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Effect of Phytocompounds from the Heartwood of *Acacia confusa* on Inflammatory Mediator Production

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Acacia confusa Merr. (Leguminosae) is traditionally used as a medicinal plant in Taiwan. In the present study, anti-inflammatory activity of extracts from the heartwood of *A. confusa* were investigated for the first time. Results demonstrated that ethanolic extracts of *A. confusa* heartwood strongly suppressed NO production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. Among all fractions derived from ethanolic extracts, the EtOAc fraction exhibited the best inhibitory activity. Following column chromatography and reverse-phase high-performance liquid chromatography, 13 specific phytocompounds including 5 new flavonoids (i.e., 7,8,3′,4′-tetrahydroxy-4-methoxyflavan-3-ol, 7,8,3′,4′-tetrahydroxyflavone, 7,8,3′-trihydroxy-3,4′-dimethoxyflavone, 7,3′,4′-trihydroxyflavone, and 7,3′,4′-trihydroxy-3-methoxyflavone) were isolated and identified from the EtOAc fraction. In addition, melanoxetin (3,7,8,3′,4′-pentahydroxyflavone), a major compound in the EtOAc fraction, markedly suppressed LPS-induced NO and prostaglandin E_2 (PGE_2) production. Moreover, melanoxetin completely suppressed gene expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) at 50 and 100 μM, respectively. This is the first report to identify the inhibitory bioactivities of melanoxetin on iNOS and COX-2.

KEYWORDS: ***Acacia confusa*; melanoxetin; nitric oxide; prostaglandin E_2; inducible nitric oxide synthase; cyclooxygenase-2**

INTRODUCTION

Many plant infusions frequently used in domestic medicine have anti-inflammatory and pharmacological properties ascribed to the presence of phenolic compounds, especially flavonoids. Flavonoids are polyphenolics occurring in a wide range of plants, with over 8000 chemically distinct compounds identified (*1*), and are plentiful in the heartwood of several *Acacia* species (*2, 3*). Secondary metabolites of the genus *Acacia* have been characterized, and this has been thoroughly reviewed by Seigler (*4*). However, little information is available concerning the chemical compositions and biological activities of *Acacia confusa*.

A. confusa, a species indigenous to Taiwan, is widely distributed over the hills and lowlands of Taiwan and is traditionally used as a medicinal plant (*5*). In a previous study, we found that the ethanolic extracts of the heartwood not only contain a large amount of polyphenols but also show an excellent antioxidant activity (*6*). However, the application of this plant to inflammatory disease has not been supported by pharmacological investigations to date. Numerous studies have revealed that improper up-regulation of inducible nitric oxide synthase (iNOS) and/or cyclooxygenase-2 (COX-2) was associated with the pathophysiologic features of certain types of human cancers as well as inflammatory disorders (*7–9*). Therefore, the identification of phytochemicals that can suppress or down-regulate the functions of iNOS and/or COX-2 may lead to the discovery of important anti-inflammatory therapeutics.

The aim of this study was to investigate the inhibitory effects of natural phytochemicals, particularly melanoxetin, from *A. confusa* heartwood on NO and prostaglandin E_2 (PGE_2) production in RAW 264.7 macrophages. In addition, we sought underlying mechanisms leading to the suppression of NO and PGE_2 generation after melanoxetin treatment.

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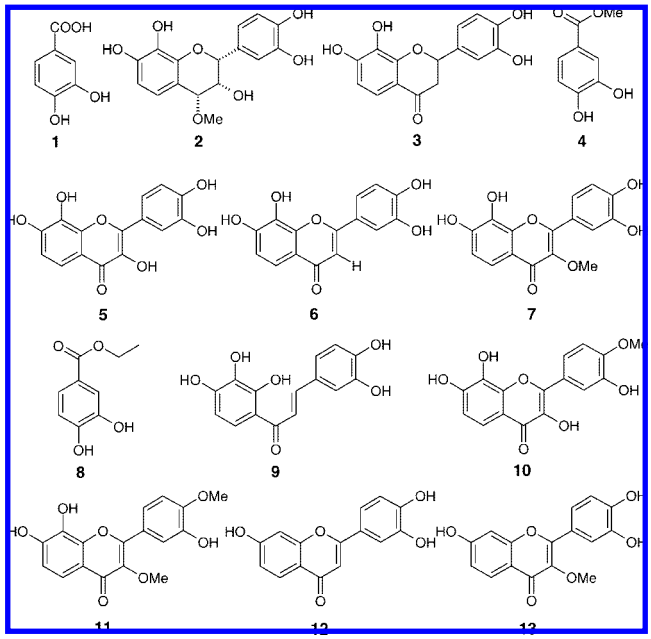

Figure 1. Chemical structures of phytocompounds isolated from the EtOAc soluble fraction of *A. confusa* heartwood: **1**, 3,4-dihydroxybenzoic acid; **2**, 7,8,3′,4′-tetrahydroxy-4-methoxyflavan-3-ol; **3**, 7,8,3′,4′-tetrahydroxyflavanone; **4**, 3,4-dihydroxybenzoic acid methyl ester; **5**, melanoxetin; **6**, 7,8,3′,4′-tetrahydroxyflavone; **7**, transilitin; **8**, 3,4-dihydroxybenzoic acid ethyl ester; **9**, 3,4,2′,3′,4′-pentahydroxy-*trans*-chalcone; **10**, 3,7,8,3′-tetrahydroxy-4′-methoxyflavone; **11**, 7,8,3′-trihydroxy-3,4′-dimethoxyflavone; **12**, 7,3′,4′-trihydroxyflavone; **13**, 7,3′,4′-trihydroxy-3-methoxyflavone.

