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ScienceDirect
Journal of Ethnopharmacology 113 (2007) 45–53
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Comparative anti-inflammatory characterization of wild fruiting body, liquid-state fermentation, and solid-state culture of *Taiwanofungus camphoratus* in microglia and the mechanism of its action

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Received 14 November 2006; received in revised form 7 February 2007; accepted 26 March 2007
Available online 18 May 2007

Abstract

Taiwanofungus camphoratus (syn. *Antrodia camphorata*), a medicinal mushroom in Taiwan, is reputed to provide several therapeutic benefits, but the wild fruiting body is very rare. In this study, we used *Taiwanofungus camphoratus* extracts from wild fruiting bodies and two types of artificial cultivation (solid-state culture and liquid-state fermentation) to examine their anti-inflammatory effects in microglia cells and their possible roles in protection against neurodegenerative diseases. First, EOC13.31 microglia was treated with various kinds of *Taiwanofungus camphoratus* extracts and lipopolysaccharide (LPS) and interferon-γ (IFN-γ) to evaluate the iNOS expression. Western blot and RT-PCR analysis showed that among the various kinds of extracts from wild fruiting bodies, methanol extracts were the most potent inhibitors of iNOS expression. Secondly, the potency of methanol extracts could be ranked as follows: extracts of wild fruiting body > solid-state culture > liquid-state fermentation. To clarify the mechanisms involved, methanol extracts from fruiting body were found to inhibit the phosphorylation of extracellular signal-regulated protein kinases (ERK), c-Jun NH2-terminal protein kinases (JNK) and signal transducer and activator of transcription-1 (STAT-1) induced by LPS/IFN-γ. Methanol extracts from fruiting body also inhibited NF-κB activation through the prevention of inhibitor κB (IκB) degradation. Moreover, methanol extracts from wild fruiting body inhibited both the iNOS and cyclooxygenase-2 (COX-2) expression induced by β-amyloid in microglia in a dose-dependent manner. In an animal model, we confirmed that methanol extracts from fruiting bodies were able to suppress ear edema, indicating that they have anti-inflammatory activity *in vivo*. These results suggest that *Taiwanofungus camphoratus* exhibits an anti-inflammatory activity that might contribute to the prevention of neurodegenerative diseases.

Keywords: Inflammation; iNOS; Microglia; *Taiwanofungus camphoratus*

Abbreviations: iNOS, inducible nitric oxide synthase; NO, nitric oxide; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; IκB, inhibitor κB; IFN-γ, interferon-γ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; COX-2, cyclooxygenase 2; WC, cold water extracts from wild fruiting bodies of *Taiwanofungus camphoratus*; WH, hot water extracts from wild fruiting bodies of *Taiwanofungus camphoratus*; WM, methanol extracts from wild fruiting bodies of *Taiwanofungus camphoratus*; LM, methanol extracts from liquid-state fermentation of *Taiwanofungus camphoratus*; SM, methanol extracts from solid-state culture of *Taiwanofungus camphoratus*

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doi:10.1016/j.jep.2007.03.037

1. Introduction

Microglia are the resident macrophage-like cells of the brain that secrete proinflammatory factors and reactive oxygen species in response to immunological stimuli and play a broad role in the brain's innate immunity and inflammatory neuropathologies (Nelson et al., 2002). Activated microglia changes their morphology and release inflammatory cytokines that activate and recruit other leukocytes to the brain lesion, resulting in neuronal damage (Hayes et al., 1988). Sustained overactivation of microglia is observed in several neurodegenerative diseases, such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, and HIV-associated dementia (McGeer et al., 1988; Raine, 1994; Kreutzberg, 1996; Banati et al., 1998). Several molecules found to be involved in microglia activation include lipopolysaccharide (LPS), interferon-γ (IFN-γ), β-amyloid, CD40L, chemokines, neurotransmitters, and gangliosides, as well as several proteases such as thrombin, tissue plasminogen activator, and matrix metalloproteinase-3. Upon stimulation of LPS and IFN-γ, microglia transduces signals through toll-like receptors and activates a variety of intracellular signaling molecules, such as protein tyrosine kinases, mitogen-activated protein kinases, protein kinase C, small G proteins, signal transducer, and activator of transcription (STAT) (Jones et al., 2001; Takeuchi and Akira, 2001; Kim et al., 2005). Several transcription factors are involved in such activated microglia, including NF-κB, C/EBP, the Fos/Jun families, and STAT, and these results in the induction of inducible nitric oxide synthase (iNOS), TNF-α, cyclooxygenase-2 (COX-2), and other cytokine genes (Sweet and Hume, 1996).

Taiwanofungus camphoratus (syn. *Antrodia camphorata*) is a *Ganoderma-like* fungus, which belongs to the *Polyporaceae*, *Basidiomycotine* family, and grows in a unique host, the endemic perennial tree *Cinnamomun kanehirai* (Bull camphor tree) in Taiwan. The Chinese name for *Taiwanofungus camphoratus* is niu-chang-chih or niu-chang-ku, and its scientific name results from the fact that it is a native fungus in Taiwan. It is known as a folk medicine in Taiwan and is used to treat abdominal pain, diarrhea, drug intoxication, hypertension, and skin itching and to improve immune system and liver function (Tsai and Liaw, 1985). Several biological activities have been reported for *Taiwanofungus camphoratus*, including anti-inflammation, antioxidation, vasorelaxation, and anti-hepatitis B surface antigen activities. Several bioactive ingredients have been identified in it, such as triterpenoid sesquiterpene lactone, steroid and polysaccharide (Chen et al., 1995; Chiang et al., 1995; Cherng et al., 1996; Shen et al., 2003), but many unknown ingredients remain to be discovered. Previous studies have demonstrated the biological function of *Taiwanofungus camphoratus* from wild fruiting body, solid-state culture, or liquid-state fermentation. Because the wild fruiting body of *Taiwanofungus camphoratus* is rare in nature and difficult to obtain, artificial cultivation was developed as a substitute. At present, solid-state culture and liquid-state fermentation of *Taiwanofungus camphoratus* are used to obtain fruiting bodies and mycelia, as well as useful cellular materials for human requirements. However, very little data on the difference between the wild fruiting body of *Taiwanofungus camphoratus* and artificial cultivation of *Taiwanofungus camphoratus* exists with regard to biological functions and medical effectiveness.

