SUPPLEMENTARY MATERIAL

Two new proanthocyanidin trimers isolated from *Cistus incanus* L. demonstrate potent anti-inflammatory activity and selectivity to cyclooxygenase isoenzymes inhibition

K.A. Mansoor^{a*}, K.Z. Matalka^b, F.S. Qa'dan^c, R. Awad^a and M. Schmidt^c

^aDepartment of Pharmaceutical Medicinal Chemistry, Faculty of Pharmacy & Medical Sciences, University of Petra, Amman, Jordan

^bDepartment of Medical Laboratories, Faculty of Health Sciences, American University of Madaba, Madaba, Jordan

^cHerbResearch, 86874 Mattsies, Germany

Abstract

Two new proanthocyanidin trimers have been isolated from *Cistus incanus* herb; gallocatechin-(4 α →6)-gallocatechin-(4 α →8)-gallocatechin (compound 1) and epigallocatechin-3-O-gallate-(4 β →8)-epigallocatechin-3-O-gallate-(4 β →8)-gallocatechin (compound 2). The structures were determined on the basis of 1D-and 2D-NMR (HSQC, HMBC) of their peracetylated derivatives, MALDI-TOF-MS and by acid-catalysed degradation with phloroglucinol. A more abundant proanthocyanidin oligomer was also isolated, purified and its chemical constitution studied by ¹³C-NMR and phloroglucinol degradation. The mean molecular weight of the polymer was estimated to be about 7 to 8 flavan-3-ol-units with a ratio of procyanidin :prodelphinidin units at 1 : 5, some of which are derivatized by gallic acid. Water extract and higher oligomeric proanthocyanidin fractions of *Cistus incanus* significantly inhibited TPA-induced oedema when applied topically at doses of 0.5 and 1 mg/ear in mice. Furthermore, the extracts and the pure compounds inhibited COX-1 and COX-2 activities. In addition, compound 2 exhibited an IC₅₀ of 4.5 μ M against COX-2 indicating its high selectivity towards COX-2.

Keywords: *Cistus incanus*; phenolic; proanthocyanidins; anti-inflammatory; COX inhibitor

1. Experimental

1.1. Plant material

Cistus incanus L. was collected in El Majdal (Jordan; 05/2005) and identified in comparison with authentic *Cistus incanus* obtained from the Botanical Garden, University of Jordan (Amman). A voucher specimen is deposited at the University of Petra, Amman-Jordan under PUAM13.

1.2. Materials

Phloroglucinol was obtained from Fluka (Amman, Jordan). COX-1 and COX-2 were purchased from Sigma (Munich, Germany). 1-¹⁴C-labeled arachidonic acid (>200 μ Ci (370 kBq), NEN) was purchased from New England Nuclear Co. (Boston, USA). Other chemicals and reagents were purchased from Roth Chemicals (Dubai, UAE).

1.3. Extraction and isolation

Air-dried material (4 kg) was exhaustively extracted with 30 L of hot water (40 °C) and the combined extracts were evaporated *in vacuo* to dryness (534 g). A portion (100 g) of the water-extract was applied to CC on Sephadex LH-20 (55 x 900 mm) and successively eluted with 5 L EtOH-H₂O and 20 L MeOH-H₂O (1:1) to afford 10 fractions; then acetone/water (3:7) was used to elute the higher oligomeric fraction (OPF; 377 g). Gallocatechin-(4 α →6)-gallocatechin-(4 α →8)-gallocatechin (**1**): Fraction 5 (7950 - 8200 ml, 220 mg) achieved from Sephadex LH-20 column was subjected to chromatography on MCI gel CHP 20P (25 x 450 mm) with a 10 - 60% MeOH linear gradient (15 ml/subfraction) to afford an amorphous white powder (subfractions 41 - 47, 31 mg); [α]_D²⁰ = - 53.6° (C 0.17, MeOH). 8 mg were acetylated to give **1a**: MALDI-TOF-MS: *m/z* = 1873 [M + Na]⁺. - CD: [θ]₂₇₂ +7588°, [θ]₂₅₄ - 890°, [θ]₂₃₇ -256802°, [θ]₂₁₇ -77214°, [θ]₂₁₃ -61077°, [θ]₂₀₉ - 40677°. ¹H

