Ethanolic Extracts of *Antrodia cinnamomea* Mycelia Fermented at Varied Times and Scales Have Differential Effects on Hepatoma Cells and Normal Primary Hepatocytes

Y.-S. CHEN, J.-H. PAN, B.-H. CHIANG, F.-J. LU, AND L.-Y. SHEEN

ABSTRACT: Mycelia of *Antrodia cinnamomea* (AC), an edible fungus native to Taiwan, were produced by submerged fermentation with various fermentation times in 250 mL, 5 and 500 L fermentors and were evaluated for the effect of fermentation products on the viabilities of Hep3B and HepG2 hepatoma cells and normal primary rat hepatocytes. The results showed that the ethanolic extracts of AC mycelia (from 250 mL fermentation for 8 wk and 5 and 500 L fermentations for 4 wk) possessed high antihepatoma activity. The IC_{50} of ethanolic extract of AC mycelia fermented for 8 wk in a 250 mL fermentor against Hep3B and HepG2 cells were 82.9 and 54.2 μg/mL, respectively. Furthermore, the IC_{50} for Hep3B and HepG2, treated with ethanolic extract of AC mycelia fermented for 4 wk in the 5 L fermentor were 48.7 and 3.8 μg/mL, respectively. Those treated with ethanolic extract of AC mycelia fermented for 4 wk in the 500 L fermentor were 36.9 and 3.1 μg/mL, respectively. No adverse effects of all samples on normal primary rat hepatocytes were observed.

Keywords: *Antrodia cinnamomea*, fermentation times and scales, Hep3B, HepG2, normal primary hepatocytes

Introduction

Antrodia cinnamomea (AC) is a traditional and prized Taiwanese medicinal supplement that is well known for its anticancer and antidote functions (Wang and Huang 2002). In 1990s, a number of novel triterpenoids and steroid acids were found in the fruit body of this edible fungus (Chen and others 1995; Cherng and others 1995, 1996; Chiang and others 1995; Yang and others 1996; Shen and others 1997; Wu and others 1997). AC only grows on the inner cavity wall of *Cinnamomum kanchirai* Hay (Lauraceae) in Taiwan. The *C. kanchirai* Hay, however, has now become scarce in Taiwan's forests and also the growth rate of AC is very slow (Wu and others 1997). Thus, at present, the edible AC products available to the local population in Taiwan are produced by large-scale fermentation.

Although AC has been used as traditional medicine by the local population in Taiwan for a long time, most of scientific research articles related to its pharmacological and nutritional aspects were published only recently. AC's properties include antioxidative activity, liver protective function, antihypertensive activity, antiangiogenic activity, anti-inflammatory activity, and anticancer activity (Song and Yen 2002, 2003; Dai and others 2003; Liu and others 2004, 2007; Mau and others 2004; Shen and others 2004; Chen and others 2005; Hsu and others 2005, 2007; Song and others 2005b). According to the survey, among "main causes of death" for the year 2006 in Taiwan, malignant neoplasm continues to be on the top list of leading causes of death, and chronic liver diseases, including liver cirrhosis, were at the 7th position. Therefore, maintaining a healthy liver function is always an important issue in Taiwan. Studies indicated that AC is promising to be an effective health promoting edible fungus due to its liver protective function and antihepatoma activity. Presently, the material sources of AC that are used by researchers are manufactured from submerged fermentation and solid state fermentation in Taiwan (Chang and others 2001; Song and Yen 2002; Yang and others 2003; Mau and others 2004; Lin and Sung 2006). However, as these material sources of AC are not produced from the same fermentation process, the relations between bioactivity functions and fermentation conditions of AC are not clear. The objective of this research was to evaluate the antihepatoma activities of AC products, isolated from AC cultivated for different time intervals at different fermentation scales. Thus, the optimum fermentation conditions for AC were established for submerged cultivation, which could produce a product with enhanced antihepatoma activity.

