A New Flavanone Glycoside from the Dried Immature Fruits of *Poncirus trifoliata*

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Received March 15, 2007; accepted April 23, 2007

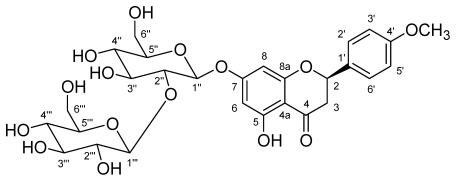
A new flavanone glycoside, (2*R*)-5-hydroxy-4′-methoxyflavanone-7-*O*-{β-glucopyranosyl-(1→2)-β-glucopyranoside} (1), was isolated from the EtOAc extract of dried immature fruit of *Poncirus trifoliata*, together with three known compounds, (2*S*)-poncirin (2), (2*S*)-naringin (3), and (2*S*)-poncirenin (4). The structure of compound 1 was elucidated by spectroscopic data analysis, including 1D and 2D NMR experiments. Among the isolates, compound 2 exhibited considerable inhibitory activity against lipopolysaccharide (LPS)-induced prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6) production, and mRNA expression in RAW 264.7 murine macrophage cells.

Key words *Poncirus trifoliata*; new flavanone; prostaglandin E_2; interleukin-6

The dried immature fruit of *Poncirus trifoliata* (L.) Raf. (Rutaceae) has been traditionally used for uterine contraction and relaxation, and treating gastrointestinal and cardiovascular diseases in China.[1)] Previous phytochemical work on the dried immature fruit of *P. trifoliata* has resulted in the isolation of numerous compounds, including various types of flavonoids, coumarines, and alkaloids.[2—12)] The extracts and some isolates of this fruit were found to have diverse biological activities such as anti-allergic,[2,13,14)] hypocholesterolemic,[3)] anti-helicobacter pylori,[4)] anti-platelet,[5)] anti-inflammatory,[15)] and prokinetic activity.[16,17)]

As a part of our research program to find new bioactive compounds from medicinal plants, dried immature fruit of *P. trifoliata* was chosen for more detailed investigation of its chemical constituents. Repeated column chromatography of an EtOAc extract of *P. trifoliata* led to the isolation of a new flavanone glycoside, namely, (2*R*)-5-hydroxy-4′-methoxyflavanone-7-*O*-{β-glucopyranosyl-(1→2)-β-glucopyranoside} (**1**), together with three known compounds, (2*S*)-poncirin (**2**),[18—21)] (2*S*)-naringin (**3**),[20—23)] and (2*S*)-poncirenin (**4**),[20,21,24,25)] which were reported previously from this plant.

Compound **1** was isolated as a white powder and gave a molecular ion peak at *m/z* 633.1790 $[M+Na]^+$ in the HR-FAB-MS, corresponding to the elemental formula $C_{28}H_{34}O_{15}$. The IR spectrum of **1** showed absorption bands at 3434 cm^{-1} for one or more hydroxyl group(s) and 1700 cm^{-1} for a conjugated carbonyl functionality.[26)] The UV spectrum of **1** exhibited absorption maxima at 226, 282, and 329 nm, indicating the presence of aromatic conjugating system(s).[26)] The ^{1}H- and ^{13}C-NMR spectra of **1** were similar to those of the known compound, poncirin,[18,19)] except for the sugar signals. The ^{1}H- and ^{13}C-NMR signals at δ_H 5.38 (1H, dd, J=12.8, 2.8 Hz)/δ_C 79.6 (C-2) and 3.23 (1H, dd, J=17.2, 12.8 Hz) and 2.88 (1H, dd, J=17.2, 2.8 Hz)/43.5 (C-3) were characteristics indicating a flavanone skeleton. Two aromatic ring systems of a flavanone skeleton were found in the ^{1}H- and ^{13}C-NMR spectra of **1**. The B ring signals of a flavanone which had a 1,4-disubstituted aromatic ring system, appeared at δ_H 7.51 (2H, d, J=8.8 Hz)/δ_C 128.8 (C-2′, C-6′) and 7.03 (2H, d, J=8.8 Hz)/114.9 (C-3′, C-5′). The aromatic signals at δ_H 6.77 (1H, d, J=2.4 Hz)/δ_C 98.4 (C-8) and 6.67 (1H, d, J=2.4 Hz)/97.1 (C-6) showed the presence of the A ring aromatic system of the flavanone in **1**. The HMBC correlations (Fig. 1) of H-2/C-1′, C-2′, H-3/C-1′, C=O, H-2′(6′)/C-3′, C-5′, C-4′, H-3′(5′)/C-1′, C-4′, H-6/C-7, C-8, and H-8/C-4a, C-7, C-8a demonstrated that compound **1** had a skeleton identical to that of poncirin. The ^{1}H-NMR signal at δ_H 3.71 (3H, s) showed three-bond connectivity with C-4′ in the HMBC experiment, therefore, the methoxy signal was assigned at C-4′. A hydrogen-bonded hydroxyl group appeared at δ_H 12.51 (1H, s) and showed two- and three-bond connec-



