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Flavonoids in Horse Chestnut (*Aesculus hippocastanum*) Seeds and Powdered Waste Water Byproducts

IRENEUSZ KAPUSTA,[†] BOGDAN JANDA,[†] BARBARA SZAJWAJ,[†] ANNA STOCHMAL,[†] SONIA PIACENTE,[‡] COSIMO PIZZA,[‡] FEDERICO FRANCESCHI,[§] CHLODWIG FRANZ,[⊥] AND WIESLAW OLESZEK*,[†]

Department of Biochemistry, Institute of Soil Science and Plant Cultivation, State Research Institute, ul. Czartoryskich 8, 24-100 Pulawy, Poland, Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Via Ponte Don Melillo, 84084 Fisciano, Salerno, Italy, Indena S.p.A., Viale Ortles, 12, 20139 Milan, Italy, Leiter des Instituts für Angewandte Botanik und Pharmakognosie, Veterinärmedizinische Universität Wien, Veterinärplatz 1, A-1210 Wien, Austria

Horse chestnut extracts are widely used in pharmacy and cosmetic industries. The main active constituents are saponins of oleane type, but seeds of horse chestnut also contain flavonoids, being glycosides of quercetin and kaempferol. Their contribution to the overall activity of the extracts was not clear. In the present work, the main flavonoids from horse chestnut seeds were isolated and their structures established with spectral methods. Seven glycosides were isolated, out of which six (**2**, **3**, **4**, **7**, **11**, **13**) were previously reported and one (**9**) was identified as a new tamarixetin 3-*O*- [β-D-glucopyranosyl(1→3)]-*O*-β-D-xylopyranosyl-(1→2)-*O*-β-D-glucopyranoside. The structures of three additional compounds **1**, **10**, and **12**, not previously reported, were deduced on the basis of their LC-ESI/MS/MS fragmentation characteristics. A new ultraperformance liquid chromatographic (UPLC) method has been developed for profiling and quantitation of horse chestnut flavonoids. The method allowed good separation over 4.5 min. Thirteen compounds could be identified in the profile, out of which di- and triglycoisdes of quercetin and kaempferol were the dominant forms and their acylated forms occurred in just trace amounts. The total concentration of flavonoids in the powdered horse chestnut seed was 0.88% of dry matter. The alcohol extract contained 3.46%, and after purification on C18 solid phase, this concentration increased to 9.40% of dry matter. The flavonoid profile and their content were also measured in the horse chestnut wastewater obtained as byproduct in industrial processing of horse chestnut seeds. The total flavonoid concentration in the powder obtained after evaporation of water was 2.58%, while after purification on solid phase, this increased to 11.23% dry matter. It was concluded that flavonoids are present in a horse chestnut extract in a relatively high amount and have the potential to contribute to the overall activity of these extracts. Industrial horse chestnut wastewater can be used to obtain quercetine and kaempferol glycosides for cosmetic, nutraceutical, and food supplement industries.

KEYWORDS: *Aesculus hippocastanum*; horse chestnut; flavonoids; UPLC; determination

INTRODUCTION

Aesculus hippocastanum is a tree which grows in Iran, Northen India, Asia Minor, and Southeast Europe from the Balkans to the Caucasus, as well as in the U.S.A. It is also widely cultivated in parks and gardens for its bright white, yellow, or red flowers (*1*). This tree produces large seeds commonly known as horse chestnuts or buckeyes. A number of reports dating from the early 18th century have indicated therapeutic properties for horse chestnuts. These have ranged from antifever to, at the end of the 19th century, antihemorrhoidal properties (*2*). Horse chestnut is most often used as a treatment for venous insufficiency. This is a condition associated with varicose veins, when the blood pools in the veins of the legs and causes aching, swelling, and a sense of heaviness.

