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Isolation and quantification of diarylheptanoids from European hornbeam (*Carpinus betulus* L.) and HPLC-ESI-MS/MS characterization of its antioxidative phenolics

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ABSTRACT

Detailed polyphenol profiling of European hornbeam (*Carpinus betulus* L.) bark, leaf, male and female catkin extracts was performed by high-performance liquid chromatography–diode array detection coupled to tandem mass spectrometry (HPLC-DAD-MS/MS). A total of 194 compounds were characterized and tentatively identified. Gallo- and ellagitannins dominated in the methanol extracts, while flavonol glycosides and methoxylated flavones prevailed in the ethyl acetate samples. In the quest for diarylheptanoids, twelve compounds were isolated by the combination of subsequent reversed-phase flash chromatographic and high-performance liquid chromatographic methods. The structural elucidation of the isolated components was performed by ultrahigh-performance liquid chromatography-Orbitrap mass spectrometry (UHPLC-Orbitrap-MS) as well as 1D and 2D nuclear magnetic resonance (NMR) spectroscopy. Six known cyclic diarylheptanoids, together with a new compound were described in *Carpinus betulus* for the first time. The occurrence of a linear diarylheptanoid and a lignan has also been unprecedented in the genus *Carpinus*. Moreover, three known flavonol glycosides were isolated. Based on the identification of characteristic fragment ions, a new mass spectrometric fragmentation pathway for *meta*,*meta*-cyclophane-type diarylheptanoids was proposed. Quantities of the four major cyclic diarylheptanoids in European hornbeam were determined by a validated UHPLC-DAD method for the first time. The antioxidant properties of the extracts and the isolated compounds were assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Contribution of the individual constituents to the total radical scavenging activity of the samples was evaluated by an off-line DPPH-HPLC-DAD method. This allowed the identification of gallo- and ellagitannin derivatives as the constituents being primarily responsible for the antioxidant capacity of the extracts.

1. Introduction

Plants are still considered as noteworthy potential sources for new drugs, but the ligneous flora is rarely referred to for the presence of possible medical agents. The genus *Carpinus* (Betulaceae) comprises approximately 35 woody species spread in Europe, Eastern Asia, North, and Central America, with the highest number of species being native to China. European hornbeam (*Carpinus betulus* L.) is a common forest tree species widespread throughout Europe [1]. It is an important raw material for the wood industry: its valuable wood is used for tools, building constructions, flooring, to prepare wooden parts of musical instruments (e.g. piano mechanisms), and as fuel wood and charcoal. Occurrence of bioactive constituents such as flavonol and flavone mono- and diglycosides were reported for *C. betulus* [2]. Hofmann and coworkers [3] characterized phenolic compounds by HPLC–MS/MS in *C. betulus* leaves, however, other parts of the plant were not analyzed. The authors investigated the seasonal changes in the antioxidant capacity of European hornbeam leaf extracts throughout the vegetation period, too. In a recent study, DPPH scavenging activity of European hornbeam bark extracts was assessed [4].

Although no specific applications of the waste (i.e. bark, leaves, etc.) resulting from processing of hornbeam wood have yet been identified, results of several studies and experiments support that *Carpinus* species could become easily affordable sources of new bioactive ingredients. The ethyl acetate and methanol extracts of *C.*

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betulus bark and leaf demonstrated in vitro growth inhibitory activity against various human cancer cell lines [5], while extracts of the cultivar *C. betulus* 'Fastigiata' presented immunosuppressive effects [6]. Although, diarylheptanoids have not yet been identified in *C. betulus*, other hornbeam species contain these compounds, e.g. the known cyclic diarylheptanoids, carpinontriols A and B as well as casuarinondiol were isolated from *Carpinus cordata* [7]. Diarylheptanoids attract interest in natural product research, due to their notable biological activities such as their cytotoxic, anti-inflammatory, anti-microbial, and antioxidant effects [8].

The aim of this study was a detailed and extensive phytochemical characterization of European hornbeam by HPLC-DAD-MS/MS. Distinct plant parts: leaf, bark, female, and male catkin samples were collected to compare their phenolic composition. *C. betulus* extracts were prepared with solvents of different polarity, in order to obtain diverse compositions of phenolics. We aimed to screen the phenolic profile of hornbeam samples with special regard to cyclic diarylheptanoids. Thus, our further aim was to confirm their plausible presence in *C. betulus* samples, reveal their structures by NMR experiments, and assess their quantities. To determine the in vitro antioxidant activity of the extracts and the isolated compounds, the DPPH assay was employed. An off-line DPPH-HPLC-DAD-MS method was applied to assess the contribution of individual constituents to the total radical scavenging activity of each extract.

2. Materials and methods

2.1. Chemicals and reagents

Chloroform, ethyl acetate, methanol, and *n*-hexane of reagent grade as well as HPLC grade methanol and acetonitrile were purchased from Molar Chemicals Kft. (Halásztelek, Hungary). Acetic acid 100% for HPLC LiChropur™, DPPH (2,2-diphenyl-1-picrylhydrazyl), rutin, trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), trifluoroacetic acid, methanol-d_4, and DMSO-d_6 for NMR measurements were acquired from Sigma-Aldrich (Steinheim, Germany). High-purity water was gained by a Millipore Direct Q5 Water Purification System (Billerica, MA, USA).

2.2. Plant material and sample preparation

For the qualitative HPLC-MS analyses, the UHPLC-DAD quantitation, and the DPPH assays, bark, leaf, female, and male catkin samples of *C. betulus* were collected in Hungary, in the Buda Hills (Budai-hegység, April 2015), Mátraháza (May 2016) and Visegrád Mountains (Visegrádi-hegység, July 2018). Authenticated samples and herbarium specimens are deposited at the Herbarium of the Department of Pharmacognosy, Semmelweis University, Budapest, Hungary. Dried and milled samples (3.0 g each) were extracted by Soxhlet extraction (6 h) with ethyl acetate and methanol (250 mL each). The extracts were distilled to dryness under reduced pressure with a rotary evaporator (Büchi Rotavapor R-200, Flawil, Switzerland) at 50 °C. The samples were redissolved in 4.0 mL methanol of HPLC gradient grade and filtered through Minisart RC 15 0.2 µm syringe filters (Sartorius AG, Goettingen, Germany). Prior to analysis, the purified samples were evaporated to dryness at 50 °C under reduced pressure and redissolved in 1.0 mL 70% (v/v) HPLC grade methanol.

2.3. Isolation procedures

For the isolation of the constituents, bark samples of *C. betulus* were collected in Hungary, in Mátraháza (May 2017) and Lajosháza (May 2019). The combined and dried samples (500 g) were ground and extracted at room temperature in ultrasonic bath with chloroform (3 × 2 L, 2 h each). In the following, the residue was extracted consecutively with solvents of increasing polarity: ethyl-acetate and then methanol (3 × 2 L for both solvents, 2 h each). The ethyl acetate extract was distilled to dryness under reduced pressure with a rotary evaporator at 50 °C. The residue was suspended in 70% (v/v) methanol (to get a concentration of 500 mg in 4 mL) and fractionated using a CombiFlash NextGen 300 + (Teledyne Isco, Lincoln, NE, USA) flash chromatograph, applying a RediSep Rf Gold C18 column (100 g, Teledyne Isco) as stationary phase. Eluent A was 0.3% acetic acid in water, eluent B was methanol, and the following gradient elution was applied at a flow rate of 60 mL/min: 30% B (0–3 min), 30–100% B (3–33 min), 100% B (33–38 min). 144 fractions (of 16 mL each) were collected. Fractions 56–60 yielded compound **177** (23.5 mg). Chromatographic separations of additional fractions were performed by semi-preparative HPLC on a Waters 2690 HPLC system equipped with a Waters 996 diode array detector (Waters Corporation, Milford, MA, USA). As stationary phase, a Luna C18 100 A (150 × 10 mm i.d., 5 µm; Phenomenex Inc; Torrance, CA, USA) column or a Kinetex C18 100 A (150 × 10 mm i.d., 5 µm; Phenomenex Inc) column was used (Fig. S1). Different gradient elution methods consisting of 0.3% acetic acid in water (eluent A) and methanol (eluent B) were applied at a flow rate of 1 mL/min. Fractions 38–41 were separated to obtain **106** (3.5 mg, t_R = 22.3 min), **114** (1.3 mg, t_R = 24.1 min), and **154** (1.5 mg, t_R = 30.0 min), using the gradient as follows: 33% B (0–20 min), 33–100% B (20–25 min), 100% B (25–33 min). Fractions 68–71 were chromatographed using the gradient 50% B (0–20 min), 50–100% B (20–23 min), 100% B (23–33 min), to yield compound **191** (2.2 mg, t_R = 24.1 min). For the chromatographic separation of fractions 61–67 to purificate **164** (0.7 mg, t_R = 13.6 min) and **187** (0.5 mg, t_R = 14.4 min), we applied a different gradient elution system consisting of 0.3% acetic acid in water (eluent A) and acetonitrile (eluent B) at a flow rate of 1 mL/min: 40–64% B (0–16 min), 64–100% B (16–17 min).