Materials and Methods

Materials

The fermentation products of AC were produced at Graduate Inst. of Food Science and Technology, Natl. Taiwan Univ. (Taipei, Taiwan). Dulbecco's Modified Eagle Medium (DMEM), antibiotic–antimycotic solution (AA), nonessential amino acid solution (NEAA), L-15 medium, insulin, transferrin, fetal bovine serum (FBS), and penicillin–streptomycin solution were purchased from Gibco Laboratories (Grand Island, N.Y., U.S.A.). Collagenase (type I) was purchased from Worthington Biochemical Co. (Lakewood, N.J., U.S.A.). Percoll was purchased from Pharmacia LKB (Piscataway, N.J., U.S.A.). N,N-dimethylfluoramide (DMF) was purchased from the Lab-Scan (Dublin, Ireland). Trypsin-EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

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tetrazolium bromide (MTT), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), reduced β-nicotinamide adenine dinucleotide (β-NADH), sodium dodecyl sulfate (SDS), phosphotungstic acid, 2-thiobarbituric acid, 1,1,3,3-tetraethoxypropane, reduced glutathione (GSH), oxidized glutathione (GSSG), iodoacetic acid, reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), sodium azide (NaN_3), glutathione reductase (GRd), and collagen were obtained from Sigma Chemical (St. Louis, Mo., U.S.A.).

Fermentation of AC in 250 mL flask

AC (BCRC35716) was obtained from Bio-resource Collection and Research Center(Hsinchu, Taiwan). The AC hyphae were activated with PDA medium at 25 °C. Then the active AC was inoculated into a 250 mL flask. The fermentation conditions were initial pH 5, malt extract 2%, peptone 0.1%, and carbon sources 2% (glucose, lactose, or galactose) cultured at 22 °C, 100 rpm for 8 wk.

Fermentation of AC in 5 and 500 L fermentors

The AC hyphae were activated with PDA medium at 25 °C. The activated AC was subcultured in a 500 mL flask. The subculture conditions were initial pH 5, malt extract 2%, peptone 0.1%, and carbon sources 2% cultured at 22 °C, 100 rpm for 4 wk. The subcultured AC (broth and mycelia) was then inoculated into a 5 or 500 L fermentor and fermented for 8 wk. The fermentation conditions were the same as subculture condition.

Preparation of samples (broth filtrates and mycelia ethanolic extracts)

Each fermentation product of AC was separated into broth and mycelia. The broth was sterilized at 121 °C for 30 min and then filtrated with 0.22 μm filter. The filtrates of broth were lyophilized. In terms of mycelia, 1 g of each sample was extracted with 95% ethanol (20 mL) at 30 °C for 24 h. The filtrates were dried under vacuum.

Hepatoma cell culture

Human hepatoma HepG2 and Hep3B cells were kindly donated by Dr. Ming Shi Shiao (Dept. of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan). Cells were cultured in complete DMEM (cDMEM) (pH 7) at 37 °C, 5% CO_2, and 90% relative humidity. The cDMEM containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin-B (1% antibiotic–antimycotic solution), and 100 μM (1% MEM nonessential amino acid solution) were used. To remove the cells from the culture dish, the cells were trypsinized using 1 mL trypsin-EDTA solution for 3 min at 37 °C.

Antihepatoma activity assay

HepG2 and Hep 3B cells were cultured in 96-well plates at a density of 1×10^4 cells/100 μL cDMEM/well. After 24 h of incubation, the medium was replaced by 100 μL new serum-free DMEM containing 0 or 100 μg/mL sample for 48 h. Then the medium was discarded, and 25 μL MTT solution (5 mg/mL PBS) and 100 μL serum-free DMEM were added to every well and reincubated for an additional 4 h. Hundred microliters of MTT lysis buffer (20 g SDS in 50 mL DMF and 50 mL water) were added to dissolve the formazan crystals formed. Then the plates were read at 570 nm in a microplate reader (Anthos reader 2001, Salzburg, Austria).

