Arch Pharm Res Vol 29, No 12, 1102-1108, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Anti-inflammatory Activity of Flavonoids from *Populus davidiana*

XinFeng Zhang, Tran Manh Hung, Phuong Thien Phuong, Tran Minh Ngoc, Byung-Sun Min[1], Kyung-Sik Song[2], Yeon Hee Seong[3], and KiHwan Bae

College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea, [1]College of Pharmacy, Catholic University of Daegu, Gyeongbuk 712-702, Korea, [2]College of Agriculture and Life Science, Kyungbuk National University, Daegu 702-701, Korea, and [3]College of Veterinary Medicine, Chungbuk National University, Cheonju 361-763, Korea

(Received July 7, 2006)

An *in vitro* bioassay-guide revealed that the methanol (MeOH) extract of the stem bark of *Populus davidiana* showed considerable inhibitory activity against cyclooxygenase (COX-1, COX-2). Continuous phytochemical study of the MeOH extract of this plant led to the isolation of ten flavonoids; sakuranetin (**1**), rhamnocitrin (**2**), 7-*O*-methylaromadendrin (**3**), naringenin (**4**), eriodictyol (**5**), aromadendrin (**6**), kaempferol (**7**), neosakuranin (**8**), sakuranin (**9**) and sakuranetin-5,4'-di-β-D-glucopyranoside (**10**). Their structures were identified on the basis of their physicochemical and spectroscopic analyses. The isolated compounds, **1-10,** were tested for their inhibitory activities against COX-1 and COX-2. Compound **7** was found to have potent inhibitory effect on COX-1 and a moderate effect on COX-2, meanwhile, compounds **1-6** showed moderate inhibition against COX-1 only. Moreover, compounds **5–8** exhibited suppressive effects on xanthine oxidase (XO). These results may explain, in part, the traditional uses of *P. davidiana* in ethnomedicine.

Key words: *Populus davidiana*, Flavonoids, Anti-inflammatory, Cyclooxygenase, Xanthine oxidase

INTRODUCTION

Inflammation is now the most common disease worldwide (Borne, 1995). Therefore, therapeutic anti-inflammatory agents have been pursued for many years. It is well known that the enzymes cyclooxygenase-1 (COX-1), -2 (COX-2) and xanthine oxidase (XO) play key roles in the pathology of inflammatory diseases (Borne, 1995). COX-1 and COX-2 are involved in inflammation through the conversion of arachidonic acid to prostaglandins and thromboxanes (Emery, 1999; Marnett *et al.*, 1999). XO is the enzyme that catalyzes the oxidation of xanthine and hypoxanthine into uric acid, which plays a pivotal role in hyperuricemia and gout diseases (Star and Hochberg, 1993).

As part of our ongoing search for anti-inflammatory and antioxidant agents from plants, the MeOH extract from the stem bark of *Populus davidiana* Dode was found to show inhibitory activity against COX-1 and COX-2. The plant *P. davidiana* [*P. tremela.* L. var. *davidiana* (Dode) Schneid.] belongs to the family Salicaceae, which is known in the North of China, Korea and the Siberian region of Russia (Wang *et al.*, 1999). The bark, branches and leaves of this plant have been used in oriental folk medicine for the treatment of various diseases, including pulmonary disease, cough, pox and variola (Bae, 1998). Moreover, the decoction of this bark is used as a remedy against body pains and as an antidote (Zhong Hua Ben Cao, 1999). Although *P. davidiana* is widely distributed in the North of Asia, the chemical constituents and biological activities have rarely been reported. Previously, phenolic glycosides and flavanoids have been reported to be constituents of this plant (Zhou *et al.*, 2002; Wang *et al.*, 2000). The MeOH extract of this plant has shown potent inhibitory activity toward nitric oxide (NO) production in a previous report (Ryu *et al.*, 2003), and some isolated flavonones from this plant, such as galangin, tectochrysin, and ombuin, have shown anti-inflammatory activity against the TNF-α release induced by LPS (Zhou *et al.*, 2002). From the repeated column chromatographic separation of the stem bark of this

Correspondence to: KiHwan Bae, College of Pharmacy, Chungnam National University, Daejeon 305–764, Korea
Tel: 82-42-821-5925, Fax: 82-42-823-6566
E-mail: baekh@cnu.ac.kr

plant, ten flavonoids have been isolated for the first time; sakuranetin (**1**), rhamnocitrin (**2**), 7-*O*-methylaromadendrin (**3**), naringenin (**4**), eriodictyol (**5**), aromadendrin (**6**), kaempferol (**7**), neosakuranin (**8**), sakuranin (**9**) and sakurenetin-5,4'-di-β-D-glucopyranoside (**10**). Moreover, these compounds were tested for their suppressive activities against COX-1, COX-2 and XO. The present paper reports the isolation and structural elucidation of ten flavonoids (**1-10**), along with their inhibitory effects on the activities of three enzymes (COX-1, COX-2 and XO).

