New sources of naturally occurring cytotoxic diarylheptanoids: Phytochemical characterization and evaluation of bioactivity

(NKFIH Project K 120342)

Final Professional Report

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Diarylheptanoids represent phenolic plant specialized (secondary) metabolites of great interest because of their anti-inflammatory and anti-tumor activities shown in various *in vitro* and *in vivo assays.* In this research, we aimed to reveal several plant species native to Hungary as potential new sources of bioactive diarylheptanoids. Therefore, systematic (qualitative and quantitative) phytochemical screening of species belonging to genera known for their diarylheptanoid constituents (*Alnus*, *Corylus*, *Carpinus*, *Juglans*, *Acer*) was proposed. Analysis of plants not related to the Betulaceae (e.g. species in the genera *Morus*, *Ficus*, *Celtis*, etc.) was also intended. Further specific aim was the comprehensive characterization of the isolated compounds by assessing their cytotoxic activities, pharmacokinetic properties, and chemical stability as well as the formulation of cyclodextrin inclusion complexes.

1. *Alnus* species (Betulaceae)

1.1. Phytochemical analyses of *Alnus* species

Diarylheptanoid compositions and contents of different parts (root, bark, leaf, involucre, catkin, fruit) of numerous *Alnus glutinosa*, *Alnus cordata*, *Alnus incana*, and *Alnus* × *spaethii* samples have been investigated using reversed-phase high-performance liquid chromatography coupled to tandem mass spectrometry (RP-HPLC-ESI-MS/MS). Samples were extracted initially by Soxhlet extraction, while later – based on our results – by ultrasound-assisted extraction, using solvents of increasing polarity (hexane, chloroform, ethyl acetate, methanol) successively. (See results of extraction optimization in section 1.3.)

We have described the presence of two cyclic diarylheptanoids (carpinontriol isomers) and that of a linear diarylheptanoid glycoside ester [1,7-bis-(3,4-dihydroxyphenyl)-5-*O*-feruloylpentosyl-heptan-3-one] in *A. glutinosa* for the first time. Additionally, we evaluated the

contribution of each compound to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of the extracts by an off-line DPPH-LC-MS method for the first time. While we only detected constituents already reported in the literature for *A. incana*, we described one biaryl-type cyclic diarylheptanoid and 21 linear diarylheptanoids in *A. cordata* for the first time, with hirsutanonol-*O*-hexoside being the main constituent. We detected linear diarylheptanoids in *Alnus* × *spaethii* for the first time: hirsutanonol-*O*-hexoside as the main compound, oregonin, rubranosides A and B, centrolobol-*O*-hexoside, alnuside, and hirsutenone.

In order to complete the phytochemical characterization of ca. 100 *Alnus* spp. extracts, we developed and validated a fast, selective, and reproducible ultra-high-performance liquid chromatography–diode-array detection (UHPLC-DAD) method for the quantitative determination of six linear diarylheptanoids: oregonin, rubranosides A and B, 1,7-bis-(3,4-dihydroxyphenyl)-5-*O*-feruloylpentosyl-heptan-3-one, hirsutanonol, hirsutenone. The analytes used as external standards have been isolated from *A. glutinosa* bark (see section 1.2.). Method validation data (regression, LOQ and LOD, precision, and accuracy) of the quantitative method are presented in Table 1.

Table 1. Method validation data of the method used for the quantitation of linear diarylheptanoids in *Alnus* spp. samples

Constituent	**Regression range (µg/ml)**	**LOD (µg/ml)**	**LOQ (µg/ml)**	**Precision (RSD%)**		**Accuracy (%)**	
		(S/N=3)	**(S/N=10)**	**Intra-day**	**Inter-day**	**Intra-day**	**Inter-day**
oregonin	0.25-250 r^2 = 0.9998	~ 0.03	~ 0.25	1.73-4.94	1.69-3.84	97.4-114.6	101.5-113.8
hirsutanolol	0.25-250 r^2 = 0.9997	~ 0.1	~ 0.25	1.10-3.34	2.57-3.69	85.3-103.3	84.5-103.1
rubranoside A	0.25-250 r^2 = 0.9999	~ 0.1	~ 0.25	0.11-0.80	5.55-10.15	100.1-107.1	83.1-105.4
rubranoside B	0.25-250 r^2 = 0.9999	~ 0.06	~ 0.25	0.39-4.22	2.09-3.03	98.6-103.4	96.1-160.2
1,7-bis-(3,4-dihydroxyphenyl)-5-*O*-feruloylpentosyl-heptan-3-one	0.2-200 r^2 = 0.9994	~ 0.1	~ 0.2	0.11-2.62	3.31-6.00	97.3-106.7	94.6-106.8
hirsutenone	0.25-250 r^2 = 0.9999	~ 0.02	~ 0.2	0.33-1.67	2.51-5.10	98.3-114.7	94.4-114.1

Results are partly unpublished, while some of them were presented as oral/poster presentations, and in diploma works:

- Alberti Á. et al. 7th BBBB International Conference on Pharmaceutical Sciences, Balatonfüred (Hungary), 5-7 October 2017
- Malcsiner P. et al. *Alnus glutinosa* fenoloid-összetételének és antioxidáns hatásának vizsgálata HPLC-DAD-ESI-MS/MS módszerrel. XLIII. Gyógyszeranalitikai Továbbképző Kollokvium, Bükfürdő (Hungary), 19-21 April 2018
- Diploma work of Eloïse Janine Silke von Broen (Semmelweis University): HPLC-MS Untersuchungen von Diarylheptanoiden in Schwarzerlen, 2019
- Diploma work of Dorottya Darabos (Semmelweis University): Kromatográfiás módszerek fejlesztése *Alnus cordata* diarilheptanoidok analízisére, 2020

1.2. Isolation of *Alnus* diarylheptanoids

The major diarylheptanoids and additional minor compounds from the ethyl acetate extracts of *A. glutinosa* bark (300 g) and leaf (100 g) have been isolated. The combined flash chromatography (silica gel and C_{18} stationary phases), preparative and semi-preparative HPLC (C_{18} stationary phase) methods yielded approximately 20-200 mg of each linear constituent as well as several grams of oregonin, and less than 1 mg of the cyclic compound. The purified analytes (shown in Fig. 1 and Table 2) have been identified by nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HR-MS).

