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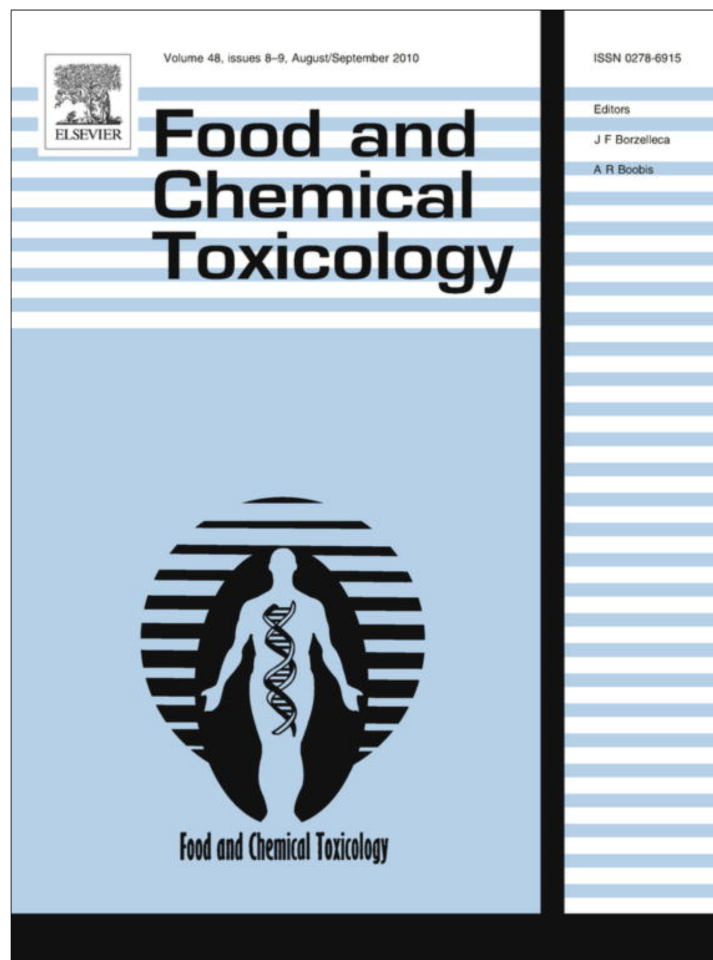

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Food and Chemical Toxicology 48 (2010) 2319–2325

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Antrodia camphorata suppresses lipopolysaccharide-induced nuclear factor-κB activation in transgenic mice evaluated by bioluminescence imaging

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ARTICLE INFO

Article history:
Received 11 September 2009
Accepted 24 May 2010

Keywords:
Antrodia camphorata
Nuclear factor-κB
Lipopolysaccharide
Transgenic mice
Anti-inflammation

ABSTRACT

In an earlier study, we found that *Antrodia camphorata* inhibited the production of lipopolysaccharide (LPS)-induced cytokines, inducible nitric oxide synthase, and cyclooxygenase-2 by blocking nuclear factor-κB (NF-κB) activation in cultured RAW 264.7 macrophages. This study was aimed at evaluating the inhibitory effects of the fermented culture broth of *A. camphorata* in terms of LPS-induced NF-κB activation in transgenic mice by using a non-invasive, real-time NF-κB bioluminescence imaging technique. Transgenic mice carrying the luciferase gene under the control of NF-κB were given *A. camphorata* (570 mg/kg, p.o.) for three consecutive days and then injected with LPS (4 mg/kg, i.p.). *In vivo* imaging showed that treatment with LPS increased the luminescent signal, whereas *A. camphorata* suppressed the LPS-induced inflammatory response significantly. *Ex vivo* imaging showed that *A. camphorata* suppressed LPS-induced NF-κB activity in the small intestine, mesenteric lymph nodes, liver, spleen, and kidney. Immunohistochemical staining revealed that *A. camphorata* suppressed production of the LPS-induced tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and NF-κB p65 subunit in these organs. Furthermore, *A. camphorata* attenuated the productions of LPS-induced TNF-α and IL-1β in serum from transgenic mice. We report the first confirmation of the anti-inflammatory action *in vivo* of this potentially beneficial mushroom.

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1. Introduction

Antrodia camphorata, a newly discovered basidiomycete in the Polyporaceae (Aphyllophorales) that causes brown heart rot in the Taiwan native tree *Cinnamomum kanehirai* Hay (*Lauraceae*), has been identified as a distinct species of the genus *Antrodia* (Zang and Su, 1990). *A. camphorata* is rare and expensive, as it grows only on the inner heart-wood wall of *C. kanehirai*. It has been used in traditional Chinese medicine for the treatment of food poisoning and drug intoxication, diarrhea, abdominal pain, hypertension, skin itches, and liver cancer (Tsai and Liaw, 1985). There is increasing evidence that *A. camphorata* possesses an extensive range of biological activity, including antioxidant, hepatoprotective, anti-hypertensive, anti-hyperlipidemic, immunomodulatory, anticancer, and anti-inflammatory (Ao et al., 2009; Hseu et al., 2002, 2004, 2007, 2008a,b; Yang et al., 2006a,b).

