FLAVONOID GLYCOSIDES FROM Aconitum baicalense

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Two known flavonoids, the 7-O-α-L-rhamnopyranosides of kaempferol and of quercetin, and also the new acylated glycoside quercetin 3-O-[O-(6-caffeoyl-β-D-glucopyranosyl)-(1→2)-β-D-glucopyranoside 7-O-α-L-rhamnopyranoside (czekanoside A) have been isolated from the epigeal part of Aconitum baicalense Turcz, ex Rapaics (A. Czekanovskyi Steinb.). Their structures have been demonstrated by the methods of IR, UV, ^1H, and ^{13}C NMR spectroscopies and FAB mass spectrometry and also with the aid of acid hydrolysis.

Plants of the genus Aconitum have been widely studied as producers of diterpene alkaloids [1]. As far as concerns flavonoids, only a few publications on this subject are known [2, 3]. At the same time, the epigeal part of the species Aconitum baicalense Turcz, ex Rapaics (A. Czekanovskyi Stein.), which is endemic for Eastern Siberia is rich in metabolites of the flavonoid type. In an aqueous ethanolic extract of this plant we have found and identified with the aid of IR, UV, PMR, and ^{13}C NMR spectroscopies, FAB mass spectrometry, and acid hydrolysis the previously described [3-5] 7-O-α-L-rhamnopyranosides of kaempferol (I) and of quercetin (II), and have also detected a new glycoside (III). In the present communication we give proofs of the structure of this glycoside obtained with the use of modern NMR-spectroscopic procedures.

The molecular mass of glycoside (III) is 934 (FAB mass spectrometry). In the products of acid hydrolysis, quercetin, caffeic acid, glucose, and rhamnose were found by the HPLC method. The assignment of the peaks of rhamnose and glucose was made from their retention times when chromatograms were recorded with different selectivities of the chromatographic system. The detection of quercetin and caffeic acid was made from their retention times and the spectral ratios $A_{350}/A_{260} = 0.70$ for quercetin and $A_{240}/A_{330} = 0.62$ for caffeic acid calculated from chromatograms of the hydrolysis products and of standard substances with detection at two wavelengths.

By analysis of the FAB mass spectrum it was established that the molecule of glycoside (III) contained two carbohydrate chains composed in the manner shown in the scheme, where the figures denote the observed masses of the fragmentary ions. This interpretation is not indisputable, since the caffeoyl and hexosyl fragments have the same mass

$$\left[\mathrm{Ca}\underset{773}{-\mathrm{O}}+\mathrm{Glc}^2-\underset{611}{\mathrm{O}}-\mathrm{Glc}^1-\overset{449}{\mathrm{O}}-\overset{433}{\mathrm{Qu}}-\underset{789}{\mathrm{O}}+\mathrm{Rha}+\mathrm{H}\right]^+ 935$$

Ca represents a caffeic acid residue and Qu quercetin.

The positions of attachment of the carbohydrate chains to the aglycon were established by UV spectroscopy with diagnostic additives. To eliminate the interfering influence of the caffeoyl residue the spectra were taken against a caffeic acid background. The shifts of the absorption bands under the action of diagnostic additives (NaOAc, NaOAc + H_3BO_3), $AlCl_3$, and $AlCl_3$ + HCl) showed the presence of free phenolic groups in positions 5, 13, and 14.