Table 2. Diarylheptanoid constituents isolated from *A. glutinosa* bark and leaf ethyl acetate extracts

Compound	MW (g/mol)	Plant source	Isolated quantity (mg)
oregonin	478.5	bark	5080
hirsutanolol	346.4	bark	18.0
rubranoside A	494.5	bark	159
rubranoside B	464.5	bark	180
1,7-bis-(3,4-dihydroxyphenyl)-5-*O*-feruloylpentosyl-heptan-3-one	654.7	bark	30.0
hirsutenone	328.4	bark	145
betulatetraol	346.4	leaf	0.5

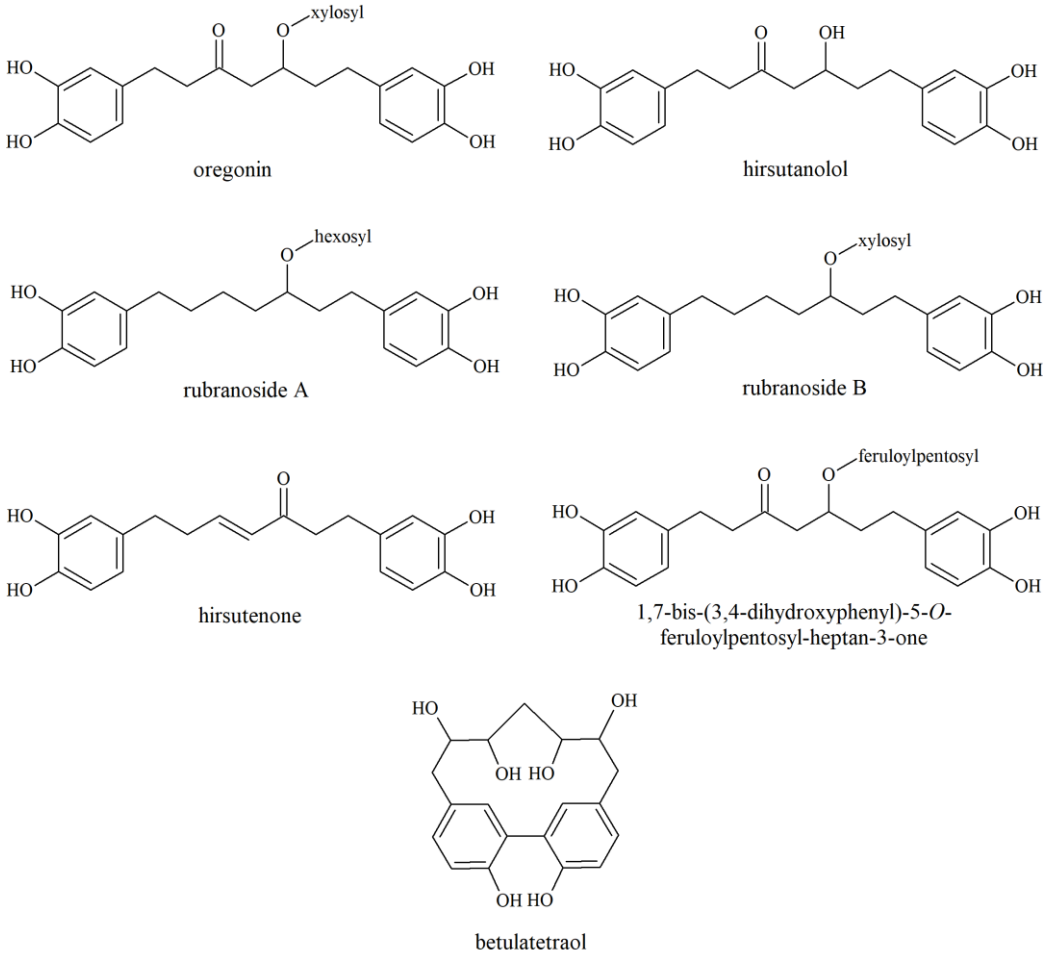


Figure 1. Diarylheptanoid constituents isolated from *A. glutinosa* bark and leaf ethyl acetate extracts

1.3. Extraction optimization

In order to achieve higher yields during the isolation of diarylheptanoids, we optimized the extraction procedure of diarylheptanoids as well as the major constituents oregonin and hirsutenone from *A. glutinosa* samples. The methods (Soxhlet extraction, ultrasound-assisted extraction, maceration) and the solvents (methanol, ethyl acetate) have been compared regarding total extractive and oregonin/hirsutenone contents. Extraction conditions for the applied methods are presented in Table 3. Quantitative analyses of the extracts were performed using our validated UHPLC-DAD method (details see in section 1.1.), and using a validated UHPLC-Orbitrap method (in collaboration with the Department of Plant Anatomy, Eötvös Loránd University).

According to our results, application of ethyl acetate as extraction solvent is favorable over that of methanol, since in addition to diarylheptanoids, numerous polar phenolic compounds are present in abundance in extracts prepared with methanol. As regards of the extraction efficiency

and oregonin content, similar results have been achieved by all methods: quantities of extractives were ca. 18% (m/m), while oregonin contents of the extracts ranged between 15-18 mg for all extraction methods. However, ultrasound-assisted extraction with extraction duration of 6 hours was by all means the fastest procedure as compared to Soxhlet extraction (3 × 6 hours) and maceration (6 days).

