Evaluation of the anti-inflammatory and anti-proliferation tumoral cells activities of *Antrodia camphorata*, *Cordyceps sinensis*, and *Cinnamomum osmophloeum* bark extracts

Yerra Koteswara Rao [a], Shih-Hua Fang [b], Yew-Min Tzeng [a,*]

[a] *Institute of Biotechnology, Chaoyang University of Technology, Wufeng 413, Taiwan, ROC*
[b] *Department of Microbiology, School of Medicine, China Medical University, Taichung 400, Taiwan, ROC*

Abstract

The extracts of chloroform (**1**) and methanol (**2**) from *Antrodia camphorata* (AC), and chloroform (**3**) and *n*-butanol (**4**) fractions of methanol extract from *Cordyceps sinensis* (CS), and hexane (**5**), ethyl acetate (**6**), and methanol (**7**) from *Cinnamomum osmophloeum* bark (CO) were evaluated for their anti-inflammatory as well as tumor-cell growth inhibitory activities in vitro. All the tested extracts dose dependently inhibited the enhanced production of inflammatory mediators such as nitric oxide (NO) through reducing inducible NO synthase expression, and cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-12 in LPS/IFN-γ activated murine peritoneal macrophages. In addition, extracts **1** from AC, and **5** and **6** from CO significantly arrest the mitogen-stimulated spleen cells in G0/G1 stage. On the other hand, all these extracts were also evaluated for their tumor-cell proliferation activities in different type of cancer cell lines such as Jurkat, HepG2, PC 3, Colon 205, and MCF 7 as well as normal PBMCs. Compared to untreated controls, the extracts **1**, **2**, and **4**–**7** were most active and inhibited Jurkat cells with IC_{50} value of 22, 40, 18, 4, 5, and 45 μg/ml, respectively. In addition, the extracts **5**, **6**, and **7** from CO showed potent growth inhibition of HepG2 and PC 3 with IC_{50} values of 35, 80, 55 μg/ml; and 42, 125, and 50 μg/ml, respectively. Similarly, the extracts **1** and **5** inhibited the growth of Colon 205 and MCF 7 cells with IC_{50} values of 65, 33; and 95 and 30 μg/ml, respectively. Interestingly, none of the tested extract has shown cytotoxicity towards normal PBMCs up to the concentration range studies (0–150 μg/ml). Taken together, these data suggest that the anti-inflammatory and anti-cancer properties of AC, CS, and CO might result from the growth inhibition of NO, TNF-α and IL-12, and tumor cells proliferation, respectively.

Keywords: *Antrodia camphorata*; *Cordyceps sinensis*; *Cinnamomum osmophloeum*; Macrophages; Anti-inflammatory; Tumor-cell proliferation

1. Introduction

Macrophages are important in non-specific host resistance to microbial pathogens and serve as central regulators of the specific immune response. The Gram-negative bacterial cell wall, in particular lipopolysaccharide (LPS), and interferon (IFN)-γ can stimulate macrophage immune cells to release inflammatory cytokines. Among them, the reactive free radical nitric oxide (NO) synthesized by inducible NO synthase (iNOS), and the pro-inflammatory cytokines, TNF-α, and interleukin (IL)-12 are important (Linton and Fazio, 2003). Moderate levels of these inflammatory mediators are important for host survival from infection, and also are required for repair of tissue injury. However, overproduction of these inflammatory mediators could be hazardous to healthy tissue and reported to be involved in the development of a number of inflammatory diseases. For example, the excess production of NO can destroy functional normal tissues during acute and chronic inflammation (Clancy et al., 1998). Extensive prior studies have demonstrated the efficacy of TNF-α and IL-12 blocking therapies in various inflammatory and autoimmune diseases such as rheumatoid arthritis (RA) and Crohn's disease (Tracey and Cerami, 1994; Barrie and Plevy, 2005). On the other hand, cancer incidence and morbidity are steadily increasing in several parts of the world even though there is a decline in certain cancers (Thun et al., 2002). It was reported that the tumor promoters recruit the inflammatory cells

* Corresponding author. Tel.: +886 4 23323000x3003; fax: +886 4 23304896.
E-mail address: ymtzeng@cyut.edu.tw (Y.-M. Tzeng).

to stimulate the reactive oxygen species and promote cancer development (Whitcomb, 2004).