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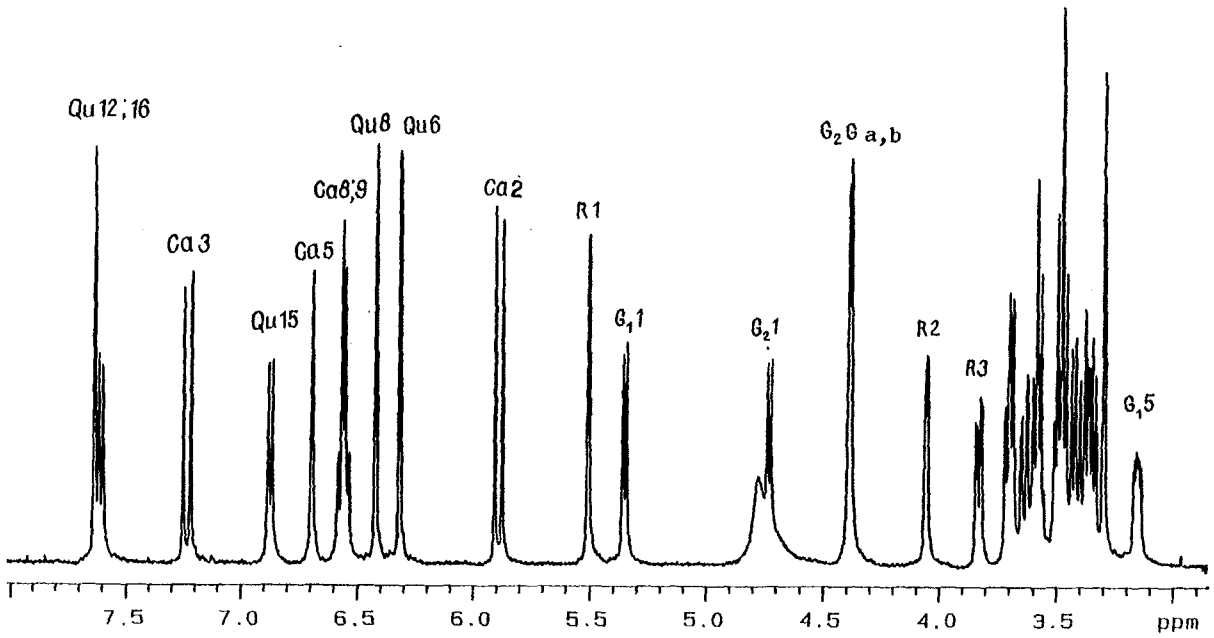


Fig. 1. Photomagnetic resonance spectrum (500 MHz) of czekanoside A.

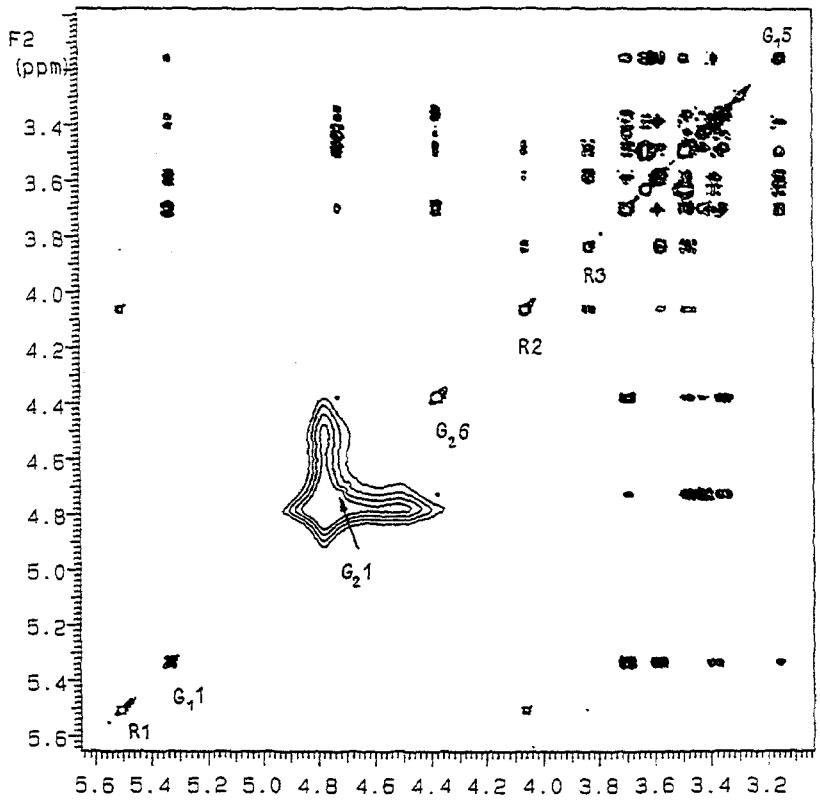


Fig. 2. Fragment of the 2D TOCSY spectrum of czekanoside A.

