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NATURAL DRUGS

COUMARINS IN HORSE CHESTNUT FLOWERS: ISOLATION AND QUANTIFICATION BY UPLC METHOD

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Abstract: The coumarins: scopoletin, esculetin and fraxetin were isolated from the flowers of horse chestnut (*Aesculus hippocastanum* L., *Hippocastanaceae*) and identified by spectrophotometric methods (UV, ^{1}H, ^{13}C NMR, ESI-MS). Their content, determined using the Ultra Performance Liquid Chromatography (UPLC), was 0.41, 0.13 and 0.05%, respectively.

Keywords: *Hippocastani flos*, coumarins: scopoletin, esculetin, fraxetin, quantification, UPLC

Aesculus hippocastanum (*Hippocastanaceae*), commonly known as horse chestnut, is not only an ornamental tree, but also a source of fruits, seeds, bark, and, less often, flowers or leaves for making medicinal preparations.

The bark extract and esculin (coumarin), as well as the seed extract and escin (a saponin mixture) from *A. hippocastanum* are used in the treatment of chronic venous insufficiency, varicose veins, hemorrhoids, postoperative edemas, burns, epidermis abrasion, skin inflammations and frostbites (1-3). The chemical constituents of the seeds and bark have been well documented, whereas the phytochemical investigations of the flowers are very limited. To date, the following flavonoids, derivatives of kaempferol: 3-*O*-α-arabinofuranoside, 3-*O*-β-glucopyranoside, 3-*O*-α-rhamnopyranoside, 3-*O*-α-rhamnopyranosyl (1→6)-*O*-β-glucopyranoside, and quercetin: 3-*O*-α-arabinofuranoside, 3-*O*-β-glucopyranoside, 3-*O*-α-rhamnopyranosyl (1→6)-*O*-β-glucopyranoside have been isolated and identified (4). The related literature also mentions the presence of tannins, coumarins, amines and amino acids (5, 6).

The aim of this study was to isolate the coumarins from the flowers of horse chestnut, identify them with the use of the UV, NMR, ESI-MS and also quantify them using Ultra Performance Liquid Chromatography (UPLC).

EXPERIMENTAL

Plant material

The flowers of *Aesculus hippocastanum* (horse chestnut), *Hippocastanaceae* were collected from the flowering trees in the Botanical Garden of Adam Mickiewicz University in Poznań in 2007 and dried in air under normal conditions. Voucher specimens were deposited at the Department of Pharmacognosy of the University of Medical Sciences in Poznań.

Extraction and isolation

The air-dried flowers of horse chestnut (600 g) were first extracted by maceration with MeOH at room temperature (the eighth day) and next, twice with MeOH (60°C, 2 h) and with 70% aq. MeOH (boiling temperature, 2 h). All of the obtained extracts had a similar qualitative composition (TLC, S_1, S_2), so they were combined. After evaporation of the solvent, the dry extract was purified by precipitation of ballast with hot water. The aqueous filtrate was successively reextracted with $CHCl_3$, and next with Et_2O and EtOAc.

The combined Et_2O and EtOAc fractions (similar qualitative composition in TLC, S_1, S_2) were separated by Medium Pressure Liquid Chromatography – MPLC (LiChroprep RP-18, eluents S_5, S_4 gradient with increasing amount of S_4). Selected

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fractions, containing compounds with blue fluorescence under $UV_{254\ nm}$ and $UV_{365\ nm}$, were separated by column chromatography on Sephadex LH-20, using S_4, and next, by preparative TLC on cellulose microcrystalline Avicel using S_5. Purification of all compounds was carried out on Sephadex LH-20, elution being performed with S_4. As a result, the following compounds were isolated: **I**, **II** and **III**.

Chromatographic analysis

TLC. Alufolien Cellulose (Merck, Germany): S_1 – HOAc–H_2O (15 : 85, v/v). Silica gel 60 (Merck, Germany): S_2 – EtOAc–HCOOH–HOAc–H_2O (100 : 11 : 11 : 26, v/v/v/v), S_3 – toluene–Et_2O (1 : 1, v/v, saturated with 10% acetic acid, upper phase) (6).