* Corresponding author. Tel. 48-81-8863421; Fax 48-81-8864547; E-mail: wo@iung.pulawy.pl
† Department of Biochemistry, Institute of Soil Science and Plant Cultivation.
‡ Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno.
§ Indena S.p.A.
⊥ Leiter des Instituts für Angewandte Botanik und Pharmakognosie, Veterinärmedizinische Universität Wien.

The principal extract and medicinal constituent of *A. hippocastanum* seeds is aescin, a mixture of triterpenoid saponin glycosides (*2*). Its components include glycosides of protoaescigenin and barringtogenol C. This can be fractionated into an easily crystallizing β-aescin mixture and the α-aescin, which is water-soluble (*3*). A number of other products have been isolated from chestnut seeds, i.e., coumarin derivatives (aesculin, fraxin, scopolin), essential oils (oleic acid, linoleic acid), and tanins (leucocyanidine, proanthocyanidin A₂) (*4*).

It is interesting that different organs of this species contain distinctive classes of the main bioactive principle: aescin in the seeds, essential oils in the leaves, and flowers and coumarins in the bark.

The extracts of horse chestnut show beneficial effects on venous insufficiency and have many positive pharmacological effects on the skin (*5*). The aescin is a potent anti-inflammatory principle, which also reduces capillary fragility and prevents leakage of fluids into surrounding tissues. These saponins have also been used in shampoos, shower foams, creams, lotions, and toothpastes.

The horse chestnut extracts have been demonstrated to be a potent scavenger of active oxygen. This showed the highest activity of 65 plant extracts tested, was more powerful than vitamin E, and exhibited a potent cell-protective effect linked to antiaging properties of antioxidants (*6*). The major principles of these activities are flavonoids present in the extracts. This group of secondary metabolites shows radical scavenging, antibacterial, and antiviral activities, and they are used in varicosis and capillary fragility. Recent work on flavonoids from the seeds of *Aesculus chinensis* demonstrated their activity against parainfluenza virus type 3 and influenza virus type A (*6*). Regarding the flavonoids from *Aesculus hippocastanum*, literature provides inconsistent data. The detailed work on structural characterization of flavonoids was performed by Hübner and co-workers and showed that they were glycosides of quercetin and kaempferol and some of them occurred as acylated forms (*8*). In the conclusion of this work, however, the authors pointed out that the concentration of these compounds in horse chestnut seeds is low (0.3%, calculated as rutin equivalent) and their contribution to the therapeutic efficacy of crude drug is unlikely. This statement remains inconsistent with the literature data cited above indicating the main role of horse chestnut flavonoids in radical scavenging activity of the extracts.

To further clarify these discrepancies and to shed more light on the possible role of horse chestnut flavonoids in overall activity of derived products, we deemed it of interest to reinvestigate their structures, to develop analytical methods for their profiling and quantitation, and to determine their concentrations in crude extracts from horse chestnut seeds and the flavonoid fraction purified by solid-phase extraction. We also analyzed the flavonoid composition and concentration in wastewater, which is a byproduct of the pharmaceutical industry producing aescin, to document whether these wastes could be effectively used as a source of flavonoid preparations.

MATERIALS AND METHODS

Spectroscopic Analysis. ESI-MS analyses were performed on a Thermo Finnigan LCQ Advantage Max ion-trap mass spectrometer with an electrospray ion source (Thermo Electron Corporation, Bellefonte, PA). Compounds were analyzed by direct injection by a syringe pump at a flow rate of 5 μL/min or by LC-MS/MS. An LC system consisting of a Finnigan Surveyor pump equipped with a gradient controller, an automatic sample injector, and a PDA detector was used. The separation was performed on a 250 × 4.6 mm i.d., 5 μm XBRIDGE C₁₈ column

(Waters, Poland). A mobile phase consisting of 0.05% acetic acid in water (B) and 0.05% acetic acid in acetonitrile (A) was used for the separation. The flow rate was kept constant at 0.5 mL/min. The system was run with a gradient program: 12% A to 50% A in 50 min. The spray voltage was set to 4.2 kV and a capillary offset voltage −60 V. All spectra were acquired at a capillary temperature of 220 °C. The calibration of the mass range (400–2000 Da) was performed in negative ion mode. Nitrogen was used as sheath gas, and the flow rate was 0.9 L/min. The maximum ion injection time was set to 200 ms. The exact masses were measured by a Voyager DE mass spectrometer. Samples were analyzed by matrix-assisted laser desorption–ionization time-of-flight (MALDITOF) mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18–39) at 2465.1989 Da and angiotensin III at 931.5154 Da as internal standard.

