UHPLC-DPPH method reveals antioxidant tyramine and octopamine derivatives in *Celtis occidentalis*

Abisola Grace Ayanlowo (Investigation) (Writing - original draft), Zsófia Garádi (Investigation) (Writing - original draft), Imre Boldizsár (Investigation) (Funding acquisition), András Darcsi (Investigation) (Writing - review and editing), Andrea Nagyné Nedves (Investigation), Bence Varjas (Investigation), Alexandra Simon (Investigation), Ágnes Alberti (Writing - review and editing) (Funding acquisition), Eszter Riethmüller (Conceptualization) (Methodology) (Writing - review and editing) (Supervision) (Project administration) (Funding acquisition)

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UHPLC-DPPH method reveals antioxidant tyramine and octopamine derivatives in *Celtis occidentalis*

Abisola Grace Ayanlowo[a], Zsófia Garádi[a], Imre Boldizsár[b], András Darcsi[a], Andrea Nagyné Nedves[a], Bence Varjas[a], Alexandra Simon[a], Ágnes Alberti[a], Eszter Riethmüller[a*]

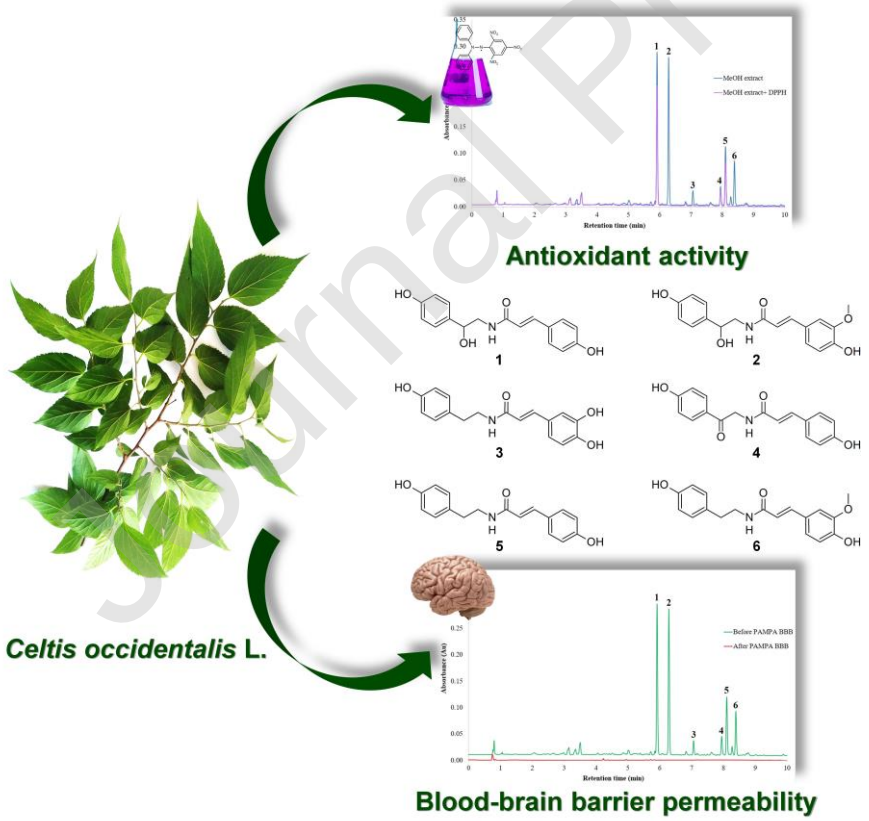
eszter.riethmuller@gmail.com

[a]Department of Pharmacognosy, Semmelweis University, Budapest, Hungary;

[b]Department of Plant Anatomy, Eötvös Loránd University, Budapest, Hungary

*Corresponding author.

Graphical abstract



HIGHLIGHTS

- UHPLC-DPPH method revealed six antioxidant compounds in *Celtis occidentalis* twigs
- The constituents were isolated by flash chromatography and semi preparative HPLC
- The compounds were first identified in *C. occidentalis* by Orbitrap® MS and NMR
- The BBB penetration capability of the compounds was tested by the PAMPA method
- No constituents in the extract were able to cross the BBB via passive diffusion

Abstract

Celtis occidentalis L. (common Hackberry, Cannabaceae) has been applied in the traditional medicine for a long time as a remedy for sore throat, aid during menstruation and for treating jaundice. Nevertheless, the phytochemical exploration of the plant is still incomplete, literature data is limited to flavonoid derivatives isolated from the leaves.

The present study reports screening approaches for bioactive compounds in *C. occidentalis* by fast and simple UHPLC-coupled assays. The UHPLC-DPPH method revealed six constituents in the methanolic extract of the twigs that had not been reported in *C. occidentalis* before. The antioxidant compounds were isolated by the means of flash chromatography and semi-preparative HPLC and identified by Orbitrap® MS and NMR spectroscopy as *N-trans-p*-coumaroyloctopamine (**1**), *N-trans*-feruloyloctopamine (**2**), *N-trans*-caffeoyltyramine (**3**), 2-*trans*-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl] prop-2-enamide (**4**), *N-trans-p*-coumaroyltryramine (**5**) and *N-trans*-feruloyltyramine (**6**).