MATERIALS AND METHODS

Reagents. Lipopolysaccharide (LPS), aspirin, sodium pyruvate, streptomycin, *N*-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, dimethyl sulfoxide (DMSO), and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals and solvents were of the highest quality available.

Extraction and Purification of Phenolic Compounds from the Heartwood of *A. confusa*. The heartwood of *A. confusa* Merr. (40-year-old) was sampled from the experimental forest of National Taiwan University in Nan-Tou County, Taiwan. The species was identified by Sheng-You Lu of the Taiwan Forestry Research Institute, and a voucher specimen (AC001) was deposited at the School of Forestry and Resource Conservation, National Taiwan University. The dried samples (1.65 kg) were cut into small pieces and soaked in 70% ethanol at ambient temperature for 7 days. The extract was decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilized. The resulting crude extract (135 g) was fractionated successively with *n*-hexane, ethyl acetate (EtOAc), butanol (BuOH), and water to yield soluble fractions of *n*-hexane (1 g), EtOAc (84 g), BuOH (20 g), and H_2O (13 g). Thirteen phytocompounds, as shown in **Figure 1**, were isolated and purified from the EtOAc fraction by semipreparative HPLC on a model PU-980 instrument (Jasco) with a 250 × 10.0 mm inside diameter, 5 μm Luna RP-18 column (Phenomenex, Torrance, CA). The mobile phase was solvent A (100% MeOH) and solvent B (ultrapure water). Elution conditions were 0–20 min of 40–60% A to B (linear gradient) and 20–28 min of 60–100% A to B (linear gradient) at a flow rate of 4 mL/min using a Jasco MD-910 photo diode array detector at 280 or 370 nm wavelength. Electron-impact mass spectrometry (EIMS) and high-resolution electron-impact mass spectrometry (HREIMS) data were collected using Finnigan MAT-95S mass spectrometry, and NMR spectra were recorded by a Bruker Avance 500 MHz FT-NMR spectrometer.

7,8,3′,4′-Tetrahydroxy-4-methoxyflavan-3-ol (2): light yellow amorphous solid; mp 106–108 °C; UV (MeOH), λ_{max}, nm (log ϵ), 283.5 (3.82); IR (KBr), ν_{max}, cm^{-1}, 3338, 1613, 1517; HREIMS, *m/z* 320.0898, $[M]^+$ calcd 320.0896 for $C_{16}H_{16}O_7$; ^{1}H NMR (500 MHz, CD_3OD), δ 7.06 (d, 1, J = 1.7 Hz, H-2′), 6.89 (dd, 1, J = 8.1, 1.7 Hz, H-6′), 6.79 (d, 1, J = 8.1 Hz, H-5′), 6.69 (d, 1, J = 8.3 Hz, H-5), 6.46 (d, 1, J = 8.3 Hz, H-6), 4.99 (br s, 1, H-2), 4.09 (d, 1, J = 2.8 Hz, H-4), 3.96 (d, 1, J = 2.8 Hz, H-3), 3.47 (s, 3, 4-OC$\underline{H}_3$); ^{13}C NMR (125 MHz, CD_3OD), δ 147.3 (C-7), 146.1 (C-3′, C-4′), 145.4 (C-9), 133.9 (C-8), 131.4 (C-1′), 123.3 (C-5), 119.8 (C-6′), 116.0 (C-5′), 115.8 (C-2′), 112.6 (C-10), 109.2 (C-6), 78.7 (C-4), 76.8 (C-2), 69.5 (C-3), 56.5 (4-O$\underline{C}H_3$).

7,8,3′,4′-Tetrahydroxyflavone (6): light yellow amorphous solid; mp 226–228 °C; UV (MeOH), λ_{max}, nm (log ϵ), 265 (4.16), 345 (4.13); IR (KBr), ν_{max}, cm^{-1}, 3226, 1640, 1613, 1574, 1517; HREIMS, *m/z* 286.0473, $[M]^+$ calcd 286.0477 for $C_{15}H_{10}O_6$; ^{1}H NMR (500 MHz, CD_3OD), δ 7.51 (d, 1, J = 8.7 Hz, H-5), 7.51 (d, 1, J = 8.9 Hz, H-6′), 7.51 (s, 1, H-2′), 6.94 (d, 1, J = 8.7 Hz, H-6), 6.91 (d, 1, J = 8.9 Hz, H-5′), 6.62 (s, 1, H-3); ^{13}C NMR (125 MHz, CD_3OD), δ 180.8 (C-4), 166.0 (C-2), 152.3 (C-7), 150.8 (C-4′), 148.5 (C-9), 147.0 (C-3′), 134.6 (C-8), 124.3 (C-1′), 118.0 (C-10), 116.8 (C-5), 116.7 (C-5′), 115.0 (C-6, C-6′), 114.4 (C-2′), 104.7 (C-3).