There is an increasing interest in using natural products to modulate immune responses and neutralize inflammatory processes, and these would suggest as a promising anti-inflammatory agents (Nam, 2006). *Antrodia camphorata* (Polyporaceae, Aphyllophorales) is a parasitic fungus on the inner heartwood wall of the endemic species *Cinnamomum kanehirai* Hay (Lauraceae) (Chang and Chou, 1995). Because of the growth rate of natural *Antrodia camphorata* in the wild is very slow and it is difficult to cultivate them in a greenhouse, the fruiting bodies are rare and expensive. It is used in folk medicine in the treatment of inflammatory skin diseases, food, alcohol, drug intoxication, diarrhea, abdominal pain, hypertension, skin itches, and liver cancer among Chinese (Tsai and Liaw, 1985). It has been reported that the fermented culture broth of *Antrodia camphorata* inhibited iNOS, COX-2, and cytokines via the NF-κB pathway (Hseu et al., 2005). It was also reported that *Antrodia camphorata* exhibited neuronal-like PC-12 cell apoptosis through a PKA/CREB-dependent pathway (Huang et al., 2005). *Cordyceps sinensis* (Chinese caterpillar fungus) is a fungus parasitic on the larvae of Lepidoptera and has been considered to be a precious tonic food and herbal medicine since ancient times in China and Taiwan (Ng and Wang, 2005). The mycelium extracts of *Cordyceps sinensis* induced human premyelocytic leukemia cell apoptosis through mitochondrion pathway (Zhang and Wu, 2007). The plant, *Cinnamomum osmophloeum* Kaneh. (Lauraceae), named as "Indigenous cinnamon tree", is one of the endemic hardwood species that grows in Taiwan at an elevation between 400 and 1500 m (Hu et al., 1985). This species has long been used in folk medicine for their interesting biological activities such as antidiabetic, anti-inflammatory, intestinal infections, astringent, and diuretic (Huang, 2003).

Continuing our screening for bioactive natural products (Fang et al., 2005; Rao et al., 2005a, 2006), and in order to validate the use of *Antrodia camphorata* (AC), *Cordyceps sinensis* (CS) fruiting bodies, and *Cinnamomum osmophloeum* bark (CO) as an anti-inflammatory agent in folk medicine, this study has been undertaken. The modulating effect of extracts from these species on the cellular inflammatory events was examined using an in vitro model LPS/IFN-γ-stimulated macrophage cells, and monitoring of the production of inflammatory cytokines, NO, TNF-α, and IL-12. In addition, to obtain some preliminary insights on the mechanism(s) of action, these extracts effect on the cell cycle progress of mice spleen cells was also examined. Furthermore, we also demonstrated that the growth inhibitory effect of these extracts on human cancer cell lines, namely, Jurkat (lymphocytic), HepG2 (hepatoma), PC 3 (hormone-resistant prostate), Colon 205 (colon), and MCF 7 (breast).

2. Materials and methods

2.1. General

Lipopolysaccharide (LPS, *Escherichia coli* 055: B5), bovine serum albumin (BSA), phosphate-buffered solution (PBS), concanavalin A (Con A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and Griess reagent were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Recombinant interferon-γ (IFN-γ) was purchased from PeproTech (Margravine, London, England). RPMI-1640 medium, Hank's balanced salt solution (HBSS), penicillin, streptomycin, L-glutamine, and fetal calf serum were purchased from Gibco BRL (Grand Island, NY, U.S.A.).

2.2. Preparation of extracts

The fruiting bodies of *Antrodia camphorata* and *Cordyceps sinensis* were obtained through solid state fermentation (SSF) process in our laboratory (accession numbers G908AC, and G908CS, respectively). The bark of *Cinnamomum osmophloeum* was collected in September 2006 from Fenglin, Hualien County in Eastern Taiwan (voucher number: YMT-05-03). The freshly collected materials were dried under shade, sliced into small pieces, pulverized using a mechanical grinder and passed through 40 mach sieve, and preserved in an airtight container for further use. The voucher specimens were deposited in the Herbarium of the Institute of Biotechnology, Chaoyang University of Technology, Taiwan.