Despite the high antioxidant activity measured in the present study and literature data suggesting potential positive effects of the compounds in the central nervous system, the PAMPA-BBB assay performed with the *Celtis* extract revealed that none of the aforementioned compounds are able to penetrate across the blood-brain barrier via transcellular passive diffusion.

Keywords: *Celtis,* UHPLC, DPPH, PAMPA, blood-brain barrier, mass spectrometry

1. Introduction

Celtis occidentalis L. (common Hackberry, Cannabaceae) is a deciduous tree commonly found and native to North America [1], but it can grow in many other regions like Africa, Europe or Australia. It has been reported that native Americans used the bark of the tree as a remedy for sore throat and aid during menstruation while the wood extract was used in treating jaundice [1]. Despite the long-standing traditional use, the phytochemical exploration of the plant is still incomplete, literature data is limited to flavonoid derivatives identified in the leaves [1].

Despite the sparse data on the phytochemistry of *C. occidentalis*, other species of the *Celtis* genus have been proved to contain several biologically active constituents, such as flavonoids, phenolic acids, anthocyanins, triterpenes, lignans and amide derivatives with antioxidant, anti-inflammatory, acetylcholinesterase inhibitory, antimicrobial and antitumor activities [1-6].

Therefore, the aim of the present study was searching for bioactive compounds in *C. occidentalis* by fast and simple UHPLC (Ultra High Performance Liquid Chromatography)-coupled assays. Coupling the DPPH (2,2-diphenyl-1-picrylhydrazyl) *in vitro* free radical scavenging assay to UHPLC separation allows the rapid identification of antioxidant constituents in the *Celtis* extract [7,8], while the transcellular passive diffusion of the compounds across biological barriers can be studied by the PAMPA (Parallel Artificial Membrane Permeability Assay) method [9].

2. Materials and methods

2.1. Plant material

The twigs of *Celtis occidentalis* L. were collected in Budapest, Hungary [47.486899,19.067525] in October, 2019. 28-28 g samples were collected from three trees after flowering stage. Plant samples were authenticated in the Department of Pharmacognosy, Semmelweis University, Budapest, where voucher specimen is deposited.

2.2. Solvents and chemicals

HPLC grade acetic and formic acid, the DPPH free radical *p*-coumaric acid, ferulic acid, caffeic acid, caffeine, rutin and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards, dimethyl sulfoxide-d_6 (99.8 atom % D with 0.03 vol.% TMS) and methanol-d_4 (99.8 atom % D), cholesterol and the porcine polar brain lipid extract were purchased from Merck (Darmstadt, Germany). *n*-Hexane and methanol of reagent grade, HPLC-MS grade acetonitrile, methanol, *n*-dodecane and dimethyl sulfoxide were obtained from Reanal-Ker (Budapest, Hungary). HPLC grade water was prepared with a Millipore Direct Q5 water purification system (Bedford, MA, USA). All aqueous eluents for HPLC were filtered through MF-Millipore membrane filters (0.45 μm, mixed cellulose esters) (Billerica, MA, USA) and degassed in an ultrasonic bath before use.

2.3. Extraction and sample preparation

Dried and milled plant sample (84 g) was pre-extracted with 840 ml of *n*-hexane in order to eliminate the apolar constituents not of our interest (e.g. chlorophyll), and consecutively with 840 ml of methanol in an ultrasonic bath at room temperature for 8-8 hours. The *n*-hexane fraction was discarded, while the methanolic extract was evaporated to dryness under reduced pressure in a rotary evaporator at 50 ºC. The dried extract (3.45 g) was dissolved in HPLC grade

70% aqueous methanol and diluted to obtain sample solution concentrations of 30 mg/ml and 3.0 mg/ml. The solutions were filtered through Phenex-RC 15 mm, 0.2 μm syringe filters (Gen-Lab Ltd, Budapest, Hungary).

2.4. UHPLC-DAD analyses

The *Celtis* samples were analyzed by a Waters Acquity Ultra High Performance Liquid Chromatogrpahy (UHPLC) system equipped with a diode array detector (DAD) (Waters Corporation, Milford, MA, USA) using an Acquity BEH C18 column (2.1×100 mm; 1,7 μm, 25 °C) with 0.1% formic acid in water as eluent A and acetonitrile as eluent B (from 95:5 to 70:30 v/v in 10 min) with a flow rate of 0.3 ml/min. The injection volume was 2 μl. UV spectra and chromatograms were recorded at 200-400 nm and the max plots were used for data evaluation.

2.5. The DPPH antioxidant activity assay

Antioxidant activity of the *Celtis* extract, the isolated compounds and the standards was determined using DPPH free radical. Stock solution was prepared by dissolving 10 mg of DPPH in 25.0 ml HPLC grade methanol (1.02 mM) and diluted with HPLC grade methanol 10-fold just before measuring. In its radical form, DPPH has absorption maxima at 515 nm. During the assay, 50 μl of the samples of five different concentrations, in three parallels were added to 2.5 ml of the free radical solution and the decrease of the absorbance was measured at 515 nm after 30 minutes of incubation at room temperature, protected from light by a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). The percentage of free radical scavenging activity was calculated as: Inhibition % = $(A_0\text{-}A/A_0) \cdot 100\%$, where A=absorbance of the free radical solution after reacting with the sample and A_0=absorbance of the solution containing only the free radical. The percentages of inhibition were plotted vs. the concentrations. The concentrations belonging to the half maximal inhibition (IC_{50} value as μg/ml and μM), were

determined by linear regression. The results are expressed as mean values and standard deviation (SD).