Table 3. Conditions of the extraction methods applied for the extraction of diarylheptanoids from *A. glutinosa* bark samples

Extraction method	Duration of extraction	Time of sampling	Further specifics
ultrasound-assisted extraction	6 hours	1, 2, 3, 4, 6 hours	change of extraction medium at time of sampling
Soxhlet extraction	3 × 6 hours	at all extraction intervals	supplementation of the extraction medium with the volume removed at sampling
maceration	6 days	1, 2, 3, 6 days	change of extraction medium at time of sampling

Additionally, a supercritical fluid extraction method was also optimized regarding extraction yield and oregonin recovery. We evaluated the influence of pressure (10, 20, 30 MPa), temperature (40, 50, 60 °C), and co-solvent content (10, 15, 20% ethanol), and a factorial experiment using a full 3^3 design was followed.

Some of the results were presented in the following publications and presentations:

- Alberti Á. et al. Characterization of diarylheptanoids: An emerging class of bioactive natural products, J Pharm Biomed Anal, 147: 13-34., 2018. https://doi.org/10.1016/j.jpba.2017.08.051
- Vesztergombi D. et al. Növényanalitikában alkalmazott szilárd-folyadék extrakciós eljárások optimalizálása *Alnus glutinosa* (Betulaceae) etil-acetáttal készült kivonatainak vizsgálata alapján. XLIII. Gyógyszeranalitikai Továbbképző Kollokvium, Bükfürdő (Hungary), 19-21 April 2018

2. *Carpinus betulus* (Betulaceae)

2.1. Phytochemical analyses of *C. betulus*

Comprehensive profiling of polyphenols in *Carpinus betulus* samples (bark, leaf, male, and female catkins) was performed by HPLC-DAD-MS/MS. Utilizing our results from the extraction optimization assays performed with *A. glutinosa* samples (see section 1.3.), the samples were extracted by ultrasound-assisted extraction, using solvents of different polarity (ethyl acetate, methanol). A total of 194 polyphenols (gallo- and ellagitannins, flavonol

glycosides and methoxylated flavones, diarylheptanoids, cinnamic acid derivatives, lignans) were tentatively identified. We reported the presence of diarylheptanoids in *C. betulus* for the first time. Several of these compounds have also been isolated (see section 2.2.).

We evaluated the antioxidant properties of the extracts and the isolated compounds as well as the contribution of the constituents to the total radical scavenging activity of the extracts in the DPPH assay. The DPPH scavenging activity of aviculin and giffonin X was determined for the first time.

In order to complete the phytochemical characterization of *C. betulus* extracts, we developed and validated a selective, reliable, and repeatable UHPLC-DAD method for the quantitative determination of the four major cyclic diarylheptanoids. We have provided quantitative data on carpinontriols A and B, giffonin X, and 3,12,17-trihydroxytricyclo-[12.3.1.$1^{2,6}$]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione in *C. betulus* extracts for the first time. The analytes used as external standards have been isolated from *C. betulus* bark (see section 2.2.).

Publication and presentation of the results:

- Felegyi-Tóth C. A. et al. Isolation and quantification of diarylheptanoids from European hornbeam (*Carpinus betulus* L.) and HPLC-ESI-MS/MS characterization of its antioxidative phenolics. J Pharm Biomed Anal, 210: 114554., 2022. https://doi.org/10.1016/j.jpba.2021.114554
- Tóth C. A. et al. Comprehensive characterisation of phenolic profile and antioxidant activity of *C. betulus*. PhD Scientific Days 2020, Budapest (Hungary), 31 August 31 – 1 September 2020
- Tóth C. A. et al. Characterisation of phenolic profile and antioxidant activity of *Carpinus betulus*. Congressus Pharmaceuticus Hungaricus XVI., Budapest (Hungary), 10-12 September 2020

2.2. Isolation of *C. betulus* diarylheptanoids

An up-scaled isolation procedure developed in the first two years of the project has been used for the isolation of *C. betulus* diarylheptanoids. According to our previous results, extractions were accomplished in ultrasonic bath for higher yields. The constituents have been isolated from the ethyl acetate and methanol extracts of *C. betulus* bark (500 g) samples. Due to the miniscule amount of diarylheptanoids and the abundant presence of other polar phenolics (e.g. galloyl esters, hydrolysable tannins), flash chromatography employing a C_{18} stationary phase and successive semi-preparative HPLC methods using a C_{18} column were combined. The purified analytes have been identified by NMR spectroscopy and HR-MS.

Seven (one new and six known) cyclic diarylheptanoids were isolated from *C. betulus* for the first time. We also described a linear diarylheptanoid and a lignan constituent that are

unprecedented in the genus *Carpinus*. Moreover, three known flavonol glycosides were also isolated, among which the esterified compound kaempferol-3-*O*-a-L-(4”-*E*-*p*-coumaroyl)-rhamnopyranoside has not been described fro *C. betulus* previously. The isolated compounds are listed in Table 4 and shown in Fig. 2.

Table 4. Diarylheptanoids and other constituents isolated from *C. betulus* bark ethyl acetate and methanol extracts

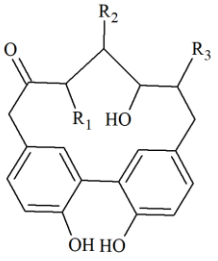
Compound	MW (g/mol)	Extract	Isolated quantity (mg)
carpinontriol A	344.4	ethyl acetate	3.5
carpinontriol B	344.4	methanol	1.7
giffonin U	360.4	ethyl acetate	1.3
giffonin X	328.4	methanol	2.0
3,12,17-trihydroxytricyclo-[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione	326.3	ethyl acetate	1.5
3,11,17-trihydroxytricyclo-[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8-one *	312.4	ethyl acetate	0.5
casuarinondiol	328.4	methanol	0.7
5-hydroxy-1,7-bis-(4'-hydroxyphenyl)-3-heptanone	314.4	ethyl acetate	0.7
aviculin	506.5	methanol	1.2
quercetin-3-*O*-rhamnopyranoside	448.4	ethyl acetate	23.5
kaempferol-3-*O*-rhamnopyranoside	432.4	ethyl acetate	2.2
kaempferol-3-*O*-α-L-(4”-*E*-*p*-coumaroyl)-rhamnopyranoside	578.5	methanol	3.3

Abbreviations: * new compound

Mass spectrometric fragmentation behavior of the isolated diarylheptanoids was studied using an UHPLC-Orbitrap method. Based on the identification of their characteristic fragment ions, a new mass spectrometric fragmentation pathway for *meta*,*meta*-cyclophane-type diarylheptanoids was also proposed.