The methanol extract of the bark sample was distilled to dryness under reduced pressure with a rotary evaporator at 50 °C. The residue was redissolved in 70% (v/v) methanol (to get a concentration of 1000 mg in 4 mL) and separated by flash chromatography as described for the ethyl acetate extract. Fractions were further separated by semi-preparative HPLC (using the same instrumentation and stationary phase as detailed above). Different gradient elutions were employed at a flow rate of 1 mL/min. Fractions 50–55 were purified with the gradient as follows (eluent A: 0.3% acetic acid in water, eluent B: methanol): 50% B (0–20 min), 50–100% B (20–22 min), 100% B (22–32 min), 6 fractions were collected. Fraction 2 (t_R = 12 min) was further chromatographed applying the following gradient elution (eluent A: 0.3% acetic acid in water, eluent B: acetonitrile): 22–24% B (0–22 min), to yield **148** (1.2 mg, t_R = 19.2 min). Fractions 56–61 from flash chromatography were separated to collect 8 fractions, with the gradient (eluent A: 0.3% acetic acid in water, eluent B: methanol): 45–50% B (0–20 min), 50–100% B (20–22 min), 100% B (22–32 min). Fraction 8 (t_R = 23 min) was chromatographed with the gradient elution (eluent A: 0.3% acetic acid in water, eluent B: acetonitrile) 35% B (0–16 min), 35–100% B (16–17 min), to yield **149** (1.7 mg, t_R = 13.7 min), **157** (2.0 mg, t_R = 14.7 min), and **161** (0.7 mg, t_R = 12.5 min). Fractions 73–75 from flash chromatography were separated to yield **185** (3.3 mg, t_R = 17.1 min), using the following gradient elution (eluent A: 0.3% acetic acid in water, eluent B: acetonitrile): 40–60% B (0–25 min). The isolation procedure is depicted in Fig. S1. Purity of the isolated substances was surveyed by HPLC-DAD-MS/MS.

2.4. HPLC-DAD-ESI-MS/MS analyses

Qualitative phytochemical screening of *Carpinus* extracts was performed with an Agilent 6410B triple quadrupole equipped with an electrospray ionization source (ESI) coupled to an Agilent 1100 HPLC system (G1379A degasser, G1312A binary gradient pump,

G1329A autosampler, G1316A column thermostat and G1315C diode array detector) (Agilent Technologies, Santa Clara, CA, USA and Waldbronn, Germany). The separation of *Carpinus* extracts was carried out on a Zorbax SB-C18 column (150 × 3.0 mm i.d., 3.5 µm; Agilent Technologies). Eluent A was 0.3% acetic acid in water and eluent B was methanol. A gradient elution was performed at a flow rate of 0.3 mL/min as follows: 10–40% B (0–35 min), 40–60% B (35–45 min), 60–100% B (45–47 min), 100% (47–50 min), 100–10% B (50–51 min), the column temperature was set to 25 °C. The injection volume was 10 µL. Nitrogen was applied as drying gas (350 °C, 9 L/min), the nebulizer pressure was 45 psi. The fragmentor voltage was set to 120 V, the capillary voltage was 3500 V. High purity nitrogen was used as collision gas, the collision energy varied between 10 and 40 eV. Full scan mass spectra were recorded in negative ionization mode in the range of *m/z* 100–1000. The MassHunter B.01.03 software was used for data acquisition and qualitative analyses.

2.5. UHPLC-ESI-Orbitrap-MS/MS conditions

High-resolution mass spectra of the isolated compounds were obtained using a Dionex Ultimate 3000 UHPLC system (3000RS diode array detector, TCC-3000RS column thermostat, HPG-3400RS pump, SRD-3400 solvent rack degasser, WPS-3000TRS autosampler), hyphenated with an Orbitrap Q Exactive Focus Mass Spectrometer equipped with electrospray ionization source (Thermo Fischer Scientific, Waltham, MA, USA). An Acquity UPLC BEH C18 column (30 × 2.1 mm i.d., 1.7 µm; Waters Corporation) was used (column temperature: 25 °C), and the mobile phase consisted of 0.1% formic acid in water (eluent A) and a mixture of 0.1% formic acid in water and acetonitrile (20:80, v/v) (eluent B). The following gradient elution was applied at a flow rate of 0.3 mL/min: 10–60% B (0.0–3.5 min), 60–100% B (3.5–4.0 min), 100% B (4.0–4.5 min), 100–10% B (4.5–7.0 min). The injection volume was 1 µL. The ESI source was operated in negative ionization mode and operation parameters were optimized automatically using the built-in software. The working parameters were as follows: spray voltage 2500 V; capillary temperature 320 °C; sheath gas (N_2), 47.5 °C; auxillary gas (N_2) 11.25 arbitrary units, and spare gas (N_2) 2.25 arbitrary units. The resolution of the full scan was of 70000, and the scanning range was between *m/z* 100–500 units. The most intense ions detected in full scan spectrum were selected for data-dependent MS/MS scan at a resolving power of 35000, in the range of *m/z* 50–500. Parent ions were fragmented with normalized collision energy of 10%, 30%, and 45%.

2.6. Quantitative UHPLC-DAD conditions

Quantities of the isolated diarylheptanoids carpinontriols A (**106**) and B (**149**), 3,12,17-trihydroxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione (**154**), and giffonin X (**157**) were determined by UHPLC-DAD. The *Carpinus* extracts were analyzed by an ACQUITY UPLC H-Class PLUS System equipped with a quaternary solvent delivery pump (QSM), an auto-sampler manager (FTN), a column compartment (CM), and a photodiode array (PDA) detector (Waters Corporation). An Acquity BEH C18 column (100 × 2.1 mm i.d., 1,7 µm; Waters Corporation) maintained at 30 °C was used as stationary phase. Eluent A was 0.3% acetic acid in water and eluent B was acetonitrile, the following gradient elution was applied (flow rate: 0.3 mL/min): 12.0–13.5% B (0.0–19.0 min), 13.5–75.0% B (19.0–25.5 min), 75.0–100.0% B (25.5–26.0 min), 100.0% B (26.0–28.0 min), 100.0–12.0% B (28.0–28.5 min). The injection volume was 2 µL. Chromatograms were recorded at 295 nm.

2.7. Validation of the quantitative method

2.7.1. Preparation of standard solutions, linearity, and selectivity

Quantitation was performed by the external standard method. Stock solutions containing 1 mg/mL of the isolated **106**, **149**, **154**, and **157** in HPLC grade methanol were prepared. For the preparation of the calibration curve, stock solutions were diluted with methanol of HPLC grade, to yield solutions with concentrations of 1, 2.5, 5, 25, 50, 100, and 250 µg/mL. Each standard solution was prepared in triplicate and injected once. Standard solutions were stored at 4 °C before injection. Linearity curves were constructed by plotting peak areas against corresponding concentrations. Slope, intercept, and correlation coefficient were determined by least squares polynomial regression analysis. Limits of detection (LOD) and quantitation (LOQ) were determined at signal-to-noise (S/N) ratios 3 and 10, respectively. The selectivity of the method was evaluated by analyzing blank samples (extracts obtained by extraction with *n*-hexane), and spiked samples (extracts fortified with standard solutions of the analytes).

2.7.2. Precision, accuracy, and repeatability

Quality control samples were prepared at 5, 50, and 250 µg/mL nominal concentrations. All samples were prepared in triplicate and injected once on the same day (intra-day precision and accuracy) or on three consecutive days (inter-day precision and accuracy). Retention time repeatability was assessed by injecting the standard solutions in six successive parallels.

2.7.3. Recovery

Extraction recovery for giffonin X (**157**) was tested in a concentration range to match with that of the target analyte in the plant sample. 1.0–1.0 g dried *C. betulus* bark samples were spiked with 0.25 mL aliquots of a solution of **157** (1.0 mg/mL) and extracted at room temperature in ultrasonic bath with ethyl acetate and methanol (3 × 10.0 mL, 30 min each), respectively. Samples were prepared in three parallels. Further sample preparation steps were the same as described in Section 2.2. Recovery (R) was calculated as $R = 100 \times (C_{found} - C_{initial})/C_{added}$, where C_{found} = measured concentration in the fortified sample, $C_{initial}$ = initial concentration in the sample, C_{added} = concentration in the standard solution used.

2.8. NMR conditions

NMR spectra of the isolated compounds were recorded in methanol-d_4 on a Varian DDR 600 (600/150 MHz) instrument equipped with a 5-mm inverse-detection gradient (IDPFG) probehead at 298 K or on a BRUKER AVANCE III HD 600 (600/150 MHz) instrument equipped with Prodigy cryo-probehead at 295 K. High temperature NMR experiments were conducted on a Bruker Avance III 400 (400/100 MHz) equipped with a PA BBO 400W1 BBF-H-D-05 Z (Billerica, MA, USA) probehead at 370 K in DMSO-d_6. The pulse programs were taken from the vendor's software library (TopSpin 3.5 or VnmrJ 3.2). ^{13}C and ^{1}H chemical shifts (δ) are given in ppm relative to the NMR solvent or relative to tetramethylsilane (TMS), while coupling constants (J) are given in ppm and in Hz, respectively. The complete ^{1}H and ^{13}C resonance assignments were achieved using 1D ^{1}H NMR, ^{13}C NMR, DeptQ, and homo- and heteronuclear 2D ^{1}H–^{1}H COSY, ^{1}H–^{13}C edHSQC, ^{1}H–^{13}C HMBC, ^{1}H–^{1}H NOESY or ^{1}H–^{1}H ROESY, and ^{1}H–^{1}H TOCSY experiments.

