

SUPPLEMENTARY MATERIAL

LC-ESI-MS/MS profiling of phenolics in the leaves of *Eleutherococcus senticosus* cultivated in the West Europe and anti-hyaluronidase and anti-acetylcholinesterase activities

Rafał Kuźniewski^a, Daniel Załuski^a, Marta Olech^b, Piotr Banaszczyk^c, Renata Nowak^b

^a*Department of Pharmacognosy, Ludwik Rydygier Collegium Medicum, Nicolaus Copernicus University, 9 Marie Curie-Skłodowska Street, 85-094 Bydgoszcz, Poland.*

^b*Department of Pharmaceutical Botany, Medical University of Lublin, 1 Chodźki Street, 20-093 Lublin, Poland.*

^c*Rogów Arboretum, Forest Experimental Station, Warsaw University of Life Sciences, Leśna 5b, 95-063 Rogów, Poland*

*Corresponding Author:

Rafał Kuźniewski E-mail: rafal.kuzniewski@o2.pl Phone: +48 52 585-38-07.

Abstract: Neither secondary metabolites of the spring leaves nor the autumn leaves of *Eleutherococcus senticosus* species cultivated in Poland, or the bioactivity are known. The richest in polyphenols were the autumn leaves (171.1 mg/g DE), while in flavonoids the spring leaves (107.9 mg/g DE). Using LC-ESI-MS/MS, protocatechuic acid has been identified as the most abundant compound in the spring and autumn leaves (200 and 70 µg/g DE, respectively). Amongst flavonoids, naringenin 7-*O*-glucoside occurred in the largest amount (20 and 10 mg/g DE in the spring and autumn leaves, respectively). The autumn leaves inhibited Hyal the strongest (74.3%), comparing to the spring leaves (33%). A weak inhibition was found towards AChE (0.64 and 5.8% for the autumn and spring leaves, respectively). To our best knowledge, no information was available on the phytochemical composition and activity of the leaves of *Eleutherococcus senticosus* cultivated in Poland.

Keywords: *Eleutherococcus*, LC-ESI-MS/MS, phenolic acids, flavonoids, Hyal, AChE

Experimental

Standards and reagents. Commercial standards of phenolic acids and flavonoids were obtained from ChromaDex (Santa Ana, CA) and Sigma (St. Louis, Mo.). o-Phenantroline, ethanol were purchased from POCH (Gliwice, Poland). Physostigmine, hyaluronidase from bovine testes type I-S, Streptococcus equi hyaluronic acid, DTNB (5,5'-Dithiobis(2-nitrobenzoic acid), ACTI (acetylthiocholine iodide) sodium phosphate buffer pH 7.0 were obtained from Sigma-Aldrich. All reagents were of analytical grade.

Plant materials. The spring and autumn leaves of *E. senticosus* (Rupr. et Maxim.) Maxim. were obtained from the arboretum in Rogów (Poland) in April and October 2016. Voucher specimen (ESS-1/16 and ESA-2/16) was deposited at the Department of Pharmacognosy, Collegium Medicum in Bydgoszcz, Poland. This species was successfully cultivated at the botanical garden in Rogów, which lies in the Central Polish Lowlands region with geographic data such as 51° 49'N and 19° 53'E. The average, long-term temperature is -20.1°C, what classified the garden to the 6bth sub-climate (according to USDA Frost Hardiness Zones) and to the second zone according to the Kórník's category. These plants are grown on the acidic, luvic, and sandy soils

Dried material extraction with 75% ethanol. The air-dried and powdered leaves (15 g each) were soaked in 150 mL 75% ethanol for 24 h. Next, the samples were subjected to triple UAE type extraction (ultrasonic bath -Polsonic, Warsaw, Poland) using 150, 2 x 100 mL of 75% ethanol. The extraction was performed at room temperature for 15 min for each cycle. Finally, 350 mL of each extract was obtained. The solvents were dried with an evaporator under vacuum conditions at 45°C and subjected to lyophilisation. The extraction yield was calculated based on the dry weight of the extract [%].

Total phenolic content (TPC). The total phenolic content of extracts was determined using the method of Singleton et al. [14]. Gallic acid was used to calculate the calibration curve (20-100 µg/mL; $y=0.0026x + 0.044$; $r^2=0.999$), and TPC was expressed as gallic acid equivalents (GAE/mL). The experiments were done in triplicate.