Microglia are the immune cells in the brain, and they play a defense and monitoring role. However, activated microglia express high levels of iNOS and its metabolite nitric oxide that significantly contribute to the pathogenesis of neurodegenerative diseases. In this paper, we used wild fruiting bodies of *Taiwanofungus camphoratus* and *Taiwanofungus camphoratus* produced by solid-state culture and liquid-state fermentation to compare their anti-inflammatory effects on microglia and to elucidate possible molecular mechanisms of anti-inflammation.

2. Materials and methods

2.1. Materials

TPA, β-amyloid protein fragment 25–35, LPS (*Escherichia coli* 0127:B8) was purchased from Sigma Chemical (St. Louis, MO). Mouse interferon-γ was purchased from R&D Systems Inc. (Minneapolis, MN).

2.2. Preparation of different extracts from Taiwanofungus camphoratus

Wild fruiting body of *Taiwanofungus camphoratus* (voucher number TC-2004-09-001) and *Taiwanofungus camphoratus* produced by liquid-state fermentation (voucher number TC-2004-09-002) and by solid-state culture (voucher number TC-2004-09-003) were provided by Well Shine Biotechnology Development Co. (Taipei, Taiwan). These specimens were deposited in the School of Medical Laboratory Science & Biotechnology, Taipei Medical University, Taipei, Taiwan. Air-dried ground powder of wild fruiting body of *Taiwanofungus camphoratus*, *Taiwanofungus camphoratus* mycelia produced by liquid-state fermentation, and solid-state culture *Taiwanofungus camphoratus* was extracted sequentially with cold water, methanol, and hot water, to get cold water extracts, methanol extracts, and hot water extracts, respectively, as described previously (Liu et al., 2006).

2.3. Partial fraction of methanol extracts from wild fruiting body of Taiwanofungus camphoratus

Methanol extracts from the wild fruiting body of *Taiwanofungus camphoratus* were dissolved in methanol and then applied to a Sephadex LH-20 column and eluted with ethanol. Six fractions were obtained, and the composition of each individual fraction was determined by HPLC analysis. We discarded the first fraction that contained the mixture of all components, and the other five fractions (CKJ-35-2–CKJ-35-6) were used to treat cells.

2.4. Cell culture

Cells of the mouse microglia cell line EOC13.31 (BCRC 60490, Food Industry Research and Development Institute, Hsinchu, Taiwan) were cultured in Dulbecco's modified

Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (Invitrogen Taiwan, Ltd., Taipei, Taiwan), 20% LADMAC conditioned medium, 100 units/ml penicillin, and 100 μg/ml streptomycin. To obtain the LADMAC conditioned medium, cells of the mouse bone marrow cell line LADMAC (BCRC 60489, Food Industry Research and Development Institute, Hsinchu, Taiwan) were cultured in minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate for 10 days, and LADMAC-conditioned medium was obtained from cell-free cultured medium. For all assays, EOC13.31 cells were plated in 60-mm dishes at 5×10^6 cells per dish and allowed to grow for 18–24 h.

2.5. Western blot

Equal amounts of total cellular protein (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto Immobilon-P membrane (Millipore, Bedford, MA) as described previously (Liu et al., 2003). The membrane was then incubated with an anti-COX-2 antiserum, anti-iNOS antiserum, anti-STAT-1 antiserum, anti-IκB antiserum, anti-phosphorylated ERK antiserum (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated JNK antiserum, anti-phosphorylated p38 antiserum (Cell Signaling Technology Inc., Danvers, MA), or α-tubulin (Invitrogen Taiwan, Ltd.). The membranes were subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence kits (ECL, Amersham).

2.6. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated and the RNA (2 μg) was reverse-transcribed at 37 °C for 1 h by adding 5 μM of random hexamer oligonucleotides (Gibco BRL), 200 units of reverse transcriptase, 2.5 mM deoxyribonucleotide triphosphates (dNTP), and 10 mM dithiothreitol. The semi quantitative PCR was carried out with 2 μl of cDNA and 23 μl of PCR mix buffer containing each primer (0.2 μM), dNTP (2.5 mM), and Taq DNA polymerase (1.25 units) (Takara Bio. Inc., Shiga, Japan). After the PCR, 10 μl of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel, and the PCR products were visualized by ethidium bromide staining. Semiquantitative PCR primers for the mouse iNOS, TNF-