NMR (CDCl₃, 600 MHz): δ 1.56 - 2.38 (m, Ac), 2.42-2.82 [*m*, H-4 (I)], 4.33-4.39 [*m*, H-4 (C) and H-4 (F)], 4.48 [*d*, *J* = 10 Hz, H-2 (F)], 4.58 [*d*, *J* = 10 Hz, H-2_R (F)], 4.67 [*d*, *J* = 10 Hz, H-2 (C)], 4.68 [*d*, *J* = 10 Hz, H-2_R (C)], 4.94 [*m*, H-2 (I)], 5.04 [*m*, H-3 (I)], 5.30-5.50 [*m*, H-3 (F)], 5.55 [*m*, H-3 (C)], 6.43 [*d*, *J* = 2.3 Hz, H-6 (A)], 6.56 [*d*, *J* = 2.3 Hz, H-8 (A)], 6.60 [*s*, H-6 or H-8 (D or G)], 6.72 [*brs*, H-2'/H-6' (B)], 6.78 [*s*, H-8 or H-6 (D or G)], 6.96 [*brs*, H-2'/H-6' (B), (H) or (G)], 7.16 [*brs*, H-2'/H-6' (B), (H) or (G)].

¹³C NMR (CDCl₃, 150 MHz): δ = 27.5 [C-4 (I)], 31.8 [C-4 (C)], 33.2 [C-4 (F)], 66.9 [C-3 (I)], 68.2 [C-3 (C)], 69.5 [C-3 (F)], 73.4 [C-2 (F)], 73.4 δ [C-2 (C)], 74.7 [C-2 (I)], 111.9 [C-8 (A)], 109.7 [C-6 (A)], 110.6 [C-4a (G)], 112.8 [C-4a (A)], 112.6 [C-4a (D)], 111.6 [C-6 (D)], 117.0 [C-8 (G)], 117.1 [C-8 (D)], 119.9 [*brs.*, C-2' (E), C-2' (H), and C-6' (B)], 121.5 [C-6' (H)], 135.3 [C-1' (E)], 134.3 [C-4' (E)], 135.1 [C-1' (H)], 135.6 [C-4' (B)], 135.7 [C-1' (B)], 141.7 [C-4' (H)], 142.0 [C-3' (H)], 142.1 [C-3' (E) and C-5' (E)], 147.1 [C-7 (D)], 148.1 [C-5 (G)], 149.4 [C-7 (A)], 150.8 [C-8a (G)], 153.4 [C-8a (D)], 155.9 [C-8a (A)].

Purified proanthocyanidin **1** (11.5 mg) was reacted with phloroglucinol (11.5 mg) in 1% HCl in EtOH (1 ml) for 15 min at room temperature with continuous shaking (Foo and Karchesy 1989). The solution was then concentrated under a stream of N₂ to dryness and purified by preparative TLC.

Epigallocatechin-3-*O*-gallat-(4 β →8)-epigallocatechin-3-*O*-gallat-(4 β →8)-gallocatechin (**2**): Fraction 6 (9200 - 9600 ml, 177 mg) achieved from Sephadex LH-20 column was subjected to chromatography on MCI gel CHP 20P (25 x 450 mm) with a 10 - 60% MeOH linear gradient (15 ml/subfraction) to afford an amorphous white powder (subfractions 22 - 28, 25 mg); [α]_D²⁰ + 109° (*c* 0.19, MeOH). 8 mg were acetylated to give **2a**: MALDI-TOF-MS: *m/z* = 2163 [M + Na]⁺. - CD: [θ]₂₇₂ -10277°, [θ]₂₅₄ + 543°, [θ]₂₃₇ -29569°, [θ]₂₁₇ + 66575°, [θ]₂₁₃ + 64188°, [θ]₂₀₉ + 70896°. - ¹H NMR (CDCl₃, 600 MHz): δ 1.92 - 2.32 (m, Ac), 2.26 [*m*, H-4 (I)], 2.61 [*m*, H-4 (I)], 4.56 [*brs*, H-4 (C)], 4.60 [*d*, *J* = 2.3 Hz, H-4 (F)], 4.84 [*m*, H-3 (C)], 4.93 [*m*, H-3 (F)], 5.24 [*m*, H-3 (I)], 5.42 [*brs*, H-2 (I)], 5.55 [*brs*, H-2 (C)], 5.71 [*brs*, H-2 (F)], 6.06 [*d*, *J* = 2.3 Hz, H-8 (A)], 6.35 [*d*, *J* = 2.3 Hz, H-6 (A)], 6.39 [*s*, H-6 (D)], 6.79 [*brs*, H-2'/H-6' (I)], 6.92 [*brs*, H-2'/H-6' (E)], 7.12 [*brs*, H-2'/H-6' (B)], 7.46 [*brs*, H-2''/H-6'' (L)], 7.61 [*brs*, H-2''/H-6'' (K)].