Hepatocytes isolation and culture

Male Narl:SD rats (6- to 8-wk old) were purchased from the Natl. Lab. Animal Center, Taiwan. The rats were housed in plastic cages with artificial 12-h light/dark cycles, and they were fed with Purina 5001 rodent chow diet (Purina, St. Louis, Mo., U.S.A.), with no water or food restriction. The method of hepatocyte isolation was as described by Sheen and others (1996). Rats were anesthetized using intraperitoneal injection with sodium pentobarbital (100 mg/kg body weight). To remove the blood, the liver was perfused via the portal vein with 150 mL of 25 mM sodium phosphate buffer (pH 7.6), which contained 3.1 mM KCl, 119 mM NaCl, 5.5 mM glucose, 1 g/L BSA, and 5 mg/L phenol red at a flow rate of 25 mL/min. For another 10 min, the liver was perfused with 200 mL of the same buffer supplemented with 80 mg collagenase, 40 mM $CaCl_2$, and 5 mg of trypsin inhibitor at a rate of 18 mL/min. The liver was then removed, sieved, washed, suspended in a Percoll buffer (Kreamer and others 1986), and centrifuged (Hermle Z300K, Germany) at 4 °C to produce a single cell suspension of hepatic parenchymal cells. Hepatocytes were washed twice with washing medium. After the final washing, the isolated hepatocytes were suspended at a density of 5×10^5 cells/mL in L-15 cell culture medium (pH 7.6), supplemented with 18 mM HEPES, 2.5% FBS, 5 mg/L insulin, 5 mg/L transferrin, 28 mM galactose, 1 μM dexamethasone, 100 units/L penicillin, and 100 mg/L streptomycin. Hepatocytes were plated at a density of 1×10^6 cells/2 mL in each 35-mm collagen-precoated culture dish (NUNC, Denmark) and incubated in a humidified incubator (NuAire, Plymouth, Minn., U.S.A.) at 37 °C in an air atmosphere. The culture medium was replaced with fresh medium at 4 h after plating. The medium was changed using the same culture medium but containing 2 g/L BSA instead of FBS at 24 h after plating.

Treatment of hepatocytes

At 24 h after plating, hepatocytes (1×10^6) were treated with 0 and 50, 100, or 200 μg/mL AC samples, which were previously dissolved in ethanol (the final concentration of ethanol was 0.5%). After 24 h of treatment, the culture medium was removed, and the hepatocytes were washed with cold PBS. Then the cells were removed with a cell scraper and collected for further analysis.

Cell viability of hepatocytes

The effect of samples on the viability of primary hepatocytes was evaluated by lactate dehydrogenase (LDH) leakage (Moldeus and others 1978). After collecting the cell-free medium, the hepatocytes were lysed with 20 mM potassium phosphate buffer containing 0.5% Triton X-100. The cells were collected and centrifuged at 10000 × *g* for 30 min at 4 °C. A supernatant was used to analyze LDH activity. The samples were mixed in 1 mL of Krebs-Henseleit buffer containing 20 g/L BSA, NADH (0.2 mM final concentration), and pyruvate (1.36 mM final concentration). The rate of change in absorbance at 340 nm due to NADH oxidation was recorded. Intra- and extracellular LDH activities were analyzed, and the percentages of leakage were counted.

Lipid peroxidation

According to the method of Fraga and others (1988), thiobarbituric acid-reactive substance (TBARS) was used to measure lipid peroxidation. The samples and standards (1,1,3,3-tetramethoxypropane) were mixed in 20 mM potassium phosphate buffer, 3% SDS solution, 0.1 N HCl, 10% phosphotungstic acid, and 0.7% thiobarbituric acid (TBA) and heated in boiling water for 30 min, then cooled to room temperature. The reactive products were extracted with *n*-butanol and analyzed with a fluorescence spectrophotometer at 515-nm excitation and 555-nm emission. The results were compared to a standard curve expressed in nanomoles of TBARS per milligram of protein.

Major component analysis by HPLC

We followed the HPLC analytical methodology, which was published by Chyr and Shiao (1991). The conditions of this HPLC analysis were as follows: Cosmosil C18 column (Nacalai Tesque, Japan), Hitachi L-7420 UV-VIS detector (Hitachi, Tokyo, Japan), UV 243 nm, gradient elution (started with 80% methanol, increased linearly to 84% methanol in 15 min, to 86% in further 15 min, to 88% in 10 min, to 94% in further 10 min, and finally to 100% in 20 min), flow speed (0.7 mL/min). The analytical concentration is 20000 ppm.

Statistical analysis

Statistical analysis was performed using 1-way analysis of variance (ANOVA) and Tukey's multiple comparison test (SAS Inst. Inc., Cary, N.C., U.S.A.) to determine significant differences among means ($P < 0.05$).

Results and Discussion

As the wild fruiting body of AC is now difficult to find in Taiwan's forests, most of the material sources of AC products are provided from submerged fermentation and solid-state fermentation. Although studies about the traditional edible AC have already been published, these studies did not use the same material sources. Moreover, the relations between bioactivity and fermentation conditions of AC are not clear. In this study, AC products were obtained via 250 mL flask, 5 and 500 L fermentation with various cultivation times, and the effects of ethanolic extracts of these AC products on the growth of hepatoma cells and normal primary rat hepatocytes were evaluated.