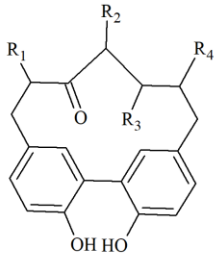
Publication and presentation of the results:

- Felegyi-Tóth C. A. et al. Isolation and quantification of diarylheptanoids from European hornbeam (*Carpinus betulus* L.) and HPLC-ESI-MS/MS characterization of its antioxidative phenolics. J Pharm Biomed Anal, 210: 114554., 2022. https://doi.org/10.1016/j.jpba.2021.114554
- Felegyi-Tóth C. A. et al. Közönséges gyertyán bioaktív vegyületeinek izolálása és szerkezetmeghatározása. 10th Jubilee Interdisciplinary Doctoral Conference, Pécs (Hungary), 12-13 November 2021



giffonin U R_1=R_2=R_3=OH

3,11,17-trihydroxytricyclo[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaen-8-one R_1=R_2=R_3=H



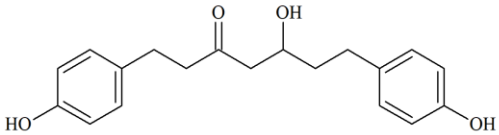
carpinontriol A R_1=R_3=R_4=OH, R_2=H

carpinontriol B R_1=H, R_2=R_3=R_4=OH

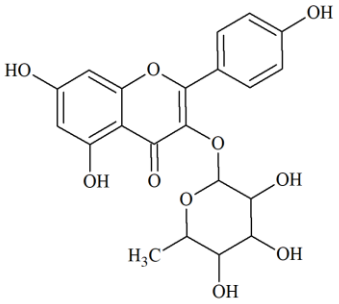
giffonin X R_1=R_2=H, R_3=R_4=OH

casuarinondiol R_1=OH, R_2=R_3=H, R_4=OH

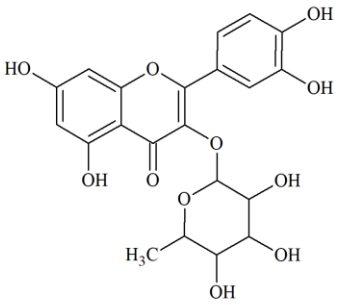
3,12,17-trihydroxytricyclo [12.3.1.1^{2,6}]nonadeca-1(18), 2(19),3,5,14,16-hexaene-8,11-dione R_1=OH, R_2=R_3=H, R_4=O



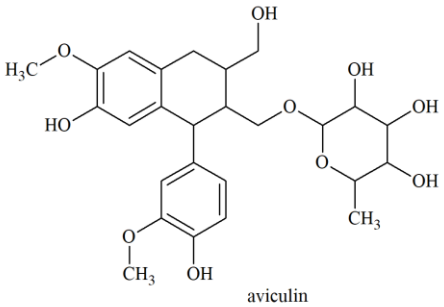
5-hydroxy-1,7-bis-(4'-hydroxyphenyl)-3-heptanone



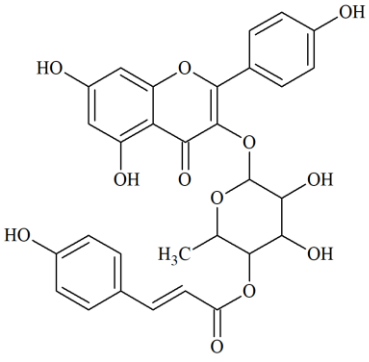
kaempferol-3-*O*-rhamnoside



quercetin-3-*O*-rhamnoside



aviculin



kaempferol-3-*O*-(4″-*E*-*p*-coumaroyl)-rhamnopyranoside

Figure 2. Diarylheptanoids and other constituents isolated from *C. betulus* bark ethyl acetate and methanol extracts

2.3. Chemical stability assay of *C. betulus* diarylheptanoids

The mid- and long-term chemical stability of the *C. betulus* bark methanol and ethyl acetate extracts was studied at different storage temperatures (-15, 5, and 22 °C). Stability studies of the methanol and aqueous solutions of the main cyclic diarylheptanoid constituents, i.e. carpinontriols A and B, giffonin X, and 3,12,17-trihydroxytricyclo-[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione were also performed. Additionally, aqueous stability of the compounds was evaluated at three physiologically relevant pH values (1.2, 6.8, and 7.4). The degradation of the four cyclic diarylheptanoids was investigated by the validated quantitative UHPLC-DAD method used for the quantitation of the compounds in *Carpinus* extracts (see section 2.1.). The structures of the degradation products were characterized by UHPLC-Orbitrap MS.

Both carpinontriol B and 3,12,17-trihydroxytricyclo-[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione showed appropriate chemical stability in the mid- and long-term assays irrespective of the storage temperature. On the other hand, only carpinontriol B was stable at all three investigated pH values, while pH-dependent degradation of the other compounds was observed. Degradation pathways for carpinontriol A and giffonin X have been proposed.