- ¹³C NMR (CDCl₃, 150 MHz): δ = 29.5 [C-4 (I)], 34.5 [C-4 (C)], 36.9 [C-4 (F)], 67.6 [C-3 (I)], 68.5 [C-3 (C)], 69.7 [C-3 (F)], 78.0 [C-2 (F)], 79.5 δ [C-2 (C)], 76.4 [C-2 (I)], 107.7 [C-8 (A)], 109.5 [C-6 (A)], 110.7 [C-4a (G)], 111.1 [C-4a (A)], 112.8 [C-4a (D)], 111.6 [C-6 (D)], 115.7 [C-8 (G)], 117.3 [C-8 (D)], 118.5 [C-2' (H) and C-6' (H)], 119.0 [C-2' (E), and C-6' (E)], 119.4 [C-2' (B) and C-6' (B)], 122.5 [C-3'' (K) and C-5'' (K)], 122.9 [C-3'' (L) and C-5'' (L)], 127.1 [C-1'' (L)], 127.3 [C-1'' (K)], 133.7 [C-1' (E)], 134.3 [C-4' (E)], 135.0 [C-1' (H)], 135.3 [C-4' (B)], 135.5 [C-1' (B)], 141.4 [C-4' (H)], 142.0 [C-3' (H)], 142.1 [C-3' (E) and C-5' (E)], 143.2 [C-3'' (K) and C-5'' (K)], 143.2 [C-3'' (L) and C-5'' (L)], 147.1 [C-7 (D)], 148.3 [C-5 (G)], 149.3 [C-7 (A)], 150.9 [C-8a (G)], 153.4 [C-8a (D)], 155.9 [C-8a (A)], 162.4 [C-1a (K)], 164.6 [C-1a (L)].

Purified proanthocyanidin **1** (14 mg) was reacted with phloroglucinol (14 mg) in 1% HCl in EtOH (1 ml) for 15 min at room temperature with continuous shaking (Foo and Karchesy 1989). The solution was then concentrated under a stream of N₂ to dryness and purified by preparative TLC.

1.4. Degradation with phloroglucinol

The oligomeric proanthocyanidin fraction (OPF) of *Cistus incanus* obtained as described above (20 g) was treated for 30 min at room temperature with phloroglucinol (15 g) with 1% HCl in EtOH (50 ml) under continuous shaking (Foo and Karchesy 1989; Foo et al. 1996). The solution was concentrated under reduced pressure (29.35 g). A portion (25 g) was fractionated on Sephadex LH-20 (55 x 900 mm) using EtOH (4 L), EtOH-MeOH 1:1 (8 L) and acetone/water 7:3 (1.8 L) to give 11 fractions (Fr). Fr.2 (1270-3250 ml, 450 mg) was subjected to chromatography on MCI-gel CHP 20P (25 x 250 mm) with a 10-80 % MeOH linear gradient (17 ml/Fr.) to afford catechin (subfr. 23-45, 255 mg). Fr. 3 (3250-4750 ml, 1190 mg) was separated by MCI-gel with the same gradient as above to afford (subfr. 12-20, 877 mg) gallocatechin and (subfr. 25-31, 32 mg) gallocatechin-3-*O*-gallate. Fr 5 (5500-5900 ml, 3340 mg) was separated on MCI to afford epigallocatechin-(4 β →2)-phloroglucinol (35-53, 2850 mg). Gallocatechin-(4 α →2) -phloroglucinol was isolated from Fr 6 (5900-6500 ml, 1075 mg) and MCI-gel chromatography (subfr. 41-67, 970 mg). Fr. 7 (6200-6700 ml, 390 mg) was subjected to chromatography on MCI-gel elution to afford epigallocatechin-3-*O*-gallate-(4 β →2)-phloroglucinol (subfr. 74-81, 170 mg). Epigallocatechin-(4 (→8) -epigallocatechin-(4 (→2) -phloroglucinol was separated from Fr. 8 (6700-7100 ml, 356 mg) and MCI-gel chromatography (subfr. 47-56, 281 mg). Fr. 9 (7100-8100 ml, 767 mg) was separated as above to yield epigallocatechin-3-*O*-gallate-(4 β →8)-epigallocatechin-(4 β →2)-

