A Bioactive Prodelphinidin from *Mangifera indica* Leaf Extract

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A new trimeric proanthocyanidin, epigallocatechin-3-*O*-gallat-(4β→8)-epigallocatechin-(4β→8)-catechin (**1**), was isolated together with three known flavan-3-ols, catechin (**2**), epicatechin (**3**), and epigallocatechin (**4**), and three dimeric proanthocyanidins, **5**–**7**, from the air-dried leaves of *Mangifera indica*. Their chemical structures were determined on the basis of 1D- and 2D-NMR spectra (HSQC, HMBC) of their peracetylated derivatives, MALDI-TOF-mass spectra, and by acid-catalyzed degradation with phloroglucinol. The isolated compounds **1**–**7** were *in vitro* tested for their inhibitory activities against COX-1 and COX-2. Compound **1** was found to have a potent inhibitory effect on COX-2, while compounds **1** and **5**–**7** exhibited moderate inhibition against COX-1.

Key words: Mangifera indica, Proanthocyanidins, COX Inhibitor

Introduction

Mango (*Mangifera indica* L.), which belongs to the family Anacardiaceae, is one of the most important fruits marketed in the world. In Jordan, Israel, and West-bank, mango is cultivated on a small scale around the Dead Sea. Extracts of *M. indica* L. have been reported to possess antiviral, antibacterial, analgesic, anti-inflammatory, and immunostimulant activities (Makare *et al.*, 2001; Scartezzini and Speroni, 2000). The mango leaves are used in the treatment of fever, diarrhoea, and diabetes (Aderibigbe *et al.*, 2001). They contain an essential oil, sugars, xanthones, flavonoids, gallates, gallotannins, ellagitannins, and benzophenones (Anjaneyulu *et al.*, 1994; Saleh and El-Ansari, 1975; Sairam *et al.*, 2003; Selles *et al.*, 2002). Several pharmacological activities, including antiphlogistic, antioxidant, antifungal, and antitumour activities, are reported for higher oligomeric and polymeric proanthocyanidins (Cos *et al.*, 2004). However, little is known about the structural designs of the flavan-3-ols and oligomeric proanthocyanidin fraction of the title plant. Such information is of importance for a better understanding of the relationship between the structure of the proanthocyanidins and their pharmacological and microbiological effects.

In the present study, seven compounds have been isolated from mango, structurally elucidated, and tested for cyclooxygenase (COX-1 and COX-2) inhibtion. The bioassay showed that compound **1** has a potent COX-2 inhibitory effect, while compounds **1** and **5**–**7** have moderate COX-1 inhibitory activities.

Results and Discussion

Phytochemical results

The ethylacetate fraction obtained from the aqueous acetone extract of the leaves of *M. indica* was chromatographed on Sephadex LH-20 and MCI gel. Three oligomeric proanthocyanidins, catechin-(4α→8)-catechin (**5**), catechin-(4α→6)-catechin (**6**), and epicatechin-(4β→8)-catechin (**7**), have been isolated for the first time in addition to the known flavanols catechin (**2**), epicatechin (**3**), and epigallocatechin (**4**). The identity of all flavanoids was established by comparison of the physical properties [1D- and 2D-NMR, circular dicroism (CD), optical rotation [α], and

MALDI-TOF-MS] of the corresponding derivatives obtained after peracetylation with authentic samples and published data (De Mello *et al*., 1996; Ploss *et al*., 2001; Qa'dan *et al*., 2003).

The remaining aqueous phase was further fractionated on Sephadex LH-20 and MCI gel columns to give compound **1**. Compound **1** showed a prominent pseudomolecular ion peak at *m*/*z* 1873 [M + Na^+] in the MALDI-TOF-mass spectrum of its peracetate **1a**, which suggested a B-type triflavanoid composed of two gallocatechin/epigallocatechin units, one catechin/epicatechin moiety, and one gallic acid acylation.

