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The Augmented Anti-Tumor Effects of *Antrodia camphorata* Co-Fermented with Chinese Medicinal Herb in Human Hepatoma Cells

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Abstract: *Antrodia camphorata*, unique fungal specie, has been used as a folk medicine in Taiwan for many years. The purpose of this study was to compare the extracts from the solid-state culture of *A. camphorata* co-fermented with Chinese medicinal herb (AC-CF) with two other extracts from fruiting bodies (AC-FB) or solid-state culture (AC-SS), for their anti-tumor effects in human hepatoma HepG2 cells. We measured *in vitro* cell proliferation, percentage of apoptosis, population distribution of cell cycles, Western blot analysis of multiple drugs resistance-1 (MDR-1), and apoptosis-related proteins in HepG2 cells treated with three different preparations of *A. camphorate* extracts. Our results showed that AC-CF had better anti-proliferation effect on human hepatoma HepG2 cells than AC-FB or AC-SS dose-dependently. In addition, AC-CF in combination with anti-tumor agents (mitomycin C or methotrexate) showed better adjuvant anti-tumor effects than AC-FB or AC-SS. We further demonstrated the augmented adjuvant anti-tumor effects of AC-CF not only through down regulation of MDR-1 expression but also through a COX-2 dependent apoptosis pathway, involving down-regulation of COX-2 and p-AKT and up-regulation of PARP-1. In conclusion, in this study, we have demonstrated a novel strategy of fermenting *A. camphorata* with Chinese medicinal herb (AC-CF), which augmented their anti-tumor effects in human hepatoma HepG2 cells as compared to the traditional ones (AC-FB or AC-SS).

Keywords: *A. camphorata;* Hepatoma; Co-Fermentation; Mitomycin.

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Introduction

Antrodia camphorata, a rare and expensive folk medicinal fungus in Taiwan, has recently been marketed in the form of nutraceuticals (Shih *et al.*, 2006; *Liu et al.,* 2007). Previous studies have shown that it can be used in treating many medical conditions such as diarrhea, hypertension, abdominal pain, and liver cancer (Liu *et al.*, 2004; Hsu *et al.*, 2005; Song *et al.*, 2005a). However, its fruiting bodies grow slowly on the inner cavity wall of rotting trunk of indigenous and rare *Cinnamonum kanehirai* (Hsu *et al.*, 2005; Yang *et al.*, 2006). Artificial cultivations such as mycelia from solid-state culture or liquid submerged fermentation were thus developed as a substitute (Song *et al.,* 2005b; Liu *et al.*, 2007). Our previous report has demonstrated that the products of artificial cultivation were not as effective as those of fruiting bodies (Chang *et al.*, 2008). Accordingly, a new strategy of co-fermenting solid/liquid cultures of *A. camphorata* with Chinese medicinal herb was developed to enhance their effects (Li *et al.,* 2006).

Hepatoma is one of the most prevalent adult malignancies in Taiwan (Chen *et al.*, 1997). Although some chemical regimens have been used to treat hepatoma in many medical institutions, the efficacy was unsatisfactory and the problems of multiple drugs resistance (MDR) were found in recurrent liver cancer (Jin *et al.*, 2007). A few studies have reported that ethyl acetate extracts from the fruiting bodies of *A. camphorate* induced apoptotic mitochondrial pathway and inhibition of NF-κB in hepatoma cells (Hsu *et al.,* 2005; Hsu *et al.,* 2007; Rao *et al.*, 2007). Our previous studies have shown that although low dosage of solid-state culture of *A. camphorate* (AC-SS) could not effectively inhibit cell proliferation of hepatoma cells, its combination therapy with anti-tumor agents showed prominent adjuvant effects through the inhibition of MDR gene expression and COX-2-dependent inhibition of p-AKT (Chang *et al.,* 2008).

