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NEW ELLAGIC ACID GLYCOSIDES FROM *Punica granatum*

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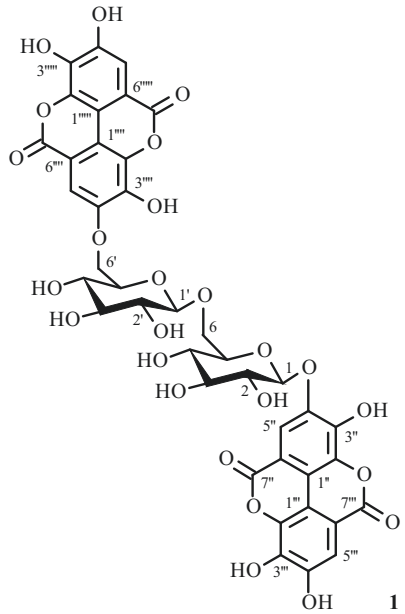
Two new ellagic acid glycosides were isolated from Punica granatum *L. (Lythraceae) pericarps and identified using UV and NMR spectroscopy and mass spectrometry as 1,6′-di-*O*-ellagoylgentiobiose (granatoside A, **1**) and 1,6-di-*O*-ellagoyl-*β*-D-glucopyranose (granatoside B, **2**). Compounds **1** and **2** exhibited* α*-glucosidase inhibitory activity.*

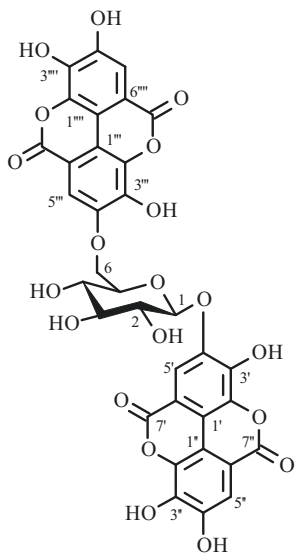
Keywords: *Punica granatum*, ellagic acid, granatoside A, granatoside B, α-glucosidase inhibitors.

Punica granatum L. (Lythraceae) is a valuable food plant that is widely used as medicine. Test results of the last decades indicate that *P. granatum* and its preparations can be used to prevent and treat type II diabetes [1]. The key mechanisms of antidiabetic action of *P. granatum* include the ability of its constituents to reduce *in vivo* oxidative stress and to affect the main digestive enzymes [2]. Phenolic compounds possessing inhibitory activity against α-glucosidase, an enzyme involved in hydrolysis of disaccharides sucrose and maltose to glucose, are especially interesting [3, 4].

Flowers and fruit of *P. granatum* were found earlier to contain various groups of such inhibitors, among which ellagitannins [5], gallotannins, and chalcones [6] were identified. Pericarp of *P. granatum* had the highest content of phenolic compounds among which tannins, ellagic acid, and ellagoyl derivatives were detected [7]. The last compound group exhibited pronounced anti-α-glucosidase activity [8]. We continued research on natural α-glucosidase inhibitors [9–13] by isolating from *P. granatum* pericarp two new ellagic acid glycosides (**1** and **2**) with anti-α-glucosidase activity.



1



2

Chromatographic separation of the EtOAc fraction of *P. granatum* pericarp isolated six known compounds including amritoside (**3**) [14], 1-*O*-ellagoylglucose (**4**) [7], punicalagins A/B (**5**/**6**) [15], granatin B (**7**) [16], and ellagic acid (**8**) [7] in addition to **1** and **2**.

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TABLE 1. PMR (500 MHz) and ^{13}C NMR Spectra (125 MHz) of **1** and **2** (Py-d_5, δ, ppm, J/Hz)