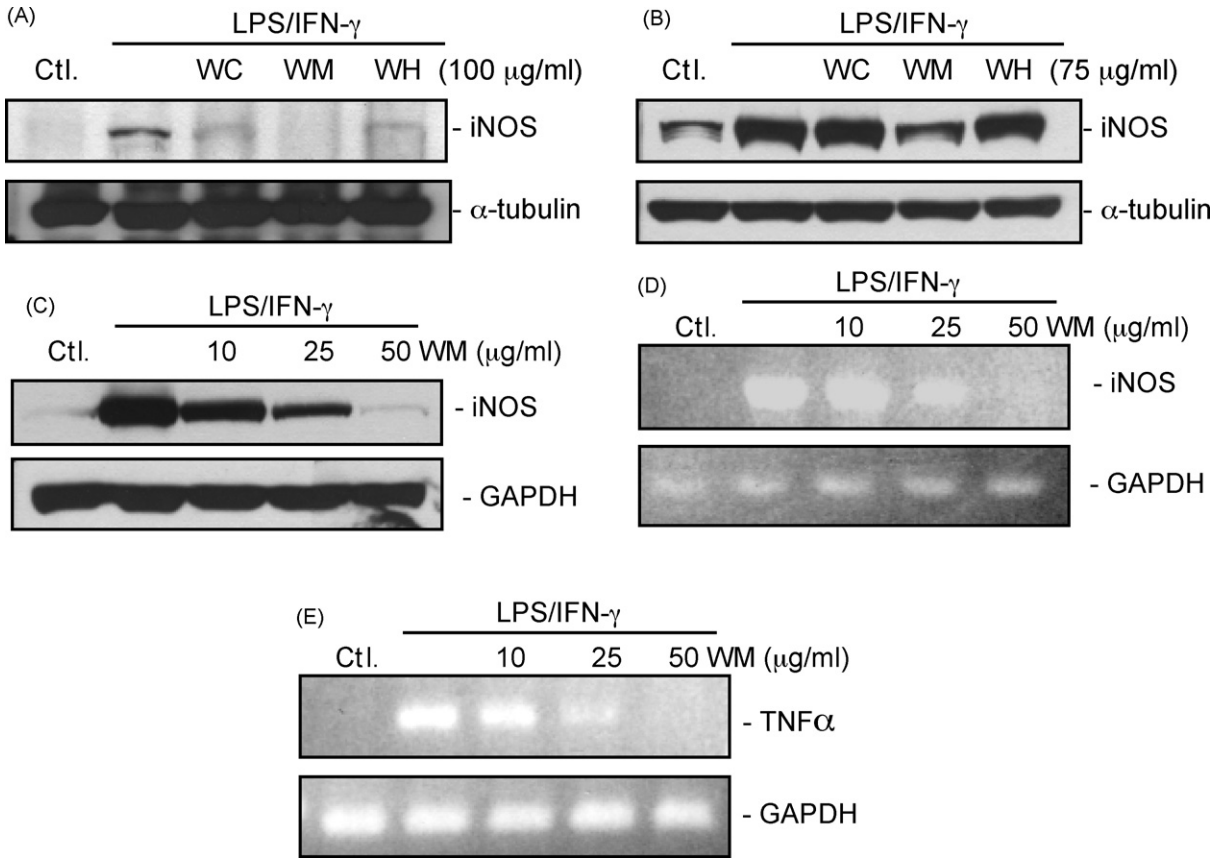

Fig. 1. Comparative effect of various extracts from wild fruiting body of *Taiwanofungus camphoratus* on the inhibition of iNOS expression induced by LPS/IFNγ in microglia. EOC13.31 cells were pretreated with (A) 100 μg/ml or (B) 75 μg/ml of WC, WM, or WH for 1 h, and then LPS (100 ng/ml) and IFN-γ (50 ng/ml) for 24 h. (C) EOC13.31 cells were treated with various concentrations of WM for 1 h and LPS (100 ng/ml) and IFN-γ (50 ng/ml) for 24 h. Total cellular protein (50 μg) was separated on 10% SDS-polyacrylamide gels and blotted with antibodies specific iNOS and α-tubulin. (D and E) EOC13.31 cells were pretreated with various concentrations of WM for 1 h, and then LPS (100 ng/ml) and IFN-γ (50 ng/ml) for 8 h. Total mRNA was prepared, and the levels of iNOS, TNF-α and GAPDH were detected by RT-PCR.

α, and GAPDH cDNA were synthesized according to the following oligonucleotide sequences: iNOS, forward primer 5′-ACACCAGGTATCAGATGTGGG-3′, reverse primer 5′-TGAAGCCCCCTCCACTTCGGTA-3′, TNF-α forward primer 5′-CGAAGGAGTTGGAGGTGTTTTCC-3′, reverse primer 5′-TTTATTGACTGAGGCACTGGGG-3′, and GAPDH, forward primer 5′-ACCACAGTCCATGCCATCAC-3′, reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′.

2.7. Measurement of mouse ear edema

Female Balb/c mice (3–4 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and kept in an animal facility for 1–2 weeks before use. All animal experimental procedures has been conducted and approved by the Institutional Animal Care and Use Committee of Taipei Medical University. Mice (three animals/group) were pretreated with the solvent (15 μl acetone), WM, or SM on both ears for 30 min and then treated with TPA (1 nmol) for 7 h. After TPA treatment, the mice were sacrificed by cervical dislocation, and ear punch biopsies were taken and weighted.

3. Statistical analysis

Data are presented as mean ± S.E., for the indicated number of independently performed experiments. Statistical analysis was done by one-way Student's *t*-test.

4. Results

4.1. Inhibition of LPS/IFN γ-activated microglia by Taiwanofungus camphoratus extracts

The three wild fruiting body extracts – cold water (WC), methanol (WM), and hot water (WH) extracts, as described in Section 2 – were used to examine the inhibitory effect on the iNOS expression in microglia. As shown in Fig. 1A, LPS/IFN-γ significantly induced iNOS expression, and 100 μg/ml of each wild fruiting body extract markedly inhibited this expression. However, only WM significantly inhibited the iNOS expression at a dose of 75 μg/ml (Fig. 1B) and did so in a dose-dependent manner (Fig. 1C). RT-PCR analysis showed that the iNOS mRNA was inhibited by WM in a dose-dependent manner (Fig. 1D), indicating the iNOS expression was inhibited at the transcription level. The expression of another proinflammatory cytokine TNF-α mRNA was examined by RT-PCR analysis, and the results showed that WM inhibited that also (Fig. 1E). These results suggest that WM has stronger anti-inflammatory activity than either WC or WH in microglia.

4.2. Comparison of methanol extracts of wild fruiting body, and of Taiwanofungus camphoratus produced through solid-state culture and liquid-state fermentation on the inhibition of iNOS expression in the LPS/IFN γ-activated microglia

Since WM appears most potent in the inhibition of iNOS expression, we compared the anti-inflammatory effects from the methanol extracts of wild fruiting body of *Taiwanofungus camphoratus* and of *Taiwanofungus camphoratus* produced through solid-state culture and liquid-state fermentation in the next experiments. As shown in Fig. 2, the inhibition of iNOS expression by individual methanol extracts could be ranked as follows: wild fruiting body > solid-state culture > liquid-state fermentation. The results suggest that solid-state culture was similar to wild fruiting body in anti-inflammatory activity.