2.9. Evaluation of the antioxidant activity

2.9.1. DPPH assay

Antioxidant activities of *C. betulus* extracts and the isolated compounds were determined by spectrophotometry in an in vitro decolorization assay using DPPH as free radical. For comparison, solutions of trolox and rutin were also studied. The following method was applied: 10 mg of DPPH was dissolved in 25.0 mL HPLC grade methanol, stock solutions were diluted with HPLC methanol just before measuring, so that the absorbance of the diluted free radical solution was approximately 0.90. Detection was carried out at 515 nm wavelength which is the characteristic absorption maximum of the DPPH radical. Hornbeam extracts of 5 different concentrations were added to the free radical solutions (2.5 mL), in triplicate. After incubation for 6 min at room temperature in the dark, the decrease in absorbance was measured with a HITACHI U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Half maximal inhibitory concentration value (IC_{50}, μg/mL) was determined for each sample [9]. Comparison between hornbeam extracts prepared with ethyl acetate and methanol was performed by oneway analysis of variance (ANOVA), followed by Tukey's post hoc HSD test.

2.9.2. DPPH-HPLC-DAD analysis

An off-line DPPH-HPLC-DAD method was applied to compare the contribution of each compound to the total antioxidant effect against DPPH [10]. Hornbeam samples (0.5 mg/mL) were mixed with a DPPH solution (1.5 mg DPPH / 1 mL HPLC methanol, prepared right before the assays) at the ratio of 1:1 (v:v). The mixtures were incubated at room temperature for 30 min, while protected from light. The control samples were made by adding methanol instead of the DPPH solution to the samples in the same ratio. The DPPH-treated samples and control samples were evaluated in 3 parallels by HPLC-DAD-MS using the same method as detailed in Section 2.4. Phenolics with antioxidant activities decompose while reacting with the DPPH radicals, thus their AUC (area under the curve) values in HPLC-DAD-MS chromatograms decrease, as compared to control samples. We calculated the changes in AUC values using the following formula: (%) = $(1\text{-}AUC_{DPPH} / AUC_{control}) \times 100$.

3. Results and discussion

3.1. HPLC-DAD-MS screening of Carpinus betulus polyphenols

HPLC-DAD-ESI-MS/MS in negative ionization mode was used to evaluate the phenolic profile of the extracts. In this study, 194 compounds were tentatively characterized by comparing their retention times, UV spectra, and mass spectrometric fragmentation patterns with data from the literature. Occurrence of the detected compounds, their chromatographic and mass spectrometric properties are listed in Supplementary Table S1. UV chromatograms of the extracts detected at 290 nm are shown in Supplementary Figs. S2-S9**.**

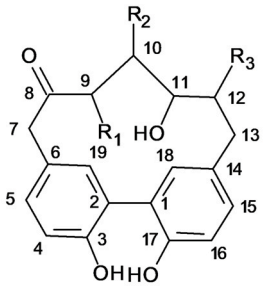
In line with literature data, gallotannins and ellagitannins prevailed in hornbeam extracts [3]. Gallic acid derivatives eluting at low retention times were characterized by their typical fragment ions at *m/z* 169 which is the deprotonated molecular ion of gallic acid, and *m/z* 125 which is created by the cleavage of the carboxyl group from gallic acid [11,12]. Compounds **8**, **16,** and **18**, characterized as galloylquinic acid isomers, could also be distinguished from the relative intensities of their fragment ions [13]. In case of 5-*O*-galloylquinic acid (**16**), the fragment ion at *m/z* 191 is dominating, while the relative intensity of the fragment ion at *m/z* 173 indicates the 4-*O*-galloylquinic acid structure for compound **18**. 3-*O*-galloylquinic acid (**8**) which showed the lowest retention time, yielded comparatively intense fragment ions both at *m/z* 169 and 191.

Gallotannins (**G**, Table S1) were found typically in methanol extracts of leaf, female, and male flower samples. The compounds contain a hexose core (mainly glucose) with its hydroxyl groups partly or completely substituted with a varying number of galloyl moieties via ester linkage. These components exhibited the representative fragment ions of gallic acid at *m/z* 169 and *m/z* 125 as well as neutral losses of 170 Da (gallic acid), 152 Da (galloyl moiety), and 134 Da (galloyl moiety losing a water molecule) [11]. Eight trigalloyl hexose isomers (**41**, **52**, **60**, **75**, **82**, **85**, **88**, **102**) were detected displaying the $[M\text{-}H]^-$ ion at *m/z* 635. The fragment ions $[M\text{-}H\text{-}170]^-$ at *m/z* 465 and $[M\text{-}H\text{-}170\text{–}152]^-$ at *m/z* 313 were generated by the cleveage of a gallic acid and a galloyl moiety, respectively. Compounds **96, 105**, **110**, and **120** presenting $[M\text{-}H]^-$ ions at *m/z* 787 were characterized as tetragalloyl hexose isomers. Pentagalloyl hexose isomers (**118**, **123**, **128**, **138**) exhibited their $[M\text{-}H]^-$ ion at *m/z* 939.

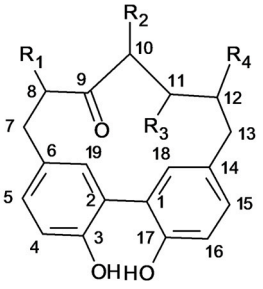
Ellagitannins (**E**, Table S1) contain hexahydroxydiphenoyl (HHDP) groups attached via ester linkage to a polyol core (e.g. glucose). These compounds were identified by the presence of the ellagic acid fragment ion at *m/z* 301, the monogalloyl hexose fragment ion at *m/z* 331, and the ellagic acid hexoside fragment ion at *m/z* 463 [11,12,14]. Compounds **6**, **20**, **23**, **30**, **38**, **43**, **51**, and **84**, with $[M\text{-}H]^-$ ions at *m/z* 633, identified as galloyl-HHDP hexose isomers, and galloyl-bis-HHDP hexoses with $[M\text{-}H]^-$ ions at *m/z* 935 (**40**, **50**, **66**, **77**, **81**, **90**) were found in the methanolic extracts of bark and flower samples. Three digalloyl-HHDP hexoses (**37**, **61**, **134**) presenting the $[M\text{-}H]^-$ ion at *m/z* 785, and five trigalloyl-HHDP hexose isomers (**89**, **109**, **127**, **129**, **144**) with the $[M\text{-}H]^-$ ion at *m/z* 937 were identified.

Glycosylated and methoxy-substituted hydroxybenzoic acid derivatives (**B**, Table S1) were present primarily in the methanolic extract of the bark sample. Their typical fragment ions included the dihydroxybenzoic acid moiety at *m/z* 153 and its fragment ion at *m/z* 109, yielded by the cleavage of the CO_2 group [11,14]. In contrast to hydroxybenzoic acids, hydroxycinnamic acid derivatives (**C**, Table S1) were representative of leaf, female, and male catkin samples. Similarly to the galloylquinic acids, the relative intensities of fragment ions in the mass spectra of the cinnamoylquinic acid isomers could facilitate their differentiation. Thus, an abundant fragment ion at *m/z* 191 indicated the identification of **65** as 5-*O*-caffeoylquinic acid, **91** as 5-*p*-*O*-coumaroylquinic acid, and **116** as 5-*O*-feruloylquinic acid [15]. The minor components **83**, **111**, and **132** displayed identical fragmentation patterns. According to the results of Jaiswal et al. [15], these compounds eluting at higher retention times were assumed as the more hydrophobic *cis* isomers of the corresponding 5-*O*-caffeoyl-, 5-*O*-coumaroyl-, and 5-*O*-feruloylquinic acids, respectively.