C atom	**1**		C atom	**2**	
	δ_H	δ_C		δ_H	δ_C
	β-D-Glucopyranose			*β*-D-Glucopyranose	
1	5.60 (1H, d, J = 8.0)	100.9	1	5.47 (1H, d, J = 8.1)	101.4
2	3.25–3.80 (4H, m)	73.4	2	3.20–3.62 (4H, m)	73.2
3		76.7	3		76.4
4		70.2	4		70.1
5		75.9	5		75.7
6	4.18 (1H, dd, J = 12.0, 2.1, H_B), 3.95 (1H, dd, J = 12.0, 5.9, H_A)	69.2	6	4.35 (1H, dd, J = 12.0, 2.1, H_B), 4.20 (1H, dd, J = 12.0, 6.2, H_A)	69.3
	6-*O*-*β*-D-Glucopyranose				
1′	4.52 (1H, d, J = 8.0)	104.2			
2′	3.25–3.80 (4H, m)	73.9			
3′		76.9			
4′		70.8			
5′		76.3			
6′	4.40 (1H, dd, J = 12.4, 2.4, H_B), 4.25 (1H, dd, J = 12.4, 6.1, H_A)	69.4			
	1-*O*-Ellagoyl			1-*O*-Ellagoyl	
1″		108.5	1′		108.6
2″		136.4	2′		136.2
3″		141.8	3′		141.9
4″		148.7	4′		148.9
5″	7.65 (1H, s)	111.8	5′	7.62 (1H, s)	111.9
6″		114.7	6′		114.8
7″		159.7	7′		159.6
1‴		107.3	1″		107.1
2‴		136.2	2″		136.0
3‴		140.2	3″		140.3
4‴		147.5	4″		148.0
5‴	7.41 (1H, s)	111.5	5″	7.37 (1H, s)	111.4
6‴		114.3	6″		114.4
7‴		159.6	7″		159.4
	6′-*O*-Ellagoyl			6-*O*-Ellagoyl	
1⁗		108.5	1‴		108.6
2⁗		136.4	2‴		136.2
3⁗		141.8	3‴		141.9
4⁗		148.0	4‴		148.9
5⁗	7.52 (1H, s)	111.8	5‴	7.53 (1H, s)	111.9
6⁗		114.7	6‴		114.8
7⁗		159.4	7‴		159.3
1⁗′		107.3	1⁗		107.1
2⁗′		136.2	2⁗		136.0
3⁗′		140.2	3⁗		140.3
4⁗′		147.0	4⁗		148.0
5⁗′	7.38 (1H, s)	111.5	5⁗	7.34 (1H, s)	111.4
6⁗′		114.3	6⁗		114.4
7⁗′		159.3	7⁗		159.1

The UV spectrum of **1** indicated that it was an ellagic acid derivative. The products of acid hydrolysis of **1** were ellagic acid and D-glucose. The ESI-MS showed a pseudo-molecular ion with *m*/*z* 909 $[M - H]^-$ and fragments with *m*/*z* 463 and 301 that were characteristic of monoglycosylated and free ellagic acid, respectively [17]. The detection of deprotonated fragments with *m*/*z* 341 (glucosylglucose) and 161 (glucose) indicated that the structure included a disaccharide. The PMR spectrum contained four 1H singlets (δ 7.65, 7.52, 7.41, 7.38) that were consistent with two ellagic acid moieties and two resonances for anomeric protons at δ 5.60 (1H, d, J = 8.0 Hz) and 4.52 (1H, d, J = 8.0 Hz) that belonged to glucose residues (Table 1).

Complete methylation of **1** followed by hydrolysis gave 2,3,4-tri-*O*-methylglucopyranose as the only permethylate decomposition product. This indicated that the glucose residues were not terminal but bonded to each other as the disaccharide 6-*O*-glucosylglucose (gentiobiose). The results also confirmed that the most probable locations of the ellagoyl moieties were gentiobiose C-1 and C-6′.

The ^{13}C NMR spectrum showed 4 resonances for lactone carbonyls at δ 159.7, 159.6, 159.4, and 159.3 ppm for two ellagic acid moieties and 12 resonances for aliphatic C atoms of two glucose residues (Table 1). The locations of the gentiobiose C-1 (δ 100.9) and C-1′ resonances (δ 104.2) indicated that their anomeric centers had the *β*-configuration. Weak-field resonances for C-6 (δ 69.2) and C-6′ (δ 69.4) confirmed that these positions were substituted. The HMBC spectrum showed correlations between gentiobiose H-1 and ellagic acid C-4″ (δ_H/δ_C 5.60/148.7) and gentiobiose H-6′ and ellagic acid C-4‴′ (δ_H/δ_C 4.25, 4.40/148.0), also indicating that the ellagic acids were bonded through C-4″ and C-4‴′ to gentiobiose C-1 and C-6′. Thus, **1** was 1,6′-di-*O*-ellagoylgentiobiose according to the results and was named granatoside A .

The spectral properties of **2** were similar to those of **1**. The ESI-MS spectrum showed a peak for a pseudo-molecular ion with *m*/*z* 747 $[M - H]^-$, i.e., 162 amu less than **1**. This result argued in favor of **2** being an analog of **1** without one of the glucoses. NMR spectra of **2** were characterized by fewer resonances (Table 1). The PMR spectrum had a single anomeric proton resonance at δ 5.47 (1H, d, J = 8.1 Hz, H-1); the ^{13}C NMR spectrum, six resonances for aliphatic C atoms of one glucopyranose. The HMBC spectrum exhibited correlations between resonances for glucose H-1 (δ 5.47) and H-6 (δ 4.20, 4.35) and ellagic acid C-4′ (δ 148.9) and C-4‴ (δ 148.9), respectively. These features established the structure of **2** as 1,6-di-*O*-ellagoyl-*β*-D-glucopyranose, which we called granatoside B.

