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Two ellagitannins from *Punica granatum* heartwood

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Abstract

In the course of studies on polyphenol metabolism in *Punica granatum* heartwood two new ellagitannins, diellagic acid rhamnosyl (1→4) glucopyranoside and 5-*O*-galloylpunicacortein D were isolated and characterized together with four known tannin metabolites, punicacortein D, punicalin, punicalagin and 2-*O*-galloylpunicalin. Structures of the isolated compounds were established by chromatography, chemical degradation, UV and 1D/ 2D $^{1}H/^{13}C$ NMR spectroscopy.

Keywords: *Punica granatum*; Punicaceae; Heartwood; Diellagic acid glycoside; 5-*O*-Galloylpunicacortein D; NMR

1. Introduction

Ellagitannins are a large group of polyphenolic compounds being widely distributed in higher plants. Increasing interest in the biological and pharmacological role of these metabolites in the therapeutic effects of traditional medicines (Haslam, 1998; Haslam et al., 1989; Okuda et al., 1989) has led to a rapid growth of knowledge in this area. Correlation of the biological activity of tannins with their structure has been achieved by their isolation using reversed-phase HPLC and by structural determination with high-resolution NMR and MS spectroscopic techniques. In our preceding paper (El-Toumy et al., 2001) we reported on the isolation and structure elucidation of ellagic acid 4-*O*-α-L-rhamnopyranoside, 6-*O*-galloyl-(α/β)-*O*-glucopyranose, 6-*O*-galloyl-2,3-*S*-hexahydroxydiphenoyl-(α/β)-D-glucopyranose, corilagin, 3,3′/-di-*O*-methylellagic acid, ellagic acid, gallic acid, methyl gallate and 3′/-*O*-methyl-3,4-methylendioxyellagic acid, isolated from the heartwood of *Punica granatum* L. (Punicaceae). Further chemical examination of the polyphenolic metabolites of this plant has now resulted in the isolation of two new ellagitannins, diellagic acid rhamnosyl(1→4) glucopyranoside (**1**) and 5-galloylpunicacortein D (**2**), in addition to punicacortein D (**3**), punicalin (**4**), punicalagin (**5**) and 2-*O*-galloylpunicalin (**6**) (Tanaka et al., 1986a,b). This paper deals with the isolation and structure determination of these tannins.

2. Results and discussion

Dry powder of *Punica granatum* heartwood was extracted with aq. ethanol (80%) under reflux. The extract obtained was concentrated in vacuo and applied to polyamide CC using H_2O–EtOH mixtures with decreasing polarity to afford fractions containing ellagitannins and gallotannins. Further purification was achieved by chromatography on microcrystalline cellulose and Sephadex LH-20 columns. Elution with aq. ethanol and/or *n*-butanol saturated with water afforded the known tannins (**3–6**) along with two new tannins, diellagic acid rhamnosyl (1→4) glucopyranoside (**1**) and 5-*O*- galloylpunicacortein D (**2**). Their structures were elucidated by established chromatographic and spectroscopic methods, in particular NMR techniques.

Compound **1** was isolated as a yellowish amorphous powder, which showed a single spot on TLC that exhibited dark purple color under UV light, gave a positive ferric chloride test (Bate-Smith, 1972) and UV spectral data characteristic of an ellagic acid derivative [UV λ_{max} (MeOH): 255, 355]. On acid hydrolysis, compound **1** afforded one mole of ellagic acid and a mixture

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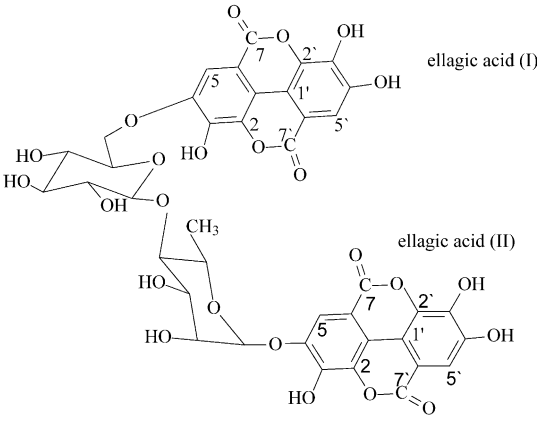
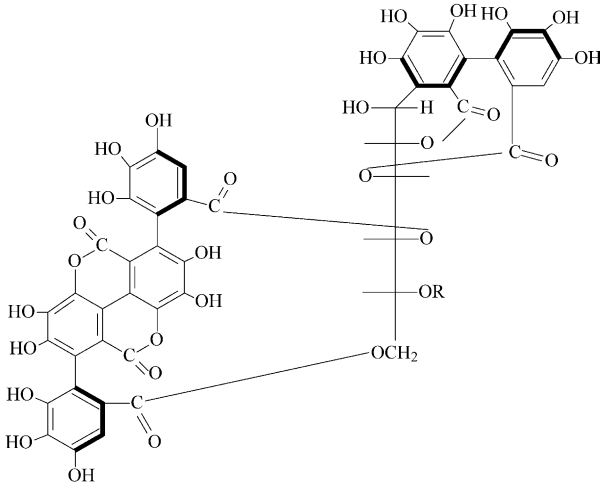