In the present study, we co-fermented the solid-state culture of *A. camphorata* with an aqueous extract of the Chinese herb, *Radix Glycyrrhizae*, traditionally used for the treatment of cough, dyspnea, epigastric pain, and detoxification and compared this new extract (AC-CF) to the other two extracts from the artificial solid-state culture (AC-SS) or fruiting bodies (AC-FB) for their anti-tumor effects on human hepatoma HepG2 cell. The possible molecular mechanisms were further elucidated by measuring the *in vitro* cell proliferation, the percentage of apoptosis, the population distribution of cell cycle, western blot analysis of multiple drugs resistance-1 (MDR-1), and apoptosis-related proteins (p-AKT, PARP-1 and COX-2) in HepG2 cells treated with these three different extracts of *A. camphorata.*

Materials and Methods

Chemicals and Reagents

Culture medium MEM (minimum essential medium), DMEM (Dulbecco's modified eagle's medium), fetal bovine serum, sodium bicarbonate, and 0.05% trypsin-EDTA were purchased from Gibco (Buffalo, NY, USA). methotrexate (MTX), mitomycin C (MMC), paclitaxel,

cisplatin, MTT [3-(4,5-dimethylthiazol-2-yle) 2,5-mdiphenyl-tetralozium bromide] and Culture medium MEM (minimum essential medium), DMEM (Dulbecco's modified eagle's medium), fetal bovine serum, sodium bicarbonate, and 0.05% trypsin-EDTA were purchased from Gibco (Buffalo, NY, USA). methotrexate (MTX), mitomycin C (MMC), paclitaxel, cisplatin, MTT [3-(4,5-dimethylthiazol-2-yle) 2,5-mdiphenyl-tetralozium bromide] and propidium iodide were bought from Sigma (Saint Louis, MI, USA). Rabbit polyclonal antibodies-MDR-1, COX-2, p-AKT and mouse monoclonal antibodies-AKT, PARP-1, β-actin and HRP-anti-mouse IgG were obtained from Santa Cruz Biotechnology (California, USA). Nitrocellulose paper membrane was from NEN Life Science Products (Boston, MA, USA). Annexin V-FITC apoptosis detection kit was from Strong Biotech Corporration (Nankang, Taipei, Taiwan).

Preparations for Culture Extracts of A. camphorate

The pulverized crude extracts from fruiting bodies of *A. camphorata* (AC-FB) used in this study were provided by Dr. Guo-Yang Lee (Southern Taiwan University, Tainan, Taiwan). The solid-state culture of *A. camphorate* (AC-SS) and the solid-state culture co-fermented with Chinese medicinal herb, *Radix Glycyrrhizae*, (AC-CF) were supplied by Blue Ocean Universal Biotech Co. (Tainan, Taiwan). Three g dried *Radix Glycyrrhizae* root powder was mixed with 100 g solid-state medium (soybean) as co-fermentation medium. These samples were extracted by the method as described previously (Hsiao *et al.*, 2003).The extracts were made in the ratio of 1:10 (v/v) with 95% ethanol in an orbital shaker and the resultant suspension was centrifuged at 5,000 rpm for 10 min. The supernatants were saved and the pellets were discarded, and then the supernatants were filtered using a 0.2 μm sterile filter and stored at -20°C for further use.

MTT Assay of Hepatoma Cells

The human hepatoma cell line, HepG2 was purchased from Food Industry Research and Development Institute (Hsin Chu, Taiwan). The cultured cells at the exponential growth phase were harvested from the culture flasks by trypsination, and then resuspended in fresh medium. The cell suspensions were dispensed into a 96-well microplate at 100 μl/well and incubated in a incubator with 5% CO_2 at 37°C. After 24 hours, 200 μl different concentrations (0$\sim$100 μg/ml) of various extracts of *A. camphorata* (AC-SS, AC-CF and AC-FB) and anti-tumor agents (mitomycin C and methotrexate) were added and incubated for 24 hours. The AC-SS and AC-CF (10 μg/ml) merged with 1 μM anti-tumor agents (mitomycin C and methotrexate); the AC-CF (10 μg/ml merged with 1 and 100 μM anti-tumor agents (paclitaxel and mitomycin C) for a 24 hour treatment to evaluate their anti-proliferation effects on hepatoma cells (HepG2). The cell proliferation in the microplate was determined with the MTT (3-(4,5-dimethylthiazol-2-yle)2,5- diphenyl-tetralozium bromide) assay (Chang *et al.*, 2008) after 24 hours of incubation. Twenty μl PBS solution containing 5 mg/ml MTT was added to each well. After incubation for 4 hours, the cells from each well were solubilized

with 100 μl DMSO for optical density reading at 570 nm. Cell proliferation activity was expressed as the percentage of MTT counts of treated cells relative to those of the control (% of control).