From these facts the conclusion followed that the carbohydrate chains were attached to the C-7 and C-3 positions of the quercetin molecule. To establish the complete structure of glycoside (III) it was necessary to determine the concrete positions of attachment of each of the carbohydrate chains and the method of linkage of the components in the longer, acylated chain. This problem was solved by NMR spectroscopy. In the first place it was necessary to assign the signals in the carbohydrate region of the ^{1}H NMR spectrum. In the spectrum taken at a working frequency of 500 MHz they showed considerable overlapping, and therefore we used the methods of two-dimensional resonance or homo- and heteronuclear COSY, TOCSY, and NOESY.

In the ordinary PMR spectrum (Fig. 1) the signal of the anomeric proton of the rhamnose residue was readily identified in the form of a broadened singlet at 5.50 ppm. From this reference point, using the 2D TOCSY spectrum (Fig. 2) we determined the signals of the other protons of the deoxyhexose residue: 4.07, 3.84, 3.59, 3.48, and 1.22. From the 2D COSY spectrum (Fig. 3) we established the vicinal links between them: 4.07 → 3.84 → 3.48 → 3.59 → 1.22. Using these results and the ordinary ^{1}H NMR spectrum we determined, where this was possible, the vicinal spin-spin coupling constants, which are given in Table 1 and confirm the assignment of the signals of the rhamnose residue. In the 2D NOESY spectrum (Fig. 4) the cross-peaks of the anomeric proton of rhamnose and of aromatic protons with chemical shifts of 6.31 and 6.42 ppm belonging to the hydrogen atoms at C-6 and C-8 of the quercetin molecule were observed. Consequently, the glycosidic bond of the rhamnose was localized at C-7 atom.

The caffeic acid residue was attached to the hydroxymethyl group of glucose Glc^2, as followed from the chemical shift of 64.8 ppm in the ^{13}C NMR spectrum. By using the method of heteronuclear two-dimensional resonance in the 2D HETCOR variant, we established

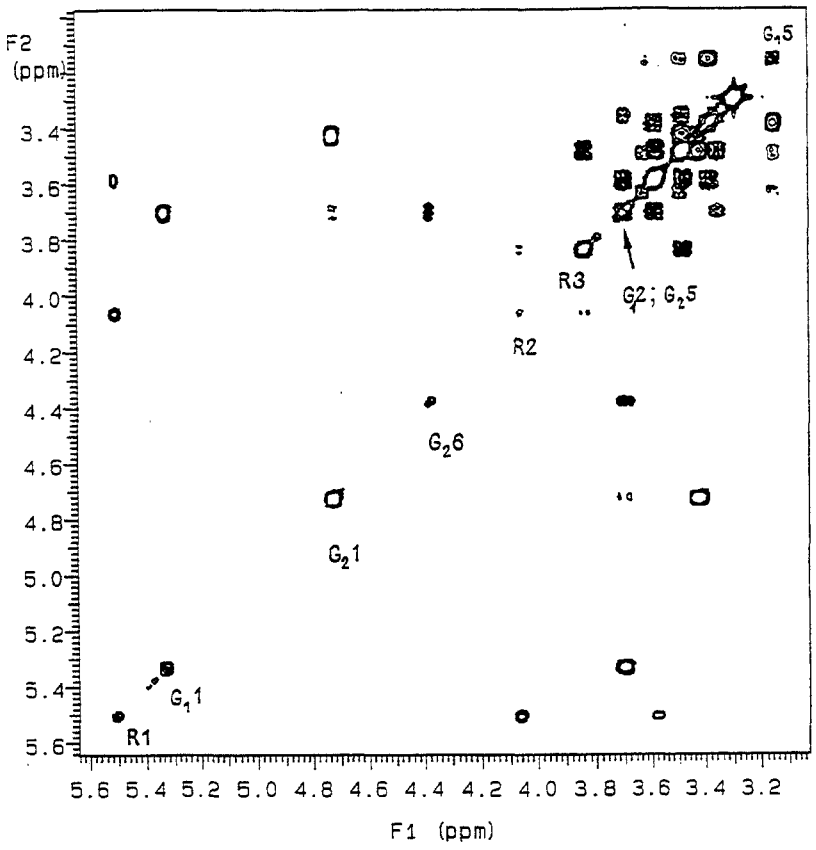


Fig. 3. Fragment of the 2D COSY spectrum of czekanoside A.