The ^{1}H NMR spectrum of **1a** in $CDCl_3$ (400 MHz) showed three sharp two-proton singlets at δ 6.84 ppm, 6.90 ppm, and 7.61 ppm and an AMX-spin system. The spectrum was very similar to that of the analogous trimeric proanthocyanidin epigallocatechin-(4β→8)-gallocatechin-(4α→8)-catechin, except the different stereochemistry of one of the extender units and

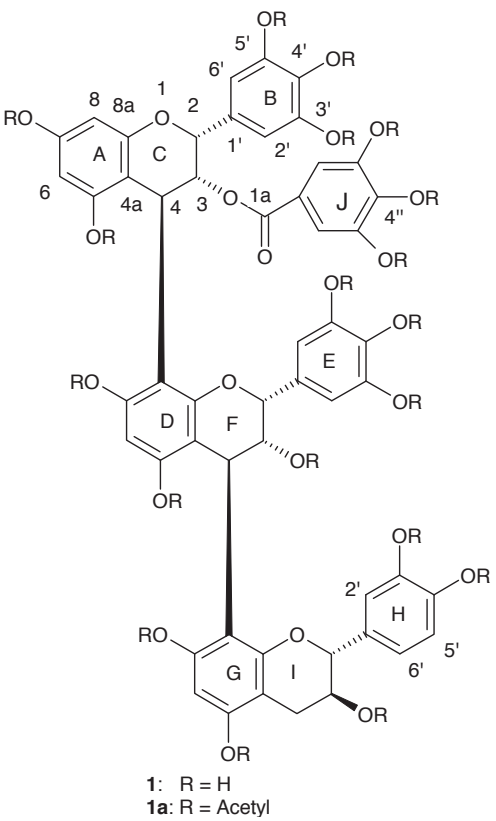

Fig. 1. Chemical structure of the new compound isolated from *Mangifera indica* leaves.

the presence of an additional galloyl moiety. The heterocyclic coupling constants $J_{2,3(C)}$ and $J_{2,3(F)}$ < 2 Hz confirmed the relative 2,3-*cis*-stereochemistry of both extender units corresponding to two epigallocatechin units. The C-4/C-8 bonding position of the interflavanoid linkages was recognized as well by ^{1}H-^{13}C long-range correlations (HMBC) of H-4 (C) with C-8a (D) and H-4 (F) with C-8a (G) (Balas and Vercauteren, 1994). In contrast, the relative 2,3-stereochemistry of the terminal unit can not be determined with the small coupling constant of the H-2 (I) proton. This observation has already been reported for the peracetylated epigallocatechin-(4β→8)-gallocatechin-(4α→8)-catechin and can be explained with conformational changes in ring I of which the catechol substituent is mainly in axial position (Qa'dan *et al*., 2003). Thus, the structure elucidation of compound **1** was corroborated by acid-catalyzed reaction in the presence of phloroglucinol (Foo and Karchesy, 1989). Compound **1** gave phloroglucinol, epigallocatechin-3-*O*-gallat-(4β→2)-phloroglucinol, epigallocatechin-(4β→2)-phloroglucinol, and a small amount of epigallocatechin-3-*O*-gallat-(4β→8)-epigallocatechin-(4β→2)-phloroglucinol as degradation products, and catechin as releasing terminal flavan-3-ol. These degradation products were identified by co-chromatography in comparison with authentic compounds. Danne *et al*. (1994) observed a reversed positive Cotton effect for galloylated (4β→8)-linked procyanidin dimers. We could observe the same effect in the CD spectrum of **1a**.

In conjunction with the optical rotation, $[\alpha]_D^{20}$ +103.3° (*c* 0.15, MeOH), compound **1** was identified as epigallocatechin-3-*O*-gallat-(4β→8)-epigallocatechin-(4β→8)-catechin (Fig. 1). To the best of our knowledge, compound **1** is here described for the first time.

Bioactivity results

The mango raw extract (RE) and the isolated compounds were tested for their inhibitory activities against cyclooxygenase (COX-I and COX-2, Table I). The RE and compounds **1**, **5**–**7** exhibited moderate inhibition against COX-1. The RE and compound **1** were found to have potent COX-2 inhibitory effects, with IC_{50} values of 39.7 μM and 28.4 μM, respectively, compared to the positive control aspirin (IC_{50} = 18.0 μM). Further studies

Table I. IC_{50} values of isolated compounds as inhibitors of COX-1 and COX-2[a].

Compound	COX-1 [μM]	COX-2 [μM]
RE	77.3 ± 1.2	39.7 ± 0.6
1	71.1 ± 1.0	28.4 ± 0.5
2	187.3 ± 2.7	137.5 ± 3.2
3	185.4 ± 2.1	143.2 ± 1.7
4	162.1 ± 2.2	148.1 ± 1.5
5	73.7 ± 1.1	123.2 ± 2.7
6	77.2 ± 0.9	130.2 ± 1.2
7	81.2 ± 0.8	125.5 ± 2.2
Aspirin	20.5 ± 0.3	18.0 ± 0.2

[a] Values are means of data obtained from three different assays. RE, raw extract.

are neccessary to examine the possibility of using it as an anti-inflammatory drug.