NMR spectra in CD₃OD were obtained using a Bruker DRX-600 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), operating at 599.19 MHz for ¹H and 150.86 MHz for ¹³C 2D experiments: ¹H–¹H DQF-COSY (double filtered direct chemical shift correlation spectroscopy), inverse detected ¹H–¹³C HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear multiple bond connectivity) were obtained using *UXNMR* software. Selective excitation spectra, 1D-TOCSY were acquired using waveform generator-based Gauss shaped pulses, mixing time ranging from 100 to 120 ms and a MLEV-17 spin–lock field of 10 kHz preceded by a 2.5 ms trim pulse.

Plant Material. Seeds of *A. hippocastanum* were collected at the Institute of Soil Science and Plant Cultivation's park in Pulawy, Poland, in September 2005, and the specimen voucher has been deposited at the Institute. The skins of the seeds were removed and the pulp was dried, finely powdered, and used for the successive extraction.

Horse chestnut wastewater powder (WWHC) was supplied by Indena, Italy.

Extraction. *A. hippocastanum* seeds that had been powdered (250 g) were extracted with 80% EtOH at room temperature. After 48 h, the extract was filtered and the residues were extracted one more time for 24 h. The extracts were combined, and the solvent was removed under reduced pressure to produce crude extract (HCE) (63 g).

Purification. The HCE (10 g) was suspended in water and loaded onto 6 cm × 10 cm, 40–63 μm LiChroprep RP-18 glass column previously preconditioned with water. The column was washed first with water to remove polar compounds, and then with 40% MeOH to elute flavonoids (HCF). This eluate was condensed under reduced pressure at 50 °C to produce crude flavonoid powder (3.67 g). The WWHC was dissolved in water and purified the same way as HCE to produce horse chestnut wastewater flavonoids (WWHCF).

Fractionation and Separation. The HCF powder was suspended in distilled water and the solution was applied to a 3 cm × 40 cm 40–63 μm LiChroprep RP-18 glass column (Millipore Corp., Bedford, MA). The column was developed with a 0–100% linear gradient of MeOH in water (Linear Gradient Former, Beckman Instrument Inc., Palo Alto, CA). Ten-milliliter fractions were collected, checked by TLC (Cellulose, Merck), developed in 15% acetic acid, and observed under UV (366 nm). Fractions showing similar TLC patterns (55 fractions) were further analyzed by UPLC. Fractions possessing one compound were combined and evaporated to dryness. Fractions containing more than one compound were further purified on a RP-18 glass column (2 cm × 50cm, 40–63 μm) using an isocratic system (MeCN–1% H₃PO₄) optimized for each fraction based on the analytical separation. This yielded several individual compounds:

Compounds **2** (10.8 mg), **3** (12 mg), **4** (5 mg), **7** (10 mg), **11** (7 mg), and **13** (6 mg) were obtained as yellow amorphous powders. Their spectral characteristics are presented in **Table 1**.

Compound **9** (8 mg) was obtained as amorphous powder; HRMALDITOFMS *m/z* 795.2019 [M+Na]⁺ (calcd for C₃₃H₄₀NaO₂₁, 795.2038). For ¹H and ¹³C NMR, see **Table 2**.