Analyses of Cell Cycle and Apoptosis

The flow cytometric analyses of HepG2 cells treated with the different concentration (0∼100 μg/ml) of *A. camphorata* extracts (AC-CF and AC-SS), and/or anti-tumor agent, mitomycin (1 μM) were performed 24, 48, and 72 hours after treatment. The percentage of cells undergoing apoptosis and distributing in different phases of cell cycle were determined by propidium iodide (PI)–staining method using a flow cytometry. After incubation, HepG2 cells were harvested from the 6-cm culture dish by trypsination and centrifuged at 1,500 rpm for 1–2 min at 4°C and the supernatant was removed. The trypsinized cells and cell pellet were washed with PBS, fixed in 75% cold ethanol, and stored at 4°C overnight. After being washed twice with phosphate-buffered saline (PBS), the ethanol-fixed cells were incubated with 3 μl RNase (10 mg/ml) at 37°C for 30 min, and stained with 20 μl PI (20 mg/ml) in the dark. The cell suspension was then filtered through a 35 mm mesh, and analyzed by a flow cytometer (FACSCalibur; Becton Dickinson Biosciences, San Jose, CA, USA) for cell cycle population analysis within 1 hour, and cellular DNA content was calculated using CellQuest software (Becton Dickinson Biosciences). In addition, the cells were washed with PBS and resuspended in 300 μl binding buffer (Annexin V- FITC kit) containing 10 μl of Annexin V-FITC stock and 10 μl of 1mg/ml PI for the determination of phosphatidylserine (PS) exposure on the outer plasma membrane. After incubation for 15 min at room temperature in a light protected area, the samples were also analyzed by CellQuest software for the measurement of apoptosis.

Western Blot Analysis

After treatment with either anti-tumor agents (cisplatin, paclitaxel and mitomycin C) or AC-CF (10 μg/ml) for 24 hours, the HepG2 cells (1×10^5 cells) were prepared by being lysated in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris pH 7.5 and protease inhibitors). After incubation on ice for 30 min, lysates were centrifuged at 13,000 $\times$ g for 30 min. Post-nuclear supernatants were mixed with twice the volume of sample buffer and boiled for 5 min. Samples were separated on a 10% polyacrylamide gel, which was then transferred to a Nitrocellulose membrane. The membranes were blocked with BSA in PBST (11.24 g Na_2HPO_4, 2.87g NaH_2PO_4 and 0.1% Tween 20) for 2 hours at room temperature. Then, the membranes were incubated for 1 hour at room temperature in wash buffer with either the anti-MDR antibody (1:500), anti-AKT antibody (1:800), anti-p-AKT antibody (1:800), anti-COX-2 antibody (1:1,000), anti-PARP-1 antibody (1:500) or anti-β-actin antibody (1:1,000) followed by four times 10 min washes. Horseradish peroxidase-conjugated anti-mouse IgG antibody was diluted to 1:5,000 in wash buffer and incubated with blots for 1 hours at room temperature, after wash four times every 10 min, and the signals were detected by

enhanced chemiluminescence (FUJIJILM LAS-3000). β-actin signal was used to normalize protein loading.

Statistical Analysis

All results are expressed as mean $\pm$ SE. Analysis of variance (ANOVA) was used to evaluate the results. When ANOVA was significant, the significance between groups were assessed by means of unpaired Student's t-test and analyzed with SPSS10.0.

Results

The Anti-Proliferation and Adjuvant Anti-Tumor Effects of Three Extracts of A. camphorata in Combination with Anti-Tumor Agents

The anti-proliferation effects of three extracts of *A. camphorata* (AC-CF, AC-SS and AC-FB) and anti-tumor agents (mitomycin C, MMC and methotrexate, MTX) in human hepatoma HepG2 cells were tested. The extracts of AC-CF and AC-FB showed a dose-dependent inhibitory effect on the cell proliferation, which was assessed by the MTT assay. The anti-tumor agents also demonstrated at the concentrations of 10 and 100 μM. However, the extract of AC-SS showed only slight inhibitory effect (Fig. 1). In summary, the relative anti-proliferation effect of *A. camphorata* extracts in HepG2 cells was in the order of AC-CF > AC-FB > AC-SS (Fig. 1A). When combined with anti-tumor agents (MMC or MTX, 1 μg/ml), the AC-CF, at the concentration of 10 μg/ml, showed the best anti-proliferation effects among the three extracts and were better than anti-tumor agent alone (MMC or MTX) (Fig. 1B), demonstrating that AC-CF had prominent adjuvant anti-tumor effects. Furthermore, AC-CF (10 μg/ml) in combination with higher concentration of other anti-tumor agents (paclitaxel or cisplatin, 100 μg/ml) showed good adjuvant anti-tumor effects (Fig. 1C), although they were not as prominent as AC-CF was in combination with a lower concentration of paclitaxel or cisplatin (1 μM).