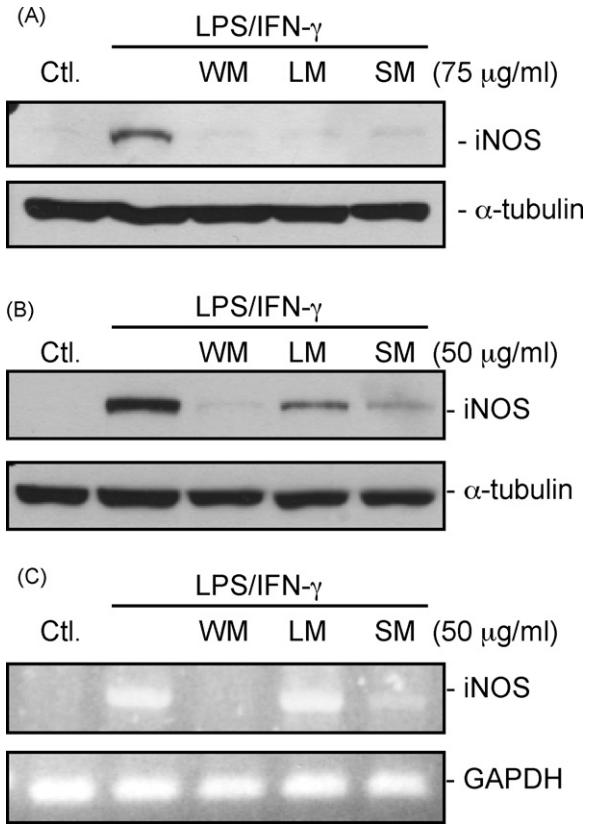

Fig. 2. Comparative effect of methanol extracts of wild fruiting body of *Taiwanofungus camphoratus* and of *Taiwanofungus camphoratus* produced through solid-state culture and liquid-state fermentation on the inhibition of iNOS expression induced by LPS/IFNγ in microglia. EOC13.31 cells were pretreated with (A) 75 μg/ml or (B) 50 μg/ml of WM, LM, or SM for 1 h, and then LPS (100 ng/ml) and IFN-γ (50 ng/ml) for 24 h (A and B) or 8 h (C). (A and B) Total cellular protein (50 μg) was separated on 10% SDS-polyacrylamide gels and blotted with antibody-specific iNOS and α-tubulin. (C) Total mRNA was prepared, and the levels of iNOS and GAPDH were detected by RT-PCR.

4.3. Methanol extracts of wild fruiting body inhibited the phosphorylation of STAT, ERK, JNK and the activation of NF-κB in the LPS/IFNγ-activated microglia

To clarify the inhibitory mechanisms involved, WM was chosen to examine the effects of *Taiwanofungus camphoratus* on the activation of MAPKs (ERK, JNK, and p38), NF-κB and STAT-1 in LPS/IFN-γ-activated microglia. The time–course experiment showed that the phosphorylation levels of STAT-1, ERK, JNK, and p38 increased after LPS/IFN-γ treatment and that WM significantly inhibited the phosphorylation of ERK and JNK, slightly inhibited STAT-1 phosphorylation, and enhanced p38 phosphorylation (Fig. 3A). After 15 min

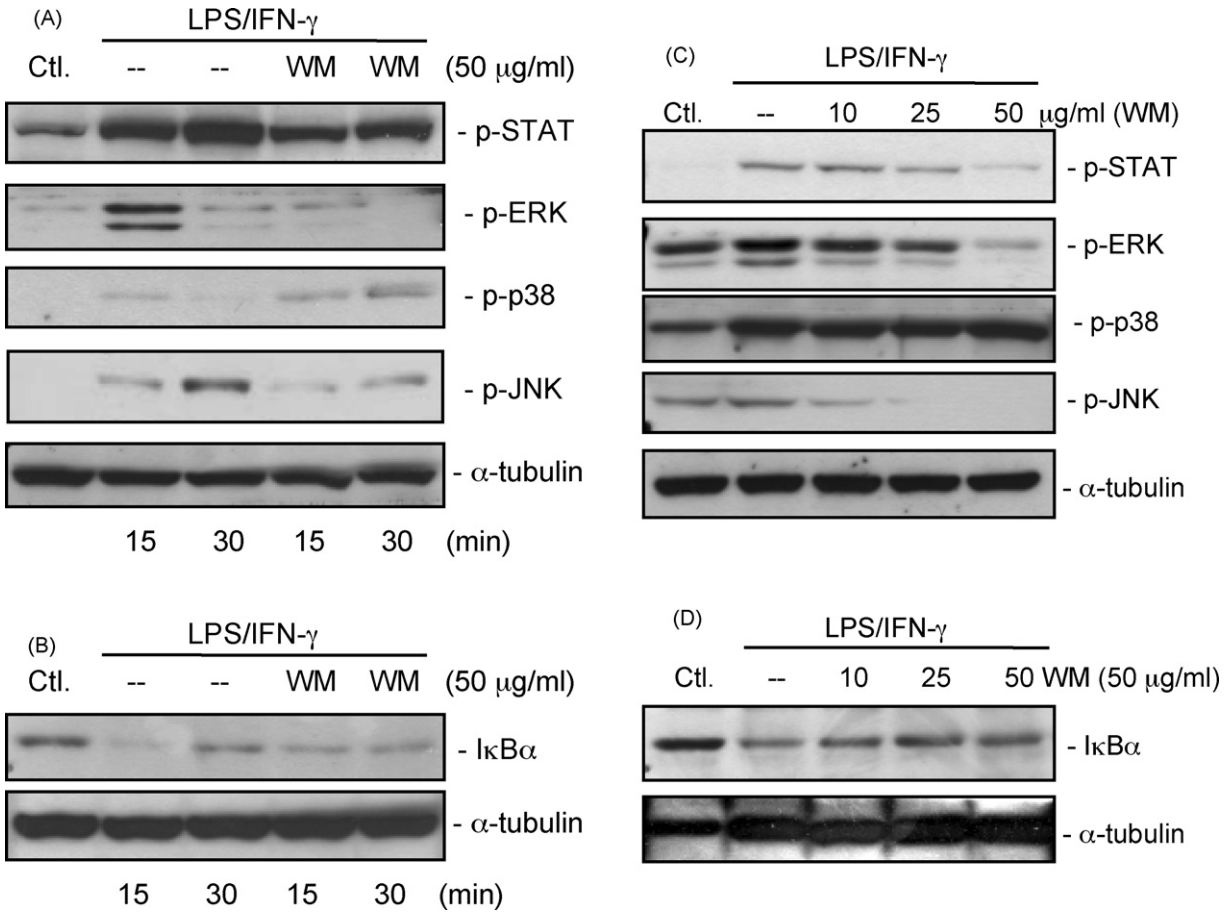

Fig. 3. Inhibitory effects of methanol extracts of wild fruiting body of *Taiwanofungus camphoratus* on the LPS/IFNγ-induced downstream signals and morphology change in microglia. (A and B) EOC13.31 cells were pretreated with 50 μg/ml of WM for 1 h, and then with LPS (100 ng/ml) and IFN-γ (50 ng/ml) for the indicated time. (C and D) EOC13.31 cells were pretreated with various concentrations of WM for 1 h, and then with LPS (100 ng/ml) and IFN-γ (50 ng/ml) for 20 min. Total cellular protein (50 μg) was separated on 10% SDS-polyacrylamide gels and blotted with antibody-specific phosphor-STAT, phosphor-ERK, phosphor-p38, phosphor-JNK, IκB and α-tubulin antibodies.

of LPS/IFN-γ treatment, IκB degradation was found, and WM significantly prevented that degradation (Fig. 3B). The dose-dependent experiment also showed that WM was able to inhibit the phosphorylation of ERK, JNK, and STAT-1, prevent IκB degradation, and then block NF-κB activation (Fig. 3C and D). These results suggest that WM inhibition of iNOS expression might result from suppressing the activation of ERK, JNK, STAT-1, and NF-κB. When EOC13.31 microglia were treated with LPS/IFNγ, they became puffy and formed pseudopodia in some activated microglia (Fig. 4B), while most of the cells pretreated with WM remained small and round and formed no pseudopodia (Fig. 4D). Cells with WM only expanded to a rod- and tabulate-shaped microglia (Fig. 4C).