UPLC Analysis. The Acquity ultraperformance liquid chromatograph (Waters) consisting of binary solvent manager, sample manager, PDA detector, and *Empower Pro 2.0* software was used. The profiling was performed on an UPLC BEH C₁₈ column (1.7 μm, 50 mm × 2.1

Table 1. Ultraperformance Chromatographic Data of Analyzed Flavonoids and Their ESI/MS/MS Degradation Profiles

compound	R_t (min)	UV (nm)	ESI/MS/MS
1	1.15	266, 344	919 [M-H]⁻, 757 [M-Glc-H]⁻, 625 [M-Glc-Xyl]⁻, 595 [M-2Glc-H]⁻, 463 [M-2Glc-Xyl-H]⁻, 300 [qercetin-2H]⁻
2	1.26	265, 349	757 [M-H]⁻, 625 [M-Xyl-H]⁻, 595 [M-Glc-H]⁻, 463 [M-Glc-Xyl-H]⁻, 300 [quercetin-2H]⁻
3	1.75	255, 353	757 [M-H]⁻, 595 [M-Glc]⁻, 463 [M-Glc-Xyl-H]⁻, 301 [quercetin-H]⁻
4	1.91	255, 353	595 [M-H]⁻, 463 [M-Xyl-H]⁻, 301 [quercetin-H]⁻
5	2.27	265, 355	863 [M-H]⁻, 595 [M-Glc-105-H]⁻, 300 [quercetin-2H]⁻
6	2.48	265, 355	946 [M-H]⁻, 595 [M-Glc-189-H]⁻, 300 [quercetin-2H]⁻
7	2.54	265, 346	741 [M-H]⁻, 609 [M-Xyl-H]⁻, 447 [M-Xyl-Glc-H]⁻, 285 [kaempferol-H]⁻
8	2.68	265, 346	579 [M-H]⁻, 447 [M-Xyl-H]⁻, 285 [kaempferol-H]⁻
9	2.78	265, 355	771 [M-H]⁻, 639 [M-Xyl-H]⁻, 609 [M-Glc-H]⁻, 314 [M-Xyl-2Glc-H]⁻, 300 [quercetin-2H]⁻
10	2.95	266, 351	771 [M-H]⁻, 625 [M-Rha-H]⁻, 609 [M-Glc-H[⁻, 463 [M-Glc-Rha-H]⁻, 300 [quercetin-2H]⁻
11	3.32	255, 344	609 [M-H]⁻, 447 [M-Glc-H]⁻, [quercetin-H]⁻
12	3.65	266, 344	930 [M-H]⁻, 595 [M-335-H]⁻, 300 [quercetin-2H]⁻
13	4.14	264, 344	593 [M-H]⁻, 431 [M-Glc-H]⁻, 285 [kaempferol-H]⁻

Table 2. ^{13}C NMR and ^{1}H NMR Data of Compound **9** in CD$_3$OD[a]

carbon	^{13}C	^{1}H
2	158.2	
3	135.5	
4	178.7	
4a	106.0	
5	163.7	
6	99.6	6.23, d, J = 1.2
7	166.1	
8	93.9	6.42, d, J =1.2
8a	158.8	
1′	124.6	
2′	117.2	7.70, d, J =1.2
3′	147.4	
4′	151.7	
5′	111.4	7.07, d, J = 8.0
6′	123.0	7.75, dd, J = 1.2 and 8.0
OMe at C-4′	55.9	3.98, s
Glc$_1$ at 3		
1″	100.6	5.64, d, J = 7.5
2″	80.3	3.87, dd, J = 7.5 and 9.0
3″	87.7	3.82, dd, J = 9.0 and 9.0
4″	69.9	3.49, dd, J = 9.0 and 9.0
5″	77.9	3.30, m
6″	62.1	3.57, dd, J = 5.0 and 12.0
		3.75, dd, J = 2.5 and 12.0
Xyl at 2″glc		
1‴	104.4	4.91, d, J = 7.5
2‴	75.1	3.35, dd, J = 7.5 and 9.0
3‴	77.8	3.33, dd, J = 9.0 and 9.0
4‴	71.0	3.54, m
5‴	66.7	3.26, dd, J = 11.0 and 11.0
		3.95, dd, J = 4.5 and 11.0
Glc$_2$ at 3″glc		
1⁗	104.2	4.71, d, J = 7.5
2⁗	75.2	3.32, dd, J = 7.5 and 9.0
3⁗	77.7	3.42, dd, J = 9.0 and 9.0
4⁗	71.4	3.33, dd, J = 9.0 and 9.0
5⁗	77.8	3.39, m
6⁗	62.1	3.67, dd, J = 5.0 and 12.0
		3.92, dd, J = 2.5 and 12.0