The spots of coumarins were detected under $UV_{365\ nm}$ before and after spraying the plates with 1%

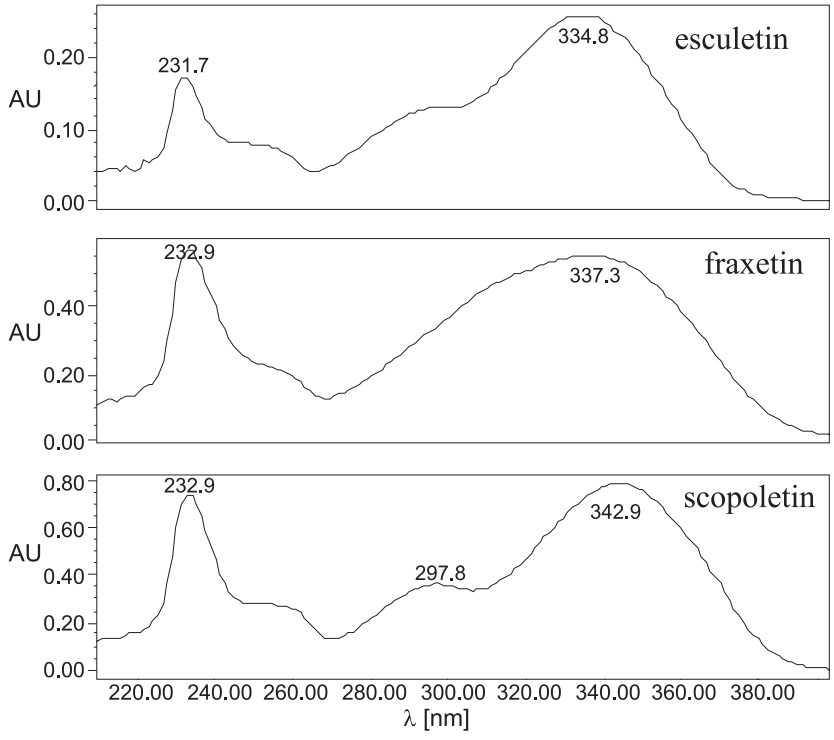


Figure 1. UV spectrum of esculetin, fraxetin, scopoletin

Table 1. ^{1}H NMR data of compounds **I**, **II**, **III** in CD_3OD, δ (ppm), *J* (Hz).

Carbon or protons number	I		II		III	
	δ C	δ H	δ C	δ H	δ C	δ H
2	162.9		164.1		163.76	
3	111.0	6.16 d (9.5)	112.5	6.12 d (9.4)	112.61	6.20 d (9.6)
4	144.7	7.77 d (9.5)	146.1	7.86 d (9.4)	146.76	7.83 d (9.6)
5	111.5	6.74		6.76 s	101.05	6.71 s
6	143.2					
7	150.8		147.2		147.17	
8	102.2	6.93 s	104.0	7.11 s		
9	149.1					
10	111.3		109.9		112.12	
6 (OCH_3)			56.9	3.90 s	56.79	3.89

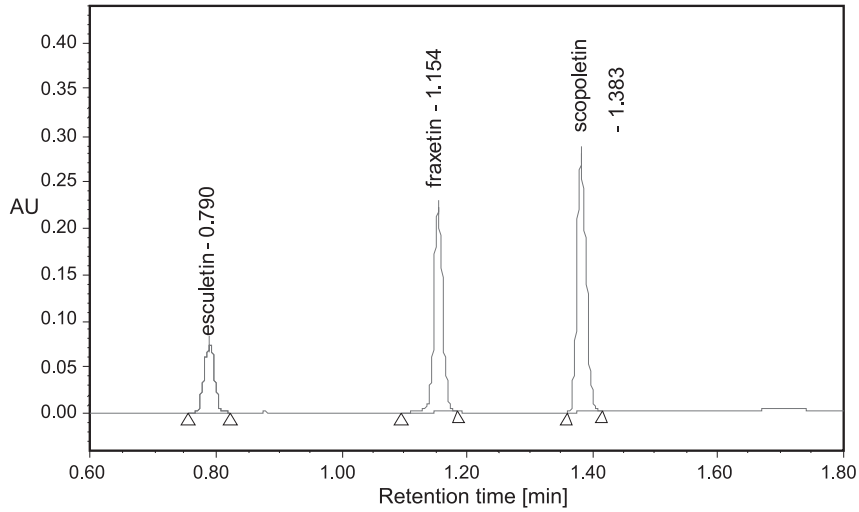


Figure 2. UPLC chromatogram of standards: esculetin (t_R = 0.790 min); fraxetin (t_R = 1.154 min); scopoletin (t_R = 1.383 min)

KOH reagents (1% methanolic KOH) (6).

PTLC. Microcrystaline cellulose Avicel® (Merck, Germany): S_4 – MeOH, S_5 – H_2O.