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doi:10.1016/j.fct.2010.05.066

Nuclear factor-κB (NF-κB) is a nuclear transcription factor that regulates the expression of various genes, including cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which have crucial roles in apoptosis, tumorigenesis, various autoimmune diseases, and inflammation (Lawrence et al., 2001). NF-κB exists in most cells as homodimeric or heterodimeric complexes of RelA (p65), c-Rel, RelB, p50, and p52 subunits (Lawrence et al., 2001). The heteromeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with the inhibitory protein IκB, and lipopolysaccharide (LPS) induces the activation of NF-κB through increasing nuclear p65 protein associated with decreased cytosolic IκB protein (Lawrence et al., 2001). Furthermore, development of the inflammatory response is controlled by various cytokines, such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), which are secreted by phagocytes (Baldwin, 1996). The production of cytokine is further controlled by the transcription factor NF-κB (Baldwin, 1996). Because of its ubiquitous role in the pathogenesis of inflammatory gene expression, NF-κB is a current target for treating various diseases (Makarov, 2000).

A wide range of human disorders, such as tumours, pneumonia, asthma and rheumatic arthritis, are associated with inflammation (Sun and Zhang, 2007). A number of studies have shown that

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A. camphorata may possess anti-inflammatory activity *in vitro* (Ao et al., 2009; Geethangili et al., 2010). In an earlier work, we found that the fermented broth of *A. camphorata* harvested from submerged cultures can inhibit the expression of LPS-induced cytokines, iNOS and COX-2 by blocking NF-κB activation in cultured macrophages (Hseu et al., 2005). However, further investigation of this activity *in vivo* is necessary to permit full exploitation of its promise. The aim of this study was to investigate the potential anti-inflammatory properties of the fermented broth of *A. camphorata* on LPS-induced NF-κB activation in transgenic mice expressing luciferase under the control of NF-κB, and bacterial LPS served as the stimulus for this established model of infection and inflammation (Ho et al., 2007; Hsiang et al., 2009). This novel approach to the detection of real-time NF-κB activity *in vivo* by the bioluminescence imaging technique allowed us to investigate the anti-inflammatory activity of *A. camphorata* during LPS challenge *in vivo* using NF-κB transgenic mice.

2. Materials and methods

2.1. Chemicals

LPS (from Escherichia coli 055:B5) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO) and dissolved in water and phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2), respectively. D-Luciferin was purchased from Xenogen (Hopkinton, MA) and dissolved in PBS at 15 mg/mL. Mouse monoclonal antibodies against p65, IL-1β, and TNF-α were purchased from Chemicon (Temecula, CA), Santa Cruz (Santa Cruz, CA), and Abcam (Cambridge, UK), respectively. All of the other chemicals were of the highest grade available and supplied either by Merck (Darmstadt, Germany) or Sigma.

2.2. Preparation of the fermented culture broth of A. camphorata

The fermented culture of *A. camphorata* was provided by Food Industry Research and Development Institute in Taiwan as previously described (Hseu et al., 2008). The *A. camphorata* culture was inoculated on potato dextrose agar and incubated at 30 °C for 15–20 days. The whole colony was then put into a flask with 50 mL of sterile water. After homogenization, the fragmented mycelial suspension was used as an inoculum. The seed culture was prepared in a 20 L fermentor (BioTop) agitated at 150 rpm with an aeration rate of 0.2 vvm at 30 °C. A 5 day culture of 15 L of mycelium inoculum was inoculated into a 250 L agitated fermentor (BioTop). The fermentation conditions were the same as those used for the seed fermentation, but the aeration rate was 0.075 vvm. The fermentation product was harvested at hour 331 and poured through a non-woven fabric on a 20-mesh sieve to separate the deep-red fermented culture broth and the mycelia, and then centrifuged at 3000*g* for 10 min followed by passage through a 0.2 μm pore-size filter. The culture broth was concentrated under vacuum and freeze-dried to a powder. The yield of dry matter from the culture broth was 18.4 g/L. For preparation of the aqueous solution, the powder samples were solubilized with 10 mM sodium phosphate buffer (pH 7.4), containing 0.15 M sodium chloride (PBS) at 25 °C. The stock solution (16 mg/mL) was stored at −20 °C before analysis of anti-inflammatory properties. The experiments were done using 2–4 different batches of the fermented culture of *A. camphorata*. Food Industry Research and Development Institute in Taiwan have standardized the preparation of different batches of *A. camphorata* using polyphenols, triterpenoids, polysaccharides, and biological activities, etc.

2.3. HPLC separation and peak identification

The HPLC profile of the fermented culture broth of *A. camphorata* was performed using a RP-18 column [COSMOSIL, $5C_{18}$-AR-II, Waters, 4.6 x 250 mm] at a flow rate of 1.0 mL/min, detected at UV 254 and 220 nm. Standard solution of the fermented culture broth from *A. camphorata* was prepared by dissolving it in water (5.0 mg/mL), filtered through 0.22 μm membrane filter and applied to HPLC analysis. The mobile phase consisted of (A) acetonitrile and (B) 0.05% trifluoroacetic acid (TFA) (v/v) using a gradient elution of 5–60% A at 5–40 min. The flow rate was maintained as 1.0 mL/min and aliquots of 20 μL were injected. We further characterized the main composition of fermented culture broth of *A. camphorata* using chromatography followed by spectral analysis. 1D and 2D NMR spectra were performed on a Bruker NMR spectrometer (Unity Plus 400 MHz) using C_5D_5N as solvent for measurement. All separations were performed on a Shimadzu LC solution program. The system included a pump (LC-20AT), an autosampler (SIL-20), a column oven (CTO-20A) and a PDA detector (SPD-M20A). LC-MS was performed using Agilent 1100 series, a mass Esquire HCT (Bruker), and a column (Agilent, Zorbax Eclipse, SB-C18, 2.1 × 150 mm, 5 μm).