[a] Assignments were confirmed by COSY, HSQC, and HMBC experiments.

mm) utilizing a gradient elution profile and a mobile phase consisting of 0.1% acetic acid in water and 40% MeCN. The column was maintained at 50 °C at a constant flow rate of 0.35 mL/min. One gram of dried and finely powdered horse chestnut seeds was extracted overnight with 50 mL of 80% EtOH at room temperature. The extract was filtered and the residues were additionally extracted twice by refluxing with 50 mL of 80% EtOH for 1 h. The extracts were combined, and the solvent was removed under reduced pressure. The

crude extract was suspended in water (10 mL), and a 2 mL portion was passed through a C$_{18}$ Sep-Pack cartridge (Waters Associates) preconditioned with water. The cartridge was washed first with water to remove sugars and then with 40% MeOH to elute phenolics. This fraction was evaporated and redissolved in MeCN–H$_2$O (2:8, 1 mL) for analyses.

For wastewater material, 100 mg of powder was suspended in water, purified on Sep-Pack, and used for analysis. The location of individual compounds in the profile was performed by spiking the extract with a purified standard. Calculations were performed on the basis of external standard curves obtained for particular compounds.

RESULTS AND DISCUSSION

The extraction of horse chestnut seeds with aqueous ethanol followed by low-pressure column chromatography afforded several individual flavonoids. Their structures were confirmed by mass spectrometry and ^{1}H and ^{13}C NMR. In this way, seven flavonoids were identified. These included six known compounds **2**, **3**, **4**, **7**, **11**, and **13** (**Figure 1**) previously identified in *A. hippocastanum* (*8*) and some also in *A. chinensis* (*7*). Their MS and NMR data were identical to those previously reported.

The pseudomolecular ion for compound **9** was at *m/z* 771. The fragmentation ions at *m/z* 639 and 609 indicated that hexose and pentose were two terminal sugars. The fragmentation patterns were similar to those observed for flavonoid **3**, but the ion corresponding to the aglycone was at *m/z* 315, which was 14 mu higher than for quercetin. Thus, the MS data suggested that the sugar sequence in compound **9** was identical to that in compound **3**, but the aglycone was methylated quercetin. This assumption was further proved by NMR experiments.

In the ^{1}H NMR spectrum of compound **9**, two signals at δ 6.23 (1H, d, J = 1.2 Hz) and 6.42 (1H, d, J = 1.2 Hz) attributable to H-6 and H-8 of a flavonoid skeleton and three signals at δ 7.07 (1H, d, J = 8.0 Hz), 7.70 (1H, d, J = 1.2 Hz), and 7.75 (1H, d, J = 1.2 and 8.0 Hz) typical of a 1,3,4-trisubstituted ring B were evident, along with a signal at δ 3.98 (3H, s), indicative of a methoxy group. Further features were the signals of three anomeric protons at δ 5.64 (1H, d, J = 7.5 Hz), 4.91 (1H, d, J = 7.5 Hz), and 4.71 (1H, d, J = 7.5 Hz). The remaining sugar signals were overlapped in the region between δ 3.95 and 3.26. On the basis of ^{1}H and ^{13}C NMR data (**Table 2**), the aglycone of **9** was identified as 4′-*O*-methylquercetin, known as tamarixetin. One-dimensional TOC-SY spectra obtained by selectively irradiating the anomeric protons at δ 5.64 (H-1Glc$_1$) and 4.71 (H-1Glc$_2$) showed in both