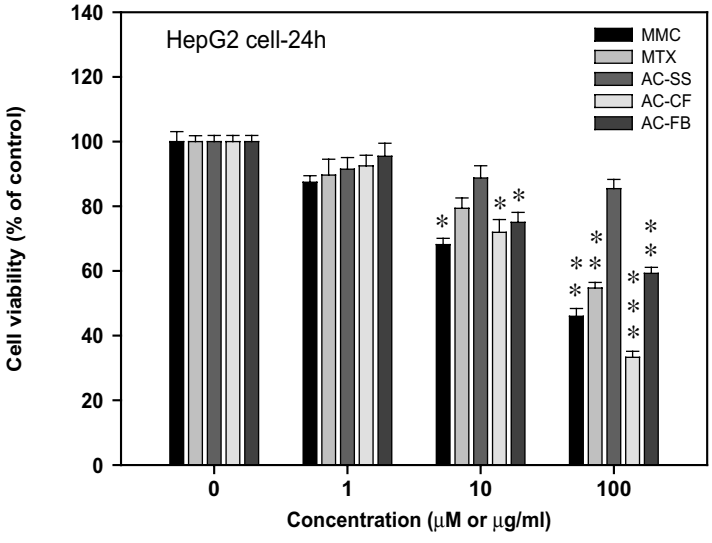
The Analyses of Cell Cycle and Apoptosis in HepG2 Cells Treated with AC-CF in Combination with Mitomycin C

The HepG2 cells were treated with different concentrations of AC-CF for 24–72 hours to test whether the growth inhibitory effect of AC-CF was related to arrest of the cell cycle. When cells were treated with 0, 1, 10 and 100 μg/ml of AC-CF for 72 hours, the period of HepG2 cells at G0 phase was 9.14%, 6.31%, 26.74% and 76.62% respectively (Fig. 2). In parallel, the ratio of apoptosis was 0.89%, 1.21%, 5.31% and 10.32% respectively in a dose-dependent manner (Fig. 2B). AC-CF (10 μg/ml) in combination with MMC markedly augmented the anti-tumor effect of MMC (1 μM), as evidenced by an increased period of G0 phase (54.59% vs. 24.28%, $p < 0.05$) and apoptosis ratio (14.37% vs. 5.95%, $p < 0.05$) (Figs. 3A and B). In contrast, AC-SS (10 μg/ml) in combination with MMC did not show

a significant adjuvant effect (% of cells in G0 phase, 35.99% vs. 24.28%; % of cells in apoptosis, 7.2% vs. 5.95%).

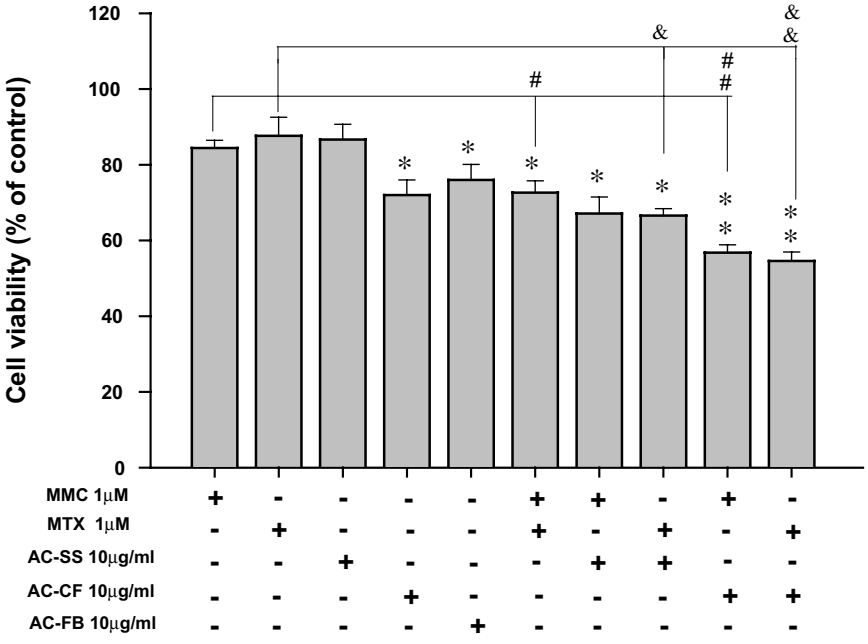
Western Blot Analysis for Apoptosis Related Proteins