2.4. Luciferase activity assay

Recombinant HepG2/NF-κB cells carrying the NF-κB-driven luciferase gene were constructed as previously described (Hsiang et al., 2005). HepG2/NF-κB cells were treated with 0, 50, 100, 150, 200, and 250 μg/mL of the fermented broth of *A. camphorata* in the presence or absence of 100 ng/mL LPS for 24 h. Cells were then lyzed in 350 μL of Triton lysis buffer (50 mM Tris–HCl, 1% (v/v) Triton X-100, 1 mM dithiothreitol, pH 7.8) and centrifuged at 12,000*g* for 2 min at 4 °C. The luciferase activity was measured by mixing 20 μL of cell lysate with 20 μL of luciferase reagent (470 μM luciferin, 33.3 mM dithiothreitol, 270 μM coenzyme A, 530 μM ATP, 20 mM Tricine, 1.07 mM $(MgCO_3)4.Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, pH 7.8) and determined with a luminometer (FB15, Zylux Corp., Maryville, TN). The relative NF-κB activity was calculated by dividing the relative luciferase unit (RLU) of treated cells by the RLU of untreated cells.

2.5. MTT assay

Cell viability was monitored by the MTT colorimetric assay. Briefly, cells ($1.0x10^5$ cells/96-well dish) were treated with 0, 50, 100, 150, 200, and 250 μg/mL of the fermented broth of *A. camphorata* in the presence or absence of 100 ng/mL LPS for 24 h. A one-tenth volume of 5 mg/mL MTT was then added to the culture medium. After incubation at 37 °C for 4 h, an equal cell culture volume of 0.04 M HCl in isopropanol was added to dissolve the MTT formazan, and the absorbance at 570 nm (A_{570}) was measured. Cell viability (%) was calculated as:
(A_{570} of treated cells/A_{570} of untreated cells) × 100.

2.6. Animal experiments

Transgenic mice carrying the luciferase gene driven by five NF-κB-responsive elements were constructed as previously described (Ho et al., 2007). All transgenic mice were crossed with wild type F1 mice to yield NF-κB-Luc heterozygous mice with the FVB genetic background. Transgenic mice were kept in cages in a pathogen-free isolation facility with a 12 h light/12 h dark cycle. Mice were provided with rodent chow and water *ad libitum*. All experiments were conducted in accordance with the guidelines of the China Medical University Animal Ethics Research Board. A total of nine male transgenic mice (12 weeks old) were divided randomly into three groups of three animals: (1) control, no treatment; (2) treatment with LPS; and (3) treatment with LPS plus *A. camphorata*. Transgenic mice were gavaged *A. camphorata* dissolved in PBS buffer at a dose of 570 mg/kg for three consecutive days and then injected with LPS (4 mg/kg, i.p.), while the control group received vehicles (PBS buffer) only. At 4 h after the injection, mice were imaged for the luciferase activity, and subsequently sacrificed for *ex vivo* imaging and immunohistochemical staining.

2.7. In vivo and ex vivo bioluminescence imaging

For *in vivo* imaging, mice were anaesthetized with isoflurane and injected with d-luciferin (150 mg /kg body weight, i.p.). After 5 min, mice were placed supine in the chamber and imaged for 5 min by the IVIS Imaging System® 200 Series (Xenogen) with the camera set at the highest sensitivity. Photons emitted from bodies were quantified using the Living Image® software (Xenogen). For *ex vivo* imaging, mice were anaesthetized and injected (i.p.) with luciferin. Five minutes later, the mice were sacrificed and organs were removed immediately, placed in the IVIS system and imaged with the setting used for the *in vivo* studies. The intensity of the signal from tissues was quantified as the sum of all photons/s.

2.8. Immunohistochemical staning

For immunohistochemical staining, organs were removed, fixed in 10% phosphate-buffered formalin solution for 2 days, rinsed in saline, and dehydrated in a series of increasing concentration of ethanol (50%, 70%, and 95% (v/v) for 30 min each). The dehydrated samples were ten embedded in paraffin and cut into 5 μm thick sections. Sections were deparaffinized in xylene and rehydrated as described above. Endogenous peroxidase activity was blocked by immersing the sections in 3% (v/v) hydrogen peroxide in methanol for 15 min followed by two washes with PBS. The non-specific binding was blocked with 1% (w/v) bovine serum albumin at room temperature for 1 h. The sections were then incubated with anti- IL-1β, anti-TNF-α, and anti-p65 antibodies overnight at 4 °C. The slides were incubated with biotinylated secondary antibody (Zymed Laboratories, South San Francisco, CA) for 20 min at room temperature. Finally, slides were incubated with avidin–biotin complex reagent and stained with 3,3′-diaminobenzidine according to the manufacturer's protocol (Histostain®-Plus Kit, Zymed Laboratories).

2.9. Cytokine enzyme-linked immunosorbent assay (ELISA)

IL-1β and TNF-α were quantified by ELISA with the OptEIA^{TM} mouse IL-1β and TNF-α sets (Pharmingen, San Diego, CA). Sera from transgenic mice were placed into wells that were coated with monoclonal antibody against IL-1β or TNF-α. After three washes with 0.05% (v/v) Tween 20 in PBS, peroxidase-conjugated avidin, bio-

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tinylated antibody against IL-1β or TNF-α and chromogenic substrate were added to each well in that order. The absorbance at 405 nm was measured in an ELISA plate reader.

2.10. Statistics

Data are presented as mean ± SD. Student's *t*-test was used for comparison of the two experiments. A value of $p < 0.05$ was set as the level of statistical significance.

3. Results

This study investigates the anti-inflammatory effects of the fermented culture broth of *A. camphorata* harvested from submerged cultures *in vivo* and *ex vivo* using NF-κB transgenic mice.

