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PHENYLPROPANOIDS FROM CALLUS TISSUE OF *Ajuga turkestanica*

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Phenylpropanoids 3,4-dihydroxy-β-*phenylethoxy*-O-α-*L-arabinopyranosyl-(1→2)*-α-*L-rhamnopyranosyl-(1→3)-4*-O-*caffeoyl*-β-*D-glucopyranoside* (**1**) *and 3,4-dihydroxy*-β-*phenylethoxy*-O-α-*L-arabinopyranosyl-(1→2)*-α-*L-rhamnopyranosyl-(1→3)-4*-O-*feruloyl*-β-*D-glucopyronoside* (**2**) *were isolated for the first time during studies of the chemical composition of* Ajuga turkestanica *(Regel) Briq. callus tissue.*

Keywords: *Ajuga turkestanica*, callus tissue, phenylpropanoids, glucose, rhamnose, arabinose.

New sources of biologically active compounds must be discovered without damaging natural reserves because of the rising demand for herbal preparations. Therefore, biotechnological approaches to the production of secondary metabolites from isolated plant tissues are highly promising [1].

Cell cultures are currently widely used in basic research because biosynthetic processes can be examined without correlated interactions and monitoring of other tissues and the whole organism. Model systems are developed by characterizing the physiology of the organ and studying its sensitivity to various hormones.

Ajuga turkestanica (Regel) Briq. is a perennial herbaceous plant of the family Lamiaceae. The species produces ecdysteroids with broad spectra of pharmacological activity [2] and is endemic to mountains of the Hissar Range.

Previously, *A. turkestanica* cell culture obtained from plant ovaries was found to biosynthesize *in vitro* ecdysterone and turkesterone [3, 4].

The goal of the present work was to study secondary metabolites produced by *A. turkestanica* callus tissue originating in leaves, to isolate them pure, to identify them, and to elucidate their structures.

Callus tissue was obtained from leaves of wild *A. turkestanica* collected in 2014 in Surxondaryo Region near Baysun village. The culture was grown in medium without added growth regulators and characteristically gave high yields of crude mass. The growth index in the second year of cultivation was 9.29.

HPLC found that the MeOH extract of biomass from the third year of cultivation contained mainly phenolic compounds with insignificant contents of ecdysterone and turkesterone. The MeOH extract of *A. turkestanica* callus tissue afforded several phenylpropanoid compounds, two of which were phenylpropanoid glycosides lavandulifolioside (**1**) [5] and leonoside A (**2**) [6].

Compound **1** was a dark-yellow resin. Its structure was elucidated by analyzing PMR and ^{13}C NMR spectra and HSQC and HMBC experiments. PMR spectra taken in C_5D_5N exhibited resonances characteristic of caffeic acid, a 3,4-dihydroxyphenylethanoid, and three monosaccharides [5].

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TABLE 1. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2** (Py-d_5, δ, ppm, J/Hz)

C atom	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
		Aglycon		
1	130.86	–	130.86	–
2	117.96	7.22 (m)	117.97	7.22 (m)
3	147.65	–	147.66	–
4	146.15	–	146.16	–
5	117.01	7.18 (m)	117.01	7.18 (m)
6	120.94	6.75 (dd, J = 8.1, 2.0)	120.94	6.76 (dd, J = 8.1, 2.2)
7	36.62	2.98 (2H, t, J = 7.5)	36.62	2.99 (2H, t, J = 7.9)
8	71.81	3.91 (dd, J = 16.2, 7.5) 4.30 (dd, J = 16.2, 7.5)	71.83	3.92 (m); 4.31 (m)
		Glucose		
1′	104.76	4.81 (d, J = 7.8)	104.79	4.85 (d, J = 7.8)
2′	76.13	4.02 (dd, J = 8.5, 8.0)	76.15	4.04 (m)
3′	80.84	4.47 (m)	80.81	4.52 (m)
4′	70.62	5.69 (t, J = 9.7)	70.71	5.72 (t, J = 9.5)
5′	76.81	3.97 (m)	76.83	4.03 (m)
6′	62.55	4.11 (m); 4.20 (d, J = 12.1)	62.60	4.15 (m); 4.22 (m)
		Rhamnose		
1″	102.14	6.41 (s)	102.12	6.44 (s)
2″	82.47	4.77 (d, J = 3.2)	82.48	4.78 (br.s)
3″	73.27	4.49 (m)	73.28	4.50 (m)
4″	74.87	4.13 (m)	74.86	4.14 (m)
5″	70.49	4.40 (m)	70.48	4.43 (m)
6″	19.45	1.58 (d, J = 6.1)	19.42	1.62 (d, J = 6.1)
		Arabinose		
1‴	108.01	5.18 (d, J = 7.5)	108.03	5.18 (d, J = 5.9)
2‴	73.76	4.51 (m)	73.75	4.51 (m)
3‴	75.23	4.10 (m)	75.24	4.10 (m)
4‴	70.17	4.23 (m)	70.17	4.22 (m)
5‴	67.84	3.72 (d, J = 12.0) 4.25 (dd, J = 12.0, 2.2)	67.87	3.72 (d, J = 12.3) 4.24 (m)
		Caffeic acid		Ferulic acid
1⁗	127.44	–	127.06	–
2⁗	116.26	7.64 (br.s)	112.01	7.36 (br.s)
3⁗	148.14	–	149.42	–
4⁗	151.06	–	151.64	–
5⁗	117.18	7.20 (m)	117.26	7.18 (m)
6⁗	122.80	7.20 (m)	124.29	7.27 (d, J = 8.1)
7⁗	147.22	8.03 (d, J = 15.8)	146.91	8.03 (d, J = 15.8)
8⁗	115.22	6.73 (d, J = 15.8)	115.61	6.77 (d, J = 15.8)
9⁗	167.47	–	167.40	–
3⁗-OCH_3	–	–	56.29	3.78 (s)