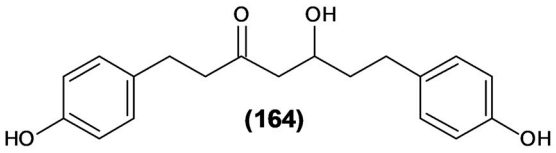
In accordance with previous studies [2,3], flavonol and flavone derivatives occurred in the flower and leaf extracts (**F**, Table S1) mainly in their glycosidic form. Cleavage of a hexose, a deoxyhexose or a pentose sugar moiety during the collision-induced dissociation (CID) of flavonoid glycosides resulted in neutral losses of 162, 146, and 132 Da, respectively [16]. The glycosylation site of flavonol glycosides could also be deduced. Flavonol-3-*O*-glycosides favour the homolytic cleavage of the saccharide moiety during their CID in negative ionization mode. Thus, the relative abundance of the radical aglycone ion $[Y_0\text{-}H]^{\bullet-}$ (deriving from a homolytic cleavage) was higher in their mass spectra than that of the aglycone anion $[Y_0]^-$ [17]. Peak **155** presenting the $[M\text{-}H]^-$ ion at *m/z* 463 was identified as myricetin-3-*O*-deoxyhexoside, based on the relative abundance of its $[M\text{-}H\text{-}147]^{\bullet-}$ ion at *m/z* 316. Analogously, **135** and **153** displayed their $[M\text{-}H]^-$ ions at *m/z* 479 and 449, respectively, and the $[M\text{-}H\text{-}163]^{\bullet-}$ and $[M\text{-}H\text{-}133]^{\bullet-}$ ions at *m/z* 316. Therefore, **135** and **153** were identified as myricetin-3-*O*-hexoside and myricetin-3-*O*-pentoside, respectively. Quercetin- and kaempferol-3-*O*-monoglycoside derivatives (**160**, **163**, **169**, **176**, **177**, **191**) were characterized similarly [16–19].



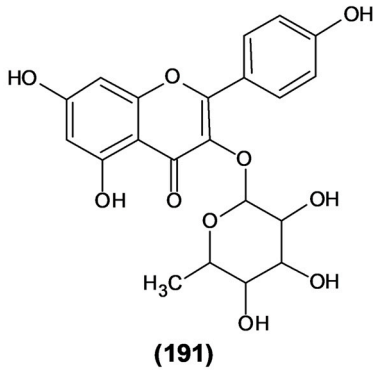
(114) R_1=R_2=R_3=OH
(187) R_1=R_2=R_3=H



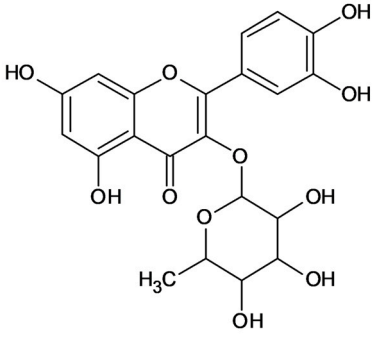
(106) R_1=R_3=R_4=OH, R_2=H
(149) R_1=H, R_2=R_3=R_4=OH
(154) R_1=OH, R_2=R_3=H, R_4=O
(157) R_1=R_2=H, R_3=R_4=OH
(161) R_1=OH, R_2=R_3=H, R_4=OH



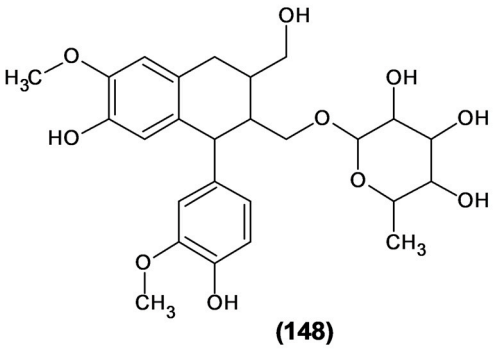
(164)



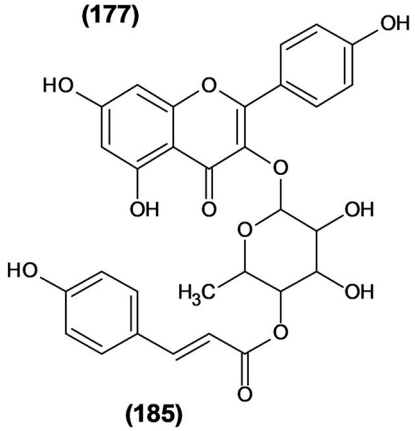
(191)



(177)



(148)



(185)

Fig. 1. Compounds isolated from *C. betulus* bark.

Compounds **185** and **186** showed complex UV spectra with absorption maxima at 267, 317, and 345 nm. In their mass spectra, two successive losses of 146 Da and the aglycone anion at *m/z* 285 could be observed, thus the constituents were supposed to be kaempferol-dideoxyhexoside isomers. However, as a result of a more rigorous analysis, one of the 146 Da losses was later characterized as a coumaroyl moiety (coumaric acid-H_2O). This presumption was confirmed by the presence of the fragment ion at *m/z* 163, which could be assigned to the $[M\text{-}H]^-$ ion of coumaric acid. Thus, **185** and **186** were established as kaempferol-deoxyhexoside coumaroyl ester isomers [20]. NMR analysis of the isolated **185** confirmed the proposed structure (see Section 3.2.).

Methoxylated flavones as well as their glycosylated and sulfated derivatives were detected in bark samples. Neutral losses of 15 Da referred to the cleavage of methyl radicals ($-CH_3^{\bullet}$) indicating the presence of methoxy groups in the molecule [21]. Accordingly, compound **179** exhibiting fragment ions at *m/z* 315 and 300 was assumed as a methoxyflavone derivative. Constituents **159**, **181**, and

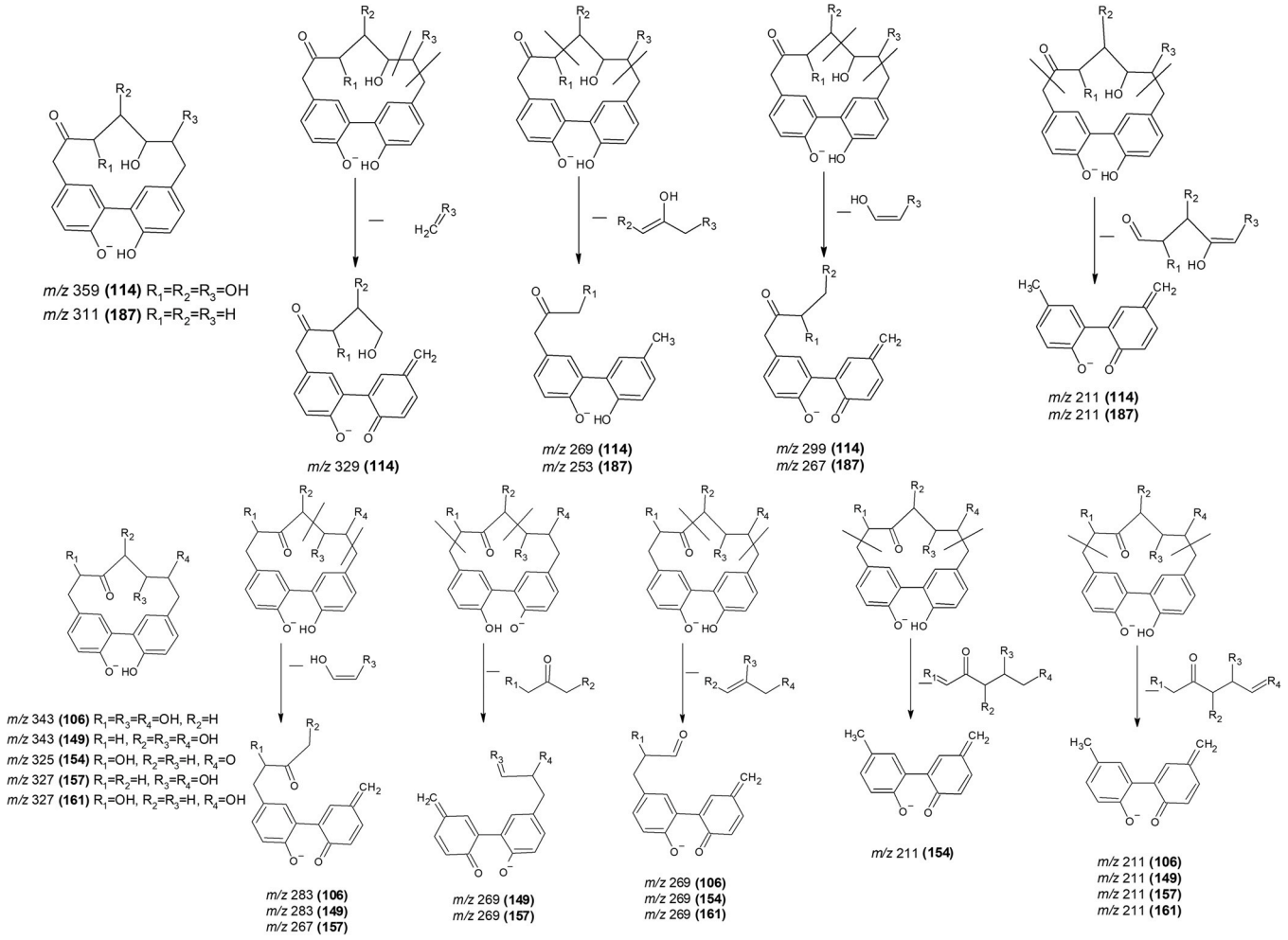


Fig. 2. Proposed mass spectrometric fragmentation pathways of cyclic diarylheptanoids isolated from *C. betulus* bark.