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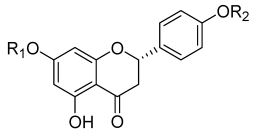

2 R_1 = β-Glu-(1→2)-α-Rha R_2 = CH_3
3 R_1 = β-Glu-(1→2)-α-Rha R_2 = H
4 R_1 = β-Glu R_2 = CH_3

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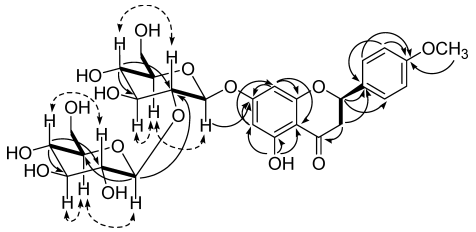

Fig. 1. Important COSY (———), NOESY (- - - - -), and HMBC (———) Correlations of **1**

tivities with C-4a, C-5, and C-6, indicating its position was at C-5. The presence of two sets of β-glucopyranosides was identified by the ^{1}H- and ^{13}C-NMR data of **1**. Its stereochemistry was confirmed by the NOESY spectrum as shown in Fig. 1. Two anomeric proton signals at δ_H 5.73 and 5.36 had large coupling constants, J=7.6 and 8.0 Hz, respectively, indicating β configuration. In the HMBC spectrum of **1**, two anomeric proton signals at δ_H 5.73 and 5.36 exhibited three-bond connectivities with the ^{13}C-NMR signals at δ_C 166.8 (C-7) and 84.6 (C-2″), respectively. Thus, the structure of **1** was elucidated as the new compound, 5-hydroxy-4′-methoxyflavanone-7-*O*-{β-glucopyranosyl-(1→2)-β-glucopyranoside}. The relative stereochemistry of **1** was proposed using a computer-aided, energy-minimized molecular modeling program (CAChe 5.0^{TM}). The energy-minimized stereostructure of **1** showed a dihedral angle of 162.8° between H-2α and H-3β which corresponded to the large coupling constant ($^{3}J_{HH}$=9—13 Hz) from the Karplus correlation graph.[27] A calculated dihedral angle between H-2α and H-3α was 72.5°, which was correlated with relatively small coupling constant ($^{3}J_{HH}$=2—3 Hz). These computational calculation were compared to the actual coupling constants observed in the ^{1}H-NMR spectrum for H-2α (δ_H 5.38, dd, J=12.8, 2.8 Hz), H-3β (δ_H 3.23, dd, J=17.2, 12.8 Hz), and H-3α (δ_H 2.88, dd, J=17.2, 2.8 Hz). The configuration at C-2 in the molecule of **1** was determined as *R* by a circular dichroism (CD) experiment. CD is a powerful tool for determining the absolute configuration of flavonoids. In the CD spectra of flavanones, a positive cotton effect ($n\rightarrow\pi^*$ transition) at 300—350 nm and a negative cotton effect ($\pi\rightarrow\pi^*$ transition) at 280—290 nm indicated a 2*S* configuration. A negative cotton effect at 300—350 nm and a positive cotton effect at 280—290 nm indicated a 2*R* configuration.[20] In the reference for CD curves of mixtures with varying amounts of 2*S* and 2*R*-narigin,[21] it was reported that lessening of the 2*S* characteristics resulted in the negative CD band near 330—350 nm. Specifically, the 60% 2*S* and 40% *2R* mixture of naringin displayed slightly positive cotton effects at both near 350 and 280 nm in the reference.[20] In relation to this phenomenon, compound **1** can be considered as a mixture of about 60% 2*R* and 40% 2*S* due to the slightly positive cotton effects both at 353 and 282 nm (Fig. 2) in the CD spectrum of **1**, which were in the opposite direction with the reference.