3.1. A. camphorata inhibited NF-κB activation induced by LPS in recombinant HepG2/NF-κB cells

In this study, recombinant HepG2/NF-κB cells were used to investigate the potential anti-inflammatory properties of *A. camphorata*. To mimic the bacteria-induced inflammation process, HepG2/NF-κB cells were stimulated with bacterial endotoxin, LPS, for 24 h. As shown in Fig. 1, LPS-activated NF-κB activity in HepG2 cells and the relative NF-κB activity reached a 6-fold induction at 100 ng/mL of LPS. The incubation of cells with *A. camphorata* (50–250 μg/mL) and LPS resulted in a significant, dose-dependent reduction of NF-κB activation (Fig. 1). Cell viability was not affected by LPS alone or by LPS plus *A. camphorata* (Fig. 1). These findings indicated that *A. camphorata* was capable of inhibiting LPS-induced NF-κB activity in HepG2 cells.

3.2. In vivo inhibition of LPS-induced NF-κB activation by A. camphorata

We monitored the effect of *A. camphorata* on the LPS-induced NF-κB activity in transgenic mice. Transgenic mice carry the luciferase gene driven by a promoter with five NF-κB-responsive elements and therefore the luciferase activity reflects the NF-κB trans-activity as reported (Ho et al., 2007; Hsiang et al., 2009). Mice were given *A. camphorata* (p.o.) for three consecutive days and then injected (i.p.) with LPS. Four hours later, the bioluminescence was observed in LPS-treated mice when compared to the mock injected group (Fig. 2). LPS stimulated the luminescent signal throughout the body and an intense signal was emitted in the abdominal region, whereas *A. camphorata* suppressed LPS-induced NF-κB activation in transgenic mice.

We monitored the effect of *A. camphorata* on the LPS-induced NF-κB activity in various organs. *Ex vivo* imaging showed that luminescence from all organs was greatly increased following induction by LPS (Fig. 3). The highest level of intensity was observed in the small intestine, while moderate intensity was observed in liver, mesenteric lymph nodes, spleen, and kidney. *A. camphorata* suppressed LPS-induced bioluminescent signals in these organs. These data suggested that *A. camphorata* treatment blocked NF-κB activation in response to LPS in the NF-κB transgenic mice.

3.3. A. camphorata inhibited LPS-induced IL-1β, TNF-α, and p65 expression

To assess the effect of *A. camphorata* on the pro-inflammatory cytokines, IL-1β and TNF-α expression in different organs was examined by immunohistochemical staining. As shown in Fig. 4, the expression of IL-1β and TNF-α were stimulated in the small intestine, liver, mesenteric lymph nodes, spleen, and kidney in mice treated with LPS, while their expression was reduced in these organs in mice treated with LPS plus *A. camphorata*. To demonstrate the correlation between *in vivo* cytokine production and NF-κB activation, we used a monoclonal antibody selective for the active form of the NF-κB p65 subunit. The antibody binds selectively to the nuclear localization sequence of p65, which is masked by its inhibitor, IκB, in unstimulated cells. As shown in Fig. 4, treatment of transgenic mice with LPS resulted in increased production of p65 protein in these organs and this phenomenon was inhibited significantly by *A. camphorata*.

3.4. Inhibitory effect of A. camphorata on LPS-induced IL-1β and TNF-α productions from serum

In order to further investigate whether *A. camphorata* suppressed cytokine production in transgenic mice, we measured the levels of IL-1β and TNF-α in serum by cytokine ELISA. As shown in Fig. 5, LPS increased IL-1β and TNF-α production, while *A. camphorata* decreased the LPS-induced production of cytokines.

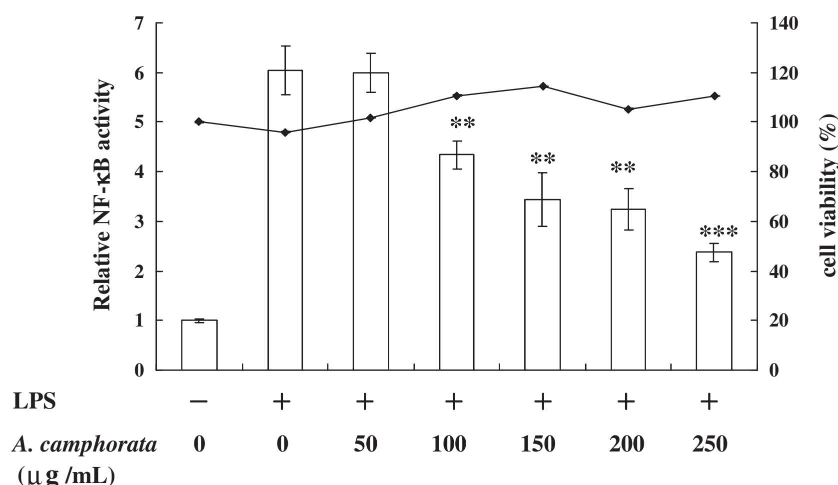

Fig. 1. The inhibitory effect of the fermented culture broth of *A. camphorata* on LPS-induced NF-κB activation in recombinant HepG2/NF-κB cells. Cells were starved for 24 h in serum-free medium and treated with 0, 50, 100, 150, 200, and 250 μg/mL of *A. camphorata* in the presence or in the absence of LPS (100 ng/mL). The luciferase activity and cell viability were determined at 24 h. The bars represent the relative NF-κB activity, which is presented for comparison with the RLU relative to untreated cells. The lines represent cell viability during treatment. The results are given as the mean ± SD of three assays. **$p < 0.01$ and ***$p < 0.001$, compared with the group treated with LPS.