1



2 R = O—C(=O)—(3,4,5-trihydroxyphenyl)

3 R = H

of two sugars that were identified by TLC as D-glucose and L-rhamnose. The ^{1}H NMR spectrum of **1** showed four one-proton singlets at δ 7.66, 7.55, 7.39 and 7.38, suggesting the presence of two ellagic acid moieties. The presence of a disaccharide moiety was supported by the ^{1}H NMR spectrum, which showed two anomeric proton signals at δ 5.47 (1 H, *d*, $J_{1,2}$ = 1.2 Hz) and 4.69 (1 H, *d*, $J_{1,2}$ = 6.6 Hz), which were assigned to H-1 of L-rhamnose and H-1 of glucose, respectively. The double doublet at δ 4.02 (J = 3.3, 1.2 Hz) is due to H-2 of L-rhamnose and the doublet at δ 1.14 (3H, J = 6 Hz) was assigned to the methyl of rhamnose. The interglycosidic (1→4) linkage between rhamnose and glucose is β, as shown by the coupling constant of the anomeric proton signal of glucose (δ 4.69, J = 6.6 Hz). The assignments of the recorded proton resonances were confirmed by COSY-NMR measurements. The ^{13}C NMR spectra of **1** showed resonances for 30 carbon signals due to some overlapping signals for quaternary carbons. The ^{13}C NMR chemical shifts of the four lactone carbonyls C-7, C-7′ (I) and C-7 and C-7′ (II) resonated at δ 159.78, 159.52, 159.61 and 159.44, respectively, which indicated that the compound has two ellagic acid moieties. Compound **1** has 12 aliphatic carbons, i.e. 6 carbon signals of glucose and the remaining 6 carbon signals of rhamnose. Two aliphatic carbon signals appeared at δ 104.71 and 99.95 that were assigned to the anomeric carbons of C-1 glucose and C-1 rhamnose. The carbon signal C-6 of glucose at δ 66.04 appeared to be shifted downfield approximately +4 ppm, suggesting substitution at this site. The carbon signal C-4 of rhamnose at δ 73.12 appeared to be shifted down approximately +3 ppm to suggest that rhamnose was linked with glucose at this position. The position of the glycosidic linkage to the aglycone was confirmed on the basis of NOESY spectra of **1** showing that the anomeric proton of rhamnose (δ 5.47) correlated with H-5 (δ 7.55) of ellagic acid (II). This interaction is only possible when the sugar residue is glycosidically linked at C-4. Thus, **1** was unambiguously identified as diellagic acid rhamnosyl (1→4) glucopyranoside.

Compound **2** was isolated as a pale yellow amorphous powder which showed a single spot on TLC, exhibited dark purple under UV light, and was identified as an ellagitannin by its color reactions with ferric chloride (dark blue) and sodium nitrite-acetic acid (reddish-brown) reagents (Bate-Smith, 1972). When treated with methanolic hydrochloride at room temperature, **2** afforded many degradation products, in particular punicacortein D (**3**) and gallic acid (Fig. 1), suggesting that **2** is a *C*-glycosidic ellagitannin having a galloyl group. The ^{1}H NMR spectrum of **2** showed the presence of one galloyl group at δ 6.99 (2H, *s*), three one-proton singlets at δ 6.46, 6.73 and 6.95 and seven aliphatic protons. The aliphatic signal pattern was typical of a *C*-glycosidic ellagitannin, which showed the characteristic pattern of an open-chain glucose due to ^{1}H NMR spectral data displaying two signals at δ 5.01 (1 H, *d*, J = 3 Hz) and δ 4.57 (1 H, *t*, J = 8.4 Hz) which were assigned to H-1 and H-3. The double doublet at δ 4.47 (J = 8.4, 3 Hz) is due to H-2 of glucose; two protons as multiplets at δ 4.33 and 4.28 were assigned to H-5 and H-4 respectively. The position of the galloyl group was determined on the basis of the following evidence: (i) comparison of ^{1}H NMR data of compounds **2** and **3** clearly showed that a multiplet signal appeared downfield at δ 4.33 and (ii) the assignment of the multiplet to H-5 indicated that the additional galloyl group was attached to this position. The configuration at the C-1 position in **2** had been determined on the basis of the coupling constant between H-1 and H-2 (J = 3 Hz) (Okuda et al., 1983;