Three known compounds were identified as (2*S*)-poncirin (**2**),[18—21] (2*S*)-naringin (**3**),[20—23] and (2*S*)-poncirenin (**4**)[20,21,24,25] by physical and spectroscopic methods as well as by comparison of their data with those of published values. The configurations at C-2 in poncirin (**2**) and poncirenin (**4**) were determined as 2*S* by CD experiments (Fig. 2) and comparison of their data with those of published values.[20,21] The

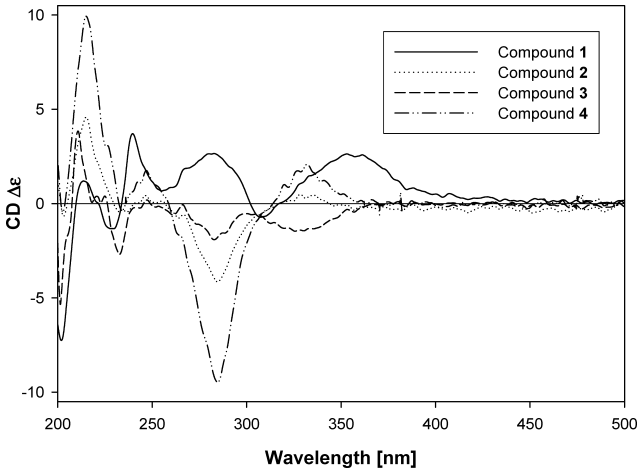

Fig. 2. Circular Dichroism Spectrum of Compounds Isolated from *P. trifoliata*

CD spectrum of naringin (**3**) showed the negative CD bands both at 284 and 332 nm due to lessening of 2*S* character. Therefore, naringin (**3**) isolated from *P. trifoliata* in the present study, was determined as the isomer mixtures of about 60% 2*S* and 40% 2*R* by comparison with the reference.[21]

All compounds were evaluated for their inhibitory activities on lipopolysaccharide (LPS)-induced prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6) production in RAW 264.7 macrophages. Cyclooxygenases (COX) produce various types of prostaglandins (PGs), which have been implicated in various physiological events including progression of inflammation, immunomodulation, and transmission of pain.[28] Two COX isoenzymes were identified: COX-1, the constitutive enzyme makes PGs that protect the stomach and kidney from damage and COX-2, the inducible enzyme induced by inflammatory stimuli such as cytokines produces PGs that contribute to the pain and swelling of inflammation. IL-6 is one of the most important inflammatory cytokines and its production is induced by several factors, TNF-α, IL-1β as well as the bacterial endotoxin, LPS. IL-6 acts as an endogenous pyrogen in addition to its multiple effects on the immune system and hematopoiesis.[29] Thus, compounds that inhibit LPS-induced COX-2 and IL-6 expressions might be an important target for anti-inflammation.

In the present study, compounds **1**—**4** dose-dependently inhibited the production of PGE_2 with IC_{50} values of 41.0, 21.0, 83.6, and 162.6 μM (positive control: NS398, IC_{50} 10±2 μM), respectively, and reduced the production of IL-6 with IC_{50} values of 112.1, 49.9, 105.7, and 157.0 μM, respectively. These compounds did not affect the cell viability of RAW 264.7 cells in either the presence or absence of LPS at 200 μM. Compounds **1** and **2** have very similar structures, which are 5-hydroxy-4′-methoxyflavanone with a 7-*O*-glycoside. However, **2** possessing glucopyranosyl-(1→2)-rhamnopyranoside showed more potent inhibitory activity on PGE_2 production than **1** with glucopyranosyl-(1→2)-glucopyranoside. Although **2** and **3** have the same sugar pattern, **2** having a 4′-methoxy group instead of a hydroxy group in **3** was more potent. On the evaluation of inhibitory activity on IL-6 production, **2** also showed more potent inhibition than **1** and **3**, which exhibited activity that was similar each other. Differing from the sugar patterns of **1**—**3**, **4** which has one