183 presented fragment ions at *m/z* 328, 313, and 298 which denoted the cleavage of two methyl radicals, thus, these compounds were characterized as dimethoxyflavone derivatives. Similarly, compounds **188** and **190** with $[M-H]^-$ ions at *m/z* 343 and fragment ions at *m/z* 328, 313, 298 were identified as trimethoxyflavones. Both **174** and **158** displayed a neutral loss of 80 Da which indicated the cleavage of a SO_3 moiety [22], therefore, they were recorded as trihydroxy-dimethoxyflavone-*O*-sulfate and its pentoside, respectively.

Constituents **48** and **54** exhibiting $[M-H]^-$ ions at *m/z* 289 were identified as flavan-3-ol derivatives catechin or epicatechin, due to their typical fragment ion $[M-H-CO_2]^-$ at *m/z* 245, deriving from the decarboxylation of catechin or epicatechin [11]. Compound **115** with its pseudomolecular ion at *m/z* 305 and $[M-H-OH-CO_2]^-$ ion at *m/z* 245 was referred to as gallocatechin or epigallocatechin. Peaks **107** and **152** presenting their $[M-H]^-$ ions at *m/z* 441 and fragment ions at *m/z* 289, 245, 169, and 125 were tentatively characterized as catechin gallate or epicatechin gallate [11].

The UV spectra of several constituents (**D**, Table S1) were similar to those of gallic acid derivatives (λ_{max} = 280–290 nm), however, their mass spectra did not display the characteristic fragment ions at *m/z* 169 and 125. Although cyclic diarylheptanoids, also exhibiting intense UV absorption in this range, have not yet been detected in *C. betulus*, we hypothesized their presence due to their occurrence in other *Carpinus* species [7]. Compounds **106** and **149** were presumed as carpinontriols A and B, respectively, since their mass spectra showed a fragmentation pattern similar to that previously described for hazelnut diarylheptanoids [20]. The base peak at *m/z* 269 was ascribed to a rearrangement of the deprotonated compound and the subsequent opening of the diarylheptanoid cycle, resulting in the neutral loss of a hydroxy-propan-2-one unit. However, the formation of further typical fragment ions has not been reported in the literature. According to our ESI-MS/MS experiments, the presence of the fragment ion at *m/z* 211 seems to be universal among cyclic diarylheptanoids with a *meta*,*meta*-cyclophane structure. Analogously to the above mentioned, after a rearrangement of the pseudomolecular ion and the subsequent cleavages of two C-C bonds (C7-C8 and C12-C13), a neutral loss of a diversely hydroxylated oxopentanal (**106**, **149**, **161**, **157**), and pentenal (**114**, **187**), or oxopentanedial (**154**) molecule occurs which results in the formation of the fragment ion at *m/z* 211. Similarly, the cleavages of two C-C bonds (C7-C8 and C9-C10) lead to the neutral loss of an ethenol or ethene-diol unit. This results in the formation of the additional characteristic fragment ions at *m/z* 299 (**114**), 283 (**106**, **149**) or 267 (**157**, **187**). Our NMR results later confirmed the presumed structures of the isolated cyclic diarylheptanoids (see Section 3.2.). Their structures and proposed mass spectrometric fragmentation pathways are shown in Figs. 1 and 2, respectively. In parallel to the isolated diarylheptanoids, compounds **94**, **103**, **108**, **119**, **124**, **136**, **140**, **143**, **171**, and **173** also exhibited typical fragment ions at *m/z* 269 and 211, thus we assumed their structures as cyclic diarylheptanoids, too.

Furthermore, **74**, **139**, **145**, and **164** were characterized as linear diarylheptanoids, previously unprecedented in *Carpinus* species. The deprotonated molecular ion $[M-H]^-$ of **164** was detected at *m/z* 313 and its typical fragment ions at *m/z* 207, 163, 149 (Fig. 3), thus the

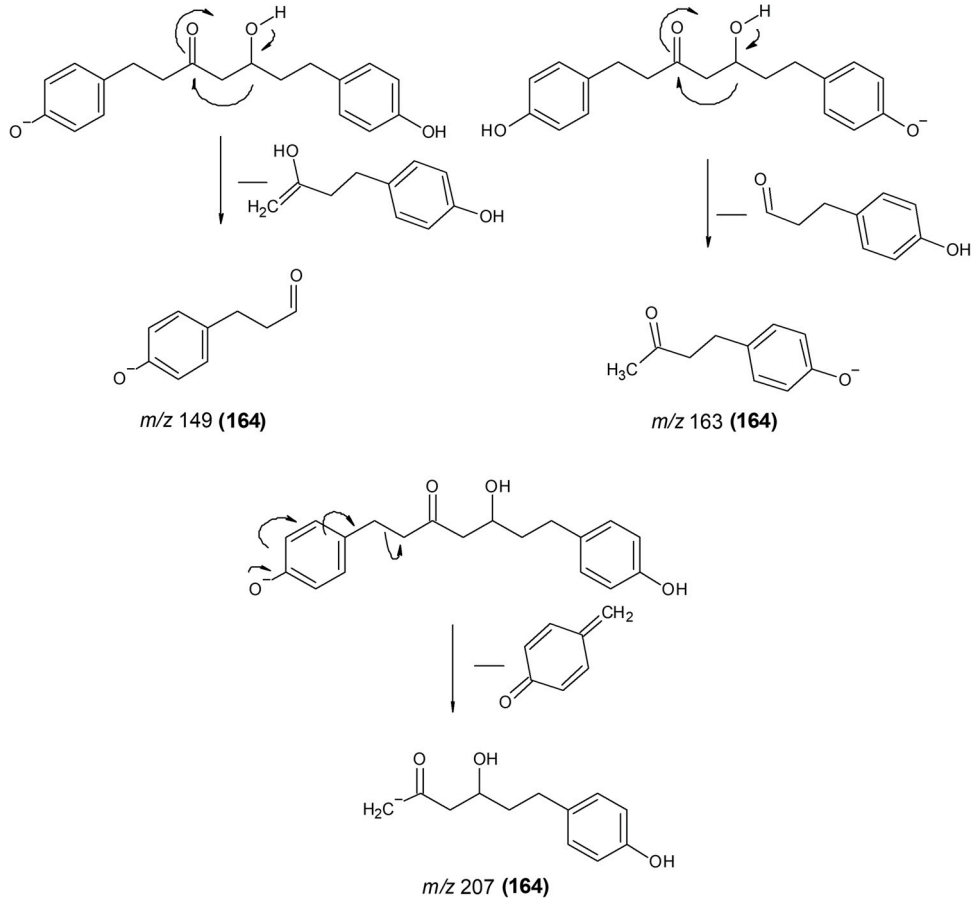


Fig. 3. Proposed mass spectrometric fragmentation pathway of the linear diarylheptanoid 5-hydroxy-3-platyphyllone isolated from *C. betulus* bark.

component was indicated as 5-hydroxy-1,7-bis-(4′-hydroxyphenyl)−3-heptanone (5-hydroxy-3-platyphyllone) [23]. Compound **145** presented a neutral loss of 150 Da, while peaks **74** and **139** showed a neutral loss of 180 Da, indicating the cleavage of a pentose and a hexose moiety from the hydroxyl group on the linear C_7 chain, respectively [23]. Based on these data, **145** was tentatively characterized as oregonin, while compounds **74** and **139** were denoted as linear diarylheptanoid hexosides.

Finally, the UV spectrum of **148** was similar to those of diarylheptanoids or gallic acid derivatives, however, their characteristic fragment ions at *m/z* 211 or 169 were not presented in the mass spectrum of **148**. According to the neutral losses observed during the CID of **148**, the presence of a deoxyhexose moiety [M-H-146]$^-$, a hydroxyl group connected to a saturated chain [M-H-146–18]$^-$, and two methoxy groups [M-H-146–18–15–15]$^-$ could be deduced. However, further conclusions could not be drawn, therefore, NMR analysis was necessary to determine the structure of **148** (see Section 3.2.).

3.2. Structural elucidation of the isolated compounds

In order to unambiguously identify their structures, eight diarylheptanoids (**106**, **114**, **149**, **154**, **157**, **161**, **164**, **187**), one lignan (**148**), and three flavonoids (**177**, **185**, **191**) were isolated by C_{18} flash chromatography followed by multiple successive C_{18} semi-preparative HPLC separations. Their structures were established by 1D and 2D NMR experiments as well as HR-ESI-MS (Orbitrap) analyses. Fig. 1 presents the structures of the isolated constituents, Table 1 summarizes the high-resolution mass spectrometric data of the diarylheptanoid-type compounds, while their ^{1}H NMR and ^{13}C NMR data are shown in Tables S2 and S3.

The ^{1}H and ^{13}C NMR spectra of **106** and **149** were similar to each other indicating isomeric structures of cyclic diarylheptanoids. Both structures contained one carbonyl, three oxymethine, and three methylene groups in the aliphatic chain. Based on the correlations of the 2D spectra, both **106** and **149** possess the carbonyl group in C-9 position, while the three hydroxyl groups were located at positions C-8, C-10, C-12 or C-10, C-11, C-12, respectively. Based on literature data [7], **106** and **149** were identified as carpinontriols A and B, respectively.

