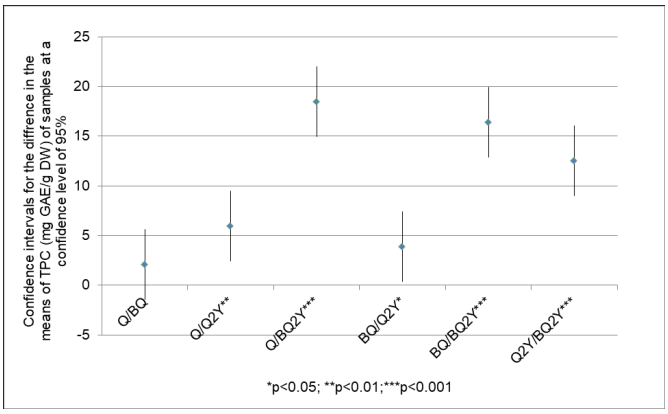

Figure 2. Graphical display of pair-wise comparisons of Tukey HSD/Kramer test for TPC. Any confidence intervals that do not include zero provide evidence of a difference between the groups.

The significant difference according to the Tukey HSD/Kramer test was observed between the Q and Q2Y, BQ and BQ2Y extracts, which showed a substantial increase in polyphenol content in the xylem and especially in the bark of *Q. robur* stumps after two years of degradation. This finding suggests that degrading *Q. robur* stumps could be a promising source of polyphenols for bio-based industries.

The relationship between TPC and H_2O_2 scavenging activity of the Q, BQ, Q2Y, and BQ2Y extracts was examined. Regression analysis revealed that TPC, H_2O_2 scavenging activity, and DPPH scavenging activity of extracts were not in a statistically significant relationship (R^2=0.99, Signf. F=0.07, p > 0.05). However, when examining the correlation between TPC and H_2O_2 scavenging activity, there was a strong negative correlation (correlation coefficient = -0.92721). This indicates that higher polyphenol content corresponds to a lower concentration of the extract required to scavenge 50% of H_2O_2. Due to its high polyphenol content, BQ2Y exhibited H_2O_2 scavenging activity at the lowest concentrations compared to the other extracts, as illustrated in Figure 3. It can also be seen that extracts BQ and Q2Y, between which a small difference in TPC was observed by the Tukey HSD/Kramer test, showed similar H_2O_2 removal activity.

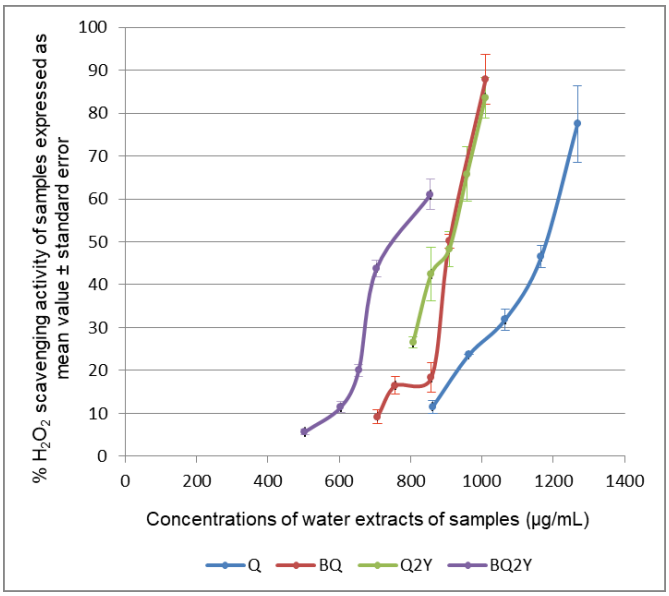

Figure 3. The dependence of %H_2O_2 scavenging activity on the sample concentration in stump water extracts from oak: xylem of freshly cut *Q. robur* (Q), bark of a freshly cut *Q. robur* (BQ), xylem of degraded *Q. robur* (Q2Y), bark of degraded *Q. robur* (BQ2Y)

