



COMPARATIVE QUANTIFICATION OF ESCIN FROM DIFFERENT PRODUCTS

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The paper aimed to quantify the saponins expressed in escin, the major bioactive constituent of *Aesculus hippocastanum*, from five different pharmaceutical formulations: two types of tablets, and one type of gel, tincture, and glycerol-hydroalcoholic extract. The products are part of over the counter drugs and dietary supplements categories. Two spectrophotometric methods were used to quantify the escin, after separation from the other active ingredients and excipients. The first method, calibration curve method, is based on the reaction between the oxidized triterpenoid saponins and vanillin and records the absorbance at 560 nm. The limit of detection, limit of quantification, and the RSD values were calculated. The second method is based on the molar absorptivity of escin. Both methods have proved to be suitable for the determination of escin in different products.

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Introduction

Saponins are secondary metabolites of plants with important pharmacological properties, like antibacterial,¹⁻³ cytotoxic,^{3,4} immunostimulant,⁵ antidiabetic,⁶ anti-inflammatory and antiulcerogenic,⁷ antioxidant⁸ etc. Saponins are amphiphilic compounds and tend to form mixed aggregates in solution, making their analysis difficult.⁹ For saponins analysis, the literature provides several types of methods, among—including spectrophotometric methods,¹⁰ TLC,^{11,12} HPLC (with different detection methods: UV, MS, ELSD),¹³⁻¹⁷ gas chromatography¹⁸ and capillary electrophoresis¹⁹.

Escin is a complex mixture of triterpenoid saponin glycosides, which is mainly found in *Aesculus hippocastanum* (horse-chestnut).²⁰ The mixture consists of α -escin and mainly β -escin isomers.²¹ The actions of escin reported in various studies are anti-inflammatory,²¹ anti-edematous,²¹ venotonic,^{21,22} anti-cancer,^{20,23} and antiallergic²⁴ properties. Spectrophotometric methods,²⁵ TLC,^{26,27} HPLC,^{28,29} etc. can be used for analysis of escin.

The purpose of this paper was to determine the escin by fast, simple, cheap spectrophotometric methods that are easily available to most laboratories. The samples taken for testing included horse-chestnut extract in combination with flavonoids like diosmin, rutin, hesperidin, or with acerola fruit extract (*Malpighia glabra*), butcher's broom (*Ruscus aculeatus*), common bilberry (*Vaccinium myrtillus*), centella (*Centella asiatica*) and vitamin C. The product package provides their utilization especially for: restoring and maintaining the tonus of the vascular walls, the functionality and elasticity of the veins, the capillary resistance and permeability, the vascular elasticity and blood circulation of the legs, and for relief of leg heaviness.

Experimental

Materials

Escin, standard substance, was supplied from Merck (Germany). All the reagents used were analytical grade reagents. The products for testing, dietary supplements and over the counter drugs, were purchased from pharmacy and herbal stores and they consisted of two products in the form of coated tablets, sample 1 comprising of dried horse-chestnut extract 250 mg/per unit expressed in 50 mg/tablet escin, butcher's broom extract (*Ruscus aculeatus*), common bilberry (*Vaccinium myrtillus*), centella (*Centella asiatica*), vitamin C, hesperidin and sample 2 comprising of dried horse-chestnut extract 200 mg/tablet, micronized diosmin, rutin trihydrate, acerola fruit extract (*Malpighia glabra*). Sample 3 was gel type consisting of *Aesculus hippocastanum* extract, *Ruscus aculeatus* extract, *Centella asiatica* extract and *Vaccinium myrtillus* extract. Sample 4 was of tincture type, an extract from horse-chestnut seeds (20 g %) in 70% (v / v) ethanol. Sample 5 was a glycerol-hydroalcohol extract of fresh horse-chestnut sprouts (45 % ethanol) (1.5 mL unit).

A Jasco V 530 double beam UV-Vis spectrophotometer with a scan range of 400-800 nm was used.

Sample Processing

For the Sample 1 and 2, prior to analysis, 20 tablets were weighed, and their average mass was calculated, after which they were crushed into a fine homogenised powder. Further, some quality parameters were tested i.e., disintegration time and friability, according to European Pharmacopoeia.³⁰

By performing the disintegration test in water, no tablet must disaggregate for 30 min. After that, the operation was repeated, replacing water with 0.1 M hydrochloric acid and monitoring the time for tablet disintegration. Each determination was repeated on five tablets and the mean value of the determinations was calculated. For the friability test, because the analyzed tablets had an individual mass greater than 0.65 g, 10 tablets were used. The determinations were repeated twice and the average of the results was calculated.

Preparation of Solutions

To a quantity of powder corresponding to one tablet, 30 mL of 70 % (v/v) ethanol was added and the mixture was extracted by magnetic stirring for 60 min at 50 °C. The extract was filtered through Whatman paper in a 50 mL volumetric flask, the filter was washed several times with ethanol and the flask was filled to the mark. Dilutions suitable for determination were made.