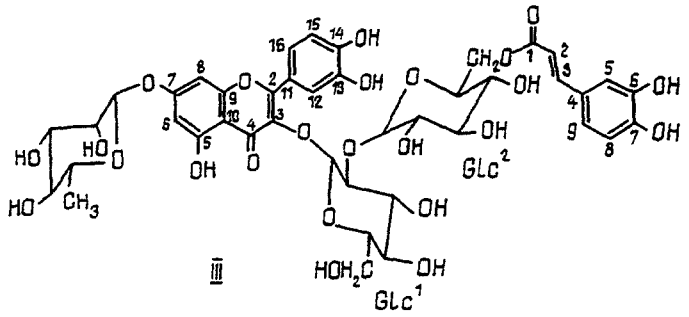
that in the proton spectrum the methylene part of this group was represented by an unresolved narrow multiplet at 4.31 ppm (2 H). Starting from this we made an assignment of all the other proton signals of the glucose residue Glc^2 (see Table 1). The remaining signals of the protons of Glc^1 could be assigned on the basis of the resonance of the anomeric proton at 5.33 ppm or of the characteristic isolated multiplet at 3.15 ppm corresponding to the hydrogen atom at C-5.

It can be seen from the NOESY spectrum (Fig. 4) that the signal of the anomeric proton of the glucose residue Glc^1 at 5.33 ppm had no other cross-peaks whatever apart from those belonging to the same sugar residue. Consequently, it was this which formed a glycosidic bond with the C-3 position of quercetin. So far as concerns the anomeric signal of the glucose residue Glc^2, it had an additional cross-peak linking it with the 3.71 ppm signal assigned to the C-2 position of Glc^1 and coinciding with the resonance of the H-5 proton of the Glc^2 residue. This coincidence of the two signals, of which the latter must necessarily give a NOE signal with the anomeric proton does not permit the position of glycosylation to be established from the NOESY spectrum alone. However, as is clear from the CH correlation diagram it is precisely the C-2 signal of Glc^1 glucosyl residue (δ, H-2 = 3.71 ppm) that was shifted from its usual position (76-77 ppm) to 85.1 ppm, and glycosylation had taken place at the C-2 atom.

Thus, substance (I) had the chemical structure of quercetin 3-O-[O-(6-caffeoyl-β-D-glucopyranosyl)-(1→2)-β-D-glucopyranoside] 7-O-α-L-rhamnopyranoside, and it has been given the name of czekanoside A. The ^{13}C NMR spectrum agrees with the proposed structure. The assignments made in Table 2 are made on the basis of literature information [6-9] and on the basis of the 2D HETCOR heteronuclear resonance spectrum.

TABLE 1. Chemical Shifts and SSCCs of the Protons of Czekanoside A

Proton	δ, ppm	J, Hz	NOE
		Quercetin (Qu)	
6	6,31 d	2,0	R : H-1
8	6,42 d	2,0	R : H-1
12	7,63d	2,0	
15	6,87d	8,4	
16	7,61dd	2,0; 8,4	
		Caffeoate (Ca)	
2	5,89 d	15,8	Ca : H-5; H-9
3	7,23 d	15,8	Ca : H-5; H-9
5	6,69 br.s		G_1 : r.-3; Ca : H-2;H-3
8	6,57 d	7,9	
9	6,54 dd	1,6; 7,9;	Ca : H-2;H-3
		Rhamnosyl (R)	
1	5,50 br.s		
2	4,07 br.s		Qu : H-6; H-8
3	3,84 dd	3,5; 9,4;	
4	3,48 t	9,4	R : H-5
5	3,59 m		
6	1,22 d	6,1	R : H-3
		Glucosyl-1 (G_1)	
1	5,33 d	7,6	G_1 : H-3; H-5; H-6a
2	3,71 dd	7,6; 9,1;	
3	3,59 t	9,1; 9,1;	Ca : H-5
4	3,36 dd	9,1; 9,1;	
5	3,16 m	2,3; 5,1; 9,4	G_1 : H-1; H-3
6a	3,64 dd	2,3 12,0	
6b	3,50		
		Glucosyl-2 (G_2)	
1	4,70d	7,6	G_2 : H-3; H-5; G_1 : H-2; H-4
2	3,43 dd	7,6; 9,2	
3	3,48 t	9,2	G_2 : H-1
4	3,39 t	9,2	
5	3,71		G_2 : H-1; H-3
6a, b	4,31 m		G_2 : H-4



