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HPLC Analysis of Esculin and Fraxin in Horse-Chestnut Bark *(Aesculus hippocastanum L.)*

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Selective and sensitive HPLC method was used for simultaneous determination of esculin and fraxin in a methanolic extract of horse-chestnut bark (*A. hippocastanum L.*). The samples were separated on a LiChrospher RP 18 column (150 × 4 mm i.d.) with the mobile phase consisting of acetic acid, 1%, and methanol (84:16 *v*/*v*) at a flow rate of 1.0 mL/min and quantified by measuring the UV absorbance at 340 nm. The assay of esculin and fraxin is linear over the range 0.02–2 mg/mL. It was shown that the bark gathered from older branch sections (5 cm in diameter) during the four seasons contained the greatest amount of esculin (3.6–6%) and fraxin (1.5–2.6%). Nevertheless, the bark of horse-chestnut tree (40 cm in diameter) was found to be the richest source of esculin (7.9%) as well as of fraxin (3.1%). In contrast, the bark of young shoots contained the lowest amount of esculin (0.4–0.8%) and fraxin (0.2–0.3%).

Key words: Horse-chestnut bark, esculin, fraxin, seasonal variation, reversed-phase high- performance liquid chromatography, UV detection

INTRODUCTION

Commonly known as a decorative tree, horse-chestnut (*A. hippocastanum L.*, *Hippocastanaceae*) is also a valuable medicinal plant used in popular as well as in official medicine. Almost every part of this species (folium,

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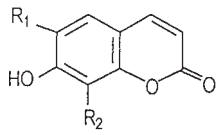
flos, semen, cortex) represents a certain plant drug that might be applied for healing diverse disorders. Modern phytotherapy pays special attention to the drug Semen hippocastani with venoprotective properties based on the activity of saponine escine.[1,2]

It is interesting that different organs of this species contain a distinctive class of the main bioactive principle: escine in the seeds, flavonoids in leaves and flowers[3] and coumarins in the bark.[4,5] Characteristic active components in horse-chestnut bark (*Cortex hippocastani*) are the coumarin glycosides esculin and fraxin.

These substances possess various biological activities. The dominant component esculin is a well-known natural UV-B protective agent and one of the phytomedicines used for the treatment of various peripheral vascular disorders.[6,7] In recent years there has been evidence of antiflogistic,[8–11] cytostatic,[12,13] and antimutagenic properties[14] of esculin or its aglycone esculetin. Likewise, spasmolytic and diuretic properties are attributed to the accompanying glycoside fraxin.



ESCULIN: R_1 = O - glucose; R_2 = H

FRAXIN: $R_1 = OCH_3$; R_2 = O - glucose

Figure 1. Structural formula of esculin and fraxin.

Different analytical methods have been proposed for the quantitative determination of 7-hydroxycoumarins in drugs or in phytopreparation: spectrophotometry,[15,16] densitometry,[17] spectrofluorimetry,[18–21] and high-performance liquid chromatography.[22–25]

Literature data about the content of esculin in the horse-chestnut bark vary in the wide range from 0.7% to 4%. However, it is known that the content of active component in drugs does not depend only on the analytical technique applied but rather on the growing period and the age of the plant part collected.

Since an accurate and precise quantitative method is a prerequisite for gathering plant material of appreciable quality as well as for pharmacological examination of standardized drug preparations, we applied an isocratic HPLC method to the separation and determination of esculin and fraxin in

the extract of horse-chestnut bark without previous purification. The content of these substances was followed throughout the four seasons in bark samples of different ages.

EXPERIMENTAL

Material and Methods

Plant material: The bark of horse-chestnut (*A. hippocastanum L.*) was collected in the Botanical garden of medicinal plants (Faculty of Pharmacy and Biochemistry, University of Zagreb) during the four seasons from several sections of branches, *i.e.* from the apex with 1 cm in diameter to the part of 5 cm thickness. In addition, bark samples from a trunk of 40 cm in diameter as well as young shoots grown from seeds and tree-stump were taken in summer. In order to find out the distribution of coumarin glycoside throughout the bark tissue, one sample of the bark was divided in two sections: an external (cork) and an internal one. All samples were air-dried and chrushed to powder.