For sample 3, to a specific weighed quantity of gel, 50 mL of 70 % (v/v) ethanol was added. The mixture was stirred for 60 minutes at 50 °C. The extract was filtered in a 50 mL volumetric flask, washing the filter several times and filling the flask to the mark. Dilutions suitable for determination were made. The analyses of samples 4 and 5 were made after suitable dilutions with 70% (v/v) ethanol.

Methods

The quantitative analysis of saponins expressed in escin was carried out by UV-Vis spectrophotometry using the calibration curve method and the molar absorptivity method.

In the first procedure, the samples were treated with 8 % vanillin (alcoholic solution) and 72 % H₂SO₄ after which the mixture was incubated at 70 °C for 10 minutes. After a rapidly cooling on ice to room temperature, the absorbance of the solutions was measured at 560 nm. From the stock standard solution (0.1 g %), the standard scale solutions were prepared in the range 1-10 mg/L.³¹ In the second procedure, the samples were treated with Folin-Ciocalteu reagent (1:10 dilution), after which 7.5 % Na₂CO₃ solution was added. The intensity of the blue color obtained was measured at 760 nm after a reaction time of 2 h.³² For both methods the determinations were made in triplicate, within 3 consecutive days.

Results and discussion

The quality parameters (disintegration time, friability, average weight) for the samples 1 and 2 are presented in table 1.

Table 1. Quality parameters for the analysed tablets

Samples	Disintegration time (min) average ± SD	Friability g % average ± SD	Average weight (g)
Sample 1	29.65 ± 0.43	0.4095 ± 0.0017	1.3663
Sample 2	14.77 ± 0.48	0.2790 ± 0.0011	1.0540

According to the European Pharmacopoeia, the coated tablets should not disintegrate in water for at most 30 min but must disaggregate in 0.1 mol hydrochloric acid in 30 min. No tablet has disaggregated in water. As can be seen from the data obtained, all analyzed samples are included in the specifications limits of the pharmacopoeia for coated tablets, obtaining a range of 28.22 - 29.08 for sample 1, and

an interval of 14.29 - 15.25 for sample 2. If we should make a hierarchy based on the time of active substance release from the tablets and the availability of the substance for absorption resulting in a faster action, the quickest release of the active substance is obtained in the case of Sample2 as compared to Sample 1.

According to European Pharmacopoeia, regarding the friability parameter, the maximum mass loss considered acceptable is 1% of the mass of the tablets to be determined. As can be seen from table 1, all the tablets under analysis complied with the limits.

The basic principle of the first method is the reaction between oxidized triterpenoid saponins using sulfuric acid as an oxidizing agent and vanillin. In order to determine the total saponins expressed in escin in the analyzed samples, the calibration curve was design by plotting the mean values of absorbances of escin standard solutions versus concentrations. The statistical parameters for the analysis were presented in table 2.

Table 2. Statistical data for escin determination

Statistical parameters	Values
Correlation Coefficient (r^2)	0.9992
Standard error	0.0101
Intercept	0.6622
Slope	0.1105
Limit of detection	0.3041
Limit of quantification	0.9214

The calibration curve has a very good linearity in the range of analysis. The system precision was determined using a 5 mg L⁻¹ solution, in 6 replicates. The SD (standard deviation of the mean) and % RSD (relative standard deviation) were calculated (Table 3).

Table 3. Experimental data for precision of the system

No.	Absorbance
1.	1.2012
2.	1.1998
3.	1.2218
4.	1.2107
5.	1.1967
6.	1.2028
Average	1.2055
SD	0.0092
% RSD	0.7678

RSD was 0.7678 %, being lower than 2 %, the value proposed by the European standards, so we can say that the system is precise.³³ Accuracy of the method was investigated by using three concentration levels, in triplicate. In table 4 are presented the experimental data of three consecutive days.

The recovery of the determination in three consecutive days was in the range of 97 – 100.6 % and the RSD values in the range 0.1149 – 0.9830 %. The RSD values were lower than 5 %, so the method is accurate.³³

Table 4. Experimental data for the accuracy of the method.

Theoretical conc. mg L ⁻¹	Day 1		Day 2		Day 3	
	Average calculated conc.	% Recovery	Average calculated conc.	% Recovery	Average calculated conc.	% Recovery
2.5	2.51	100.4	2.47	98.8	2.49	99.6
5	5.03	100.6	4.85	97.0	4.99	99.8
7.5	7.53	100.4	7.39	98.5	7.49	99.8
Average		100.46	Average	98.1	Average	99.73
SD		0.1154	SD	0.9643	SD	0.1154
% RSD		0.1149	% RSD	0.9830	% RSD	0.1157

Table 5. Escin mg tablet⁻¹ in sample 1.

Stated concentration	Method 1			Method 2		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
50 mg tablet ⁻¹	51.12	50.87	50.78	51.01	48.97	49.78
	51.60	49.23	50.84	50.98	49.35	48.99
	51.43	49.87	50.91	50.24	49.86	50.10
Average ± SD/day	51.38 ± 0.24	49.99 ± 0.82	50.84 ± 0.06	50.74 ± 0.43	49.39 ± 0.44	49.62 ± 0.57
Average ± SD/sample	50.73 ± 0.7			49.91 ± 0.72		

Table 6. Escin mg tablet⁻¹ in sample 2.

