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Anti-inflammatory Activities of Coumarins Isolated from *Angelica gigas* Nakai on LPS-stimulated RAW 264.7 Cells

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Abstract

Five kinds of coumarin compounds were successfully purified from *Angelica gigas* Nakai by using recycling-preparative HPLC and identified as decursin (1), decursinol angelate (2), 7-demethylsuberosine (3), marmesin (4), and decursinol (5) by NMR analyses. None of the purified compounds in ethanol showed DPPH radical scavenging activity, while the *A. gigas* extract (AGNEX) displayed a significant level of activity. Interestingly, compounds 3 in phosphate buffered saline (PBS) showed good $ABTS^+$ radical scavenging activity (IC_{50}=8.1 μg/mL) as did compounds 4 and 5. The anti-inflammatory activities of the purified compounds were evaluated and compared using the NO concentration assay and western blot analysis on LPS-stimulated RAW 264.7 cells. NO production was significantly suppressed by all the compounds in a dose-dependent manner among which compounds 1, 2, and 3 showed very good activities with IC_{50} values of 7.4, 6.5, and 7.6 μg/mL, respectively. Treatment with compounds 1-5 effectively suppressed the expression levels of iNOS, IL-1β, and COX-2, which are responsible for promoting the inflammatory process. Thus, the ethanol extract and coumarin compounds of *A. gigas* Nakai hold promise for use as potential anti-inflammatory agents.

Key words: *Angelica gigas* Nakai, coumarin, recycling-preparative HPLC, NMR, anti-inflammatory.

INTRODUCTION

Angelica gigas Nakai (also known as Cham-Danggui in Korea) is a perennial plant belonging to the Umbelliferae family, and the root has been traditionally used in Korean folk medicine as a tonic. In addition, it is regarded by herbalists as 'Female Ginseng' due to its hemopoietic and health-promoting activities (1).

Many studies have examined the pharmacological properties of *A. gigas* Nakai such as its antibacterial, anticancer, antitumor, antioxidant, anti-inflammatory, neuroprotective, anti-dementia, inhibition of platelet aggregation, and blood coagulation activity (2-12). Based on these pharmacological properties, efforts have been made to isolate the active constituents from this plant. This work has led to the isolation of many coumarins among which two pyranocoumarins, decursin and decursinol angelate, were identified as the major active components. These two components constituted 4.56% and 3.68%, respectively, of the total compounds isolated from the dried root (13-15). Interestingly, decursin and decursinol angelate are found in large quantities in only *A. gigas* Nakai (Korean danggui) and only low amounts are found in *A. sinensis* Diels (Chinese danggui) or *A. acutiloba* Kitagawa (Japanese danggui), which means Korean danggui is an excellent source for decursin and decursinol angelate (15,16). The bioactivities of decursin have been studied extensively due to its anticancer, antibacterial, anti-inflammatory, neuroprotective properties, etc. (2-6,8,9). Recently, the decursin isomer, decursinol angelate and other coumarin compounds have also received attention due to its potent pharmacological effects against various diseases (2,3,6,9,10,12). However, information on the anti-inflammatory properties of decursinol angelate and other coumarin compounds is limited.

In this study, five types of major coumarins were successfully isolated from *A. gigas* Nakai by a simple and rapid procedure that used recycling preparative chromatography. In addition, the anti-inflammatory activities of these compounds were compared and evaluated using a radical scavenging assay, NO concentration assay and western blot analyses of inducible nitric oxide synthase

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(iNOS), interleukin-1β (IL-1β) and Cyclooxygenase 2 (COX-2) protein expression in LPS-stimulated RAW 264.7 cells.

MATERIALS AND METHODS

Plant and cell line

The root of *A. gigas* Nakai was purchased from the local market of Korea. The murine macrophage RAW 264.7 cell line was obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 2 mM penicillin.

Chemicals

Dulbecco's modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were from Gibco BRL (Gaithersburg, MD, USA). Lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), glutamine, penicillin, Griess reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were from the Sigma (St. Louis, MO, USA). Bradford protein assay reagent was from Bio-Rad (Hercules, CA, USA). PRO-PREP™ protein extraction solution was from iNtRON Biotechnology (Seongnam, Korea). Nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany). SDS-polyacrylamide gel, running buffer, and transfer buffer were from Invitrogen (Carlsbad, CA, USA). The anti-mouse iNOS antibody was from BD Transduction Laboratories (Lexington, KY, USA). Alkaline phosphatase conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Anti-rabbit IL-1β polyclonal IgG antibody, anti-goat Actin polyclonal IgG antibody, anti-goat COX-2 polyclonal IgG antibody, bovine anti-goat IgG-AP secondary antibody, and bovine anti-goat IgG-HRP-linked secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bovine anti-rabbit IgG-HRP-linked antibody was from Cell Signaling Technology (Danvers, MA, USA). HPLC-grade acetonitrile (ACN) and water were from Burdick & Jackson (Morristown, NJ, USA). Ethanol (95%) was from DUKSAN (Ansan, Korea). Other chemicals were of analytical grade.