The powdered fruiting bodies of *Antrodia camphorata* (10 g) were extracted with $CHCl_3$ (200 ml) and MeOH (200 ml) successively at room temperature (3 days twice). After filtration, the solvent was evaporated under reduced pressure obtaining the $CHCl_3$ and MeOH extracts [AC-C (**1**) and AC-M (**2**)] in yield of 23.6% and 8.7% (w/w), respectively. On the other hand, powdered dried fruiting bodies of *Cordyceps sinensis* (15 g) were extracted with methanol using soxhlet. The methanol extract (4.6 g) was separated into $CHCl_3$, *n*-butanol (*n*-BuOH), and water-soluble fractions. The $CHCl_3$ and *n*-BuOH fractions were concentrated in vacuum to obtained extracts **3** (CS-C, 5.7%) and **4** (CS-B, 3.5%), w/w, respectively. The powdered bark of *Cinnamomum osmophloeum* (100 g) was extracted with 600 ml of hexane, ethyl acetate (EtOAc), and MeOH separately and successively with Soxhlet extractor for 6 h at 60 °C. The extracts were filtered through Whatman filter paper No. 1 and concentrated under vacuum to get crude viscous extracts **5** (CO-H, 13.2%), **6** (CO-EA, 6.5%), and **7** (CO-M, 4.1%, w/w), respectively. The solvent-free extracts were used for the present study.

2.3. Mice

BALB/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) and maintained in the Animal Center of China Medical University. The animal room was at a 12-h light:12-h dark cycle with constant temperature and humidity. All mice used were 8 weeks of age. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals (NRC, U.S.A.) and approved by the Committee of China Medical University.

2.4. Cell culture

Animals were sacrificed by cervical spine dislocation. Mouse peritoneal excluded macrophages were obtained from mice by

lavage with 10 ml of cold Hank's balanced salt solution (HBSS; Life Technologies, GIBCO-BRL, Gaithersburg, MD) per mouse at 3 days after intraperitoneal (i.p.) injection of 2 ml of 3% thioglycollate in saline (1.5 ml per mouse, Difco, Detroit, MI). The spleen was removed and crushed into a single cell suspension, and red blood cells lysed with Tris-buffered ammonium chloride before washing (three times) with HBSS. Jurkat (human lymphocytic cancer cell line, ATCC TIB 152), PC-3 (human prostate cancer cell line, ATCC CRL 1435), HepG2 (human hepatoma cancer cell line, ATCC HB 8065), and Colon 205 (human colon cancer cell line, ATCC CCL 222) were obtained from American Type Culture Collection (Rockville, MD) and cultured with complete RPMI-1640 medium (Gibco BRL) supplemented with 10% fetal calf serum, antibiotics, L-glutamine. Blood was collected from healthy volunteers, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. The cell numbers were determined with a hemocytometer, and viabilities were assessed by Trypan Blue dye exclusion. Cells were seeded at a density of 2×10^6 cells/ml and incubated at 37 °C in humidified 5% CO_2/95% air to allow macrophages adherence. Two hours later, the non-adherent cells were removed by washing with warmed PBS and the remaining cells (90% macrophages, judged by non-specific esterase stain) were incubated with medium containing various concentrations of test extracts. All the tested extracts were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO added to medium was 0.1% (v/v). Control cells were grown under identical conditions but were not exposed to the test extracts or mitogen. All culture materials were disposable and free of endotoxin.

2.5. Determination of nitric oxide

Supernatant nitrite concentrations were quantified as a marker of NO secretion using a commercial kit (R&D Systems, Minneapolis, MN) based on the Griess colorimetric assay (Fang et al., 2005). Concentrations of nitrites secreted by untreated-stimulated cells were regarded as 100%, and nitrite concentrations secreted by treated-stimulated cells were compared with those secreted by untreated-stimulated cells.

2.6. Cell cycle analysis

Cell cycle analysis was performed by the method described previously (Fang et al., 2006). Cells were washed in PBS and stained with 20 μg/ml propidium iodide in 0.1% Triton X-100, and 0.1 mM EDTA. Cell suspensions were analyzed with a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA). The percentage of cell cycle distribution in the G0/G1, S, and G2/M phases was determined using the MODFIT software (Becton Dickinson).

2.7. Western blot analysis

Cells were cultured in a 10 mm plate in the presence of different concentrations of extracts for 24 h. Cells were washed with PBS, harvested, and homogenized. The lysate was centrifuged at 15,000 × *g* for 30 min, and the supernatant was collected to determine the protein concentrations. Electrophoresis was performed using 8% SDS-PAGE and transferred to immobilon polyvinylidene difluoride membranes (Millipore, Germany). The membrane was incubated overnight at 4, blocking with 1% BSA and then incubated with anti-iNOS and anti-α-tublin antibodies (Santa Cruz Biotechnology Inc., U.S.A.). Expression of protein was detected by enhanced chemiluminescence (ECL) using Hyperfilm and ECL reagent (Amersham, UK). The intensity of the bands was scanned and quantified by AlphaImager TM 2200.

