Full-length article

Modulation of inflammation-related genes of polysaccharides fractionated from mycelia of medicinal basidiomycete *Antrodia camphorata*[1]

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Abstract

Aim: To investigate the effect of water soluble-ethanol precipitation fraction (AC-1) and alkaline extraction-isoelectric precipitation fraction (AC-2) from *Antrodia camphorata* (Polyporaceae, Aphyllophorales) on lipopolysaccharide (LPS)-induced gene activation in mouse macrophages. **Methods:** The AC-1 and AC-2 fractions were prepared, and their effects on LPS-induced gene expression were monitored by Western blotting and RT-PCR. **Results:** Our results indicated that AC-2, but not AC-1 dose-dependently (50–200 mg/L) inhibited LPS-induced nitric oxide production as well as the protein and the mRNA expression of the inducible nitric oxide synthase (iNOS) gene. Neither AC-1 nor AC-2 inhibited LPS-induced cyclooxygenase-2 gene expression. Using the cytokine array assay, it showed that AC-2 also had the ability to inhibit LPS-induced the protein expression of interleukin (IL)-6, IL-10, the monocyte chemoattractant protein (MCP)-5, and regulated upon activation, normal T-cell expressed, and presumably secreted (RANTES). Like iNOS, AC-2 inhibiting LPS-induced IL-6 and IL-10 secretion resulted from inhibiting their mRNA expression. **Conclusion:** It was suggested that alkaline extraction-isoelectric precipitated the polysaccharide fraction of *A camphorata* and had the ability to inhibit LPS-induced iNOS, IL-6, IL-10, MCP-5, and RANTES expression in mouse macrophages.

Introduction

Antrodia camphorata is a medicinal mushroom in Taiwan well-known as a folk medicine for the treatment of intoxication caused by alcohol or drugs, and is also used in the treatment of diarrhea, abdominal pain, hypertension, skin itching, and liver cancer[1]. It specifically parasitizes on the inner cavity of the wood of the endemic species *Cinnamomum kanehirai* and grows extremely slowly in nature. Therefore, the submerged liquid culture using the parasitic hyphae of *A camphorata* for the production of mycelia has being become one of the most important methods. The biological functions of *A camphorata* have been studied in various bioassays. In the research of aqueous extracts from *A camphorata* mycelia, the suppression of oxidative hemolysis and lipid/protein peroxidation in erythrocytes has been demonstrated[2]. The inhibition ability of its fermented filtrate on H_2O_2-induced lipid peroxidation and its extracts on the CCl_4-induced rat liver damage was also reported[3,4]. The extracts also showed a concentration-dependent inhibition of *N*-formyl-methionyl-leucyl-phenylalanine or phorbol 12-myristate 13-acetate-induced reactive oxygen species production in peripheral human neutrophils or mononuclear cells[5]. Moreover, the fermented culture broth of *A camphorata* has been found to have inhibitory capabilities on the production of lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α, interleukin (IL)-1β, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression[6]. Water extracts of submerged culture of *A camphorata* have being shown to have antitumor activity[7–10]. Polysaccharides extracted from fruiting bodies or cultured mycelia of *A camphorata* exhibit antihepatitis B

virus activity[11]. As the polysaccharide components of mushroom species have been established as the most promising pharmacologically-active anti-tumor portion[12,13], the partially-purified polysaccharide component from *A camphorata* mycelia was also found to have antitumor effects on both the *in vitro* and *in vivo* model[14]. Fungous LPS from *A camphorata* was found to inhibit bacterial LPS-induced intercellular adhesion molecule-1 expression and the subsequent monocyte adhesion process in vascular endothelial cells[15]. However, there is no report about the anti-inflammatory activity of the *A camphorata* polysaccharide fraction.

Cells of the monocytic/macrophage lineage play an important role in response to inflammation and infection, as the main players performing innate immune response and contributors rendering adaptive immune response. Bacterial LPS, a component of the gram-negative bacteria cell wall, activates macrophages to secrete pro-inflammatory cytokines, such as IL-6, TNF-α, and secondary mediators such as leukotrienes, prostaglandins (PG) and nitric oxide (NO). The iNOS is responsible for high output formation of NO by macrophages against invading microorganisms[16] or tumor cells[17]. Although large production of NO by iNOS may promote host defensive potency, it also contributes to septic and hemorrhagic shock, rheumatoid arthritis and chronic infections[18]. The COX-2 is an essential enzyme in the production of inflammatory PG, and is inducible in activated macrophages, fibroblasts, and several other cell types. *In vivo,* the expression of COX-2 is observed in chronic inflammatory conditions such as arthritis[19] and human colon cancer tissue[20]. *In vitro*, COX-2 expression was induced in response to stimuli such as LPS and growth factors[21,22]. Therefore, the screening of chemopreventive products from natural resources with the functions focused on inhibiting NO and PGE$_2$ production would be an effective and direct method.

The anti-inflammatory effects of the polysaccharide fractions from *A camphorata* have never been studied. The objective of this study was to investigate the effects of the polysaccharides from the sequential extractions of *A camphorata* mycelia on LPS-induced inflammation-related gene expression by using mouse macrophages.