Extraction and purification of coumarins from *A. gigas* Nakai

Dried and powdered root of *A. gigas* Nakai (1 kg) was extracted with 5 L of 95% ethanol for 24 hr at room temperature. Extracts were filtered through Whatman No. 1 filter paper, and were concentrated using a rotary evaporator (R-200, Büchi Labortechnik AG, Flawil, Switzerland) under reduced pressure.

The coumarin compounds were purified from the *A. gigas* Nakai ethanol extract (AGNEX) using recycling preparative HPLC (LC-9104, JAI, Tokyo, Japan) and the overall purification procedure is shown in Fig. 1. For the purification of decursin and decursinol angelate, the concentrated extract (20 g) was dissolved in 30 mL of 70% acetonitrile/water and filtered with a 0.45 μm membrane filter. Three milliliters of sample was injected to the JAIGEL ODS-AP column (20 × 500 mm, JAI) at a flow rate of 4 mL/min. Isocratic elution was applied using 70% acetonitrile/water as the mobile phase, and the peaks were detected using a RI and UV/Vis detector at 328 nm. The eluate containing decursin and decursinol angelate was recycled 9 times until the two compounds were completely separated. Finally, 5.3 g of decursin (**1**) and 2.7 g of decursinol angelate (**2**) were obtained.

The purification of other minor coumarins from AGNEX was carried out in two steps. First, the AGNEX solution was injected into the JAIGEL ODS-AP column and five fractions were collected without using the recycling mode by grouping adjacent peaks that had shorter retention times than decursin and decursinol angelate. Among them, fraction 5 contained a pure compound that was indentified as 7-demethylsuberosine (**3**, 250 mg). Fraction 3 (136.6 mg) was then purified further using recycling preparative HPLC with 40% acetonitrile/water as the eluent. After recycling the peaks, 21.6 mg of marmesin (**4**) and 18.2 mg of decursinol (**5**) were obtained. The chemical structures of the purified compounds were confirmed by NMR analysis.

NMR analysis

^{1}H-NMR, ^{13}C-NMR spectra of **1**, **3**, **4**, and **5** were

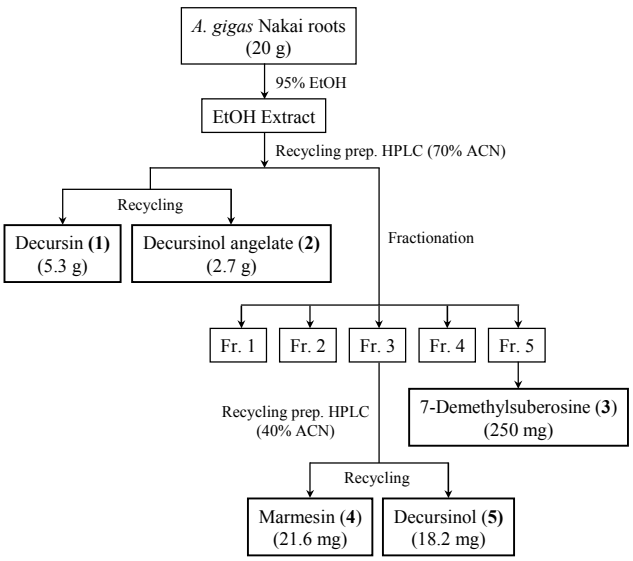

Fig. 1. Procedure used to purify coumarins from the roots of *A. gigas* Nakai.

measured in MeOD on a JNM-ECX 400 NMR Spectrometer (JEOL, Japan) at 300MHz. ^{1}H-NMR, ^{13}C-NMR, HMBC and HMQC spectra of **2** were recorded on a 600MHz High Resolution NMR Spectrometer, Bruker AV-600 (Germany) in MeOD.