2.6. The UHPLC-DPPH assay

100 µl of the diluted *Celtis* extract (3.0 mg/ml) dissolved in methanol and 100 µl of the DPPH solution (1.2 mg/ml in methanol) were mixed and incubated for 30 minutes at room temperature, protected from light. Then the reaction mixture was directly analyzed by UHPLC-DAD. The control sample was prepared by the addition of 100 µl methanol to 100 µl of the extract.

2.7. The fractionation of the *Celtis* extract by flash chromatography

The *Celtis* extract was fractionated by flash chromatography (CombiFlash Nextgen 100, Teledyne Isco, USA) on two RediSep Rf Gold® 15.5g Reversed-phase C18 columns (Teledyne Isco, USA) eluting with 0.1% formic in water as eluent A and methanol as eluent B (from 100:0 to 70:30 v/v in 2 min, to 30:70 v/v in 10 min, to 0:100 v/v in 2 min and 0:100 v/v for 10 min, with a flow rate of 10 ml/min). Fourteen fractions (A-N) were obtained on the basis of UV chromatograms (290 nm). Fractions C, D and E were further purified by semi preparative HPLC.

2.8. Purification and isolation by semi preparative HPLC

Fractions C and E were purified by a Hanbon Newstyle NP7000 semi preparative HPLC (Hanbon Sci. & Tech. CO. Jiangsu, China) using a Gemini C18 column (150 x 21.2 mm, 5µm, 25 ºC, Phenomenex Inc.; Torrance, CA, USA), in the case of fraction C, using 0.1% formic acid in water as eluent A and methanol as eluent B (from 70:30 to 55:45 v/v in 30 min), while in the case of fraction E, 0.1% formic acid in water as eluent A and acetonitrile as eluent B (from 80:20 to 60:40 v/v in 30 min) with a flow rate of 10 ml/min. Fraction D was separated by a

Waters 2690 HPLC system with a Waters 996 diode array detector (Waters Corporation, Milford, MA, USA) equipped with a Luna C18 100 A reversed phase column (150 × 10.0 mm, 5 µm ; Phenomenex Inc; Torrance, CA, USA) using 0.1% formic acid in water as eluent A and methanol as eluent B with a flow rate of 3.0 ml/min. Fraction C yielded compounds **1** (4.5 mg) and **2** (2.5 mg), compound **3** (1.7 mg) was obtained from fraction D, while compounds **4** (1.1 mg), **5** (1.0 mg) and **6** (1.2 mg) were isolated from fraction E.

2.9. Structure elucidation by UHPLC-Orbitrap® Mass Spectrometry

For obtaining high resolution mass spectrometric data of the isolated compounds (**1-6**), 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) was used hyphenated with a Orbitrap® Q Exactive Focus Mass Spectrometer equipped with electrospray ionization (Thermo Fischer Scientific, Waltham, MA, USA). The same chromatographic method was applied as described above. The electrospray ionization source was operated in positive 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; auxiliary gas (N_2), 11.25 arbitrary units; spare gas (N_2), and 2.25 arbitrary units. The resolution of the full scan was of 70000, and the scanning range was between 120 and 500 *m/z* units. Parent ions were fragmented with normalized collision energy of 10%, 30%, and 45%.

2.10. Structure elucidation by NMR spectroscopy

NMR spectra were recorded in deuterated methanol (Methanol-d_4) or in deuterated dimethylsulfoxide (DMSO-d_6) on a BRUKER AVANCE III HD 600 (600/150 MHz) instrument equipped with Prodigy cryo-probehead at 295 K, or on a Varian DDR 600 (600/150 MHz) equipped with a 5-mm inverse-detection gradient (IDPFG) probehead. at 298 K. The

pulse programs were taken from the Bruker or the Varian software library (TopSpin 3.5 or VnmrJ 3.2). Chemical shifts (δ) and coupling constants (J) are given in ppm and in Hz, respectively. ^{13}C and ^{1}H chemical shifts are given in ppm relative to the NMR solvent or relative to tetramethylsilane (TMS) where internal standard was used. The complete ^{1}H and ^{13}C assignments were achieved with widely accepted strategies based on ^{1}H NMR, ^{13}C NMR, DeptQ, ^{1}H-^{1}H COSY, ^{1}H-^{13}C edHSQC and ^{1}H-^{13}C HMBC measurements.