III

In the NOESY spectrum of glycoside (III) there is a cross-peak revealing long-range interaction of the H-5 proton of caffeic acid with H-3 of the glucose residue Glc^1 (Fig. 4). The conformation in which the spatial propinquity of these atoms is realized is probably stabilized by hydrogen bonds between the phenolic and hydroxy groups of the monosaccharide residue Glc^1 and the caffeoate residue.

EXPERIMENTAL

Melting points were determined on a Kofler stage and are uncorrected. Angles of rotation were measured on a Polamat A polarimeter. Electronic spectra were taken in methanol on SF-4A, SF-16, and Specord UV-VIS spectrometers. Vibrational spectra were recorded in KBr tablets on a UR-20 instrument. Jeol RX 90Q and Bruker WP-200sv radiospectrometers were used for recording the NMR spectra of substances (I) and (II), while a Varian VXR-500S spectrometer was used for compound (III). HMDS was employed as standard.

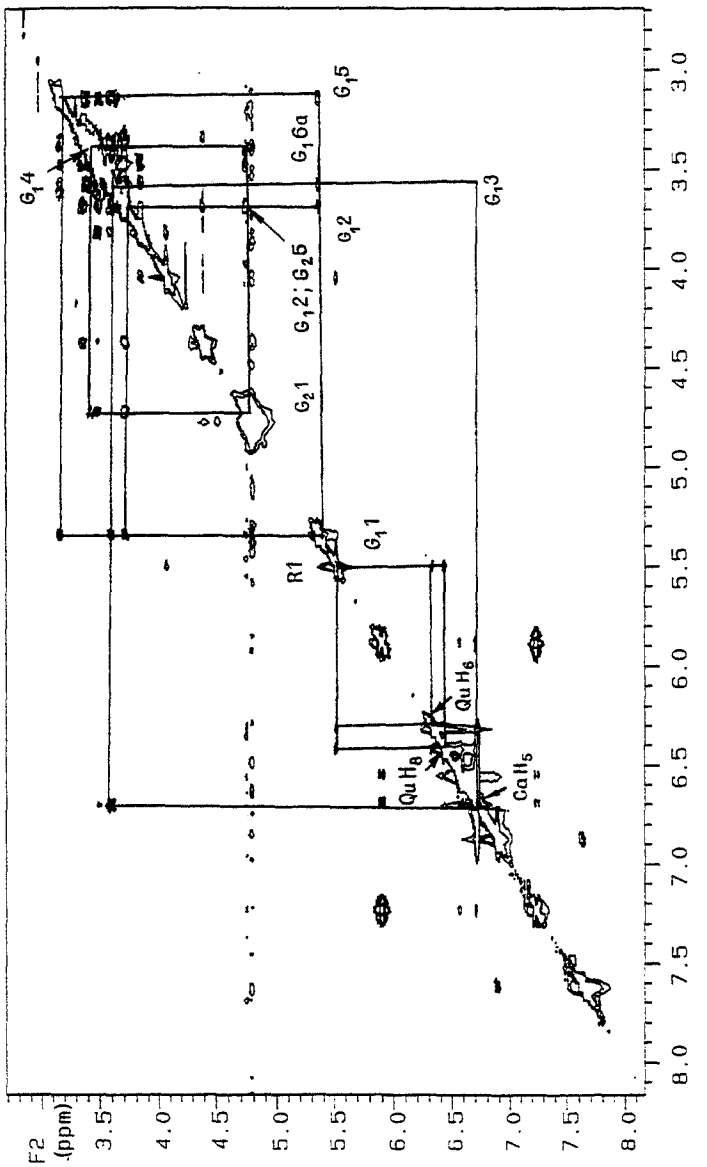


Fig. 4. Fragment of the 2D NOESY spectrum of czekanoside A.