DPPH radical scavenging activity

The DPPH radical scavenging activity of AGNEX and the isolated compounds was measured as described by Han et al. (17) with minor modifications. 10 μL of each sample in ethanol was added to 1.0 mL of 1×10^{-4} M DPPH ethanol solution and 10 μL of ethanol was added instead of the sample for the control. The absorbance at 517 nm was measured after the solution was maintained in the dark for 60 min. Ascorbic acid was used as a positive control. A lower absorbance of the reaction mixture indicated higher DPPH radical scavenging activity, which was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \left(1-\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right)\times100$$

ABTS^{+} radical scavenging activity

The ABTS^{+} radical scavenging activity of AGNEX and the isolated compounds was measured using the methods of Han et al. (17) with some modifications. ABTS was dissolved in water to a concentration of 7 mM. ABTS^{+} radicals were produced by reacting the ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixtures to stand in the dark at room temperature for 16 hr before use. The ABTS^{+} radical solution was diluted with ethanol for AGNEX or with 0.01 M phosphate buffered saline (PBS, pH 7.4) for the purified compounds to reach an absorbance of 0.70 ($\pm$0.02) at 734 nm and equilibrated at 30°C for 30 min. An ethanolic solution (10 μL) of the samples with various concentrations was mixed with 1.0 mL of diluted ABTS^{+} radical solution and 10 μL of ethanol was added instead of sample for the control. After incubation at room temperature for 20 min, the absorbance at 734 nm was measured. Ascorbic acid was used as a positive control. A lower absorbance of the reaction mixture indicates higher ABTS^{+} radical scavenging activity. The ABTS^{+} radical scavenging activity was calculated as follows:

$$\text{ABTS}^{+}\text{ radical scavenging activity (\%)} = \left(1-\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right)\times100$$

Cell culture and treatment

The murine macrophage RAW 264.7 cell line was cultured in DMEM supplemented with 10% FBS, 2 mM penicillin, and 2 mM L-glutamine. Cells in 10-mm dishes (5×10^{6} cells/dish) or 24 well plates (5×10^{5} cells/well) were preincubated with and without the indicated concentrations of the extract or purified compounds for 2 hr, and then incubated with LPS (1 μg/mL) for 20 hr at 37°C in a humidified atmosphere containing 5% CO_2. LPS treatment was not used in the negative control.

Cell viability

Cell viability was assessed by measuring the uptake of the supravital dye neutral red by viable cells (18). After culturing the cells as described previously, the medium was removed and replaced with 0.5 mL of fresh medium containing 1.14 mM neutral red. After incubation for 3 hr, the medium was removed and the cells were washed twice with PBS (pH 7.4). The incorporated neutral red was released from the cells by incubation for 15 min at room temperature in the presence of 1 mL of cell lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM dithiothreitol (DTT), and Triton X-100 (1%, v/v)] containing acetic acid (1%, v/v) and ethanol (50%, v/v). To measure the amount of dye taken up, the cell lysis products were centrifuged and the absorbance of the supernatant was measured spectrophotometrically at 540 nm.

NO concentration assay

Nitrite accumulation in the culture medium, which is an indicator of NO production, was measured according to the Griess reaction (19). One hundred microliters of each medium supernatant was mixed with 50 μL of 1% sulphanilamide (in 5% phosphoric acid) and 50 μL of 0.1% naphthylenediamine dihydrochloride, and then incubated at room temperature for 10 min. The absorbance was measured at 550 nm and the nitrite concentration was calculated using a sodium nitrite ($NaNO_2$) standard curve.

Western blot analysis

Western blot analysis was assayed by the method of Park et al. (20) with slight modifications. Cells (5×10^{6} cells/dish) in 10-mm dishes were preincubated with or without the indicated concentrations of compounds for 2 hr, and then incubated with LPS (1 μg/mL) for 20 hr. The cells were washed twice with PBS, and the cell pellet was harvested by centrifugation at 16,000$\times g$ for 10～20 sec and resuspended in 400 μL of PRO-PREP^{TM} solution. Cell lysis was induced by incubation for 20 min on ice. After centrifugation at 16,000$\times g$ and 4°C for 5 min, the supernatant was transferred to a fresh 1 mL tube. The protein content in the lysate supernatant was determined using the Bradford protein assay reagent (Bio-Rad).

The protein sample (50 μg) from each lysate was separated on a 10% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membranes. Membranes were

blocked over night at 4°C with 5% nonfat dry milk in a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20. The membranes were then incubated over night at 4°C with a 1:1000 dilution of anti-mouse iNOS antibody, 1:200 dilution of anti-rabbit IL-1β polyclonal IgG antibody, 1:100 dilution of anti-goat COX-2 polyclonal IgG antibody, and a 1:500 dilution of anti-goat Actin polyclonal IgG antibody in blocking buffer. After the membranes were washed, they were further incubated with a 1:1000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG secondary antibody, 1:1000 dilution of anti-rabbit IgG-HRP secondary antibody, 1:1000 dilution of anti-goat IgG-HRP secondary antibody, and a 1:500 dilution of bovine anti-goat lgG-AP secondary antibody for 2 hr at room temperature. The blots were finally developed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/ nitroblue tetrazolium (NBT) color developing solution or the signals were detected by electrochemiluminescence (ECL). Data were quantified using the Gel Doc EQ System (Bio-Rad). All signals were normalized to the protein levels of the housekeeping gene, Actin, and expressed as a ratio.