phloroglucinol (subfr. 56-63, 51 mg) and epigallocatechin-3-*O*-gallate-(4 β →8)-epigallocatechin-3-*O*-gallate-(4 β →2)-phloroglucinol epigallocatechin-3-*O*-gallate (subfr. 13-20, 185 mg).

All compounds were identified after acetylation by comparing their physical data (NMR, MS, and CD) with those of authentic samples and published values (Danne et al. 1994; Fletcher et al. 1977; Foo and Porter 1978; Petereit et al. 1989; Porter et al. 1982; Foo and Porter 1983; Qa'dan 1999; Qa'dan et al. 2006).

1.5. Structural analysis

¹H NMR spectra were recorded in CDCl₃ (except for the oligomers, see Figure 1) on a Varian Gemini 200 (200 MHz) and on a Bruker AM 600 (600 MHz) relative to CHCl₃. ¹³C- NMR were recorded at 150 MHz. CD data were obtained in MeOH on a Jasco J 600. MALDI-TOF mass spectrometer: LAZARUS II (home built), N₂-laser (LSI VSL337ND) 337 nm, 3 ns puls width, focus diameter 0.1 mm, 16 kV acceleration voltage, 1 m drift length, data logging with LeCroy9450A, 2.5 ns sampling time and expected mass accuracy $\pm$ 0.1 %, sample preparation: acetylated compounds were deposited from a solution in CHCl₃ on a thin layer of 2,5-dihydroxybenzoic acid (DHB) crystals. Analytical TLC was carried out on aluminium sheets (Kieselgel 60 F₂₅₄, 0.2 mm, Merck) using acetone-toluene-formic acid (60 : 30 : 10, system A). Compounds were visualized by spraying with vanillin-HCl reagent and 1% ethanolic FeCl₃ solution. Preparative TLC was performed on silica gel plates (Kieselgel 60 F₂₅₄, 0.5 mm Merck) using system A. Acetylation was performed in pyridine-acetic acid anhydride (1:1.2) at ambient temp for 24 h.

1.6. Animals

Female Swiss-Albino mice (22–30 g) were housed in standard environmental conditions, fed with standard pellet diet and water *ad libitum*. The experimental protocol was approved by the Institute Animal Ethics Committee (IAEC) and the experiments were carried out in accordance with the guidelines of CPCSEA given on animal experimentation.

1.7. Anti-inflammatory activity

The anti-inflammatory activities of the water extract (WE) and the higher oligomeric proanthocyanidin fraction (OPF) were evaluated as per the method described previously (Hernandez et al. 2001; Ukiya et al. 2007). The animals were divided into groups of six per cage ($n = 6$). To the untreated group, only 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma, USA) (2.5 μ g dissolved in 20 μ L of acetone) was applied topically to the right ear on both sides of the ear surfaces with a micropipette. In the treatment groups, TPA and extracts/compounds were applied simultaneously to the right ear. Indomethacin (Sigma, USA) at a dose of 0.5 mg/ear was used as a positive control. Ear thickness was measured before (T_0) and after (T_1) induction of inflammation using a digital calliper (Mutitoyo, Japan). The maximum oedema was reached 4 h after application of TPA. The oedema was expressed as an increase in the ear thickness due to the TPA application ($T_1 - T_0$), and the oedema inhibition was expressed as the percentage of thickness reduction referred to the untreated group.