Materials and methods

Materials LPS (*Escherichia coli* 055:B5) and anti-β-actin antibodies were purchased from Sigma-Aldrich (St Louis, MO, USA). SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce (Rockford, IL, USA). Antibodies against COX-2, Sp1 and TLR4 were purchased from Santa Cruz (Santa Cruz, CA, USA). The antibody against NF-κB p65 was from Abcam (Cambridge, UK). The antibody against iNOS/NOS II was from Upstate (Lake Placid, NY, USA). PRO-PREP™ Protein Extraction Solution was from iNtRON Biotechnology (Kyungki-Do, Korea). Bio-Rad protein assay was purchased from Bio-Rad (Hercules, CA, USA). TRIzol reagent and SuperScript II were from Invitrogen (Carlsbad, CA, USA).

Cell culture RAW 264.7 cells were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured at 37 °C in a 5% CO$_2$ atmosphere in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum FBS (Biowest, Rue de la Caille, Nuaille, France). The endotoxin concentration of FBS was below 0.15 ng/mL, which was certificated by Biowest.

Pretreatment of the mycelial powder Prior to the extraction of the polysaccharides, a supercritical fluid carbon dioxide (SC-CO$_2$, 99.5% in purity) extraction was performed to remove some inherently existing oil soluble substances in order to minimize the interference to the polysaccharides, according to a previous report[23]. Briefly, 100 g of the lyophilized mycelial powder was weighed and added with 5% n-hexane to serve as a modifier. The extraction was carried out at 60 °C and 5000 psi in a supercritical gluid extraction apparatus (ISCO SFX™ 2-10, Isco, Lincoln, NE, USA) attached with a modified extraction vessel. In the beginning, a dynamic continuous extraction was adopted at a flow rate of 1 mL SC-CO$_2$/min for 1 h, and was then followed by a static extraction for an additional 1 h. The oil soluble extracts were collected in 95% ethanol. The residue (ACR$_1$) remaining in the extraction vessel was used in the subsequent experimentations.

Preparation of AC-1 and AC-2 The method of Ker *et al*[23] was followed for the preparation of the polysaccharide fraction AC-1 and AC-2. For the AC-1 fraction, ACR$_1$ (100 g) was extracted with a reflux 3 times with 2000 mL double distilled water (DDW) at 90 °C and was constantly stirred at 400 r/min for 2 h. The extracts were filtered with aspiration after cooling; the residue (ACR$_2$) was kept for further experimentation. One mol/L HCl was added to the filtrate to adjust the pH to 4.0, and then a 2 fold volume of ethanol (95%) was added to precipitate the water-soluble polysaccharides, which were collected and further purified in 400 mL of hot water (100 °C). Finally, the water-soluble polysaccharides were precipitated with the addition of a 3 fold volume of ethanol (95%), and then collected and lyophilized (AC-1). For the AC-2 fraction, the residue ACR$_2$ was added with 1000 mL of 2% NaOH and extracted 3 times, and constantly stirred at 400 r/min and refluxed. The extracts were filtered with aspira-

tion after cooling; the residue was stored for the other experimentation. The filtrate was collected and adjusted to pH 4.0 with 12 mol/L H_2SO_4, and left to stand overnight. The sediment was collected, dialyzed and lyophilized to recover the isoelectric precipitate (AC-2). The yields of the polysaccharide of *A camphorata* were 2.92% (*w/w*) in the AC-1 fraction, and 10.38% (*w/w*) in the AC-2 fraction. The average molecular masses of AC-1 and AC-2 were 508 kDa and 394 kDa by gel permeation chromatography analysis.

Cell viability assay RAW 264.7 cells were plated in a 6 well plate at 2.8×10^6 cells per well and allowed to adhere to the plate overnight; then the culture medium were refreshed by new medium containing AC-1 or AC-2. After the introduction of AC-1 or AC-2 for 1 h, the cells were stimulated with LPS (500 µg/L). The cell numbers were counted after 18 h of exposure to LPS. Cell viability was examined using trypan blue exclusion and counted using hemocytometer and phase contrast microscopy.

Nitrite determination RAW 264.7 cells were cultured in a 24 well plate at a density of 5×10^5, 1 d before LPS treatment. The cells were treated with AC-1 or AC-2 1 h before the introduction of 500 µg/L LPS. After LPS treatment for 18 h, the extracellular medium containing nitrite ion (NO_2^-) was used as an indication of NO production, and the amount of NO_2^- in the culture medium was determined according to the colorimetric method by using Griess reagent. The isolated supernatants were incubated with an equal volume of Griess reagent and incubated at room temperature for 10 min. Absorbance at 540 nm was then read and compared with known standard solutions of $NaNO_2$.

Protein extraction RAW 264.7 cells were precultured in a 3.5 cm dish for cell lysate extraction 1 d before LPS treatment. The cells were treated with each polysaccharide 1 h prior to the introduction of 500 µg/L LPS. The total cell lysates were prepared by lysing the cells in buffer containing PRO-PREP™ Protein Extraction Solution containing 10 mmol/L NaF and 1 mmol/L orthovanadate at 4 °C for 15 min and centrifuged at $7500 \times g$ at 4 °C for 30 s. The supernatants containing the protein extracts were stored at -80 °C for stabilization.

