

IN VITRO ANTI-INFLAMMATORY ACTIVITY OF THE COMPONENTS OF *AMOMUM TSAO-KO* IN MURINE MACROPHAGE RAW 264.7 CELLS

Chun Whan Choi^{1, #}, Ju Young Shin^{1, #}, Changon Seo¹, Seong Su Hong¹, Eun-Kyung Ahn¹, Young Hoon Jung¹, Joa Sub Oh^{2*}

¹Bio-Center, Gyeonggi Institute of Science & Technology Promotion, Gyeonggi-do 16229, Korea. ²College of Pharmacy, Dankook University, Chungnam 31116, Korea. [#]These authors contributed equally to this work.

*Corresponding Author E-mail: jsoh@dankook.ac.kr

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Abstract

Background: Plants still remain the prime source of drugs for the treatment of inflammation and can provide leads for the development of novel anti-inflammatory agents.

Material and methods: An *in vitro* bioassay guide revealed that the 80% ethanol (EtOH) extract of the whole plant, *Amomum tsao-ko* (Zingiberaceae), displayed anti-inflammatory activity after assessing its effects on murine macrophage RAW 264.7 cells.

Result: Phytochemical study of the 80% EtOH extract of *Amomum tsao-ko* led to the isolation of eight compounds: 4-hydroxy-3-methoxy-benzoic acid (**1**), *meso*-hannokinol (**2**), (+)-hannokinol (**3**), coumaric acid (**4**), 4-hydroxy-benzoic acid (**5**), (+)-epicatechin (**6**), (-)-catechin (**7**), and myrciaphenone A (**8**). The results indicated that two of the isolated components, (+)-epicatechin (**6**) and (-)-catechin (**7**), inhibited the production of nitric oxide (NO) significantly in lipopolysaccharide treated RAW 264.7 cells.

Conclusion: LPS-induced interleukin tumor necrosis factor-alpha (TNF- α), IL-1 β and IL-10 production was also decreased in a dose-dependent manner. In addition, western blot analysis revealed that (+)-epicatechin (**6**) and (-)-catechin (**7**) reduced the expression of inducible nitric oxide synthase and inhibited nuclear localization of nuclear factor kappa-B (NF- κ B).

Keywords: *Amomum tsao-ko*, Anti-inflammatory activity, Phytochemical, Cytokines, NF- κ B

Introduction

Amomum tsao-ko (*A. tsao-ko*) Crevost et Lemaire is a plant of the genus *Amomum* in the family Zingiberaceae, native to several countries Asia and Africa such as southern China, northern Viet Nam and Ethiopia. The dried fruit of *A. tsao-ko* used as an eliminate phlegm, warm the spleen, reduce abdominal pain, dyspepsia, and vomiting agent in Oriental traditional medicine (Lim et al., 2013; Zhao et al., 2010). Tsaokoin, a bicyclic nonane, and antioxidants, including hannokinol diarylheptanoids, have been isolated from this plant (Teresita et al., 2000). An antibacterial, spiroketal aculeatin (Moon et al., 2004), an antimalarial, diterpene peroxide (Kamchonwongpaisan et al., 1995), and eicosenones (Donga et al., 1988) have been identified from other species of the genus *Amomum*. Although the plant *A. tsao-ko* has long been used as a spice and perfume in addition to its medicinal usage, very few studies have reported its anti-inflammatory constituents (Lee et al., 2008). As a result of our ongoing search for novel bioactive natural products from medicinal plants, the 80% ethanol (EtOH) extract of the powdered fruit of *A. tsao-ko* was found to show significant anti-inflammatory activity in lipopolysaccharide (LPS)-treated RAW 264.7 cells. To investigate the anti-inflammatory properties of *A. tsao-ko* Ext. and its main components, we performed their effects on the survival and immune status of RAW 264.7 murine macrophage cells. Cell viability was determined using a MTT assay after treatment with various concentrations of the isolated constituents. Inhibition of NO production and iNOS expression were measured by reaction with Griess reagent and western blot analysis in LPS-induced RAW 264.7 cells, respectively. Constantly, we investigated the effects of compounds **6** ((+)-epicatechin) and **7** ((-)-catechin) on tumor necrosis factor (TNF)- α , IL-1 β , and IL-10, which are related to inflammatory response at both reverse

transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). NF- κ B is known to play an important role in gene expression on inflammation (Eliopoulos et al., 2002). The present study confirmed that the inhibitory effects of (+)-epicatechin and (-)-catechin were mediated via inhibition of nuclear localization of NF- κ B, which were also determined by western blot analysis.

Materials and Methods

General Procedures

Ultraviolet (UV) spectra were obtained using a Shimadzu UV-1650PC spectrometer. Nuclear magnetic resonance (NMR) spectra were measured on a Bruker Advance (700MHz 54mm Asecd shielded Magnet). Chemical shifts were expressed in terms of values. Electrospray ionization (ESI) mass spectra were obtained using a LTQ Orbitrap XL (Thermo Scientific) mass spectrometer. Preparative high performance liquid chromatography (prep-HPLC) was performed using a Shimadzu system (LC-8A pump and Diode Array Detector) and a YMC-Pack octadecyl (ODS) A column (250 \times 20 mm i.d.), using a gradient solvent system of methanol MeOH-water (0:100-100:0) at a flow rate of 8 mL/min. Medium pressure liquid chromatography (MPLC; Combi Flash RF, Teledyne ISCO) separations were performed using a RediSep Rf C18 column (50g-150 g C18 Reverse Phase with a flow rate of 40mL-85 mL/min). Open column chromatography was performed using silica gel (Kieselgel 60, 70-230 mesh Merck) and thin layer chromatography (TLC) was performed using pre-coated silica gel 60 F254 (0.25 mm, Merck).