Publication and presentation of the results:

- Felegyi-Tóth C. A. et al. Stability, membrane permeability and cytotoxicity studies of cyclic diarylheptanoids from European hornbeam (*Carpinus betulus* L.) (manuscript under review)
- Felegyi-Tóth C. A. et al. Mid- and Long-Term Stability Study of Cyclic Diarylheptanoids from *Carpinus Betulus*. 33rd International Symposium on Chromatography – ISC 2022, Budapest (Hungary), 18-22 September 2022
- Diploma work of Tímea Heilmann (Semmelweis University): Közönséges gyertyán diarilheptanoidjainak stabilitásvizsgálata, 2022

3. *Corylus maxima*

3.1. Isolation of *C. maxima* diarylheptanoids

Ultra-sound assisted extraction of *C. maxima* leaf (150 g) samples was performed using ethyl acetate followed by methanol as extraction solvents. For the separation of the constituents, combinations of silica gel and reversed-phase (C_{18}) flash chromatography with semi-preparative C_{18} HPLC methods were used. The purified analytes have been identified by NMR spectroscopy and HR-MS.

Altogether seven diarylheptanoids were isolated: the linear compounds hirsutanonol-5-*O*-β-D-glucopyranoside, platyphyllonol-5-*O*-β-D-xylopyranoside, platyphyllenone; and the cyclic derivatives alnusonol-11-*O*-β-D-glucopyranoside, alnusone, giffonin F, and carpinontriol B (Fig. 3 and Table 5). Cyclic diarylheptanoids have been reported in *C. maxima* for the first time.

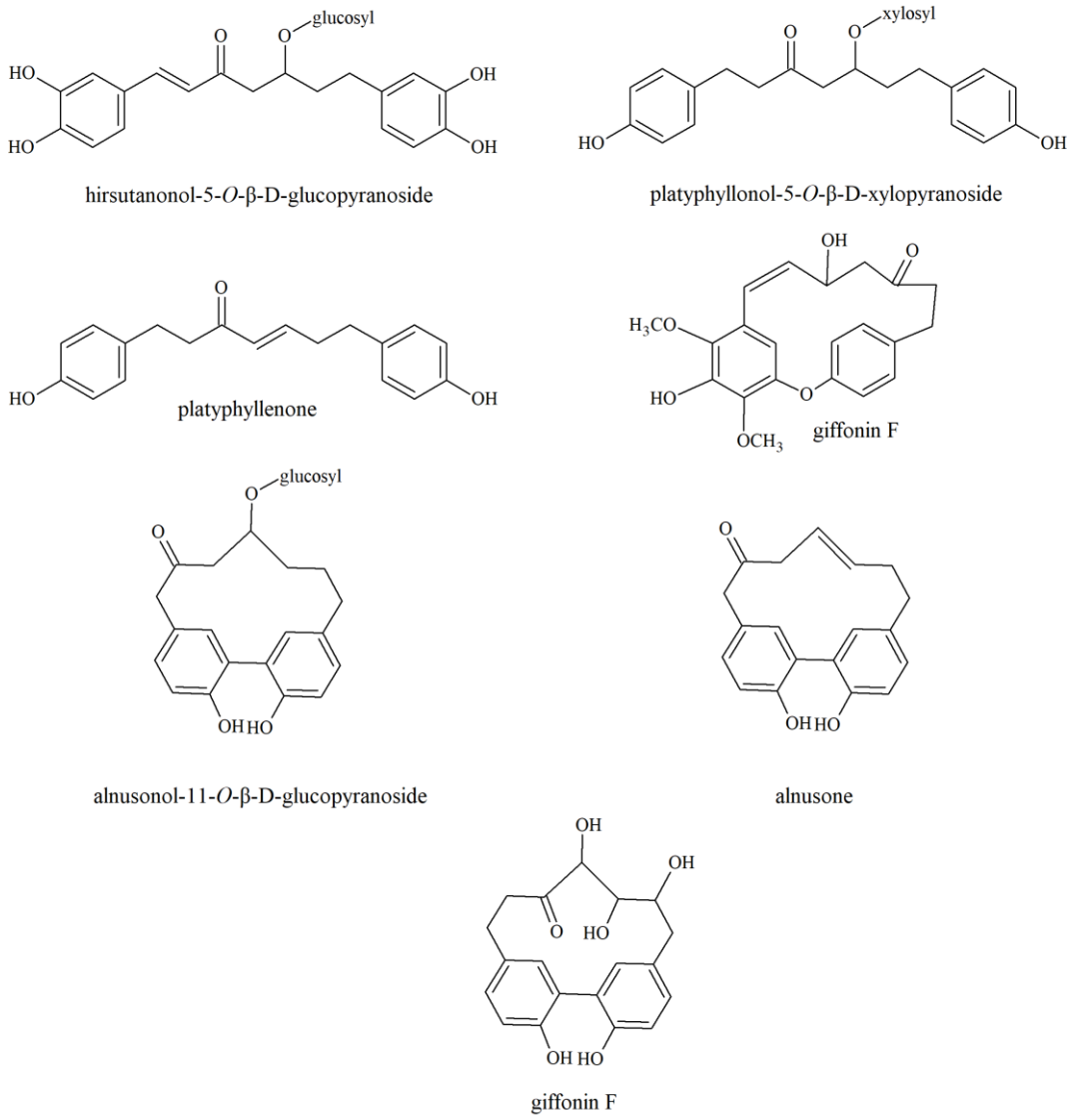


Figure 3. Diarylheptanoid constituents isolated from *C. maxima* leaf ethyl acetate and methanol extracts

Table 5. Diarylheptanoid constituents isolated from *C. maxima* leaf ethyl acetate and methanol extracts

Compound	MW (g/mol)	Extract	Isolated quantity (mg)
hirsutanonol-5-*O*-β-D-glucopyranoside	508.5	methanol	7.0
platyphyllonol-5-*O*-β-D-xylopyranoside	446.5	ethyl acetate	4.4
platyphyllenone	296.4	methanol	2.5
alnusonol-11-*O*-β-D-glucopyranoside	474.5	ethyl acetate	1.5
alnusone	294.4	methanol	1.9
giffonin F	370.4	ethyl acetate	8.9
carpinontriol B	344.4	ethyl acetate	5.2

3.2. Chemical stability assay of *C. maxima* diarylheptanoids

The aqueous stability of the isolated compounds and that of other characteristic diarylheptanoids of *C. maxima* oregonin and hirsutenone, was evaluated at pH 1.2, 6.8, and 7.4. A validated UHPLC-DAD method was utilized for the determination of compound concentrations. The structures of the degradation products were characterized by UHPLC-Orbitrap MS.

