QUANTITATIVE HPLC DETERMINATION OF MAIN FLAVONOID CONTENT OF *Rhododendron adamsii* LEAVES AND STEMS

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The content of main flavonoids from Rhododendron adamsii *R. leaves and stems was determined quantitatively using HPLC. It was found that myricetin and quercetin dominated the identified compounds (myricetin, quercetin, dihydroquercetin, rutin) in leaves; dihydroquercetin, in stems (1.1, 1.0, and 2.5 mass% of raw material, respectively). Dihydroquercetin and rutin were found for the first time in* R. adamsii.

Key words: *Rhododendron adamsii*, HPLC, flavonoids, myricetin, quercetin, dihydroquercetin, rutin.

Certain plants of the genus *Rhododendron* (Ericaceae) have a long history of use as folk medicine in various countries. *Rhododendron adamsii* Rehder is used as a stimulant and tonic in Buryatia, Mongolia, and China [1, 2]. Decoctions and tinctures of it are used for colds and cardiac diseases; as a diuretic agent for cardiac edema, and as a adaptogen [3]. Essential oils [4], ursolic and oleanolic acids [5], phenolcarboxylic acids, coumarins, flavonoids, and other compounds from the aerial part of *R. adamsii* have been observed among the biologically active compounds and studied [6, 7]. Of the aforementioned group of compounds, flavonoids, which exhibit antioxidant, capillary-, hepato-, and onco-protective activity, are widely distributed in the plant world [8].

The goal of our work was to determine the major flavonoids from leaves and stems of *R. adamsii* and to establish their mass content.

Spectrophotometric determination of total flavonoids is the most common method for quantitative determination of them in plant material [9]. Reversed-phase HPLC with UV or mass-spectrometric detection is the most effective method for quantitative determination of the flavonoid content in plant material [10, 11].

Two-dimensional paper chromatography found previously that the aerial part of *R. adamsii* contains the flavonoids myricetin, quercetin, azaleatin, avicularin (quercetin-α-L-arabinoside), hyperoside (quercetin-3-galactoside), and myricitrin (myricetin-3-rhamnoside) [7]. However, the experimental details were not reported. The quantitative content of flavonoids in *R. adamsii* was determined by spectrophotometry based on quercetin at 430 nm for plant runners [6, 12]. It was reported [6] that the flavonoid content in *R. adamsii* runners is 3.17 ± 0.09 mass%. It was shown [12] that the mass fraction of flavonoids in raw material varied from 2.0 to 3.0% depending on the collection site. We established using HPLC that the major flavonoids of leaves and stems of *R. adamsii* were myricetin, quercetin, and dihydroquercetin (DHQ) and rutin, which were observed for the first time in this plant.

We used multiple extractions with aqueous ethanol (70%) and monitoring of the completeness of the extraction by HPLC to extract total flavonoids from *R. adamsii* because they are present as aglycons and glycosides. Analytical chromatograms of the resulting extracts were used to determine the native flavonoids. Total aglycons were determined after acid hydrolysis of the extracts.

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TABLE 1. Spectral Peak Ratios for Myricetin, Rutin, Quercetin, and DHQ in Standard and *R. adamsii* Extracts and Their Content

Sample	A 260/360	A 280/360	A 300/360	A 320/360	Content, mass %
Myricetin	0.899	0.369	0.389	0.443	
NH (leaves)	1.099	0.486	0.486	0.552	1.66±0.06
H (leaves)	0.892	0.344	0.373	0.446	1.13±0.04
NH (stems)	1.075	0.513	0.560	0.584	1.10±0.04
H (stems)	0.912	0.386	0.409	0.458	1.06±0.04
Rutin	1.168	0.430	0.497	0.590	
Leaves	1.177	0.626	0.603	0.668	1.08±0.05
Stems	1.224	0.751	0.826	0.745	0.77±0.03
Quercetin	0.997	0.377	0.382	0.430	
NH (leaves)	1.156	0.562	0.496	0.512	0.17±0.01
H (leaves)	1.022	0.430	0.389	0.446	1.01±0.04
NH (stems)	1.034	0.455	0.692	0.612	0.30±0.01
H (stems)	1.009	0.426	0.426	0.454	0.98±0.06
DHQ	0.203	0.972	0.316	0.052	
NH (leaves)	0.189	1.283	0.504	0.202	0.40±0.02
H (leaves)	0.174	1.023	0.361	0.078	0.33±0.04
NH (stems)	0.201	1.204	0.436	0.101	1.70±0.01
H (stems)	0.212	0.994	0.340	0.066	2.46±0.11

NH, nonhydrolyzed extract; H, hydrolyzed extract.