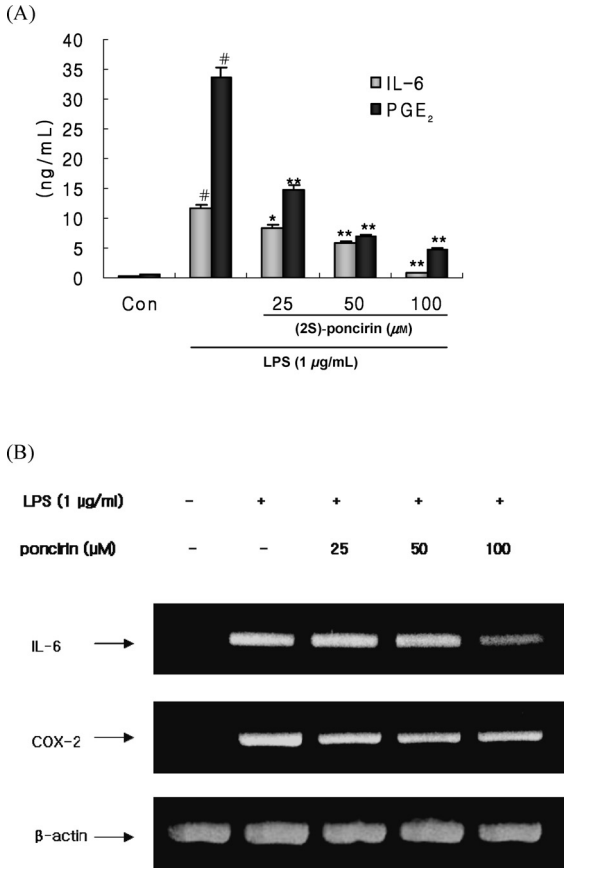

Fig. 3. The Effects of (2*S*)-Poncirin (**2**) on LPS-Induced PGE_2 and IL-6 in RAW 264.7 Macrophage Cells

(A) Cells were pretreated with different concentrations (25, 50, 100 μM) of **2** for 1 h and then LPS 1 μg/ml was added and the cells were incubated for 24 h. Control (Con) values were obtained in the absence of LPS or **2**. The values shown represent the means±S.D. of three independent experiments. $^{\#}p<0.05$ *vs.* control group, $^{*}p<0.05$ *vs.* 1 μg/ml LPS only treated-group. (B) Total RNA was prepared for the RT-PCR analysis of COX-2 and IL-6 gene expression from RAW 264.7 macrophages pretreated with different concentrations (25, 50, 100 μM) of **2** for 1 h and then with LPS 1 μg/ml for 4 h. COX-2 specific sequences (721 bp) and IL-6-specific sequences (142 bp) were detected by agarose gel electrophoresis. PCR of β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiment was repeated three times and similar results were obtained.

glucopyranoside showed the weakest activity in the present assay systems. Therefore, we assume that the 4′-methoxy group and 7-*O*-sugar pattern played important roles in the inhibitory activity on PGE2 and IL-6 production. (2*S*)-Poncirin (**2**) which was the most active among the tested compounds, showed dose-dependent activity as shown in Fig. 3A.

To determine if the observed inhibitory effect of **2** on these inflammatory mediators (PGE_2 and IL-6) was related to the modulation of COX-2 and IL-6 induction, we examined their mRNA expression levels by RT-PCR. In response to LPS, COX-2 and IL-6 were markedly upregulated, and compound **2** significantly inhibited these COX-2 and IL-6 mRNA inductions (Fig. 3B). In unstimulated RAW 264.7 cells, COX-2 and IL-6 mRNA were undetectable. In general, these results are consistent with the profile of the inhibitory effect of compound **2** on PGE_2 and IL-6 release (Fig. 3A).

(2*S*)-Poncirin (**2**) showed considerable activity in this assay system, and thus further study of its mechanism of action is encouraged for the discovery of an anti-inflammatory agent. This is the first report on the evaluation of inhibitory activity on LPS-induced PGE_2 and IL-6 production of compounds **1**, **2**, and **4**, although the general anti-inflammatory activities of the two latter compounds have been already reported.[24,30]

Experimental

General Optical rotations were measured with a JASCO P-1010 polarimeter at 25 °C. Circular dichroism measurements were performed using a JASCO J-715 CD/ORD spectropolarimeter. UV and IR spectra were recorded on a Hitachi U-3000 spectrophotometer and a Bio-Rad FTS 135 FT-IR spectrometer, respectively. 1D and 2D NMR experiments were performed on a Varian Unity INOVA 400 MHz FT-NMR instrument with tetramethylsilane (TMS) as internal standard. FAB-MS was obtained on a JEOL JMS 700 Mstation HR-MS spectrometer operating at 70 eV. Silica gel (230—400 mesh, Merck, Germany) and RP-18 (ODS-A, 12 nm, S-150 μm, YMC, Japan) were used for column chromatography. Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F_{254} (silica gel, 0.25 mm layer thickness, Merck, Germany) and RP-18 F_{254s} (Merck, Germany) plates, with visualization under UV light (254, 365 nm) and 10% (v/v) sulfuric acid spray followed by heating (120 °C, 5 min).