Total flavonoid content (TFC). The TFC in investigated samples was determined using FeCl₃ and DNPH colorimetric methods [15]. TFC were expressed as means (±S.E.) mg of quercetin equivalent (QEs/mL for FeCl₃ method; 20-100 µg/mL; $y=0.0041x + 0.236$;

$r^2=0.999$) and as means (\pm S.E.) mg of hesperetin equivalent (HEs/mL for DNPH method; 250-1000 μ g/mL; $y=6.374x - 0.098$; $r^2=0.988$). The experiments were done in triplicate.

LC-ESI-MS/MS conditions of analysis of phenolic acids. The samples were analyzed according to modified method previous described by Załuski et al. (Załuski et al., 2017). For this purpose an Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a binary gradient solvent pump, a degasser, an autosampler and column oven connected to a 3200 QTRAP Mass spectrometer (AB Sciex, USA) equipped with an electrospray ionisation source (ESI) and a triple quadrupole-ion trap mass analyzer was used. The separation of the analytes was carried out on a Zorbax SB-C18 column (2.1 x 50 mm, 1.8- μ m particle size; Agilent Technologies, USA) maintained at 25 °C, using 3 μ L injections. The solvents used were: water containing 0.1% HCOOH (solvent A) and methanol containing 0.1% HCOOH (solvent B). The following gradient elution program at a flow rate of 370 μ L min⁻¹ was applied: 0 – 1 min – 5% B; 2 – 4 min – 20% B; 8 – 9.5 min – 70 % B; 11.5 – 15.5 min – 5% B. Mass spectrometer was controlled by the Analyst 1.5 software. ESI worked in the negative-ion mode with the curtain, nebulizer and turbo-gas (all nitrogen) set at 30, 60 and 60 psi, respectively. The ion spray needle voltage was –4500 V and capillary temperature 400 °C. For each compound the optimum conditions of Multiple Reaction Mode (MRM) were determined in the direct infusion mode. Triplicate injections were made for each standard solution and sample. The analytes were identified by comparing retention time and m/z values obtained by MS and MS² with the mass spectra from corresponding standards tested under the same conditions. The calibration curves obtained in MRM mode were used for quantification of all analytes. The identified phenolic acids were quantified on the basis of their peak areas and comparison with a calibration curve obtained with the corresponding standards. Linearity ranges for calibration curves were specified. The limits of detection (LOD) and quantification (LOQ) for phenolic compounds were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

LC-ESI-MS/MS conditions of analysis of flavonoids. An Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a binary gradient solvent pump, a degasser, an autosampler and column oven connected to 3200 QTRAP Mass spectrometer (AB Sciex, USA) was employed. Chromatographic separations were carried out at 25°C, on a Eclipse XDB-C18 column (4.6 x 150 mm, 5- μ m particle size; Agilent Technologies, USA) with a mobile phase consisting of water containing 0.1% HCOOH (solvent A) and acetonitrile

containing 0.1% HCOOH (solvent B), using 5 μ l injections. The flow rate was 450 μ L min⁻¹ and the gradient was as follows: 0 – 1 min – 18% B; 1.5 – 5.5 min – 20% B; 7 – 10 min – 25 % B; 13 - 15 min – 60% B, 17 – 21 min – 18%B. The QTRAP-MS system was equipped with electrospray ionisation source (ESI) operated in the negative-ion mode. ESI worked at the following conditions: capillary temperature 500 °C, curtain gas at 25 psi, nebulizer gas at 50 psi, negative ionisation mode source voltage –4500 V. Nitrogen was used as curtain and collision gas. For each compound the optimum conditions of Multiple Reaction Mode (MRM) were determined in the infusion mode. The data was acquired and processed using Analyst 1.5 software (AB Sciex, USA). Triplicate injections were made for each standard solution and sample. The analytes were identified by comparing retention time and m/z values obtained by MS and MS² with the mass spectra from corresponding standards tested under the same conditions. The calibration curves obtained in MRM mode were used for quantification of all analytes. The identified phenolic acids were quantified on the basis of their peak areas and comparison with a calibration curve obtained with the corresponding standards. Linearity ranges for calibration curves were specified. The limits of detection (LOD) and quantification (LOQ) for phenolic compounds were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