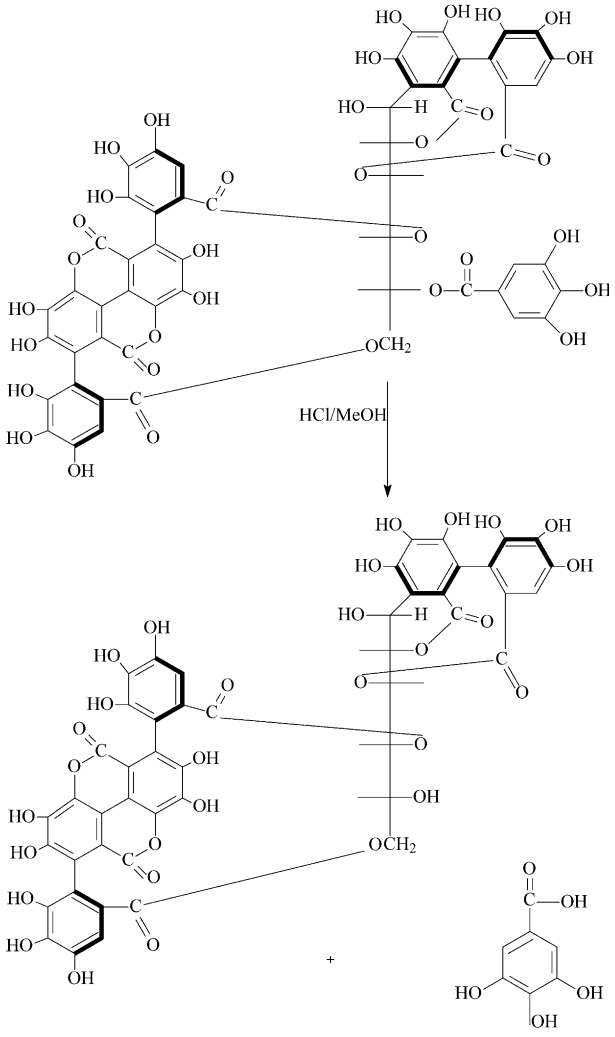


Fig. 1.

Mayer et al., 1969, 1971). Assignments of the recorded proton resonances were confirmed by COSY-NMR measurements. The ^{13}C NMR spectra of **2** exhibited seven carbonyl signals among which the chemical shifts δ 158.25 and 157.97 of two clearly separated resonances (attributable to lactonic carbons) were consistent with those of a tetraphenyl (gallagyl) group (Mayer et al., 1977). The five carbon resonances localized in the spectrum at δ 165.40, 166.10, 168.34, 169.60 and 169.81 were assigned to carbonyl carbons. The appearance of six aliphatic carbon signals and the absence of an anomeric carbon signal indicated that compound **2** possesses a C_6-polyalcohol core, which agrees with the results of Tanaka et al. (1986b). In the ^{13}C NMR spectra of compounds **2** and **3,** a distinct signal appeared downfield at 69.29, and the assignment of this signal to C-5 indicated that the galloyl group was attached to this site. Thus, **2** was unambiguously identified as 5-*O*-galloylpunicacortein D.

3. Experimental

3.1. General

UV-analyses were run in analytically pure MeOH on a Shimazu UV 160-A model and 4 ml quartz cells (1 cm optical pathway). ESI-MS were measured with a Bruker Daltonics FT-ICR mass spectrometer (Billerica, MA, USA). NMR spectra were recorded on a Varian Mercury-300 NMR spectrometer using TMS as internal standard. TLC was carried out on alumina sheets coated with cellulose F (Merck) using solvent systems A (6% AcOH) and B (*n*-BuOH–AcOH–H_2O, 4:1:5, upper layer). Compounds were visualized by exposure to UV_{254} sprayed with $FeCl_3$ and $NaNO_2$–AcOH reagents.