The cyclic diarylheptanoid aglycones alnusone and carpinontriol B were stable at all investigated pH values, while a pH-dependent decomposition of the other compounds was observed. In accordance with the results from the stability studies of *C. betulus* diarylheptanoids, carpinontriol B showed good aqueous stability.

Publication and presentation of the results:

- Felegyi-Tóth C. A. et al. Membrane Permeability and Aqueous Stability Study of Linear and Cyclic Diarylheptanoids from *Corylus maxima*. Pharmaceutics, 14(6): 1250., 2022. https://doi.org/10.3390/pharmaceutics14061250
- Diploma work of Zsófia Tóth (Semmelweis University): Diarilheptanoidok előfordulása a Betulaceae családban. *Corylus maxima* Mill. fitokémiai vizsgálata, 2021

4. Further species not related to the Betulaceae family

Diarylheptanoids are abundant constituents of *Acer* (Sapindaceae) and *Juglans* (Juglandaceae) species, however, literature data regarding diarylheptanoids in *Juglans nigra*, *Acer campestre*, *Acer platanoides*, and *Acer pseudoplatanus* are lacking. Therefore, our aim was to confirm the presence of diarylheptanoid constituents in these species which is plausible, due to the

chemotaxonomic relationship with other species belonging to the genera *Acer* and *Juglans*. We also aimed to identify and report new sources of cytotoxic diarylheptanoids in other plant genera. Our preliminary results indicated that diarylheptanoid-type compounds might occur in plants not related to plant families known for their diarylheptanoids. Accordingly, we performed the phytochemical screening of potential new sources of diarylheptanoids, i.e. that of plants from the families Moraceae (genera *Morus*, *Ficus*, *Maclura*, *Broussonetia*), Cannabaceae (genus *Celtis*), and Fabaceae (genera *Anthyllis* and *Lathyrus*).

4.1. *Juglans nigra* (Juglandaceae) and *Acer species* (Sapindaceae)

Bark, leaf, flower, and fruit samples of different horticultural varieties of *A. campestre*, *A. platanoides*, and *A. pseudoplatanus* have been collected. *J. nigra* pericarp as well as leaf and bark samples have also been collected. Analytical and preparative-scale extractions have been carried out in an ultrasonic bath by successive extractions with chloroform, ethyl acetate and methanol. For the analysis of the samples, qualitative UHPLC-Orbitrap methods have been applied.

Evaluation of the results from *Acer* and *Juglans* analyses and tentative identification of the constituents is partly completed. According to the first results, catechin oligomers (procyanidins), gallotannins, and methoxylated flavones are abundant in the *Acer* samples, while naphthalene derivatives, gallo- and ellagitannins prevail in the *Juglans* extracts. Diarylheptanoids can be detected as minor constituents, e.g alnusonol-*O*-pentoside in *A. campestre* bark samples, asadanin (MW 344.4 g/mol) in *J. nigra* pericarp. We reported data on the phytochemical properties of *A. campestre* and *A. pseudoplatanus* extracts for the first time. The comprehensive characterization of the compounds is still in progress.

The preparative-scale extraction of *J. nigra* has also been accomplished, in order to be able to isolate its representative constituents. Ultra-sound assisted extraction of *J. nigra* pericarp (100 g) samples was performed using chloroform, ethyl acetate, and methanol. For the separation of the constituents, combinations of reversed-phase (C_{18}) flash chromatography and semi-preparative C_{18} HPLC methods have been used. Identification of the purified analytes by NMR spectroscopy and HR-MS is in progress. The isolation procedure is still ongoing, while the following compounds have been purified so far: gallic acid (MW 170.1 g/mol), ellagic acid (MW 302.2 g/mol), tetragalloyl-hexose (MW 788.6 g/mol), myricetin-3-*O*-galloyl-rhamnoside (MW 616.5 g/mol), and 5-hydroxy-2,3-dihydro-1,4-naphtalenedione (MW 176.2 g/mol).

Publication and presentation of the results:

- The preparation of a manuscript regarding *J. nigra* is in progress.
- Diploma work of Rita Osztie (Semmelweis University): Új farmakognóziai adatok kertészeti fafajok potenciálisan antiproliferatív fenoloidjairól, 2023

4.2. Species belonging to the Moraceae family

Leaf, bark, and fruit samples of the following species have been collected: *Morus alba*, *Ficus* carica, *Maclura pomifera*, and *Broussonetia papyrifera*. In addition, *in vitro* callus cultures of *M. alba* have also been generated.

Our preliminary results indicated the presence of diarylheptanoids in *M. alba* leaf and bark samples. We isolated the linear diarylheptanoids oregonin, hirsutanonol, and hirsutenone from the ethyl acetate extracts of the samples by the combination of silica gel column chromatography, reversed-phase (C_{18}) preparative HPLC, Sephadex LH20 column chromatography, and silica gel preparative thin-layer chromatography (TLC). However, attempts to repeat the procedure were not successful. Specimens collected from the same plants or from plants growing in other habitats have also been studied unavailingly.

In case of the *in vitro* callus cultures, we evaluated the effects of culture conditions, i.e. those of different nutrient media, on the production of polyphenolic compounds. Diarylheptanoids have not been detected in the samples, however, presence of numerous stilbenes, chalcones and prenylated flavones has been shown. We developed a validated HPLC-DAD-ESI-MS/MS method for the quantification of mulberroside A (a stilbene diglycoside) and kuwanon H (a prenylated flavone).