Plant Material

Dried *A. tsao-ko* was purchased from the Kyungdong Oriental Herbal Market in Korea (August 2012) and identified by one of the authors (Prof. Joa Sub Oh). A voucher specimen (G47) was deposited at the Natural Products Research Laboratory, Gyeonggido Business and Science Accelerator.

Extraction and Isolation

Dried *A. tsao-ko* (9.6 kg) was extracted with 5 L of 80% EtOH at room temperature for 5 days. After filtration with a cotton ball, the filtrate was combined and evaporated to dryness to give 538.5 g of dark syrupy extract, which was suspended in water (10 L) and partitioned with equal volumes of dichloromethane (CH₂Cl₂), ethylacetate (EtOAc), and *n*-butanol (*n*-BuOH) successively to give a CH₂Cl₂ soluble fraction (146 g), an EtOAc soluble fraction (33.5 g), a *n*-BuOH soluble fraction (85 g), and a residual aqueous fraction (267 g). The EtOAc soluble fraction (33.5 g) was subjected to silica gel column (\varnothing = 5.0 \times 100 cm) chromatography and eluted with MeOH in CH₂Cl₂ in a step-gradient manner (1% to 50%) to afford seven fractions (F1: 1.7 g, F2: 0.98 g, F3: 2.6 g, F4: 2.4 g, F5: 4.0 g, F6: 4.0 g, and F7: 17.5 g). The fraction F2 (0.98 g) was further purified using ODS column (\varnothing = 1.0 \times 80 cm) chromatography to give 18.8 mg of **1**. Fraction F3 (2.4 g) was also purified by silica gel column chromatography eluted with MeOH in CH₂Cl₂ (1% to 50%) in a stepwise gradient manner to produce five fractions (F31-F35). F32 was further purified using RP-18 column chromatography to produce 5.6 mg of **2** and 7.3 mg of **3**. F4 (2.4 g) was purified with RP-18 column chromatography to produce 16.1 mg of compound **4** and 27.7 mg of **5**. F5 (4.0 g) was purified with RP-18 column chromatography to produce 9.4 mg of **6** and 10.4 mg of **7**. Repeated RP-18 chromatography of F6 (4.0 g) with step-gradient elution of MeOH in water resulted in the purification of 4.3 mg of **8**.

Compound **1** - white amorphous powder; EI-MS *m/z*: 168 [M]⁺; ¹H-NMR (700 MHz, MeOH-*d*₄): δ 7.58 (1H, d, *J* = 1.2 Hz, H-3), 7.57 (1H, dd, *J* = 1.8 & 7.5 Hz, H-5), 6.85 (1H, d, *J* = 7.5 Hz, H-6), 3.91(3H, s, OCH₃); ¹³C-NMR (175 MHz, MeOH-*d*₄): δ 168.9 (C-7), 151.1 (C-1), 147.2 (C-2), 123.8 (C-5), 122.0 (C-4), 114.4 (C-3), 112.4 (C-6), 55.0 (C-OMe).

Compound **2** - white amorphous powder; ESI-MS *m/z*: 317 [M+H]⁺; ¹H-NMR (700 MHz, MeOH-*d*₄): δ 6.99 (4H, d, *J* = 8.4 Hz, H-2', H-6', H-2'', H-6''), 6.67 (4H, d, *J* = 8.4 Hz, H-3', H-5', H-3'', H-5''), 3.78 (2H, m, H-3, H-5), 2.68-2.50 (4H, m, H₂-1, H₂-7), 1.69-1.63 (4H, m, H₂-2, H₂-6), 1.53 (2H, dd, *J* = 6.5 & 5.8 Hz, H₂-4); ¹³C-NMR (175 MHz, MeOH-*d*₄): δ 32.2 (C-1), 41.4 (C-2), 68.7 (C-3), 45.6 (C-4), 68.7 (C-5), 41.4 (C-6), 32.2 (C-7), 134.5 (C-1'), 130.3 (C-2'), 116.1 (C-3'), 156.3 (C-4'), 116.1 (C-5'), 130.3 (C-6'), 134.5 (C-1''), 130.3 (C-2''), 116.1 (C-3''), 156.3 (C-4''), 116.1 (C-5''), 130.3 (C-6'').

Compound **3** - white amorphous powder; ESI-MS *m/z*: 317 [M+H]⁺; ¹H-NMR (700 MHz, MeOH-*d*₄): δ 6.99 (4H, d, *J* = 8.4 Hz, H-2', H-6', H-2'', H-6''), 6.67 (4H, d, *J* = 8.4 Hz, H-3', H-5', H-3'', H-5''), 3.74 (2H, m, H-3, H-5), 2.63-2.52 (4H, m, H₂-1, H₂-7), 1.66-1.70 (4H, m, H₂-2, H₂-6), 1.60 (2H, dd, *J* = 6.5 & 5.8 Hz, H₂-4); ¹³C-NMR (175 MHz, MeOH-*d*₄): δ 30.5 (C-1), 39.5 (C-2), 69.5 (C-3), 43.4 (C-4), 69.5 (C-5), 39.5 (C-6), 30.5 (C-7), 133.0 (C-1'), 128.9 (C-2'), 114.7 (C-3'), 154.9 (C-4'), 114.7 (C-5'), 128.9 (C-6'), 133.0 (C-1''), 128.9 (C-2''), 114.7 (C-3''), 154.9 (C-4''), 114.7 (C-5''), 128.9 (C-6'').