We introduced a spectral-chromatographic index relating the mass of compound placed on the chromatographic column and the peak area on the chromatogram, called the specific mass extinction, to determine the mass content of flavonoids by HPLC. This index was calculated as the numerical value of the peak area on the chromatogram per μg of compound (see Experimental). It was characteristic for the used chromatograph (Milikhrom A-02) and can be used in other investigations without using standards. The specific mass extinction and mass content for myricetin, quercetin, and rutin was calculated at 360 nm; for DHQ, at 280 nm because the first three are flavonol derivatives and have absorption maxima in the UV spectrum at 360-380 nm whereas DHQ is a flavanonol (dihydroflavonol) with characteristic absorption at 280-300 nm [13].

According to HPLC, myricetin (about 1.7 mass%) dominated the native flavonoids in leaves and stems of *R. adamsii*. However, the spectral ratios of the peak for this compound on the chromatogram did not agree with the values for the standard (Table 1).

After hydrolysis, the spectral ratios of the myricetin peak were close to the standards whereas the compound content in leaves was about 1.1%. Spectral ratios of the myricetin peak on the chromatogram of the stem extract, like for leaves, differed from the standards by 1-5% whereas the content of this flavonoid in stems was about 1.1 mass%. Apparently, other glycosidic compounds with retention times similar to that of myricetin but with UV spectra different from that of it were present in both leaves and stems of *R. adamsii*. Myricetin itself was present in the plant mainly in the non-glycosylated form (myricitrin content was insignificant). Its total content was less than 1.1%.

The rutin (quercetin-3-rhamnosylglucoside) content that was determined by HPLC was about 1.1 and 0.8% of the leaf and stem mass, respectively. Spectral ratios of the peak corresponding to the retention time of this flavonoid differed from those of the standard (Table 1). This may indicate that other compounds with retention times similar to that of rutin were present.

For this reason, the rutin content was known to be less than the values in Table 1. It could only be determined by isolating the pure compound.

Rutin decomposed during acid hydrolysis into quercetin, rhamnose, and glucose. As a result, the mass fraction of quercetin increased in the hydrolyzed extracts compared with the non-hydrolyzed ones (Table 1).

TABLE 2. Specific Mass Extinction of Flavonoids and Their Mass Content in *R. adamsii*, %

Compound	ε_{360}, AU×μL/μg	Leaves		Stems	
		NH	H	NH	H
Myricetin	42.64	1.66±0.06	1.13±0.04	1.10±0.04	1.06±0.04
Quercetin	63.63	0.17±0.01	1.01±0.04	0.30±0.01	0.98±0.06
DHQ	33.87[a]	0.40±0.02	0.33±0.04	1.70±0.01	2.46±0.11
Rutin	25.90	1.08±0.05	-	0.77±0.03	-

[a]Value for ε_{280} is given.

The content of native quercetin in leaves and stems was about 0.2 and 0.3%, respectively. Certain spectral ratios for the peak of this compound differed by 1.2-1.5 times from the standards. Apparently other flavonoids with a structure similar to quercetin (in particular, azaleatin) were present in *R. adamsii* and contributed to the spectral ratios of the peak. The spectrum of the quercetin peak after acid hydrolysis became similar to the standard. Its content increased noticeably. This was explained primarily by the presence of rutin in the plant material. However, as noted above, its content in leaves and stems was less than 1.1 and 0.8 mass%, respectively.

Because the aglycon quercetin accounts for one half the mass of the whole rutin molecule, the contribution of the latter to the increase of quercetin concentration was about 0.6% in leaves and 0.4% in stems. The remaining 0.2-0.3% of the mass increase of the quercetin fraction was probably the result of hydrolysis of its other glycosides, in particular, monoglycosides of avicularin (quercetin-α-L-arabinoside) and hyperoside (quercetin-3-galactoside), which have been observed in *R. adamsii* earlier [7]. However, their total content was less than 0.3-0.5%. We performed an additional extraction of the raw material by aqueous ethanol with added HCl in order to determine the carbohydrates formed by acid hydrolysis of the glycosides, including flavonoids. The evaporated extract was treated with ethylacetate. Total water-soluble compounds were treated with 2,4-dinitrophenylhydrazine to modify the carbohydrates formed after hydrolysis. HPLC showed that the aqueous fraction contained glucose, rhamnose, arabinose, and galactose. This confirmed the hypothesis about the presence in the plant of azaleatin and hyperoside in addition to rutin.

According to HPLC, the DHQ content in leaves of *R. adamsii* was 0.4% before hydrolysis and 0.3% after hydrolysis. However, its determination was complicated by the presence of another compound with a similar retention time but different UV spectrum. This was evident in the shape of the peak for this compound on the chromatogram and the values of its spectral ratios (Table 1).

Because spectral ratios of the DHQ peak after hydrolysis differed from the standards as before whereas the content of this compound was practically constant, it can be assumed that DHQ and the component accompanying it were present in leaves in the free form and were not glycosylated. The presence of the accompanying compound prevented an accurate determination of DHQ in the plant leaves. It this instance it could only be assumed that it was less than 0.4%.