Nuclear extract preparation The cells from the 10 cm dishes were washed twice with phosphate-buffered saline PBS and scraped in 1 mL of PBS. The cells were collected by centrifuging at $7500 \times g$ for 30 s, resuspended in 0.4 mL of buffer A [10 mmol/L *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid HEPES, pH 7.9, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.5 mmol/L EDTA] at 4 °C for 10 min. Then nuclei were pelleted by centrifugation at $7500 \times g$ for 30 s. The pellets were resuspended in 0.1 mL of buffer C [20 mmol/L HEPES,

pH 7.9, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, and 25% glycerol (*v/v*)] at 4 °C for 20 min. The suspension was centrifuged by centrifugation at $7500 \times g$ for 2 min. The supernatants were collected and stored at -80 °C until use. Both buffers A and C contained the following protease inhibitors: 0.5 mmol/L phenylmethylsulfonyl, 1 mmol/L orthovanadate, 2 µg/mL pepstatin A, and 2 µg/mL leupeptin.

Western blotting Protein concentration was determined by using the Bio-Rad protein assay reagent. The extracted protein (30 µg) was separated in 8% SDS-PAGE and transferred to a polyvinylidene fluoride PVDF membrane. After blotting, the membrane was incubated with specific primary antibodies overnight at 4 °C, then further incubated for 1 h with a horseradish peroxidase HRP-conjugated secondary antibody and eventually incubated with SuperSignal West Pico Chemiluminescent Substrate for 2 min. The bounded antibodies were detected by Kodak Digital Science™ (Image Station 4000 MM, New Haven, CT, USA).

RT-PCR RAW 264.7 cells were cultured in a 3.5 cm dish the day before LPS treatment. The cells were treated with each inhibitor 1 h prior to the introduction of 500 µg/L LPS. The RNA was extracted with TRIzol reagent and detected by RT-PCR technique. RT was performed on 2 µg of total RNA by random primers (9 mers) and SuperScript II, then 1/20 volume of reaction mixture was pooled, followed by PCR with mouse COX-2 specific primers (5'-CAGCAAATCCT-TGCTGTTCC-3' and 5'-TGGGCAAAGAATGCAAACATC-3'), mouse iNOS specific primers (5'-GTCAACTGCAAGA-GAACGGAGAAC-3' and 5'-GAGCTCCTCCAGAGGGTAG-GCT-3'), mouse IL-6 specific primers (5'-AGTAAGTTCCT-CTCTGCAAGAGACT-3' and 5'-CACTAGGTTTGCCGAGTA-GATCTC-3'), mouse IL-10 specific primers (5'-CGTCGGATC-CGCCATGCCTGGCTCACCACTGCT-3' and 5'-CGTCTCTA-GATTAGCTTTTCATTTTGATCA-3'), or β-actin specific primers (5'-CCTAAGGCCAACCGTGAAAA-3' and 5'-TCT-TCATGGTGCTAGGAGCCA-3'). Cycle numbers of PCR were 25 cycles for each primer. The RT-PCR products were separated on 1% agarose gel and analyzed.

Protein chip assay RayBio Mouse Cytokine Antibody Array I was purchased from RayBiotech (Norcross, GA, USA). It was employed to assay cell culture conditioned medium and used according to the manufacturer's instructions. The confluent cells were replaced from medium containing 10% FBS to serum-free medium; in the meantime, AC preparations were added into the cells. LPS was added 1 h after AC treatment. The conditioned medium was collected after LPS treatment for 9 h. Twenty-two different cytokines were evaluated: granulocyte-colony stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor

(GM-CSF), IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40/p70, IL-12p70, IL-13, IL-17, interferon-γ, monocyte chemoattractant protein-1 (MCP-1), monocyte chemoattractant protein-5 (MCP-5), regulated upon activation, normal T-cell expressed, and presumably secreted (RANTES), stem cell factor(SCF), sTNFR1, TNF-α, thrombopoietin, and vascular endothelial growth factor. The bounded cytokines were detected by biotin-labeled antibodies and horseradish peroxidase-labeled streptavidin, and were detected by SuperSignal West Pico Chemiluminescent Substrate for 2 min. The bounded antibodies were analyzed by Kodak Digital Science™ (Image Station 4000MM).

Statistical analysis Differences among the data of the LPS-treated control and further treatments with each polysaccharide fractions were analyzed by Student's *t*-test. Statistical probability was expressed as *P*<0.05.

Results

Effects of AC-1 and AC-2 on the viability of RAW 264.7 cells Before testing the effects of AC-1 and AC-2 on LPS-induced nitrite and COX-2 expression, we tested the effects of AC-1 and AC-2 on cell viability. After 18 h treatment of AC-1 or AC-2 and LPS in mouse macrophage RAW 264.7 cells, cell numbers were counted individually. The results presented in Figure 1 demonstrate that cell numbers were not affected by AC-1 at 100 mg/L or AC-2 at 50 mg/L. A minute decrease was found at 200 mg/L of AC-1 mg/L or 100 mg/L, and 200 mg/L of AC-2 (Figure 1). After statistical analysis, we suggested that there was no significant cytotoxicity under 200 mg/L of AC-1 or AC-2. Further study on the inflammation-related gene expression affected by AC-1 and AC-2 under the concentration of 200 mg/L was conducted.