7,8,3′-Trihydroxy-3,4′-dimethoxyflavone (11): light yellow amorphous solid; mp 211–213 °C; UV (MeOH), λ_{max}, nm (log ϵ), 259.5 (4.33), 346.5 (4.25); IR (KBr), ν_{max}, cm^{-1}, 3184, 1660, 1602, 1567, 1513; HREIMS, *m/z* 310.0734, $[M]^+$ calcd 310.0739 for $C_{17}H_{14}O_7$; ^{1}H NMR (500 MHz, CD_3OD), δ 7.77 (dd, 1, J = 8.6, 2.1 Hz, H-6′), 7.74 (d, 1, J = 2.1 Hz, H-2′), 7.54 (d, 1, J = 8.8 Hz, H-5), 7.08 (d, 1, J = 8.6 Hz, H-5′), 6.94 (d, 1, J = 8.8 Hz, H-6), 3.94 (s, 3, 4′-OC$\underline{H}_3$), 3.77 (s, 3, 3-OC$\underline{H}_3$); ^{13}C NMR (125 MHz, CD_3OD): δ 177.0 (C-4), 157.5 (C-2), 152.0 (C-7), 151.6 (C-4′), 147.7 (C-9), 147.6 (C-3′), 141.2 (C-3), 134.4 (C-8), 124.8 (C-1′), 122.4 (C-6′), 118.6 (C-10), 116.8 (C-5), 116.5 (C-2′), 115.2 (C-6), 112.3 (C-5′), 60.4 (3-O$\underline{C}H_3$), 56.4 (4′-O$\underline{C}H_3$).

7,3′,4′-Trihydroxyflavone (12): light yellow amorphous solid; mp 294–296 °C; UV (MeOH), λ_{max}, nm (log ϵ), 235.5 sh (4.27), 316 sh (4.13), 344.5 (4.25); IR (KBr), ν_{max}, cm^{-1}, 3207, 1660, 1621, 1567, 1513; HREIMS, *m/z*, 270.0498, $[M]^+$ calcd 270.0528 for $C_{15}H_{10}O_5$; ^{1}H NMR (500 MHz, CD_3OD), δ 7.96 (d, 1, J = 8.7 Hz, H-5), 7.40 (d, 1, J = 8.9 Hz, H-6′), 7.39 (s, 1, H-2′), 6.95 (s, 1, H-8), 6.92 (d, 1, J = 8.7 Hz, H-6), 6.90 (d, 1, J = 8.9 Hz, H-5′), 6.62 (s, 1, H-3); ^{13}C NMR (125 MHz, CD_3OD), δ 180.3 (C-4), 166.1 (C-2), 165.0 (C-7), 159.7 (C-9), 150.8 (C-4′), 147.1 (C-3′), 127.7 (C-5), 124.0 (C-1′), 120.2 (C-6′), 117.2 (C-10), 116.8 (C-5′), 116.3 (C-6), 114.1 (C-2′), 105.2 (C-3), 103.5 (C-8).

7,3′,4′-Trihydroxy-3-methoxyflavone (13): light yellow amorphous solid; mp 225–227 °C; UV (MeOH), λ_{max}, nm (log ϵ), 242 sh (4.21), 321 sh (4.07), 349.5 (4.17); IR (KBr), ν_{max}, cm^{-1}, 3192, 1660, 1605, 1563, 1505; HREIMS, *m/z* 300.0625, $[M]^+$ calcd 300.0634 for $C_{16}H_{12}O_6$; ^{1}H NMR (500 MHz, CD_3OD), δ 7.99 (d, 1, J = 8.7 Hz, H-5), 7.64 (d, 1, J = 2.2 Hz, H-2′), 7.55 (dd, 1, J = 8.4, 2.2 Hz, H-6′), 6.92 (d, 1, J = 8.7 Hz, H-6), 6.91 (d, 1, J = 8.4 Hz, H-5′), 6.91 (s, 1, H-8), 3.76 (s, 3, 3-OC$\underline{H}_3$); ^{13}C NMR (125 MHz, CD_3OD), δ 176.5 (C-4), 164.8 (C-7), 158.7 (C-9), 157.9 (C-2), 149.8 (C-4′), 146.5 (C-3′), 141.2 (C-3), 127.8 (C-5), 123.3 (C-1′), 122.3 (C-6′), 117.7 (C-10), 116.5 (C-2′), 116.4 (C-6), 116.2 (C-5′), 103.1 (C-8), 60.3 (3-O$\underline{C}H_3$).

Cell Culture. The murine monocyte/macrophage cell line RAW 264.7 was obtained from the Culture Collection and Research Center (CCRC), HsinChu, Taiwan. RAW 264.7 cells were cultured in 75 or 150 cm^2 plastic flasks with Dulbecco's modified Eagle's medium (DMEM, Gibico/BRL, Grand Island, NY) supplemented with 1 mM sodium pyruvate, penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal bovine serum (Gibico/BRL). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Measurement of NO and PGE_2. RAW 264.7 cells were seeded in 96-well plates at 2 × 10^5 cells/well and grown for 2 h for adherence. The adhered cells were incubated for 24 h, with or without 1 μg/mL of LPS, in the absence or presence of the test extracts or compounds (DMSO as a vehicle). The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction (*10*). Briefly, 100 μL of culture supernatant or sodium nitrite standard underwent reaction with an equal volume of Griess reagent [a mixture of 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride in water and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid] in a 96-well plate. After 10 min at ambient temperature, the absorbance