Compound **4** - white amorphous powder; EI-MS *m/z*: 164 [M]⁺; ¹H-NMR (700 MHz, MeOH-*d*₄): δ 7.57 (1H, d, *J* = 15.8 Hz, H-7), 7.44 (2H, d, *J* = 8.5 Hz, H-3 & 5), 6.80 (2H, d, *J* = 8.5 Hz, H-2 & 6), 6.29 (1H, d, *J* = 15.8 Hz, H-8); ¹³C-NMR (175 MHz, MeOH-*d*₄): δ 159.7 (C-1), 115.4 (C-2), 129.6 (C-3), 126.0 (C-4), 129.6 (C-5), 115.4 (C-6), 144.8 (C-7), 115.1 (C-8), 171.5 (C-9).

Compound **5** - white amorphous powder; EI-MS *m/z*: 138 [M]⁺; ¹H-NMR (700 MHz, MeOH-*d*₄): δ 7.87 (2H, d, *J* = 8.8 Hz, H-3 & 5), 6.82 (2H, d, *J* = 8.8 Hz, H-2 & 6), 6.80 (2H, d, *J* = 8.5 Hz, H-2 & 6), 6.29 (1H, d, *J* = 15.8 Hz, H-8);

^{13}C -NMR (175 MHz, MeOH- d_4): δ 162.0 (C-1), 114.6 (C-2), 131.6 (C-3), 121.4 (C-4), 131.6 (C-5), 114.6 (C-6), 168.7 (C-7).

Compound **6** - white amorphous powder; EI-MS m/z : 290 $[\text{M}]^+$; ^1H -NMR (700 MHz, MeOH- d_4): δ 7.00 (1H, d, $J = 1.9$ Hz, H-2'), 6.82 (1H, d, $J = 1.9$ Hz & 8.1 Hz, H-6'), 6.78 (1H, d, $J = 8.1$ Hz, H-5'), 5.96 (1H, d, $J = 2.1$ Hz, H-6), 5.94 (1H, d, $J = 2.1$ Hz, H-8), 4.84 (1H, s, H-2), 4.20 (1H, m, H-3), 2.89 (1H, dd, $J = 4.63$ & 16.6 Hz, H-4a), 2.76 (1H, dd, $J = 2.93$ & 16.6 Hz, H-4b); ^{13}C -NMR (175 MHz, MeOH- d_4): δ 78.5 (C-2), 66.1 (C-3), 27.9 (C-4), 156.6 (C-5), 95.0 (C-6), 156.3 (C-7), 94.5 (C-8), 156.0 (C-9), 98.7 (C-10), 130.9 (C-1'), 113.9 (C-2'), 144.5 (C-3'), 144.4 (C-4'), 114.5 (C-5'), 118.0 (C-6').

Compound **7** - white amorphous powder; EI-MS m/z : 290 $[\text{M}]^+$; ^1H -NMR (700 MHz, MeOH- d_4): δ 6.85 (1H, d, $J = 1.9$ Hz, H-2'), 6.78 (1H, d, $J = 8.1$ Hz, H-5'), 6.74 (1H, dd, $J = 1.9$ & 8.1 Hz, H-6'), 5.94 (1H, d, $J = 2.1$ Hz, H-6), 5.87 (1H, d, $J = 2.1$ Hz, H-8), 4.58 (1H, d, 7.6 Hz, H-2), 3.99 (1H, m, H-3), 2.87 (1H, dd, $J = 7.6$ & 16.0 Hz, H-4a), 2.52 (1H, dd, $J = 8.6$ & 16.0 Hz, H-4b); ^{13}C -NMR (175 MHz, MeOH- d_4): δ 81.5 (C-2), 67.4 (C-3), 27.1 (C-4), 156.5 (C-5), 94.9 (C-6), 156.2 (C-7), 94.1 (C-8), 155.5 (C-9), 94.4 (C-10), 130.8 (C-1'), 113.8 (C-2'), 144.9 (C-3'), 144.8 (C-4'), 114.7 (C-5'), 118.6 (C-6').

Compound **8** - white amorphous powder; ESI-MS m/z : 331 $[\text{M}+\text{H}]^+$; ^1H -NMR (700 MHz, MeOH- d_4): δ 6.20 (1H, d, $J = 2.2$ Hz, H-4), 5.97 (1H, d, $J = 2.2$ Hz, H-6), 5.05 (1H, d, $J = 7.8$ Hz, H-1'), 2.71 (3H, s, H-8); ^{13}C -NMR (175 MHz, MeOH- d_4): δ 203.4 (C-7), 166.3 (C-1), 164.9 (C-5), 161.2 (C-3), 105.3 (C-2), 100.6 (C-1'), 96.7 (C-4), 93.9 (C-6), 77.1 (C-5'), 77.0 (C-3'), 73.3 (C-2'), 69.7 (C-4'), 61.0 (C-6'), 32.1 (C-8).