MATERIALS AND METHODS

Plant material

The stem bark of *P. davidiana* Dode. was collected at Yangu, Kangwon, Korea in August 2002, and identified by one of the authors, Prof. KiHwan Bae. A voucher specimen (CNU 255) has been deposited at the Herbarium of the College of Pharmacy, Chungnam National University.

General experimental procedures

Melting points were determined on a Kofler micro-hotstage; UV spectra were obtained with a Beckman Du-650 UV-VIS recording spectrophotometer; IR spectra were obtained on a Jasco Report-100 type spectrometer from KBr discs; MS were carried out with a JEOL JMS-HX/HX110A tandem mass spectrometer. ^{1}H-NMR (300 MHz, 400 MHz, and 600 MHz) and ^{13}C-NMR (75 MHz, 100 MHz, and 150 MHz) were recorded on Bruker DRX 300 and JEOL 400 spectrometers. The chemical shifts were referenced to δ using TMS as an internal standard. Analytical TLC was performed on pre-coated silica gel 60 F_{254} plates (Merck) or RP-18 F_{254}. Silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck) and Sephadex LH-20 (Amersham Biosciences) were used as the stationary phases for column chromatography.

Chemicals

Xanthine, xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), nitroblue tetrazolium chloride (NBT), glutathione, *l*-epinephrine hematin and apigenin were purchased from Sigma chemical Co. (St. Louis, MO). COX-1 and COX-2 were purchased from the Cayman Chemical (USA). 1-^{14}C arachidonic acid (50 μCi, NEC-661, NEN) was purchased from BMS (Amersham, NEN). All other reagents were of analytical grade.

Extraction and isolation

The stem bark of *P. davidiana* (4.8 kg) was extracted three times with hot MeOH (20 L × 3) for 5 h, and the extract concentrated under reduced pressure. The residue (1.3 kg) obtained was suspended in water and successively partitioned with hexane, ethyl acetate (EtOAc) and butanol (BuOH), to afford hexane-soluble (47 g), EtOAc-soluble (770 g) and BuOH-soluble (360 g) fractions, respectively. The EtOAc-soluble fraction was subjected to silica gel column chromatography, using $CHCl_3$ and MeOH mixtures of increasing polarity, to yield seven fractions (E1-E7). Fraction E2 was applied to a silica gel column and eluted with hexane-EtOAc (5:1) to yield ten subfractions (E2.1-E2.10). Subfractions E2.4, E2.5 and E2.6 were dissolved in $CHCl_3$ and crystallized to afford three compounds **1** (1.1 g), **2** (27 mg) and **3** (126 mg), respectively. Fraction E3 was subjected to chromatography on a silica gel column, with $CHCl_3$-acetone of increasing polarity, to give compound **4** (560 mg). Fraction E4 was subjected to silica gel column chromatography, using mixture of $CHCl_3$ and acetone of increasing polarity, to give eight subfractions (E4.1-E4.8). Subfraction E4.4 was subjected to chromatography on a Sephadex LH-20 column, and eluted with MeOH, to give three fractions, with compounds **5** (65 mg) and **6** (126 mg) crystallized from the first and second fractions, respectively. The third fraction was subjected to chromatography on an ODS column, using medium-pressure liquid chromatography (MPLC, Shimadzu), and eluted with MeOH-H_2O (1:1), furnishing compound **7** (48 mg) after crystallization with MeOH. Fraction E5 was subjected to silica gel column chromatography, using mixtures of $CHCl_3$-MeOH-H_2O of increasing polarity (15:1:0.1→1:1:0.1), to yield seven fractions (E5.1-E5.7); subfraction E5.5 was further separated on an YMC column, using MeOH-H_2O (10, 30, 50, and 100%), to give four fractions. The 30% eluted fraction was applied to a silica gel column and eluted with $CHCl_3$-MeOH (8:1), and then purified on an RP-18 column, using MeOH-H_2O (2:3) as the eluting solvent, to give compounds **8** (260 mg) and **9** (560 mg). The BuOH-soluble fraction was subjected to silica gel column, and eluted with $CHCl_3$-MeOH-H_2O mixtures of increasing polarity (9:1:0.1→0:1:0.5), to give six fractions (B1-B6). Fractions B6 was repeatedly subjected to an YMC column, and eluted with 10%→50% MeOH, to give thirteen subfractions (B6.1-B6.13); subfraction B6.9 was purified on a Sephadex LH-20 column, and eluted with MeOH, to yield compound **10** (120 mg).