TABLE 2. Chemical Shifts of the Carbon Atoms of Czekanoside A

C atom	Fragments				
	Ca	Qu	Rha	Glc¹	Glc⁴
1	169.0		99,7	100.7	106,3
2	114.5	157,6	71,6	85,1	76,1
3	146,8	135,3	72,1	77,6	77,7
4	127,4	179,7	73,7	71,9	71,1
5	115.0	162.5	71,0	78,1	75,7
6	146,3	100,5	19.0	62,3	64,8
7	149,1	163,2			
8	146,2	95,3			
9	123,8	156,6			
10		107,3			
11		122,9			
12		116,2			
13		145,9			
14		149,8			
15		117,5			
16		122,7			

Mass spectra were obtained on a LKB-2091 instrument with an Iontech FAB ion source with ionization by Xe atoms having an energy of 6 keV at a discharge current of 1 mA.

For column chromatography and thin-layer chromatographies we used Woelm polyamide; the plates were visualized by $AlCl_3$, $FeCl_3$, or diazotized sulfanilic acid. Analysis by the HPLC method was carried out on a Milikhrom-1 chromatograph.

Two-Dimensional NMR Spectroscopy. 2-D NMR spectra were recorded on the Varian VXR-500S spectrometer fitted with a SUN 3/50 computer with the standard VNMR equipment. We used the following methods to obtain two-dimensional $^1H-^1H$ and $^{13}C-^1H$ spectra:

COSY – the standard relayn program. Dimensions of the matrix 1 K × 0.5 K. Width of spectrum 262 Hz. In the pulse sequence we used two 90° pulses (COSY-90). The relaxation delay was 1.8 s. The Fourier transformation of the free-induction decay signal was multiplied by a bell-shaped function with zero shift.

DQCOSY – the standard dqcosy program in the phase-sensitive variant. The size of the matrix 2 K × 1 K. Width of the spectrum 2673 Hz. Before Fourier transformation a Gaussian function with zero shift was used for weighting.

TOCSY – the standard tocsy program in the phase-sensitive variant. Dimensions of the matrix 2 K × 1 K. Width of the spectrum 2673 Hz. Values for the 90°-reading pulses and the trim pulse 22 μs, mixing time [sic] 0.05 ms. Relaxation 1.8 s. To bring the spin system to equilibrium we used the sspyl sequence: 90°–HS–90°. Before Fourier transformation, a Gaussian function with zero shift was used for weighting.

NOESY – the standard noesy program in the phase-sensitive variant. Dimensions of the matrix 1 K × 0.5 K. Width of the spectrum 2673 Hz. Width of the 90° pulse 22 μsec. Mixing time 0.25 s. Relaxation delay 2 s. We used the sspyl: 90°–HS–90°. Filling time 8 h. A Gaussian function with zero shift was used for weighting.

2D $^{13}C-^1H$ – the standard hetcor program. Dimensions of the matrix 2 K × 0.25 K. Width of the spectrum for ^{13}C 6000 Hz, and for ^1H 1150 Hz. Values for the ^{13}C 90° pulse 12.4 μs and for the ^1H 90° pulse 18 μs. Relaxation delay 2 s. The delay τ was adjusted to a value of $J = 140$ Hz. For weighting we used a bell-shaped function with a shift of 90° or without shift in the F2 region and a bell-shaped function with zero shift in the F1 region. In a number of cases we used suppression of the signal of the residual protons of the solvent by presaturation for 1 s.

Isolation of the Glycosides. The leaves and flowers of Aconitum baicalense (5.8 kg) were extracted three times with 60% ethanol. The extract was evaporated to a volume of ~0.5 liter and was treated successively with chloroform, ethyl acetate, and butanol. The ethyl acetate fraction (24.4 g) was subjected to chromatography on a column of polyamide in the $CHCl_3$–CH_3OH system three times. In the final stage, with elution by this mixture in a ratio of 25:1, compound (I) was obtained, while an eluent with a ratio of 50:3 yielded compound (II). The butanolic fraction (450 g) contained a complex mixture of flavonoid

oligosides. It was separated by repeated chromatography on a polyamide column using as eluents mixtures of methanol with water and with chloroform. In this way we obtained a fraction enriched with compound (III). For further purification we carried out precipitation with water from methanolic solution, and the crystalline substance so obtained was subjected to preparative high-performance liquid chromatography with 40% methanol on a column of Nucleosil-5, C_{18} (Bruker LC instrument, isocratic regime, UV detection at 260 nm).

