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MEDICINAL PLANTS

DETERMINATION OF TOTAL FLAVONOIDS IN SIBERIAN HAWTHORN FRUIT

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A quantitative determination method for total flavonoids in hawthorn fruit was developed using differential spectrophotometry (analytical wavelength 412 nm) and state standard sample hyperoside and was used to analyze a series of raw material samples. It was shown that the flavonoid content in hawthorn fruit varied in the range 0.112 – 0.183% (calculated as hyperoside). Statistical processing of the test results indicated that the error of a single determination of total flavonoids in hawthorn fruit at 95% confidence probability was ± 4.87%.

Keywords: Siberian hawthorn, *Crataegus sanguinea* Pall., fruit, flavonoids, hyperoside, spectrophotometry, standardization.

Fruit and flowers of Siberian hawthorn (*Crataegus sanguinea* Pall.) and other pharmacopoeial species are widely used in medicine as cardiotonics and hypocholesterolemia agents to treat various cardiovascular diseases [1 – 3]. Flavonoids are the dominant biologically active compounds in this plant although other compounds, e.g., saponins, contribute to the pharmacological effect [4]. Hawthorn fruit is standardized for flavonoid content (>0.06% calculated as hyperoside) [5]. However, in our opinion, the quantitative determination method for total flavonoids in raw material from this plant is extremely complicated and involves many steps that inevitably cause losses of analytes and; therefore, diminish the analytical results. Furthermore, the multi-step sample preparation procedure in the pharmacopoeial method is also used for hyperoside state standard sample (SSS). This also not only complicates the method but also affects the accuracy. Therefore, improvement of the chemical standardization of hawthorn fruit is crucial.

The goal of the present research was to develop a quantitative determination method for total flavonoids in hawthorn fruit.

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EXPERIMENTAL PART

We studied samples of Siberian hawthorn (*C. sanguinea*) fruit and flowers collected in 2010 – 2012 in Samara Oblast (Gavrilova Polyana town and Prosvet village) in addition to a commercial sample of hawthorn fruit (OAO Krasnogorskleksredstva, 2012, batch 10112). The method development included a determination of the optimum extraction conditions for hawthorn fruit. Thus, the extractant was EtOH (70%) at a raw material:extractant ratio of 1:30 with extraction for 60 min at 85 – 90°C on a water bath (Table 1). It should be noted that the total flavonoid content was >3 times less than that of the optimum version if EtOH (95%) was used as the extractant as in the pharmacopoeial method [5] (Table 1). This could be explained by the poor solubility of hyperoside in 95% EtOH. For this reason, 70% EtOH is recommended for preparation of the hyperoside SSS solution rather than 95% EtOH (see Note in the method).

UV spectra of aqueous EtOH extracts of the raw material were studied during development of the quantitative determination method for total flavonoids in hawthorn fruit. Spectra were recorded using a Specord 40 spectrophotometer (Analytik Jena).

Complexation by dissolved $AlCl_3$ was used to develop the quantitative determination method for total flavonoids in order to avoid contributions to the optical density by other

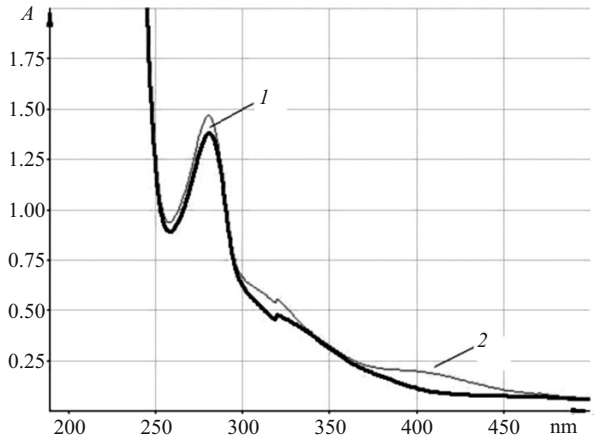

Fig. 1. UV spectra of aqueous EtOH extracts from Siberian hawthorn fruit: extract (*1*) and extract with added $AlCl_3$ (*2*).