Sakuranetin (1)

White needles, mp 151-153°C; $[\alpha]_D^{25}$ -7.0° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε): 216.0 (4.31), 288.0 (4.11) nm; IR (KBr) ν_{max} cm^{-1}: 3400 (OH), 1640 (C=O), 1520, 1450 (C=C), 1320, 1305 (C-O-C), 1200, 1060, 840, 760 (Ar-H); ^{1}H-NMR (300 MHz, methanol-d_4) δ (ppm): 5.37 (1H, dd, *J* = 3.0, 12.9 Hz, H-2), 2.74 (1H, dd, *J* = 3.0, 17.1 Hz, Heq-3), 3.15 (1H, dd, *J* = 12.9, 17.1 Hz, Hax-3), 6.06 (1H, d, *J* = 2.4 Hz, H-8), 6.04 (1H, d, *J* = 2.4 Hz, H-6), 3.81 (3H, s, 7-OCH_3), 7.33 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.83 (2H, d, *J* = 8.4 Hz, H-3', 5'); ^{13}C-NMR (75 MHz, methanol-d_4) δ (ppm):

79.6 (C-2), 43.0 (C-3), 197.2 (C-4), 164.2 (C-5), 94.7 (C-6), 168.5 (C-7), 93.9 (C-8), 163.7 (C-9), 103.1 (C-10), 129.9 (C-1'), 115.3 (C-3', 5'), 128.1 (C-2', 6'), 158.1 (C-4), 55.2 (7-OCH_3).

Rhamnocitrin (2)

Yellow needle crystal, mp 202–203°C; UV (MeOH) λ_{max} (log ε): 268.0 (4.24), 366.0 (4.26) nm; IR (KBr) ν_{max} cm^{-1}: 3250 (OH), 1660 (C=O), 1605, 1590, 1500 (aromatic ring), 1410, 1350, 1280, 1220, 1160 (C-O), 980, 820 (Ar-H); ^{1}H-NMR (300 MHz, methanol-d_4) δ (ppm): 6.31 (1H, d, *J* = 2.1 Hz, H-6), 6.59 (1H, d, *J* = 2.1 Hz, H-8), 6.93 (2H, d, *J* = 9.0 Hz, H-3', 5'), 8.12 (2H, d, *J* = 9.0 Hz, H-2', 6'), 3.89 (3H, s, 7-OCH_3); ^{13}C-NMR (75 MHz, methanol-d_4) δ (ppm): 161.2 (C-2), 135.5 (C-3), 176.4 (C-4), 159.7 (C-5), 97.5 (C-6), 166.0 (C-7), 91.7 (C-8), 157.1 (C-9), 104.6 (C-10), 122.6 (C-1), 129.8 (C-2', 6'), 115.3 (C-3', 5'), 147.5 (C-4'), 55.4 (7-OCH_3).

7-*O*-methylaromadendrin (3)

White needles crystal, $[\alpha]_D^{25}$ +31.9° (*c* 0.5, MeOH); mp 192-193°C; UV (MeOH) λ_{max} (log ε): 218 (4.54), 291 (4.31) nm; IR (KBr) ν_{max} cm^{-1}: 3350 (OH), 1630 (C=O), 1520, 1460 (aromatic ring), 1365, 1290, 1260, 1150, 1080, 1005, 840; ^{1}H-NMR (300 MHz, methanol-d_4) δ (ppm): 3.81 (3H, s, 7-OCH_3), 4.58 (1H, d, *J* = 11.7 Hz, H-2), 5.02 (1H, d, *J* = 11.7 Hz, H-3), 6.09 (1H, d, *J* = 2.4 Hz, H-8), 6.05 (1H, d, *J* = 2.4 Hz, H-6), 6.85 (2H, d, *J* = 8.4 Hz, H-3', 5'), 7.37 (2H, d, *J* = 8.4 Hz, H-2', 6'); ^{13}C-NMR (75 MHz, methanol-d_4) δ (ppm): 55.3 (7-OCH_3), 84.1 (C-2), 72.7 (C-3), 198.0 (C-4), 164.0 (C-5), 95.0 (C-6), 168.8 (C-7), 94.1 (C-8), 163.4 (C-9), 101.6 (C-10), 128.2 (C-1'), 129.4 (C-2', 6'), 115.2 (C-3', 5'), 158.3 (C-4').