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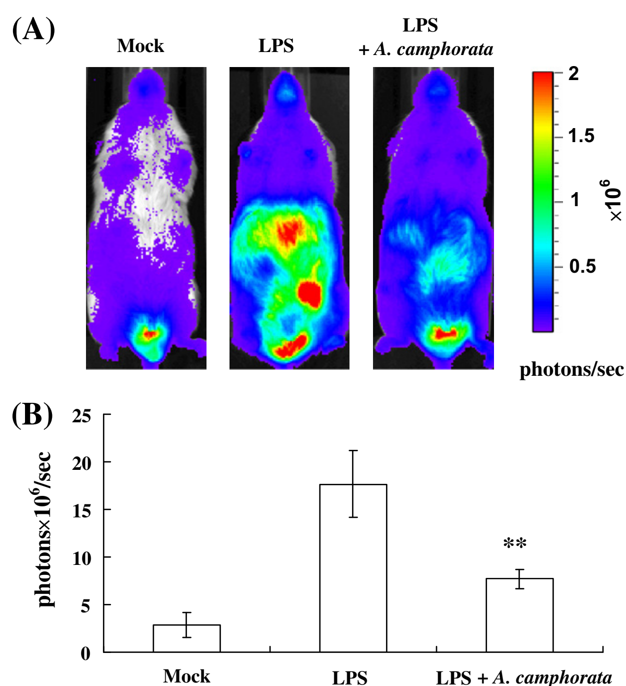

Fig. 2. Anti-inflammatory activity of *A. camphorata* on LPS-induced NF-κB activity in living transgenic mice shown by bioluminescence imaging. Transgenic mice were given *A. camphorata* (570 mg/kg, p.o.) for three consecutive days and then injected with LPS (4 mg/kg, i.p.). Four hours later, mice were anaesthetized and luciferin was administered. After 10 min, the mice were imaged using IVIS 200 and the photons from the whole animal were counted. (A) Diagrams show the bioluminescent signal emitted from the whole body. The colour overlay on the image represents the light (photons/s) emitted from the animal as indicated by the colour scales. (B) Quantification of photon emission from the whole body. Values are expressed as the mean ± SD of three mice. $^{**}p < 0.01$, compared with the group treated with LPS.

These results suggested that *A. camphorata* inhibited the expression of LPS-induced IL-1β and TNF-α in mice.

3.5. HPLC metabolite profiling of the fermented culture broth of *A. camphorata*

As shown in Fig. 6, the HPLC profile of the fermented culture broth of *A. camphorata* was performed using a RP-18 column. Many chromatographic peaks were exhibited, suggested that various kinds of compounds were included in the fermented culture broth of *A. camphorata* (Fig. 6.). The major compound in the *A. camphorata* was obtained at retention times of 19.1 min. The identification of 2,3-dimethoxy-5-methyl-1,4-benzoquinone was characterized by comparison of their spectral data (NMR and LC-MS) with analogous information reported in the literature (Chen et al., 2007b; Wu et al., 2007). According to the results of HPLC analysis, the amounts of the compound 2,3-dimethoxy-5-methyl-1,4-benzoquinone in the fermented culture broth of *A. camphorata* were 17.3% (254 nm) and 13.5% (220 nm), respectively.

4. Discussion

Inflammation is related to multiple human pathologies, including immune deficiencies, diabetes and atherosclerosis as well as tumours (Sun and Zhang, 2007). Therefore, research and development of anti-inflammatory agents is a promising approach to the treatment of these diseases. In an earlier study, we showed the inhibitory effects of a fermented culture broth of *A. camphorata* (25–100 μg/mL) from submerged culture in terms of LPS-induced nitric oxide (NO) and prostaglandin E2 production, and iNOS and COX-2 expressions through blocking NF-κB activation, without appreciable cytotoxicity toward the RAW 264.7 macrophage cells (Hseu et al., 2005). The anti-inflammatory activity of the culture medium was lower than that of *A. camphorata* in submerged culture, indicating that anti-inflammatory components of the fungus must be derived from secondary metabolites of the mycelia (Hseu et al., 2005). These results, mainly from experiments at the cellular or molecular level *in vitro*, suggested that *A. camphorata* is a potent inhibitor of inflammation by blocking NF-κB activation. We used *in vivo* bioluminescent imaging to analyse the anti-inflammatory activity of the fermented culture broth of *A. camphorata* in mice and we report here the first confirmation of the anti-inflammatory action *in vivo* of this potentially beneficial mushroom.

NF-κB is a potent pro-inflammatory signal transduction molecule, and NF-κB activation amplifies the expression of multiple chemokine and cytokine genes involved in the innate and adaptive immune responses (Lee and Burckart, 1998; Csizmadia et al., 2001). However, our knowledge of the role of NF-κB in the mediation of inflammatory response remains limited because of the difficulties encountered in obtaining *in vivo* data. A quantitative method using bioluminescence imaging with transgenic mice for