RESULTS AND DISCUSSION

Purification and identification of coumarins from *A. gigas Nakai*

For the purification of coumarins, 1 kg of dried and powdered root from *A. gigas* Nakai was extracted with 95% ethanol and 85 g of extract (AGNEX) was obtained. The coumarins were purified simply by using recycling preparative HPLC from AGNEX instead of silica column chromatography, which has been the conventional method most often used to purify coumarin compounds (13). By using this method, a fraction containing several compounds with similar retention times could be re-injected into the column using the recycling mode until a good separation of each compound was achieved. Recently, Kim et al. (21) reported purifying decursin and decurisol angelate from *A. gigas* Nakai using recycling preparative HPLC in a single step. In this study, five coumarins, including decursin and decursinol angelate, were successfully purified using the recycling mode from AGNEX without the need for conventional column chromatography (Fig. 1). First, decursin (**1**) and decursinol angelate (**2**) were purified from AGNEX using the recycling mode together with five fractions that eluted earlier. Fr. 5 consisted of a pure compound (**3**), and compounds **4** and **5** were purified from Fr. 3.

The chemical structures of these components were confirmed by comparing the UV, ^{1}H, ^{13}C and 2D-NMR spectral data to published spectra. The structures of the compounds were shown in Fig. 2.

Compound **1** appeared as white amorphous powder. UV (MeOH): 204, 220, and 328 nm. ^{1}H-NMR (MeOD, 300 MHz) δppm: 7.79 (d, 9.3), 7.32 (s), 6.70 (s), 6.19 (d, 9.3), 5.63 (m), 5.10 (t, 4.8), 3.23 (dd, 18, 4.8), 2.86 (dd, 18, 4.8), 2.11 (d, 1.2), 1.86 (d, 1.2), 1.37 (s), and 1.35 (s). ^{13}C-NMR (MeOD, 300 MHz) δppm: 163.3 (C2), 114.3 (C3), 145.5 (C4), 130.5 (C5), 117.8 (C6), 158.0 (C7), 105.0 (C8), 155.3 (C9), 113.5 (C10), 28.7 (C2'), 78.1 (C3'), 70.6 (C4'), 25.2 (C4'-CH3), 23.6 (C4'-CH3), 167.0 (C1"), 116.3 (C2"), 159.9 (C3"), 27.5 (C4"), and 20.4 (C3"-CH3). These data were consistent with those reported in reference (22) and this compound was identified as decursin.

Compound **2** appeared as white amorphous powder. UV (MeOH): 204, 220, and 328 nm. ^{1}H-NMR (MeOD, 600 MHz) δppm: 7.83 (d. 9.6), 7.37 (s), 6.75 (s), 6.22 (d, 9.6), 6.11 (m), 5.18 (t, 4.8), 3.27 (dd, 18, 4.8), 2.94 (dd, 18, 4.8), 1.83 (m), 1.80 (m), 1.40 (s), and 1.39 (s). ^{13}C-NMR (MeOD, 600 MHz) δppm: 163.5 (C2), 113.7 (C3), 145.7 (C4), 130.7 (C5), 117.9 (C6), 158.2 (C7), 105.1 (C8), 155.6 (C9), 114.5 (C10), 28.9 (C2'), 78.2 (C3'), 71.6 (C4'), 25.3 (C4'-CH3), 24.0 (C4'-CH3), 168.5 (C1"), 128.8 (C2"), 140.2 (C3"), 16.0 (C4"), and 20.8 (C2"-CH3). These data were consistent with those reported in reference (22) and this compound was identified as decursinol angelate.

Compound **3** appeared as amorphous powder with orange yellow color. UV (MeOH): 204, 220, and 328 nm. ^{1}H-NMR (MeOD, 300 MHz) δppm: 7.79 (d. 9.3), 7.23 (s), 6.67 (s), 6.13 (d, 9.3), 5.31 (m), 3.30 (m), 1.74 (s), and 1.70 (s). ^{13}C-NMR (MeOD, 300 MHz) δppm: 164.0 (C2), 112.0 (C3), 146.3 (C4), 134.0 (C5), 123.0 (C6), 160.9 (C7), 102.6 (C8), 155.4 (C9), 112.9 (C10), 28.6 (C1'), 127.9 (C2'), 129.4 (C3'), 26.0 (C4'), and 17.8 (C3'-CH3). Compound **3** was identified as 7-demethylsuberosine.