2.11. Investigation of blood-brain barrier permeability by the PAMPA-BBB method

The test solutions were prepared with dimethyl sulfoxide (DMSO) at the concentration of 10.0 mM for the standards and at 200 mg/ml for the *Celtis* extract. These were diluted with PBS (Phosphate Buffer Saline; pH = 7.4) to obtain the donor solutions (270.0 µl buffer+3.0 µl DMSO solution) and filtered through Phenex-RC 15 mm, 0.2 µm syringe filters (Gen-Lab Ltd, Budapest, Hungary). A parallel artificial membrane permeability assay (PAMPA) system was used to determine the effective permeability (Pe) for the compounds of interest. Each well of the top plate (96-well polycarbonate based filter donor plates (Multiscreen™-IP, MAIPN4510, pore size 0.45 µm; Millipore)) was coated with 5 µl of porcine polar brain lipid extract (PBLE) solution (16.0 mg PBLE + 8.0 mg cholesterol dissolved in 600.0 µl *n*-dodecane), then 150.0 µl of the filtrate was placed on the membrane. The bottom plate (96-well PTFE acceptor plates (Multiscreen Acceptor Plate, MSSACCEPTOR; Millipore)), was filled with 300.0 µl buffer solution (0.01 M PBS buffer, pH= 7.4). The donor and acceptor plates were fit, and then the sandwich system was incubated at 37 °C for 4 hours in a Stat-Fax 2200. After the incubation the PAMPA plates were separated and the compound concentrations in the donor (CD(t)) solution, as well as in the donor solution at zero time point (CD(0)) were determined by UPLC-DAD. Since in our experiment no peaks were detected in the acceptor phase, concentrations (CA(t)) were not determined, thus calculation of the effective permeability according to Avdeef [10] was not performed.

All experiments were performed in three triplicates on three consecutive days (n=9), caffeine standard was used as positive, while rutin standard as negative control.

3. Results and Discussion

3.1. The UHPLC-DPPH assay

Based on the results of the *in vitro* antioxidant activity test using DPPH free radical, it could be concluded that the *Celtis* extract possessed notable free radical scavenger activity (with IC_{50} value of 60.54 μg/ml). In order to gain information about the constituents presumably responsible for the activity of the extract, the DPPH assay was coupled to UHPLC separation. It has previously been demonstrated that the chromatographic peak areas of constituents presenting antioxidant activity significantly decrease after the addition of free radical solution to the extract, whereas no change can be observed regarding the peaks of compounds without scavenging activity [7,8]. Therefore, the applied UHPLC-coupled assay allows the rapid identification of the antioxidant constituents in such complex mixtures as plant extracts.

Six main compounds were detected in the *Celtis* extract by UHPLC-DAD (Figure 1). Based on the decrement in the peak areas after the addition of the DPPH free radical, compounds **2**, **3** and **6** were supposed to possess high, while compounds **1**, **4** and **5** moderate scavenging capacity.

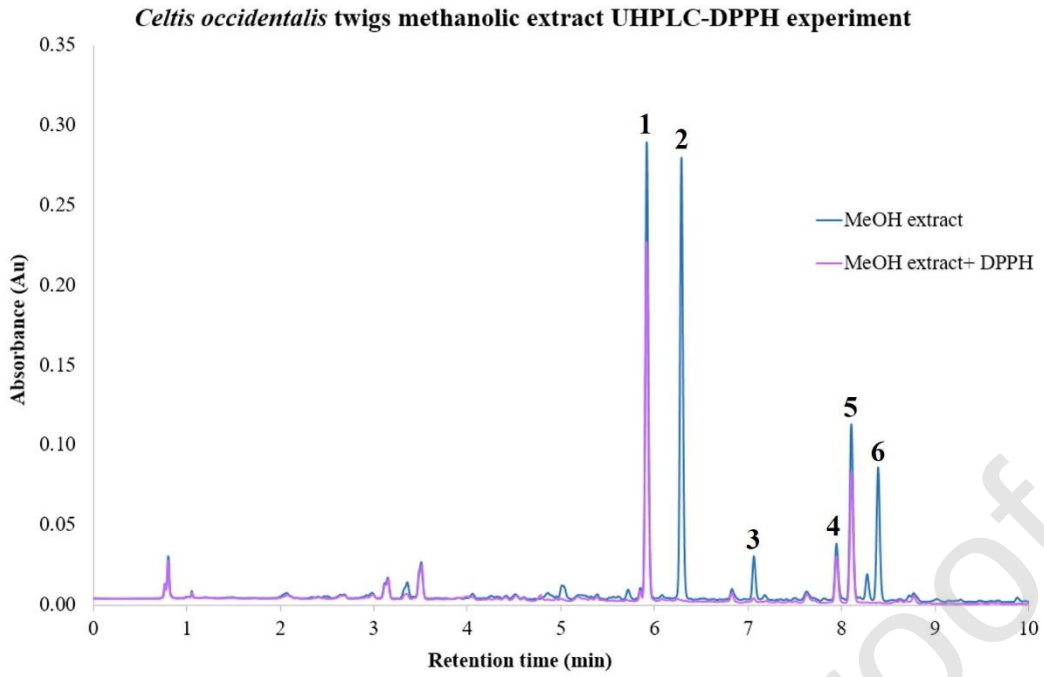


Figure 1. UHPLC-DAD chromatograms of the control sample and the sample after spiking with DPPH of *Celtis occidentalis* twigs methanolic extract.