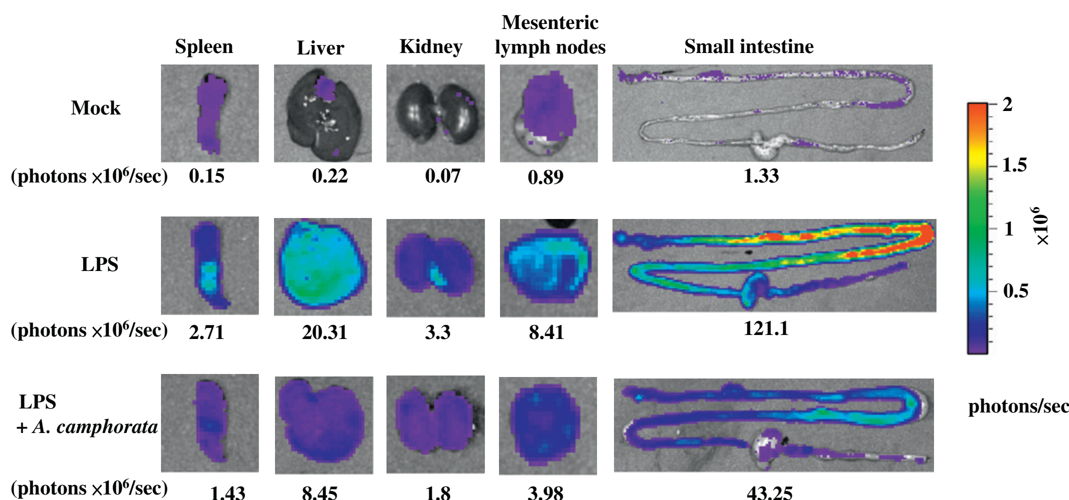

Fig. 3. The inhibitory effect of *A. camphorata* on LPS-induced bioluminescence in mouse organs. Transgenic mice were given *A. camphorata* (570 mg/kg, p.o.) for three consecutive days and then injected with LPS (4 mg/kg, i.p.). After whole-body imaging, the animals were sacrificed by cervical dislocation, the organs were excised rapidly and subjected to imaging. The colour overlay on the image represents the light (photons/s) emitted from the organs, as indicated by the colour scales. The quantified photon signal (photons/s) is shown below each image.

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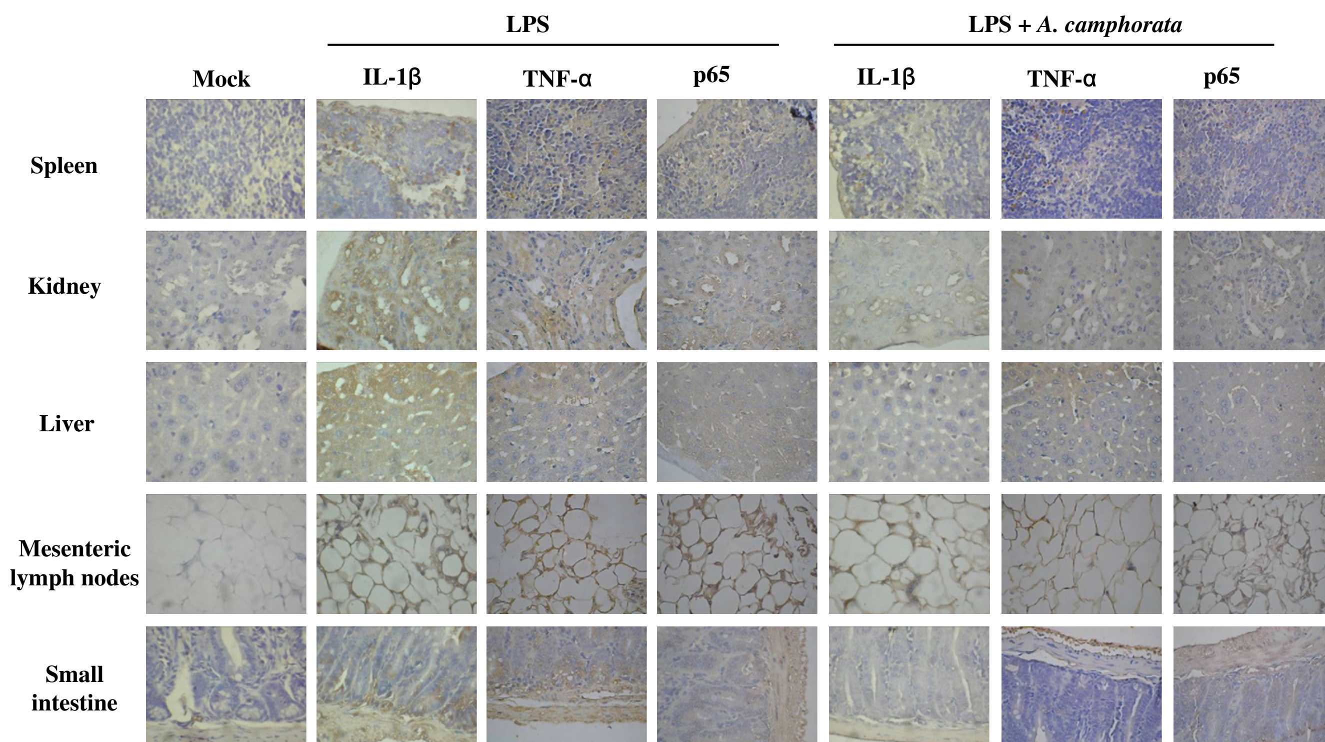

Fig. 4. *A. camphorata* suppresses LPS-induced cytokine productions and p65 expression in mouse organs as determined by immunohistochemical staining. Transgenic mice were given *A. camphorata* (570 mg/kg, p.o.) for three consecutive days and then injected with LPS (4 mg/kg, i.p.). Sections of spleen, kidney, liver, small intestine and mesenteric lymph nodes were stained with antibody against IL-1β, TNF-α, and p65 (magnification 100×).