The effect of ethanolic extract of AC from 250 mL flask fermentation product on the antihepatoma activity

AC was submerged and fermented in 250 mL flask with 2% glucose as carbon sources for 1, 2, 4, 6, and 8 wk. These mycelia and broth were separated by filter paper, extracted with 95% ethanol for 24 h at 30 °C, and lyophilized afterward. The effects of the ethanolic extracts of AC mycelia and broth (fermentation of 1, 2, 4, 6, and 8 wk) on the cell viability of HepG2 and Hep3B cells were evaluated. The result showed no adverse effect on cell viability of HepG2 and Hep3B cells that were treated with ethanolic extracts from 1, 2, and 4 wk mycelia and broth from all the weeks (data not shown). However, the treatment with ethanolic extracts of AC mycelia from 6 and 8 wk fermentations could significantly decrease the cell viability of HepG2 and Hep3B cells in a dose-dependent manner (Table 1). The IC_{50} of ethanolic extract of fermentation of 8 wk AC mycelia to inhibit HepG2 cells growth was 54.2 μg/mL, but to inhibit Hep3B cells was about 82.9 μg/mL. The results showed that the extent of inhibition was more profound in HepG2 cells than Hep3B cells.

The effects of ethanolic extracts of AC from 5 and 500 L fermentation products on the antihepatoma activity

The subcultured AC was inoculated into a 5 L fermentor and fermented for 4 and 8 wk. The fermentation conditions were initial pH 5, malt extract 2%, peptone 0.1%, and carbon source (glucose) 2% cultured at 22 °C, 100 rpm. The results showed that the treatment with ethanolic extract of 4 and 8 wk fermentation mycelia of AC (5FAC4-Em and 5FAC8-Em) could significantly decrease the cell viability of HepG2 and Hep3B cells in a dose-dependent manner (Table 2). However, 5FAC4-Em and 5FAC8-Em possessed similar ability to inhibit proliferation of HepG2 and Hep3B cells. The IC_{50} of Hep3B and HepG2 treated with 5FAC4-Em were 48.7 and 3.8 μg/mL, respectively, while those treated with 5FAC8-Em were 52.6 and 2.9 μg/mL, respectively. The inhibition activity of 5FAC4-Em and 5FAC8-Em against HepG2 cells is comparatively higher then Hep3B cells. The results indicate that the ethanolic extracts of 4 weeks' AC mycelia from 5 L fermentor (5FAC4-Em) could

Table 1—The effect of ethanolic extracts of AC mycelia from 250-mL flask fermentation on the growth inhibition of Hep3B and HepG2 cells.

		Concentrations of treatment (μg/mL)					
		0.1	1	10	100	200	
Cells	Fermentation time (wk)	Cell viability (%)					IC_{50} (μg/mL)
Hep3B	1	102.5 ± 8.1^{a}	103.8 ± 6.9^{a}	111.4 ± 6.4^{a}	108.7 ± 7.2^{a}	109.7 ± 5.9^{a}	–
	2	100.1 ± 5.8^{a}	103.2 ± 6.9^{ab}	110.7 ± 6.4^{b}	94.7 ± 4.8^{b}	96.3 ± 8.7^{b}	–
	4	103.8 ± 10.9^{a}	103.8 ± 4.9^{a}	114.7 ± 5.7^{a}	90.5 ± 5.0^{b}	82.0 ± 6.1^{b}	–
	6	99.6 ± 6.0^{a}	90.6 ± 6.3^{ab}	81.4 ± 8.2^{bc}	73.8 ± 6.1^{c}	48.9 ± 5.6^{d}	199.5
	8	103.2 ± 5.2^{a}	91.8 ± 4.1^{a}	75.9 ± 13.5^{a}	42.3 ± 1.4^{c}	30.2 ± 13.5^{c}	82.9
HepG2	1	107.5 ± 2.9^{a}	102.4 ± 38^{a}	100.7 ± 5.2^{a}	111.6 ± 3.9^{a}	109.1 ± 4.9^{a}	–
	2	100.7 ± 5.1^{a}	96.6 ± 7.5^{a}	99.3 ± 3.3^{a}	94.9 ± 7.1^{ab}	87.3 ± 6.4^{b}	–
	4	98.7 ± 7.2^{a}	99.8 ± 4.8^{a}	90.3 ± 5.7^{b}	86.0 ± 6.6^{b}	73.9 ± 3.7^{c}	–
	6	97.5 ± 7.5^{a}	89.9 ± 10.5^{ab}	85.1 ± 4.5^{b}	68.0 ± 4.1^{c}	41.8 ± 9.2^{d}	168.2
	8	107.5 ± 2.9^{a}	89.4 ± 2.5^{b}	47.1 ± 2.6^{c}	27.6 ± 1.5^{d}	2.9 ± 0.4^{e}	54.2

Values bearing different letter in the same row are significantly different ($P < 0.05$) by 1-way ANOVA and Tukey's multiple comparison test ($n = 6$).