Kaempferol 7-O-α-L-rhamnopyranoside (I). $C_{21}H_{20}O_{10}$, mp 218-221°C (from methanol). IR spectrum, ν_{max}, cm^{-1}: 3420, 2930, 1650, 1580, 1480. UV spectrum (λ_{max}, nm: 367, 320 sh., 265; 255 (lg ε 4.09, 3.82, 4.07, 4.07); +CH_3ONa: 435, 270, 245; +$AlCl_3$: 426, 359, 267, 260 sh.; +$AlCl_3$–HCl: 423, 354, 278 sh., 269; 258; +NaOAc: 410 sh., 385, 255; +NaOAc–H_3BO_3: 370, 255. FAB–MS, m/z: 433 $(M + H)^+$, 287 $(M + H - dhex)^+$.

PMR (DMSO), δ, ppm: 1.11 (d, CH_3 of rhamnose, 5.52 (s, H-1 of rhamnose); 6.4 (d, H-6); 6.8 (d, H-8); 6.91 (d, H-13 and H-15); 8.08 (d, H-12 and H-16).

Quercetin 7-O-α-L-rhamnopyranoside (II). $C_{21}H_{20}O_{11}$, mp 258°C (from methanol). IR spectrum, ν_{max}, cm^{-1}: 3420, 2930, 1650, 1580, 1480. IR spectrum, ν_{max}, nm: 370, 300 sh., 276 sh., 271 sh., 255 (lg ε 4.30; 3.99; 4.08; 4.27; 4.38); +CH_3ONa: 422, 278 sh., 272, 259 sh.; +$AlCl_3$–HCl: 431, 370 sh., 271, 267; +NaOAc: 390, 255; +NaOAc–H_3BO_3: 391, 256. FAB – MS, m/z: 449 $(M + H)^+$, 303 $(M + H - dhex)^+$. PMR (DMSO), δ, ppm, 1.11 (d, CH_3 of rhamnose; 5.52 (s), H-1 of rhamnose); 6.4 (d, H-6); 6.78 (d, H-8); 6.88 (d, H-15); 7.57 (dd, H-16); 7.70 (d, H-12); 9.54 (s, 3-OH); 12.48 (s, 5-OH).

Czekanoside A (III). $C_{42}H_{46}O_{24}$, mp 219-220°C (from water), $[\alpha]_D^{20}$ – 98.1 (c 7.3; methanol). IR spectrum, ν_{max} paraffin oil, cm^{-1}: 3000-3500, 1660, 1590, 1000-1110. UV spectrum, λ_{max}, nm: 337, 270 sh., 255 (lg ε 4.44; 4.35; 4.46); +CH_3ONa: 383, 310 sh., 265; +NaOAc: 383, 253; +NaOAc–H_3BO_3: 365, 260; +$AlCl_3$: 440, 350, 300 sh., 275; +$AlCl_3$–HCl: 405, 340, 300 sh., 275. UV spectrum against a caffeic acid background, λ_{max}, nm: 360, 255; +NaOAc: 275, 255; +NaOAc–H_3BO_3: 370; +$AlCl_2$: 440, 340, 280; +$AlCl_3$–HCl: 405, 270.

FAB–MS: m/z: see scheme.

PMR (CD_3OD), δ, ppm: given in Table 1.

^{13}C NMR (CD_3OD), ppm: given in Table 2.

Hydrolysis of Glycosides (I) and (II). A solution of 5.4 mg of compound (I) or (II) in 0.5 ml of methanol was heated with 0.7 ml of a 4.5% solution of hydrochloric acid in the boiling water bath for 2.5 h. Completeness of hydrolysis was checked on polyamide and Silufol in the chloroform–methanol (3:1) system. The cooled hydrolysate was filtered, and the residue (aglycon) was washed with water and dried. The aglycons obtained were identified by TLC in comparison with known samples, and by mass spectrometry and UV spectroscopy. After the neutralization of the filtrate by the anion-exchange resin AV-17 (OH^-), the sugars were identified by TLC on silica gel in the chloroform–methanol–water (70:23:4) system, the spots being revealed by vanillin in orthophosphoric acid, and by PC in the butanol–pyridine–water (6:4:3) system with revelation by aniline phthalate.