While the extracts did not demonstrate significant hydrogen peroxide scavenging activity (Table 1) compared to ascorbic acid (IC_{50}=129.83±0.06 µg/mL), they exhibited similar antioxidant activity against H_2O_2 when compared to the study conducted by Valensia-Alvires *et al*. (2018) [48]. Specifically, the antioxidant activity of water extracts from various *Quercus spp*. bark samples ranged from 597 to 1102 µg/mL, similar to the *Q. robur* bark stump extracts analyzed in this study. When comparing the H_2O_2 scavenging activity of methanolic extracts of Vinca major (IC_{50} 600 µg/mL) [49], a plant traditionally used in folk medicine, to our study, we found that the BQ2Y extract exhibited similar antioxidant activity. It is important to note that methanol extraction yields a higher polyphenol content compared to water extraction, which

contributes to the antioxidative capacity of the extract [30]. This indicates a significant H_2O_2 scavenging potential in the extracts derived from degrading *Q. robur* stump bark. When comparing the H_2O_2 antioxidant activities of water extracts of *Mollugo cerviana* and *Bierberstinia multifida* (IC_{50} 1480.3 µg/mL and 1364 µg/mL, respectively) [38,50], the extracts examined in this research demonstrated higher antioxidant activity against H_2O_2. This finding highlights the potential benefits of using wood and bark from tree stumps, as well as decaying stumps, to obtain extracts with significant antioxidant properties. Additionally, using water as an environmentally friendly solvent enhances these benefits.

Table 1. H_2O_2 scavenging activities of water extracts of *Q. robur* stumps expressed as IC_{50} value ± standard error of regression, and DPPH scavenging expressed as mean value ± standard deviation in two different units of measurement

Sample	IC_{50}* (H_2O_2), µg/mL of extract	DPPH*, mg TE**/g dry wood	DPPH*, %
Q	1103.65±9.12	631.70±0.68[a]	79.82±0.09[a]
BQ	906.42±15.94	562.61±17.86[b]	72.23±2.38[b]
Q2Y	923.91±4.37	658.44±12.70[a]	83.98±1.67[a]
BQ2Y	682.39±8.87	720.33±1.41[c]	91.97±0.19[c]

*The measurements were performed in triplicate; a, b, c indicates significant difference between DPPH scavenging activities among samples at p<0.05 (post-hoc Tukey-Kramer test at a confidence level of 95%);
**Trolox Equivalent

In comparing our findings with those of Dudonne *et al.* (2009) [51], we observed that the DPPH scavenging activity in extracts taken from freshly cut *Q. robur* stumps was slightly lower, measuring 79.82%, compared to the 88.60% reported by Dudonne *et al.* (2009). This difference is important because antioxidant activity correlates with the extract's composition, which can vary due to factors such as the age of the wood, its geographical origin, environmental conditions, and the specific location of the sampling on the tree itself. Consequently, we expect to see variations in the percentage of antioxidant activity among water extracts from the same species. In comparison to the findings reported by Smailagić *et al.* (2019) [40], which indicated that the DPPH scavenging activity of the 60% (*v/v*) ethanol extract of *Q. robur* heartwood ranged from 283.15 to 473.92 mmol TE/kg, the DPPH scavenging activity observed in this study was higher. This result aligns with existing literature suggesting that the lower parts of a tree, particularly stumps, contain a greater amount of extractives, which enhances their antioxidant potential [14,45].

Extracts of degrading *Q. robur* stump exhibited higher DPPH antioxidant activity than those of freshly cut ones. After conducting an ANOVA, we found a significant difference in the means of DPPH scavenging activity across all extracts ($F(3.8) = 106.07$, $p < 0.001$). To further analyze these differences, we performed the Tukey HSD/Kramer test, the results of which are illustrated in Figure 4.