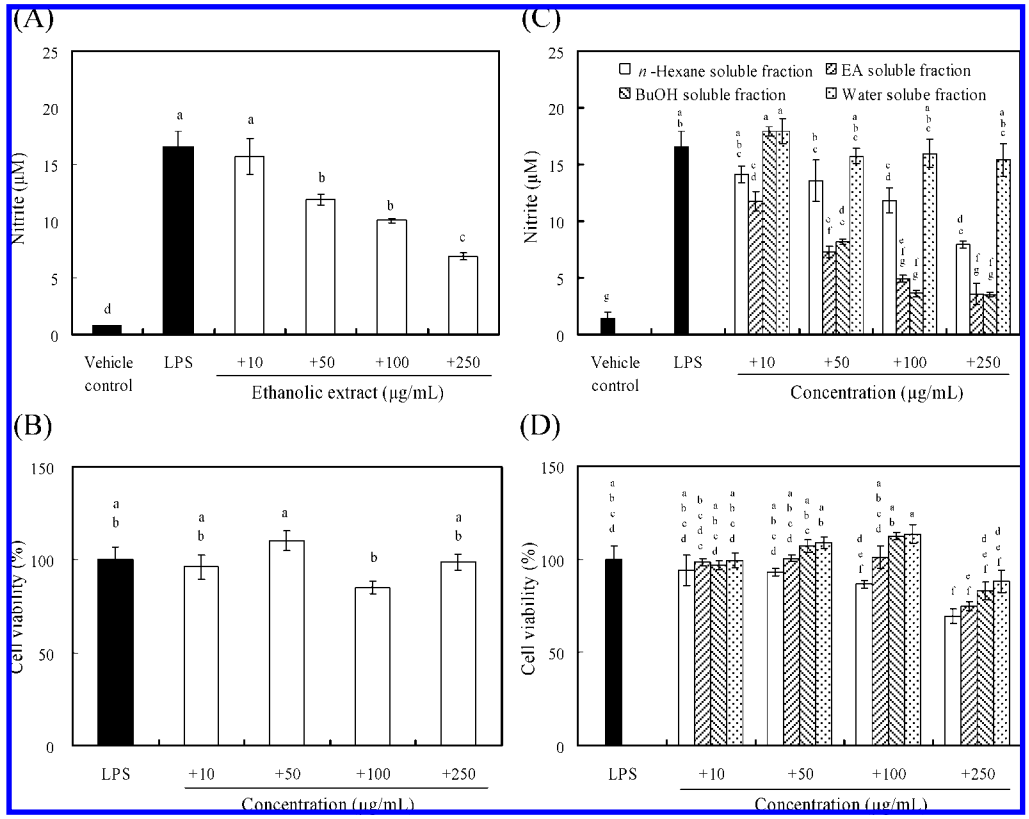

Figure 2. Effect of ethanolic extract and its derived soluble fractions of *A. confusa* on the nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells: (**A**) effect of ethanolic extract on LPS-induced nitric oxide production; (**B**) cytotoxicity of ethanolic extract on RAW 264.7 cells in the presence of LPS, measured by the MTT assay; (**C**) effect of various fractions of ethanolic extract on LPS-induced nitric oxide production; (**D**) cytotoxicity of various fractions of ethanolic extract on cells in the presence of LPS, measured by the MTT assay. Results are mean ± SD ($n = 3$). Different letters indicate significant differences among groups ($p < 0.05$).

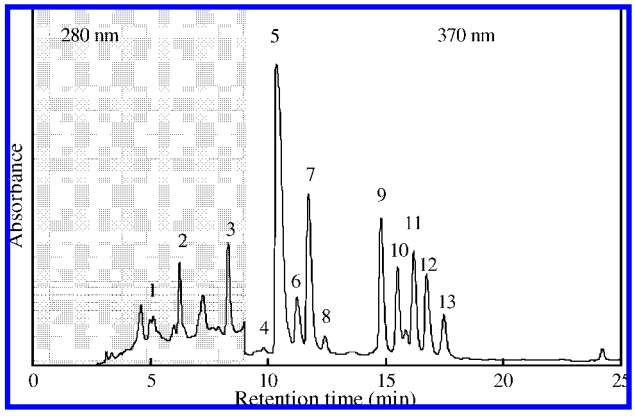

Figure 3. HPLC chromatogram of EtOAc fraction from the heartwood of *A. confusa*.

at 550 nm was measured by use of a microtitration plate reader (Labsystems, Helsinki, Finland).

For PGE_2 determination, RAW 264.7 cells were seeded in 96-well plates at 1×10^4 cells/well and incubated for 18 h. Cells were pretreated with 500 μM of aspirin for 3 h to inactivate endogenous cyclooxygenase-1 (COX-1) based on the method reported by Hwang et al. (*11*). Then, cells were washed twice with phosphate-buffered saline (PBS) and further incubated for 16 h in fresh DMEM with or without 1 μg/mL of LPS in the absence or presence of the test samples. After incubation, supernatants were collected to measure PGE_2 concentration with monoclonal antibody by enzyme-linked immunosorbent assay (ELISA) as specified by the manufacturer (Cayman Chemical, Ann Arbor, MI).

Cell Viability (MTT Assay). Cell respiration as an indicator of cell viability was determined on the basis of mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (*12*). After culture supernatants were collected for NO or PGE_2 measurement, culture wells were washed once with 1 mL of PBS to remove the remaining test samples. Cells were then incubated with 0.5 mg/mL MTT for 4 h at 37 °C in a 5% CO_2 incubator. The medium was aspirated, and the insoluble formazan product was dissolved in DMSO (250 μL) for 2 h in the dark. The extent of MTT reduction was quantified by measuring the absorbance at $\lambda = 570$ nm.