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Rockville, MD, USA). Penicillin-streptomycin was purchased from Invitrogen (Carlsbad, CA, USA). *N*-(1-naphthyl) ethylenediamine dihydrochloride and sulfanilamide were purchased from Merck Millipore (Billerica, MA, USA). Lipopolysaccharide from *Escherichia coli* serotype 0111:B4, bovine serum albumin, RIPA buffer, protease inhibitor cocktail, Dexamethasone and N^G -methyl-L-arginine acetate salt (L-NMMA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-iNOS antibody was purchased from Abcam (Cambridge, UK). Anti- β -actin, anti-NF- κ B p65, anti-NF- κ B p50 and rabbit IgG-horseradish peroxidase conjugated secondary antibodies were purchased from Cell Signaling (Danvers, MA, USA). Goat polyclonal anti-Lamin B and goat IgG-horseradish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture

RAW 264.7 mouse macrophage cells (TIB-71) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM supplemented with 10% Korean Cell Line Bank were cultured in DMEM supplemented with 10% FBS and 1% penicillin (100 U/mL)-streptomycin (100 $\mu\text{g}/\text{mL}$) in a humidified incubator with 5% CO_2 at 37°C.

Cell viability

RAW 264.7 cells were seeded on 96-well plates (5×10^4 cells/well), were treated with *A. tsao-ko* EtOH extracts or either of its main components for 1 h prior to LPS (1 $\mu\text{g}/\text{mL}$) stimulation for 24 h. MTT solution (5 mg/mL) was added to each well After 2 h of incubation at 37°C with 5% CO_2 , the supernatant was removed and dissolved in DMSO. The absorbance of each well was measured at a wavelength of 540 nm using a SpectraMax 190PC microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data are presented as the mean \pm standard deviation of three replicates.

Nitric oxide production assay

RAW 264.7 cells (5×10^4 cells/well in 96-well plates) were examined with *A. tsao-ko* EtOH extracts or either of its main components for 1 h prior to LPS (1 $\mu\text{g}/\text{mL}$) stimulation for 24 h. Nitrite in culture medium was measured by using Griess reagent (*N*-(1-naphthyl) ethylenediamine dihydrochloride and sulfanilamide). Absorbance was subsequently measured at 540 nm, using a SpectraMax 190PC microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells were seeded on 6-well plates (1×10^6 cells/well), were treated with main components ((+)-epicatechin and (-)-catechin) for 1 h prior to LPS (1 $\mu\text{g}/\text{mL}$) stimulation for 24 h. The total RNA was extracted using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The integrity of the RNA was evaluated using agarose gel electrophoresis and ethidium bromide staining. Briefly, 1 μg RNA was used as a template for each RT-PCR, using the SuperScript[®] III First-Strand Synthesis System and Platinum[®] PCR SuperMix which contains Taq DNA polymerase (Invitrogen; Thermo Fisher Scientific, Inc.), according to the

manufacturer's protocol. RT-PCR amplification was performed using MyGene™ Series Peltier Thermal Cycler (LongGene® Scientific Instruments Co., Ltd., Hangzhou, China) and AccuPower® Pfu PCR PreMix (Bioneer Corporation). The following conditions were used for each PCR reaction: 95°C for 5 min (1 cycle); 95°C for 30 sec, 55°C for 40 sec, and 72°C for 1 min (30 cycles); and a final extension phase at 72°C for 10 min. The following primers (Bioneer Corporation, Daejeon, Republic of Korea) were used for PCR amplification: TNF- α , 5'-CTGAGA CAATGAACGCTACA-3' (sense) and 5'-TTCTTCCACATCTATGCCAC-3' (antisense); IL-1 β , 5'-CTTTGAAGAAGAGCCCCATCC-3' (sense) and 5'-TTTGTCGTTGCTTGGTTCTC-3' (antisense); IL-10, 5'-CCTGGTAGAAGTGATGCCCCAGGCA-3' (sense) and 5'-CTATGCAGTTGATGA AGATGTCAA-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CAG GTACCAGGAGAGTG-3' (sense) and 5'-GTAGACTCCACGACATCTC-3' (antisense). PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Bands were captured using a ChemiDoc™ XRS system (Bio-Rad Laboratories) and quantified using Quantity One software version 4.6.3 (Bio-Rad Laboratories). Data was compared with the housekeeping gene GAPDH.

Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells (1×10^6 cells/well in 6-well plates) were examined with main components ((+)-epicatechin and (-)-catechin) for 1 h prior to LPS (1 μ g/mL) stimulation for 24 h. Levels of TNF- α , IL-1 β , and IL-10 in cell culture supernatants were quantified using platinum TNF- α , IL-1 β , and IL-10 ELISA kits (eBioscience, Inc., San Diego, CA, USA), according to the manufacturer's instructions.

Western blot analysis

Following treatment as indicated, cells were washed twice with PBS and lysed with RIPA buffer containing protease inhibitor cocktails. Cell lysates were clarified at $13,000 \times g$ for 10 min at 4°C, and the supernatants were subjected to Western blot analysis as described previously. All western blot analyses are representative of at least three independent experiments.

Preparation of nuclear extract

RAW 264.7 cells were plated at a density of 1×10^6 cells/well in 6-well plates and treated with main components ((+)-epicatechin and (-)-catechin) for 1 h prior to LPS (1 μ g/mL) stimulation for 30 min. cells were washed twice with ice-cold PBS prior to trypsinization and centrifugation at $90 \times g$ and 4°C for 5 min. Cells were then centrifuged at $20,000 \times g$ and 4°C for 5 min and resuspended in 200 μ l buffer (10 mM HEPES at pH 7.9, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF and 0.1 mM EDTA). After incubation on ice for 10 min, cells were lysed by the addition of 12.5 μ l of 10% NP-40. Cells were then centrifuged at $20,000 \times g$ for 2 min at 4°C, and the supernatants were collected as cytosolic extract. Pellets were resuspended in 50 μ l of extraction buffer (20 mM HEPES at pH 7.9, 0.4 M NaCl, 1 mM DTT, 1 mM PMSF, 1 mM EDTA and 1% NP-40) and incubated on ice for 10 min. Nuclear extract was collected by centrifugation at $15,000 \times g$ for 15 min at 4°C.