Anti-hyaluronidase studies. The ability of the extracts to inhibit Hyal was determined by the spectrophotometric method of Yus et al. [17]. The extracts concentration was 1.0 mg/mL in 10% water ethanol solution. The final concentration in the reaction's mixture was 22 µg/0.161 mL. The activity was determined on the basis of precipitation of undigested HA with albumin. 50 µL of enzyme in acetate buffer pH 4.5, 50 µL of sodium phosphate buffer (50 mM, pH 7.0; with 77 mM NaCl and 1 mg/mL of albumin) and 22 µL of the analyzed samples were combined. All the reaction mixtures were incubated at 37 °C for 10 min. Next, 50 µL of HA (0.3 mg/mL of acetate buffer pH 4.5) was added and incubated at 37 °C for 45 min. The undigested HA was precipitated with 1 mL acid albumin solution made up 0.1% bovine serum albumin in 24 mM sodium acetate and 85 mM acetic acid. The mixture was kept at room temperature for 10 min., the absorbance of the reaction mixture was measured at 600 nm (Multi-Detection Microplate Reader Synergy™ HT – BioTek). Aescin was used as the positive control at the following concentration 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 mg/0.161 mL, the absorbance in the absence of enzyme was used as the blind control. All assays were done in triplicates. The percentage of inhibition was calculated as:

$$\% \text{ inhibition} = [(AB - AE)/(AS - AE)] \times 100$$

whereas:

AB - absorbance of the enzyme+substrate+extract

AE - absorbance of the enzyme+substrate sample

AS - absorbance of the enzyme+ substance sample

Anti-acetylcholinesterase studies. The ability of the extracts to inhibit AChE was determined by the spectrophotometric method of Ellman et al. (Ellman et al., 1961). The extract concentration was 1.0 mg/mL in 10% water ethanol solution. The final concentration in the reaction's mixture was 22 µg/0.2 mL. Physostigmine was used as the positive control at the following concentration 2, 3, 4, 15, 30, 40 µg/0.195 mL. Every assay was done in triplicate.

Statistical analysis. Determinations were performed by triplicate. The obtained data were subjected to statistical analysis using Statistica 7.0. (StatSoft, Cracow). The evaluations were analyzed for one-factor variance analysis. Statistical differences between the treatment groups were estimated by Spearman's (R) and Person's (r) test. All statistical tests were carried out at significance level of $\alpha = 0.05$.

References

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TABLES

Table S1. LC-ESI-MS/MS analytical results of phenolic acids, including retention times, mass-to-charge ratio (m/z) and fragments obtained with given collision energy. Compounds confirmed by comparison with authentic standards.

Compound	Peak no.	T _R (min)	[M-H] ⁻	Products of [M-H] ⁻	Colision energy (eV)
Gallic acid	1	0.74	168.7	124.9	- 14
				78.9	- 36
Protocatechuic acid	2	1.70	152.9	107.8	- 38
				80.9	- 26
Gentisic acid	3	2.70	152.8	107.9	- 36
				81	- 30
4-OH-benzoic acid	4	3.26	136.8	92.9	- 18
Vanillic acid	5	4.49	166.8	107.9	- 18
				123	- 12
Caffeic acid	6	4.65	178.7	134.9	- 16
				88.9	- 46
Syringic acid	7	5.26	196.9	181.9	- 12
				122.8	- 24
p-Coumaric acid	8	5.60	162.7	119	- 14
				93	- 44

Ferulic acid	9	5.77	192.8	177.9	- 12
				133.9	- 16
Salicylic acid	10	5.80	136.8	93	- 16
				75	- 48
Veratric acid	11	5.80	180.7	136.9	- 12
				121.9	- 18
Synapic acid	12	5.81	222.8	148.9	- 20
				121	- 36
3-OH-cinnamic acid	13	5.82	162.8	119	- 14
				91	- 36
Rosmarinic acid	14	5.97	358.7	160.8	- 20
				132.6	- 44

Compounds confirmed by comparison with authentic standards.

Table S2. LC–MS/MS data for the calibration curves and LODs, LOQs values for each analyzed phenolic acid.