4.4. Methanol extracts of wild fruiting body inhibited iNOS and COX-2 expression in the β-amyloid-activated microglia

β-Amyloid is an important mediator in the activation of microglia and contributes to neurotoxicity in the brain. We next examined whether WM could inhibit the inflammation responses induced by β-amyloid. As shown in Fig. 5, 25 μM of β-amyloid significantly induced both iNOS and COX-2 expression in microglia, and WM inhibited the expression induced by β-amyloid in a dose-dependent manner. These results suggest that WM is able to act against several inflammatory stimuli, such as LPS/IFN-γ and β-amyloid, and might be useful for prevention of inflammation in the brain.

4.5. Methanol extracts of wild fruiting body and solid-state culture of Taiwanofungus camphoratus inhibited TPA-induced ear edema in mice

To examine whether *Taiwanofungus camphoratus* can inhibit inflammation in animals, mice ears were treated with TPA and/or methanol extracts of *Taiwanofungus camphoratus*, and the ear edema was evaluated. Various concentrations of WM or SM were tested for their ability to inhibit TPA-induced ear edema. As shown in Fig. 6, WM had the best effect on the inhibition of ear edema induced by TPA. Application of 0.5, 1.0, or 3.0 mg of WM inhibited TPA-induced ear edema by 34.8%, 70.4%, or 88.7%, respectively. SM also exhibited strong inhibition on TPA-induced ear edema, but it was less effective than WM. The results suggest that both WM and

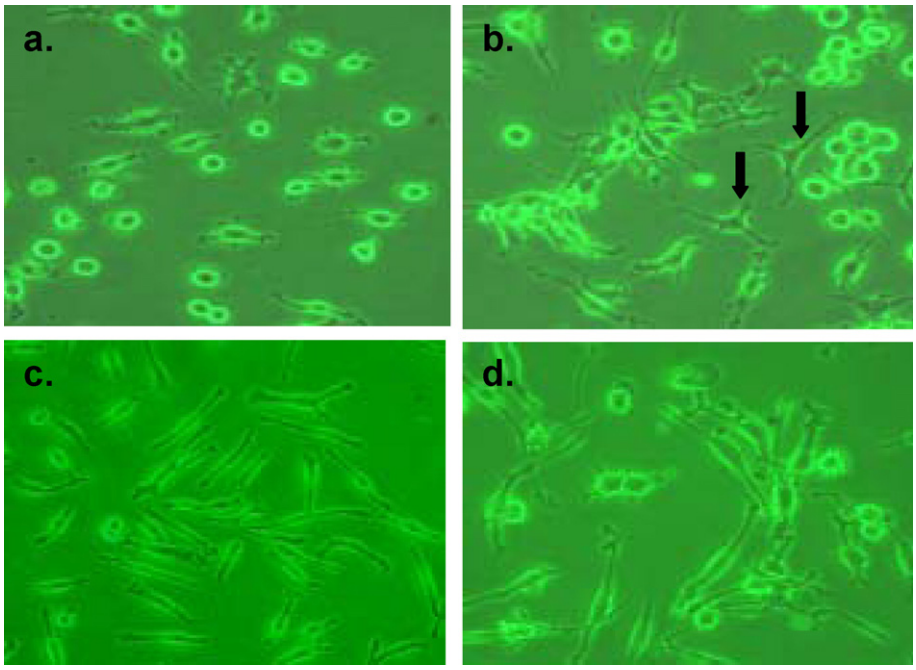

Fig. 4. Inhibitory effects of *Taiwanofungus camphoratus* wild fruiting body methanol extracts on the LPS/IFNγ-induced morphology changes in microglia. EOC13.31 cells were pretreated with 50 μg/ml of WM for 1 h, and then LPS (100 ng/ml) and IFN-γ (50 ng/ml) for 24 h, and then fixed and photographed (200×): (a) control, (b) LPS/IFN-γ, (c) WM alone and (d) LPS/IFN-γ + WM. Arrow indicates the pseudopodia in activated microglia.

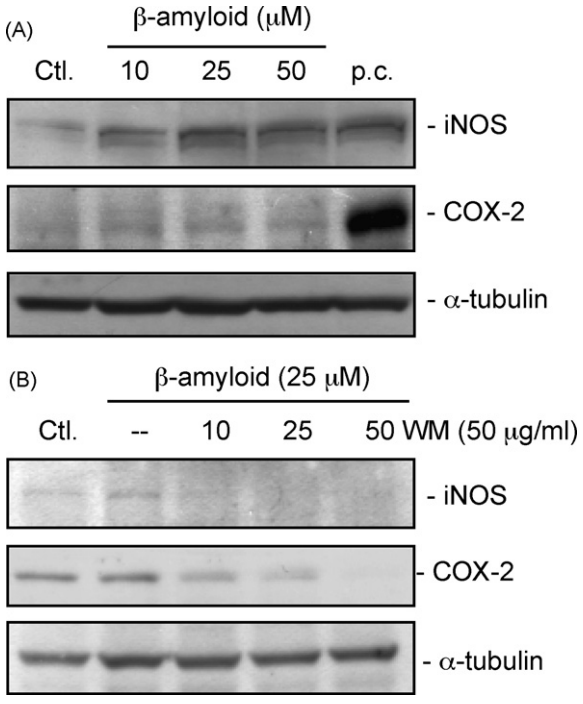

Fig. 5. Inhibitory effects of methanol extracts of wild fruiting body of *Taiwanofungus camphoratus* on the β-amyloid-induced iNOS and COX-2 expression in microglia. (A) EOC13.31 cells were treated with various concentrations of β-amyloid for 24 h. (B) EOC13.31 cells were pretreated with various concentrations of WM for 1 h, and then 25 μM β-amyloid for 24 h. Total cellular protein (50 μg) was separated on 10% SDS-polyacrylamide gels and blotted with antibody-specific iNOS, COX-2 and α-tubulin antibodies.