Figure 1. Chemical structures of isolated flavonoids.
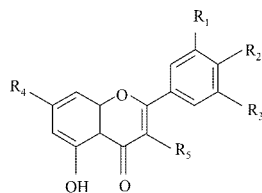
Comp.	R_1	R_2	R_3	R_4	R_5
1	OH	Glc	H	OH	Xyl-Rha-Glc
2	OH	Glc	H	OH	Xyl (1-2)Glc
3	OH	OH	H	OH	Xyl (1-2)[Glc (1-3)]Glc
4	OH	OH	H	OH	Xyl (1-2)Glc
5	OH	Glc-nicotynoyl	H	OH	Xyl (1-2)Glc
6	OH	Glc-indolin-2-on-3hydroxy-3-acetyl	H	OH	Xyl (1-2)Glc
7	H	OH	H	OH	Xyl (1-2)[Glc (1-3)]Glc
8	H	OH	H	OH	Xyl (1-2)Glc
9	OCH3	OH	H	OH	Xyl (1-2)[Glc (1-3)]Glc
10	OCH3	Glc	H	OH	Xyl (1-2)[Glc (1-3)]Glc
11	OH	OH	H	OH	Glc-Rha
12	OH	Glc-indolin-2-on-3-acetyl	H	OH	Xyl (1-2)Glc
13	H	OH	H	OH	Glc-Rha

cases the typical spin system of a β-glucopyranosyl unit, while 1D-TOCSY resulting from the selective irradiation of the signal at δ 4.91 yielded the subspectrum of a β-xylopyranosyl unit. HSQC experiments, which correlated all the proton resonances with those of each corresponding carbon, allowed the identification of the glycosidation sites. Glycosidation shifts were observed for C-2 Glc_1 (δ 80.3) and C-3 Glc_1 (δ 87.7). On the basis of the HMBC correlations between H-1 Glc_1 (δ 5.64) and C-3 of the aglycone (δ 135.5), H-1 Xyl (δ 4.91) and C-2 Glc_1 (δ 80.3), H-1 Glc_2 (δ 4.71) and C-3 Glc_1 (δ 87.7), compound **9** was determined as tamarixetin 3-*O*-[β-D-glucopyranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→2)-*O*-β-D-glucopyranoside and to the best of our knowledge this is a new compound not identified in any other plant species.

The isolated compounds were used for the development of the chromatographic method for their profiling and determination, as no chromatographic profile of flavonoids in horse chestnut had been published so far. The ultraperformance liquid

chromatography (UPLC) was used successfully to separate all the compounds. The separation was performed in a very short run, lasting just 4.5 min (**Figure 2**). Spiking the extract with isolated flavonoids allowed their location in the UPLC profile. It was evident that all isolated compounds were dominant flavonoids of horse chestnut extract, but careful examination of the profile with a photodiode array detector showed that it contained several minor compounds showing typical flavonoid absorption spectra. To characterize, at least temporarily, these minor flavonoids, LC-MS/MS experiments were performed, using the same type of column as in the UPLC technique. This allowed us to obtain exactly the same sequence in the profile but during a 50 min run, and the unknown compounds could be characterized from the MS/MS fragmentation patterns in comparison to the isolated flavonoids.

Thus, compound **1** had a pseudomolecular ion at m/z 919, which was by 162 mu units higher than for flavonoid **2**. The fragmentation ions at m/z 757 [M-hexose-H]$^-$, 625 [M-hexose-pentose]$^-$, 595 [M-2hexoses-H]$^-$, and 463 [M-2hexoses-pentose-H]$^-$ indicated that the molecule contained three terminal sugars: 2 hexoses and a pentose. The aglycone ion at m/z 300 and not 301 as usually occurs for quercetin provided information that one of the terminal sugars was in the C3′ position. Such a fragmentation had been reported previously for ring B-substituted quercetin; in an ESI ion source, two protons of C3′ and C4′ are lost when quercetin is substituted with a sugar (9). A similar effect was observed in the present research for 3′Glc-substituted compounds, e.g., flavonoid **2**. Substitution at the C3′ position was also evident from the UV spectrum. The maximum absorption of band II was shifted from 255 nm characteristic for C3′ unsubstituted compounds, e.g., **3** and **4**, to 265 nm characteristic for substituted flavonoids, e.g., **2**. On the basis of these deductions and taking into account that all isolated horse chestnut flavonoids contained glucose and xylose, the structure of flavonoid **1** was proposed to be quercetin 3-*O*-[β-D-glucopyranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-3′-*O*-β-D-glucopyranoside. This compound had not been reported before in any plant species.