MPLC. LiChroprep RP-18 – 40–63 µm (Merck, Germany): S_5–S_4.

Identification

Spectral analysis (UV, ^{1}H NMR, ^{13}C NMR, ESI-MS)

UV spectra were recorded on a UV/VIS Perkin Elmer Lambda 35 spectrometer, in MeOH.

^{1}H (300 MHz) and ^{13}C (75.5 MHz) NMR spectra were recorded in CD_3OD-d_4 on a Varian Unity–300 MHz Spectrometer with TMS as an internal standard; chemical shifts are given in δ (ppm).

The ESI-MS were obtained using a Waters/Micromass (Manchester, United Kingdom) ZQ Mass spectrometer connected with HPLC (Waters typ 2690; Milford, USA) and spectrometer UV Photodiode Array Detector Waters 996 (λ = 200–500). ESI-MS analyses were performed in positive and negative ionization modes.

6,7-Dihydroxycoumarin (esculetin) (I)

Yellow amorphous powder (4 mg); m. p. 270°C; TLC R_f S_2 = 0.99, S_3 = 0.21. UV λ_{max}: MeOH 231.7, 334.8 (Fig. 1). ESI-MS (negative): m/z 177 $[M - H]^-$. ESI-MS (positive): m/z 179 $[M + H]^+$ calculated for $C_9H_6O_4$. ^{1}H and ^{13}C NMR data (Table 1).

7-Hydroxy-6-methoxycoumarin (scopoletin) (II)

Yellow amorphous powder (7 mg); m. p. 205°C; TLC R_f S_2 = 0.87, S_3 = 0.32. UV λ_{max}: MeOH 232.9, 297.8, 342.9 (Fig. 1). ESI-MS (negative): m/z 191 $[M - H]^-$. ESI-MS (positive): m/z 193 $[M + H]^+$ calculated for $C_{10}H_8O_4$. ^{1}H and ^{13}C NMR data (Table 1).

7,8-Dihydroxy-6-methoxycoumarin (fraxetin) (III)

White amorphous powder (3 mg); m. p. 232°C; TLC R_f S_2 = 0.80, S_3 = 0.05. UV λ_{max}: MeOH 232.9, 337.3 (Fig. 1). ESI-MS (negative): m/z 207 $[M - H]^-$. ESI-MS (positive): m/z 209 $[M + H]^+$ calculated for $C_{10}H_8O_5$. ^{1}H and ^{13}C NMR data (Table 1).

Quantification of coumarins by UPLC method

Standards and reagents

Esculetin, fraxetin and scopoletin were of HPLC-grade purity from Sigma-Aldrich (USA). Methanol (MeOH), acetonitrile (ACN), water (H_2O) and acetic acid (HOAc) were of HPLC grade purity (POCh, Gliwice, Poland).

Instrumentation for UPLC analysis

RP-UPLC was performed on an Acquity UPLC (Waters, USA) system comprising a pump (LO6UPB301M), an autosampler (KOHUPS072M), a thermostated column compartment, a vacuum degasser, a PDA-detector (KO6UPD883H), a 10-µL sample injector.

The extract was separated on 2.1 mm × 100.0 mm, 1.7 µm particle, Acquity UPLC™ BEH C18 (Waters).

UPLC procedure

The mobile phase consisted of solvent A [1% (v/v) HOAc in H_2O] and solvent B [ACN] with the elution profiles as follows: 0–0.5 min.: 95% A; 0.5–1.5 min: 85–75% A; 1.5–2.5 min: 75–70% A; 2.5–3.0 min: 70–95% A. The flow rate was 0.75 mL/min, the injection volume 5 µL, the temperature 45°C.

The coumarins were detected at 254 nm, corresponding to the λ_{max} of the analyzed compounds in the methanol solution.

Calibration curve

A standard stock solution of esculetin, scopoletin and fraxetin (10.00 µg/mL) was prepared in MeOH. One milliliter of each standard solution was taken, and then, all of them were mixed. One milliliter of the mixture contained: 3.33 µg of esculetin, 3.33 µg fraxetin and 3.33 µg of scopoletin. The obtained mixture of the standard substances was used to prepare calibration curves of esculetin, fraxetin and scopoletin. For each sample, three replicate assays were performed. The retention times for esculetin, fraxetin and scopoletin were 0.792–0.797, 1.154–1.158 and 1.375–1.385 min, respectively (Fig. 2).