Stated Concentration	Method 1			Method 2		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Not stated	39.48	38.50	39.72	38.94	38.10	38.89
	39.56	38.27	39.61	38.87	38.77	38.01
	39.87	38.19	38.48	38.25	38.12	38.23
Average ± SD/day	39.63 ± 0.2	38.32 ± 0.16	39.27 ± 0.68	38.68 ± 0.37	38.33 ± 0.38	38.37 ± 0.45
Average ± SD/sample	39.07 ± 0.67			38.46 ± 0.19		

Table 7. Escin mg 100 g⁻¹ gel in sample 3.

Stated Concentration	Method 1			Method 2		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Not stated	698.73	695.67	696.28	691.67	686.28	687.67
	697.45	695.82	696.55	691.82	686.55	687.25
	697.23	695.32	697.27	691.32	686.27	687.32
Average ± SD/day	697.80 ± 0.81	695.60 ± 0.25	696.70 ± 0.51	691.60 ± 0.25	686.36 ± 0.15	687.41 ± 0.22
Average ± SD/sample	696.70 ± 1.1			688.45 ± 2.77		

Table 8. Escin mg 100 g⁻¹ tincture in sample 4.

Stated Concentration	Method 1			Method 2		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Not stated	368.67	367.25	368.25	366.23	366.79	367.74
	367.26	368.01	368.15	367.76	368.27	367.10
	369.10	368.54	368.92	368.10	367.75	366.98
Average ± SD/day	368.34 ± 0.96	367.93 ± 0.64	368.44 ± 0.41	367.36 ± 0.99	367.60 ± 0.75	367.27 ± 0.40
Average ± SD/sample	368.23 ± 0.27			367.41 ± 0.17		

When applying the validated method to the analyzed samples, the total saponins value was expressed in mg escin/tablet and the values are shown in table 5 for sample 1 and table 6 for sample 2. For the samples 3 and 4, the total

saponins value was expressed in mg escin/100 g sample and the values are shown in table 7 and 8. For sample 5, the results are presented in table 9, expressed in mg escin/unit (1.5 mL).

Table 9. Escin mg/unit glycerol-hydroalcoholic extract in sample 5.

Stated Concentration	Method 1			Method 2		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Not stated	0.2218	0.2035	0.2156	0.2226	0.2110	0.2232
	0.2305	0.2189	0.2333	0.2145	0.2045	0.2024
	0.2018	0.2301	0.2006	0.1958	0.2232	0.2115
Average ± SD/day	0.2180 ±	0.2175 ±	0.2165 ±	0.2109 ±		
	0.014	0.013	0.016	0.013	0.2129 ± 0.01	0.2123 ± 0.01
Average ± SD/sample	0.2173 ± 0.007			0.2120 ± 0.001		

The proposed spectrophotometric method is based on the reduction of phosphomolybdotungstic acid from the Folin-Ciocalteu reagent by escin, in the presence of sodium carbonate, to obtain a blue product. The molar absorptivity method was applied for calculation, knowing from the literature that for escin $\epsilon = 1.0439 \times 10^4 \text{ Lmol}^{-1}\text{cm}^{-1}$.³² The results are presented for each sample in tables 3-7.

According to the leaflet, Sample 1 contains 250 mg of horse-chestnut extract/unit, corresponding to an escin content of 50 mg/tablet. If we consider a deviation according to Romanian Pharmacopoeia, Compressi Monograph, this would be $\pm 7.5\%$,³⁴ which means 46.25-53.75 mg escin/tablet. If we consider a deviation according to European Pharmacopoeia,³³ this would be $\pm 5\%$, which means 47.5-52.5 mg escin/tablet. Our results meet both national and European requirements.

According to the leaflet, the analyzed tablets from sample 2 contain 200 mg of horse-chestnut/tablet extract but the amount of escin mg/tablet is not specified. If we take into account the first analyzed product, we can consider that the 200 mg extract contains 40 mg escin/tablet, implicitly a 37-43 mg escin/tablet range according to Romanian Pharmacopoeia and 38-42 mg escin/tablet range according to European Pharmacopoeia. In this case also, we can consider that the results comply with both national and European requirements.

On samples 3, 4 and 5, the quantity of escin is not specified in the leaflet, so no comparison can be made with the declared quantity. But, we noted that between the two methods of analysis used, the differences are insignificant.

Conclusions

We analyzed five products in the over the counter and dietary supplements categories by two spectrophotometric methods using calibration curve and molar absorptivity methods. The methods were simple, easy to use and cheap. In the case of products that have the concentration stated on the label, the results comply with the limits imposed by regulations. For products that did not have the concentration specified on the label, a comparison could not be made. Thus, we draw attention to the need to include the concentration of active substances on the product label, even if they are part of the category of supplements.

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