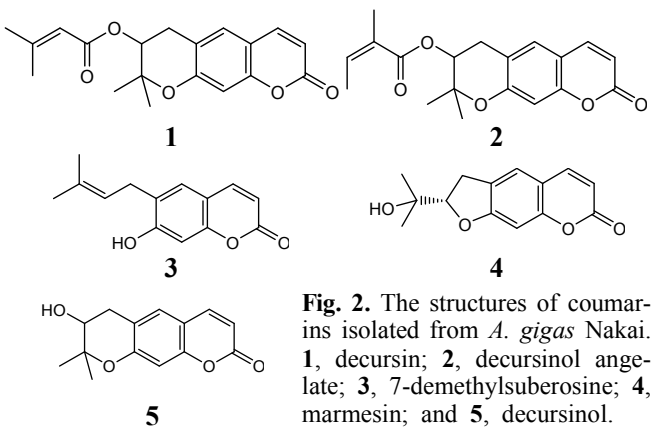

Fig. 2. The structures of coumarins isolated from *A. gigas* Nakai. **1**, decursin; **2**, decursinol angelate; **3**, 7-demethylsuberosine; **4**, marmesin; and **5**, decursinol.

Compound **4** appeared as amorphous powder with light yellow color. UV (MeOH): 204, 220, and 328 nm. ^{1}H-NMR (MeOD, 300 MHz) δppm: 7.81 (d. 9.3), 7.36 (s), 6.68 (s), 6.16 (d, 9.3), 4.74 (m), 3.23 (m), 3.30 (m), 1.280 (s), and 1.215 (s). ^{13}C-NMR (MeOD, 300 MHz) δppm: 165.2 (C2), 112.1 (C3), 146.2 (C4), 127.3 (C5), 125.0 (C6), 163.7 (C7), 111.9 (C8), 156.8 (C9), 114.0 (C10), 72.3 (C2'), 98.1 (C3'), 92.5 (C1"), 30.2 (C2"), 25.9 (C1"-CH3). These data were consistent with those reported in reference (23) and this compound was identified as marmesin.

Compound **5** appeared as white amorphous powder. UV (MeOH): 204, 220, and 328 nm. ^{1}H-NMR (MeOD, 300 MHz) δppm: 7.79 (d. 9.3), 7.31 (s), 6.66 (s), 6.17 (d, 9.3), 3.80 (dd, 5.1, 7.2), 3.06 (dd, 5.1, 17.7), 2.79 (dd, 7.2, 17.7), 1.35 (s), and 1.30 (s). ^{13}C-NMR (MeOD, 300 MHz) δppm: 163.5 (C2), 113.2 (C3), 145.7 (C4), 130.5 (C5), 119.3 (C6), 158.4 (C7), 104.8 (C8), 155.2 (C9), 114.0 (C10), 31.5 (C2'), 79.7 (C3'), 69.7 (C4'), 25.9 (C4'-CH3), and 21.7 (C4'-CH3). These data were consistent with those reported in reference (23), and this compound was identified as decursinol.

DPPH radical scavenging activity

The DPPH radical, which can be readily scavenged by antioxidants, is stable with a maximum absorption at 517 nm (24). Since the DPPH radical assay can accommodate many samples in a short time period and is sensitive enough to detect active ingredients at low concentration, the DPPH radical scavenging assay has been widely used to evaluate the anti-radical activity of various samples (17,25). As shown in Fig. 3A, the AGNEX displayed a dose-dependant DPPH radical scavenging activity with an IC_{50} of 1.29 mg/mL. However, compared with ascorbic acid, which was used as the positive control, compounds **1**～**5** showed very low activities (Fig. 3B).

ABTS^{+} radical scavenging activity

The ABTS^{+} radical scavenging assay, which uses a specific absorbance (734 nm) at a wavelength remote from the visible region and requires a short reaction time, can be used in both organic and aqueous solvent systems and can also be used as an index reflecting the antioxidant activity of the test samples (17,26). Hence, ABTS^{+} radical scavenging activities of AGNEX and the