Plant Material Dried immature fruit of *Poncirus trifoliata* (L.) Raf. (Rutaceae) purchased from Kyungdong Oriental Herbal market, Seoul, Korea, in December 2004 was identified by Dr. Chang-Soo Yook, Emeritus Professor, College of Pharmacy, Kyunghee University, Korea. A voucher specimen (EA239) was deposited at the Natural Product Chemistry Laboratory, College of Pharmacy, Ewha Womans University, Korea.

Extraction and Isolation Dried immature fruit of *P. trifoliata* (3 kg) was boiled in distilled water (3×15 l) at 80 °C for 3 h, and the aqueous extract was filtered, concentrated *in vacuo*, and freeze-dried to give a powder. This aqueous extract powder was extracted with EtOH (7×3 l) overnight at room temperature. The extracts were concentrated *in vacuo* at 40 °C to afford an EtOH-soluble residue (500 g), which was then suspended in water (3 l) and separated with *n*-hexane (3×3 l), EtOAc (3×3 l), and *n*-BuOH (2×3 l), sequentially. The EtOAc extract (130 g) was separated by vacuum liquid column chromatography (ϕ 10 cm; silica gel 230—400 mesh, 550 g) using gradient mixtures of MeOH in CH_2Cl_2 (0→100%) as mobile phases, affording 7 fractions (FI—FVII). Fraction V (7 g), eluted with 3—7% MeOH in CH_2Cl_2 from the first separation, was subjected to reversed-phase column chromatography (ϕ 3 cm; ODS-A, 100 g) using a gradient solvent system of MeOH–water (5 : 5→10 : 0), providing **4** (170 mg, 0.0057%). Fraction VII (60 g), eluted with 20—100% MeOH in CH_2Cl_2 from the first separation, was subjected to reversed-phase column chromatography (ϕ 7 cm; ODS-A, 500 g) using a gradient solvent system of MeOH–water (3 : 7→10 : 0), and **2** (23 g, 0.77%) and **3** (6 g, 0.2%) were isolated from the second and sixth fractions, respectively. The fourth fraction (940 mg) from this column was subjected again to reversed-phase column chromatography (ϕ 2 cm; ODS-A, 15 g), with MeOH–water (4 : 6→5 : 5) as the solvent system, providing **1** (11 mg, 0.00038%).

(2*R*)-5-Hydroxy-4′-methoxyflavanone-7-*O*-{β-glucopyranosyl-(1→2)-β-glucopyra-noside} (**1**): White amorphous powder. $[\alpha]_D^{23}$ +166.9° (*c*=0.13, MeOH). UV λ_{max} (MeOH) nm (log ε): 226 (4.56), 282 (4.35), 329 (3.68). IR (KBr) cm^{-1}: 3434, 2917, 1700, 1612, 1520, 1253, 1082. CD (*c*=0.2 mM, MeOH) $\Delta\varepsilon_{226}$ −1.5, $\Delta\varepsilon_{282}$ +2.6, $\Delta\varepsilon_{307}$ −0.9, $\Delta\varepsilon_{353}$ +2.6. ^{1}H-NMR (400 MHz, pyridine-d_5) δ: 12.51 (1H, s, 5-OH), 7.51 (2H, d, *J*=8.8 Hz, H-2′, H-6′), 7.03 (2H, d, *J*=8.8 Hz, H-3′, H-5′), 6.77 (1H, d, *J*=2.4 Hz, H-8), 6.67 (1H, d, *J*=2.4 Hz, H-6), 5.73 (1H, d, *J*=7.6 Hz, H-1″), 5.39 (1H, d, *J*=8.0 Hz, H-1‴), 5.38 (1H, dd, *J*=12.8, 2.8 Hz, H-2α), 4.55 (1H, m, H-6‴), 4.45 (3H, m, H-6‴, H-4″, H-6″), 4.37 (1H, t, *J*=7.6 Hz, H-2″), 4.35 (3H, m, H-6″, H-3″, H-3‴), 4.29 (1H, t, *J*=9.0 Hz, H-4‴), 4.14 (1H, *J*=8.0 Hz, H-2‴), 4.07 (1H, m, H-5″), 4.02 (1H, m, H-5‴), 3.71 (3H, s, 4′-OCH_3), 3.23 (1H, dd, *J*=17.2, 12.8 Hz, H-3β), 2.88 (1H, dd, *J*=17.2, 2.8 Hz, H-3α). ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 197.3 (C-4), 166.8 (C-7), 164.8 (C-8a), 163.7 (C-5), 160.8 (C-4′), 131.5 (C-1′), 128.8 (C-2′, C-6′), 114.9 (C-3′, C-5′), 107.4 (1‴), 104.6 (C-4a), 100.2 (C-1″), 98.4 (C-8), 97.1 (C-6), 84.6 (C-2″), 79.6 (C-2), 79.3 (C-5″), 79.2 (C-5‴), 78.7 (C-4‴), 78.2 (C-4″), 77.0 (C-2‴), 71.7 (C-3‴), 70.9 (C-3″), 62.8 (C-6‴), 62.3 (C-6″), 55.6 (4′-OCH_3), 43.5 (C-3). LR-FAB-MS *m/z* (rel. int.): 633 ([M+Na]$^+$, 5), 609 (5), 531 (5), 413 (15), 381 (50), 254 (35), 176 (100). HR-FAB-MS *m/z*: 633.1790 [M+Na]$^+$ (Calcd for $C_{28}H_{34}O_{15}Na$ 633.1795).