2.8. Cytokine analysis

Cytokines (TNF-α and IL-12) were analyzed from cell culture medium of macrophage cells by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions.

2.9. Determination of inhibition of tumor-cell proliferation

The MTT assay was used to measure the ability of extracts to inhibit human cancer cell proliferation (Rao et al., 2005b). The cell cultures were exposed to various concentrations of the extracts during a 72 h growth period. Cell proliferation was measured by the ability of viable cells to reduce MTT to formazan based on the ability of living cells to utilize Thiazolyl Blue and convert it into purple formazan, which absorbs light at 570 nm and could be analyzed spectrophotometrically. Measurement was performed in triplicate. The absorbance was measured using the Beckman UV–vis spectrophotometer.

2.10. Statistical analyses

All data are expressed as mean ± S.D. and accompanied by four distinct experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnetts post hoc test, and the significant difference was set at $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

3.1. Growth inhibition of NO, TNF-α, and IL-12

As a first step, the cytotoxic activity of tested extracts on macrophage cells in the absence and presence of LPS/IFN-γ was assessed using MTT assay. Co-treatment of both unstimulated and stimulated macrophage cells with these extracts did not affect the cell viability (data not shown). These results suggest that the effects on activated macrophage cells induced by LPS/IFN-γ were unlikely due to the toxic effects of tested extracts. The productions of NO, TNF-α, and IL-12 from unstimulated cells were not detectable. Moreover, pretreatment of unstimulated cells with extracts over 48 h did not result in

Table 1
The inhibitory effect of crude extracts **1**–**7** on the NO, TNF-α and IL-12 production and cell viability in LPS/IFN-γ activated murine peritoneal macrophages

Extract	Conc. (μg/ml)	Inhibition (%)			OD_{570}
		NO	TNF-α	IL-12	
	0				0.412
AC-C (**1**)	3.125	27.2*	34.8*	20.1*	0.425
	6.25	51.2**	53.8**	51.8**	0.432
	12.5	65.7**	71.1***	64.5**	0.416
	25	92.8***	89.2***	76.3***	0.422
AC-M (**2**)	6.25	3.1	2.5	5.3	0.429
	12.5	20.2*	19.2*	26.6*	0.425
	25	35.2*	41.9**	58.7**	0.418
	50	69.5***	68.1***	73.6***	0.421
CS-C (**3**)	25	4.7	1.1	−2.4	0.426
	50	38.8*	32.5*	29.1*	0.452
	75	59.5**	52.6**	51.4**	0.416
	100	70.7***	68.1***	66.6***	0.442
CS-B (**4**)	25	2.2	9.4	−1.2	0.431
	50	51.4**	38.4**	29.4**	0.438
	75	55.5**	46.6**	38.4**	0.419
	100	72.3***	73.4***	66.8**	0.425
CO-H (**5**)	0.5	5.4	1.4	−4.3	0.436
	1	4.1	7.4	3.4	0.427
	2.5	30.6*	33.2*	39.8*	0.428
	5	93.7***	86.6***	75.8***	0.431
CO-EA (**6**)	2.5	45.8**	38.7**	31.6**	0.435
	5	78.6***	62.5**	61.5**	0.441
	10	89.6***	76.6***	64.8**	0.428
	12.5	92.1***	88.6***	79.4***	0.431
CO-M (**7**)	10	15.7	3.1	11.4	0.426
	20	22.5*	14.9	32.9*	0.425
	40	55.4**	51.6**	59.8**	0.431
	60	67.2***	67.1***	69.4***	0.438

The significant difference was set at $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ compared to LPS/IFN-γ activated murine peritoneal macrophages.

any change in the production of NO, TNF-α, and IL-12 (data not shown).