Table 2—The effects of ethanolic extracts of AC mycelia from 5 L fermentation on the growth inhibition of Hep3B and HepG2 cells.

		Concentrations of treatment (μg/mL)				
		0.1	1	10	100	
Cells	Fermentation time (wk)	Cell viability (%)				IC_{50} (μg/mL)
Hep3B	4	78.8 ± 1.6^{a}	71.4 ± 2.2^{a}	53.6 ± 6.3^{b}	5.2 ± 4.1^{c}	48.7
	8	92.0 ± 4.8^{a}	74.2 ± 8.8^{b}	44.1 ± 1.2^{c}	9.6 ± 1.3^{d}	52.6
HepG2	4	85.0 ± 8.6^{a}	56.2 ± 6.0^{a}	30.2 ± 3.3^{b}	15.2 ± 5.8^{b}	3.8
	8	59.8 ± 3.8^{a}	29.8 ± 1.5^{b}	23.8 ± 1.5^{c}	17.1 ± 3.1^{d}	2.9

Values bearing different letter in the same row are significantly different ($P < 0.05$) by 1-way ANOVA and Tukey's multiple comparison test ($n = 6$).

Table 3—The effect of ethanolic extracts of AC mycelia from 500 L fermentation on the growth inhibition of Hep3B and HepG2 cells.

Cells	Fermentation time (wk)	Concentrations of treatment (μg/mL) 0.1	1	10	100	200	IC_{50} (μg/mL)
		Cell viability (%)					
Hep3B	1	94.1 ± 2.9[a]	91.1 ± 4.6[a]	96.6 ± 3.6[a]	69.3 ± 3.2[b]	50.3 ± 8.1[c]	196.8
	2	105.8 ± 3.2[a]	84.3 ± 5.3[b]	59.0 ± 1.8[c]	43.6 ± 1.6[d]	40.5 ± 2.0[d]	78.9
	3	65.9 ± 4.9[a]	54.5 ± 4.0[b]	43.1 ± 7.5[c]	42.4 ± 2.1[c]	37.7 ± 2.1[c]	48.6
	4	76.8 ± 4.9[a]	63.3 ± 4.9[b]	40.1 ± 2.0[c]	29.3 ± 3.3[d]	16.6 ± 5.6[e]	36.9
	6	70.2 ± 3.1[a]	58.1 ± 4.7[b]	40.3 ± 3.0[c]	29.8 ± 3.9[d]	3.4 ± 2.2[e]	27.0
HepG2	1	89.5 ± 2.3[a]	89.7 ± 8.2[a]	93.4 ± 7.6[a]	64.6 ± 7.9[b]	27.8 ± 8.1[c]	135.5
	2	74.2 ± 8.4[a]	69.6 ± 9.3[a]	63.9 ± 5.7[a]	20.8 ± 1.7[b]	7.6 ± 1.3[c]	54.0
	3	90.3 ± 5.1[a]	82.4 ± 7.5[b]	39.6 ± 3.1[c]	15.4 ± 0.1[d]	20.7 ± 2.5[d]	17.5
	4	77.5 ± 9.9[a]	64.8 ± 5.6[b]	16.4 ± 1.3[c]	19.7 ± 5.8[c]	19.9 ± 2.0[c]	3.1
	6	91.7 ± 9.6[a]	61.6 ± 9.7[b]	22.0 ± 4.8[c]	14.8 ± 5.7[c]	2.9 ± 1.4[d]	5.1

Values bearing different letter in the same row are significantly different ($P < 0.05$) by 1-way ANOVA and Tukey's multiple comparison test ($n = 6$).

significantly decrease the cell viability of HepG2 and Hep3B cells, in contrast to the ethanolic extract of 4 wk mycelia from the 250 mL flask, which did not show any inhibition. In the 250-mL flask fermentor, only ethanolic extract of 8 wk AC mycelia had inhibition activity against HepG2 and Hep3B cells.