The aromatic protons of caffeic acid appeared at weak field as a 2H multiplet at 7.20 ppm (H-5⁗ and H-6⁗); a broad 1H singlet at 7.64 ppm (H-2⁗); and two olefinic resonances of the acyclic chain as two doublets at 8.03 and 6.73 ppm with spin–spin coupling constant (SSCC) J = 15.8 Hz. These data were confirmed by resonances in the ^{13}C NMR spectrum of **1** at 147.22 (C-7⁗) and 115.22 ppm (C-8⁗) (Table 1).

The SSCC (J) of 15.8 Hz indicated that H-7⁗ and H-8⁗ were positioned *trans* relative to each other. The PMR spectrum showed resonances for aromatic protons of the 3,4-dihydroxyphenylethanoid part of the compound as multiplets at 7.18 (H-5) and 7.22 ppm (H-2) and a doublet of doublets at 6.75 ppm (H-6) with *meta*- and *ortho*-constants (J = 2.0, 8.1 Hz). Furthermore, the spectrum had a 2H triplet at 2.98 ppm (J = 7.5 Hz, H-7) and two doublets of doublets at 3.91 (J = 16.2, 7.5 Hz, H-8a) and 4.30 ppm (J = 16.2, 7.5 Hz, H-8b) for the ethanoid moiety.

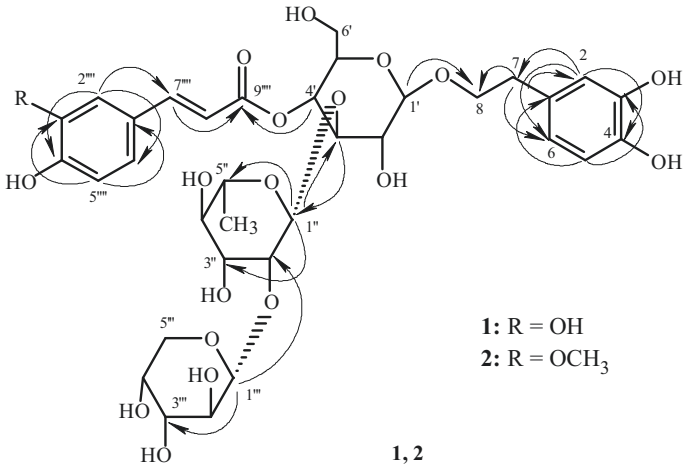

Fig. 1. Chemical structures of **1** and **2** and key cross-peaks in an HMBC experiments.

Anomeric protons of the three carbohydrates resonated at 4.81 ppm as a doublet with SSCC J = 7.8 Hz (H-1′), at 6.41 as a singlet (H-1″), and at 5.18 as a doublet with SSCC J = 7.5 (H-1‴). The three anomeric protons confirmed that the compound was a trioside. The other carbohydrate proton resonances were observed at 5.69–3.97 ppm; rhamnose methyl protons, as a doublet at 1.58 ppm (J = 6.1 Hz).

^{13}C NMR and HSQC spectra of **1** showed resonances for 34 C atoms represented by seven quaternary including one carbonyl, 22 methine, four methylene, and one methyl C atom.