Table 1 summarizes the effects of tested extract on NO secretion of stimulated cells. According to nitrite assay, the extracts **1** from AC; and **5** and **6** from CO strongly inhibited NO production in a dose-dependent manner, and the inhibition reached 92.8%, 93.7%, and 92.1% at concentration of 25, 5, and 12.5 μg/ml, respectively. Interestingly, in the tested two fungal fruiting bodies, AC extracts reduced nitrite levels more than CS. Thus, 25 and 50 μg/ml, of extracts **1** and **2** from AC inhibited NO release by 92.8, and 69.5, respectively, while relatively at higher concentration 100 μg/ml of extracts **3** and **4** from CS limited NO secretion by 70.7% and 72.3%, respectively (Table 1). In addition, to study the possible inhibitory mechanism of these extracts on NO production, we compared the NO production from macrophages in which inducible NO synthase (iNOS) expression had already been induced. We found that the inhibitory action of all the extracts was lost, except **1**, which had 30% inhibition, when peritoneal macrophages had been pretreated with LPS/IFN-γ for 24 h (data not shown).

Next, treatment of macrophage cells with LPS/IFN-γ for 24 h significantly enhanced TNF-α, and IL-12 productions, but co-treatment with extracts **1**–**7** suppressed such enhancement by increasing concentrations of the extracts, and the inhibition pattern was similar to that seen in the NO measurement. As shown in Table 1, the extracts **1**, **5**, and **6** extracts significantly blocked the production of TNF-α by 89.2%, 86.6% and 88.6%; and IL-12 by 76.3%, 75.8%, and 79.4% at concentration of 25, 5, and 12.5 μg/ml, respectively (Table 1). In addition, activated IL-12 production was enhanced by a low concentration, but inhibited by high concentrations of the extracts **3**, **4**, and **5**. To determine whether there was an association between the modulated cytokines and the decreased NO production, Pearson's correlation analysis was performed. Significant positive correlations were observed between the decreased production of NO and TNF-α, or IL-12 levels ($R=0.74$; $R=0.68$, $p<0.01$, $p<0.05$, respectively). The results suggest that these inflammatory mediators may be co-regulated by tested extracts.

3.2. Western blot analysis

Next, to study the inhibition mechanism of NO production, we monitored the iNOS protein expression using Western blotting in the activated macrophages with these extracts at their approximate IC_{50} concentration. Each extract showed distinctive inhibitory effects regarding the iNOS protein expression of the NO (Fig. 1), although some showed different patterns from secreted levels. Thus, the ratio of iNOS to α-tublin protein expression of **3** and **5**–**7** was noticeably decreased from 1.24 to 0.54, 0.67, 0.63, and 0.76, while **2** and **4** had moderate inhibition to 0.82 and 0.81, respectively (Fig. 1).

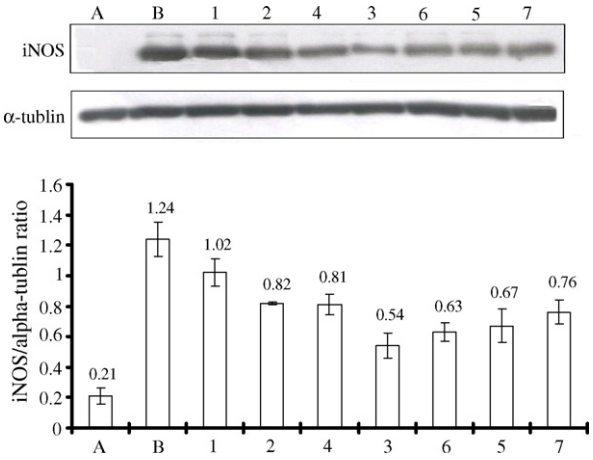

Fig. 1. Effects of the extracts **1**–**7** on the iNOS protein expression in activated murine macrophages. Cells were cultured in the absence (lane A) or presence (lane B) of LPS/IFN-γ. The approximate IC_{50} values of extracts **1** (6.25 μg/ml, lane 1), **2** (50 μg/ml, lane 2), **3** (75 μg/ml, lane 3), **4** (50 μg/ml, lane 4), **5** (4 μg/ml, lane 5), **6** (3 μg/ml, lane 6), and **7** (40 μg/ml, lane 7) were added with LPS/IFN-γ to stimulate macrophages. After 24 h, cells were washed with PBS, harvested, and homogenized. Each lane contained 40 μg protein extract. Electrophoresis was performed using 8% SDS-PAGE and detected with anti-iNOS and anti-α-tublin antibodies, respectively. The intensity of the bands was scanned and quantified by AlphaImager TM 2200.