Naringenin (4)

White needles crystal, $[\alpha]_D^{25}$ -24.0° (*c* 0.5, MeOH); mp 241-242°C; UV (MeOH) λ_{max} (log ε): 225.0 (4.43), 290.0 (4.26) nm; IR (KBr) ν_{max} cm^{-1}: 3200 (OH), 1620 (C=O), 1520, 1495 (aromatic ring), 1460, 1315 (C-O-C), 1250, 1180, 1160, 1070, 970, 830 (Ar-H); ^{1}H-NMR (300 MHz, methanol-d_4) δ (ppm): 5.34 (1H, dd, *J* = 3.0, 12.9 Hz, H-2), 2.70 (1H, dd, *J* = 3.0, 17.1 Hz, Heq-3), 3.12 (1H, dd, *J* = 12.9, 17.1 Hz, Hax-3), 5.91 (1H, d, *J* = 2.1 Hz, H-8), 5.89 (1H, d, *J* = 2.1 Hz, H-6), 7.33 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.83 (2H, d, *J* = 8.4 Hz, H-3', 5'); ^{13}C-NMR (75 MHz, methanol-d_4) δ (ppm): 79.5 (C-2), 43.0 (C-3), 196.8 (C-4), 164.4 (C-5), 96.1 (C-6), 167.3 (C-7), 95.2 (C-8), 163.9 (C-9), 102.4 (C-10), 130.1 (C-1'), 115.3 (C-3', 5'), 128.0 (C-2', 6'), 158.0 (C-4').

Eriodictyol (5)

White needles crystal, mp 255-257°C; $[\alpha]_D^{25}$ -1.9° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε): 214.0 (4.69), 289.0 (4.27); IR (KBr) ν_{max} cm^{-1}: 3350 (OH), 1620 (C=O), 1450 (aromatic ring), 1260, 1160, 1080, 820; ^{1}H-NMR (300 MHz, methanol-d_4) δ (ppm): 5.29 (1H, dd, *J* = 3.0, 12.9 Hz, H-2), 2.71 (1H, dd, *J* = 3.0, 17.1 Hz, Heq-3), 3.08 (1H, dd, *J* = 17.1, 12.9 Hz, Hax-3), 5.91 (1H, d, *J* = 2.1 Hz, H-8), 5.89 (1H, d, *J* = 2.1 Hz, H-6), 6.93 (1H, s, H-2'), 6.80 (2H, d, *J* = 1.2 Hz, H-5', 6'); ^{13}C-NMR (75 MHz, methanol-d_4) δ (ppm): 79.5 (C-2), 43.1 (C-3), 196.8 (C-4), 164.4 (C-5), 95.1 (C-6), 167.3 (C-7), 96.1 (C-8), 163.8 (C-9), 102.3 (C-10), 130.8 (C-1'), 113.7 (C-2'), 145.9 (C-3'), 145.5 (C-4'), 115.2 (C-5'), 118.2 (C-6').

Aromadendrin (6)

White needles crystal, mp 228-230°C; $[\alpha]_D^{25}$ +34.0° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε): 214.0 (4.22), 293.0 (4.01); IR (KBr) ν_{max} cm^{-1}: 3400 (OH), 1620 (C=O), 1520, 1460 (aromatic ring), 1370, 1260, 1200, 1160, 1080, 1020, 830; ^{1}H-NMR (300 MHz, methanol-d_4) δ (ppm): 4.99 (1H, d, *J* = 11.7 Hz, H-2), 4.56 (1H, d, *J* = 11.7 Hz, H-3), 5.94 (1H, d, *J* = 2.1 Hz, H-8), 5.89 (1H, d, *J* = 2.1 Hz, H-6), 7.37 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.85 (2H, d, *J* = 8.4 Hz, H-3', 5'); ^{13}C-NMR (75 MHz, methanol-d_4) δ (ppm): 84.0 (C-2), 72.6 (C-3), 197.5 (C-4), 164.3 (C-5), 96.3 (C-6), 167.7 (C-7), 95.3 (C-8), 163.5 (C-9), 100.8 (C-10), 128.3 (C-1'), 129.4 (C-2', 6'), 115.1 (C-3', 5'), 158.2 (C-4').

Kaempferol (7)

Yellow powder, mp 230-232°C; UV (MeOH) λ_{max} (log ε): 269.0 (3.93), 364.0 (3.99) nm; IR (KBr) ν_{max} cm^{-1}: 3350 (OH), 1660 (C=O), 1610, 1500 (aromatic ring), 1380, 1310, 1250, 1180, 1090, 820; ^{1}H-NMR (300 MHz, methanol-d_4) δ (ppm): 6.19 (1H, *J* = 2.1 Hz, H-6), 6.40 (1H, *J* = 2.1 Hz, H-8), 6.91 (2H, *J* = 8.7 Hz, H-3', 5'), 8.09 (2H, *J* = 8.7 Hz, H-2', 6'); ^{13}C-NMR (75 MHz, methanol-d_4) δ (ppm): 147.0 (C-2), 136.1 (C-3), 176.3 (C-4), 161.5 (C-5), 98.3 (C-6), 164.5 (C-7), 93.5 (C-8), 157.2 (C-9), 103.5 (C-10), 122.7 (C-1'), 130.4 (C-2', 6'), 115.2 (C-3', 5'), 159.5 (C-4').