RT-PCR Analysis. RAW 264.7 cells were seeded in six-well plates at 3×10^6 cells/well. The cells were treated with various concentrations of test compounds for 1 h and then incubated for 6 h in fresh DMEM with or without 1 μg/mL of LPS. After three washings with ice-cold PBS, the cells were harvested, and total RNA was extracted with use of TRIzol reagent (Life Technologies, Gaithersburg, MD) as specified by the manufacturer. For each RT-PCR reaction, 2 μg of total RNA was used to synthesize first-strand cDNA with Superscript reverse transcriptase (Life Technologies). Reverse transcriptase-generated cDNA encoding for murine iNOS and COX-2 were amplified by PCR. Oligonucleotide primers for iNOS and COX-2 were 5′-CAGAAGCAGAATGTGACCATC-3′ (sense), 5′-CTTCTGGTCGATGTCATGAGC-3′ (antisense); and 5′-ACTCACTCAGTTTGTTGAGTCATTC-3′ (sense), 5′-TTTGATTAGTACTGTAGGGTTAATG-3′ (antisense), respectively. The cDNA sequence of glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) as an internal control was also amplified using the following primers: 5′-CCATCAATGACCCCTTCATTGACC-3′ (sense) and 5′-GAAGGCCATGCCAGTGAGCTTCC-3′ (antisense). For PCR amplification the following conditions were used: for iNOS and GA3PDH, 94 °C for 2 min for one cycle and then 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min for 30 cycles; for COX-2, 94 °C for 2 min for one cycle and then 94 °C for 15 s, 55 °C for 1 min, and 72 °C for 1 min for 30 cycles. The amplified PCR products were analyzed with 2% agarose gel electrophoresis and visualized with ethidium bromide staining.

Table 1. ^{13}C NMR Spectral Data and HMBC Correlations of Five New Phytocompounds Isolated from the Heartwood of *A. confusa* (CD_3OD)

	2		6		11		12		13	
no.	δ_C	HMBC	δ_C	HMBC	δ_C	HMBC	δ_C	HMBC	δ_C	HMBC
2	76.8	H-4 H-2′ H-6′	166.0	H-3	157.5	H-2′ H-6′	166.1	H-3 H-2′ H-6′	157.9	H-2′ H-6′
3	69.5		104.7		141.2	OCH_3	105.2		141.2	OCH_3
4	78.7	H-5 OCH_3	180.8	H-5	177.0	H-5	180.3	H-3 H-5	176.5	H-5
5	123.3	H-4	116.8		116.8		127.7		127.8	
6	109.2		115.0		115.2		116.3	H-8	116.4	H-8
7	147.3	H-5	152.3	H-5	152.0	H-5 H-6	165.0	H-5 H-8	164.8	H-5 H-8
8	133.9	H-6	134.6	H-6	134.4	H-6	103.5	H-6	103.1	H-6
9	145.4	H-4 H-5	148.5	H-5	147.7	H-5	159.7	H-5 H-8	158.7	H-5 H-8
10	112.6	H-3 H-6	118.0	H-3 H-6	118.6	H-6	117.2	H-3 H-6	117.7	H-6 H-8
1′	131.4	H-2 H-5′	124.3	H-3 H-5′	124.8	H-5′	124.0	H-5′	123.3	H-2′ H-5′
2′	115.8	H-2 H-6′	114.4	H-6′	116.5	H-6′	114.1	H-6′	116.5	H-6′
3′	146.1	H-5′	147.0	H-2′ H-5′	147.6	H-5′	147.1	H-2′ H-5′	146.5	H-2′ H-5′
4′	146.1	H-2′ H-6′	150.8	H-2′ H-6′	151.6	H-2′ H-6′ OCH_3	150.8	H-2′ H-5′ H-6′	149.8	H-2′ H-5′ H-6′
5′	116.0		116.7		112.3		116.8		116.2	
6′	119.8	H-2 H-2′	115.0	H-2′	122.4	H-2′	120.2	H-2′	122.3	H-2′

Statistical Analyses. All results are expressed as mean ± SD ($n = 3$). Statistical analyses were performed using ANOVA with Scheffe's test, and $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Effects of Phytochemicals from *A. confusa* Heartwood on NO Production in RAW 264.7 Cells. NO is a critical signaling molecule produced at inflammatory sites by iNOS, which is often expressed in response to LPS, interferon-γ, and various proinflammatory cytokines (*13*). Several physiological events by which intracellular elevated NO can exert genotoxic effects after reacting with oxygen include formation of carcinogenic *N*-nitroso compounds, DNA strand breakage, and nitrosative deamination of DNA bases (*14, 15*). Thus, maintenance of a tight regulation of NO production is important for human health. In our previous study, the ethanolic extract (*6*) and phenolic compounds (*16*) from the heartwood of *A. confusa* exhibited excellent inhibitory activity against reactive oxygen species (ROS). To determine further the effects of those extracts on NO production, different concentrations of test samples were incubated with LPS-activated RAW 264.7 macrophages. As shown in **Figure 2A**, the nitrite level in cultured supernatant of RAW 264.7 cells was markedly elevated, from 0.8 to 16.6 μM, after 24 h of treatment with LPS. An ethanolic extract of *A. confusa* heartwood significantly inhibited LPS-induced NO production in a dose-dependent manner. Relative to LPS treatment only, 28% of NO production was inhibited in the ethanolic extract-treated cells at the concentration of 50 μg/mL. To evaluate whether the inhibition of NO production was possibly caused by the cytotoxic effect of test extracts, the viability of test cells was determined by the MTT assay. **Figure 2B** shows that the ethanolic extract of heartwood shows no significant cytotoxicity to RAW 264.7 cells at all test concentrations. Thus, the inhibition of NO production in LPS-stimulated RAW 264.7 cells by the ethanolic extract of *A. confusa* heartwood was not due to cytotoxicity.