3.3. Cell cycle analyses

To examine whether the growth inhibition of inflammatory mediators in response to extracts **1–7** administration was associated with cell cycle arrest, the cell cycle distribution was assessed in the murine spleen cells after mitogen stimulation. The FACS analysis of control cells showed prominent G0/G1, followed by S and G2/M phases. In the treated group, a typical subdiploid peak was observed after 48 h of culture, which implied the presence of cells with fragmented DNA. From the results of flow cytometric analysis, it was observed that there was a significantly increased percentage at the G0/G1 phase, along with a dramatically decreased cell population of the S phase (Table 2). Particularly, the decreased cell population in G0/G1 phase by Con A was arrested by increasing concentrations of extracts **1** from AC, and **5** and **6** from CO by 27.71%, 22.88%, and 27.16%, at 5, 2.5, and 25 μg/ml, respectively. Further, the rest of the extracts have moderate effect on cell cycle analysis (Table 2).

3.4. Inhibition of tumor-cell proliferation

In addition to the anti-inflammatory effects of extracts **1–7** on the murine macrophages, we further studied for their in vitro growth inhibitory activities in different type of cancer cell lines containing examples of lymphocytic (Jurkat), hepatoma (HepG2), prostate (PC 3), colonic (Colon 205), and breast (MCF 7). In order to determine the degree of growth inhibition of these extracts towards healthy cells, experiments were also carried out under the same experimental conditions in normal PBMCs. All the results presented were obtained by two independent methods: cell density measurement—Trypan Blue exclusion method; and cellular viability assessment—MTT colorimetric assay. A treatment period of 3 days was selected since the control cells were still in the exponential growth phase at this time. Typical data extracted from the multiple experiments are presented in Table 3. The anti-proliferative effect of CO extracts on all the tested tumor cells was stronger than that of AC and CS compared with the DMSO vehicle control. Among tested, the extracts **1**, **2**, and **4–7** were most active and inhibited Jurkat cells with IC_{50} (50% growth inhibition) value of 22, 40, 18, 4, 5, and 45 μg/ml, respectively (Table 3). In addition, the extracts **5**, **6**, and **7** from CO showed significant growth inhibition of HepG2 and PC 3 with IC_{50} values of 35, 80, 55 μg/ml; and 42, 125, and 50 μg/ml, respectively (Table 3). Similarly, the extracts **1** and **5** inhibited the growth of colonic and breast tumor cell lines (Colon 205 and MCF 7) with IC_{50} values of 65, 33; and 95 and 30 μg/ml, respectively (Table 3). Furthermore, the extract **4** from CS exhibited moderate growth inhibition in MCF 7 with an IC_{50} value of 100 μg/ml. Interestingly, all the extracts tested had negligible growth inhibition in normal PBMCs in the concentration range studies (0–150 μg/ml). The molecular mechanisms involved in the growth inhibition of the tested extracts are now being explored.

Table 2
Effect of crude extracts **1–7** on the cell cycle distribution of spleen cells[a]

Extract	Conc. (μg/ml)	G0/G1	S	G2/M
Spleen		94.53	0.84	4.63
Con A		63.78	30.73	5.49
AC-C (**1**)	1.25	72.89*	18.76**	8.35
	2.5	78.35*	13.41**	8.24
	5	91.49***	5.43***	3.08
AC-M (**2**)	25	63.3	26.7*	9.99*
	50	72.16*	17.89**	9.95*
	75	76.67*	14.53**	8.8
CS-C (**3**)	25	61.9	30.94	7.16
	50	81.71**	12.09**	6.21
	75	82.65**	9.55***	7.8
CS-B (**4**)	25	66.59	28.67	4.74
	50	73.92*	16.49**	9.59*
	100	89.73**	3.67***	6.6
CO-H (**5**)	0.625	79.13*	10.26**	10.62*
	1.25	83.84**	7.4***	8.76
	2.5	86.66**	7.68***	5.66
CO-EA (**6**)	5	60.67	31.53	7.8
	10	63.21	26.1*	10.69*
	25	90.94***	9.03***	0.03**
CO-M (**7**)	25	71.76*	15.17**	13.07*
	50	71.07*	16.22**	12.7*
	75	86.87**	5.53***	7.6

The significant difference was set at $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ compared to cell treated with Con A only.

