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Ellagitannin oligomers and a neolignan from pomegranate arils and their inhibitory effects on the formation of advanced glycation end products

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ABSTRACT

Two new ellagitannin oligomers, pomegraniins A (**7**, tetramer) and B (**8**, pentamer), and a new glucose ester of neolignan, pomegralignan (**19**), together with six known ellagitannins, were isolated from the arils and pericarps of *Punica granatum* L. (pomegranate). The structures of the new compounds were elucidated based on spectroscopic analyses and chemical evidence. The known ellagitannins included oligomers such as oenothein B (**4**), eucalbanin B (**5**), and eucarpanin T_1 (**6**), in addition to the known ellagitannin monomers such as punicalagin (**1**), punicalin (**2**), and punicacortein C (**3**). This paper therefore represents the first report concerning the isolation of ellagitannin oligomers from pomegranate. Examination of the inhibitory activities of the polyphenolic constituents from pomegranate towards the formation of advanced glycation end products (AGEs) revealed that all ellagitannins tested were more potent inhibitors than aminoguanidine, which was used as a positive control, and pomegraniin A (**7**) showed the most potent effect.

1. Introduction

Punica granatum L. (pomegranate) is a shrub or a small tree belonging to the Lythraceae (Punicaceae) family (Curro, Caruso, Distefano, Gentile, & La Malfa, 2010) that was originally distributed in Iran and Turkey, although the cultivation of *P. granatum* has been widely extended to temperate regions including China and the United States. The fruit of this plant can be divided into the pericarp and the edible aril (pulp). The pericarp has been used as an antidiarrhoetic, haemostatic and vermifuge agent in China (Cuccioloni et al., 2009). Other parts of pomegranate have also been used as traditional medicines, including the bark and root, which have been used for the treatment of parasitic disease in Ayurvedic medicine (Ismail, Sestili, & Akhtar, 2012), and the flower, which has been used as a herbal supplement for diabetes mellitus in Unani medicine (Huang et al., 2005). Recent biological studies have demonstrated that pomegranate possesses a diverse range of functions, including antioxidant (Noda, Kaneyuki, Mori, & Packer, 2002), anti-inflammatory (Larrosa et al., 2010), anticancer (Seeram et al., 2007), antimicrobial (Al-Zoreky, 2009), anti-diabetic (Li et al., 2005) and anti-mutagenic activities (Wongwattanasathien, Kangsadalampai, & Tongyonk, 2010), which effectively support these traditional applications. Phytochemical studies of various parts of the pomegranate have revealed the presence of ellagitannins, flavonoids, and alkaloids (Rahimi, Arastoo, & Ostad, 2011; Wang, Ding, Liu, Xiang, & Du, 2010). Among the ellagitannins, which are predominantly contained in the pericarp, bark and flowers, punicalagin (**1**) and punicalin (**2**) are well-known as the main components of the pericarp and bark (Tanaka, Nonaka, & Nishioka, 1986a). The numerous biological properties of pomegranate are therefore believed to be related to these ellagitannins and the related polyphenols, such as ellagic acid.

The glycation reaction is initiated by the non-enzymatic reaction of free amino groups on a protein with reducing sugars to form Amadori products. Further complex reactions of the Amadori products lead to the production of advanced glycation end-products (AGEs). The accumulation of AGEs has been related to the pathogenesis of diabetes, osteoporosis, arteriosclerosis, and Alzheimer's disease, as part of the aging process (Nagai, Mori, Yamamoto, Kaji, & Yonei, 2010), suggesting that inhibiting the formation of AGEs could represent a promising strategy for preventing the progress of several age-related diseases. In the current study, we have investigated the isolation and characterisation

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of polyphenols from the edible part of pomegranate, and the inhibitory effects of these compounds on the formation of AGEs.