Stem extract that was not hydrolyzed contained more than one compound with the retention time of DHQ because spectral ratios of its peak also differed from the standards. After hydrolysis, the contribution of the interfering compounds decreased and spectral ratios approached the standards. The DHQ content in stems after hydrolysis was unexpectedly high (about 2.5 mass % of raw material). The content of native DHQ was less than 1.7%.

The HPLC investigation identified the main flavonoids from leaves and stems of *R. adamsii* and determined their mass content. DHQ and rutin were observed for the first time in this plant. The major flavonoids of leaves were myricetin and quercetin (including as the glycosides). Stems typically had a high content of DHQ. A spectrophotometric method for determining total flavonoid content calculated as quercetin will have a systematic error because the DHQ—$AlCl_3$ complex had an absorption maximum at 310-315 nm; the quercetin—$AlCl_3$ complex, at 430 nm [14]. This is due to the presence in the plant material of dihydroflavonols and depends on the total amount of these compounds. Multi-wavelength detection is most reliable for identifying the compounds for quantitative determination of flavonoids in total extracts using HPLC with UV detection.

EXPERIMENTAL

Plant Material and Chromatographic Analyses. We used plant material from OOO Travy Sibiri that was collected in July 2005 near Arshan village of Irkutsk Oblast′ and dried in air. Dry raw material was separated into leaves and stems and ground to particle size 0.5-1.0 mm. The ground raw material was stored in a hermetically sealed container under Ar until used in the experiment. The moisture content of ground leaves and stems was 2.0 and 3.0%, respectively.

Chromatographic analysis of standard flavonoids and plant extracts by HPLC was performed on a Milikhrom A-02 chromatograph (ZAO Institute of Chromatography, EkoNova, Novosibirsk) using a column (2 × 75 mm) packed with reversed-phase sorbent Prontosil-120-5-C18 AQ (5 μm) and thermostatted at 35°C. The eluent was a linear gradient from CF_3COOH (0.1%) to methanol (100%). The elution rate was 150 μL/min; analysis time, 20 min. Multi-wavelength detection was used. Scanning used wavelengths 260, 280, 300, 320, and 360 nm. The resulting chromatograms were processed using the Multikhrom program 1.5x-E (ZAO Ampersand, Moscow).

Carbohydrates were analyzed as hydrazones by HPLC using an Agilent 1100 liquid chromatograph (Agilent Technologies, USA) with an Agilent 1100 G1315B diode-array detector, G1313A autosampler, G1311A four-channel pump, G1322A degasser, and ChemStation software for processing chromatographic data. The column (4.6 × 150 mm) was packed with Zorbax SB-C18 (5 μm) reversed-phase sorbent. The elution conditions were a linear gradient of CH_3CN:CF_3COOH (0.05%) (from 0 to 20% CH_3CN in 0-25 min; from 20 to 70% CH_3CN, 25-35 min) at eluent flow rate 1.0 mL/min and detection at 360 nm.

Standard flavonoids were myricetin, quercetin, and rutin (ChemaPol, Czech Rep.) and DHQ (OOO Khimiya Drevesiny, Irkutsk) of at least 95% purity.

Determination of Specific Mass Extinction of Standards. A weighed portion (about 1 mg, m_0, accurate weight) was dissolved in methanol (1000 μL, V_0). An aliquot of the resulting solution (2 μL, V_1) was analyzed by HPLC. The specific mass extinction (ελ) was calculated using the formula $\varepsilon\lambda = (S\lambda \cdot V_0)/(1000 \cdot m_0 \cdot V_1)$, where $S\lambda$ is the peak area of the compound on the chromatogram at the selected wavelength λ (AU × μL) and 1000 is a coefficient for converting mg to μg. Table 2 gives the results.

Content of Native Flavonoids in Raw Material. About 50 mg (accurate weight) of ground raw material was extracted with ethanol (70%, 7 × 1 mL) by shaking on a shaker at room temperature. Three experiments were carried out in parallel including an experiment for monitoring the completeness of extraction of flavonoids from raw material. Extracts were combined and evaporated until the solvent was fully evaporated. The evaporated extract was dissolved in methanol (1000 μL). A sample (10 μL) of the resulting solution was analyzed by HPLC. Compounds were identified by comparing retention times and spectral ratios of compound peaks with the values of these characteristics for standards.

Determination of Total Flavonoids after Hydrolysis. An aliqout (200 μL) of the methanol solution of the extract that was used to determine native flavonoids was treated with HCl (conc., 31.2 μL), heated in a sealed tube at 90°C for 4 h, and cooled to room temperature. An aliquot of the resulting hydrolysate (10 μL) was analyzed. Table 2 gives the content of native flavonoids and their total content.

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