To clarify whether inhibiting the expression level of MDR protein might play a role in the adjuvant anti-tumor effect of the AC-CF, we measured the expressions of MDR-1 and apoptosis related proteins, COX-2, Akt, p-Akt, and PARP-1 in HepG2 cells by Western blotting. Our analyses showed that treatment with paclitaxel (10 μM), cisplatin (10 μM) or mitomycin C (1 μM) would increase the level of MDR-1 from a basal level of 0.75 to 1.25, 1.40, and 1.11 respectively. As expectated, AC-CF (10 μg/ml) markedly inhibited the expression of MDR-1 protein from 1.25 to 0.89 in HepG2 cells treated with paclitaxel (10 μM) or from 1.40 to 0.80 with cisplatin (10 μM) (Fig. 4). However, AC-CF inhibited the expression of MDR-1 protein less in HepG2 cells treated with MMC (1 μM) than paclitaxel or cisplatin (Fig. 4A), suggesting that inhibition of MDR-1 expression might not be the

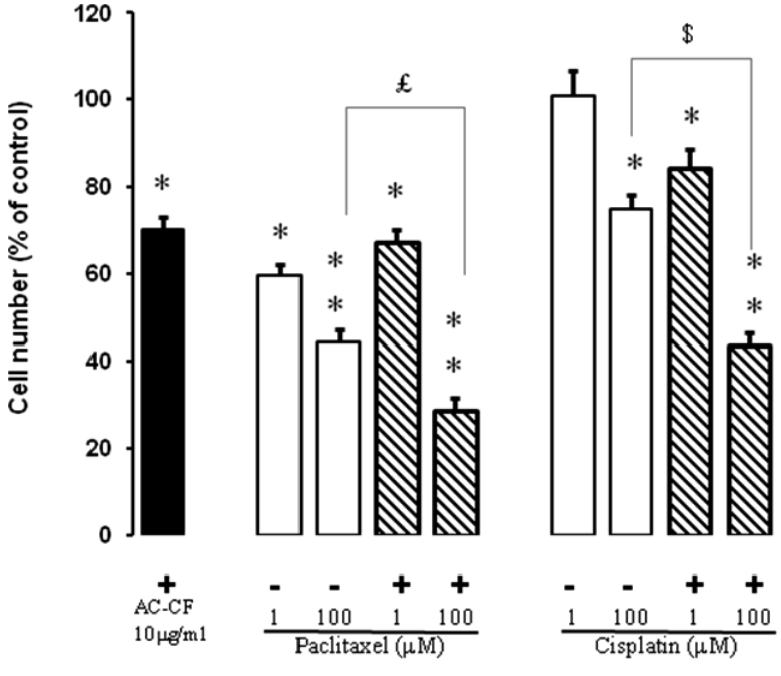


(A)

Figure 1. The anti-proliferation and adjuvant effects of *A. camphorata* extracts in combination with anti-tumor agents. The HepG2 cells were treated with different concentrations of *A. camphorata* extracts (ACs) and anti-tumor agents (0∼100 μg/ml) to evaluate their viabilities. The MTT assay of HepG2 cells treated with ACs (AC-SS, AC-CF or AC-FB) or anti-tumor agents (mitomycin C, MMC or methotrexate, MTX) (A). The adjuvant effects of ACs in combination with anti-tumor agents (B). The adjuvant effect of AC-CF (10 μg/ml) in combination with different concentrations of anti-tumor agents (cisplatin and paclitaxel) on HepG2 cells (C). $*$: denotes significance compared to the untreated (the control) with student's t-test: $^{*}p < 0.05$ and $^{**}p < 0.01$. #: denotes significant difference between MMC and ACs-treated cells with student's t-test: $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. &: denotes significant difference between MTX and ACs-treated cells with a Student's t test: $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$. \$ and £: denote a significant variation ($p < 0.05$) in ACs-treated cells as compared to paclitaxel and cisplatin, respectively with student's t-test.