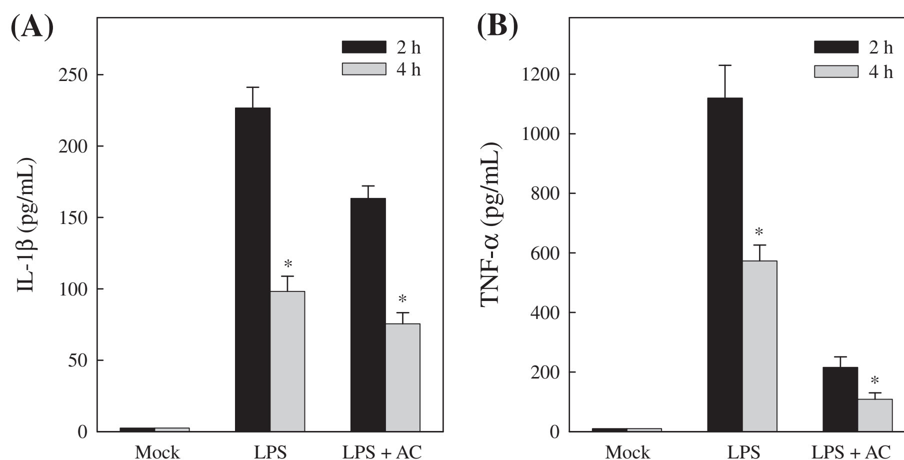

Fig. 5. The inhibitory effect of *A. camphorata* on the LPS-induced IL-1β and TNF-α productions from mouse serum. Transgenic mice were given *A. camphorata* (570 mg/kg, p.o.) for three consecutive days and then injected with LPS (4 mg/kg, i.p.). The levels of IL-1β (A) and TNF-α (B) were measured using commercial ELISA kits. Values are expressed as the mean ± SD of at least three independent experiments. $^{*}p < 0.05$, compared with the group treated with LPS. AC: *A. camphorata*.

evaluating NF-κB activation *in vivo* was developed recently (Roth et al., 2006; Sadikot and Blackwell, 2005). Earlier, we demonstrated that the non-invasive imaging technique can be used to monitor real-time inflammation in the transgenic mice (Ho et al., 2007; Hsiang et al., 2009). The transgenic mice were engineered to possess a luciferase gene driven by five NF-κB-responsive elements. In these genetically modified mice (referred to here as NF-κB-Luc mice), the production and intracellular accumulation of luciferase are dependent on NF-κB-activated gene transcription; therefore, bioluminescence imaging using NF-κB-Luc mice provides a useful *in vivo* reporter-based assay system in response to a variety of inflammatory signals, including LPS (Sadikot and Blackwell, 2005). We tested the *in vitro* effect of the fermented culture broth of *A. camphorata* in HepG2/NF-κB cells and the results showed that the fermented culture broth of *A. camphorata* suppressed LPS-induced NF-κB activity. We used an NF-κB-Luc transgenic mouse model to study the expression and regulation of the NF-κB gene *in vivo*. Luciferase-positive cells and NF-κB-positive cells were found in LPS-stimulated mice and *A. camphorata* inhibited LPS-mediated luciferase expression in the transgenic mice. Further, *ex vivo* imaging analysis revealed decreased expression of the luciferase gene in mice after treatment with *A. camphorata*, which resulted from inhibition of the NF-κB p65 protein in the *A. camphorata*-treated mice, as shown by immunohistochemical staining.

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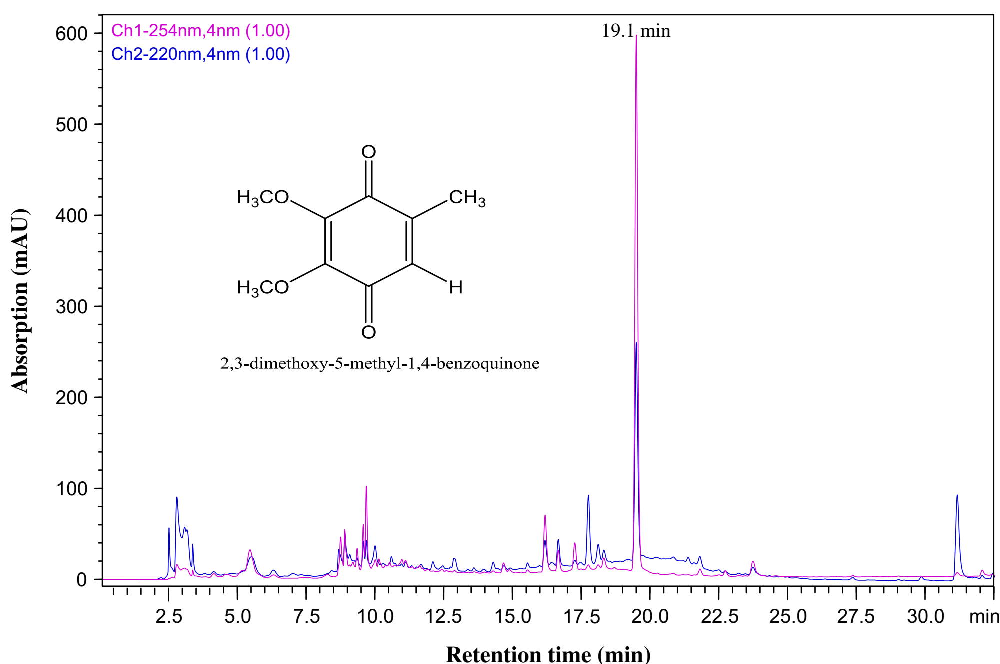

Fig. 6. The HPLC profile of the fermented culture broth of *A. camphorata*. The chemical profile of *A. camphorata* was performed using a RP-18 column and detected at UV 254 and 220 nm (see Section 2.).