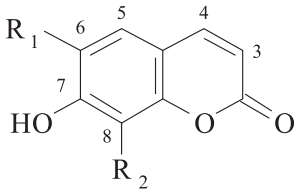
The calibration curves were defined for each compound in the range of sample quantity 0.01665–0.09999 µg. The linearity of the dependence of response on concentration was verified by regression analysis (Empower 2 Software – number of licence W6PKNK819OM).

The calibration curves of the dependence between the peak area and concentration of the standards were described by equations: $y = 1.3 \cdot 10^6 x$ ($r = 0.99899$) for esculetin, $3.31 \cdot 10^6 x$ ($r = 0.99879$) for fraxetin, $y = 4.13 \cdot 10^6 x$ ($r = 0.99932$) for scopoletin, where r = correlation coefficient.

The parameters of the equations of the least-squared regression and the times of standards are presented in Table 2.

Sample preparation for UPLC analysis

The dried and pulverized *Hippocastani flos* was accurately weighed (1000.0 mg) and extracted with 40 mL of MeOH for 1 h on a boiling H_2O bath under reflux. The obtained extract was filtered into a 100 mL volumetric flask and the plant material was again extracted with a new portion of MeOH (40 mL for 20 min). The combined extracts were diluted with MeOH to 100 mL. An extract of concentration corresponding to 10 mg of the plant material in 1 mL of the extract was obtained. A 5 mL portion of the extract was filtered through a 13 mm, 0.45 µm pore (PTFE syringe filter (Whatman, UK). The filtrate (5 µL) was injected into the UPLC apparatus, corresponding to 50 µg of the plant material in the sample. Analysis was performed after two sepa-



	R_1	R_2
Esculetin (**I**)	OH	H
Scopoletin (**II**)	OCH_3	H
Fraxetin (**III**)	OCH_3	OH

Figure 3. Structure of esculetin, scopoletin and fraxetin

Table 2. Parameters of the equations of the least-squared regression and the times of standards.

Standard	RSD [%]	R^2	Range of sample quantity [µg]	Retention time [min]
Esculetin	2.7294	0.9990	0.01665–0.09999	0.792–0797
Fraxetin	2.9817	0.9988	0,01665–0,09999	1.154–1.158
Scopoletin	2.2823	0.9993	0,01665–0,09999	1.375–1.385

Table 3. Results of the quantitative determination of esculetin, fraxetin ad scopoletin in flowers of *A. hippocastanum* using UPLC.

	Retention time [min]	Content of coumarins [% of dry weight]	S	S_x	RSD	μ
Esculetin	0.785–0.787	0.1273	0.0012	0.0005	0.0093	0.1273 ± 0.0012
Fraxetin	1.149–1.150	0.0507	0.0012	0.0005	0.0228	0.0507 ± 0.0012
Scopoletin	1.364–1.365	0.4133	0.0101	0.0041	0.0244	0.4133 ± 0.0106

Values in parentheses are relative standard deviation RSD (%) (n = 6, $t_{\alpha,f}$ (α = 0.05, f = 5) = 2.571)

rate extractions of each sample, and each extract was diluted and injected in triplicate (Table 3).

RESULTS AND DISCUSSION

The combined methanol and methanol-water extracts from the flowers of *Aesculus hippocastanum* L., after evaporation of the solvent, were partitioned between CH_3Cl, Et_2O, EtOAc. The combined Et_2O and EtOAc fractions, containing a complex of coumarin compounds, together with other constituents, were first separated by Medium Pressure Liquid Chromatography – MPLC (LiChroprep RP-18, eluent S_5–S_4 gradient with increasing amount of S_4). After the chromatographic analysis (TLC; S_1, S_2, S_3), appropriate fractions were separated by column chromatography on Sephadex LH-20, using S_4, and, next, by preparative TLC on cellulose microcrystalline Avicel using S_5. Fractions containing chromatographically homogeneous compounds were purified on Sephadex LH-20 column eluted with S_4. As a result, three compounds were isolated: **I**, **II** and **III** (Fig. 3).

The identification of the isolated coumarins was carried out by the spectroscopic methods: UV, ^{1}H, ^{13}C NMR and ESI-MS, and confirmed by co-chromatography with the standards (S_1, S_2, S_3).

Compounds **I**, **II** and **III** gave blue fluorescence, typical for coumarin compounds, under $UV_{254\ nm}$ and $UV_{365\ nm}$, changing into intensive blue (**I**, **II**) or yellow and blue (**III**) after spraying with KOH/$UV_{365\ nm}$ and NA-reagent/$UV_{365\ nm}$, respectively.