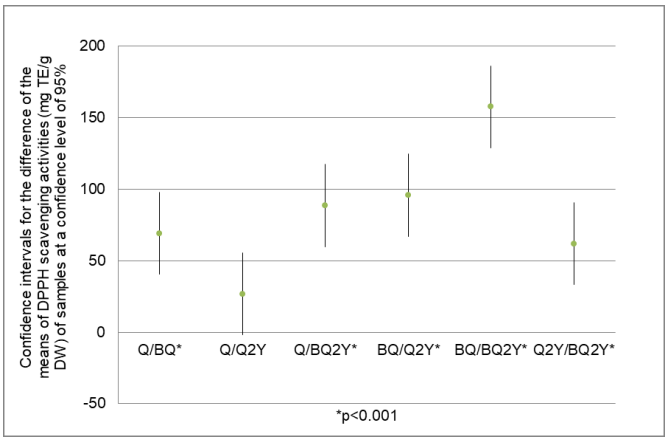

Figure 4. Graphical display of pair-wise comparisons of Tukey HSD/Kramer test for DPPH scavenging activity. Any confidence intervals that do not include zero provide evidence of a difference between the groups

The Tukey HSD/Kramer test indicated a statistically significant difference among all extracts except for Q and Q2Y. The most notable difference was between BQ and BQ2Y. This indicates that the bark extracts of partially decomposed *Q. robur* stumps possess not only significant antioxidant potential but also a greater potential in the bark than in the wood.

Both the DPPH and H_2O_2 scavenging assays demonstrated that the decaying *Q. robur* stumps exhibited the highest scavenging activity. This, along with the highest total phenolic content in these extracts, suggests that they are a valuable source of phenolic compounds and possess significant antioxidant capacity. Additionally, the TPC and scavenging assay values for the Q and BQ extracts were comparable to those reported in other studies on the wood and bark of *Q. robur*. Therefore, these ma-

terials remain valuable sources of phenolic compounds and extractives with antioxidant properties derived from wood remains.

HPTLC-based chemical profiling and fingerprinting

Distinct chromatographic profiles of the extracts were achieved using a mobile phase consisting of ethyl acetate, *n*-hexane, formic acid, and water (11:2:1:0.5, *v/v/v/v*), optimized to maximize the separation of phenolic compounds and terpenoids in aqueous extracts from fresh and biodegraded *Q. robur* xylem and bark. Separated compounds were identified based on their characteristic coloration, UV fluorescence, or post-derivatization appearance (Figure 5).

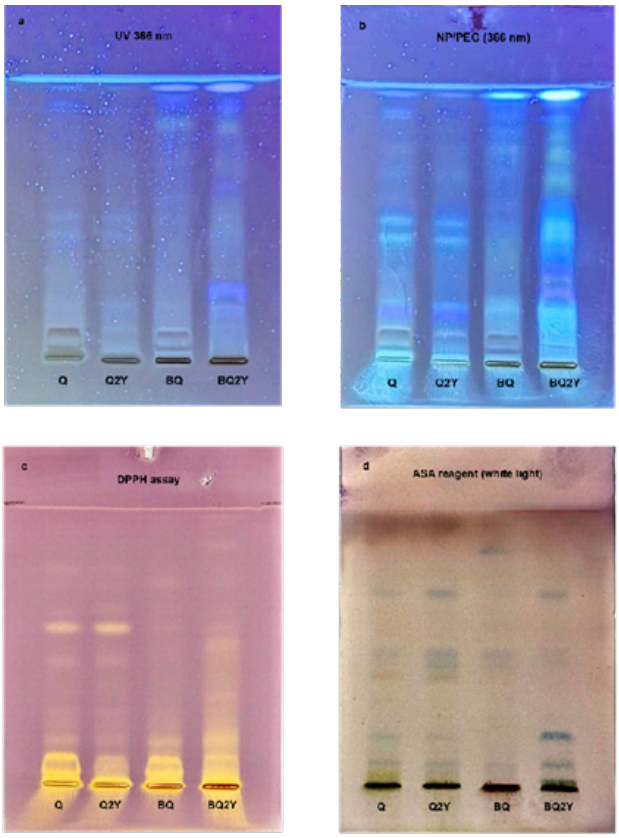

Figure 5. HPTLC fingerprinting of Q, Q2Y, BQ, and BQ2Y extracts visualized under (a) UV 366 nm, (b) with NP/PEG reagent, UV 366 nm, (c) DPPH scavenging assay, white light, and (d) with ASA reagent, white light. Mobile phase, ethyl acetate: *n*-hexane: formic acid: water (11:2:1:0.5 *v/v/v/v*)