Effects of AC-1 and AC-2 on LPS-induced NO production NO synthesized by iNOS has been implicated as a mediator of inflammation. The inhibitory effects of AC-1 and AC-2 on LPS-induced NO production were investigated. As shown in Figure 2, NO production was greatly increased after LPS treatment for 18 h. Only AC-2, but not AC-1, showed a dose-dependent reduction of NO production between 50 and 200 mg/L. This suggests that the AC-2 fraction has the ability to inhibit LPS-induced NO production.

Effects of AC-1 and AC-2 on LPS-induced expression of the iNOS protein and mRNA In general, iNOS is not present in the resting cells, but is induced by various stimuli. Increased expression of iNOS has been associated with inflammatory disorders. Because LPS-induced NO production could be reduced by AC-2, we further tested the effects
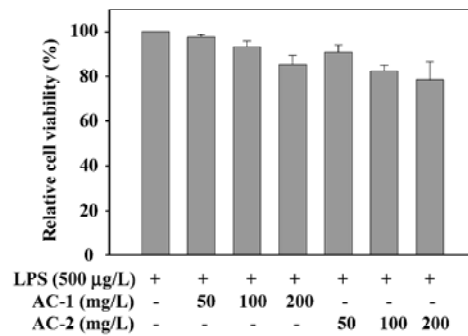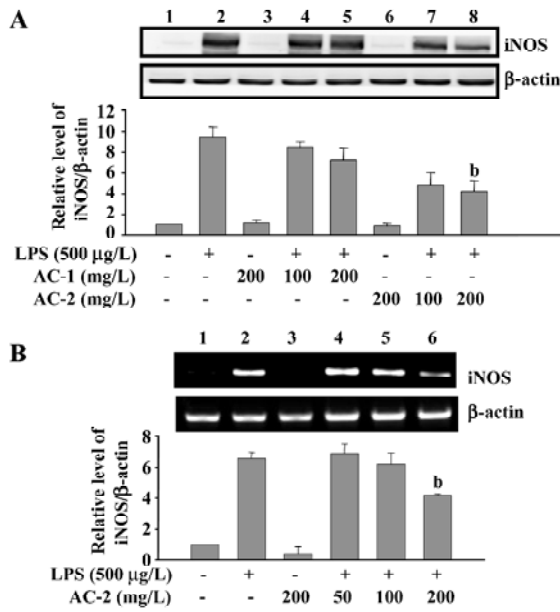

Figure 1. Effects of AC-1 and AC-2 on the cell viability of RAW 264.7 macrophages. Aliquots of 2.8×10^6 of the macrophage cell line RAW 264.7 were cultured in a 6 well plate with 10% FBS supplemented with DMEM medium 1 d before the addition of AC-1, AC-2, and LPS. The cells were treated with 0, 50, 100 and 200 mg/L of AC-1 or AC-2 for 1 h, then LPS (500 µg/L) was added for 18 h. Cell viability was monitored by using phase contrast microscopy. The cells were harvested and the cell number was determined by using a hemocytometer and trypan blue exclusion. Results are presented as mean±SD of 3 independent experiments.
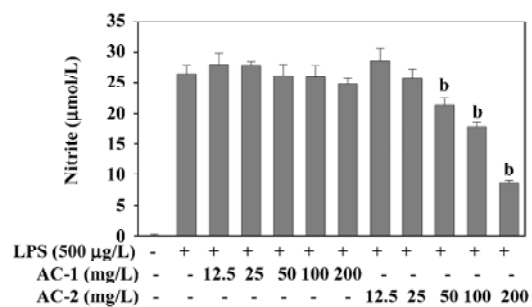
Figure 2. Effects of AC-1 and AC-2 on LPS-induced NO production in RAW 264.7 cells. Aliquots of 5×10^5 of the macrophage cell line RAW 264.7 were cultured in a 24 well plate with 10% FBS supplemented with DMEM medium 1 d before the addition of AC-1, AC-2, and LPS. AC-1 or AC-2 was added 1 h prior to the treatment of LPS for 18 h. The extracellular medium containing NO_2^- was determined by the Griess reagent system. Measurements were performed in triplicate and presented as mean±SD. Statistically significant difference compared to LPS-activated cells (^bP<0.05).

of AC-1 and AC-2 on the LPS-induced iNOS protein and mRNA expression. As shown in Figure 3, AC-2, but not AC-1, dose-dependently inhibited the LPS-induced iNOS protein (Figure 3A) and mRNA (Figure 3B) expression in RAW 264.7 cells. This suggests that the inhibitory effects of AC-2 on LPS-induced NO production is due to the suppression of LPS-induced iNOS gene expression.