SM exhibited anti-inflammatory activities both *in vitro* and *in vivo*. To determine which ingredients were the most effective components in the WM, the extracts were separated by Sephadex LH-20 and five fractions were obtained (CKJ-35-2–6). Of these, CKJ-35-2 appeared to inhibit both iNOS and COX-2 expression most potently in LPS/IFNγ-activated microglia in the following order: CKJ-35-2 > CKJ-35-3 > CKJ-35-4 > CKJ-35-5 > CKJ-35-6 (Fig. 7).

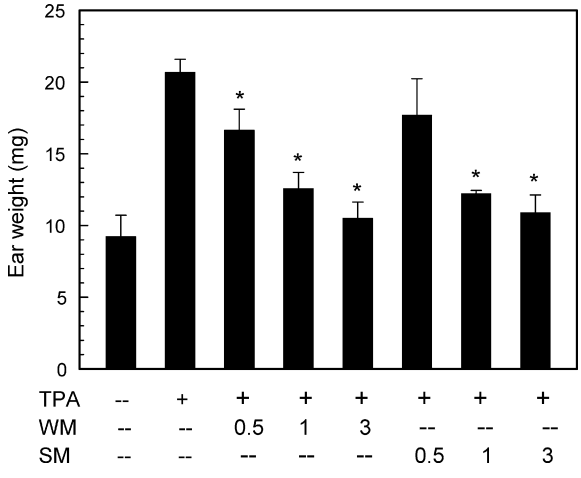

Fig. 6. Inhibitory effects of methanol extracts of wild fruiting body of *Taiwanofungus camphoratus* and of *Taiwanofungus camphoratus* produced through solid-state culture on TPA-induced ear edema. Both ears of mice were pretreated topically with vehicle (15 μl acetone), WM, or SM for 30 min, then treated with TPA (1 nmol). After 7 h of TPA treatment, the mice were sacrificed, and 6.5 mm diameter ear punches were weighed. Data represented the mean ± S.E. of triplicate tests. *Statistically different from TPA alone group ($P < 0.05$, Student's *t*-test).

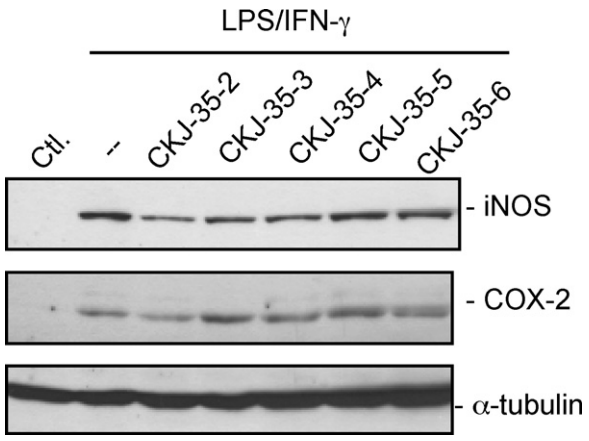

Fig. 7. Effects of five fractions of methanol extracts of wild fruiting body of *Taiwanofungus camphoratus* on the LPS/IFNγ-induced iNOS and COX-2 expression in microglia. EOC13.31 cells were pretreated with five fractions (CKJ-35-2, CKJ-35-3, CKJ-35-4, CKJ-35-5 and CKJ-35-6) of WM (12.5 μg/ml) for 1 h, and then LPS (100 ng/ml) and IFN-γ (50 ng/ml) for 24 h. Total cellular protein (50 μg) was separated on 10% SDS-polyacrylamide gels and blotted with antibody-specific iNOS, COX-2 and α-tubulin antibodies.

5. Discussion and conclusion

The wild fruiting body of *Taiwanofungus camphoratus* is restricted to Taiwan forests and is well known as an effective and expensive folk remedy for many human diseases. Two artificial methods of cultivating *Taiwanofungus camphoratus* were developed to compensate for the scarcity of wild fruiting bodies. However, there has been a dearth of papers on the comparison between wild fruiting body and artificially cultivated *Taiwanofungus camphoratus* with regard to their biological functions. In this study, we have investigated wild fruiting body of *Taiwanofungus camphoratus* and *Taiwanofungus camphoratus* produced through solid-state culture and liquid-state fermentation and their anti-inflammatory activity in mice microglia. We demonstrated that WM significantly inhibited iNOS, COX-2, and TNF-α expression in LPS/IFNγ- or β-amyloid-activated microglia. Methanol extracts from solid-state culture produced results similar to those for wild fruiting body, but they were less effective on the anti-inflammatory activity in microglia. These findings provide further insight into the various anti-inflammatory activities among these three specimens of *T. camphorates*.

Previous studies have reported that extracts from wild fruiting body or artificially cultivated *T. camphorate* exhibited antioxidative, anti-inflammatory, and anti-tumor activities as well as protective effects against liver toxicity and neuron damage. In the antioxidative experiment, water extract and other solvent extracts of mycelia (products from liquid-state fermentation) exhibited antioxidative activities in preventing the depletion of glutathione and ATP in erythrocytes (Hseu et al., 2002), preventing CuSO4-oxidized LDL (Yang et al., 2006a), and preventing lipid peroxidation (Song and Yen, 2002; Hsiao et al., 2003). Several reports demonstrated that extracts from wild fruiting body had a strong anti-tumor activity in many types of human tumor cells. These extracts have been found to inhibit the growth of bladder cancer cells, arrest the cell cycle in the G2/M phase (Peng et al., 2006), and inhibit the growth of MCF-7 human breast cancer cells and HepG2 and PLC/PRF/5 human hepatoma cells through the induction of apoptosis (Hsu et al., 2005; Yang et al., 2006b). More studies using the extracts from liquid-state fermentation have demonstrated anti-tumor activity. The extracts from mycelia induced apoptosis of human hepatoma cells HepG2 and Hep3B through regulation of the Fas pathway and activation of caspases cascades (Song et al., 2005a, 2005b). Other reports showed that the extracts from liquid-state fermentation induced apoptosis in human leukemia U937 and HL-60 cells (Liu et al., 2004; Hseu et al., 2004). Mycelia extracts also reduced liver fibrosis and hepatic toxicity induced by CCl_4 in rat (Hsiao et al., 2003; Song and Yen, 2003; Lin et al., 2006). Ethanol extracts from solid-state culture significantly inhibited the proliferation of human lung carcinoma cells A549 by inducing endoplasmic reticulum stress, but not the proliferation of primary human fetal lung fibroblast MRC-5 cells (Wu et al., 2006).