(B)



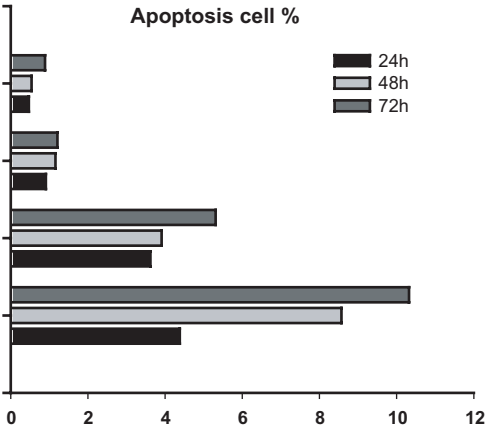
(C)

Figure 1. (*Continued*)

	G0(%)			G1(%)			S(%)			G2/M(%)		
AC-CF	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
Control	5.27	4.96	9.14	40.18	50.15	41.98	40.95	23.40	20.56	10.47	18.07	25.12
1μg/ml	4.40	4.00	5.31	29.45	48.91	50.66	59.13	24.39	21.18	5.86	18.03	19.85
10μg/ml	12.40	17.70	26.74	25.84	34.97	30.87	48.83	22.83	23.43	10.63	22.46	18.21
100μg/ml	26.77	47.60	75.52	48.49	35.67	14.97	23.02	16.80	9.65	0.81	0.24	0.02

(A)

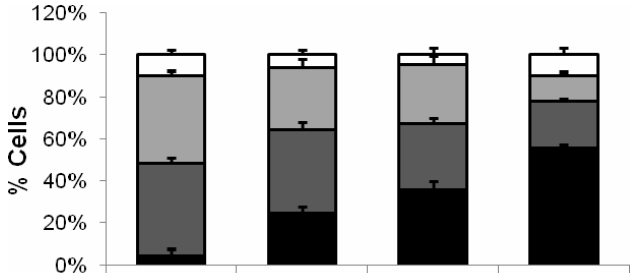
HepG2 cell	24h	48h	72h
Control	0.47	0.54	0.89
AC-CF 1μg/ml	0.91	1.16	1.21
AC-CF 10μg/ml	3.62	3.91	5.31
AC-CF 100μg/ml	4.38	8.57	10.32



(B)

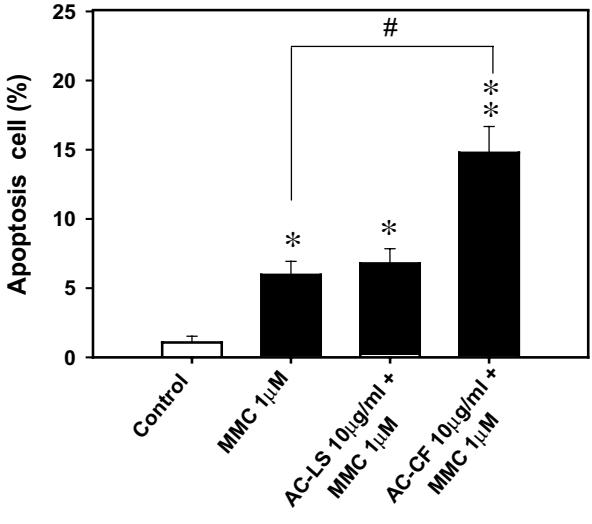
Figure 2. The analysis of cell cycle and apoptosis in AC-CF treated HepG2 cells. After 24, 48 and 72 hours incubation, AC-CF-treated HepG2 cells (0∼100 μg/ml) were analyzed for the population percentage in the G0, G1, S and G2/M phases of the cell cycle (A). The table (left panel) and the bar (right panel) represented a ratio of apoptosis treated with AC-CF in HepG2 cells at 24, 48 and 72 hours, respectively (B). Data are represented as percentage of untreated controls. Values are means for two independent experiments performed in triplicate.

only pathway by which AC-CF exerted its adjuvant anti-tumor effects. By analyzing the expressions of apoptosis-related proteins, we further found that the HepG2 cells treated with AC-CF (10 μM) showed a significant decrease in the expression of COX-2 protein, whereas the down-stream related proteins remained unchanged as compared to the vehicle control. In contrast, other than a decreased expression of COX-2, down-regulation of p-AKT and up-regulation of PARP-1 were found in HepG2 cells treated with MMC (1 μM), which was further augmented when MMC (1 μM) was combined with AC-CF (10 μM) (Fig. 4B).