Effects of AC-1 and AC-2 on LPS-induced expression of COX-2 protein and mRNA Arachidonic acid is released from

Figure 3. Effects of AC-1 and AC-2 on LPS-induced iNOS expression in RAW 264.7 cells. (A) aliquots of 4×10^6 of the macrophage cell line RAW 264.7 were cultured in a 6 cm dish with DMEM supplemented with 10% FBS 1 d before LPS treatment. AC-1 and AC-2 were added 1 h prior to the addition of LPS. The total cell lysates were extracted 15 h later and analyzed by Western blotting. (B) aliquots of 2.8×10^6 of the macrophage cell line RAW 264.7 were cultured in a 3.5 cm dish with 10% FBS supplemented with DMEM medium 1 d before the addition of AC-1, AC-2, and LPS. The inhibitors were added 1 h prior to introduction of LPS. Total RNA was extracted 9 h later. Total RNA (2 µg) was applied to detect iNOS mRNA by the RT-PCR technique as described. The RT-PCR products were separated on 1% agarose gel and digitally imaged after staining with ethidium bromide. The traces represent the means from 3 independent experiments, which are presented as mean±SD. The ratio of iNOS to β-actin expression observed with the control is set at 1. ^bP values are calculated by comparing with the LPS-treated group.

the cell membrane catalyzed by phospholipase A_2, converted into PGH_2 by cyclooxygenase, and further metabolized to PGE_2. The effects of AC-1 and AC-2 on the expression of the COX-2 protein and mRNA were investigated. As shown in Figure 4, 200 mg/L AC-1 or 200 mg/L AC-2 did not suppress either the LPS-induced COX-2 protein (Figure 4A) or mRNA (Figure 4B) expression. Furthermore, we detected the PGE_2 concentration in the culture medium. We also found that that 200 mg/L AC-1 or 200 mg/L AC-2 did not inhibit LPS-induced PEG_2 production (data not shown). These data suggest that AC-1 and AC-2 had no inhibitory effect on LPS-induced COX-2 gene expression and PGE_2 production.
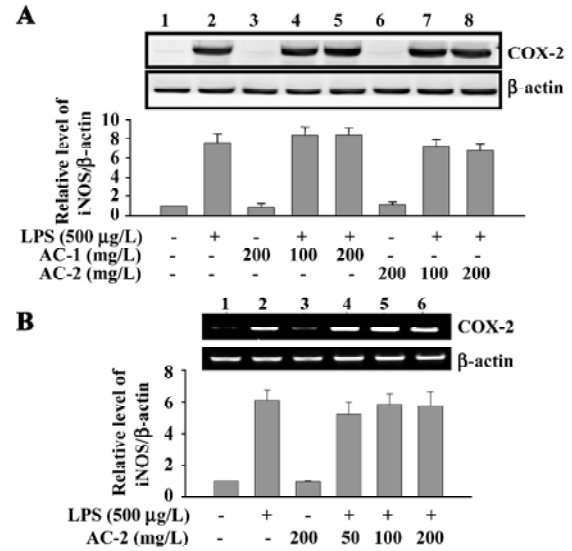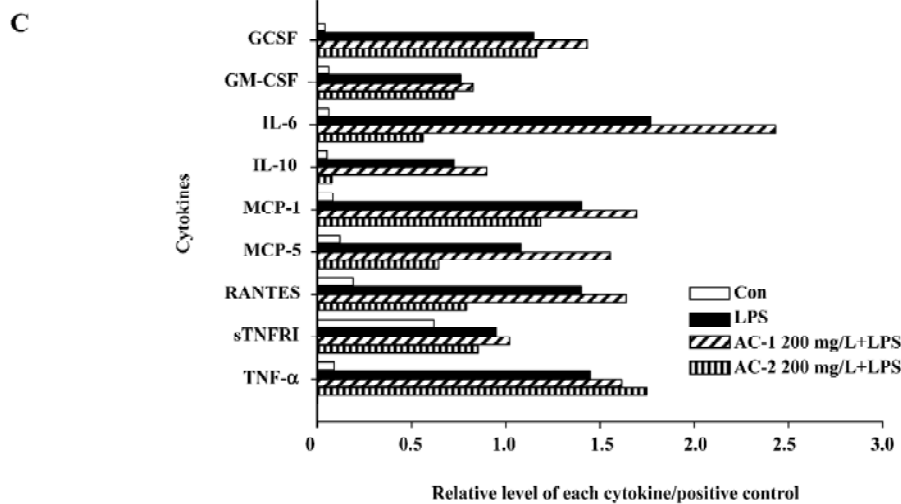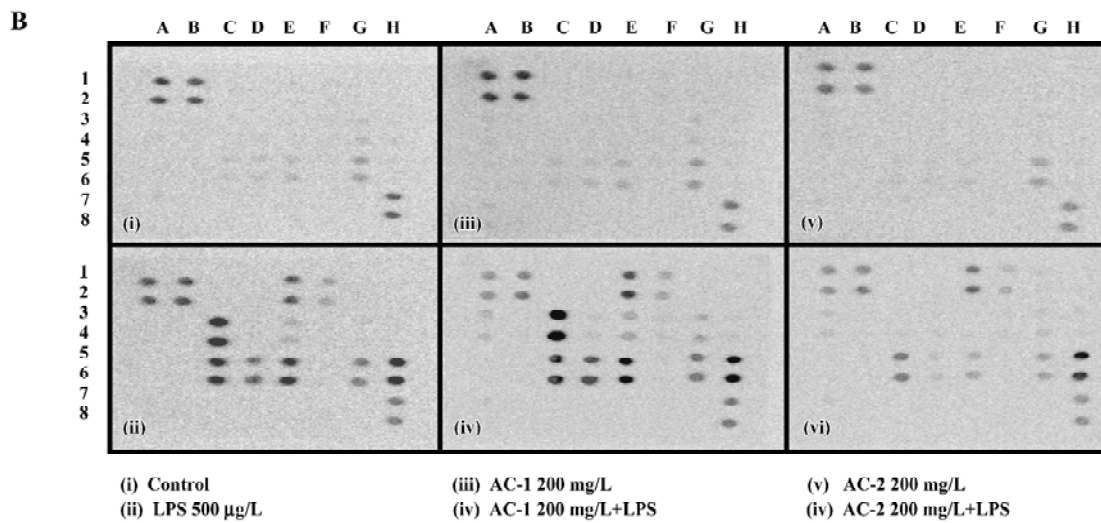