Regarding the anti-inflammatory effect of *Taiwanofungus camphoratus*, Hseu et al. demonstrated that extracts from liquid-state fermentation could inhibit iNOS and COX-2 expression through the NF-κB pathway in mouse macrophage RAW264.7 cells (Hseu et al., 2005). Zhankuic acid isolated from ethanol extracts of wild fruiting body has shown anti-inflammatory effects induced by fMLP and TPA in human neutrophils (Shen et al., 2004). However, the underlying molecular mechanisms are not well understood. In this study, we used both wild fruiting body and artificially cultivated *Taiwanofungus camphoratus* and different extraction solvents, and compared their anti-inflammatory activity. Our results showed the methanol extracts from wild fruiting body exhibited more potency than water extracts on the anti-inflammatory activity in microglia. Several functional components of the wild fruiting body have been isolated, including fatty acids, lignans, phenyl derivatives, sesquiterpenes, steroids, and triterpenoids (Chen et al., 1995; Chiang et al., 1995; Cherng et al., 1996; Shen et al., 2003). The methanol extract is rich in triterpenoids, steroids, and some phenyl derivatives, but lacks polysaccharides, while the water extract is rich in monosaccharide, nucleosides, amino acids, and polysaccharides, and has modest amounts of phenyl compounds and triterpenoids. Several reports have demonstrated that triperpenes inhibit inflammation in many assays (Safayhi and Sailer, 1997; Takada and Aggarwal, 2003; Nam, 2006). In our methanol extracts of wild fruiting body, the triterpenoids might be the major active compounds suppressing inflammation. Our results also showed that extracts from solid-state culture were similar to wild fruiting body in anti-inflammatory activity, but liquid-state fermentation was less effective. A previous report by Chen et al. (2001) showed that solid-state fermentation contained high amounts of triterpenoids and that the triterpenoid HPLC profile was similar to that of wild fruiting body. On the other hand, liquid-state fermentation made it very difficult to produce triterpenoids. Different amounts of triterpenoids in the extracts may possibly result in different levels of anti-inflammatory activity. Although several reports have demonstrated that extracts of *Taiwanofungus camphoratus* produced by liquid-state fermen-

tation have multiple biological functions as described in above, our results indicate it has fewer anti-inflammatory effects in microglia. This might be attributable to the poor triterpenoid content that results from liquid-state fermentation. In Fig. 7, the CKJ-35-2 fraction showed the most potent inhibition of iNOS and COX-2 expression. However, the specific chemical behind this inhibition of inflammation has not yet been identified.

Another report indicated that methanol extract of wild fruiting body contains several diterpenoids and these exhibited a neuroprotective effects against β-amyloid in primary neonatal cortical neurons (Chen et al., 2006). The adenosine component from the *Taiwanofungus camphoratus* produced from liquid-state fermentation prevented PC 12 cells from serum deprivation-induced apoptosis (Lu et al., 2006). These results together with our finding suggest that extracts from *Taiwanofungus camphoratus* are able to protect against neurotoxicity through several different mechanisms and may be useful for prevention of neurodegenerative diseases.

Acknowledgments

This work was supported by the Agriculture and Food Agency, Council of Agriculture, Republic of China (93AS-5.1.3-FD-Z3 and 94AS-12.1.3-FD-Z1), Topnotch Stroke Research Center Grant, Ministry of Education, Republic of China and the Well Shine Biotechnology Development Co., which provided the *Taiwanofungus camphoratus* samples.

References

Banati, R.B., Daniel, S.E., Blunt, S.B., 1998. Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. Movement Disorders 13, 221–227.

Chen, C.H., Yang, S.W., Shen, Y.C., 1995. New steroid acids from *Antrodia cinnamomea*, a fungal parasite of *Cinnamomum micranthum*. Journal of Natural Products 58, 1655–1661.

Chen, C.J., Su, C.H., Lan, M.H., 2001. Study on solid cultivation and bioactivity of *Antrodia camphorata*. Fungal Science 16, 65–72.

Chen, C.C., Shiao, Y.J., Lin, R.D., Shao, Y.Y., Lai, M.N., Lin, C.C., Ng, L.T., Kuo, Y.H., 2006. Neuroprotective diterpenes from the fruiting body of *Antrodia camphorata*. Journal of Natural Products 69, 689–691.

Cherng, I.H., Wu, D.P., Chiang, H.C., 1996. Triteroenoids from *Antrodia cinnamomea*. Phytochemistry 41, 263–267.

Chiang, H.C., Wu, D.P., Cherng, I.H., Ueng, C.H., 1995. A sesquiterpene lactone, phenyl and biphenyl compounds from *Antrodia cinnamomea*. Phytochemistry 39, 613–616.

Hayes, G.M., Woodroofe, M.N., Cuzner, M.L., 1988. Characterisation of microglia isolated from adult human and rat brain. Journal of Neuroimmunology 19, 177–189.

Hseu, Y.C., Chang, W.C., Hseu, Y.T., Lee, C.Y., Yech, Y.J., Chen, P.C., Chen, J.Y., Yang, H.L., 2002. Protection of oxidative damage by aqueous extract from *Antrodia camphorata* mycelia in normal human erythrocytes. Life Sciences 71, 469–482.

Hseu, Y.C., Yang, H.L., Lai, Y.C., Lin, J.G., Chen, G.W., Chang, Y.H., 2004. Induction of apoptosis by *Antrodia camphorata* in human premyelocytic leukemia HL-60 cells. Nutrition and Cancer 48, 189–197.

Hseu, Y.C., Wu, F.Y., Wu, J.J., Chen, J.Y., Chang, W.H., Lu, F.J., Lai, Y.C., Yang, H.L., 2005. Anti-inflammatory potential *of Antrodia Camphorata* through inhibition of iNOS, COX-2 and cytokines via the NF-kappaB pathway. International Immunpharmacology 5, 1914–1925.

Hsiao, G., Shen, M.Y., Lin, K.H., Lan, M.H., Wu, L.Y., Chou, D.S., Lin, C.H., Su, C.H., Sheu, J.R., 2003. Antioxidative and hepatoprotective effects of *Antrodia camphorata* extract. Journal of Agricultural and Food Chemistry 51, 3302–3308.

Hsu, Y.L., Kuo, Y.C., Kuo, P.L., Ng, L.T., Kuo, Y.H., Lin, C.C., 2005. Apoptotic effects of extract from *Antrodia camphorata* fruiting bodies in human hepatocellular carcinoma cell lines. Cancer Letter 221, 77–89.

Jones, B.W., Means, T.K., Heldwein, K.A., Keen, M.A., Hill, P.J., Belisle, J.T., Fenton, M.J., 2001. Different Toll-like receptor agonists induce distinct macrophage responses. Journal of Leukocyte Biology 69, 1036–1044.