As for the inhibitory effects of various soluble fractions derived from the ethanolic extract on NO production, data in **Figure 2C** show that except for the water soluble fraction, all of the test samples exhibited significant and differential inhibitory activities against NO generation. The IC_{50} values of *n*-hexane, EtOAc and BuOH soluble fractions were 208, 31, and 42 μg/mL, respectively. Of these, the EtOAc fraction exhibited the strongest activity. The addition of 10, 50, 100, and 250 μg/mL EtOAc extract to LPS-stimulated cells resulted in decreased NO production, by 71, 44, 30, and 22%, respectively, as compared to that of the cells treated with LPS alone. In addition, the MTT assay revealed that concentrations up to 100 μg/mL produced no cytotoxic effects on cells treated with EtOAc extract (**Figure 2D**).

In our previous study, the EtOAc extract showed a stronger antioxidant activity than other fractions (*6*). These results indicate that antioxidant and anti-inflammatory activities of ethanolic extract of *A. confusa* heartwood have been effectively enriched in the EtOAc fraction. Moreover, the EtOAc fraction had 84- and 4.2-fold more mass than the *n*-hexane and BuOH fractions, respectively. Thus, it was further investigated in this study for its phytochemical characteristics. **Figure 3** shows the HPLC chromatogram of the EtOAc fraction, from which 13 phenolic compounds (**1**−**13**) were isolated and identified as 3,4-dihydroxybenzoic acid (**1**), 7,8,3′,4′-tetrahydroxy-4-methoxyflavan-3-ol (**2**), 7,8,3′,4′-tetrahydroxyflavanone (**3**), 3,4-dihydroxybenzoic acid methyl ester (**4**), melanoxetin (**5**), 7,8,3′,4′-tetrahydroxyflavone (**6**), transilitin (**7**), 3,4-dihydroxybenzoic acid ethyl ester (**8**), 3,4,2′,3′,4′-pentahydroxy-*trans*-chalcone (**9**), 3,7,8,3′-tetrahydroxy-4′-methoxyflavone (**10**), 7,8,3′-trihydroxy-3,4′-dimethoxyflavone (**11**), 7,3′,4′-trihydroxyflavone (**12**), and 7,3′,4′-trihydroxy-3-methoxyflavone (**13**), respectively. Among these, the ^1H, ^{13}C NMR, and MS spectral data of compounds **1**, **3**−**5**, and **7**−**10** were in good agreement with the published values (*16–22*). Additionally, five flavonoids, including compounds **2**, **6**, **11**, **12**, and **13**, were for the first time isolated

from nature and reported here as new natural products. All ^{13}C signals and heteronuclear multiple bond correlations (HMBC) of these new phytocompounds are shown in **Table 1**. Furthermore, the relative stereochemistry of compound **2** was assigned on the basis of coupling constants and NOESY data. Particularly important was the NOESY correlation seen from H2 (a broad singlet at δ_H 4.99) to H3 (δ_H 3.96), which placed these two protons in vicinal axial–equatorial orientations. In addition, H2 proton also showed NOESY correlation to H4 (δ_H 4.09), and so H4 could be assigned to an axial orientation. Accordingly, this assigned stereochemistry confirmed compound **2** as (2,3-*cis*-3,4-*cis*)-7,8,3′,4′-tetrahydroxy-4-methoxyflavan-3-ol.

On the other hand, melanoxetin and transilitin were found as two major flavonoids in the EtOAc fraction. The contents of melanoxetin and transilitin were determined as 33.4 ± 1.1 and 24.5 ± 2.0 μg/mg of dry weight of EtOAc extract, respectively. The effect of the two specific flavonoids of *A. confusa* on the inhibition of NO generation in LPS-stimulated RAW 264.7 cells was further investigated. Quercetin, one of flavonoids known for its anti-inflammatory activity (*23, 24*), was used in parallel as a positive control. **Figure 4A** shows that melanoxetin and quercetin strongly inhibited LPS-induced NO production in a concentration-dependent manner, with IC_{50} values of 6.9 and 6.4 μM, respectively. In contrast, transilitin showed poor inhibitory effects (IC_{50} > 100 μM). Except for quercetin, which exhibited a slight cytotoxicity at 100 μM, no effects were observed for melanoxetin and transilitin at the same tested concentrations (**Figure 4B**). This result implies that the 3-methoxyl moiety in the C-ring of transilitin significantly attenuated its inhibitory activity on NO production.

Effect of Melanoxetin on PGE_2 Release from LPS-Activated Macrophages. The activation of macrophages plays a critical role in the inflammatory process by releasing a variety of inflammatory mediators (*11*). Prostaglandins (PGs) are one of the potent modulators in both inflammation and immune responses. High levels of PGE_2 can affect the activities of several cell types, including neurons, glia, and endothelial cells, and can regulate microglia/macrophage and lymphocyte functions during inflammatory and immune processes (*25*). In addition, as compared with the surrounding normal tissues, elevated levels of PGs, especially PGE_2, have also been found in many human cancers (*26*). Thus, phytochemicals that can effectively inhibit the abnormal production of PGE_2 may be potential anti-inflammatory and cancer chemopreventive agents. In the present study, the accumulation of PGE_2 in RAW 264.7 cells was increased from 31.6 to 1383.7 pg/mL by LPS treatment. However, melanoxetin and quercetin inhibited the LPS-induced accumulation of PGE_2 in a dose-dependent manner. **Figure 5** shows that, at a dose of 100 μM, melanoxetin markedly suppressed (~60%) the PGE_2 production in LPS-stimulated RAW 264.7 cells. Quercetin exhibited 98% inhibition of PGE_2 production, but slight cytotoxicity was observed at the same concentration (data not shown). Thus, the hydroxyl moieties in the A-ring of flavonoids play a role and exhibit differential effects on PGE_2 production in macrophages (i.e., the 5,7-dihydroxyl groups in quercetin possess higher activity than the 7,8-dihydroxyl groups in melanoxetin).