Fluorescent zones were observed under UV light at 366 nm (Figure 5a), where certain compounds exhibited fluorescence upon excitation by long-wavelength UV radiation. Under 366 nm UV light, aromatic compounds and conjugated systems appeared as blue- and violet-fluorescent zones on the HPTLC plate. For compounds that did not fluoresce under UV light, the plates were treated with chemical reagents for visualization (Figure 5b,c) [52]. Flavonoids were revealed by NP/PEG derivatization, and the resulting HPTLC fingerprint was analyzed under 366 nm UV light (Figure 5b). The NP/PEG reagent enhances the natural fluorescence of flavonoids, isoflavonoids, and phenolic acids, producing distinct color patterns under UV light that reflect their molecular structures [53]. Yellow-green and orange fluorescence is characteristic of flavones and certain flavonols, while dark green fluorescence indicates some flavones. Phenolic carboxylic acids show a fluorescent blue color [53]. The biodegraded bark extract, BQ2Y, was found to have the highest flavonoid content, as indicated by the presence of more fluorescent zones, followed by the Q2Y extract with slightly lower flavonoid content (Figure 5b). In general, the wood and bark extracts of freshly cut stumps are poorer in flavonoids. Thus, the intense blue fluorescent zones in the BQ2Y extract indicate a rich content of phenolic acids at $R_F \approx 0.90\text{-}0.97$ (such as gallic acid, caffeic acid, ferulic acid, and/or *p*-coumaric acids), and in the R_F range of 0.45-0.55 (such as cinnamic acid derivatives) [6]. In addition, light blue zones above the start, in the range of $R_F \approx 0.04\text{-}0.19$, indicate the presence of phenolic acids. Pale yellow-green ($R_F \approx 0.6\text{-}0.8$) fluorescent zones indicate the presence of flavonoids.

The antioxidant activity of all samples was assessed using the HPTLC-DPPH• scavenging assay, revealing multiple zones of activity. In HPTLC, antioxidants form yellow zones on a purple backdrop, while spectrophotometric analysis measures activity through the decrease in violet coloration. HPTLC offers the advantage of visualizing individual antioxidant compounds [54]. Among all the extracts, BQ2Y exhibited the highest antioxidant potential, with moderate activity observed in Q2Y and Q, and the lowest in BQ (Figure 5c) [14]. Spectrophotometric tests confirmed these results. In particular, the observed bands at $R_F \approx 0.03\text{-}0.16$, 0.32, 0.51, and 0.56 played a significant role in the antioxidant activity.

Nevertheless, it is important to acknowledge that other secondary plant metabolites, such as terpenoids, also contribute to the overall observed antioxidant activity [55]. ASA is commonly used to detect terpenoids through color differentiation [53]. The resulting colors reflect the chemical nature of the compounds. After derivatization and heating, compounds visualized under white light reveal that extracts from stump wood (BQ2Y and Q2Y), which have undergone two years of natural degradation, contain more terpenoids, as indicated by the presence of blue, purple, or brown zones (Figure 5d).

Conclusion

Degrading *Q. robur* stumps, as well as fresh ones, particularly their bark, show a high content of polyphenols and antioxidant capacity against H_2O_2 and DPPH. HPTLC and spectrophotometric analyses revealed that bark and wood water extracts from *Q. robur* stumps, naturally degraded in the forest for two years, exhibited the highest levels of antioxidant potential and phenolic content. In addition to the higher antioxidant potential, the phenolic profile of the extracts of the bark and wood of

partially degraded stumps was richer compared to fresh stump extracts. This suggests that, aiming to maximize wood utilization, partially degrading *Q. robur* stumps could be a valuable source of biologically active polyphenols for industries. Furthermore, the water extraction procedure uses a non-toxic solution and demonstrates high efficiency, highlighting its potential for large-scale extraction.

In further work, it is necessary to detect polyphenolic and other biologically active compounds, such as terpenoids and alkaloids, which are present in aqueous extracts of fresh and degraded tree stumps. Considering the higher antioxidant potential and richer phenolic profile of the extracts of the bark and xylem samples of the partially degraded stump compared to the fresh *Q. robur* stump, it is especially interesting to detect these new low-molecular-weight components, which are probably released by the degradation of structural compounds.