2. Materials and methods

2.1. General

Optical rotations were recorded on a Jasco DIP-1000 polarimeter (Jasco, Tokyo, Japan). UV and CD spectra were recorded on Jasco V-530 and Jasco J-720 W spectrometers, respectively. The ^{1}H and ^{13}C NMR spectra were measured in acetone-d_6-D_2O (9:1) at 24 ± 1 °C on a Varian NMR System (600 MHz for ^{1}H NMR and 151 MHz for ^{13}C NMR; Palo Alto, CA) and a VXR-500 (500 MHz for ^{1}H NMR and 126 MHz for ^{13}C NMR) system. Chemical shifts have been reported in δ (ppm) values relative to the solvent peaks (δ_H: 2.04; δ_C: 29.8) on a tetramethylsilane scale. The standard pulse sequences programmed for the instrument were used for the different 2D NMR experiments (i.e., ^{1}H–^{1}H COSY, NOESY, HSQC, and HMBC). The J_{CH} value was set at 8 Hz in the HMBC experiment. Mass spectra were obtained on a Bruker MicrOTOF II spectrometer (Bruker, Billerica, MA) using an electrospray ionisation (ESI) source in negative-ion mode. Normal-phase HPLC was conducted on a YMC-Pack SIL A-003 column (YMC Co., Ltd., Kyoto, Japan) column (250 × 4.6 mm i.d.) using a mobile phase composed of n-C_6H_{14}:MeOH:THF:HCOOH (55:33:11:1) containing oxalic acid (450 mg/L) (system A) and n-C_6H_{14}:MeOH:THF:HCOOH (47:39:13:1) containing oxalic acid (450 mg/L) (system B) (flow rate, 1.5 mL/min; detection, UV 280 nm) at 24 ± 1 °C. Preparative normal-phase HPLC was performed using system B without oxalic acid. Reversed-phase HPLC was performed on an Inertsil ODS-3 (GL Sciences, Tokyo, Japan) column (250 × 4.6 mm i.d.) using a mobile phase composed of H_2O:CH_3CN:HCOOH (74:25:1 or 72:25:3) (flow rate, 1.0 mL/min; detection, UV 280 or 305 nm) at 40 °C (system C), as well as a J'sphere ODS H-80 (YMC Co., Ltd.) column (250 × 4.6 mm i.d.), using a mobile phase composed of 0.01 M H_3PO_4:0.01 M KH_2PO_4:CH_3CN (44:44:12) (system D) and 0.01 M H_3PO_4:0.01 M KH_2PO_4:EtOH:EtOAc (47.5:47.5:3:2) (system E) (flow rate, 1.0 mL/min; detection, UV 280 nm) at 40 °C. Preparative reversed-phase HPLC was performed on a YMC-Pack ODS A-302 (YMC Co., Ltd.) column (250 × 4.6 mm i.d.) with H_2O:CH_3CN:HCOOH (97:2:1, 95:3:2 or 93:2:5). Purifications by column chromatography were conducted on Diaion-HP-20 (Mitsubishi Kasei Co., Tokyo, Japan), Toyopearl HW-40 (coarse grade) (Tosoh Co.), MCI GEL CHP-20P (75–150 μm) (Mitsubishi Kasei Co.), and YMC-gel ODS-AQ 120–50S (YMC Co., Ltd.).

2.2. Plant material

Dried pericarps of *P. granatum* were obtained from Maechu Co. (Nara, Japan). Fresh arils of *P. granatum* cultivated in the Medicinal Plant Garden of Okayama University were collected in October 2011. A voucher specimen (OPH-POM 011) was deposited in the Herbarium of the Faculty of Pharmaceutical Sciences at Okayama University.

2.3. Extraction and isolation

The dried pericarps (4 kg) of *P. granatum* were homogenised with 70% aqueous acetone (3 × 22 L), and the concentrated solution (15 L) was extracted sequentially with Et_2O (3 × 15 L), EtOAc (3 × 15 L), and water-saturated n-BuOH (3 × 15 L), to give the respective extracts as well as a water-soluble portion. The fractions were collected on the basis of normal- and/or reversed-phase HPLC analysis. A portion (624 g) of the water-soluble extract (1222 g) was chromatographed over Diaion HP-20 (80 × 15 cm i.d.) using H_2O, 10%, 30%, 50% aqueous MeOH, MeOH, and 70% aqueous acetone as the eluent. A portion (3.0 g) of the 10% MeOH fraction (29.6 g) was further chromatographed over Toyopearl HW-40 (coarse grade) (35 × 2.2 cm i.d.) with aqueous MeOH and 70% aqueous acetone, to afford punicalagin (**1**) (0.17 g, 0.013% of pericarp extract) and punicalin (**2**) (1.1 g, 0.084%) from the 50% and 30% MeOH fractions, respectively. The 50% MeOH fraction from the Toyopearl HW-40 separation was further chromatographed over MCI GEL CHP-20P (25 × 1.1 cm i.d.) with aqueous MeOH to give punicacortein C (**3**) (30 mg, 0.0023%) from the 50% MeOH fraction. The 30% MeOH fraction (78 g) from the Diaion HP-20 separation was purified by column chromatography over Diaion HP-20 (80 × 15 cm i.d.) with aqueous MeOH and Toyopearl HW-40 (coarse grade) (35 × 2.2 cm i.d.) with aqueous MeOH, to give punicalagin (**1**) (1.47 g, 0.11%) (Tanaka et al., 1986a).