Effect of Melanoxetin on iNOS mRNA and COX-2 mRNA Levels in LPS-Activated RAW 264.7 Macrophages. Nitric oxide synthase (NOS) catalyzes NO synthesis in biological systems. Three distinct isoforms of NOS have been isolated, including a neuronal (nNOS), an endothelial (eNOS), and an endotoxin- or cytokine-inducible (iNOS) form (*27*). Both nNOS and eNOS, often grouped together as constitutive NOS (cNOS), are usually constitutively expressed, and their activities are regulated by intracellular calcium concentrations via calmodulin. iNOS is usually not detectable in healthy tissues but is expressed after immunological challenge or injury. Enhanced expression of iNOS and its enzymatic activity have been observed in many human tumor tissues and in inflammatory disorders (*7–9, 13*). In this study, the effect of melanoxetin on iNOS gene expression was also investigated. As shown in **Figure 6A**, unstimulated RAW 264.7 cells (vehicle control) showed barely detectable

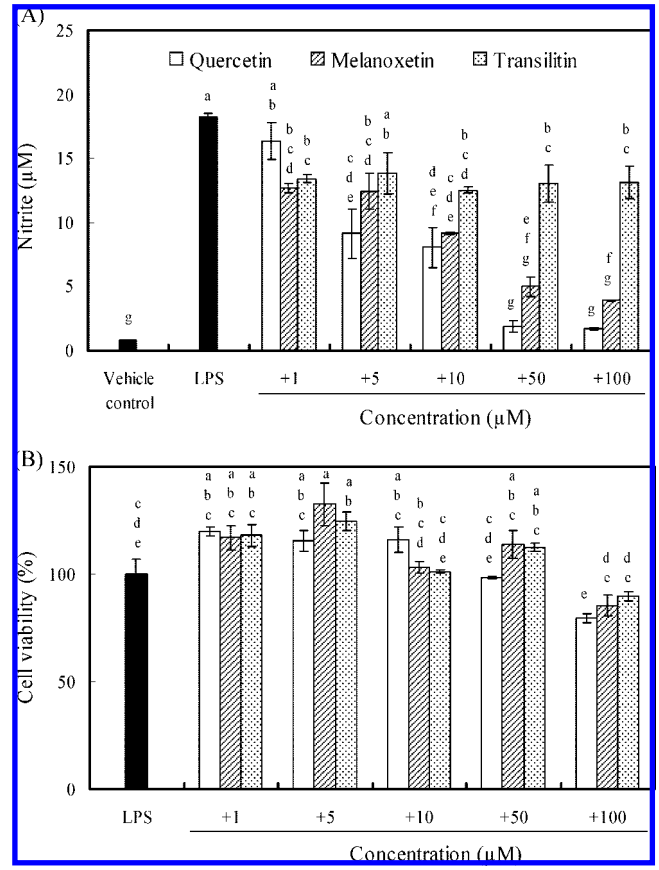

Figure 4. Effect of melanoxetin, transilitin, and quercetin on the nitric oxide production of lipopolysaccharide-stimulated RAW 264.7 macrophage cells: (**A**) concentration-dependent inhibition of nitric oxide production; (**B**) cytotoxicity of phytocompounds on cells in the presence of LPS, measured by the MTT assay. Results are mean ± SD (n = 3). Different letters indicate significant differences among groups (p < 0.05).

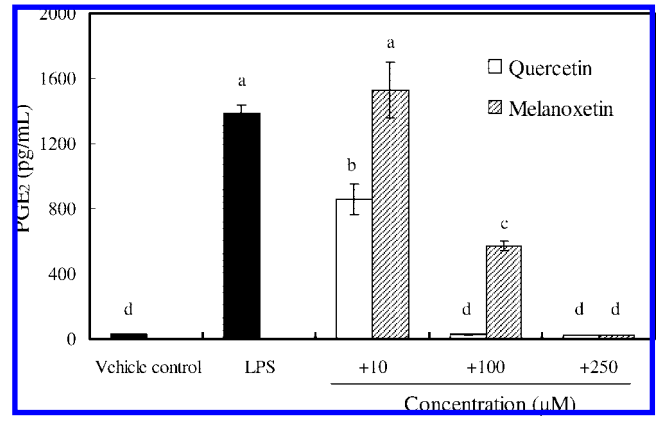

Figure 5. Effect of melanoxetin and quercetin on the prostaglandin E_2 production of lipopolysaccharide-stimulated RAW 264.7 macrophage cells. Prostaglandin E_2 in the culture medium was measured as described under Materials and Methods. Results are mean ± SD (n = 3). Different letters indicate significant differences among groups (p < 0.05).