Statistical analysis

Statistical analysis was performed by a Student's t-test using Microsoft Excel 2007 software (Microsoft Corporation, Redmond, WA, USA). Results are presented as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LPS, a component of the cell wall of gram-negative bacteria, increases the levels of pro-inflammatory cytokines, including NO, TNF- α , IL-10, and IL-1 β , in macrophages and monocytes. It also induces diverse disease-related inflammatory responses (Willeaume et al. 1995). As shown in Table 1, we showed that 80% EtOH extract of the powdered fruit of *A. tsao-ko* decreased LPS-induced NO production in RAW 264.7 cells.

Table 1: Inhibitory effects of *A. tsao-ko* extract and isolated fractions on LPS-induced NO production RAW 264.7 cells

Fractions	NO IC ₅₀ (μ g/mL)	MTT IC ₅₀ (μ g/mL)
80% EtOH ext.	59.5 ± 1.8^a	>100
CH ₂ Cl ₂	<25	92.8 ± 1.6
EtOAc	<25	>100

BuOH	73.1 ± 3.3	>100
H ₂ O	>25	>100
PC ^b	27.3 ± 0.3 μM	>100

^aThe experiments were repeated in triplicate; values are expressed as mean ± SD. ^b*N*-Monomethyl-L-arginine (LNMMA) was used as a positive control.

The concentration of extract required for 50% inhibition (IC₅₀) of activity was 59.5 μg/mL. Among the four fractions obtained by serial solvent partition of *A. tsao-ko* (CH₂Cl₂ soluble fraction, EtOAc soluble fraction, *n*-BuOH soluble fraction, and remaining aqueous fraction), the CH₂Cl₂ and the EtOAc soluble fraction displayed potent inhibitory activity (IC₅₀ < 25 μg/mL). The concentration of *A. tsao-ko* Ext. and the four fractions that is possibly cytotoxic to RAW 264.7 cells was determined by MTT assay. Cytotoxicity effect was not caused by CH₂Cl₂ soluble fraction, EtOAc soluble fraction, *n*-BuOH soluble fraction, and aqueous fraction in LPS-induced RAW 264.7 cells. Therefore, bioassay guided purification of the active fraction, i.e., the EtOAc soluble fraction of *A. tsao-ko*, was conducted to purify the active components that display anti-inflammatory activity against LPS-treated RAW 264.7 cells.

Repeated column chromatography on silica gel and a RP-18 column of the EtOAc soluble fraction led to the isolation of eight compounds: 4-hydroxy-3-methoxy-benzoic acid (**1**) (Gonzalez-Baro 2008), *meso*-hannokinol (**2**) (Lee et al. 2008), (+)-hannokinol (**3**) (Lee et al. 2008), coumaric acid (**4**) (Salum et al. 2010), 4-hydroxy-benzoic acid (**5**) (Sivakumar et al. 2010), (+)-epicatechin (**6**) (Teresita et al. 2000), (-)-catechin (**7**) (Teresita et al. 2000), and myrciaphenone A (**8**) (Gupte 2009) (Fig. 1).

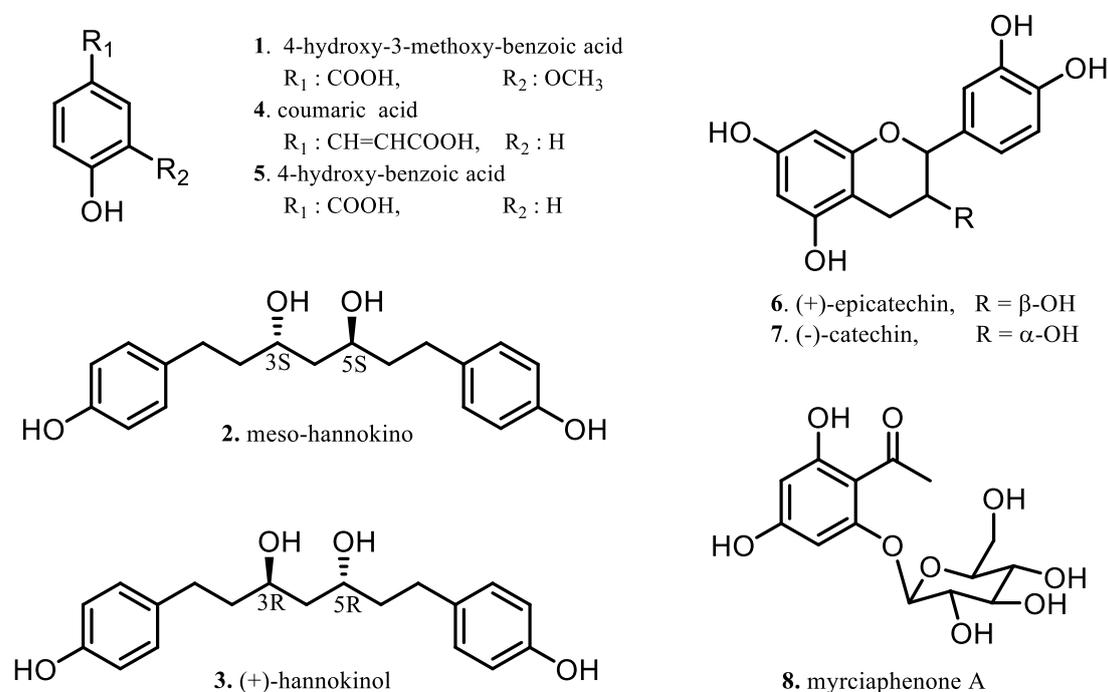


Figure 1: Structures of compounds isolated from the EtOAc fraction (1-8)

NMR and mass spectra were analyzed to determine the structures of the compounds. In addition, all physical and spectroscopic data obtained in the present study were compared with those of previously published manuscripts. Isolated components were tested for their inhibitory effect on NO production in LPS-stimulated RAW 264.7 cell culture system.