Table 3
Anti-proliferative activity of extracts **1–7** in human tumor cell lines

Extract	IC_{50} (μg/ml)				
	Jurkat	HepG2	PC 3	Colon 205	MCF 7
AC-C (**1**)	22***	150*	>150	65**	95**
AC-M (**2**)	40***	>150	>150	>150	>150
CS-C (**3**)	>150	>150	>150	>150	>150
CS-B (**4**)	18***	>150	150*	>150	100*
CO-H (**5**)	4***	35**	42**	33***	30***
CO-EA (**6**)	5***	80**	125**	>150	125**
CO-M (**7**)	45**	55***	50**	>150	150*

Results are means ± S.D. of duplicate analysis of three replications. The significant difference was set at $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

4. Discussion

The species *Antrodia camphorata*, *Cordyceps sinensis*, and *Cinnamomum osmophloeum* bark are an active oriental herbal medicine used in various inflammatory diseases. In spite of their famous legacy, the pharmacological effects have not been fully explored from the anti-inflammatory view point. In this study, therefore, we investigated the modulatory effects of these species extracts on macrophage-mediated inflammatory phenomena such as NO and cytokines, TNF-α, and IL-12 production, in order to understand their anti-inflammatory action.

Inflammatory stimuli such as LPS/IFN-γ cause inflammatory liver damage and septic shock due to production of high levels of NO and cytokines in the process of macrophage activation, which mediates tissue responses in different phases of inflammation and autoimmune disorders in a sequential and concerted manner (Clancy et al., 1998; Kox et al., 2000). To sustain an

inflammatory response, an elaborate interaction between both innate and adaptive immune response is required (Clancy et al., 1998). NO also plays a role in the pathogenesis of rheumatoid arthritis (RA), and increased levels have been observed in various immunological diseases, which may become self-destructive (Macmicking et al., 1997). On the other hand, TNF-α and IL-12 are known as the main pro-inflammatory cytokines secreted during the early phase of acute and chronic inflammatory diseases such as asthma, RA, septic shock (Tracey and Cerami, 1994; Barrie and Plevy, 2005). It also has been reported that the excessive production of NO, and TNF-α could cause various inflammatory diseases and also contributes to secondary damage that further disease progression (Kox et al., 2000). For example, in Alzheimer's disease, upregulation of TNF-α could be induced by amyloidal peptide that exacerbates a neurotoxic environment (Rosenberg, 2005). Furthermore, in the autoimmune disorders, production of NO and TNF-α plays a role in demyelination and cell loss, in addition, improper upregulation of NO and TNF-α is associated with the pathogenesis of inflammatory disease in the central nervous system (Keegan and Noseworthy, 2002). Thus, inhibition of NO and cytokine (TNF-α and IL-12) production or function serves as a key mechanism in the control of inflammation.

This study shows for the first time, the extracts from AC, CS, and CO significantly blocked the cytokines, TNF-α, and IL-12 secretion were accompanied by a decreased NO production from macrophage cells stimulated by LPS/IFN-γ (Table 1). Moreover, iNOS expression was clearly inhibited, but the production of NO remained unaffected when the enzyme had been previously synthesized. These activities were not attributed to cell cytotoxicity of the tested species to macrophages, thus the anti-inflammatory actions of these species may be due to the modulation of macrophage-mediated inflammatory events such as cytokines, TNF-α, and IL-12 secretion and the production of inflammatory mediator NO. These results are consistent with those of Shen et al. (2004a), who found that a crude ethanol extract from the fruiting bodies of AC exhibited immunomodulating effects in human leukocytes; in addition, the fermented culture broth also inhibited iNOS, COX-2, and cytokines via the NF-κB pathway (Hseu et al., 2005). This study also further supports the anti-inflammatory effects AC fruiting bodies in isolated peripheral human neutrophils (Shen et al., 2004b). On the other hand, our results on TNF-α secretion appear to diverge from those of Ka et al. (2006), who reported that CS induced the production of IL-1β, IL-6, IL-10, and TNF-α from peripheral blood mononuclear cells. Moreover, it has recently been reported that the immunomodulatory and antitumour effects of an exopolysaccharide fraction from cultivated CS (Zhang et al., 2005). However, these prior studies were conducted using blood mononuclear cells, including monocytes and lymphocytes, whereas we used a homogeneous macrophage cell line. This may be due to differences in the compounds tested, the cells used, and/or the experimental design.