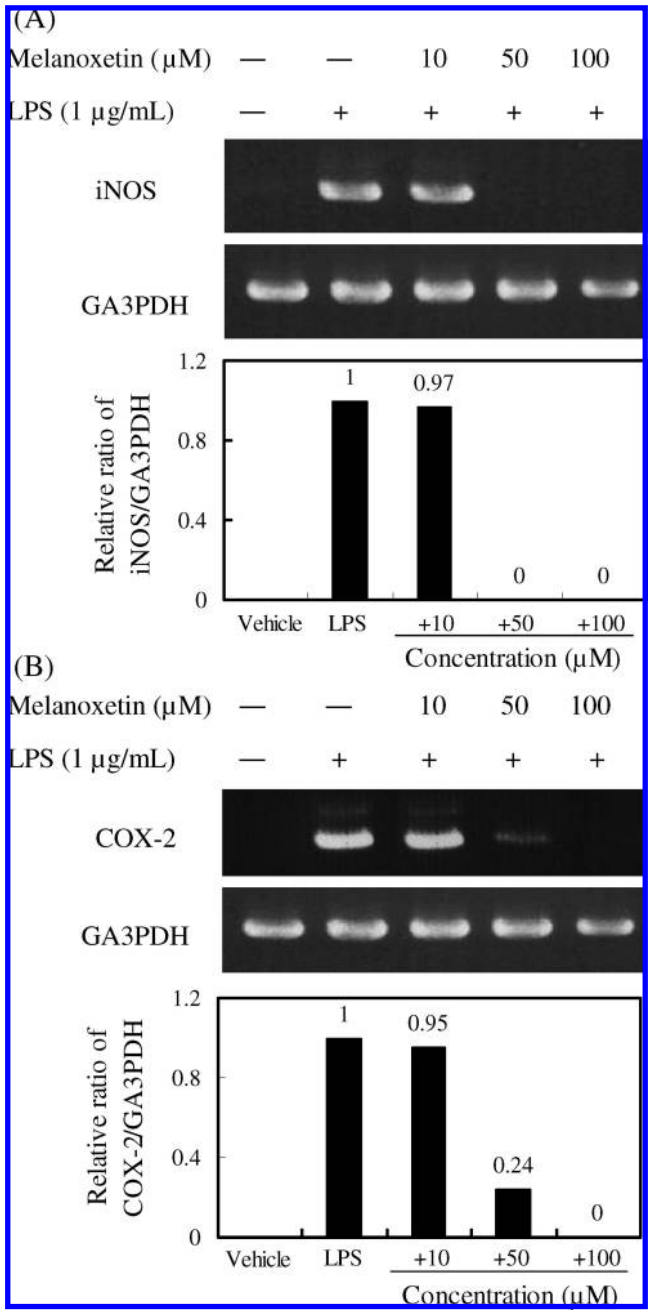

Figure 6. Effect of melanoxetin on the expression of iNOS mRNA (**A**) and COX-2 mRNA (**B**) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Cells were incubated with or without LPS (1 μg/mL) in the presence of the indicated concentrations of melanoxetin.

iNOS mRNA. In contrast, 6 h of incubation with LPS resulted in a large increase in iNOS mRNA expression. However, at 50 μM, melanoxetin completely suppressed the iNOS mRNA expression. The proposed mechanisms associated with the reduction of NO production are scavenging of NO, suppression of iNOS enzyme activity, inhibition of iNOS gene expression, and/or down-regulation of iNOS enzyme by modulation of enzyme activities related to signal transduction, etc. (*28–32*). In the present study, melanoxetin inhibited NO production in macrophages via one or more of these mechanisms.

Cyclooxygenase (COX) is the rate-limiting enzyme in PG synthesis and exists as two isoforms: constitutive (COX-1) and inducible (COX-2) (*29*). Like iNOS, COX-2 is an important enzyme that mediates inflammatory processes. Multiple lines of compelling evidence support COX-2 playing a role in the development of tumors (*13*). Thus, aberrant or excessive expression of iNOS and COX-2 is implicated in inflammatory disorders and the pathogenesis of cancer. Melanoxetin not only showed strong inhibitory activity on iNOS mRNA expression but also significantly inhibited the COX-2 mRNA expression in LPS-stimulated macrophages. **Figure 6B** shows that melanoxetin suppressed the LPS-induced COX-2 mRNA expression in a dose-dependent manner. Approximately 5 and 76% reductions were observed at 10 and 50 μM, respectively, as determined by densitometry analysis. At concentrations up to 100 μM, melanoxetin could completely inhibit the expression of COX-2 mRNA in LPS-stimulated cells. Thus, one of the mechanisms of melanoxetin inhibition of NO and PGE_2 production in LPS-stimulated macrophages is mediated by the down-regulation of iNOS and COX-2 mRNA expression. Further studies on intracellular signaling cascades leading to COX-2 and iNOS reduction by melanoxetin are of interest. In addition, in vivo pharmacological research on the anti-inflammatory activity of melanoxetin should also be addressed.

Phytochemicals, especially flavonoids and phenolic acids, are of current interest because of their important biological and pharmacological properties, including reactive oxygen species (ROS) scavenging and anti-inflammatory, anticancer, antimutagenic, and anticarcinogenic activities (*33–36*). Rice-Evans and Miller (*37*) have demonstrated the potential role of phytocompounds, including phenylpropanoids, flavonoids, and phenolic acids, as important contributing factors to dietary antioxidant activity. The heartwood of *A. confusa* contains abundant phenolic compounds, especially flavonoids, and shows significant antioxidant and anti-inflammatory activities. Thus, heartwood extracts or the derived phytocompounds from *A. confusa* have great potential to prevent diseases caused by the overproduction of reactive oxygen species and inflammatory disorders.

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