Until now, the only known di-*O*-ellagoyl derivative was 1,6′-di-*O*-ellagoyl-(4-*O*-glucosyl)rhamnopyranose, which was isolated from *P. granatum* wood [18]. Compounds **3**–**8** were previously isolated from *P. granatum* [5–7].

Biological studies of **1** and **2** showed that they were capable of inhibiting *α*-glucosidase with IC_{50} values of 52.0 ± 1.7 and 92.4 ± 3.6 μM, respectively (IC_{50} of acarbose, 84.2 ± 3.0 μM). The inhibition efficiency of unsubstituted ellagic acid was much lower (154.3 ± 5.2 μM). This indicated that the carbohydrate residues (gentiobiose, glucose) had a positive influence on the anti-*α*-glucosidase activity of the ellagic-acid derivatives. Earlier, several ellagic-acid glycosides were shown to have greater inhibitory effects on *α*-glucosidase than the deglucosylated analogs. Thus, 3,3′-dimethoxyellagic acid and its more active 4-*O*-*β*-D-xylopyranoside were isolated from *Terminalia superba* Engl. & Diels (Combretaceae) [8]. The *α*-glucosidase inhibition for 3,4′-dimethoxyellagic acid 3′-*O*-*β*-D-xylopyranoside was greater than that of acarbose [19]. Thus, new compounds **1** and **2** were isolated from *P. granatum* pericarp and could display hypoglycemic activity by suppressing absorption of carbohydrates from the intestines and lowering postprandial elevation of blood glucose levels [20]. Ellagic-acid glycosides could be recommended as promising antidiabetic agents.

EXPERIMENTAL

P. granatum was cultivated under cover at experimental plantings of the IGEB, SB, RAS (2014). Pericarps from ripe *P. granatum* fruit were dried under vacuum to 8–10% moisture (of the air-dried raw material mass) and used in the work.

Column chromatography (CC) used reversed-phase silica gel (RP-SiO_2) and Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA). Spectrophotometric studies used an SF-2000 spectrophotometer (OKB Spectr, St. Petersburg, Russia). Mass spectrometric studies were performed in an LCMS-8050 TQ mass spectrometer (Shimadzu, Columbia, MD, USA). Conditions: electrospray ionization (ESI), negative-ion mode, ESI interface temperature 300°C, desolvation line temperature 250°C, heating block temperature 400°C, spray-gas (N_2) flow rate 3 L/min, heating-gas (air) flow rate 10 L/min, collision-inducing dissociation gas (CID, Ar) pressure 270 kPa, Ar flow rate 0.3 mL/min, capillary potential +25 kV, field potential 3.5 kV, mass scan range (*m*/*z*) 100–1000. NMR spectra were recorded on a VXR 500S NMR spectrometer (Varian, Palo Alto, CA, USA). Preparative HPLC used a Summit liquid chromatograph (Dionex, Sunnyvale, CA, USA) equipped with an RP-18 LiChrospher column (250 × 10 mm, ∅ 10 μm, Supelco, Bellefonte, PA, USA), mobile phase H_2O (A) and MeCN (B), flow rate (ν) 1 mL/min, column temperature 30°C, and UV detector at 270 nm. Analytical HPLC used a Milichrom A-02 microcolumn liquid chromatograph (EcoNova, Novosibirsk, Russia) equipped with a ProntoSIL-120-5-C18 AQ column (2 × 75 mm, ∅ 5 μm, Metrohm AG, Herisau, Switzerland).

Extraction and Fractionation. A weighed portion (1.2 kg) of milled raw material was extracted (2×) with EtOH (60%) (1:20) in an ultrasonic bath (100 W, 35 kHz) at 45°C for 90 min. The resulting extract was filtered and concentrated