Solvents and reference substances: Methanol for chromatography, LiChrosolv, was obtained from Merck (Darmstadt, Germany). Glacial acetic acid, analytical grade, obtained from Kemika (Zagreb, Croatia) and distilled deionised water were purified using the Milli-Q system (Millipore, Bradford, MA, USA). The mobile phase, consisting of 1% acetic acid in water and methanol (84:16, *v/v*), was degassed with helium for 15 minutes before analyses.

Esculin sesquihydrate, 98% (HPLC), was purchased from Fluka (BioChemika, Buchs, Switzerland) and fraxin was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Drug extracts and standard solutions: Optimal extraction procedure was established during preliminary studies, varying the degree of fineness of powdered drug and the concentration of methanol in water between 80% and 100% as well as changing the drug and the solvent ratio. Optimal extraction was performed by heating 0.50 g of fine powdered drug with 20 mL of methanol on a water bath under reflux for one hour. The methanol extract was filtered quantitatively into a volumetric flask and the volume was adjusted to 25 mL with methanol.

Esculin and fraxin were dissolved in methanol to give stock solutions of approximately 5 mg/mL of free base equivalents. The stock solutions were further diluted with methanol to provide solutions in the range concentrations of 0.2–2.0 mg/mL in order to establish the linearity of the method. All standard solutions were stored at 4 °C.

HPLC analyses: Separation and determination of esculin and fraxin was performed with a PU liquid chromatographic system (Pye Unicam, Cambridge, UK) on a HPLC column (125 × 4 mm i.d.) and precolumn (20 × 4 mm i.d.) packed with LiChrospher RP 18, 5 m (Merck, Darmstadt, Germany). The flow rate of the solvent was 1 mL/min. Injection volume was 5 μL. The column eluent was monitored by a PU 4025 UV detector at 340 nm. Chromatograms were recorded on a PU 4811 computing integrator. A weighted least squares regression was fitted to each calibration curve. The fitting algorithm does not force the curve through zero.

RESULTS AND DISCUSSION

It is generally cited for drugs named »cortex« that they are gathered in spring from young stems and branches. Contrary to expectation, in preliminary semiquantitative TLC analysis, we observed that the older horsechestnut bark (*Aesculus hippocastanum L.*) from thicker parts of branches contained more esculin than younger sections. In order to establish the content variability of the dominant coumarin glycosides esculin and fraxin in the drug *Cortex hippocastani,* we collected bark samples during the four seasons from easily accessible branches.

Extraction procedure includes the process of continuous extraction of finely powdered drug with methanol. Pure methanol was chosen in order to avoid simultaneous extraction of polar ballast substances from the plant material. The advantage of continuous extraction is that only a small volume of solvent is necessary and tedious evaporation of large volumes of solvent can be avoided. In addition, this is also a much more efficient and more rapid procedure than percolation or general successive extraction.

For the separation and determination of esculin and fraxin in methanolic extracts of bark samples, high-performance liquid chromatography in the reversed-phase mode was applied as a reliable, accurate and sensitive method. The coumarins absorb light at 340 nm, which conveniently excludes most other aromatic chromophores and enables selective detection of the desired compounds.

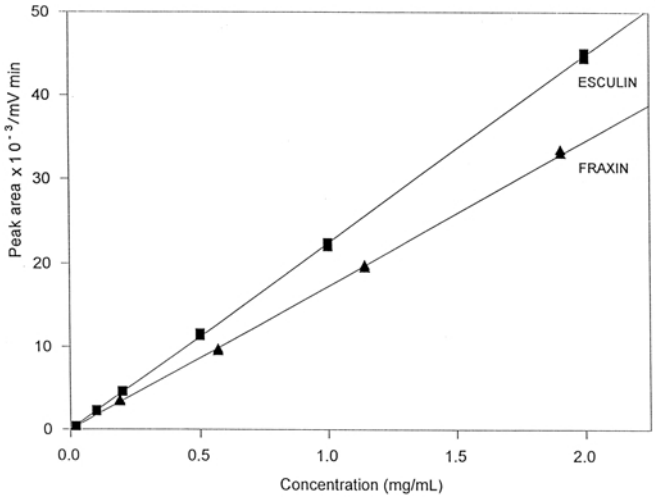


Figure 2. Linearity plot and linear regression analysis data of UV detector signal versus standard solutions of esculin (E) and fraxin (F).