Compound	LOQ [ng μL^{-1}]	LOD [ng μL^{-1}]	r^2	Linearity range [ng μL^{-1}]
Gallic acid	0.10	0.05	0.9994	0.10 – 10.00
Protocatechuic acid	0.02	0.01	0.9991	0.025 – 3.13
Gentisic acid	0.015	0.008	0.9993	0.025 – 25.00
4-OH-benzoic acid	0.10	0.05	0.9971	0.10 - 2.50
Vanillic acid	0.20	0.10	0.9999	0.2 - 50
Caffeic acid	0.08	0.04	0.9972	0.08 - 1.25
Syringic acid	0.10	0.05	0.9997	0.1 – 50.00
p-Coumaric acid	0.061	0.018	0.9971	0.10 – 10.20
Ferulic acid	0.025	0.01	0.9997	0.025 – 5.00
Salicylic acid	0.02	0.01	0.9986	0.02 - 0.50
Veratric acid	0.70	0.40	0.9977	0.50 – 25.00
Synapic acid	0.025	0.007	0.9987	0.025 – 5.00
3-OH-cinnamic acid	0.05	0.02	0.9994	0.05 - 2.50
Rosmarinic acid	0.01	0.005	0.9985	0.025 – 25.00

Analyte ^a	Peak no.	T _R (min)	[M-H] ⁻	Products of [M-H] ⁻	Collision energy (eV)
Luteolin 3,7-diglucoside	1	6.25	609.1	285.0	-50
				447.0	-32
Rutin (Quercetin 3- <i>O</i> -rutinoside)	2	8.08	608.7	299.6	-46
				270.9	-60
Hyperoside (Quercetin 3- <i>O</i> -galactoside)	3	9.20	462.7	299.7	-28
				254.7	-42
Luteolin 7- <i>O</i> -glucoside	4	9.35	446.8	284.8	-30
				132.9	-78
Isoquercetin (Quercetin 3- <i>O</i> -glucoside)	5	9.55	462.7	299.7	-30
				270.7	-44
Kaempferol 3- <i>O</i> -rutinoside	6	9.70	592.7	284.8	-38
				226.7	-68
Naringin (Naringenin 7- <i>O</i> - rhamnosidoglucoside)	7	11.40	579.1	151.0	-54
				271.0	-42
Astragalin (Kaempferol 3- <i>O</i> -glucoside)	8	11.70	446.7	254.8	-40
				226.8	-54
Quercitrin (Quercetin 3- <i>O</i> -rhamnoside)	9	12.20	446.8	299.7	-30
				270.7	-40
Apigenin 7- <i>O</i> -glucoside	10	12.70	430.7	267.7	-38
				116.9	-84
Luteolin 4'- <i>O</i> -glucoside	11	12.90	447.0	285.1	-28
				133.0	-74
Naringenin 7- <i>O</i> -glucoside	12	13.12	432.7	270.8	-22
				118.9	-64

Table S3. LC-ESI-MS/MS analytical results of flavonoids, including retention times, mass-to-charge ratio (*m/z*) and fragments obtained with given collision energy. Compounds confirmed by comparison with authentic standards.

Table S4. LC–MS/MS data for the calibration curves and LODs, LOQs values for each analyzed flavonoid.

Compound	Calibration curve	r^2	LOD [ng mL ⁻¹]	LOQ [ng mL ⁻¹]	Linearity range [ng mL ⁻¹]
Luteolin 3,7-diglucoside	$y = 656x + 1.85e+003$	0.9999	0.02	0.05	0.5 - 2500
Rutin	$y = 245x - 309$	0.9999	2.5	5	5 - 5000
Hyperoside	$y = 295x + 6.52e+003$	0.9988	10	20	20 - 2500
Luteolin 7-glucoside	$y = 701x + 6.65e+003$	0.9998	2.5	5	5 - 2500
Isoquercetin	$y = 308x + 5.35e+003$	0.9993	10	20	20 - 2500
Kaempferol 3-rutinoside	$y = 266x + 127$	0.9999	5	10	10 - 5000
Naringin	$y = 730x - 192$	0.9999	2	5	5 - 2500
Astragalin	$y = 385x + 1.58e+003$	0.9999	3	7.5	10 - 2500
Quercetrin	$y = 453x + 5.05e+003$	0.9998	5	10	10 - 5000
Apigenin 7-glucoside	$y = 902x + 4.77e+003$	0.9996	2	4	5 - 1000
Luteolin 4-glucoside	$y = 1.32e+003x + 2e+004$	0.9996	1	2.5	8 - 2500
Naringenin 7-glucoside	$y = 402x + 7.72e+003$	0.9996	0.01	0.04	0.1 - 1000