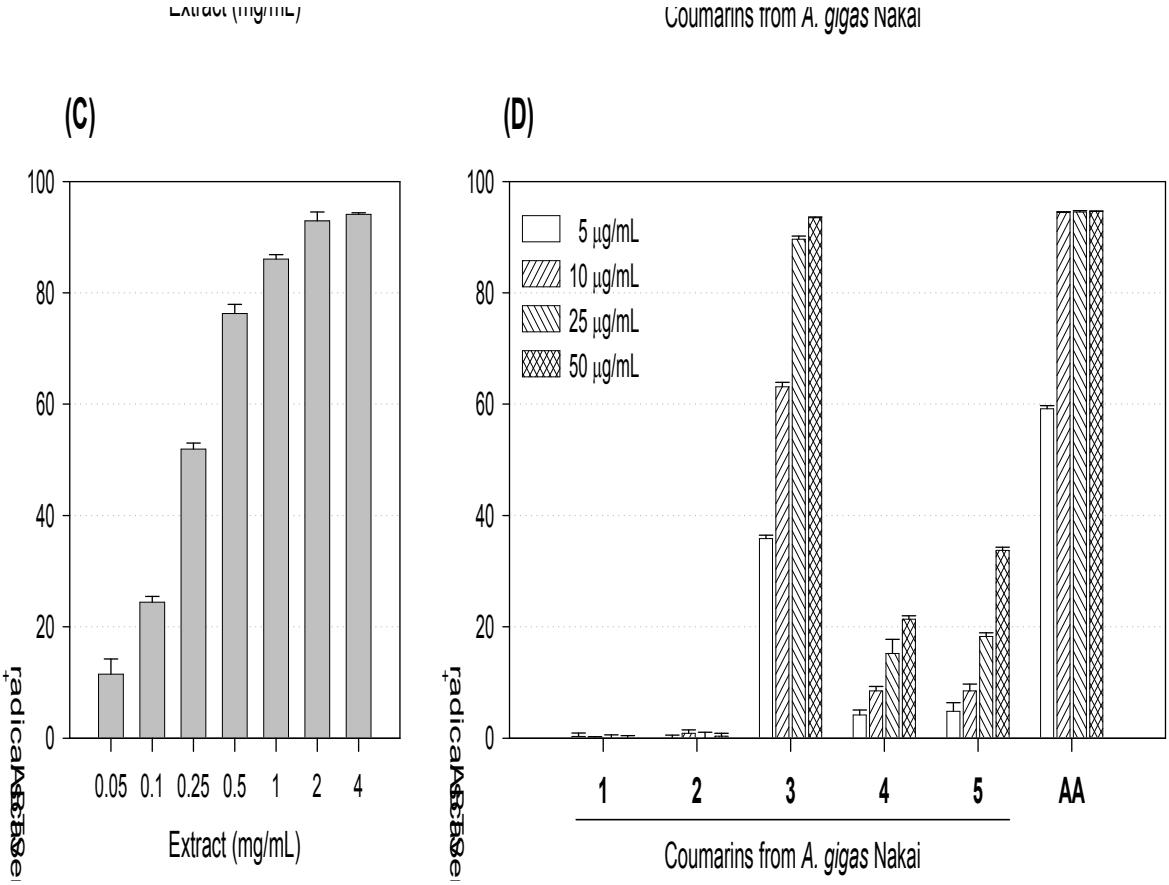

Fig. 3. DPPH and ABTS^{+} radical scavenging activities of ethanol extract (A and C) and purified coumarins (B and D) from *A. gigas* Nakai. **1**, decursin; **2**, decursinol angelate; **3**, 7-demethylsuberosine; **4**, marmesin; **5**, decursinol; and AA, ascorbic acid (positive control). Data represent the means±SD of triplicate experiments.

isolated coumarin compounds diluted in PBS were measured and compared. As shown in Fig. 3C and 3D, AGNEX and compound **3** exerted relatively good $ABTS^+$ radical scavenging activity with an IC_{50} of 0.29 mg/mL and 8.1 μg/mL, respectively. Compounds **4** and **5** showed weak $ABTS^+$ radical scavenging activity and compounds **1** and **2** showed no $ABTS^+$ radical scavenging activity.

Suppression of NO production in LPS-stimulated RAW 264.7 cells

NO is a compound produced from l-arginine via nitric oxide synthase (NOS) and is an important cellular second messenger. It reacts with oxygen in water and its reactive intermediates to yield other compounds (e.g. NO_2), moderately stable anions (NO_2^-), very stable anions (NO_3^-), unstable higher oxides (e.g. N_2O_3), and unstable peroxides (e.g. $ONOO^-$), which play an important role in the processes of inflammation (20,27-30). In this study, NO production was significantly suppressed by AGNEX and compounds **1**～**3** in a dose-dependent manner with an IC_{50} of 10.3, 7.4, 6.5, and 7.6 μg/mL, respectively (Fig. 4). However, compounds **4** and **5** only weakly suppressed NO production with an IC_{50} of 17.4 and 16.7 μg/mL, respectively.

Cell viability of all samples was more than 100% at all concentrations tested as assessed by the neutral red assay, which supports the notion that the suppressive effects of AGNEX and compounds on NO production are not due to cell death from the sample treatment (data not shown).