Because the lifetime of inducted air filter is no more than 2 mo in the 500 L fermentation, the cultivation time of AC was only for 6 wk. One liter of AC fermentation product was sampled at 1, 2, 3, 4, and 6 weeks. These samples were extracted with 95% ethanol for 24 h at 30 °C and lyophilized. The effect of these ethanolic extracts of AC mycelia (500FAC1-Em, 500FAC2-Em, 500FAC3-Em, 500FAC4-Em, and 500FAC6-Em) on the cell viability of HepG2 and Hep3B cells was also evaluated. The results showed that these samples could significantly decrease the cell viability of HepG2 and Hep3B cells in a dose-dependent manner (Table 3). Again, the inhibition activity of these samples against HepG2 cells was comparatively higher than that of Hep3B cells. The IC_{50} of 500FAC1-Em, 500FAC2-Em, 500FAC3-Em, and 500FAC4-Em decreased as the cultivation time increased. However, the IC_{50} of ethanolic extract of 6 wk AC mycelia (500FAC6-Em) was not less than 500FAC4-Em.

Figure 1 indicates the variation of morphology of HepG2 and Hep3B cells with 5FAC4-Em treatment. There were marked differences between 10, 100, 200, and 400 μg/mL and control treatment on the morphology of HepG2 and Hep3B cells. A similar result was observed on the morphology of HepG2 and Hep3B cells with 500FAC6-Em treatment (data not shown).

The results from cell viability studies showed that Hep3B and HepG2 cells were more inhibited on treatment with the ethanolic extract of AC mycelia fermented for 8 wk in the 250 mL flask than those fermented for 1, 2, 4, and 6 wk. The IC_{50} of ethanolic extract of AC mycelia from the 500 L fermentation (500FAC1-Em, 500FAC2-Em, 500FAC3-Em, and 500FAC4-Em) also decreased with cultivation time. These results indicate that the antihepatoma activity of AC is related to the cultivation time of fermentation. Similar results have been reported on fermented soy food (doenjang) where increasing the fermentation period when making doenjang, increases its antitumor and antimetastatic effect *in vivo* (Jung and others 2006). Doenjang fermented for 24 mo exhibited a 2- to 3-fold increase in the antitumor effects on sarcoma-180-injected mice and antimetastatic effects in colon 26-M 3.1 cells in mice compared with the 3- or 6-mo fermented doenjang.

The AC mycelia needed 8 wk of submerged fermentation in a 250 mL flask to exhibit an inhibitory effect against HepG2 and Hep3B cells. However, the ethanolic extracts of AC mycelia fermented only for 4 wk in the 5 and 500 L fermentors possessed significant inhibitory effect against Hep3B and HepG2 cells. These results indicated that the antihepatoma activity of AC products is also related to the scale of fermentation fermentor. When the volume was changed from the 250 mL fermentor into a 5 L fermentor, the cultivation time could be decreased from 8 to 4 wk. Moreover, the ethanolic extracts of AC mycelia fermented for 4 wk in a 5 L fermentor have slightly lower inhibitory effect against Hep3B