Among the isolated compounds, compounds **6** ((+)-epicatechin) and **7** ((-)-catechin) showed the highest activity (IC₅₀ = 70.6 μM and IC₅₀ = 73.3 μM, respectively) against NO production without cytotoxicity (Table 2, Fig. 2).

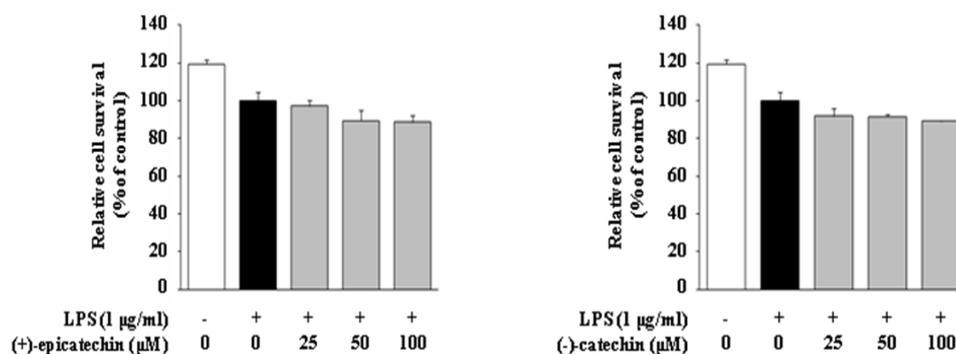
Table 2: Inhibitory effect of compounds 1-5 on LPS-induced NO production RAW 264.7 cells

Compounds	IC ₅₀ value (μM)	MTT assay IC ₅₀ (μM)
1	>100 ^a	>100
2	>100	>100
3	>100	>100
4	>100	>100
5	>100	>100
6	70.57 ± 0.3	>100
7	73.32 ± 0.5	>100
8	>100	>100
PC ^b	25.29 ± 0.4	>100

^aThe experiments were repeated in triplicate; values are expressed as mean ± SD.

^b*N*-Monomethyl-L-arginine (LNMMA) was used as a positive control.

A.



B.

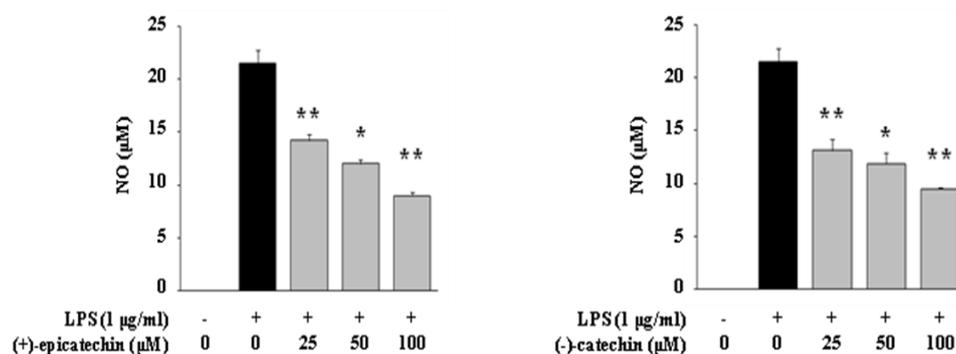


Figure 2: Effect of (+)-epicatechin (6) and (-)-catechin (7) on cytotoxicity and inhibition of NO production in **RAW 264.7 macrophages**. Cells were examined with (+)-epicatechin and (-)-catechin for 1 h prior to LPS (1 μg/mL) stimulation for 24 h. A. Cytotoxicity of (+)-epicatechin and (-)-catechin was determined by MTT assay. B. NO production was determined by measuring the concentrations of NO₂⁻ and NO₃⁻ in cell culture supernatants. The bars represent the mean ± SD of three independent experiments. Statistical significance is indicated (**P* < 0.05, ***P* < 0.01, compared with LPS-treated cells).

Constantly, we conducted the effect of (+)-epicatechin and (-)-catechin on iNOS expression induced by LPS using western blot analysis. As shown in Fig. 3, (+)-epicatechin and (-)-catechin effectively inhibited LPS-induced expression of iNOS at protein level in RAW264.7 cells.

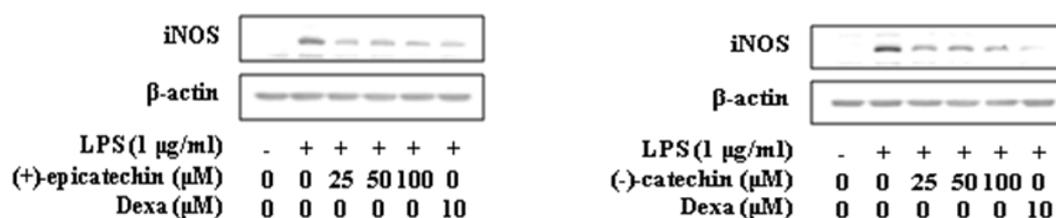


Figure 3: Effect of (+)-epicatechin and (-)-catechin on the expression of iNOS in LPS-stimulated RAW 264.7 cells. Expression levels of iNOS protein were determined by western blot. β-actin served as internal control western blot. Values represent the mean ± SD of three independent replicates.