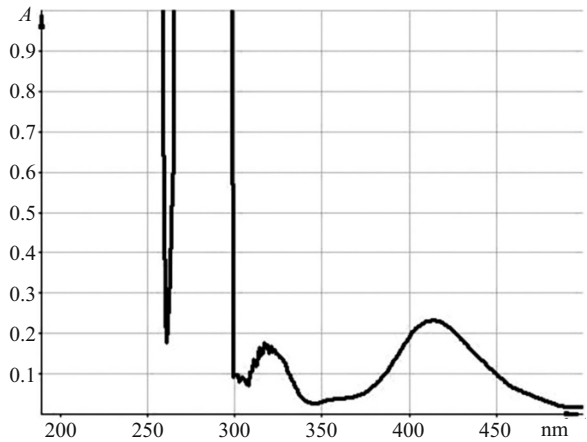

Fig. 2. UV spectrum of aqueous EtOH extract from Siberian hawthorn fruit (differential spectrum).

types of compounds [6], including hydroxycinnamic acids typical of this plant. As a rule, their principal absorption maximum is in the range 280 – 330 nm [7]. The long-wavelength band of flavonoids, in particular, flavonols (hyperoside etc.), undergoes a bathochromic shift after complexation. This was observed in the UV spectrum as an absorption maximum at 400 – 412 nm (Fig. 1) and was confirmed using differential spectrophotometry (Fig. 2).

The study of hyperoside SSS UV spectra showed that the solution of this standard in the presence of $AlCl_3$ had an absorption maximum at 412 nm, including in the differential spectrum (Figs. 3 and 4).

Therefore, hyperoside could be used as the SSS in the analytical method. Furthermore, UV spectra of the fruit and flower extracts of Siberian hawthorn (Fig. 5) differed sharply (fruit were characterized by a strong absorption maximum at 285 ± 2 nm). This could be used as a diagnostic for this raw material. However, the Qualitative Reactions section calls for TLC detection of hyperoside in hawthorn fruit and flowers [5], which in this instance is a common and not a distinguishing signature.

Furthermore, we developed previously a determination method for flavonoids in hawthorn flowers [8]. It was used to analyze a series of hawthorn flower samples and showed that the total flavonoid content varied in the range 1.41 – 1.84% (calculated as hyperoside). This allowed the total flavonoid content to be recommended preliminarily as >0.5% [8].

Quantitative determination method for total flavonoids in hawthorn fruit. The analytical sample was ground to a particle size passing through a 1-mm sieve. Ground raw material (~1 g, accurate weight) was placed into a 50-mL flask with a ground-glass joint and treated with EtOH (70%, 30 mL). The flask was closed with a stopper and weighed on

TABLE 1. Completeness of Total Flavonoid Extraction from Hawthorn Fruit as a Function of Conditions

Sample No.	Extractant	Raw material:extractant ratio	Extraction time, min	Total flavonoid content (calculated as hyperoside and absolute dry raw material), %
1.	40% EtOH	1:30	60	0.033 ± 0.001
2.	50% EtOH	1:30	60	0.044 ± 0.001
3.	60% EtOH	1:30	60	0.087 ± 0.002
4.	70% EtOH	1:30	60	0.158 ± 0.002
5.	80% EtOH	1:30	60	0.072 ± 0.002
6.	95% EtOH	1:50	60	0.045 ± 0.001
7.	70% EtOH	1:30	45	0.078 ± 0.002
8.	70% EtOH	1:30	90	0.156 ± 0.002
9.	70% EtOH	1:30	120	0.150 ± 0.002
10.	70% EtOH	1:20	60	0.137 ± 0.002
11.	70% EtOH	1:50	60	0.160 ± 0.001

TABLE 2. Total Flavonoid Contents in Various Hawthorn Samples

Sample No.	Raw material characteristics	Total flavonoid content (calculated as hyperoside and absolute dry raw material), %	
		developed method	pharmacopoeial method
1.	Commercial sample (OAO Krasnogorskleksredstva, 2012, batch 10112)	0.158 ± 0.002	0.063 ± 0.002
2.	Samara Oblast (Prosvet village), Aug. 18, 2011	0.137 ± 0.002	0.056 ± 0.001
3.	Samara Oblast (Gavrilova Polyana town), Aug. 25, 2011	0.183 ± 0.002	0.071 ± 0.002
4.	Samara Oblast (Prosvet village), Aug. 20, 2010	0.112 ± 0.001	0.051 ± 0.001
5	Samara Oblast (Gavrilova Polyana town), Aug. 23, 2010	0.161 ± 0.002	0.065 ± 0.002