1.8. Effect on cyclooxygenase -1 and -2

The effect on cyclooxygenase-1 and -2 (COX-1 and COX-2) was determined by measuring PGE₂ production. The reaction mixtures were prepared in tris(hydroxymethyl) aminomethane-HCl buffer(pH 8.0), containing glutathione (350 μ M), epinephrine (350 μ M), hematin (1.5 μ M), enzyme (COX-1or COX-2, 50 μ l) and various concentrations of the water extract (WE), higher oligomeric proanthocyanidin fraction (OPF) or isolated compounds. 1-¹⁴C arachidonic acid (10 μ l) was added to start the reaction. The mixture was first incubated for 30 min at 37 °C, then the reaction was terminated by adding the reaction mixture (20 μ l) to 30 μ M indomethacin (200 μ l). Arachidonic acid and its radio-labelled metabolites were separated and determined by reversed-phase HPLC using berthold radioactivity monitor (Pharma Tech R&D, Amman, Jordan). Inhibition refers to the reduction of PGE₂ formation in comparison to a blank run without inhibitor. ASA was used as a positive control. The results are means of three independent experiments.

1.9. Statistical analysis

Numerical data are expressed as mean % $\pm$ standard error mean (S.E.M). Statistical differences between the treatments and the untreated groups were evaluated by one way ANOVA followed by Dunnett's test. $p < 0.05$ was considered to be significant.

Spectra of isolated compounds

The ^{13}C NMR spectrum of the purified oligomeric fraction (OF; obtained s. Exp)

2

R= H= Compound 2
 R= Acetyl = Compound 2a

^1H NMR

(CDCl_3 , 600 MHz)

R= H= Compound 2
 R= Acetyl= Compound 2a

^{13}C NMR (CDCl_3 , 150 MHz)

^{13}C NMR (CDCl_3 , 150 MHz)

R= H= Compound 1
R= Acetyl= Compound 1a

References

- Danne A, Petereit F, Nahrstedt A. 1994. Flavan-3-ols, prodelphinidins and further polyphenols from *Cistus salvifolius*. *Phytochemistry*. 37 (2):533-538.
- Fletcher AC, Porter LJ, Haslam E, Gupta RK (1977) Plant proanthocyanidins. Part 3. Conformational and configurational studies of natural procyanidins. *J Chem Soc, Perkin Trans (I)*14:1628-1637.
- Foo LY, Porter LJ. 1978. Prodelphinidin polymers: Definition of structural units. *J Chem Soc., Perkin Trans (I)*10:1186-1190.
- Foo LY, Porter LJ. 1983. Synthesis and conformation of procyanidin diastereoisomers. *J Chem Soc, Perkin Trans (I)*:1535-1543.
- Foo LY, Karchesy JJ. 1989. Procyanidins polymers of douglas fir bark: Structure from degradation with phloroglucinol. *Phytochemistry*. 28:3185-3190.
- Foo LY, Newman R, Waghorn G, McNabb WC, Ulyatt MJ. 1996. Proanthocyanidins from *Lotus corniculatus*. *Phytochemistry*. 41:617-624.
- Hernandez V, Recio MC, Manez S, Prieto JM, Giner RM, Rios JL. 2001. A mechanistic approach to the in vivo anti-inflammatory activity of sesquiterpenoid compounds isolated from *Inula viscosa*. *Planta Med*. 67: 726-731.
- Petereit F. 1992. Polyphenolische inhaltsstoffe und untersuchungen zur entzuendungshemmenden aktivitaeten der traditionellen arzneipflanze *Cistus incanus* L. (Cistaceae) [Dissertation]. Muenster: University Muenster-Germany.
- Porter LJ, Newman RH, Foo LY, Wong H, Hemingway RW. 1982. Polymeric proanthocyanidins. ¹³C NMR studies of procyanidins. *J. Chem Soc Perkin Trans. (I)*:1217-1221.
- Qa'dan F (1999) Analytik oligomererproanthocyanidineaus *Cistus albidus* (Cistaceae) [Dissertation]. Muenster: University Muenster-Germany.
- Qa'dan F, Petereit F, Mansoor K, Nahrstedt A. 2006. Antioxidant oligomeric proanthocyanidins from *Cistus salvifolius*. *Nat Prod Res*. 20 (13):1216-1224.
- Ukiya M, Akihisa T, Yasukawa K, Koike K, Takahashi A, Suzuki T, Kimura Y. 2007. Triterpene Glycosides from the flower petals of sunflower (*Helianthus annuus*) and their anti-inflammatory activity. *J Nat Prod*. 70:813-816.