These results indicate that treated with (+)-epicatechin and (-)-catechin (25-100 μM) significantly suppress LPS-induced NO production related to down-regulating iNOS expression in RAW264.7 macrophages. NF- κB plays a crucial role in general inflammatory reaction by controlling the activation of iNOS. The activation of NF- κB caused translocation of active NF- κB p50 and p65 from the cytoplasm to nucleus (Chen et al. 1995). In this study, we investigated whether (+)-epicatechin and (-)-catechin could suppress the translocation of NF- κB (p65 and p50) into the nucleus. We showed that (+)-epicatechin and (-)-catechin inhibited the translocation of NF- κB (p65 and p50) into the nucleus (Fig. 4).

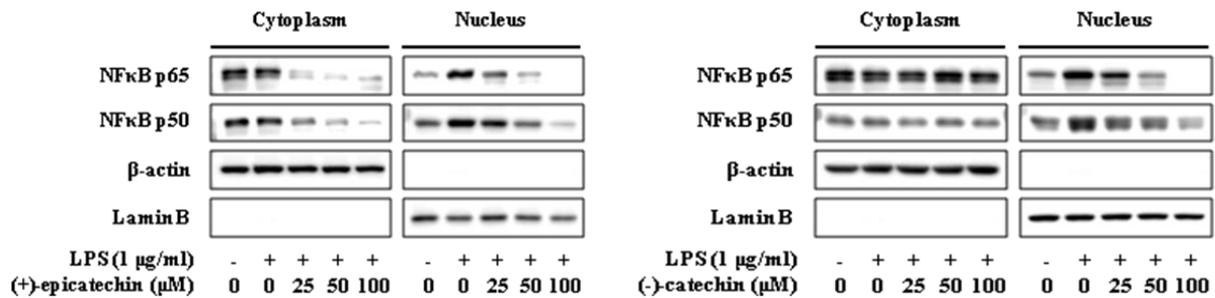
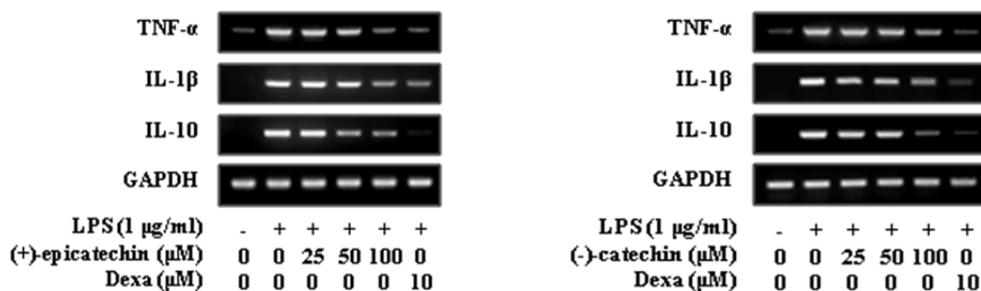


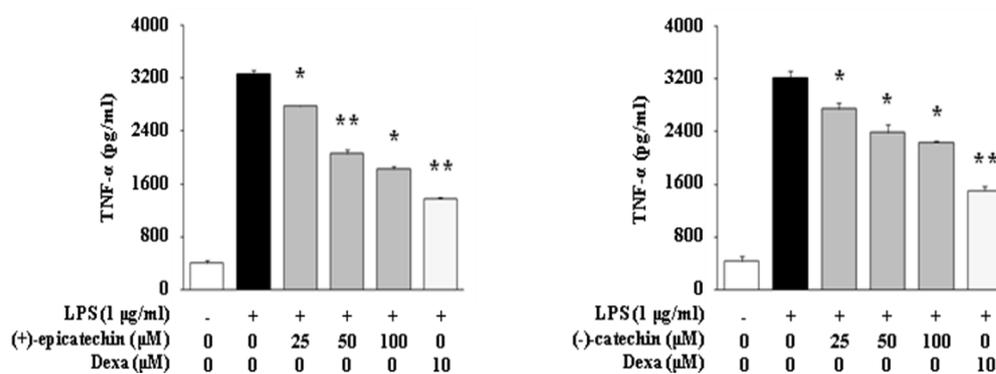
Figure 4: Regulatory effects of (+)-epicatechin and (-)-catechin on NF- κB nuclear localization in LPS-stimulated RAW 264.7 cells. Cells were pretreated with (+)-epicatechin and (-)-catechin for 1 h, followed by LPS (1 $\mu\text{g/ml}$) stimulation for 30 min. Cytosolic and nuclear extracts were analyzed via western blot with anti-NF- κB p65, anti-NF- κB p50, anti- β -actin or anti-Lamin B antibodies. β -actin served as a marker for the cytoplasm and Lamin B for the nucleus. Results shown are representative of at least three independent experiments.