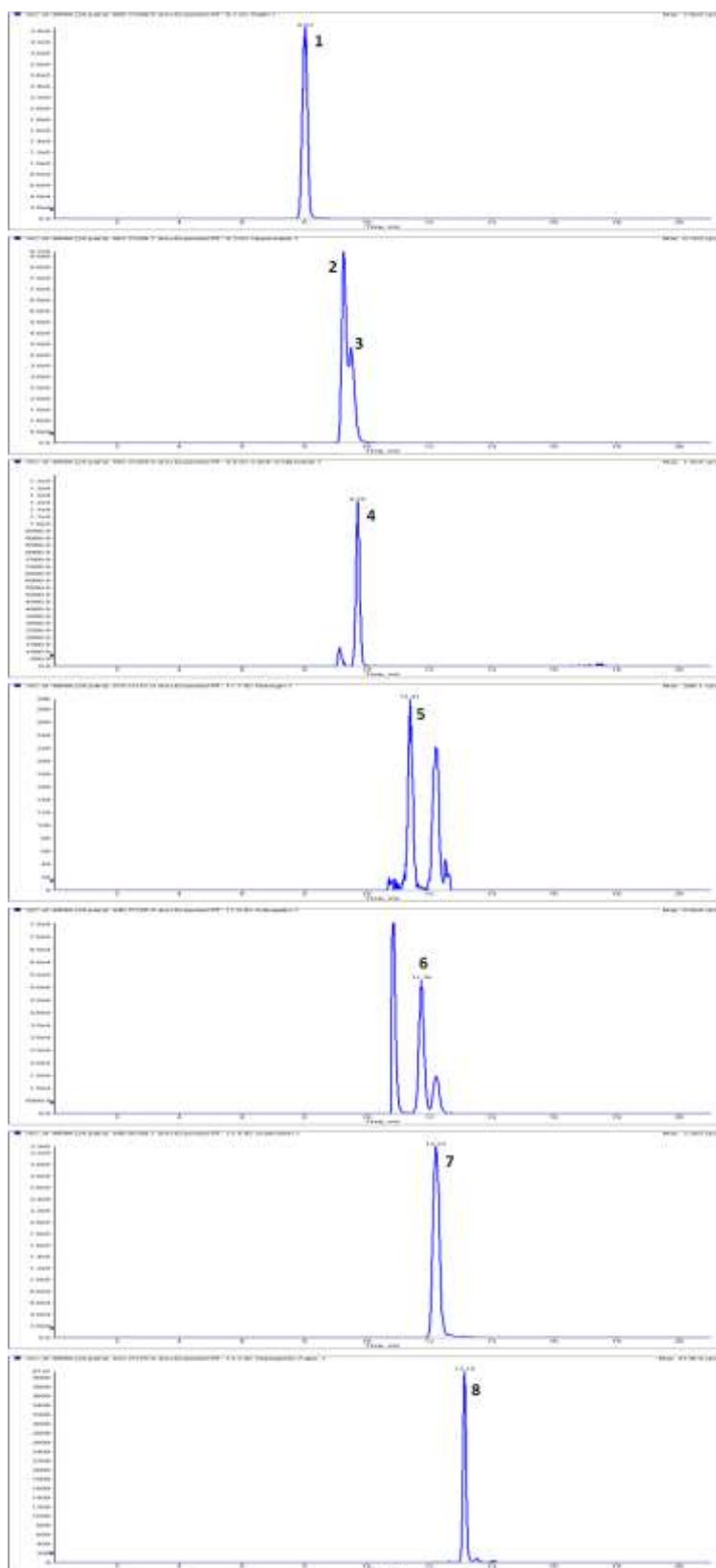


Figure S1. An exemplary chromatogram of flavonoids in the leaves of *E. senticosus*: 1. rutin; 2. hyperoside; 3. isoquercetin; 4. keampferol-3-*O*-rutinoside; 5. naringin; 6. astragalin; 7. quercetrin; 8. naringenin-7-*O*-glucoside.