The linearity of the method was tested with a series of standard solutions of esculin and fraxin. Calibration lines obtained in the concentration range of 0.2 – 2.0 mg/mL are presented in Figure 2. For esculin it is of type $y = 22.365\,x + 0.0183$ with correlation coefficient $r = 0.9999$ and for fraxin $y = 17.398\,x + 0.1242$ with correlation coefficient $r = 0.9997$.

Representative chromatograms, one of methanolic extracts of horse-chestnut bark as well as one of esculin and fraxin standard solutions are presented in Figure 3.

Each analytical result shown in Table I represents the mean value (CV 2%) of three parallel HPLC determinations of coumarin contents in tested bark samples.

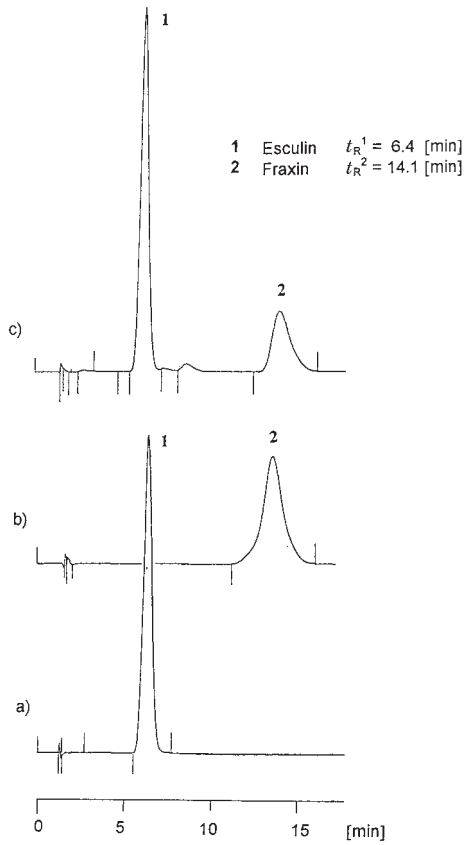


Figure 3. Representative chromatograms of esculin (a) and fraxin (b) standard solutions and of methanolic extracts of horse-chestnut bark (c). For chromatographic conditions see text.

TABLE I

Content (g%) of esculin (E) and fraxin (F) in horse-chestnut bark from branch sections of different thickness (1–5 cm) during the four seasons

	1 cm		2 cm		3 cm		4 cm		5 cm	
Season	E	F	E	F	E	F	E	F	E	F
Spring	1.06	0.45	2.44	0.96	2.44	1.11	3.50	1.76	4.10	1.86
Summer	3.01	1.04	4.75	1.77	4.21	1.83	4.56	2.04	5.96	2.62
Autumn	1.66	0.71	1.98	0.86	2.88	1.25	3.15	1.33	3.61	1.50
Winter	2.53	0.89	2.61	1.19	3.28	1.48	4.14	1.72	4.11	1.53

It is obvious that the highest content of esculin and fraxin was found in the oldest bark samples (branch diameter of 5 cm) during the whole growing period. The values differed from 3.61% in autumn to 5.96% in summer for esculin and from 1.53% to 2.62% for fraxin. In the same period, the coumarin content in the youngest bark (branch diameter of 1 cm) varied in a range from 1.06% to 3.01% for esculin and from 0.45% to 1.04% for fraxin.

As regards the quantities of esculin and fraxin throughout the seasons, it was found that all summer samples were the richest plant material (Figure 4). In contrast, the samples from autumn or spring contained the lowest amount of these substances. It is also evident that the ratio of fraxin and esculin varied from about 1:2 to 1:3 in all samples analyzed.