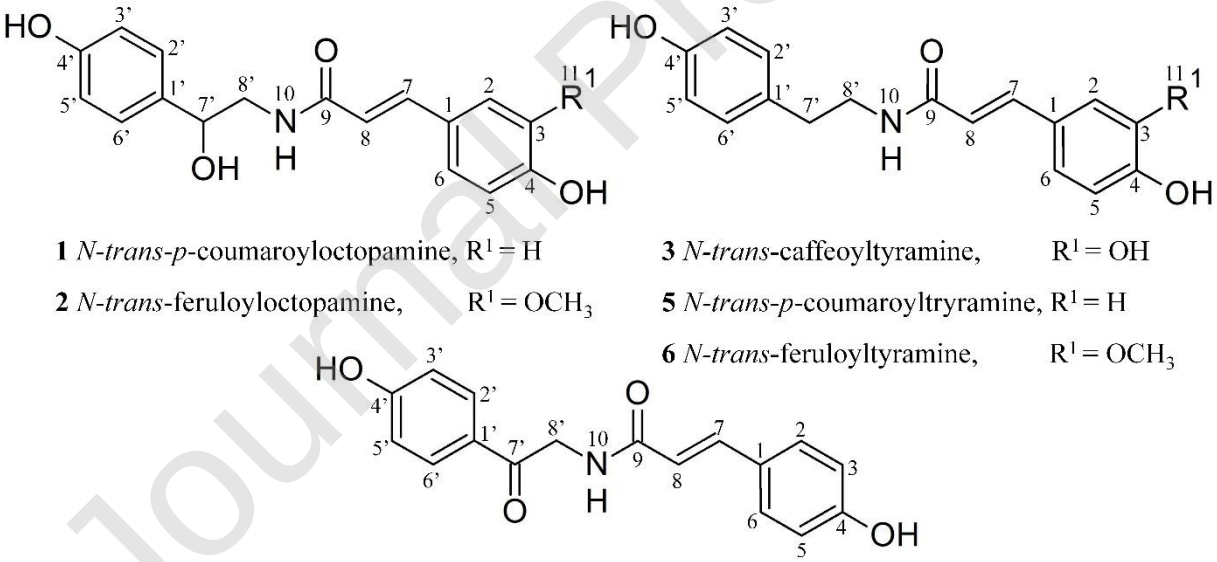
For further investigation of the antioxidant activity, and in order to gain information about the structures of the aforementioned compounds, isolation was carried out by flash chromatographic fractionation followed by semi preparative HPLC purification.

3.2. Structure elucidation by UHPLC-Orbitrap® MS and NMR

The isolated compounds were identified by Orbitrap® mass spectrometry hyphenated to UHPLC separation and by NMR spectroscopy. In the mass spectrometric experiments all the six compounds exhibited molecular ions $[M+H]^+$ with even *m/z* values, indicating the presence of odd number of nitrogen atoms in their molecules (Table 1).

In the mass spectra of compounds **1**, **4** and **5** characteristic fragment ions at *m/z* 147.0437, 119.0492, 91.0547 (Table 1) were detected suggesting a coumaroyl moiety [11]. The calculated molecular formula of compound **5** ($[M+H]^+$ *m/z* 284.1270, $C_{17}H_{17}NO_3$) together with the aforementioned product ions suggested the compound to be *N-trans-p*-coumaroyltyramine

(Figure 2), a compound that had previously been isolated from *Celtis africana* [6]. This presumption was further confirmed by NMR spectroscopy. The ^{1}H NMR spectrum showed resonances at δ 7.37 (d, *J*=8.6 Hz, 2H, H-2, H-6), 7.00 (d, *J*=8.3 Hz, 2H, H-2', H-6'), 6.78 (d, *J*=8.6 Hz, 2H, H-3, H-5) and 6.67 (d, *J*=8.3 Hz, 2H, H-3', H-5') ppm which indicated the presence of two *para*-substituted aromatic rings. The resonance at δ 8.02 (br t, *J*=5.7 Hz, 1H, H-10) ppm with no HSQC correlation confirmed the amide structure. The resonances at δ 2.63 (m, 2H, H-7') and 3.31 (m, 2H, H-8') ppm revealed the presence of two methylene units. Furthermore, two olefinic ^{1}H resonances appeared at δ 7.30 (d, *J*=15.7 Hz, 1H, H-7) and 6.39 (d, *J*=15.7 Hz, 1H, H-8) ppm, their coupling constant suggested *trans* configuration of the double bond. The presence of two extra olefinic ^{1}H resonances with lower relative intensities at δ 6.48 (d, *J*=13.0 Hz, H-7) and 5.75 (d, *J*=13.0 Hz, H-8) ppm and smaller coupling constant values suggested the presence of the cis isomer as well. The ^{1}H NMR resonances were similar to that of previously reported [12].



1 *N-trans-p*-coumaroyloctopamine, R^1 = H
2 *N-trans*-feruloyloctopamine, $R^1 = OCH_3$
3 *N-trans*-caffeoyltyramine, R^1 = OH
5 *N-trans-p*-coumaroyltryramine, R^1 = H
6 *N-trans*-feruloyltyramine, $R^1 = OCH_3$

4 2-*trans*-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl] prop-2-enamide

Figure 2. Structure of the isolated compounds (**1-6**)

In the case of compound **1**, besides the aforementioned product ions, a characteristic ion formed by the neutral loss of 18.0105 amu ($[M+H]^+$ *m/z* 300.1221 → $[M-H_2O+H]^+$ *m/z* 282.1116)

(Table 1) from the molecular ion was also detected in the mass spectrum, suggesting the presence of a primary or secondary hydroxyl group in the molecule. This was also confirmed by the calculated molecular formula differing from that of compound **5** in one oxygen atom ($C_{17}H_{17}NO_4$). The ^{1}H NMR spectrum of compound **1** showed analogous resonances with compound **5** however instead of the methylene group adjacent to the aromatic ring an oxymethine resonance at δ 4.76 (m, 1H, H-7') ppm appeared indicating the presence of a hydroxyl group in the aliphatic chain. Thus, the compound was identified as *N-trans-p*-coumaroyloctopamine (Figure 2), although octopamine derivatives have not been reported in *Celtis* species yet. The ^{1}H NMR spectrum was similar to that of previously reported [13].