In case of *Ficus* carica, *Maclura pomifera*, and *Broussonetia papyrifera*, the same extraction procedure has been applied as for the *Acer* and *Juglans* samples: ultrasound-assisted extraction has been performed using chloroform, ethyl acetate, and methanol, successively. For the qualitative analyses UHPLC-DAD-QTOF methods have been applied. Evaluation of the results and tentative identification of the constituents is in progress.

Publication and presentation of the results:

- Pénzes R. et al. Separation and identification of diarylheptanoids from *Morus alba* L. PHYTOPHARM 2017, Graz (Austria), 2-5 July 2017
- Alberti Á. et al. Isolation and Structural Identification of Diarylheptanoids from *Morus alba*. 11th Balaton Symposium on High-Performance Separation Methods, Siófok (Hungary), 6-8 September 2017

4.3. Species belonging to the Cannabaceae and Fabaceae family

Although no diarylheptanoids have been detected in *Celtis occidentalis* (Cannabaceae) samples, some alkylamides unprecedented in this species have been isolated from the twigs (84 g): *N*-trans-*p*-coumaroyltryamine, *N*-*trans*-caffeoyltyramine, *N*-*trans*-feruloyltyramine, *N*-*trans*-feruloyloctopamine, a new octopamine ester *N*-*trans*-*p*-coumaroyoctopamine, and 2-*trans*-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl]prop-2-enamide. The DPPH scavenging activity of the alkylamides has been determined. Additionally, their ability to penetrate across the blood-brain barrier via transcellular passive diffusion was also assessed by the PAMPA-BBB assay.

Aerial parts of *Anthyllis vulneraria* and *Lathyrus tuberosus* (both belonging to the Fabaceae) have also been investigated. Again, no diarylheptanoids have been detected in the samples, however, both species displayed a large numbers of flavonol-*O*-glycosides showing an extensive diversity. Besides, the antioxidant, cytotoxic, and wound-healing properties of the extracts were also evaluated.

Publication and presentation of the results:

- Ayanlowo G. et al. UHPLC-DPPH method reveals antioxidant tyramine and octopamine derivatives in *Celtis occidentalis*. J Pharm Biomed Anal, 191: 113612., 2020. https://doi.org/10.1016/j.jpba.2020.113612
- Diploma work of Abisola Grace Opeyemi Ayanlowo (Semmelweis University): Phytochemical characterization and anatioxidant activity of *Celtis occidentalis* L., 2020
- Csepregi R., et al. Cytotoxic, Antimicrobial, Antioxidant Properties and Effects on Cell Migration of Phenolic Compounds of Selected Transylvanian Medicinal Plants. Antioxidants, 9(2): 166., 2020. https://doi.org/10.3390/antiox9020166
- Csepregi R. et al. Cytotoxic, Antimicrobial, Antioxidant Properties and Effects on Cell Migration of Phenolic Compounds of *Lathyrus tuberosus* L. (manuscript ready for submission)
- Alberti Á. Erdélyi gyógynövények fitokémiai szűrővizsgálata. Erdélyi népi gyógyászat – hagyományoktól az alkalmazásig, Pécs (Hungary), 5-6 April 2019

5. Cyclodextrin complexation study of diarylheptanoids by affinity capillary electrophoresis

According to the literature and our results from the stability studies, diarylheptanoids possess poor stability and low aqueous solubility, which may limit their therapeutical applications. Cyclodextrins (CDs) are capable of improving the bioavailability of other compounds, as their inner cavity can accommodate apolar guest molecules through inclusion complexation. Our aim

was to optimize the pharmacokinetic properties of five diarylheptanoids by CD inclusion complexation: the linear diarylheptanoids hirsutenone, rubranosides A and B from *A. glutinosa* as well as the cyclic constituents carpinontriol A and giffonin X from *C. betulus*.

In our preliminary study, a capillary electrophoretic (CE) method for the separation of the target analytes has been developed. Diarylheptanoids, being phenolic compounds, deprotonate at pH values above 8 and can be therefore successfully evaluated by capillary electrophoresis. The background electrolyte has been optimized, the 20 mM borate buffer (pH 9.0) has been the most appropriate. The buffer system is suitable for the analysis of both linear and cyclic diarylheptanoids. The capillary electrophoretic method demonstrated satisfactory separation for the sample containing all five diarylheptanoids, with baseline separation for all the peaks ($R_s > 1.5$).

In the continuation of the study, the complex stability constants of the inclusion complexes were determined by affinity capillary electrophoresis, applying three native and six derivatized CDs. The background electrolytes contained the CDs in the 0-60 mM concentration range.

With the cyclic diarylheptanoids carpinontriol A and giffonin X only the native γ-CD was able to form (low stability) complexes. Contrary to that, all the studied native and derivatized CDs exhibited remarkable complex formation with the linear analytes. All three native CDs (α-, β-, and γ-CD) were capable of forming complexes with the linear diarylheptanoids, although the β-CD cavity size was the most suitable for the linear guest molecules.

Besides the cavity size, the type of substituents also influenced the complex formation. The substitution of the CD skeleton with neutral substituents, like methyl or hydroxypropyl groups, resulted in decreased complex stabilities in case of all CDs. The hydroxypropylated β-CD analogues were able to form the most stable complexes with all three linear diarylheptanoids, resulting in complex stabilities with magnitude of 1000 M^{-1}. Outstanding stabilities could be observed with rubranoside B (5235±980), rubranoside A (2080±770), and hirsutenone (900±75) applying this CD derivative, which is widely used as a pharmaceutical excipient. Based on our results, this "ideal excipient" was selected for the follow-up solubility and stability studies of CD-based preparations.