(2*S*)-Poncirin (**2**): White powder. $[\alpha]_D^{23}$ −81.6° (*c*=0.18, MeOH). CD (*c* 0.2 mM, MeOH) $\Delta\varepsilon_{246}$ +0.4, $\Delta\varepsilon_{284}$ −4.2, $\Delta\varepsilon_{335}$ +0.5.

(2*S*)-Naringin (**3**): Pale yellow powder. $[\alpha]_D^{23}$ −74.5° (*c*=0.22, MeOH). CD (*c* 0.5 mM, MeOH) $\Delta\varepsilon_{247}$ +0.2, $\Delta\varepsilon_{284}$ −1.8, $\Delta\varepsilon_{332}$ −0.1.

(2*S*)-Poncirenin (**4**): White powder. $[\alpha]_D^{23}$ −159.5° (*c*=0.11, MeOH). CD

(c 0.2 mM, MeOH) $\Delta\varepsilon_{247}$ +1.8, $\Delta\varepsilon_{287}$ −9.2, $\Delta\varepsilon_{332}$ +2.1.

PGE_2 and IL-6 Assay PGE_2 and IL-6 levels in macrophage culture media were quantified using enzyme immunoassay (EIA) kits according to the protocols provided by R&D Systems (Minneapolis, MN, U.S.A.).

MTT Assay for Cell Viability Cytotoxicity after 24 h of continuous exposure to the various concentrations of compounds was measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT).

RNA Preparation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology) according to the manufacturer's instructions. From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM dNTP, and oligo (dT_{12-18}) 0.5 μg/μl. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect COX-2, IL-6 and β-actin (as an internal standard) gene expression using a thermal cycler (Perkin-Elmer Cetus, Foster City, CA, U.S.A.). The reactions were carried out in a volume of 25 μl containing (final concentration) 1 unit of *Taq* DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95 °C, thirty amplification cycles were performed for COX-2 (1 min of 94 °C denaturation, 1 min of 60 °C annealing, and 1 min 72 °C extension) and IL-6 (1 min of 94 °C denaturation, 1 min of 57 °C annealing, and 1 min 72 °C extension). PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea): sense strand COX-2, 5′-GGAGAGACTATCAAGATAGT-3′ anti-sense strand COX-2, 5′-ATGGTCAGTAGACTTTTACA-3′; sense strand IL-6, 5′-GAGGATACCACTCCCAACAGACC-3′; anti-sense strand IL-6, 5′-AAGTGCATCATCGTTGTTCATACA-3′; sense strand β-actin, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′, anti-sense strand β-actin, 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Acknowledgments This work was supported by a grant from the Brain Korea 21 Project and grant No. R15-2006-020-00000-0 from the National Core Research Center (NCRC) program of the Ministry of Science & Technology (MOST) and the Korea Science and Engineering Foundation (KOSEF) through the Center for Cell Signaling & Drug Discovery Research at Ewha Womans University.

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