The calculated molecular formula of compound **4** ($[M+H]^+$ *m/z* 298.1063, $C_{17}H_{15}NO_4$) differed from that of compound **1** in two hydrogen atoms, therefore the presence of a carbonyl group instead of a hydroxyl group was supposed in C7' position (Figure 2) The ^{1}H NMR spectrum of the compound was similar to that of compound **1**. The ^{1}H NMR spectrum confirmed the lack of the oxymethine group, while the ^{13}C NMR and the HMBC spectra of compound **4** supported the presence of a carbonyl group in the C7' position at δ 193.5 ppm, therefore the compound was identified as 2-*trans*-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl] prop-2-enamide, although the proposed structure was found neither in Reaxys nor in Pubchem nor in SciFinder databases, which allows us to draw the conclusion that compound **4** can be considered a new compound.

In the mass spectra of compounds **2** and **6** characteristic fragment ions at *m/z* 177.0542, 145.0281, 117.0335, 89.0390 (Table 1) were detected suggesting a feruloyl moiety [11]. The calculated molecular formula of compound **6** ($[M+H]^+$ *m/z* 314.1374, $C_{18}H_{19}NO_4$) together with the aforementioned product ions corresponded to *N-trans*-feruloyltyramine (Figure 2), a compound previously isolated from *Celtis africana* [6], which presumption was further confirmed by NMR spectroscopy. The ^{1}H NMR spectrum of compound **6** showed similar ^{1}H

NMR pattern to compound **5**, however compound **6** exhibited resonances at δ 7.11 (d, $^{4}J_{H,H}$=1.9 Hz, 1H, H-2), 6.97 (dd, $^{3}J_{H,H}$ =8.2 Hz, $^{4}J_{H,H}$ =1.9 Hz, 1H, H-6) and 6.79 (d, $^{3}J_{H,H}$ =8.2 Hz, 1H, H-5) ppm. These three aromatic resonances along with their coupling constants indicated a 1,2,4-trisubstitued aromatic moiety. The resonance at δ 3.80 (s, 3H, H-11) ppm suggested a methoxy group at position 11. The ^{1}H NMR resonances were analogous to literature data [12]. In the case of compound **2**, besides the aforementioned product ions, a characteristic ion formed by the neutral loss of 18.0105 amu ($[M+H]^{+}$ *m/z* 330.1325 → $[M-H_2O+H]^{+}$ *m/z* 312.1219) (Table 1) from the molecular ion was also detected, suggesting the presence of a primary or secondary hydroxyl group in the molecule, which was also confirmed by the calculated molecular formula differing from that of compound **6** in one oxygen atom ($C_{18}H_{19}NO_5$). The ^{1}H NMR spectrum of compound **2** had analogous resonances with compound **6**, however it presented an oxymethine signal at δ 4.53 (dd, *J*=8.0, 4.7 Hz, 1H, H-7') ppm rather than a methylene group. This indicated the presence of a hydroxyl group in the aliphatic moiety. Thus, the compound was identified as *N-trans*-feruloyloctopamine (Figure 2). The ^{1}H NMR spectrum was similar to that of published earlier [14].

The calculated molecular formula ($[M+H]^{+}$ *m/z* 300.1220, $C_{17}H_{17}NO_4$) of compound **3** indicated it being an isomer of *N-trans-p*-coumaroyloctopamine (compound **1**), but the product ions in the mass spectrum at *m/z* 163.0385, 145.0281, 121.0647, 107.0493 (Table 1) suggested compound **3** being a caffeic acid derivative [11], thus it was identified as *N-trans-p*-caffeoyltyramine (Figure 2), a compound previously isolated from *C. africana* [6]. Using the 1D and 2D NMR data acquired in the case of compound **3** confirmed its structure as the desmethyl derivative of compound **6**. The NMR spectra was identical to that of a previous report [15].

All the ^{1}H, ^{13}C, COSY, HSQC and HMBC NMR spectra measured for compounds **1-6** can be found in the **Supporting information.**

It has to be noted that although the NMR studies confirmed the presence of both the *cis* and *trans* isomers of the compounds in the isolated samples, spiking the extract with the mixture of the two isomers, presenting peaks with baseline separation (resolution > 2 in all cases) by UHPLC, clearly showed that only the *trans* isomers were originally present in the extract. The interconversion between the *cis*-*trans* isomers might have occurred rapidly due to unavoidable exposure to UV light during the isolation process.