In the case of compound **114**, the ^{1}H NMR resonances confirmed the macrocyclic diaryl structure. However, the resonance assignment of the aliphatic chain failed in CD_3OD at 295 K, due to the minute amount of the isolated compound. Compared to the literature [24], all the detected ^{1}H and ^{13}C resonances were in good agreement with that of giffonin U.

The ^{1}H NMR spectrum of compound **157** indicated the presence of two 1,2,4-trisubstituted aromatic rings. The resonances at δ 4.47 (dd, J=11.4, 4.0 Hz, 1H, H-12) and δ 4.20 (m, 1-H, H-11) ppm revealed the presence of two oxymethine groups. In addition, eight more aliphatic resonances recommended the presence of four methylene units. The ^{13}C NMR spectrum showed one carbonyl resonance at δ 212.0 ppm. The characteristic multiplicities and splitting patterns suggested the cyclic diarylheptanoid structure. The correlations of the 2D spectra revealed that the carbonyl group was in C-9 position and the hydroxyl groups were in C-11 and C-12 positions. Based on these data, the structure of **157** was established as 11-oxo-3,8,9,17-tetrahydroxy-[7,0]-metacyclophane (giffonin X) [25].

The aromatic resonances in the ^{1}H NMR spectrum of **161** indicated a macrocyclic diaryl structure, while the resonances in the aliphatic region suggested the presence of four methylene and two oxymethine groups (at 4.39 and 4.04 ppm, respectively) in the

Table 1
HR-MS data of the isolated diarylheptanoid compounds [a].

No.	$[M-H]^-$ (*m/z*) experimental	$[M-H]^-$ (*m/z*) calculated	Error (ppm)	Fragment ions (*m/z*)	Molecular formula	Structure
106	343.1184	343.1187	2.23	283, 269, 257, 239, 225, 211, 197, 193, 183	$C_{19}H_{20}O_6$	carpinontriol A
114	359.1134	359.1136	2.42	329, 299, 269, 257, 239, 211, 197, 193	$C_{19}H_{20}O_7$	giffonin U
149	343.1186	343.1187	2.84	284, 283, 270, 269, 239, 211. 197, 193	$C_{19}H_{20}O_6$	carpinontriol B
154	325.1079	325.1081	2.46	307, 269, 239, 211, 209, 197, 193, 183, 113	$C_{19}H_{18}O_5$	3,12,17-trihydroxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18), 2(19),3,5,14,16-hexaene-8,11-dione
157	327.1238	327.1238	3.48	300, 269, 268, 267, 241, 239, 211, 197, 193, 183	$C_{19}H_{20}O_5$	giffonin X
161	327.1235	327.1238	- 0.9	283, 269, 267, 253, 239, 225, 211, 197, 193	$C_{19}H_{20}O_5$	casuarinondiol
164	313.1437	313.3682	0.78	254, 251, 241, 239, 227, 211, 210, 207, 189, 163, 149	$C_{19}H_{22}O_4$	5-hydroxy-1,7-bis-(4′-hydroxyphenyl)- 3-heptanone
187	311.1287	311.1289	3.00	286, 267, 253, 241, 211, 197	$C_{19}H_{20}O_4$	3,11,17-trihydroxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaen-8-one

[a] For NMR spectral data and structural elucidation of compounds see Section 3.2. Operating conditions for Orbitrap MS can be found in Section 2.5.

heptane chain. Furthermore, the ^{13}C spectrum indicated the presence of a carbonyl group (δ 220.1 ppm). Based on additional 2D correlations, the hydroxyl groups are located at the C-8 and C-12 positions, while the carbonyl group is located at the C-9 position. This structure was previously published in the literature as casuarinondiol [7].

The ^1H spectrum of compound **154** in DMSO-d_6 at 295 K showed very broad unresolved resonances, without any coupling patterns, therefore no structural information could be deduced. After the addition of trifluoroacetic acid and recording the spectra at higher temperatures (at 335 K and 370 K), the ^1H spectrum showed the characteristic pattern of cyclic diarylheptanoid resonances in the aromatic region. However, the aliphatic resonances could not be assigned due to significantly broad resonances. Nevertheless, comparing the NMR data with those found in the literature [26], the 3,12,17-trihydroxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione structure was proposed for compound **154**.

The ^1H NMR spectrum of **187** showed aromatic resonances at δ 7.05 (dd, $^3J_{H,H}$=8.3 Hz, $^4J_{H,H}$ =2.5 Hz, 1H, H-5), 7.04 (dd, $^3J_{H,H}$ =8.3 Hz, $^4J_{H,H}$ =2.5 Hz, 1H, H-15), 6.80 (d, $^3J_{H,H}$ =8.3 Hz, 1H, H-4), 6.78 (d, $^3J_{H,H}$ =8.3 Hz, 1H, H-16), 6.79 (d, $^4J_{H,H}$ =2.5 Hz, 1H, H-18) and 6.60 (d, $^4J_{H,H}$ =2.5 Hz, 1H, H-19) ppm. These two separated ABX coupling patterns (also confirmed by 2D COSY experiment) indicated the presence of two 1,2,4-trisubstituted aromatic rings. The ^1H resonance at δ 4.20 (m, 1H, H-11) ppm and its HSQC correlation to ^{13}C resonance at δ 67.4 ppm revealed the presence of an oxymethine group. In addition, the aliphatic resonances at 3.19 (m, 1H, H-9a), 3.02 (dd, $^2J_{H,H}$ =13.2 Hz, $^3J_{H,H}$ =3.6 Hz, 1H, H-10a), 2.99 (m, 2H, H-7), 2.90 (m, 1H, H-9b), 2.88 (m, 2H, H-13), 2.68 (m, 1H, H-10b), 2.46 (m, 1H, H-12a), and 1.80 (m, 1H, H-12b) ppm along with their HSQC correlations recommended the presence of five methylene units. Four of these -CH_2- units constitute a spin system with that of the oxymethine resonance. The ^{13}C NMR spectrum revealed a carbonyl resonance at δ 212.0 ppm, which separates the additional methylene unit from that of the aforementioned spin system confirming a heptane skeleton. Thorough inspection of the HMBC crosspeaks revealed that the carbonyl group is located at the C-8 position while the hydroxyl can be placed at position C-11. Further HMBC correlations between the aromatic rings confirmed the cyclic diarylheptane skeleton, therefore compound **187** could be assigned as 3,11,17-trihydroxytricyclo [12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaen-8-one, a newly isolated and identified diarylheptanoid.

The ^1H NMR spectrum of compound **164** indicated the presence of two *para*-substituted aromatic rings. The resonance at δ 4.00 (m, 1H, H-5) ppm suggested the presence of one oxymethine group. Furthermore, five methylene units were identified. The ^{13}C NMR spectrum showed a single carbonyl resonance at δ 211.9 ppm. Based on all these informations, a linear diarylheptanoid structure was proposed. The 2D spectra determined the position of the carbonyl group at C-3 and the hydroxyl group at C-5. The ^1H and ^{13}C resonances were analogous to literature data [27], thus, **164** was identified as 5-hydroxy-1,7-bis-(4′-hydroxyphenyl)−3-heptanone (5-hydroxy-3-platyphyllone).

Based on the ^1H, ^{13}C, and additional 2D spectra, compound **148** was identified as a lignan glycoside, aviculin. The NMR spectra was identical to that of a previous report [28]. Presence of lignan-type compounds in *Carpinus* species was established for the first time. The compound **185** was confirmed as kaempferol-3-*O*-(4"-*E*-*p*-coumaroyl)-rhamnopyranoside by comparing the NMR spectroscopic data (^1H and ^{13}C resonances) with those found in the literature [29]. The coupling constant of the two olefinic ^1H resonances suggested *trans* configuration of the double bond. Based on their ^1H NMR spectra, compounds **191** and **177** were identified as kaempferol-3-*O*-rhamnoside (afzelin) and quercetin-3-*O*-rhamnoside (quercitrin), respectively. The ^1H resonances were similar to those published earlier [30].

Table 2
Quantitative determination of the main diarylheptanoids in *Carpinus betulus* extracts (data are expressed as mg/g dry extract).

Compound	Quantity ± SD (mg/g dry extract)			
	BE	**BM**	**ME**	**MM**
Carpinontriol A (**106**)	19.13 ± 0.10	13.94 ± 0.26	n.d.	3.55 ± 0.05
Carpinontriol B (**149**)	6.44 ± 0.18	4.16 ± 0.15	7.60 ± 0.12	16.25 ± 0.19
3,12,17-Trihydroxytricyclo [12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione (**154**)	16.04 ± 0.12	11.05 ± 0.02	n.d.	n.d.
Giffonin X (**157**)	18.07 ± 0.03	9.97 ± 0.10	n.d.	n.d.

Abbreviations: BE: bark ethyl acetate extract, BM: bark methanol extract, ME: male catkin ethyl acetate extract, MM: male catkin methanol extract, n.d.: not detected.

Table 3
Method validation: regression, LOQ and LOD of the quantitative method.