Here, we describe the *in vivo* anti-inflammatory mechanisms mediated by *A. camphorata* that are based on the inhibition of LPS-mediated activation of NF-κB.

NF-κB activation is necessary for the expression of various cytokines in response to LPS (Lawrence et al., 2001; Baldwin, 1996). Cumulative evidence indicates that an abnormality in the production or function of cytokines, such as IL-1β and TNF-α, has an essential role in many inflammatory lesions (De Nardin E., 2001). Inflammatory stimuli, such as LPS, induce cytokines in the process of macrophage activation, which mediates tissue responses in different phases of inflammation in a sequential and concerted manner (Laskin and Pendino, 1995). Thus, inhibition of cytokine production or function serves as a key mechanism in the control of inflammation. We correlated the bioluminescent imaging with cytokine activity and showed that *A. camphorata* was capable of suppressing the LPS-induced IL-1β and TNF-α productions in mouse plasma and organs. In agreement with these results, we have shown that *A. camphorata* inhibits IL-1β and TNF-α productions in RAW 264.7 cells stimulated by LPS (Hseu et al., 2005). Because IL-1β and TNF-α have crucial roles in the pathogenesis of the inflammation process, these findings suggest that *A. camphorata* has anti-inflammatory activity.

It has been shown that natural antioxidant compounds directly inhibit the expression of NF-κB-dependent cytokines iNOS and COX-2 and thereby reduce inflammation (Ma et al., 2003; Surh et al., 2001). Our earlier study suggested that *A. camphorata* has protective antioxidant properties (Hseu et al., 2002). The suppressive effects of these antioxidant compounds on the production of the inflammatory mediators are associated with their antioxidant activity. The antioxidant NF-κB inhibitors restrict the production of inflammatory mediators through suppression of their gene expression and thus prevent inflammatory diseases. The potential inhibition of the generation of reactive oxygen species (ROS) induced by *A. camphorata* is in accord with inhibition of NF-κB activation and cytokine expression and, thus, reduced inflammation.

There is increasing evidence that the compounds identified in *A. camphorata* are predominantly polysaccharides, triterpenoids, steroids, benzenoids, and maleic/succinic acid derivatives (Ao et al., 2009). The reported yields of polysaccharides, crude triterpenoids and total polyphenols in the fermented *A. camphorata* broth were 23.2, 47, and 67 mg/g, respectively, whereas no polysaccharide, crude triterpenoid, or polyphenol was detected in the dry matter of the culture medium (Song and Yen, 2002). It has been demonstrated that polysaccharides from submerge-cultured *A. camphorata* mycelia possess anti-inflammatory activity in RAW264.7 macrophages (Wu et al., 2007; Chen et al., 2007a). Moreover, methanolic extracts from the mycelia of *A. camphorata* exhibit an anti-inflammatory effect by suppressing ROS production in human leukocytes (Shen et al., 2004a). Further, methanolic extracts from the fruiting body of *A. camphorata* inhibit the iNOS, COX-2 and TNF-α expression induced by LPS/interferon-γ (IFN-γ) or β-amyloid in microglia (Liu et al., 2007). The anti-inflammatory properties of both chloroform and methanol extracts from the fruiting body of *A. camphorata* might result in the inhibition of NO, TNF-α and IL-12 production in LPS/IFN-γ-activated murine peritoneal macrophages (Rao et al., 2007). It has been shown that antrocamphin A (benzenoid) and antcin A and B (steroids) isolated from the *A. camphorata* fruiting body exhibit potent inhibition against *N*-formylmethionyl-leucyl-phenylalanine-induced superoxide production with IC_{50} values less than 10 μM (Chen et al., 2007b). Moreover, zhankuic acid (A, B, and C) and anticin K (steroids) from the *A. camphorata* fruiting body have potent anti-inflammatory effects by inhibiting ROS production and firm adhesion by neutrophils in human leukocytes (Shen et al., 2004b). These observations confirm and extend the anti-inflammatory action of these polysaccharides, benzenoids, and/or steroids from *A. camphorata*. It is reasonable to suggest, therefore, that *A. camphorata* metabolizes the culture medium and releases active components during fermentation by the submerged culture. The results imply that natural polysaccharides, benzenoids, and/or steroids might act as anti-inflammatory agents, rendering them the most effective fraction of *A. camphorata*. In this study, 2,3-dimethoxy-5-methyl-1,4-benzoquinone was isolated from the fermented culture broth of *A. camphorata* as determined by HPLC (Fig. 6), and this compound is suggested to possess significant anti-inflammatory activity *in vitro* (Chen et al., 2007b; Geethangili et al., 2010). Further

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bioassay-directed fractionations leading to the identification and purification of the compounds responsible for the anti-inflammatory effect of *A. camphorata* are warranted.

In summary, we showed that the bioluminescence imaging technique can be used to monitor real-time inflammation in mice and to demonstrate the anti-inflammatory activity of *A. camphorata in vivo*. This anti-inflammatory effect involves down-regulation of the production of cytokines IL-1β and TNF-α via the suppression of NF-κB activation. In conclusion, *A. camphorata* exhibits anti-inflammation potential both *in vitro* and *in vivo* by regulation of the immune system process and physiological response to stimuli. Further investigation using other animal models and humans is required to confirm these promising results. These data provide important knowledge that could aid the development of food and drug products for human use.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the National Research Program for Genomic Medicine, National Science Council (NSC 96-2628-B-039-030-NY3), the Committee on Chinese Medicine and Pharmacy, Department of Health (CCMP 97-RD-201) and the China Medical University (CMU97-190, CMU96-207, and CMU97-CMC-004).

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