Neosakuranin (8)

Yellow powder, mp 107-108°C; UV (MeOH) λ_{max} (log ε): 207.0 (4.44), 371 (4.35) nm; IR (KBr) ν_{max} cm^{-1}: 3400, 1620, 1500, 1450, 1350, 1220, 1070, 830; ^{1}H-NMR (600 MHz, DMSO-d_6) δ (ppm): 7.67 (2H, d, *J* = 7.8 Hz, H-2, 6), 6.83 (2H, d, *J* = 9.0 Hz, H-3, 5), 7.96 (1H, d, *J* = 15.6 Hz, H-7), 7.65 (1H, d, *J* = 15.6 Hz, H-8), 6.17 (1H, d, *J* = 2.4 Hz, H-3'), 6.32 (1H, d, *J* = 2.4 Hz, H-5'), 5.16 (1H, d, *J* = 7.8 Hz, H-1"), 3.16-3.48 (4H, m, H-2", 3", 4", 5"), 3.73 (1H, dd, *J* = 1.8, 15.4 Hz, Hax-6"), 3.47 (1H, m, Heq-6"), 3.81 (3H, s, 4'-OCH_3). ^{13}C-NMR (150 MHz, DMSO-d_6) δ (ppm): 126.6 (C-1), 131.5 (C-2), 116.4 (C-3), 160.2 (C-4), 116.4 (C-5), 131.5 (C-6), 144.0 (C-7), 124.4 (C-8), 193.0 (C-9), 107.1 (C-1'), 165.7 (C-2'), 94.3 (C-3'), 166.1 (C-4'), 95.7 (C-5'), 160.5 (C-6'), 100.9 (C-1"), 70.2 (C-2"), 77.9 (C-3"),

74.1 (C-4"), 77.3 (C-5"), 61.1 (C-6"), 56.2 (4'-OCH_3).

Sakuranin (9)

White amorphous powder, mp 199-201°C; $[\alpha]_D^{25}$ -105° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε): 230.0 (4.20), 283.0 (4.42) nm; IR (KBr) ν_{max} cm^{-1}: 3350 (OH), 1680, 1610, 1560, 1520, 1460, 1360, 1260, 1200, 1160, 1090, 830, 760; ^{1}H-NMR (600 MHz, DMSO-d_6) δ (ppm): 5.45 (1H, dd, *J* = 3.0, 13.2 Hz, H-2), 3.15 (1H, dd, *J* = 16.8, 13.2 Hz, Hax-3), 2.68 (1H, dd, *J* = 2.4, 16.8 Hz, Heq-3), 6.54 (1H, d, *J* = 2.4 Hz, H-6), 6.35 (1H, d, *J* = 2.4 Hz, H-8), 7.35 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.81 (2H, d, *J* = 8.4 Hz, H-3', 5'), 4.78 (1H, d, *J* = 7.2 Hz, H-1"), 3.40–3.50 (4H, m, H-2", 3", 4", 5"), 3.76 (1H, dd, *J* = 5.4, 10.8 Hz, Hax-6"), 3.49 (1H, m, Heq-6), 3.81 (3H, s, 7-OCH_3). ^{13}C-NMR (150 MHz, DMSO-d_6) δ (ppm): 78.9 (C-2), 44.9 (C-3), 190.6 (C-4), 160.6 (C-5), 98.4 (C-6), 166.1 (C-7), 96.5 (C-8), 164.8 (C-9), 106.8 (C-10), 129.3 (C-1'), 115.6 (C-3'), 128.8 (C-2'), 158.2 (C-4'), 128.8 (C-6'), 116.4 (C-5'), 103.6 (C-1"), 73.9 (C-2"), 78.0 (C-3"), 70.4 (C-4"), 76.3 (C-5"), 61.3 (C-6"), 56.3 (7-OCH_3).

Sakurenetin-5, 4-di-β-D-glucopyranoside (10)

White amorphous powder, mp 201-202°C; UV (MeOH) λ_{max} (log ε): 226.0 (4.38), 286.0 (4.15) nm; IR (KBr) λ_{max} cm^{-1}: 3400 (OH), 2900, 1605, 1560, 1505, 1280, 1080, 1040; ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 5.51 (1H, dd, *J* = 3.0, 12.0 Hz, H-2), 3.15 (1H, dd, *J* = 12.0, 16.0 Hz, Hax-3), 2.68 (1H, dd, *J* = 2.4, 16.0 Hz, Heq-3), 6.52 (1H, d, *J* = 2.4 Hz, H-6), 6.35 (1H, d, *J* = 2.4 Hz, H-8), 7.44 (2H, d, *J* = 8.0 Hz, H-2', 6'), 7.06 (2H, d, *J* = 8.0 Hz, H-3', 5'), 4.76 (1H, d, *J* = 7.0 Hz, H-1"), 4.89 (1H, d, *J* = 7.5 Hz, H-1'"), 3.79 (3H, s, 7-OCH_3); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 78.1 (C-2), 44.5 (C-3), 190.0 (C-4), 160.1 (C-5), 98.0 (C-6), 165.7 (C-7), 96.0 (C-8), 164.2 (C-9), 106.4 (C-10), 131.9 (C-1'), 128.1 (C-2'), 116.2 (C-3'), 157.5 (C-4'), 116.2 (C-5'), 128.1 (C-6'), 100.2 (C-1"), 73.2 (C-2"), 77.1 (C-3"), 69.7 (C-4"), 75.8 (C-5"), 60.7 (C-6"), 103.0 (C-1'"), 73.5 (C-2'"), 77.7 (C-3'"), 69.9 (C-4'"), 76.6 (C-5'"), 60.9 (C-6'"), 55.9 (7-OCH_3).