	Control (HepG2)	Mitomycin (MMC) 1μM	AC-SS 10μg/ml+ MMC 1μM	AC-CF 10μg/ml+ MMC 1μM
G2/M	9.72	6.35	4.77	9.76
S	41.80	29.41	28.28	12.04
G1	44.07	39.95	31.82	21.96
G0	4.37	24.28	35.99	54.59

(A)



(B)

Figure 3. The adjuvant effects of AC-CF or AC-SS in combination with mitomycin C on cell cycle and apoptosis. The population percentage in the G0, G1, S and G2/M phases of HepG2 cells treated with AC-CF or AC-SS in combination with MMC (A). The ratio of apoptosis of HepG2 cells treated with AC-CF or AC-SS in combination with MMC (B). Data are represented as percentage of untreated controls. Values are means ± SE for two independent experiments performed in triplicate. Data analysis was made by Cell Quest software. *Compared to the control, $p < 0.05$. #Compared to the MMC, $p < 0.05$.

Discussion

In this study, we co-fermented solid-state culture of *A. camphorata* with an aqueous extract of the Chinese medicinal herb, *Radix Glycyrrhizae*, traditionally used for the treatment of

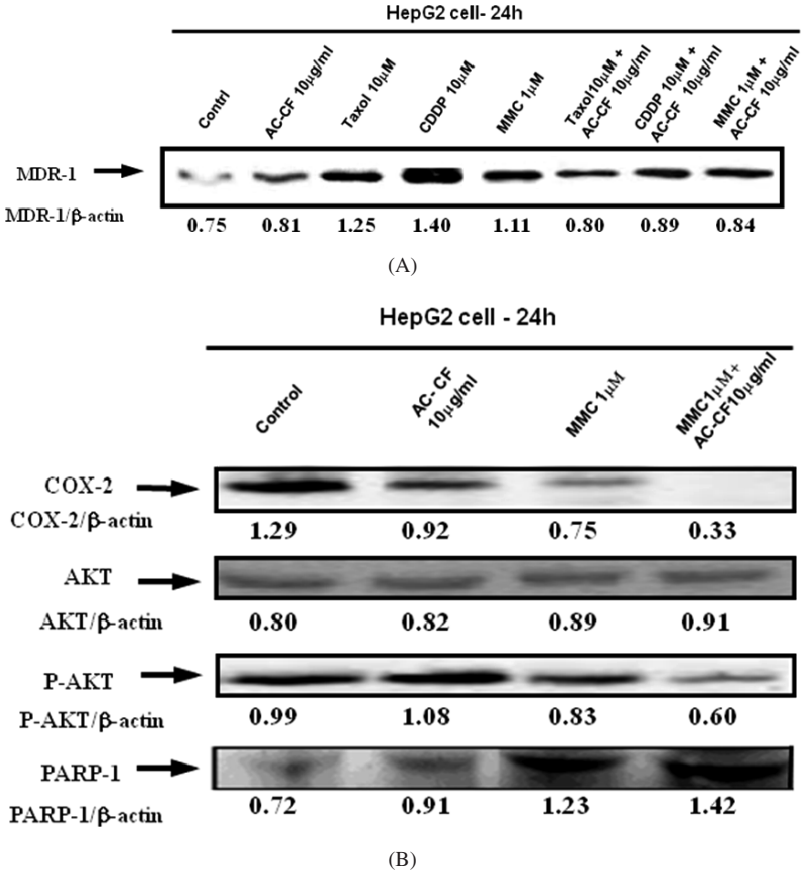


Figure 4. Western blot analysis for MDR-1 and apoptosis related proteins. The expression of MDR-1 in HepG2 cells treated with AC-CF (10 μg/ml) combined with anti-tumor agents (paclitaxel, cisplatin or MMC) for 24 hours were assayed by Western blotting (A). The expressions of apoptosis related proteins (COX-2, AKT P-AKT and PARP-1) in HepG2 cells treated with AC-CF (10 μg/ml) in combination with MMC for 24 hours (B). All ratios (by β-actin) of the MDR-1, COX-2, AKT, P-AKT and PARP-1 proteins were shown in each row below.

cough, dyspnea, epigastric pain, and detoxification, and have demonstrated that this new product of AC-CF had better anti-proliferation effects on human hepatoma HepG2 cells than the extracts from fruiting bodies (AC-FB) or from solid-state culture (AC-SS). By analyzing cell cycle and apoptosis of HepG2 cells, we have found that the period of HepG2 cells at G0 phase was 9.14%, 6.31%, 26.74% and 76.62% respectively after being treated with AC-CF for 72 hours at the concentration of 0,1,10 and 100 μg/ml, and in parallel, the ratio of apoptosis was 0.89%, 1.21%, 5.31% and 10.32% respectively in a dose dependent manner (Fig. 2). These findings are consistent with previous reports which showed that the anti-proliferation effects of *A. camphorata* on several tumor cells are through induction of apoptosis (Wu *et al.*, 2006; Yang *et al.*, 2006; Lu *et al.,* 2007). Our previous study also showed that although AC-SS alone did not inhibit cell proliferation on human hepatoma