Western blot analysis

It has been proposed that iNOS mediates high output production of NO causing cell injury through the generation of potent reactive radicals, such as peroxynitrite, and is closely related with the inflammation process (20,29,30). Cyclooxygenase 2 (COX-2) is a predominant cyclooxygenase at sites of inflammation in the arachidonic acid pathway. COX-2 catalyzes the inducible production of prostaglandins (PGs), which clearly represents an important step in the inflammatory process. Development of COX-2 inhibitors represents a major advance in the therapy of inflammation (29,31). Other mediators of inflammation are interleukins (e.g. IL-1β), which can increase leukocyte recruitment. Adhesion molecules, such as PGE, PAF and PGI_2, also play important roles in the process of inflammation (8,32).

We investigated the effects of coumarin compounds on the expression of iNOS, IL-1β, and COX-2 protein in LPS-stimulated RAW 264.7 cells to elucidate the anti-inflammatory mechanisms of these compounds. Pretreatment with AGNEX and compounds **1**～**5** significantly suppressed the expression of iNOS and IL-1β in a dose-dependent manner while the suppression of COX-2 expression was relatively weak (Fig. 5). However, the expression of the housekeeping gene, Actin, was not effected. These results suggest that the purified coumarins effectively inhibit LPS-inducible iNOS, IL-1β and COX-2 expression in murine macrophage, which is one of the major factors controlling the anti-inflammatory activities of *A. gigas* Nakai.

The anti-inflammatory activities of the *A. gigas* Nakai extract and decursin have already reported by Kim et al. (8). According to this reference, decursin suppressed NO production and suppressed the levels of MMP-9,

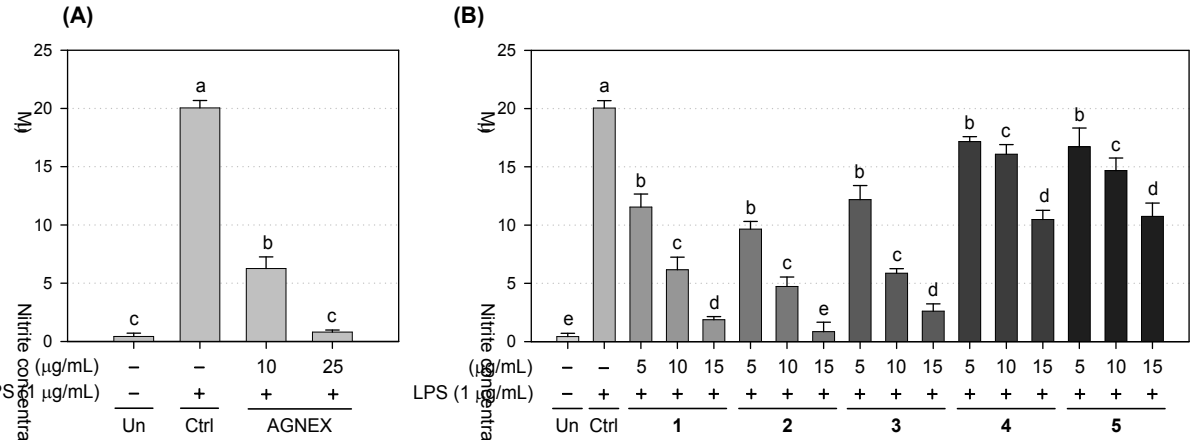

Fig. 4. Effects of (A) ethanol extract and (B) purified coumarins from *A. gigas* Nakai on NO production in LPS-stimulated RAW 264.7 macrophages. Un, untreated; Ctrl, control; AGNEX, ethanol extract from *A. gigas* Nakai; **1**, decursin; **2**, decursinol angelate; **3**, 7-demethylsuberosine; **4**, marmesin; and **5**, decursinol. Cells (5×10^5/well) in 24-well plates were preincubated with the indicated concentrations of extract or purified coumarins for 2 hr, and then incubated with LPS (1 μg/mL) for 20 hr. Untreated (Un) indicates the negative control that was not subjected to LPS treatment. Data represent the means±SD of triplicate experiments. Values marked by different letters are significantly different at $p<0.05$.