Enzymatic hydrolysis of 10

Naringinase (100 mg, from almond) was added to a suspension of **10** (5 mg) in 50 mM acetate buffer (pH 7.0), and the mixture stirred at 37°C for 48 h (Min *et al.*, 2004). The reaction mixture was extracted with EtOAc. The aglycone showed the same TLC pattern as compound **1** ($CHCl_3$-MeOH, 20:1). Glucose in the water layer was revealed by TLC (EtOAc-MeOH-H_2O-AcOH, 65:20:15:15). The spot on the TLC plate was visualized using an anisaldehyde-H_2SO_4 reagent.

Cyclooxygenase (COX-1, COX-2) assay

The experiments were performed according to the method reported by Noreen, with slight modification (Noreen *et al.*, 1998). 10 μL enzyme (COX-1, 3.0 units, 0.43 μg protein; COX-2, 3.0 units, 0.39 μg protein) was activated on ice for 4 min with 170 μL of a cofactor solution, consisting of 1.3 mg/mL reduced hematin (Sigma, U.S.A.), 1.3 mg/mL *l*-epinephrine (Sigma, U.S.A.) and 3 mg/mL reduced glutathione (Sigma, U.S.A.) in Tris-HCl buffer (pH 8.0). An aliquot (10 μL) of either the test solution (compound dissolved in DMSO) or vehicle (DMSO) was added to the reaction tube and preincubated on ice for 10 min. After initiating the reaction by the addition of 10 μL (0.02 μCi) 1-^{14}C arachidonic acid (50 μCi, NEC-661, NEN), the mixture was incubated for 20 min at 37°C. The reaction was quenched by the addition of 10 μL 2M HCl. The prostaglandins produced and the un-metabolized arachidonic acid were extracted with ethyl ether and separated by TLC (developing system, $CHCl_3$-MeOH-acetic acid, 18:1:1). The authentic gross count of the ^{14}C-labelled PGE_2 was measured using electronic autoradiography. The inhibitory effects of the test samples were shown by the amount of ^{14}C-labelled PGE_2 produced compared to that of the DMSO control (% control). The IC_{50} values were calculated from four test concentrations using the method described by Wu (Wu *et al.*, 1992).

Superoxide radical assay

Superoxide radical scavenging activity was assayed using the NBT reduction method (Valentao *et al.*, 2002), with some modification. The assay mixture (495 μL) consisted of 50 mM sodium carbonate buffer (pH 7.8), 50 mM xanthine, 50 μM nitro blue tetrazolium (NBT) and 0.1 mM EDTA in the presence or absence of the test compound. The reaction was initiated by the addition of 5 μL xanthine oxidase (20 nM). The increase in absorbance at 560 nm was read after 5 min using a spectrophotometer (Shimadzu UV-1240). The superoxide radical scavenging activity was expressed by the decrease in the NBT reduction of the test group compared to that of the control group, and calculated using the following equation:

$$\text{Scavenging activity (\%)} = 100 \times [(Ac - As)/Ac]$$

Where Ac and As were the absorbance of the control (without test compound) and sample (test compound), respectively. The IC_{50} values were defined as the concentration able to scavenge 50% of the radicals produced, and calculated using the method reported by Wu *et al.* (Wu *et al.*, 1992).

Effect on xanthine oxidase activity

The effect of the lyophilized infusion on the XO activity was evaluated by measuring the formation of uric acid from xanthine, using a spectrophotometer (Shimadzu UV-

1240) at room temperature (Valentao *et al.*, 2002). The reaction mixtures contained the same proportion of components as in the enzymatic assay for the superoxide radical scavenging activity, except for the NBT, in a final volume of 500 μL. The absorbance was measured at 295 nm for 5 min. The IC_{50} values were calculated from five test concentrations using the method described by Wu (Wu *et al.*, 1992).