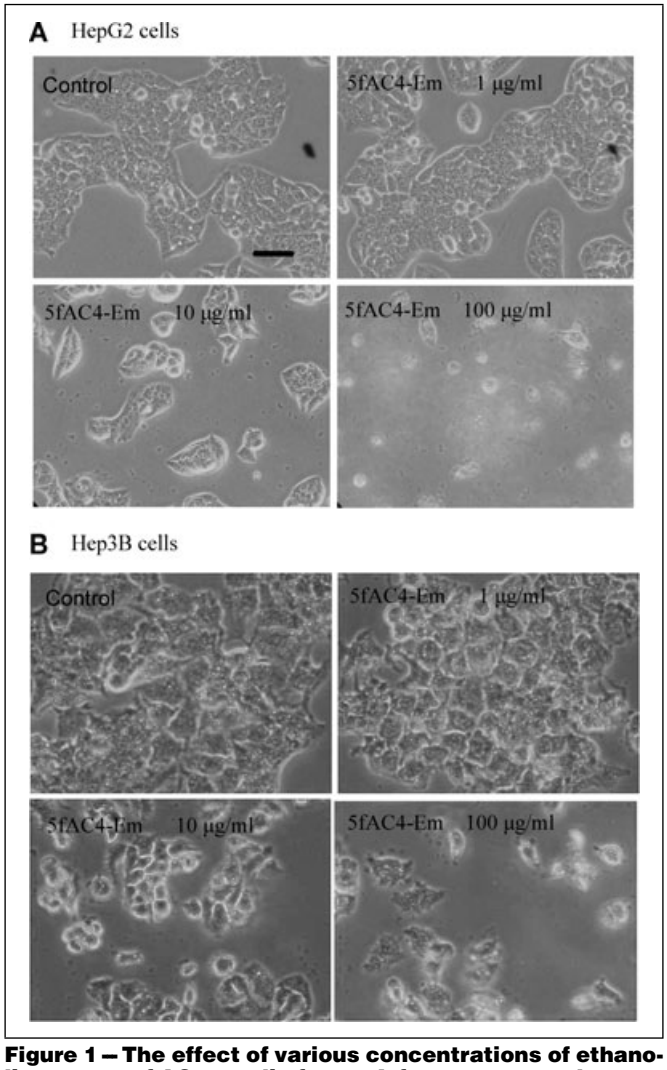

Figure 1—The effect of various concentrations of ethanolic extract of AC mycelia from 5 L fermentors on the morphology of (A) HepG2 and (B) Hep3B cells (200×). ▬ is 100 μm in length

and HepG2 cells than the samples from a 500 L fermentor. The tank pressure of the 500 L fermentor probably reduced the growth of AC. Therefore, according to these results, we suggest that the cultivation time requires 4 wk in a large fermentor, and the ethanolic extract of AC mycelia possessed antihepatoma activity. Many reports related to anticancer activity of AC have been published in recent years (Hseu and others 2004; Liu and others 2004; Nakamura and others 2004; Hsu and others 2005; Song and others 2005a, 2005b). However, these studies did not use the same material sources of AC. Moreover, the relationship between bioactivity and bioactive compounds of AC are not clear.

Effect of ethanolic extract of AC mycelia from 5 and 500 L fermentation on the growth of primary rat hepatocytes

In this study, we used liver-perfusion method to isolate normal primary rat hepatocytes from male Narl:SD rats (6 to 8 wk old) and to evaluate the effect of 5FAC4-Em and 500FAC6-Em on the growth of normal primary rat hepatocytes. Figure 2 indicates the variation of morphology of normal primary rat hepatocytes with 5FAC4-Em and 500FAC6-Em treatment. There were no marked differences between 1, 10, and 100 μg/mL and control treatment on the morphology of normal primary rat hepatocytes. However, the growth of normal primary rat hepatocytes was inhibited significantly at 200 and 400 μg/mL treatment with 5FAC4-Em and 500FAC6-Em.

In addition, to evaluate the cell viability of normal primary rat hepatocytes by assessing the percentage of LDH leakage (Figure 3). The result indicated no ill effect of normal primary rat hepatocytes at 1, 10, and 100 μg/mL of 5FAC4-Em and 500FAC6-Em treatment. For hepatoma cell lines, however, the IC_{50} of 5FAC4-Em and 500FAC6-Em were 48.7 and 27 μg/mL for Hep3B cells and 3.8 and 5.1 μg/mL for HepG2 cells, respectively. These dose concentrations are well below the 100 μg/mL dose concentration of 5FAC4-Em and 500FAC6-Em treatment. Thus, ethanolic extracts of AC mycelia possessing inhibiting activity on HepG2 and Hep3B cells did not show any adverse effect on normal primary rat hepatocytes, but the LDH leakages of normal primary rat hepatocytes were