C3A and PLC/PRF/5 cells when combined with cisplatin or mitomycin C, it might exert anti-proliferation effects by increasing the distribution of the cell cycle in stage G0/G1 and by inducing higher apoptosis ratio (Chang *et al.,* 2008). In the present study, we further demonstrated that AC-CF (10 μg/ml) in combination with anti-tumor agents (MMC or MTX, 1 μg/ml) had the most prominent adjuvant anti-tumor effects on HepG2 cell among the three AC extracts (Fig. 1B). The resistance of human hepatoma to most chemical drugs has been reported in many clinical studies (Chen *et al.*, 1997). MDR-1, a member in the multidrug resistance family, is associated with resistance to paclitaxel and cisplatin, which were reportedly associated with the multiple drug resistance (MDR) in many cancers (Kamazawa *et al.*, 2002; Komuro *et al.,* 2005; Zalipsky *et al.*, 2007). Our previous study has shown that AC-SS could improve the efficacies of some anti-tumor agents through decreasing the MDR-1 expression in human hepatoma C3A and PLC/PRF/5 cells (Chang *et al.*, 2008). In the present study, we further found that AC-CF (10 μg/ml) markedly inhibited the expression of MDR-1 protein in HepG2 cells treated with paclitaxel (10 μM) or cisplatin (10 μM) (Fig. 4), and that AC-CF inhibited the expression of MDR-1 protein less in HepG2 cells treated with mitomycin C (1 μM) than with paclitaxel or cisplatin (Fig. 4A), suggesting that the inhibition of MDR-1 expression could only partially explain the adjuvant anti-tumor effect of AC-CF, especially when combined with mitomycin C. Recent studies have shown that the induction of apoptosis was associated with regulation of COX-2, p-AKT, PARP-1 proteins (Leng *et al.*, 2003; Kardosh *et al.*, 2005; Zatelli *et al.*, 2007). A relevant study also showed that mitomycin-induced apoptosis is reduced by up-regulating the expression of COX-2 and Bcl-2 genes, resulting in the activation of some apoptosis pathways in some cancers (Park *et al.*, 2000; Pirnia *et al.*, 2002). To further elucidate the possible mechanisms of adjuvant effects of AC-CF combined with mitomycin C, we analyzed the expressions of apoptosis-related proteins by Western blot analysis. As expected, it was found that other than through the inhibition of MDR-1 expression, the adjuvant anti-tumor effects of AC-CF might be through down- regulation of COX-2 and p-AKT, and up-regulation of PARP-1 (Fig. 4B). Taking all these results together, we suggested that the combination treatment of AC-CF with mitomycin C inhibited the growth of Hep G2 cells not only through the inhibition of MDR-1 expression but also through a COX-2 dependent pathway of apoptosis.

In this present study, we have used the most popular Chinese herb, *Radix Glycyrrhizae*, which was often used in many traditional Chinese medicinal formulations. *Radix Glycyrrhizae* (licorice root) is one of the commonly used Chinese herbs already identified by the National Cancer Institute as possessing many pharmacological properties (Wang and Nixon, 2001). It has been reported that licochalcone-A of licorice root could induce apoptosis in MCF-7 and HC-60 cell lines (Rafi *et al.*, 2000). Moreover, isoliquiriligenin of licorice root is widely suggested as a candidate agent for the treatment of prostate cancer. However, its anticancer activity was shown at high concentration. In this study, *A. camphorata* was cultured in a medium supplemented with a low proportion (3%, w/w) of licorice root powder and we have demonstrated that even in a relatively low dosage of licorice root, it could augment the anti-tumor effects of *A. camphorata*. In future studies, another Chinese herb with specific pharmacological properties other than licorice root might be chosen to ferment with *A. camphorata* or other medicinal mushrooms or fungi. In this way, it is expected that

many new and derived ingredients in the co-fermentation could be produced and would show better therapeutic potentials than the original ones.

In conclusion, we have demonstrated a novel strategy of fermenting *A. camphorata* with Chinese medicinal herb (AC-CF), which may augment their anti-tumor effects in human hepatoma HepG2 cells as compared to the traditional ones (AC-FB or AC-SS).

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