Figure 4. Effects of AC-1 and AC-2 on LPS-induced COX-2 expression in RAW 264.7 cells. (A) aliquots of 2.8×10^6 of the macrophage cell line RAW 264.7 were cultured in a 3.5 cm dish with DMEM supplemented with 10% FBS 1 d before LPS treatment. AC-1 or AC-2 was added 1 h prior to the addition of LPS. The total cell lysates were extracted 5 h later and analyzed by Western blotting. The traces represent the means from 3 independent experiments, which are presented as mean±SD. (B) aliquots of 2.8×10^6 of the macrophage cell line RAW 264.7 were cultured in a 3.5 cm dish with 10% FBS supplemented with DMEM medium 1 d before the addition of AC-1, AC-2 and LPS. The AC-1 or AC-2 was added 1 h prior to the introduction of LPS. Total RNA was extracted 5 h later. Total RNA (2 µg) was applied to detect COX-2 mRNA by the RT-PCR technique as described. The RT-PCR products were separated on 1% agarose gel and digitally imaged after staining with ethidium bromide. The traces represent the means from 3 independent experiments, which are presented as mean±SD. The ratio of COX-2 to β-actin expression observed with the control is set at 1.

Effects of AC-1 and AC-2 on LPS-induced cytokine expression In order to widely observe the effects of AC-1 and AC-2 on LPS-induced cytokine expression, the mouse cytokine antibody array (Figure 5A) was applied. When the cells were incubated with AC-1 or AC-2 for 10 h, neither AC-1 nor AC-2 changed the cytokine expression pattern, compared to the control cells (Figure 5Bi, iii, v). After LPS treatment for 9 h, the protein expression of GCSF, GM-CSF, IL-6, IL-10, MCP-1, MCP-5, RANTES, sTNFRI, and TNF-α all increased (Figure 5Bi, ii). Each LPS-increased dot was quantified and the result is shown in Figure 5C. It indicated that AC-2, but not AC-1, inhibited LPS-induced protein expression of IL-6, IL-10, MCP-5 and RANTES, and neither AC-1 nor AC-2 inhibited LPS-induced GCSF, GM-CSF, MCP-1, sTNFRI, and
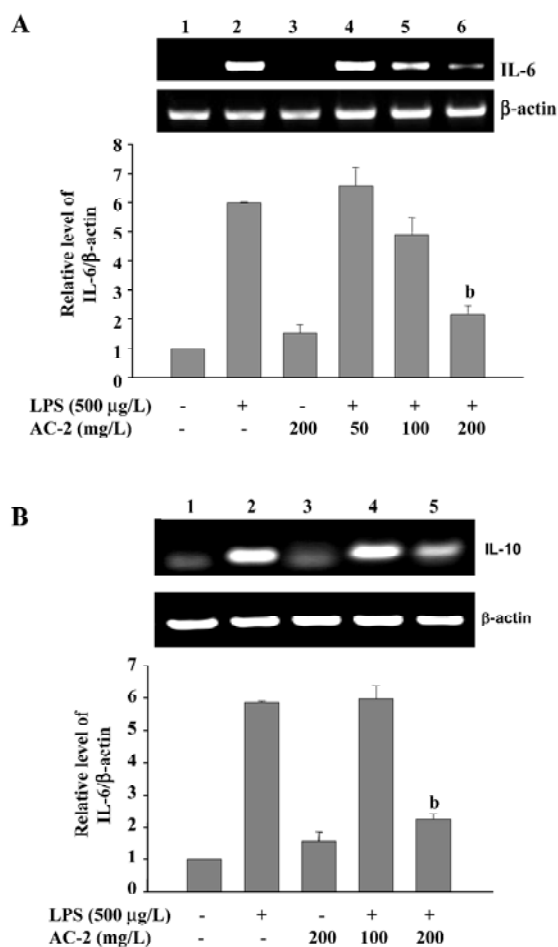
A