Compounds **5** and **6** showed UV spectra and m/z 300 characteristic for C3′-substituted quercetin glycosides. Their pseudomeolecular ions were found at m/z 863 and 946, respectively. In the MS/MS spectrum, fragmentation ions at m/z 595 were found in both flavonoids. This suggested that these two flavonoids differ in the substitution at ring B, as strong ion m/z 595 was indicative of sugar sequence 3-*O*-glc-xyl as observed in flavonoid **4**. The loss of 267 mu in compound **5**
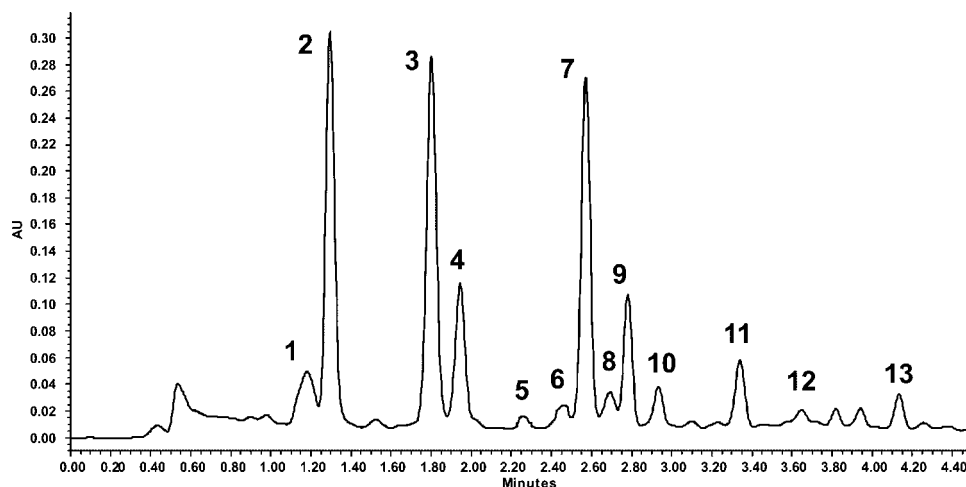

Figure 2. Ultraperformance liquid chromatography profile of horse chestnut flavonoids.

Aesculus hippocastanum flavonoids

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Table 3. Total Concentration of Flavonoids in Horse Chestnut Seed, Horse Chestnut Wastewater, and Their Fractions

sample	fraction	concentration of flavonoids (% dry matter)
horse chestnut seed	HCP	0.88
	HCE	3.46
	HCEF	9.40
horse chestnut waste water	WWHC	2.58
	WWHCF	11.23

was interpreted as a fragment 162 + 105 mu, which corresponds to 6-*O*-nicotynoyl-glucose. Similarly, the loss of 351 mu in compound **6** was interpreted as a fragment 162 + 189, which corresponds to 6-*O*-[indolin-2-on-3-hydroxy-3-acetyl]glucose. These two acylated quercetin glycosides were previously identified in horse chestnut (*8*).