RESULTS AND DISCUSSION

The MeOH extract of the stem bark of *P. davidiana* showed considerable inhibitory effect towards COX-1 and COX-2, with inhibitory values of 97 and 67.2% (the final concentration 1 mg/mL), respectively. Repeated column chromatography of the EtOAc-soluble and BuOH-soluble fractions of the MeOH extract led to the isolation of ten compounds (**1**-**10**). On the basis of their physicochemical and spectroscopic analyses, and by comparison of these data with those published in the literature, nine known compounds **1**-**9** were identified as sakuranetin (Vasconcelos *et al.*, 1998), rhamnocitrin (Scio *et al.*, 2003; Lin *et al.*, 2002), 7-*O*-methylaromadendrin, naringenin (S. Ibrahim, 2003; Vasconcelos *et al.*, 1998), eriodictyol (Lee *et al.*, 2003; Parejo *et al.*, 2004), aromadendrin (Lee *et al.*, 2003; El-Sohly *et al.*, 1999), kaempferol (Kim *et al.*, 2002), neosakuranin (Thapliyal *et al.*, 1993; Yoshinari *et al.*, 1990) and sakuranin (Yoshinari *et al.*, 1990), respectively. Accordingly, this is the first report on the presence of flavonoids **1**-**9** from this plant.

Compound **10** was obtained as a white amorphous powder. Its UV spectrum showed maximum absorptions at 226.0 and 286.0 nm, and the IR spectrum disclosed absorption peaks at 3400, 2900, 1605, 1560, 1505, 1280, 1080 and 1040 cm^{-1}. The ^1H- and ^{13}C-NMR spectra showed a similar skeleton to that of compound **9**, with the exception of an additional sugar moiety. The linkage of one sugar was deduced as being located at the C-5 position, similar to that of compound **9**, which was supported by the HMBC long-range correlation between δ_H 4.76 (H-1) and δ_C 160.1 (C-5), as shown in Fig. 2. Another D-glucose moiety linkage was also found from the HMBC correlation between δ_H 4.89 (H-1) and δ_C 157.5 (C-4). Enzymatic hydrolysis of compound **10** yielded a monosaccharide unit, which was identified by co-TLC with an authentic sample. The sugar moiety was identified as *β*-D-glucopyranoside, based on the $J_{1,2}$ value of the anomeric proton at 7.5 Hz (δ 4.89) and 7.0 Hz (δ 4.76). Therefore, the structure of compound **10** was determined to be sakurenetin-5,4'-di-*β*-D-glucopyranoside. Although a previous report (Reichel *et al.*, 1969) has chemosynthetically demonstrated the structure of this compound, this is the first report of the isolation, spectral data and physical property of compound **10** from a natural source.

The inhibitory effects of the isolated compounds on COX-1 and COX-2 were tested *in vitro,* according to the method reported by Noreen *et al.* (Noreen *et al.*, 1998), with slight modification, the results of which are shown in Table I. Among the tested compounds, the flavonol, kaempferol (**7**), was the strongest inhibitor of the COX-1-catalyzed PG biosynthesis, with an IC_{50} value of 7.5 μM, and also showed moderate inhibition of the COX-2 activity, with an IC_{50} value of 269.2 μM. Another flavonol, rhamnocitrin (**2**), showed slightly less potent activity (IC_{50} 50.2 μM) than **7**, due to the presence of a methoxy group at the C-7 position (Jang *et al.*, 2004). Five compounds, **1** and **3**-**6**, exhibited less inhibitory effects on the COX-1-catalyzed PG biosynthesis, with IC_{50} values of 196.1, 102.2, 336.2, 477.3 and 257.7 μM, respectively, but not on COX-2-catalyzed PG biosynthesis. Three flavonoid glycosides, compounds **8**-**10**, were found to be much less active, with inhibitions of 25.6, 44.6 and 20.1%, respectively, at a test concentration of 20 mM. This indicates that the sugar moiety significantly reduces the inhibitory activity of

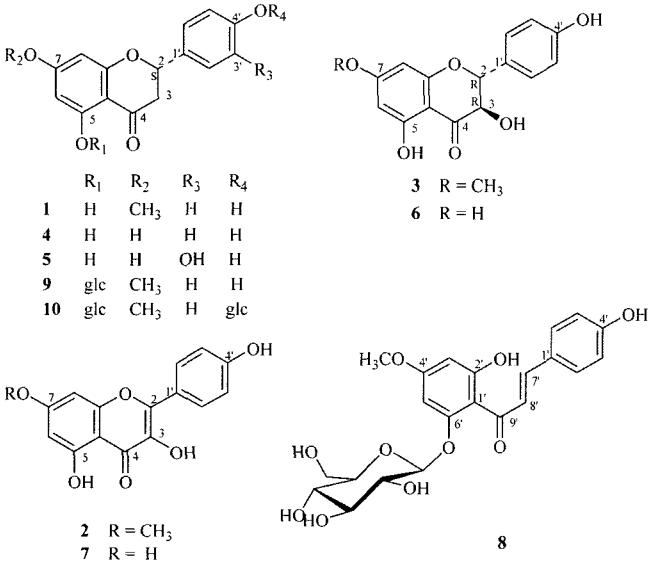

Fig. 1. The chemical structures of compounds **1**-**10** isolated from *P. davidiana*