	A	B	C	D	E	F	G	H
1	POS	POS	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
2	POS	POS	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p70	IL-12 p70	IL-13
4	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p70	IL-12 p70	IL-13
5	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
6	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
7	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos
8	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos

(i)   Control
(ii) LPS 500 μg/L
(iii) AC-1 200 mg/L
(iv) AC-1 200 mg/L+LPS
(v)   AC-2 200 mg/L
(iv) AC-2 200 mg/L+LPS

Figure 5. Cytokine profile of AC-1 and AC-2 on LPS-activated RAW 264.7 cells. (A) each cytokine is represented by duplicate spots in the location shown. (B) aliquots of 2.8×10^6 of the macrophage cell line RAW 264.7 were cultured in a 3.5 cm dish with DMEM supplemented with 10% FBS 1 d before LPS treatment. AC-1 or AC-2 was added with changing fresh serum-free medium 1 h prior to the treatment of LPS. After LPS treatment for 9 h, the medium were collected for cytokine array assay. (C) the ratio of average intensity of each cytokine spot to each positive spot on the same chip is shown.
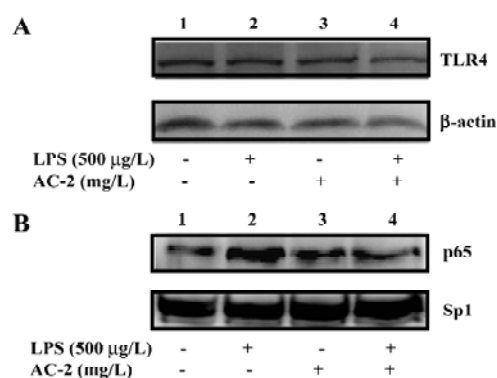
TNF-α protein secretion in mouse macrophages.

Effects of AC-2 on LPS-induced IL-6 and IL-10 mRNA expression We further identified whether AC-2 inhibited LPS-induced mRNA expression of IL-6 and IL-10. The mRNA expression of IL-6 and IL-10 were monitored by RT-PCR. AC-2 dose-dependently inhibited LPS-induced IL-6 mRNA expression (Figure 6A); it also inhibited IL-10 mRNA expression (Figure 6B). The inhibitory effect on LPS-induced IL-6

and IL-10 gene expression by AC-2 is important in the regulation of IL-6 and IL-10 genes in mouse macrophages.

Effects of AC-2 on TLR4 and LPS-induced NF-κB translocation Because AC-2 has the ability to repress some specific gene activation by LPS, we further identified the effects of AC-2 on the upstream linkage of LPS signaling. TLR4 is a membrane receptor for the recognition of LPS. After binding to LPS, it will activate the NF-κB pathway to induce the expression of many inflammatory genes[24]. Next, we analyzed whether TLR4 and NF-κB were affected by LPS and AC-2. As shown in Figure 7A, the protein expression of the TLR4 receptor was unchanged after LPS and AC-2 treatment. NF-κB p65 translocation is one of the important signal pathways activated by LPS; therefore, the effect of AC-2 on LPS-induced NF-κB p65 translocation was monitored. It showed that LPS increased the nuclear translocation of p65, and AC-2 inhibited LPS-induced p65 translocation (Figure 7B).

Figure 6. Effects of AC-2 on LPS-induced IL-6 and IL-10 mRNA expression in RAW 264.7 cells. Aliquots of 2.8×10^6 of the macrophage cell line RAW 264.7 were cultured in a 3.5 cm dish with 10% FBS supplemented with DMEM medium 1 d before the addition of AC-1, AC-2, and LPS. The AC-1 or AC-2 was added 1 h prior to the introduction of LPS. Total RNA was extracted 9 h later. Total RNA (2 μg) was applied to detect IL-6 (A) and IL-10 (B) mRNA by the RT-PCR technique as described. The RT-PCR products were separated on 1% agarose gel and digitally imaged after staining with ethidium bromide. The traces represent the means from 3 independent experiments, which are presented as mean±SD. The ratio of IL-6 or IL-10 to β-actin expression observed with the control is set at 1. $^b P$ values are calculated by comparing with the LPS-treated group.

Figure 7. Effects of AC-2 on protein expression of TLR4 and on LPS-induced NF-κB p65 translocation. Aliquots of 2.8×10^6 of the macrophage cell line RAW 264.7 were cultured in a 3.5 cm dish with 10% FBS supplemented with DMEM medium 1 d before the addition of AC-1, AC-2, and LPS. The AC-1 or AC-2 was added 1 h prior to the introduction of LPS. (A) the total cell lysates were extracted 9 h later and analyzed by Western blotting using anti-TLR4 and β-actin (as an internal control) antibodies. (B) the nuclear extracts were prepared 20 min after LPS treatment and analyzed by Western blotting using anti-NF-κB p65 and Sp1 (as an internal control) antibodies.