Next, we examined the effect of (+)-epicatechin and (-)-catechin on inflammatory cytokines, such as TNF- α , IL-1 β , and IL-10 in LPS-stimulated RAW 264.7 cells. In this study, we measured the expression levels of these inflammatory cytokines by RT-PCR and ELISA analysis. As shown in Fig. 5, (+)-epicatechin and (-)-catechin inhibited the expression of TNF- α , IL-1 β , and IL-10 in a dose-dependent manner at both the mRNA and protein levels in RAW264.7 cells. These results showed that (+)-epicatechin and (-)-catechin have a potential activity on anti-inflammatory activity through the down-regulation of the NF- κB signaling pathway.

A.



B.



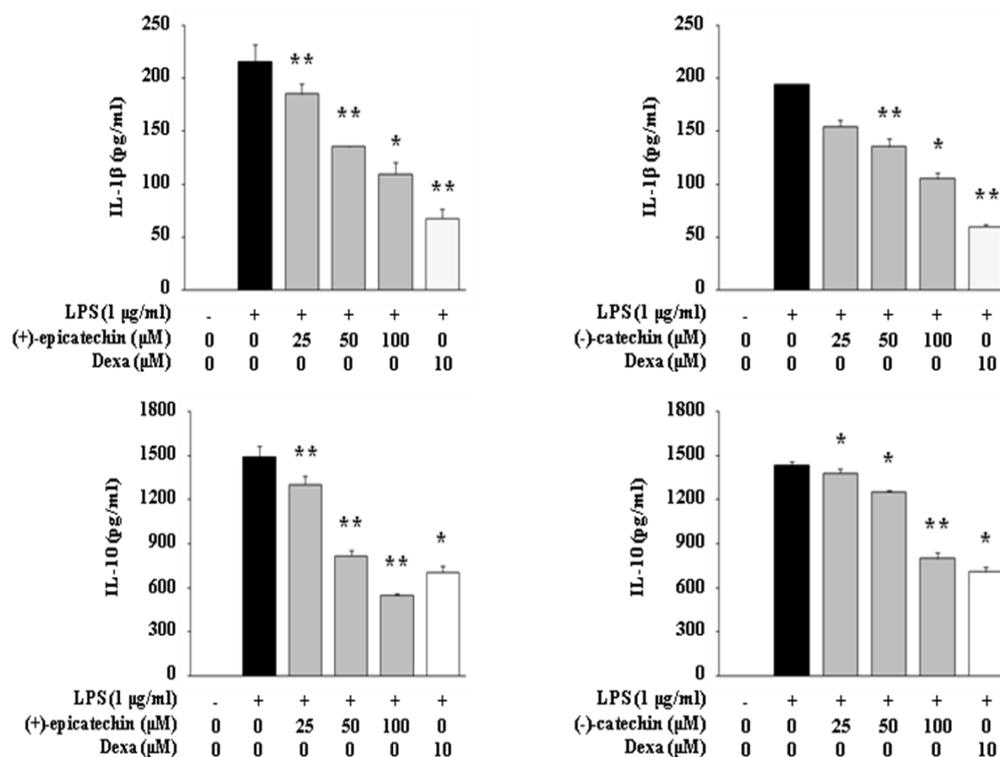


Figure 5: Effects of (+)-epicatechin and (-)-catechin on the expression and secretion of various cytokines in RAW 264.7 cells. A. The expression of TNF- α , IL-1 β , and IL-10 mRNA was determined by RT-PCR analysis. B. Cell culture supernatant was subjected to TNF- α , IL-1 β , and IL-10 cytokine ELISA as described in Materials and methods. Values represent the mean \pm SD of three independent experiments. Statistical significance is indicated (* $P < 0.05$, ** $P < 0.01$, compared with LPS-treated cells).

Discussion

Macrophages play an important role in both passive and active immunity and regulate anti-inflammatory mediators including NO and pro-inflammatory cytokines (Alleva et al., 2002; Eliopoulos et al., 2002). iNOS (NOS II), one of the isoforms of the nitric oxide synthase (NOS) family, is found in macrophages and hepatocytes. iNOS is not expressed in most resting cells; however, upon exposure to endogenous and exogenous stimulators, such as LPS or pro-inflammatory cytokines like TNF- α , IL-1 β and IL-10 the expression of the iNOS gene is increased and triggers several disadvantageous cellular responses, causing diseases, including inflammation, sepsis, and stroke (Nathan et al., 1992; Marletta et al., 1993; Duval et al., 1996). In order to extend the understanding of the anti-inflammatory effects of (+)-epicatechin and (-)-catechin, which showed the potent inhibitory activity, on the expression of inflammatory proteins, iNOS expression and the production of NO were evaluated in LPS-stimulated RAW 264.7 cells. Treatment with (+)-epicatechin and (-)-catechin inhibited NO production and iNOS expression in a dose-dependent manner in LPS-stimulated RAW 264.7 cells (Fig. 3). TNF- α is an element of the innate immune response against stimulus and IL-1 β is an important factor in the acute inflammation response (Strandberg et al., 2005). NF- κ B activation leads to release of pro-inflammatory proteins production. NF- κ B transcription factors are in the cytoplasm as an inactive state and when stimulation, NF- κ B transfer to nuclear localization (Wu et al., 2008).

Altogether, the results indicated that (+)-epicatechin and (-)-catechin, isolated from *A. tsao-ko*, effectively inhibited NO production in LPS-stimulated RAW 264.7 cells by suppression of iNOS expression and the production of inflammatory cytokines, such as TNF- α , IL-1 β and IL-10. In addition, we confirmed that (+)-epicatechin and (-)-catechin have anti-inflammatory activity via down-regulating nuclear localization of NF- κ B.

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Conflict of Interest: Authors have declared that no competing interests exist.

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