No.	t_R (min)	$[M+H]^+$ *m/z* exp.	Error (ppm)	Molecular formula	Product ions	Compound
1	6.0	300.1221	-3,214	$C_{17}H_{17}NO_4$	282.1116 147.0437 119.0492 91.0547	***N*-*trans*-*p*-coumaroyloctopamine**
2	6.3	330.1325	-3.390	$C_{18}H_{19}NO_5$	312.1219 177.0542 145.0281 117.0335 89.0390	*N*-*trans*-feruloyloctopamine
4	8.0	298.1063	-3.571	$C_{17}H_{15}NO_4$	147.0437 119.0491 91.0547 65.03934	2-*trans*-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl]prop-2-enamide
5	8.2	284.1270	-3.942	$C_{17}H_{17}NO_3$	147.0437 119.0491 91.0547	*N*-*trans*-*p*-coumaroyltyramine

					177.0541	
					145.0280	
					121.0647	
6	8.5	314.1374	-4.026	$C_{18}H_{19}NO_4$	117.0335	*N-trans*-feruloyltyramine
					93.0703	
					89.0390	

Table 1. Orbitrap® MS data of compounds **1-6.**

3.3. Antioxidant activity of the isolated compounds

The antioxidant activity of the isolated compounds was determined by *in vitro* tests using DPPH free radical. The IC_{50} values were compared to Trolox, *p*-coumaric acid, ferulic acid and caffeic acid standards. The results are in good agreement with the conclusions drawn from the UHPLC-DPPH experiment (Table 2).

Sample	**IC_{50} (µg/ml) DPPH**	**IC_{50} (µM) DPPH**
Trolox	4.9±0.1	19.6±0.4
p-Coumaric acid	161.3±9.7	983±59.2
Ferulic acid	6.8±0.3	35.2±1.7
Caffeic acid	4.6±0.3	25.6±1.7
Methanolic extract	60.4±0.3	-
N-trans-p-coumaroyloctopamine (**1**)	412.7±10.2	1380.3±34.1
N-trans-feruloyloctopamine (**2**)	30.9±0.6	94.04±1.8
N-trans-caffeoyltyramine (**3**)	9.5±0.4	31.6±1.4

2-trans-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl]prop-2-enamide (**4**)	349.7±15.4	1177.4±51.9
N-trans-p-coumaroyltyramine (**5**)	389.9±16.3	1377.7±57.6
N-trans-feruloyltyramine (**6**)	15.0±0.6	47.9±1.9

Table 2. DPPH scavenging activity of the *Celtis* extract, isolated compounds and standards

N-trans-caffeoyltyramine (**3**) was shown to possess the highest radical scavenging activity followed by *N-trans*-feruloyltyramine (**6**) and *N-trans*-feruloyloctopamine (**2**), all exceeding the activity of the whole extract. The coumaroyl derivatives (compounds **1**, **4**, and **5**) exerted relatively low activity compared to the whole extract and Trolox standard as well (Table 2).

These aforementioned results do not come as a surprise, since the scavenging ability of hydroxycinnamic acid derivatives against free radicals strongly depends on the number of hydroxyl groups on the benzene ring (two in the case of caffeic acid compared to one in *p*-coumaric acid) and the *ortho*-substitution with the electron donor methoxy group (in the case of ferulic acid) that increase the stability of the phenoxy radical, resulting in higher scavenger activity.

Our results regarding the DPPH scavenging capacity of *N-trans*-caffeoyltyramine (**3**) and *N-trans*-feruloyltyramine (**6**) (Table 2) are in good accordance with literature data as well: Al-Taweel et al. reported IC_{50} values of 26.3±0.32 μM and 33.2±0.14 μM, respectively [6]. However, the IC_{50} value of *N-trans-p*-coumaroyltyramine (**5**) showed significant difference when compared to the results of the previously mentioned study (62.0±0.15 μM vs. 1377.7±57.6 μM). Furthermore, in our experiments, the other *p*-coumaric acid derivatives (compounds **1** and **4**) possessed relatively high IC_{50} values as well, similar to that of *N-trans-p*-coumaroyltyramine (**5**) (Table 2). Since literature data regarding the DPPH scavenging activity

of *p*-coumaric acid is, to put it mildly, diverse (Table 3), it was determined together with the isolated compounds as well as ferulic acid and caffeic acid standards. As it can be seen in Table 2, all the isolated derivatives showed significantly lower activity than the corresponding phenolic acids, and for *p*-coumaric acid the IC_{50} value of 983±59.2 μM was measured. Based on these observations, the results regarding the *p*-coumaric acid derivatives (compounds **1**, **4** and **5**) were found to be acceptable despite the significantly different result for *N-trans-p*-coumaroyltyramine (**5**) of Al-Taweel et al. [6].

Concentration / Inhibitory %				Incubation time	Reference
caffeic acid	**ferulic acid**	***p*-coumaric acid**	**DPPH**		
no data	no data	762.2 μM / 6.99%	1500 μM	30 min	[16]
39.6 μM / 50.0%	60. 56 μM / 50.0%	7819.8 μM / 50.0%	75 μM	30 min	[17]
166.7 μM / 32.1%	no data	182.9 μM / 5.3%	80 μM	10 min	[18]
no data	no data	182.9 μM /55.6%	25 μM	30 min	[19]
20.0 μM / 49.6.0%	20.0 μM / 27.3%	20.0 μM / 7.0%	100 μM	30 min	[20]

Table 3. Literature data regarding the DPPH scavenging activity of *p*-coumaric acid, ferulic acid and caffeic acid

It is also worth mentioning, that interestingly, compounds **2, 3** and **6** showed significantly higher antioxidant activity than the whole extract, which suggest antagonistic interactions between the constituents.

3.4. Investigation of blood-brain barrier permeability by the PAMPA-BBB method

Besides the antioxidant activity reported here, the isolated compounds have been shown to possess other beneficial biological effects as well, for instance *N-trans-p*-coumaroyl tyramine

from *C. chinensis* reversibly inhibited acetylcholinesterase activity in a dose-dependent manner (IC_{50} 34.5 µg/ml) [5].