Compound	Regression equation	r^2	Regression range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Carpinontriol A (**106**)	y = 88.99x + 177.77	0.9997	1–250	0.15	0.5
Carpinontriol B (**149**)	y = 95.80x + 228.73	0.9995	1–250	0.1	0.3
3,12,17-Trihydroxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione (**154**)	y = 43.17x - 16.79	0.9999	1–250	0.2	0.6
Giffonin X (**157**)	y = 86.71x + 177.24	0.9996	1–250	0.15	0.5

3.3. Quantitative analysis and method validation

There are currently no literature data regarding the quantitative analysis of diarylheptanoids in *C. betulus*. Thus, additional aim of this study was to determine the quantities of the major diarylheptanoid constituents in hornbeam extracts: carpinontriols A (**106**) and B (**149**), 3,12,17-trihydroxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione (**154**), and giffonin X (**157**) by an UHPLC-DAD method.

Ethyl acetate and methanol extracts of all samples (bark, leaf, female, and male catkins) were analyzed. In accordance with the results of the qualitative screening, the evaluated diarylheptanoids were not detected in leaf and female flower extracts. Quantities of the studied compounds in hornbeam bark and male catkin methanol and ethyl acetate extracts ranged from 3.55 to 19.13 mg/g dry extract, results are shown in Table 2. Compound **149** was present in all bark and male catkin extracts, being the most abundant diarylheptanoid of male catkin samples. Compound **106** was the chief diarylheptanoid in both bark extracts, while in bark ethyl acetate samples, **157** was present in the second highest concentration.

The linearity regression equations, correlation coefficients (r^2), linearity ranges, LOD and LOQ values of the method are shown in Table 3. Excellent linearity was achieved ($r^2 \geq 0.9995$) in the range of 1–250 μg/mL for all analytes. The LOD and LOQ values were within the ranges of 0.1–0.2 μg/mL and 0.3–0.6 μg/mL, respectively. Intra-day and inter-day precision evaluated at low, mid, and high concentration ranges was also acceptable (0.16–3.33 RSD%), while intra- and inter-day accuracy results varied from 80.31% to 107.06% (Table 4). The extraction recovery rate of **157** was 96.29% ± 1.36% for the ethyl acetate extract, and 114.91% ± 2.19% in case of the methanol extract. These results indicate that the method was reliable and repeatable. Retention time repeatability was suitable for all four compounds, relative standard deviation ranged from 0.18% to 0.58% (n = 6). In order to evaluate the selectivity of the method, blank samples (hexane extracts which do not contain the analytes of interest) were compared to extracts spiked with **106**, **149**, **154**, and **157**. No co-elution was observed at the retention times of the analytes of interest, indicating that this method provides good selectivity.

3.4. DPPH scavenging activity

Antioxidant capacities of hornbeam bark, leaf, male, and female catkin extracts prepared with methanol and ethyl acetate were compared. Table 5 summarizes the results of the DPPH scavenging assay, data are expressed as means ± SD. Antioxidant activities of hornbeam extracts prepared with methanol were significantly different ($p < 0.001$) as compared with those of the ethyl acetate extracts (results are shown in Fig. 4), however, a trend in relation to the extraction solvent could not be found. Overall, the leaf methanol extract showed the best antioxidant capacity, while male catkin methanol extract was also effective in the test. Both samples exhibited radical scavenging activities similar to those of the well-known antioxidant compounds quercetin and trolox. Our results correspond with literature data, where *C. betulus* leaf and bark extracts showed medium to high DPPH neutralizing activity [3,4].

We also investigated the antioxidant activities of the constituents isolated from *C. betulus* samples. For comparison, reference compounds with known radical scavenging activity were also studied, results are shown in Table 5. In accordance with literature data, the potent antioxidant activity of quercitrin (**177**) was comparable to other quercetin glycosides, like rutin. On the other hand, afzelin (**191**), carpinontriols A (**106**) and B (**149**), casuarinondiol (**161**), and 5-hydroxy-3-platyphyllone (**164**) showed weak radical scavenging activity [7,31,32]. According to literature data, coumaroyl flavonol glycosides show potent free radical scavenging activity [33]. However, kaempferol-3-*O*-(4″-*E*-*p*-coumaroyl)-rhamnoside (**185**) exhibited no radical scavenging activity at the concentration of 250 μg/mL. Although some of its structural characteristics such as the lack of unsubstituted OH groups (due to the absence of the catechol group

Table 4
Method validation: Precision and accuracy of the quantitative method.

Nominal conc (μg/mL)	Precision (RSD%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
Carpinontriol A (106)				
5	0.53	0.75	80.95	81.69
50	1.81	1.48	105.22	106.54
250	0.16	0.24	99.62	99.63
Carpinontriol B (149)				
5	0.96	1.65	80.31	81.34
50	0.73	0.88	104.92	105.02
250	0.59	0.80	99.43	99.61
3,12,17-Trihydroxytricyclo [12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione (154)				
5	0.76	2.38	107.68	107.38
50	1.37	2.06	97.27	98.43
250	0.43	3.33	100.14	103.74
Giffonin X (157)				
5	2.84	1.69	81.10	81.81
50	0.72	1.81	105.54	107.06
250	1.56	1.44	99.48	100.29

Table 5
DPPH scavenging activity of *C. betulus* extracts, constituents isolated from the bark, and reference compounds (Data are expressed as means ± SD).

Extracts	IC_{50} ± SD (μg/mL)
Bark ethyl acetate extract (**BE**)	9.0 ± 0.3
Bark methanol extract (**BM**)	10.7 ± 0.3
Leaf ethyl acetate extract (**LE**)	14.0 ± 0.4
Leaf methanol extract (**LM**)	5.5 ± 0.2
Female catkin ethyl acetate extract (**FE**)	9.4 ± 0.2
Female catkin methanol extract (**FM**)	11.9 ± 0.7
Male catkin ethyl acetate extract (**ME**)	13.3 ± 0.5
Male catkin methanol extract (**MM**)	7.6 ± 0.3
Isolated constituents	
Carpinontriol A (**106**)	77.2 ± 4.5
Carpinontriol B (**149**)	123 ± 10
Giffonin X (**157**)	138 ± 11
Casuarinondiol (**161**)	> 250
5-Hydroxy-3-platyphyllone (**164**)	121 ± 9
Aviculin (**148**)	23.8 ± 0.9
Quercitrin (**177**)	6.9 ± 0.5
Afzelin (**191**)	> 250
Kaempferol-3-*O*-(4″-*E*-*p*-coumaroyl) rhamnopyranoside (**185**)	> 250
Reference compounds	
Trolox	5.3 ± 0.2
Rutin	7.3 ± 0.3

in the B ring and the glycosylation at C3-OH) may result in a lower scavenging capacity, these can not explain the contradiction with the literature. To the best of our knowledge, the DPPH scavenging activity of aviculin (**148**, IC_{50} 23.8 ± 0.9 μg/mL) and giffonin X (**157**, IC_{50} 138 ± 11 μg/mL) was determined for the first time.

In order to assess the contribution of the individual antioxidant constituents to the total antioxidant activity of *C. betulus* extracts, an off-line DPPH-HPLC-DAD-MS method was applied. Upon reaction with DPPH, phenolics which can neutralize DPPH$^{\bullet}$ by providing hydrogen atoms or by electron donation, will be oxidized to form free radicals, and subsequently stable quinoidal structures. As a consequence of this structural change, peak areas (peak intensities) of these antioxidants will decrease in the HPLC chromatogram [10]. Chromatograms of hornbeam samples were compared before and after reacting with DPPH. The antioxidant effect was characterized by the decrease of the intensity (area under the curve, AUC) values in percentage. The compounds which reduced the peak intensity by more than 20% were considered as potential antioxidants [10]. Values are means of intensity reductions determined for each extract containing the specific compound. Results are presented in Supplementary Table S4. Representative HPLC-UV chromatograms demonstrating untreated and DPPH-treated bark methanolic samples are shown in Fig. 5. According to the results of our HPLC-MS/MS analyses, the leaf sample was dominated by the presence of gallic acid derivatives and ellagitannins. It was presumed that galloyl hexoses of different polymerization degrees as well as galloyl-HHDP hexose derivatives could contribute significantly to the total antioxidant activity, since they are well known for their strong radical scavenging effect [34]. The increasing number of galloyl moieties in the constituents correlated with higher antioxidant capacities. Monogalloyl hexoses (e.g. **4** and **11**) exerted lower reduction in peak intensities as compared to tri-, tetra-, or pentagalloyl hexose isomers (e.g. **85**, **105**, and **138**, respectively). On the other hand, digalloylshikimic acid isomers (**87** and **99**), and digalloylquinic acid (**58**) showed lower reduction in AUC values as compared to their monogalloyl counterparts (e.g. **34** and **16**, respectively). In case of ellagitannins, the galloyl:HHDP rate of the compounds determines the antioxidant capacity. In accordance with literature data [35], galloyl-bis-HHDP hexose isomers (e.g. **40**, **50**, **81**) did not show antioxidant activity as compared to galloyl-HHDP hexoses (e.g. **38**, **43**, **51**, **84**). Flavonol glycosides, and in particular quercetin derivatives, prevailed in *C. betulus* extracts. The aglycone quercetin (**192**) bears all structural criteria of a potent antioxidative compound [34]. However, in case of other flavonol derivatives, the glycosidation of the C3-OH group (e.g. **163** and **177**), the methylation of free hydroxyl groups (e.g. **179** and **188**), or the lack of a catechol moiety in B ring (e.g. **160** and **191**) resulted in lower free radical scavenging activities. Hydroxycinnamic acid derivatives bearing two hydroxyl groups in the *ortho* position (caffeic acid derivatives, **65** and **83**) showed higher radical scavenging ability than those containing only one hydroxyl group (coumaric acid derivatives, **111**). Methylation of hydroxyl groups in ferulic acid derivatives (**116** and **132**) also leads to the reduction in the radical scavenging activity [34]. In agreement with literature data [7] and our results from the radical scavenging assay of the isolated compounds, diarylheptanoids in the *C. betulus* extracts (**114**, **143**, **149**) exhibited moderate antioxidant effect.