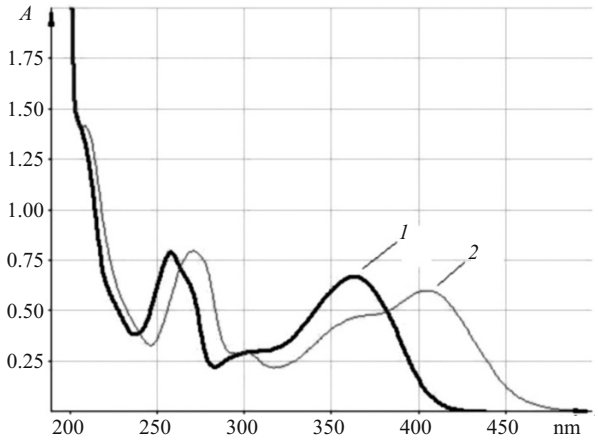

Fig. 3. UV spectrum of EtOH solution of hyperoside SSS: hyperoside SSS (*1*) and hyperoside SSS with added $AlCl_3$ (*2*).

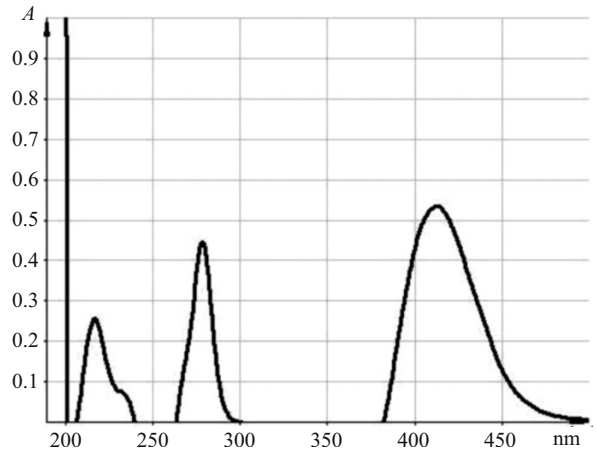

Fig. 4. UV spectrum of hyperoside SSS solution (differential spectrum).

an analytical balance to an accuracy of ± 0.0001 g. The flask was connected to a reflux condenser and heated on a boiling-water bath (gently boiling) for 60 min. Then, the flask with the extract was cooled for 30 min, closed with the same stopper, weighed again, and restored to the initial mass using solvent. The resulting extract was filtered (red band). Test solution was prepared as follows. Extract (5 mL) was placed into a 25-mL volumetric flask, treated with $AlCl_3$ solution (2 mL, 3%) in EtOH, and adjusted to the mark with EtOH (95%) (test solution A). The reference solution was prepared under the same conditions but without adding $Alcl_3$ (reference solution A). Optical density was measured at 412 nm on a spectrophotometer. The optical density of hyperoside SSS solution was measured in parallel at 412 nm. The solution was prepared analogously to the test solution (see Note).

Note. Preparation of hyperoside SSS solution. Hyperoside (~0.02 g, accurate weight) was placed into a 50-mL volumetric flask, dissolved in EtOH (30 mL, 70%) with heating on a water bath, cooled to room temperature, and adjusted to the mark with EtOH (70%) (hyperoside solution A). Hyperoside solution A (1 mL) was placed into a 25-mL volumetric flask, treated with $AlCl_3$ solution (1 mL, 3%) in EtOH, and adjusted to the mark with EtOH (95%) (hyperoside test solution B). The reference solution was prepared as follows. Hyperoside solution A (1 mL) was placed into a 25-mL volumetric flask and adjusted to the mark with EtOH (95%) (hyperoside reference solution B).