Discussion

A camphorata (Polyporaceae, Aphyllophorales), the cause of brown heart rot of *Cinnamomum kanahirai* Hay in Taiwan, is a new basidiomycete and a scarce traditional medicine that has attracted great attention due to its antioxidant and antitumor effects *in vivo* and *in vitro*. It was identified in 1990 as a traditional Chinese medicine in Taiwan. The present study was undertaken to elucidate the pharmacological and

molecular effects of the partially purified polysaccharide fraction from *A camphorate* on LPS-induced inflammatory mediators in macrophages. The results indicate that AC-2 is an effective inhibitor of LPS-induced inflammatory mediators such as IL-6, IL-10, MCP-5, RANTES, and NO. This indicates that the polysaccharide fraction of *A camphorate* appears to have some potential bioactive compounds in inhibiting LPS-induced inflammatory gene expression, including IL-6, IL-10, iNOS, MPC-5, and RANTES.

IL-6 was originally identified as a B-cell differentiation factor, but it is now known to play a central role in host defense because it is released in response to infection, burns, trauma and tumor, and its functions range from key roles in acute phase protein induction to B- and T-cell growth and differentiation[25]. IL-6 can induce a variety of acute-phase proteins, such as fibrinogen, serum amyloid A, and the C-reactive protein in human hepatocytes[26]. IL-6-deficient mice also show a severely defective inflammatory acute-phase response after tissue damage or infection[27]. An unregulated, high-level production of IL-6 could generate an undesired inflammatory state, a circumstance that can cause various diseases. Several reports indicate that IL-6 is implicated in the pathogenesis of a number of human disorders, including rheumatoid arthritis and inflammatory bowel disease[28,29]. AC-2, a partially purified polysaccharide fraction from *A camphorate* could inhibit LPS-induced IL-6 gene expression, which could provide a therapeutic clue in IL-6-related diseases.

IL-10 was discovered as a cytokine synthesis inhibiting factor, and its principal function is the activity inhibition of Th1 cells[30]. IL-10 has multiple biological functions on different cell types. In macrophages, IL-10 inhibits ligand-induced activation and the production of pro-inflammatory cytokines from macrophages[31,32]. IL-10 inhibits the proliferation as well as cytokine synthesis of CD4^{+} T-cells[33]. Other immunosuppressive effects have also been reported on eosinophils[34], neutrophils[35], and dendritic cells[36]. In contrast to these immunosuppressive effects, IL-10 has been shown to have the abilities of immunostimulation on cytotoxic T-cells[37], and is a growth costimulator for thymocytes and mast cells[38]. In this study, AC-2 also inhibited LPS-induced IL-10 gene expression.

Besides IL-6 and IL-10, AC-2 also reduced LPS-induced iNOS gene expression and NO release in RAW 264.7 cells. The expression inhibition of the iNOS protein responsible for NO inhibition by *A camphorata* had been reported[6]. Here we suggested for the first time that *A camphorata* reduced LPS-induced NO production by inhibiting iNOS mRNA expression. Although nanomolar concentrations of

NO play an important physiological role as a defense molecule in the immune system[39], overproduction of NO, predominantly via the upregulation of iNOS in macrophages, contributes to numerous pathological processes, including inflammation[40] and atherosclerosis[41]. The mechanism responsible for NO inhibition of several plant extracts had been reported, including direct scavenging of NO[42], suppression of iNOS activity[43], or the reduction on iNOS gene expression[44]. In the present data, the AC-2 fraction of *A camphorata* expressed anti-inflammatory effects via iNOS gene inhibition. This inhibition may in part be through inhibiting LPS-activated NF-κB translocation (Figure 7B). This data suggests that the AC-2 polysaccharide fraction of *A camphorate* could be an attractive candidate for adjunctive therapy in gram-negative bacterial infections.

AC-2 also has the ability to repress the secretion of MCP-5 and RANTES. MCP-5 is a potent monocyte active chemokine that is involved in allergic inflammation[45]; RANTES is also an important chemoattractant for many immune cells[46]. Some molecules inhibit LPS-activated macrophages by interfering LPS binding to cell surface[47,48]. In this study, it is not likely that AC-2-inhibited cytokine expression is due to interferring LPS binding to its receptor TLR4 because the protein expression of TLR4 does not change after AC-2 treatment (Figure 7A). LPS-induced COX-2, GCSF, GM-CSF, MCP-1, sTNFRI, and TNF-α were not reduced simultaneously. Although the inhibition of COX-2 protein expression by *A camphorata* had been reported[6], there was no such bioactive constituent in this polysaccharide fraction of *A camphorata*. It also suggests that the bioactive compounds for inhibiting COX-2 and iNOS existed in different components of *A camphorata*. However, the characterization of candidate compounds that mediate the inhibition of LPS-induced NO, IL-6, IL-10, MCP-5, and RANTES secretion requires further study.

In conclusion, the present study shows that the fractionated polysaccharides AC-2 of *A camphorata* inhibits the production of IL-6, IL-10, MPC-5, RANTES, and NO in LPS-stimulated mouse macrophages. This inhibition of IL-6, IL-10, and iNOS was mediated by transcriptional downregulation of IL-6, IL-10, and iNOS genes. Because IL-6, IL-10, MCP-5, RANTES, and NO are thought to be associated with acute and chronic inflammation diseases, the inhibitory effects of *A camphorata* on LPS-induced IL-6, IL-10 MCP-5, RANTES, and NO might provide a partial anti-inflammation function in LPS-induced inflammatory conditions.

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