Taken in consideration the aforementioned effect together with the antioxidant activity of the *Celtis* constituents, in order to get information about the ability of the compounds to reach the central nervous system, the investigation of their transcellular passive diffusion across the blood-brain barrier (BBB) was found to be reasonable. For this purpose, the PAMPA-BBB assay was chosen that had previously been validated for natural compounds [9]. Coupling the PAMPA-BBB method to UHPLC separation allowed the rapid simultaneous investigation of the membrane penetration capability of the constituents present in the *Celtis* extract. As it can be seen in Figure 3, none of the compounds were detected in the acceptor phase of the PAMPA-BBB model, indicating their inability to cross the BBB via passive diffusion.

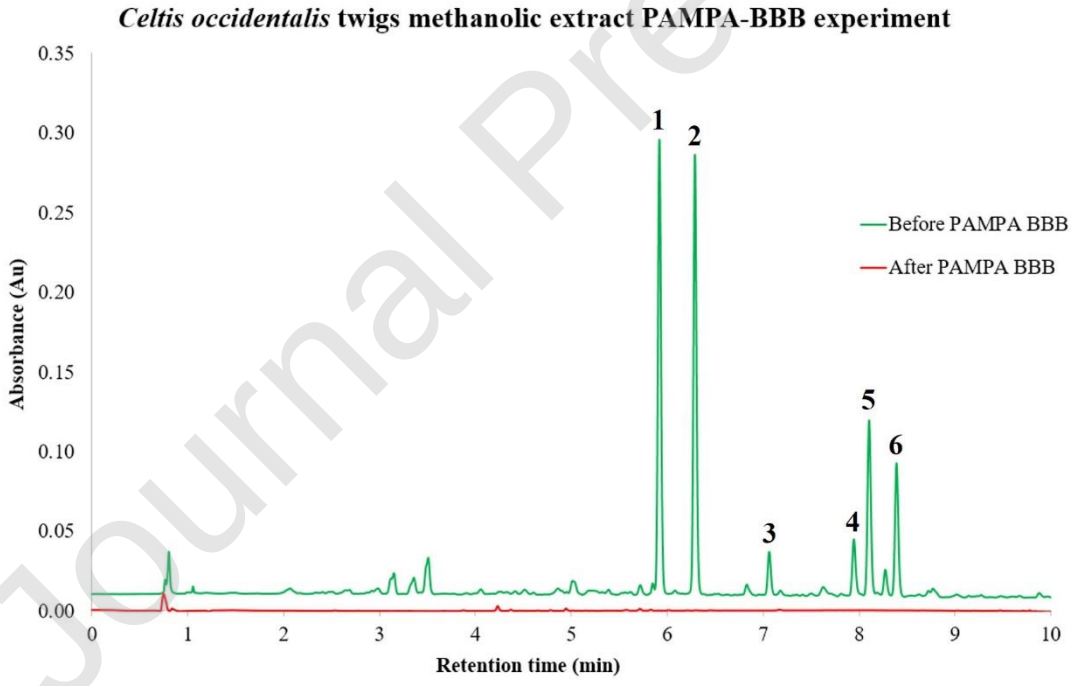


Figure 3. Result of the PAMPA BBB experiment of *Celtis occidentalis* twigs methanolic extract

4. Conclusions

The UHPLC-coupled DPPH assay followed by isolation and structure elucidation allowed the identification of six antioxidant constituents in *Celtis occidentalis* twigs methanolic extract: *N-trans-p*-coumaroyloctopamine (**1**), *N-trans*-feruloyloctopamine (**2**), *N-trans*-caffeoyltyramine (**3**), 2-*trans*-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl] prop-2-enamide (**4**), *N-trans-p*-coumaroyltryramine (**5**) and *N-trans*-feruloyltyramine (**6**).

All the six compounds are reported in *Celtis occidentalis* for the first time, *N-trans-p*-coumaroyloctopamine (**1**) and *N-trans*-feruloyloctopamine (**2**) have not been identified in *Celtis* species yet, while 2-*trans*-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl] prop-2-enamide (**4**) can be considered a new compound.

Besides the antioxidant activity reported here, literature data indicates further beneficial pharmacological effects of the isolated compounds, for instance *N-trans-p*-coumaroyloctopamine (**1**) and *N-trans*-feruloyloctopamine (**2**) from garlic skin have been reported to attenuate oxidative stress and liver pathology in rats with non-alcoholic steatohepatitis [13], while *N-trans-p*-coumaroyltyramine (**5**) from *C. chinensis* has been shown to reversibly inhibit acetylcholinesterase activity in a dose-dependent manner [5].

The recent study highlights the importance of *C. occidentalis* as a new natural source of the aforementioned compounds with potential beneficial biological effects, and also reports compound **4**, a new natural compound with antioxidant activity and pharmacological perspectives that can be explored in the future. However, the result of the PAMPA-BBB experiments indicates that none of the compounds are able to penetrate across the BBB via passive diffusion, thus, a special emphasis should be placed on this issue in future studies aiming the investigation of the potential effects of the compounds in the central nervous system.

CRediT author statement

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Declaration of interests

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.

Conflict of interest

The authors declare no competing financial interest.

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