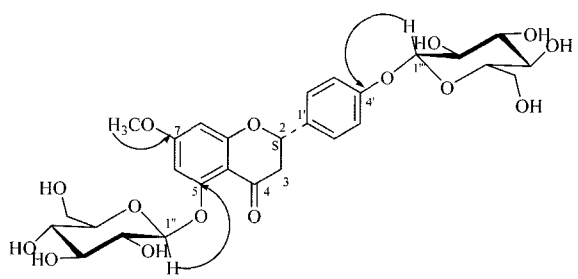

Fig. 2. The selected HMBC correlation of compound **10**

Table I. Inhibitory activities of the isolated compounds, **1-10**, against COX-1 and COX-2

Compounds	COX-1, IC_{50} (μM)[a]	COX-2, IC_{50} (μM)[a]
Sakuranetin (**1**)	196.1	NA[b]
Rhamnocitrin (**2**)	50.2	NA
7-*O*-methylaromadendrin (**3**)	102.2	(4.6 %)[c]
Naringenin (**4**)	336.2	NA
Eriodictyol (**5**)	477.3	(47.2 %)
Aromadendrin (**6**)	257.7	NA
Kaempferol (**7**)	7.5	269.2
Neosakuranin (**8**)	(25.6 %)	NA
Sakuranin (**9**)	(44.6 %)	NA
Sakurenetin-5,4-di-β-D-glucopyranoside (**10**)	(20.1 %)	NA
Aspirin[d]	1804	
Indomethacin[d]		76.5

[a] IC_{50} values indicate the concentration able to inhibit 50% of the enzymes activity, and was calculated from regression lines using four different concentrations. [b] Non-active. [c] Values in parentheses are percent inhibitions lower than 50%, where the IC_{50} values were not determined. [d] Aspirin and indomethacin were used as positive controls.

Table II. Effects of the isolated compounds, **1-10**, on enzyme xanthine oxidase

Compound	IC_{50} (μM)[a]	
	Superoxide radical scavenging	Effect on xanthine oxidase
Eriodictyol (**5**)	9.2	23.6
Aromadendrin (**6**)	69.9	>200
Kaempferol (**7**)	8.4	11.6
Neosakuranin (**8**)	56.9	157.8
Catechin[b]	12.1	20.5
Apigenin[b]	2.4	5.1

[a] The IC_{50} values indicate the 50% inhibition concentrations, and were calculated from regression lines using six different concentrations in triplicate experiments, repeated at least three times; Compounds **1-4**, **9** and **10** were not active (IC_{50} value > 200 μM) in this assay system. [b] These compounds were used as positive controls.

these compounds.

The isolated compounds, **1-10**, were also examined for their suppressive activities against XO. From the result presented in Table II, the compounds (**1-4**, **8-10**) without either a catechol group or double bond between C-2 and C-3 were inactive or very weak active in this assay (Cos *et al.*, 1998; Nguyen *et al.*, 2006). In contrast, compounds **5** and **7** manifested strong inhibitory effects on the XO activity, with IC_{50} values of 23.6 and 11.6 μM, respectively. These results were indicative of the scavenging activity of these compounds against the superoxide radicals generated by XO (Table II), which is mainly due to their suppression of the XO activity (Cos *et al.*, 1998).

The inflammatory response involves in the production of highly reactive compounds, including reactive oxygen species (ROS), which occur due to stimulation of the enzyme XO, which catalyzes the cellular synthesis of uric acid, H_2O_2 and $O_2^{\bullet-}$ from purines (Leiro *et al.*, 2004). In several inflammatory pathologies, the XO activity may be strongly increased. Meanwhile, the inducible enzyme isoforms produced by phagocytes, such as COX-2, also play a pivotal role in inflammatory reactions via the production of inflammatory prostaglandins (Borne, 1995; Fujimoto *et al.*, 2004). Since some flavonoids from *P. davidiana* exhibited suppressive effects on these enzymes, they may act as contributing principles for the traditional uses of this remedy against inflammatory and gout diseases (Leiro *et al.*, 2004; Nguyen *et al.*, 2006).

ACKNOWLEDGEMENTS

The research was supported by a grant of the Biogreen 21 program (2006), Rural Development Administration, Republic of Korea. We are grateful to the Korea Basic Science Institute (KBSI) for supplying the NMR spectra.

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