In conclusion, three flavanols and three known dimeric proanthocyanidins have been isolated and identified from an acetone/water extract of *Mangifera indica* in addition to a new trimeric prodelphinidin. The isolated compounds showed the predominance of 2,3-*cis*-configurated flavan-3-ol units with mostly dihydroxylated B-rings. The presence of a galloylated moiety and two trihydroxylated B-rings in the isolated new compounds, and its relative higher molecular weight might be responsible for the potent inhibitory activity against COX-2 of compound **1**. The raw extract contained in addition tannins other compounds like xanthones and flavonoids which were reported to have anti-inflammatory effects (Garrido *et al*., 2001; Clavin *et al*., 2007). Future investigations should deal with the structure elucidation of the polymeric proanthocyanidin fraction, and its pharmacological testing is necessary.

Experimental

General

NMR spectra were recorded in $CDCl_3$ with a Varian Mercury 400 plus instrument. Chemical shifts were recorded relative to $CHCl_3$. CD spectra were measured in MeOH on a CD spectrometer AVIV 62A DS. Acetylation was performed in Ac_2O/pyridine (1.2:1) at ambient temperature for 24 h. MALDI-TOF-mass spectrometer: LAZARUS II (home-built); N_2-laser (LSI VSL337ND), 337 nm; puls width, 3 ns; focus diameter, 0.1 mm; acceleration voltage, 16 kV; drift length, 1 m; data logging with LeCroy9450A (Qa'dan *et al.*, 2003); sampling time, 2.5 ns; expected mass accuracy, ± 0.1%; sample preparation, acetylated compounds were deposited from a solution in $CHCl_3$ on a thin layer of 2,5-dihydroxybenzoic acid (DHB) crystals. Analytical TLC was done on silica gel GF_{254} plates (Merck) with the mobile phase EtOAc/HCOOH/H_2O (18:1:1). Compounds were visualized as red spots by spraying with vanillin/HCl. Optical rotation ([α]) was measured using a Perkin-Elmer polarimeter 241.

Plant material

Mangifera indica L. leaves were collected from nine-year-old trees grown around the Dead Sea in Jordan in July 2007 and identified in comparison with authentic *Mangifera indica* obtained from the University of Jordan, Amman. A voucher specimen is deposited at the University of Muenster, Germany (no. 1701).

Chemicals and reagents

COX-1 and COX-2 were purchased from Sigma (Munich, Germany). ^{14}C-Labeled arachidonic acid [>200 μCi (370 kBq), NEN)] was purchased from New England Nuclear Co. (Boston, USA). Other chemicals and reagents were purchased from Roth Chemicals (Muenster, Germany).

Extraction and isolation

The air-dried material (2 kg) was exhaustively extracted with Me_2CO/H_2O (7:3, 12 l), and the combined extracts were evaporated *in vacuo* to 1.5 l, filtered to remove the precipitated chlorophyll, concentrated and defatted with petroleum ether (30–50 °C). Successive extractions with EtOAc (7.5 l) gave, on evaporation of the solvent, 32.3 g of a solid. The remaining H_2O phase (WP) was evaporated to dryness (282 g). 30.0 g of the EtOAc fraction were subjected to CC on a Sephadex LH-20 column (5.5 × 68 cm) eluted with EtOH/H_2O (6 l), EtOH/MeOH (1:1, 7 l), MeOH (3 l), and acetone/H_2O (7:3, 4 l) to give 10 fractions. Fraction 3 (3800–4200 ml, 1.1 g) was subjected to chromatography on an MCI gel CHP 20 P column (25 × 250 mm) eluted with a 10–80% MeOH linear gradient (17 ml/fraction) to afford epicatechin (**3**) (subfractions 29–41, 201 mg) and catechin (**2**) (subfractions 69–89, 23 mg). Fraction 4 (4200–4770 ml, 1.5 g) was separated on an MCI gel column with the same gradient as above to

afford epigallocatechin (**4**) (subfractions 32–37, 43 mg). Fraction 5 (4770–5700 ml, 3.5 g) was separated on an MCI gel column to afford epicatechin-(4*β*→8)-catechin (**7**) (subfractions 90–105, 49 mg). Catechin-(4*α*→8)-catechin (**5**) was achieved from fraction 6 (5700–6300 ml, 20 mg) following MCI gel chromatography (subfractions 41–49, 17 mg). Catechin-(4*α*→6)-catechin (**6**) was isolated from fraction 9 (9700–9900 ml, 12 mg) following MCI gel chromatography as described above (subfractions 26–31, 18 mg). All compounds were identified after acetylation by their physical data (NMR, MS, CD) and by comparison with authentic samples and published values (De Mello *et al*., 1996; Ploss *et al*., 2001; Qa'dan *et al*., 2003).