Kaempferol (Ia). $C_{15}H_{10}O_6$, 286 (M^+), mp 258°C, UV spectrum, λ_{max}, nm: 367, 325 sh., 267.

Quercetin (IIa). $C_{15}H_{10}O_7$, 302 (M^+), mp 305-306°C. UV spectrum, λ_{max}, nm: 370, 300 sh., 257.

Acid Hydrolysis of (III). Compound (III) (31 mg) was treated with 3 ml of a mixture of 15% acetic and 15% hydrochloric acids (1:1) and the mixture was heated at 95°C for 3 h. Then, after cooling, the aqueous solution of acids was distilled off from the reaction mixture. The resulting dry residue was treated with 2 ml of water and the mixture was homogenized in an ultrasonic bath for 10 min.

To detect carbohydrates, the suspension was centrifuged for 5 min, and the supernatant liquid was neutralized with AV-17 anion-exchange resin (OH^-). The resin was filtered off, and the aqueous solution was separated to dryness. The residue was dissolved in 0.1 ml of 80% (by vol.) of aqueous acetonitrile, and the solution was analyzed on a Milikhrom-1 liquid chromatograph. The stationary phase was Silasorb-5, NH_2, and the

column dimensions 2 × 64 mm. The mobile phase was acetonitrile–water. To identify the peaks of the carbohydrates we used acetonitrile:water ratios of 80:20, 85:15, and 90:10 (vol/vol). Rate of flow 100 µl/min. Detection at 190 nm. V_r of rhamnose 4.8 min, V_r of glucose 9.6 min (at an acetonitrile:water ratio of 80:20).

To detect quercetin and caffeic acid, the homogenized aqueous suspension after acid hydrolysis was centrifuged, and the supernatant liquid was passed through a Sep-Pak C18 cartridge. The cartridge was washed with water and then with ethanol. The methanolic solution was evaporated to dryness. The residue was combined with the residue obtained on centrifugation and was dissolved in 0.1 ml of a mixture of 0.1 M KH_2PO_4 (pH 2.50) and methanol (60:40, vol/vol). The solution was analyzed on a Milikhrom-1 liquid chromatograph. The stationary phase was Silasorb-5, C18 and the column dimensions 2 × 64 mm. The mobile phase was a mixture of 0.1 M KH_2PO_4 (pH 2.50) and methanol. Elution with a stepwise gradient of methanol, the volume of each step being 400 µl. The KH_2PO_4:methanol ratios were 80:20, 60:40, and 20:80 (vol/vol) successively for each stage. The rate of flow was 100 µl/min. V_r of caffeic acid 7.9 min. Detection at 240 and 330 nm. V_r of quercetin 10.9 min. Detection at 260 and 350 nm.

LITERATURE CITED

1. N. G. Bisset, J. Ethnopharmacol., 4, No. 3, 247 (1981).
2. D. A. Young and R. W. Sterner, Phytochemistry, 20, 2055 (1981); I. I. Samokish, Khim. Prir. Soedin., 787 (1984); I. I. Samokish, A. L. Shinkarenko, and V. A. Makarov, Khim. Prir. Soedin., 439 (1969).
3. I. I. Samokish and A. L. Shinkarenko, Khim. Prir. Soedin., 441 (1969).
4. S. Asen and S. L. Emsweller, Proc. Am. Soc. Hort. Sci., 81, 530 (1962).
5. F. Kozjek, P. Lebreton, T. J. Mabry, K. R. Markham, H. Yoshioka, and G. Netein, Ann. Pharm. Fr., 26, Nos. 7-8, 513 (1968).
6. H. Ravn, S. Nishibe, M. Sasahara, and L. Xuebo, Phytochemistry, 29, 3627 (1990).
7. T. Kodama, H. Ishida, T. Kokubo, T. Yamakawa, and H. Noguchi, Agr. Biol. Chem., 54, No. 12, 3283 (1990).
8. T. Brasseur and L. Angenot, Phytochemistry, 25, 563 (1986).
9. C. Nasr, A. Lobstein-Guth, M. Haag-Berrurier, and R. Anton, Phytochemistry, 26, 2869 (1987).