Nomenclature

ANOVA Analysis of variance
ASA *p*-anisaldehyde/sulfuric acid
BQ bark of the tree stump of freshly cut *Q. robur*
BQ2Y bark of the tree stump of *Q. robur* cut two years ago
DPPH 2,2-di(4-tert-octylphenyl)-1 picrylhydrazyl
DW dry wood
FC Folin-Ciocalteu
GAE gallic acid equivalents
H_2O_2 hydrogen peroxide
HPTLC High-performance thin-layer chromatography
HSD Honest Significant Difference
IC_{50} The concentration at which the sample scavenging activity is 50%
Na_2CO_3 sodium carbonate
NP natural product
PBS phosphate-buffered saline
PEG polyethylene glycol
TE Trolox Equivalent
TPC total phenolic content
Q xylem of the tree stump of freshly cut *Q. robur*
Q2Y xylem of the tree stump of *Q. robur* cut two years ago
UV ultraviolet
Vis visible

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Izvod

UTICAJ PRIRODNE DEGRADACIJE NA FENOLNI PROFIL PANJA HRASTA LUŽNJAKA (*QUERCUS ROBUR* L.): HPTLC I SPEKTROFOTOMETRIJSKA ISPITIVANJA

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Hrast lužnjak (*Quercus robur* L.) je bogat ekstraktivima, posebno srčevina, panj i kora, što ove materijale čini značajnim za industrije zasnovane na bioresursima. Uzimajući navedeno u obzir kao i sve veću potražnju za efikasnim korišćenjem drveta, u ovoj studiji su ispitivani ekstraktivi panjeva hrasta preostalih posle sveže seče i seče stabala dve godine ranije. Tokom ove dve godine panjevi su bili izloženi vremenskim uslovima (padavine, UV zračenje i dr.) i delovanju gljivica, bakterija i drugih patogenih organizama. Ovi faktori su doprineli prirodnoj degradaciji drveta i narušavanju prvobitne hemijske strukture panjeva. Ispitivan je sadržaj ukupnih fenola, antioksidativni potencijal prema H_2O_2 i prema slobodnim radikalima, vodenih ekstrakata ksilema i kore sveže posečenih i biodegradiranih hrastovih panjeva. Ukupni sadržaj fenola je bio veći u kori i ksilemu degradiranih hrastovih panjeva sa 71,70 mg GAE/g DW i 59,17 mg GAE/g DW, respektivno. Degradirani hrastovi panjevi su imali veći anitioksidativni kapacitet prema H_2O_2 sa IC_{50} od 682,39 µg/mL i 923,90 µg/mL i prema slobodnim radikalima od 91,97% i 83,97% respektivno. Tankoslojna hromatografija visokih performansi u obliku "otiska prsta" za ekstrakte ksilema i kore otkrila je jedinjenja različitog hemijskog profila, dok je HPTLC–DPPH test procenio antioksidativni profil ekstrakata. Ovo istraživanje je pokazalo da su ekstrakti kore i drveta degradiranog panja jači antioksidansi i imaju bogatiji fenolni profil u odnosu na ekstrakte sveže posečenog panja. Degradirani hrastovi panjevi mogu predstavljati značajan izvor polifenola.

Ključne reči: *Quercus robur* L., degradirani panj, ukupan sadržaj fenola, antioksidativni potencijal, visoko–efikasna tankoslojna hromatografija (HPTLC)

CRediT authorship contribution statement:
Gordana Petković: Conceptualization, Formal analysis and investigation, Writing - original draft, Validation.
Ivana Stojiljković: Conceptualization, Methodology, Formal analysis and investigation, Writing - original draft, Funding acquisition, Writing – Reviewing and Editing, Validation.
Jasmina Popović: Conceptualization, Methodology, Formal analysis and investigation, Writing - original draft, Funding acquisition, Writing – Reviewing and Editing, Validation.

Declaration of Competing Interest
No potential conflict of interest was reported by the author(s).