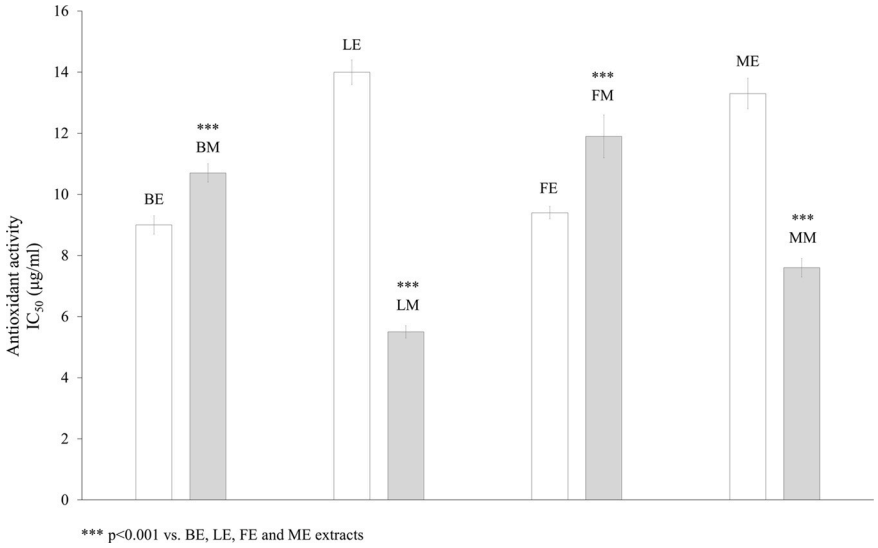


Fig. 4. DPPH scavenging activity of *C. betulus* extracts prepared with solvents of different polarity. Values are means ± SD. *** $p < 0.001$ compared with ethyl acetate extracts. Abbreviations: BE: bark ethyl acetate extract, BM: bark methanol extract, LE: leaf ethyl acetate extract, LM: leaf methanol extract, FE: female catkin ethyl acetate extract, FM: female catkin methanol extract, ME: male catkin ethyl acetate extract, MM: male catkin methanol extract.

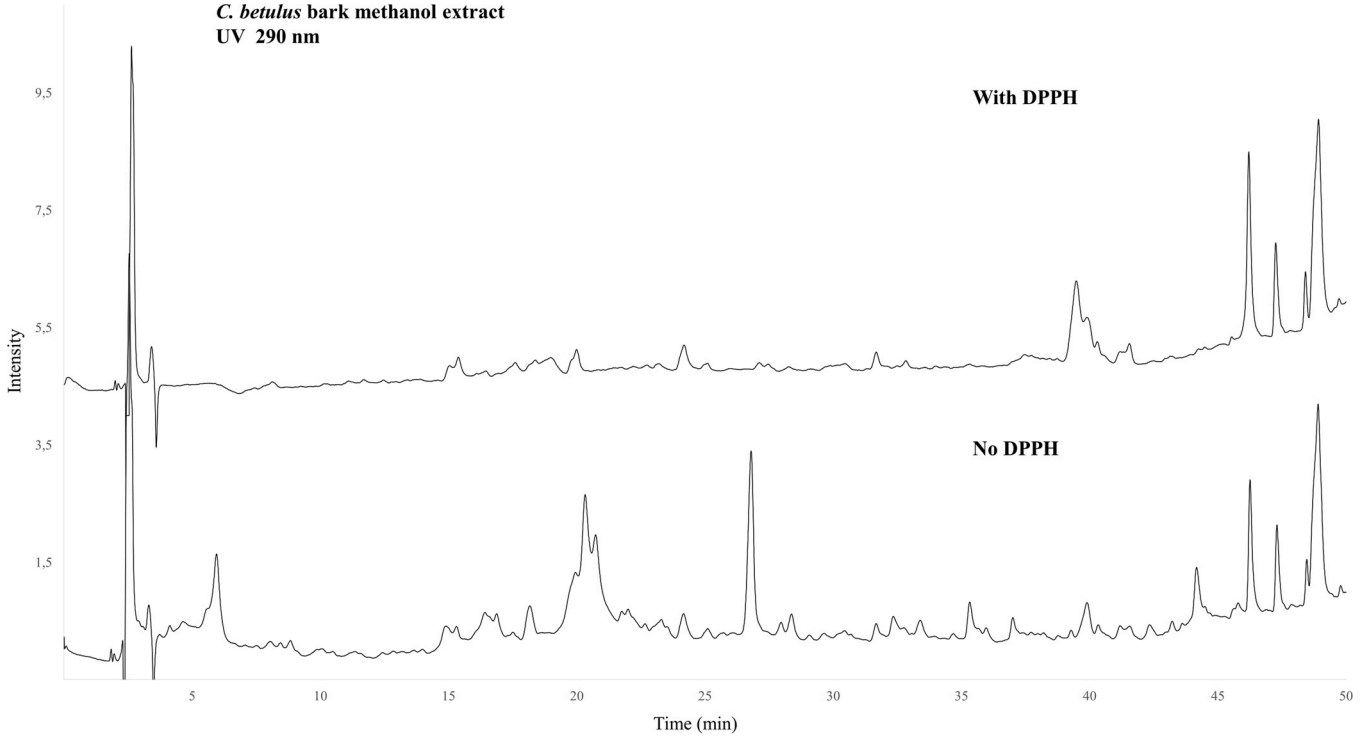


Fig. 5. Chromatograms of untreated and DPPH-treated *C. betulus* bark methanolic extract samples.

4. Conclusions

In the present study, a comprehensive profiling of phenolic compounds in *C. betulus* was performed. Distinct plant parts (bark, leaf, female, and male catkin samples) were extracted successively with solvents of increasing polarity (ethyl acetate and methanol) to obtain as extensive a range of extractives as possible. Altogether 194 polyphenols were tentatively characterized by HPLC-DAD-ESI-MS/MS. Gallo- and ellagitannins dominated in the methanol extracts, while flavonol glycosides and methoxylated flavones prevailed in the ethyl acetate samples. Seven cyclic diarylheptanoids (**106**, **114**, **149**, **154**, **157**, **161**, **187**) were isolated from *C. betulus* for the first time, with 3,11,17-trihydroxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaen-8-one (**187**) being a new compound. We also described the occurrence of linear diarylheptanoid (**164**) and lignan (**148**) constituents in the genus *Carpinus* for the first time. Three known flavonol glycosides (**177**, **185**, **191**) were also isolated. A new mass spectrometric fragmentation pathway of cyclic diarylheptanoids with a *meta,meta*-cyclophane structure was proposed. Additionally, this is the first report of quantitative data regarding the main diarylheptanoids in *C. betulus* extracts. A selective, reliable, and repeatable UHPLC-DAD method was developed and validated to determine the contents of **106**, **149**, **154**, and **157**. Compound **106** prevailed both in bark ethyl acetate and methanol extracts, while **149** was the main diarylheptanoid of male catkin extracts. The antioxidant properties of the extracts and the isolated compounds were assessed by the DPPH assay. Leaf and male catkin methanol extracts showed the highest antioxidant activity. The DPPH scavenging activity of aviculin (**148**) and giffonin X (**157**) was determined for the first time. Potential antioxidant compounds in *C. betulus* extracts contributing to the total radical scavenging activity of the samples were indicated using an off-line DPPH-HPLC method. According to our results, hydrolyzable tannins may be responsible for the antioxidant capacity of *Carpinus* extracts.

CRediT authorship contribution statement

Csenge Anna Felegyi-Tóth: Investigation, Data curation, Writing – original draft, Visualization. **Zsófia Garádi**: Investigation, Data curation, Writing – original draft. **András Darcsi**: Investigation. **Orsolya Csernák**: Writing – original draft, Validation. **Imre Boldizsár**: Investigation, Data curation. **Szabolcs Béni**: Writing – review & editing. **Ágnes Alberti**: Conceptualization**,** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2021.114554.

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