A portion (100 g) of the water phase was successively subjected to CC on a Sephadex LH-20 column (55 × 900 mm) with 5 l EtOH/H_2O and 10 l MeOH/H_2O (1:1) to afford 8 fractions.

Epigallocatechin-3-O-gallat-(4β→8)-epigallocatechin-(4β→8)-catechin (**1**): Fraction 5 (7800–8200 ml, 360 mg) achieved from Sephadex LH-20 CC was subjected to chromatography on an MCI gel CHP 20P column (25 × 450 mm) with a 10–60% MeOH linear gradient (16 ml/subfraction) to afford an amorphous white powder (subfractions 22–28, 21 mg); $[\alpha]_D^{20}$ +103.3° (*c* 0.15, MeOH). 8 mg were acetylated to give **1a**: MALDI-TOF-MS: *m/z* = 1873 $[M + Na]^+$. – CD: $[\theta]_{272}$ –11341°, $[\theta]_{254}$ +675°, $[\theta]_{237}$ –30157°, $[\theta]_{217}$ +68734°, $[\theta]_{213}$ +65215°, $[\theta]_{209}$ +70896°. – ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.92–2.33 (m, Ac), 2.28 [*m*, H-4 (I)], 2.62 [*m*, H-4 (I)], 4.55 [*brs*, H-4 (C)], 4.62 [*d*, *J* = 2.3 Hz, H-4 (F)], 4.86 [*m*, H-3 (C)], 4.95 [*m*, H-3 (F)], 5.23 [*m*, H-3 (I)] 5.41 [*brs*, H-2 (I)], 5.57 [*brs*, H-2 (C)], 5.73 [*brs*, H-2 (F)], 6.05 [*d*, *J* = 2.3 Hz, H-8 (A)], 6.24 [*dd*, *J* = 2.3 and 8.3 Hz, H-6' (H)], 6.33 [*d*, *J* = 2.3 Hz, H-6 (A)], 6.38 [*s*, H-6 (D)], 6.44 [*d*, *J* = 2.1 Hz, H-2' (H)], 6.84 [*brs*, H-2'/H-6' (B)], 6.90 [*brs*, H-2'/H-6' (E)], 7.05 [*d*, *J* = 8.3 Hz, H-5' (H)], 7.61 [*brs*, H-2'/H-6' (J)]. – ^{13}C NMR ($CDCl_3$, 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) and C-2 (F)], 73.4 [C-2 (C)], 74.7 [C-2 (I)], 107.9 [C-8 (A)], 109.7 [C-6 (A)], 110.6 [C-4a (G)], 111.0 [C-4a (A)], 111.5 [C-6 (D)], 112.6 [C-4a (D)], 115.5 [C-8 (G)], 117.1 [C-8 (D)], 118.5 [C-2'(H), C-2' (E), and C-6' (E)], 119.4 [C-2' (B) and C-6' (B)], 121.6 [C-6' (H)], 122.5 [C-3'' (J) and C-5'' (J)], 123.1 [C-5' (H)], 127.1 [C-1'' (J)], 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'' (J) and C-5'' (J)], 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)], 162.4 [C-1a (J)].

Purified proanthocyanidin **1** (10 mg) was reacted with phloroglucinol (10 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 N_2 to dryness and purified by preparative TLC. The main phloroglucinol adduct was further purified by preparative TLC on cellulose (*t*-BuOH/CH_3COOH/H_2O).

Effect on cyclooxygenase-1 and -2

The effect on cyclooxygenase-1 and -2 (COX-1 and COX-2) was determined by measuring the PGE2 production. The reaction mixtures were prepared in tris(hydroxymethyl)aminomethane-HCl buffer (pH 8.0), containing glutathione (350 μM), epinephrine (350 μM), hematin (1.5 μM), enzyme (COX-1 or COX-2, 50 μl), and various concentrations of extracts or isolated compounds. For starting the reaction, 1–^{14}C arachidonic acid (10 μl) was added. The mixture was first incubated for 30 min at 37 ºC, then the reaction was terminated by adding the reaction mixture (20 μl) to 30 μM indomethacin (200 μl). Arachidonic acid and its radiolabeled metabolites were separated and determined by reversed-phase HPLC using a 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. Aspirin was used as a positive control. The results are means of three independent experiments.

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