Flavonoid **10** had pseudomolecular ion at *m*/*z* 771, identical to compound **9** described above, both in the sugar chain and the aglycone part. The only difference was the intensity of the ion at *m*/*z* 609, corresponding to the loss of glucose. In compound **9**, this ion was four times stronger than the ion at *m*/*z* 639, corresponding to the loss of xylose. At the same collision energy, ions at *m*/*z* 609 and 639 in compound **10** had the same intensity. This indicated that in **10** there are two terminal sugars, glucose and xylose, but the glucose is substituted in a different position than in **9**. Comparison of intensities of these two ions in **10** and **3** showed high similarity. From these data, the structure of flavonoid **10** was deduced as tamarixetin 3-*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-xylopyrano-syl-3′-*O*-β-D-glucopyranoside.

Another minor compound in the profile marked with the number **12** showed UV spectrum characteristic for flavonoid. Its mass spectrometry fragmentation pattern was similar to those of compounds **5** and **6.** The presence of the ion at *m*/*z* 595 indicated the loss of 335 mu, which corresponds to the hexose and acylating agent with 173 mu. This acetylating agent was 16 mu lower than the indolin derivative of compound **6**. Considering the lower polarity of compound **12** in comparison to **6**, and 16 mu lower mass, it can be deduced that the acylating agent is indolin-2-on-3-acetyl, but further spectral proof is needed.

The UPLC profile of horse chestnut flavonoids showed that di- and triglycosides of quercetin and kaempferol were the major components of the mixture and the acylated forms occurred just in trace amounts. The total concentration of the flavonoids in horse chestnut seeds (HCP) was 0.88% of dry matter (**Table 3**). This remains in disagreement with the data published before (*8*), showing that the content of flavonoids was ca. 0.3% (calculated as rutin, but no information on the method of determination was presented). In the cited paper, Hübner and co-workers concluded that flavonoid concentration in horse chestnut is very low and their contribution to the therapeutic efficacy of horse chestnut-derived drugs seems unlikely. The present research performed with a precise analytical method showed that the real concentration of flavonoids in HCP is more than two times higher than previously reported. Besides, it is not a horse chestnut powder used in therapy but its extracts. The alcohol extract contained nearly 3.5% of flavonoids in relation to its dry matter, which is a considerable concentration that may influence the overall activity of the extract. This concentration could be further increased to nearly 10% of dry matter when the extract was purified by solid-phase extraction on a reverse-phase C18 column.

In the present research, we also studied the flavonoid profile and concentration in the powder obtained after evaporation of water from horse chestnut wastewater (WWHC). The large volume of WWHC remains in the process of precipitation of aescin from the horse chestnut extracts. This is waste byproduct still containing some amount of saponins and flavonoids, which can be commercially useful.

The flavonoid profile of WWHC was identical to the profile of HCE, with the total concentration of 2.54% dry matter. One-step purification of this fraction on the solid phase produced WWHCF, the product with flavonoid concentration of 11.23% dry matter. This has been a quite high amount, and the product can have commercial value. It can be used in the pharmaceutic, cosmetic, and food industries. Eleven percent concentration of flavonoids makes the product an attractive source for the nutraceutic industry as a high-flavonoid supplement. As shown above, horse chestnut flavonoids are the mixture of quercetin and kaempferol glycosides, the most desired flavonoids in our diet due to their antioxidant activity. Quercetin and kaempferol were documented to be the most potent antioxidant of all fla-vonoids, (*10*) and their daily consumption is estimated to be about 25 mg per day (*11*). Further increase of this amount in the diet can be obtained by flavonoid supplementation, and WWHCF can be a good source of this. The economic evaluation of this source should be performed. It seems logical that the WWHC, instead of being dried and powdered, can be simply passed through a solid-phase column (reversed-phase C18 or Amberlite XAD4), which retains flavonoids and lets saccharides and other strongly polar components of the WWHC matrix go through.

It can be concluded that the flavonoid content in horse chestnut seed seems to be high enough to contribute to overall activity of the extracts. The high content of flavonoids in industrial horse chestnut wastewater and the ease of their condensation and purification makes this byproduct a promising source of quercetin and kaempferol glycosides to be used in the cosmetic and food additive-producing industries.

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