Kreutzberg, G.W., 1996. Microglia: a sensor for pathological events in the CNS. Trends in Neurosciences 19, 312–318.

Kim, Y.S., Kim, S.S., Cho, J.J., Choi, D.H., Hwang, O., Shin, D.H., Chun, H.S., Beal, M.F., Joh, T.H., 2005. Matrix metalloproteinase-3: a novel signaling proteinase from apoptotic neuronal cells that activates microglia. Journal of Neuroscience 25, 3701–3711.

Lin, W.C., Kuo, S.C., Lin, W.L., Fang, H.L., Wang, B.C., 2006. Filtrate of fermented mycelia from *Antrodia camphorata* reduces liver fibrosis induced by carbon tetrachloride in rats. World Journal of Gastroenterology 12, 2369–2374.

Liu, J.D., Lin, S.Y., Ho, Y.S., Pan, S., Hung, L.F., Tsai, S.H., Lin, J.K., Liang, Y.C., 2003. Involvement of c-Jun N-terminal kinase activation in 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and prostaglandin A_1-induced apoptosis in AGS gastric epithelial cells. Molecular Carcinogenesis 37, 16–24.

Liu, J.J., Huang, T.S., Hsu, M.L., Chen, C.C., Lin, W.S., Lu, F.J., Chang, W.H., 2004. Antitumor effects of the partially purified polysaccharides from *Antrodia camphorata* and the mechanism of its action. Toxicology and Applied Pharmacology 201, 186–193.

Liu, D.Z., Liang, Y.C., Lin, S.Y., Lin, Y.S., Wu, W.C., Hou, W.C., Su, C.H., 2006. Antihypertensive activities of solid-state culture of *Taiwanofungus camphoratus* (Chang-chih) in spontaneously hypertensive rats. Bioscience, Biotechnology, and Biochemistry 71, 23–30.

Lu, M.K., Cheng, J.J., Lai, W.L., Lin, Y.R., Huang, N.K., 2006. Adenosine as an active component of *Antrodia cinnamomea* that prevents rat PC 12 cells from serum deprivation-induced apoptosis through the activation of adenosine A (2A) receptors. Life Sciences 79, 252–258.

McGeer, P.L., Itagaki, S., Boyes, B.E., McGeer, E.G., 1988. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology 38, 1285–1291.

Nam, N.H., 2006. Naturally occurring NF-kappaB inhibitors. Mini-Reviews in Medicinal Chemistry 6, 945–951.

Nelson, P.T., Soma, L.A., Lavi, E., 2002. Microglia in diseases of the central nervous system. Annals of Medicine 34, 491–500.

Peng, C.C., Chen, K.C., Peng, R.Y., Su, C.H., Hsieh-Li, H.M., 2006. Human urinary bladder cancer T24 cells are susceptible to the Antrodia camphorata extracts. Cancer Letter 243, 109–119.

Raine, C.S., 1994. Multiple sclerosis: immune system molecule expression in the central nervous system. Journal of Neuropathology and Experimental Neurology 53, 328–337.

Safayhi, H., Sailer, E.R., 1997. Anti-inflammatory actions of pentacyclic triterpenes. Planta Medica 63, 487–493.

Shen, C.C., Kuo, Y.C., Huang, R.L., Lin, L.C., Don, M.J., Chang, T.T., Chou, C.J., 2003. New ergostane and lanostane from *Antrodia camphorata*. Journal of Chinese Medicine 14, 247–258.

Shen, Y.C., Wang, Y.H., Chou, Y.C., Chen, C.F., Lin, L.C., Chang, T.T., Tien, J.H., Chou, C.J., 2004. Evaluation of the anti-inflammatory activity of zhankuic acids isolated from the fruiting bodies of *Antrodia camphorata*. Planta Medica 70, 310–314.

Song, T.Y., Yen, G.C., 2002. Antioxidant properties of *Antrodia camphorata* in submerged culture. Journal of Agricultural and Food Chemistry 50, 3322–3327.

Song, T.Y., Yen, G.C., 2003. Protective effects of fermented filtrate from *Antrodia camphorata* in submerged culture against CCl4-induced hepatic toxicity in rats. Journal of Agricultural and Food Chemistry 51, 1571–1577.

Song, T.Y., Hsu, S.L., Yeh, C.T., Yen, G.C., 2005a. Mycelia from *Antrodia camphorata* in submerged culture induce apoptosis of human hepatoma HepG2

cells possibly through regulation of Fas pathway. Journal of Agricultural and Food Chemistry 53, 5559–5564.

Song, T.Y., Hsu, S.L., Yen, G.C., 2005b. Induction of apoptosis in human hepatoma cells by mycelia of *Antrodia camphorata* in submerged culture. Journal of Ethnopharmacology 100, 158–167.

Sweet, M.J., Hume, D.A., 1996. Endotoxin signal transduction in macrophages. Journal of Leukocyte Biology 60, 8–26.

Takada, Y., Aggarwal, B.B., 2003. Betulinic acid suppresses carcinogen-induced NF-kappa B activation through inhibition of I kappa B alpha kinase and p65 phosphorylation: abrogation of cyclooxygenase-2 and matrix metalloprotease-9. Journal of Immunology 171, 3278–3286.

Takeuchi, O., Akira, S., 2001. Toll-like receptors, their physiological role and signal transduction system. International Immunpharmacology 1, 625–635.

Tsai, Z.T., Liaw, S.L., 1985. The Use and the Effect of Ganoderma. Sheng-Yun Publishers, Inc., Taichung, Taiwan, pp. 116–117.

Wu, H., Pan, C.L., Yao, Y.C., Chang, S.S., Li, S.L., Wu, T.F., 2006. Proteomic analysis of the effect of *Antrodia camphorata* extract on human lung cancer A549 cell. Proteomics 6, 826–835.

Yang, H.L., Hseu, Y.C., Chen, J.Y., Yech, Y.J., Lu, F.J., Wang, H.H., Lin, P.S., Wang, B.C., 2006a. *Antrodia camphorata* in submerged culture protects low density lipoproteins against oxidative modification. American Journal of Chinese Medicine 34, 217–231.

Yang, H.L., Chen, C.S., Chang, W.H., Lu, F.J., Lai, Y.C., Chen, C.C., Hseu, T.H., Kuo, C.T., Hseu, Y.C., 2006b. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*. Cancer Letter 231, 215–227.