Results are unpublished yet, however, they are presented in a diploma work:

- Diploma work of Dorottya Ledacs-Kiss (Semmelweis University): Diarilheptanoidok kapilláris elektroforetikus vizsgálata, 2022

6. Evaluating the cytotoxicity and other biological properties of the isolated diarylheptanoids

6.1. Cytotoxicity studies of diarylheptanoids

The cytotoxicity assays have been started in the Department of Pathology and Experimental Cancer Research during Spring 2018. *In vitro* cytotoxicity of plant extracts and isolated constituents has been investigated. *A. glutinosa* and leaf and bark methanol and ethyl acetate extracts were evaluated in HT29 and Colo205 human colon cancer cell lines by the tetrazolium dye (MTT) colorimetric assay to perform their effect-directed fractionation. The extracts exhibited cytotoxic and anti-proliferative effects at ca. 10 mg/ml concentrations with the bark ethyl acetate extract as the most effective one. In the following year, the cytotoxicity assays using the same human colon cancer cell lines have been resumed. Evaluation of *B. pendula* leaf and bark methanol and ethyl acetate extracts as well as that of the major diarylheptanoids isolated from *A. glutinosa*: oregonin, rubranosides A and B, hirsutanonol, hirsutenone, and 1,7-bis-(3,4-dihydroxyphenyl)-5-*O*-feruloylpentosyl-heptan-3-one has been started.

Due to the breakdown of the LC-MS in the Department of Pharmacognosy lasting throughout the April 2018 – June 2020 period, we could not perform a substantial part of the proposed phytochemical analyses. The LC-MS was essential during the isolation and identification of the target analytes, additionally, the failure of the instrument also delayed other proposed project elements, e.g. the *in vitro* and *in vivo* cytotoxicity and pharmacokinetic studies. The research has come to a halt again in March 2020 because of the COVID-19 pandemic. We made several attempts to resume the cytotoxicity studies in Spring 2022, however, after the first exchange of e-mails with the participant researchers, we haven't received any answer.

We searched for new collaboration partners who may be able to assess the cytotoxic activity of the isolated diarylheptanoids. In Autumn 2022, we managed to get in touch with the researchers of the Eötvös Loránd Research Network Research Group of Peptide Chemistry. They have evaluated the *in vitro* antiproliferative activity of the isolated *Carpinus* diarylheptanoids by the Alamar Blue assay. Carpinontriols A and B, giffonin X, and 3,12,17-trihydroxytricyclo-[12.3.1.1^{2,6}]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione have been studied in HT-29 human colon cancer, HepG2 human hepatocellular carcinoma, HL-60 human leukemia, U87 human glioblastoma, and A2058 human metastatic melanoma cell lines for the first time. Carpinontriol A showed antiproliferative activity against A2058 human metastatic melanoma cells (IC_{50} 14.9 μM).

6.2. Membrane permeability studies of diarylheptanoids

The ability of isolated *Carpinus* and *Corylus* diarylheptanoids to penetrate across biological membranes via transcellular passive diffusion was also assessed by the parallel artificial membrane permeability assay for the gastrointestinal tract (PAMPA-GI) and the blood–brain barrier (PAMPA-BBB) methods for the first time.

Carpinontriols A and B, giffonin X, and 3,12,17-trihydroxytricyclo-[12.3.1.$1^{2,6}$]nonadeca-1(18),2(19),3,5,14,16-hexaene-8,11-dione from *C. betulus* have been investigated. Only giffonin X was considered to be able to cross the membranes via passive diffusion in the PAMPA-BBB studies. The constituents with a higher polarity, e.g. carpinontriol B were not able to cross the lipid membranes in the applied models. Typical diarylheptanoids from *C. maxima:* oregonin, hirsutanonol-5-*O*-β-D-glucopyranoside, platyphyllonol-5-*O*-β-D-xylopyranoside, platyphyllenone, hirsutenone, alnusonol-11-*O*-β-D-glucopyranoside, alnusone, and giffonin F have also been evaluated. Platyphyllenone and alnusone possessed log *Pe* values greater than −5.0 and −6.0, respectively, indicating their ability to cross the membranes of the GIT and the BBB via passive diffusion. However, according to the results of the PAMPA-GI and PAMPA-BBB studies, only platyphyllenone can be considered to be able to cross the lipid membranes in the applied models.

Results have been partly published, current publications of the results are the following:

- Felegyi-Tóth C. A. et al. Membrane Permeability and Aqueous Stability Study of Linear and Cyclic Diarylheptanoids from *Corylus maxima*. Pharmaceutics, 14(6): 1250., 2022. https://doi.org/10.3390/pharmaceutics14061250
- Felegyi-Tóth C. A. et al. Stability, membrane permeability and cytotoxicity studies of cyclic diarylheptanoids from European hornbeam (*Carpinus betulus* L.) (manuscript under review)

7. Summary

We accomplished the comprehensive characterization of diarylheptanoids from species belonging to the Betulaceae, Moraceae, Juglandaceae, Sapindaceae, Cannabaceae, and Fabaceae families. We optimized the extraction procedure of diarylheptanoids from plant samples. We analyzed the phenolic profile of *Alnus* × *spaethii*, *C. occidentalis*, *A. campestre*, and *A. pseudoplatanus*, and described several cyclic and linear diarylheptanoids in *A. cordata* for the first time. We provided new data on the presence and quantities of diarylheptanoids in *C. betulus*. We reported the occurrence of cyclic diarylheptanoids in *C. maxima* for the first time as well. We isolated seven diarylheptanoids from *A. glutinosa*, twelve phenolic compounds

(eight diarylheptanoids) from *C. betulus*, seven diarylheptanoids from *C. maxima*, six alkylamides from *C. occidentalis*, and five phenolic compounds from *J. nigra*. Among the isolated compounds, carpinontriol A showed *in vitro* antiproliferative activity against A2058 human melanoma cells. We have also assessed the chemical stability and membrane permeability of several *Corylus* and *Carpinus* diarylheptanoids. We have formulated cyclodextrin inclusion complexes with some of the isolated diarylheptanoids, in order to improve their stability and aqueous solubility. Our results have been presented in eight scientific articles (five published and three under review/publication), three oral and seven poster presentations. According to our results, there might be a possibility of future therapeutic use of plant derived diarylheptanoids, however, further research is needed to improve their stability and bioavailability.