The ^{13}C NMR spectrum at strong field exhibited characteristic resonances for rhamnose methyl at 19.45 ppm and a phenylpropanoid methylene. The range 62–82 ppm showed 13 ^{13}C resonances characteristic of carbohydrates. The three anomeric C atoms resonated in the middle of the ^{13}C NMR spectrum at 102.14 (C-1″), 104.76 (C-1′), and 108.01 ppm (C-1‴). An analysis of the PMR and ^{13}C NMR spectral data and HSQC and HMBC experiments led to the conclusion that compound **1** contained glucose, rhamnose, and arabinose in a 1:1:1 ratio. The aromatic and weak-field parts of the ^{13}C NMR spectrum exhibited resonances for phenylethanoid and caffeic acid aromatic C atoms (Table 1).

The positions of the caffeic acid and three carbohydrates in **1** were established using HMBC and chemical shifts of C atoms. The HMBC spectrum of **1** showed cross peaks H-1′/C-8, H-3′/C-1″, H-4′/C-9″, and H-1‴/C-2″. Also, H-4′ (5.69 ppm) was shifted to weaker field in the PMR spectrum; C-3′ (80.84) and C-2″ (82.47), in the ^{13}C NMR spectrum. These data established that glucose was in the phenylpropanoid C-8 position; caffeic acid and rhamnose, on glucose C-4′ and C-3′, respectively; and arabinose, on rhamnose C-2″.

Acid hydrolysis of **1** produced D-glucose, L-rhamnose, and L-arabinose and mixtures of genin-type products.

The results as a whole gave the structure of **1** as 3,4-dihydroxy-β-phenylethoxy-*O*-α-L-arabinopyranosyl (1→2)-α-L-rhamnopyranosyl-(1→3)-4-*O*-caffeoyl-β-D-glucopyranoside isolated earlier from *Stachys lavandulifolia* [5] (Fig. 1). Compound **2** was identified as leonoside A, the ^{1}H and ^{13}C spectral data are given in Table 1 [6].

Phenylpropanoids were previously isolated from seven *Ajuga* species growing in Japan [6] and from *A. turkestanica* for the first time.

EXPERIMENTAL

General Comments. NMR spectra were recorded in C_5D_5N on a Varian VNMRS 600 at operating frequency 600 MHz for ^{1}H. The internal standard for PMR spectra was TMS (0 ppm); for ^{13}C NMR spectra, a solvent resonance (α-Py-d_5, 150.35 ppm vs. TMS). UV spectra were obtained on a Shimadzu UV-2550 spectrophotometer. Analytical HPLC used a Dionex Ultimate 3000 instrument with a UV detector and XBridge™ C18 column (4.6 × 250 mm, 5 μm). Preparative HPLC used a Shimadzu LC-20AT with a UV detector and XBridge™ Prep C18 column (10 × 150 mm, 5 μm). TLC was performed on Silufol UV 254 chromatographic plates with detection by I_2 vapor, NH_3 vapor, UV light at 254 and 365 nm, and vanillin solution (1%) in conc. H_2SO_4. Paper chromatography (PC) was carried out on Filtrak No. 11 paper using

n-BuOH–AcOH–H_2O (4:1:5, system 1) and *n*-BuOH–Py–H_2O (6:4:3, system 2). Free monosaccharides in PC were detected by spraying with aniline hydrogen phthalate.

Callus Tissue Cultivation Conditions. Callus tissue was cultivated at 26°C in the dark in Murashige–Skoog medium [7] with added sucrose (30 g/L), meso-inositol (100 mg/L), thiamine HCl (0.4 mg/L), and agar-agar (0.75%) and without added growth regulator.

Extraction and Isolation of Phenylpropanoid Triglycosides from *A. turkestanica* Callus Tissue. Air-dried and milled plant raw material (100 g) was extracted with MeOH (3 × 200 mL) at room temperature. The combined extract was evaporated *in vacuo*. The condensed residue (20 g) was chromatographed over a column (1.5 × 150 cm) with ODS (150 g) using a MeOH–H_2O gradient (100:1→1:1) to produce fractions A–C that were separated by prep. HPLC using MeOH–H_2O.

Compounds 1 and 2 were isolated by prep. HPLC using MeOH (35%)–H_2O as a dark-yellow amorphous powder, $C_{34}H_{44}O_{19}$ (**1**) and $C_{35}H_{46}O_{19}$ (**2**). Table 1 lists the PMR and ^{13}C NMR spectral data (600 MHz).

Acid Hydrolysis. Compound **1** or **2** (10 mg) was hydrolyzed by HCl (5%) in aqueous MeOH (15 mL) for 2 h on a boiling-water bath. The resulting precipitate of the aglycon was filtered off. The carbohydrate part of the hydrolysate was neutralized with $BaCO_3$ and evaporated. PC of the residue using system 2 identified D-glucose, L-rhamnose, and L-arabinose.

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