In the present study, we did not attempt to identify the components of tested extracts that are responsible for inhibiting inflammatory responses. However, AC is known to contain the ingredients: zhankuic acids, diterpenes, and other maleic and succinic acid derivatives (Nakamura et al., 2004; Shen et al., 2004b; Chen et al., 2006). On the other hand, CS is known to have sterols and nucleosides (Bok et al., 1999; Guo et al., 2006). Therefore, the observed anti-inflammatory activity may be attributed from these constituents. Although the molecular mechanism of tested species inhibiting the pro-inflammatory responses has not been elucidated, to date, several important common pathways have been identified. One of these common pathways is known to be the transcription factor, nuclear factor (NF)-κB, as it controls the expression of pro-inflammatory genes such as cytokines, adhesion molecules and cytotoxic molecule generating enzymes including iNOS (Nam, 2006). This is also the reason why many people are trying to develop potent inhibitors of NF-κB as a novel anti-inflammatory drug candidate.

Controlling the cell cycle is critical for cell growth and normal organ development. Deregulation of cell cycle progression is a signal that can initiate the apoptosis (Fang et al., 2006). In our study, based on the flow cytometry analysis, the extracts **1** from AC, and **5** and **6** from CO caused in the S stage decreased levels, while number of cells in the G0/G1 stage increased in a dose-dependent manner (Table 2). This phenomenon implied that these extracts influenced cell cycle through inhibiting DNA replication. So it suggested that inhibitive activity of extracts **1**, **5**, and **6** on spleen cells multiplication maybe related to interdiction of DNA replication in the G1-S stage and to regulation of cell mitosis cycle.

Several epidemiological and clinical studies show that NSAIDs have promising antitumor activity (Herendeen and Lindley, 2003). It is well known that the people who use acetylsalicylic acid and other NSAIDs reduce the risk of adenomatous polyps and colorectal cancer compared to non-users (Thun et al., 2002). The cytokines including TNF-α, IL-12, and others contribute to carcinogenesis by influencing the survival, growth, mutation, proliferation, differentiation, and movement of tumor and stromal cells and by regulating angiogenesis (Ohshima et al., 2005). On the other hand, the enzyme iNOS catalyzes the production of nitric oxide ($NO^{\bullet}$) from L-arginine and its expression has been reported consistently in human cancer at a variety of sites, including the bladder, prostate, oral cavity, and esophagus and less consistently at other sites such as the stomach, colon, and breast (Ohshima et al., 2005). The reported results also suggest that iNOS contributes to the promotion of carcinogenesis, and that its inhibition is a potential strategy for chemoprevention (Ma and Kinneer, 2002). Many signaling pathways and molecules (e.g. NF-κB, iNOS, etc.), play dual roles in inflammation and carcinogenesis (Ohshima et al., 2005). Some anti-inflammatory drugs have actually been tested in human clinical trials to prevent cancer and numerous pharmaceutical companies are developing new drugs targeting NF-kB, iNOS, COX-2, etc. The tested extracts showed good anti-inflammatory activity as indicated by inflammatory mediators NO, TNF-α, and IL-12, and tumor-cell proliferation inhibitions. Our results also support the earlier findings that fermented culture broth of AC to induce apoptosis and inhibit COX-2 in estrogen-non-responsive (MDA-MB-231) human breast cancer cells (Hseu et al., 2007). Furthermore, the experimental findings of CS were

in agreement with the previous report that ethyl acetate extract showed a significant inhibitory effect on the proliferation of cancer cell lines, MCF-7 and HepG2 (Wu et al., 2007), in addition to its anti-inflammatory activity (Ka et al., 2006). From these observations, the observed inflammatory mediator inhibition of tested extracts and their in vitro growth inhibitory activities of the Jurkat, colon, prostate, and breast cancer cell lines are probably related.

In summary, we have demonstrated that the anti-inflammatory effect of *Antrodia camphorata*, *Cordyceps sinensis*, and *Cinnamomum osmophloeum* bark, in line with its traditional use is due to the inhibition of macrophage-mediated inflammatory mediators such as NO and TNF-α and IL-12, and cell cycle arrest in G0/G1 phase in LPS/IFN-γ activated mouse peritoneal macrophages. These findings demonstrate the potential value of these species in the diet of patients with inflammatory and autoimmune diseases characterized by chronic hyperactivity of the immune system. Moreover, the hexane and EtOAc fraction of *Cinnamomum osmophloeum* bark are the most promising to isolate the active anti-inflammatory principles, are worthy of additional studies regarding these potential effects under proper in vivo models.

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