under vacuum (40°C) to an aqueous residue that was extracted with hexane and EtOAc to produce an EtOAc fraction (216 g) that was separated in 20-g portions over Sephadex LH-20 (5 × 100 cm) with elution by $Me_2CO–H_2O$ (100:0→0:100). Subfractions of similar composition were combined and rechromatographed over Sephadex LH-20 under the same conditions followed by separation over RP-SiO_2 (2 × 30 cm, MeCN–H_2O eluent, 100:00:100) and by preparative HPLC [gradient mode (%B): 5–90 min, 5–30%] to isolate eight compounds, i.e., **1** (12 mg), **2** (18), amritoside (1-*O*-ellagoylgentiobiose, 22 mg, **3**) [14], 1-*O*-ellagoylglucose (18 mg, **4**) [7], punicalagins A/B (hexahydroxydiphenoylgallagyl-α/β-D-glucopyranose as the total with α/β ratio 1:18, 9.7 g, **5**/**6**) [15], granatin B (galloyl hexahydroxydiphenoyldehydrohexahydroxydiphenoyl-β-D-glucopyranose, 107 mg, **7**) [16], and ellagic acid (15.2 g, **8**) [7].

Granatoside A (1). $C_{40}H_{30}O_{25}$. UV spectrum (MeOH, λ_{max}, nm): 256, 357. HR-ESI-MS, *m*/*z* 909.640 ([M – H]$^-$; calcd 909.662). ESI-MS, *m/z*: 909 [M – H]$^-$, 463 [(M – H) – $C_{14}H_5O_8$ – $C_6H_{10}O_5$]$^-$, 341 [(M – H) – 2 × $C_{14}H_5O_8$]$^-$, 301 [(M – H) – $C_{14}H_5O_8$ – 2 × $C_6H_{10}O_5$]$^-$, 161 [(M – H) – 2 × $C_{14}H_5O_8$ – $C_6H_{10}O_5$]$^-$. Table 1 lists the PMR (500 MHz, 300 K, Py-d_5, δ, ppm) and ^{13}C NMR spectra (125 MHz, 300 K, Py-d_5, δ, ppm).

Granatoside B (2). $C_{34}H_{20}O_{20}$. UV spectrum (MeOH, λ_{max}, nm): 254, 355. HR-ESI-MS, *m*/*z*: 747.503 ([M – H]$^-$; calcd 747.519). ESI-MS, *m/z*: 747 [M – H]$^-$, 463 [(M – H) – $C_{14}H_5O_8$]$^-$, 301 [(M – H) – $C_{14}H_5O_8$ – $C_6H_{10}O_5$]$^-$, 161 [(M – H) – 2 × $C_{14}H_5O_8$]$^-$. Table 1 lists the PMR (500 MHz, 300 K, Py-d_5, δ, ppm) and ^{13}C NMR spectra (125 MHz, 300 K, Py-d_5, δ, ppm).

Acid Hydrolysis of 1 and 2. The compound (2 mg) was dissolved in trifluoroacetic acid (TFA, 5%, 5 mL) in Me_2CO and heated at 100°C for 2 h. The hydrolysate was concentrated to dryness with MeOH *in vacuo*. The dry residue was dissolved in EtOH (50%, 2 mL) and passed over RP-SiO_2 (5 g) with elution successively by H_2O (40 mL, eluate I) and MeCN (40%, 60 mL, eluate II). A portion of eluate I was derivatized with 3-methyl-1-phenyl-2-pyrazolin-5-one [21] and analyzed by analytical HPLC (conditions 1). A second portion of eluate I underwent reductive amination with L-tryptophan [22] followed by analytical HPLC (conditions 2) to determine if the monosaccharides were the D- or L-form. Eluate II was analyzed by ^{13}C NMR spectroscopy and mass spectrometry. Hydrolysates of **1** and **2** contained ellagic acid (**8**) [7] and D-glucose.

Compounds 1 and 2 were methylated in K_2CO_3–DMF–MeI according to Moalin et al. [23] followed by GC-MS analysis of the hydrolysate [24].

Anti-α-glucosidase activity was determined by a microplate spectrophotometric method as described earlier [25]. The positive control was ellagic acid (≥95%, No. E2250), and acarbose (≥95%, A8980, Sigma-Aldrich).

Analytical HPLC. Conditions 1: mobile phase CH_3COONH_4 (100 mM, pH 4.5) (A) and MeCN (B); gradient mode (%B): 0–20 min, 20–26%, ν 150 μL/min; column temperature 35°C; UV detector at 250 nm. Retention times of monosaccharide derivatives with 3-methyl-1-phenyl-2-pyrazolin-5-one (t_R, min) were glucose 12.50. Conditions 2: mobile phase NaH_2PO_4 (10 mM) and $Na_2B_4O_7$ (50 mM) (1:1, pH 9.6); isocratic mode; ν 200 μL/min; column temperature 35°C; UV detector at 220 nm. Retention times of monosaccharide derivatives with L-tryptophan (t_{R-}, min) were D-glucose 8.32 and L-glucose 8.67.

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