3.2. Plant material

Punica granatum heartwood was collected from a mature tree, growing in Giza, Egypt in June 1995, and identified by Professor Dr. L. Boulos, National Research Center, Cairo, Egypt. A voucher specimen is deposited at the herbarium of the National Research Center.

3.3. Extraction and isolation

The dry powder of *P. granatum* heartwood (2 kg) was defatted with $CHCl_3$ and extracted with EtOH (80%), to yield dry extract (110 g) after removal of the solvent. The aq. extract was chromatographed on a polyamide 6S column (Riedel-De Haen AG, Seelze Hanover, Germany), with the gradient solvent system H_2O–EtOH (10:0–1:9) to afford several crude fractions. The obtained major tannin fractions from the extract were subjected to microcrystalline cellulose CC using (EtOH–H_2O, 7:1) as eluant. Fractions obtained from the cellulose column were then applied to Sephadex LH-20 (*n*-BuOH saturated with water) to afford compounds **1**–**6** in pure form.

3.4. Diellagic acid rhamnoside (1→4) glucopyranoside (*1*)

R_f-values: 0.18 and 0.45 in solvent A and B, respectively. UV λ_{max} (MeOH): 255, 355. ESI-MS: *m*/*z* 893 $[M-H]^-$. ^1H NMR (300 MHz, DMSO-d_6): δ ppm 7.66 (1H, *s*, H-5, I); 7.55 (1H, *s*, H-5, II); 7.39 (1H, *s*, H-5′, I); 7.38 (1H, *s*, H-5′, II); 5.47 (1H, *d*, J = 1.2 Hz, H-1 rhamnose); 4.46 (1H, *d*, J = 6.6 Hz, H-1 glucose); 4.02 (1H, *dd*, J = 3.3, 1.2 Hz, H-2, rhamnose); 1.14 (3H, *d*, J = 6 Hz, H-6, rhamnose) and 3.22–3.82 remaining glucosyl and rhamnosyl protons. For ^{13}C NMR (300 MHz, DMSO-d_6) data see Table 1.

Acid hydrolysis of **1**: a solution of 5 mg in 10 ml 2 N HCl (MeOH–H_2O, 1:1) was refluxed at 100 °C for 2 h.

Table 1
^{13}C NMR spectral data of compound **1** (δ ppm, in DMSO-d_6, room temp.)

Atom	Ellagic acid I	Ellagic acid II	Atom	Rhamnose	Glucose
C-1	108.81	108.81	C-1	99.95	104.71
C-2	136.28	136.28	C-2	69.23	71.85
C-3	142.19	142.19	C-3	70.17	76.21
C-4	149.47	148.90	C-4	73.12	69.73
C-5	112.65	112.65	C-5	69.93	76.21
C-6	115.76	115.76	C-6	17.85	66.04
C-7	159.78	159.61			
C-1′	106.58	106.58			
C-2′	136.28	136.28			
C-3′	140.75	140.75			
C-4′	147.70	146.57			
C-5′	112.13	112.13			
C-6′	115.22	115.22			
C-7′	159.52	159.44			

After cooling, the reaction mixture was extracted with EtOAc. The aq. hydrolysate was neutralized, concentrated and then analyzed by chromatography to prove the presence of ellagic acid, glucose and rhamnose.

3.5. 5-O-Galloylpunicacortein D (**2**)

R_f-values: 0.38 and 0.12 in solvent A and B, respectively. UV λ_{max} (MeOH): 271. ESI-MS: m/z 1235 [M-H]$^-$. ^1H NMR (300 MHz, DMSO-d_6): δ ppm 6.99 (2 H, *s*, galloyl-H); 6.46. 6.73, 6.95 (each 1H, *s*, aromatic H); 5.01 (1H, *d*, $J=3$ Hz, H-1); 4.57 (1H, *t*, $J=8.4$ Hz, H-3); 4.47 (1H, *dd*, $J=8.4$, 3 Hz, H-2); 4.28 (1H, *m*, H-4); 4.33 (1H, *m*, H-5); 3.94 (1H, *t*, $J=10.5$ Hz, H-6a); 3.11 (1H, *t*, $J=10.5$ Hz, H-6b). ^{13}C NMR (300 MHz, DMSO-d_6): 158.25, 157.95 (CO-lactones), 165.40, 166.10, 168.43, 169.60, 169.81 (CO_2), 74.12 (C1), 73.60 (C3), 69.80 (C2), 69.29 (C5), 66.26 (C4), 64.33 (C6).

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