The homogenate of the fresh arils (2 kg) of *P. granatum* with 70% aqueous acetone (3.5 L) was filtered and evaporated *in vacuo*. The extract (130 g) was separated by column chromatography over Diaion HP-20 (60 × 5.4 cm i.d.) using H_2O with increasing amounts of MeOH (H_2O → 10% → 20% → 40% → 100% MeOH) in stepwise mode followed by 70% aqueous acetone. The 50% MeOH fraction (1.27 g) was chromatographed over Toyopearl HW-40 (coarse grade) (35 × 2.2 cm i.d.) with 70% aqueous MeOH → MeOH:H_2O:acetone (7:2:1) → MeOH:H_2O:acetone (7:1:2) → 70% aqueous acetone. The 70% aqueous MeOH fraction was purified over MCI GEL CHP-20P (30 × 1.1 cm i.d.) with aqueous MeOH and Sephadex LH-20 (20 × 1.1 cm i.d.) with EtOH and MeOH to yield oenothein B (**4**) (85 mg, 0.065% of aril extract), eucalbanin B (**5**) (97 mg, 0.075%), and pomegralignan (**19**) (15 mg, 0.012%). The MeOH:H_2O:acetone (7:2:1), MeOH:H_2O:acetone (7:1:2), and 70% aqueous acetone fractions were purified by preparative normal-phase HPLC to give eucarpanin T_1 (**6**) (39 mg, 0.03%) and pomegraniin A (**7**) (11 mg, 0.0085%) and B (**8**) (10 mg, 0.0077%), respectively. The 70% aqueous acetone fraction was used as the ellagitannin oligomer fraction to estimate the molecular weights of the ellagitannin oligomers (**6**–**8**) by normal phase HPLC.

The physicochemical data for compounds **6**–**8** are described below. The NMR data for **1** and **2** are also discussed, because fully assigned NMR data have not previously been provided for these compounds.

Punicalagin (**1**): ^{1}H NMR [600 MHz, acetone-d_6-D_2O (9:1)]: δ 2.08 [dd, J = 1.8, 10.8 Hz, glucose (Glc)-H-6α], 2.17 (dd, J = 1.8, 10.8 Hz, Glc-H-6β), 2.64 (ddd, J = 1.8, 9.0, 10.8 Hz, Glc-H-5β), 3.23 (ddd, J = 1.8, 9.6, 10.8 Hz, Glc-H-5α), 4.10 (2H, t, J = 10.8 Hz, Glc-H-6α, β), 4.62 (dd, J = 7.8, 9.0 Hz, Glc-H-2β), 4.67 (d, J = 7.8 Hz, Glc-H-1β), 4.76 (br t, J = 9.6 Hz, Glc-H-4α), 4.78 (br t, J = 9.0 Hz, Glc-H-4β), 4.77 (dd, J = 3.6, 9.0 Hz, Glc-H-2α), 4.87 (t, J = 9.0 Hz, Glc-H-3β), 5.08 (d, J = 3.6 Hz, Glc-H-1α), 5.19 (t, J = 9.6 Hz, Glc-H-3α), 6.52 [s, hexahydroxydiphenoyl (HHDP)-H-2α], 6.52 (s, HHDP-H-2β), 6.60 (s, HHDP-H-3α), 6.61 (s, HHDP-3β), 6.63 (s, gallagyl-H-4α), 6.71 (s, gallagyl-H-4β), 6.98 (s, gallagyl-H-6α), 6.99 (s, gallagyl-H-6β); ^{13}C NMR [151 MHz, acetone-d_6-D_2O (9:1)]: δ 64.19 (Glc-C-6β), 64.23 (Glc-C-6α), 66.7 (Glc-C-5α), 70.7 (Glc-C-4β), 70.99 (Glc-C-4α), 72.5 (Glc-C-5β), 74.3 (Glc-C-2α), 76.5 (Glc-C-3α), 76.6 (Glc-C-2β), 78.9 (Glc-C-3β), 89.99 (Glc-C-1α), 94.2 (Glc-C-1β), 107.4 (2C, HHDP-C-2α, β), 107.5 (2C, HHDP-C-3α, β), 109.0 (gallagyl-C-4α), 109.6 (gallagyl-C-4β), 110.4, 110.5, 110.8, 111.1, 111.59 (gallagyl-C-6α), 111.62 (gallagyl-C-6β), 113.8, 113.9, 114.4, 114.5, 114.5, 114.7, 114.7, 114.7, 115.1 (2C), 117.6, 117.7, 122.0, 122.0, 123.4, 123.6, 124.4, 124.7, 124.9, 125.1, 126.1, 126.4 (2C), 126.4, 136.1 (2C), 136.2, 136.3, 136.3, 136.5, 136.6, 136.6, 136.9, 137.2, 137.6, 137.7, 138.4, 138.5, 139.1, 139.4, 143.8, 143.9, 144.1 (2C), 144.2, 144.2, 144.6, 144.7, 145.0 (2C), 145.1, 145.3, 145.4, 145.5, 145.6, 147.6, 147.7 (2C), 148.0, 158.1 (2C, lactone carbonyl-C), 158.6 (lactone carbonyl-C), 158.6 (lactone carbonyl-C), 168.2 (ester carbonyl-C-6β), 168.3 (ester carbonyl-C-