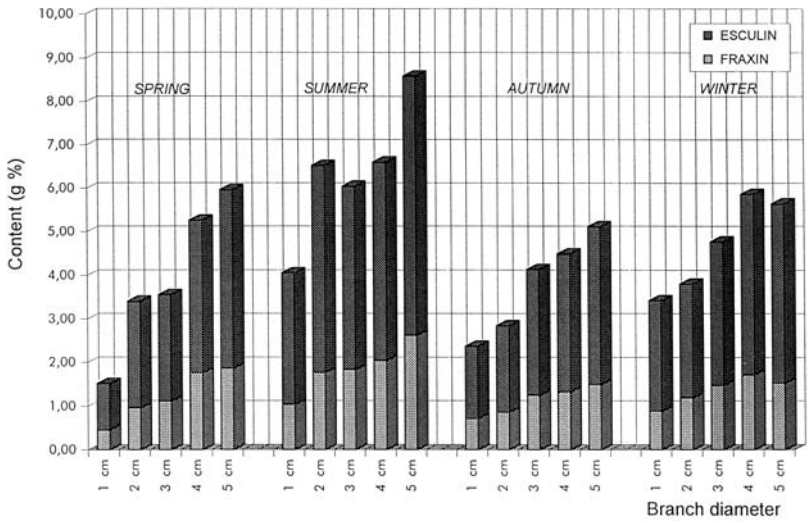


Figure 4. Seasonal variation of the total amount of esculin and fraxin in horse-chestnut bark from branch sections of different diameter.

Interesting results were obtained from some additional analyses. For example, the bark of horse-chestnut tree (40 cm in diameter) appeared to be the richest source of esculin (7.9%) as well as of fraxin (3.1%). On the other side, the lowest amount of coumarin glycosides was found in the bark from young shoots. Thus, the bark of spring shoots grown from horse-chestnut seeds contained only 0.4% esculin and 0.2% fraxin. Similarly, the bark of young shoots grown from tree-stump contained 0.8% esculin and 0.3% fraxin. The analysis of the internal and external zones of one bark sample (summer, 3 cm in diameter) showed that the external bark layer (cork) contained much more esculin (5.1%) and fraxin (1.9%) than the internal one (1.9% esculin and 1.1% fraxin).

In addition, it should be pointed out that methanolic extracts stored at 4 °C were very stable, having the same qualitative and quantitative composition after six months.

In conclusion, our research has confirmed the necessity of developing an appropriate analytical method for the quantification of plant constituents before the collecting or harvesting process. The simple and reliable method presented here can be applied successfully to the monitoring and determination of these and related hydroxycoumarins in various plant species or plant preparations.

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SAŽETAK

HPLC analiza eskulina i fraksina u kori divljeg kestena (*Aesculus hippocastanum* L.)

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Za istodobno određivanje eskulina i fraksina u metanolnom ekstraktu kore divljeg kestena (*A. hippocastanum* L.) primjenjena je selektivna i osjetljiva metoda HPLC.

Uzorci su razdvajani na koloni LiChrospher RP 18 (150 × 4 mm i.d.), s pokretnom fazom koja se sastojala od 1%-tne octene kiseline i metanola (84:16 *v*/*v*), uz protok od 1.0 mL/min i određeni mjerenjem UV apsorpcije na 340 nm. Koncentracijski odziv eskulina i fraksina linearan je u području od 0.02–2 mg/mL. Pokazalo se da starije grane (promjer 5 cm) u kori sadržavaju najveću količinu eskulina (3.6–6%) i fraksina (1.5–2.6%) kroz sva četiri godišnja doba. Ipak, najbogatiji izvor eskulina (7.9%) i fraksina (3.1%) jest kora sa stabla divljeg kestena (promjer 40 cm). Nasuprot tome, kora mladih izdanaka sadržava najmanje količine eskulina (0.4–0.8%) i fraksina (0.2–0.3%).