(A)

1 (μg/mL)	−	0	5	10	20	**1**	LPS	Protein/Actin ratio		
LPS (1 μg/mL)	−	+	+	+	+	(μg/mL)	(1 μg/mL)	iNOS	IL-1β	COX-2
						−	−	0.07±0.03[e]	0.03±0.01[e]	0.03±0.02[d]
iNOS						0	+	0.80±0.05[a]	1.29±0.05[a]	1.32±0.04[a]
IL-1β						5	+	0.48±0.03[b]	0.61±0.01[b]	1.09±0.05[b]
COX-2						10	+	0.34±0.03[c]	0.37±0.02[c]	0.96±0.08[bc]
Actin						20	+	0.25±0.03[d]	0.14±0.02[d]	0.78±0.05[c]

(B)

2 (μg/mL)	−	0	5	10	20	2	LPS	Protein/Actin ratio		
LPS (1 μg/mL)	−	+	+	+	+	(μg/mL)	(1 μg/mL)	iNOS	IL-1β	COX-2
						−	−	0.17±0.06[e]	0.06±0.06[e]	0.02±0.05[d]
iNOS						0	+	0.96±0.07[a]	1.68±0.05[a]	1.35±0.09[a]
IL-1β						5	+	0.78±0.06[b]	1.16±0.12[b]	1.10±0.05[b]
COX-2						10	+	0.68±0.04[c]	0.57±0.14[c]	1.01±0.06[bc]
Actin						20	+	0.33±0.06[d]	0.21±0.06[d]	0.77±0.06[c]

(C)

3 (μg/mL)	−	0	5	10	20	**3**	LPS	Protein/Actin ratio		
LPS (1 μg/mL)	−	+	+	+	+	(μg/mL)	(1 μg/mL)	iNOS	IL-1β	COX-2
						−	−	0.06±0.02[e]	0.01±0.01[e]	0.01±0.03[e]
iNOS						0	+	1.65±0.15[a]	1.26±0.03[a]	1.22±0.07[a]
IL-1β						5	+	1.10±0.03[b]	0.76±0.08[b]	1.05±0.03[b]
COX-2						10	+	0.67±0.01[c]	0.39±0.07[c]	0.86±0.02[c]
Actin						20	+	0.46±0.04[d]	0.18±0.04[d]	0.54±0.05[d]

(D)

4 (μg/mL)	−	0	5	10	20	**4**	LPS	Protein/Actin ratio		
LPS (1 μg/mL)	−	+	+	+	+	(μg/mL)	(1 μg/mL)	iNOS	IL-1β	COX-2
						−	−	0.08±0.00[e]	0.04±0.01[e]	0.09±0.03[e]
iNOS						0	+	0.80±0.01[a]	0.83±0.05[a]	1.83±0.07[a]
IL-1β						15	+	0.71±0.01[b]	0.72±0.02[b]	1.61±0.08[b]
COX-2						30	+	0.58±0.01[c]	0.31±0.05[c]	1.43±0.08[c]
Actin						50	+	0.45±0.01[d]	0.17±0.02[d]	0.85±0.03[d]

(E)

5 (μg/mL)	−	0	5	10	20	**5**	LPS	Protein/Actin ratio		
LPS (1 μg/mL)	−	+	+	+	+	(μg/mL)	(1 μg/mL)	iNOS	IL-1β	COX-2
						−	−	0.05±0.01[e]	0.13±0.03[d]	0.11±0.03[e]
iNOS						0	+	0.65±0.02[a]	1.75±0.08[a]	2.18±0.15[a]
IL-1β						15	+	0.56±0.01[b]	1.10±0.06[b]	1.79±0.04[b]
COX-2						30	+	0.36±0.02[c]	0.55±0.03[c]	1.44±0.08[c]
Actin						50	+	0.11±0.01[d]	0.19±0.04[d]	0.88±0.03[d]

Fig. 5. Effects of purified coumarins from *A. gigas* Nakai on iNOS, IL-1β, and COX-2 expression in LPS stimulated RAW 264.7 macrophages. (A) decursin; (B) decursinol angelate; (C) 7-demethylsuberosine; (D) marmesin; and (E) decursinol. Cells (5×10^6/dish) in 10-mm dishes were preincubated with the indicated concentrations of purified coumarins for 2 hr, and then incubated with LPS (1 μg/mL) for 20 hr. Levels of iNOS, IL-1β, and COX-2 protein expression were measured by Western blot analysis. The blot was rehybridized with antibody against Actin to verify equal loading of protein in each lane. Actin was used as an internal control. The band intensity was quantified using the Gel Doc EQ system. All signals shown in the Tables were normalized to the protein levels of the housekeeping gene, Actin, and expressed as a ratio. Data represent the means±SD of triplicate experiments. Values marked by different letters are significantly different at $p<0.05$.

iNOS, IL-1β and TNF-α expression in different kinds of cell lines. In this study, we examined the anti-inflammatory activities of five kinds of major coumarins including decursin isolated from *A. gigas* Nakai. By treatment with compounds **1**∼**5**, NO production was significantly decreased in a dose-dependent manner and the levels of iNOS, IL-1β and COX-2 expression were also suppressed.

The combined results of this study suggest that the ethanol extract and coumarin compounds isolated from *A. gigas* Nakai hold great promise for use as potential anti-inflammatory agents.

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