The total flavonoid content calculated as hyperoside and absolute dry raw material was calculated in percent (X) using the formula:

TABLE 3. Metrological Characteristics of Quantitative Determination Method for Total Flavonoid Content in Hawthorn Fruit

f	$\overline{X}$	S	P, %	$t(P,f)$	ΔX	E, %
10	0.158	0.0034	95	2.26	± 0.0077	± 4.87

$$X = \frac{D \cdot m_0 \cdot 30 \cdot 1 \cdot 25 \cdot 100 \cdot 100}{D_0 \cdot m \cdot 50 \cdot 5 \cdot 25 \cdot (100 - W)},$$

where D is the optical density of the test solution; D_0, the optical density of the hyperoside SSS solution; m, the raw material mass (g); m_0, hyperoside SSS mass (g); and W, mass loss upon drying (%).

It also seemed advantageous to calculate the total flavonoid content using the specific absorption coefficient of hyperoside (330). In this instance, the total flavonoid content in the raw material was calculated using the simplified formula:

$$X = \frac{D \cdot 30 \cdot 25 \cdot 100}{330 \cdot m \cdot 5 \cdot (100 - W)},$$

where D is the test solution optical density; 330, the specific absorption coefficient at 412 nm of the hyperoside complex with $AlCl_3$; m, the raw material mass (g); and W, the mass loss upon drying in percent.

A series of hawthorn-fruit samples were analyzed using the developed method. It was shown (Table 2) that the total

TABLE 4. Total Flavonoid Content in Hawthorn Fruit as a Function of Added Hyperoside

Initial total flavonoid content, mg/g	Added hyperoside, mg/g	Total content flavonoids, mg/g		Error	
		calc.	found	absolute, mg	relative, %
1.58	0.4 (to raw matl.)	1.98	1.95	– 0.03	– 1.54
1.58	0.8 (to raw matl.)	2.38	2.42	+ 0.04	+ 1.68
1.58	1.2 (to raw matl.)	2.78	2.83	+ 0.05	+ 1.80

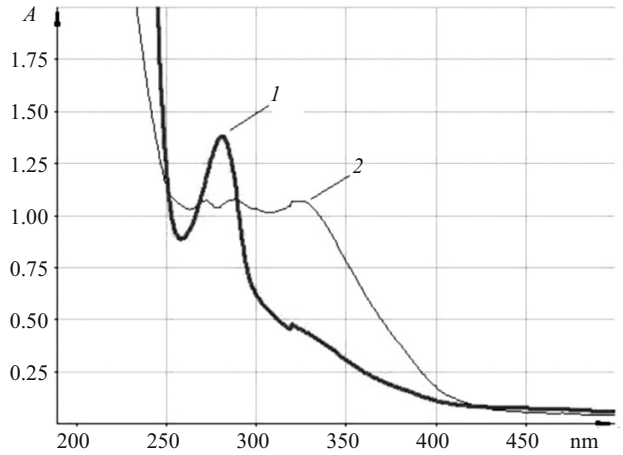

Fig. 5. UV spectrum of aqueous EtOH extract from fruit (*1*) and flowers (*2*) of Siberian hawthorn.

flavonoid content in the samples varied in the range 0.112 – 0.183% (calculated as hyperoside). This allowed the total flavonoid content to be recommended as >0.1% (instead of 0.06%). Of course, this parameter should be refined further.

Table 3 presents the metrological characteristics of the flavonoid quantitative determination method for hawthorn fruit.

Statistical processing of the test results indicated that the error of a single determination of total flavonoids in hawthorn fruit was ±4.87% with 95% confidence probability.

Tests with hyperoside SSS added to the raw material showed that the analytical error was within the error of a single determination. This indicated that the developed method did not have systematic error (Table 4).

Thus, a quantitative determination method for total flavonoids in hawthorn fruit was developed using differential spectrophotometry (analytical wavelength 412 nm). Use of hyperoside as a SSS in the developed method was justified. The total flavonoid content in the hawthorn fruit samples varied in the range 0.112 – 0.183% (calculated as hyperoside). The electronic spectral characteristics (strong absorption maximum at 285 ± 2 nm) could also be used to identify raw material and preparations of this plant. In our opinion, implementation of this method that satisfies validation requirements could objectively evaluate the quality of hawthorn raw material.

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