The UV spectra showed absorption characteristic for 6,7-dihydroxycoumarin (230–240 and 330–350 nm) (7).

In ^{1}H NMR spectra of **I**, **II** and **III**, two doublets at 7.8–7.9 ppm (H-4) and 6.2 ppm (H-3), with coupling constants J = 9.4–9.6 Hz, characteristic for *orto* configuration were present. In ^{1}H NMR spectra of **I** and **II** there were also two doublets at 6.9–7.1 ppm (C-8) and 6.7–6.8 ppm (C-5), whereas in **III**, only one singlet at 6.7 ppm (C-5) was present, which proves a different proton environment of this compound at C-8, yet the same one at C-5. The singlets at δ 3.90 ppm, in turn, provide evidence for the presence of methoxyl groups in **II** and **III**.

The signals in the spectra of ^{1}H NMR and in ^{13}C NMR are in agreement with the literature data for esculetin (**I**), scopoletin (**II**) and fraxetin (**III**) (8, 9). The ESI-MS spectra of compounds **I, II** and **III** recorded at negative and positive ion modes only showed molecular ions [M^+], which confirms the fact that the compounds are aglycones of molecular weight consistent with the proposed chemical formulas: **I** – 178, **II** – 192 and **III** – 208 m/z, which correspond to the weight of esculetin, scopoletin and fraxetin, respectively (10, 11).

As a result, simple coumarins: esculetin (6,7-dihydroxycoumarin), scopoletin (7-hydroxy-6-methoxycoumarin) and fraxetin (7,8-dihydroxy-6-methoxycoumarin) were isolated from the horse chestnut flowers and identified for the first time.

From among the numerous analytical methods of quantitative determination of coumarin compounds, the following are used: gas-liquid chromatography, polarography, electrophoresis-spectrophotometry, high performance capillary electrophoresis (HPCE) (12), TLC-densitometry (13). Most frequently, however, coumarins are determined with the use of reversed-phase high performance liquid chromatography (RP-HPLC), on C18 silica columns and with the isocratic or gradient elution with different mobile phases and with UV detection and different wavelength (13, 14–17). A variety of HPLC is the ultra performance liquid chromatography UPLC, in which higher pressure (1000 bar) is used to isolate and analyze the mixture of compounds. The advantage of UPLC over HPLC is greater sensitivity and resolution as partition is carried out with stationary phase molecules of smaller diameter (1.7 μm) in comparison with classical HPLC (5 μm), speed (the time of a single analysis is less than 1.5 min) and a much smaller

amount of reagents to be used, which may be especially useful in serial determination, since it enables to determine a lot of samples in a very short time.

In this study, UPLC was used to determine the content of the previously isolated and identified coumarin compounds. This is the first time when the method has been used to determine this group of compounds.

In order to quantitatively determine coumarins in the flowers of horse chestnut, mixed standards of esculetin, fraxetin and scopoletin were used to prepare calibration curves. Each calibration curve consisted of four different concentrations, showed good linearity ($r > 0.9988$) and was defined for each compound in the range of sample quantity 0.01665–0.09999 µg. Precision of integrated peak areas (RSD = 2.2823–2,29817%) were found to be satisfactory.

The methanol extract from the investigated material of concentration equal to 10 mg of the material/1 mL was subjected to analysis. The UPLC analysis was performed on C18 columns, with acetic acid in water and acetonitrile (in different gradient elution) as the mobile phase and detection at 254 nm (UPLC procedure).

As a result of the study, simple coumarins: scopoletin (0.4133 ± 0.0106%), esculetin (0.1273 ± 0.0012%) and fraxetin (0.0507 ± 0.0012%) (Table 3) were quantitatively determined in the flowers of horse chestnut using the UPLC method for the first time.

The coumarins, whose presence in the flowers of horse chestnut was demonstrated, display a variety of biological activity: esculetin and esculin (glycoside of esculetin) inhibit coagulation of blood, enhance the tones of veins and stimulate blood reflux (12). Scopoletin exerts nonspecific spasmolytic effects and reduces blood pressure (18). Esculetin, fraxetin and scopoletin also have anti-inflammatory activity; they are inhibitors of the proinflammatory lipoxygenase and cyclooxygenase pathways of arachidonate metabolism (19, 20).

The coumarins in the flowers of horse chestnut, as compounds with proven wide biological activity, may not only synergistically work with flavonoids, mainly within the range of blood vessel tightening, antithrombotic and anti-inflammatory activity, but also influence its pharmacological activity.

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