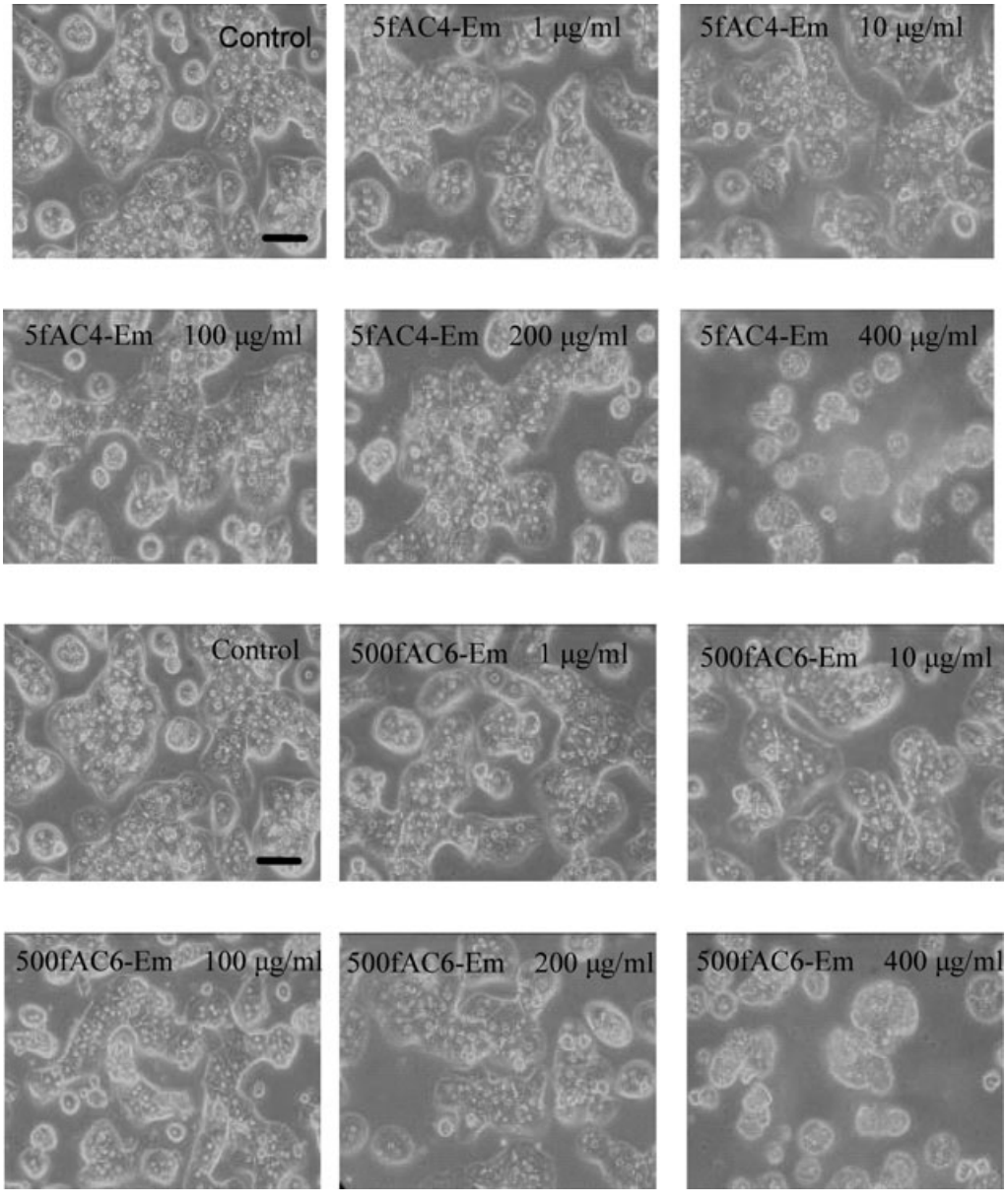

Figure 2—The effect of various concentrations of ethanolic extract of AC mycelia from 5 and 500 L fermentors on the morphology of primary rat hepatocytes (200×). — is 100 μm in length.

quite significant when treated with 200 and 400 μg/mL of 5FAC4-Em and 500FAC6-Em.

The lipid peroxidation of normal primary rat hepatocytes with various concentrations of 5FAC4-Em and 500FAC6-Em treatments was evaluated. The TBARS values of normal primary rat hepatocytes showed no significant difference from 0 to 200 μg/mL of 5FAC4-Em and 500FAC6-Em treatments. This result indicated that the ethanolic extracts of AC mycelia would not cause lipid peroxidation in normal primary rat hepatocytes (Figure 4).

Human hepatocytes in primary culture provide the closest *in vitro* model to human liver and the only model that can produce a metabolic profile of a given drug that is similar to that found *in vivo*. However, their availability is limited due to the restricted access to suitable tissue samples (Gomez-Lechon and others 2003). In this study, normal human hepatocytes were replaced by normal primary rat heptatocytes. The results showed that 1, 10, and 100 μg/mL of ethanolic extract of AC possessing antihepatoma activity on HepG2 and Hep3B cells, which did not have any adverse effect on normal primary rat hepatocytes. In addition, HepG2 is a kind of wild type hepatoma cells. Hep3B is a kind of delete-p53 hepatoma cells. This variation may be the reason for a comparatively higher inhibition activities of FAC4-Em, FAC8-Em and 500FAC4-Em against HepG2 cells than with Hep3B cells.

The HPLC chromatograms of the ethanolic extracts of AC mycelia from 500 L fermentor

To evaluate the major activity component of AC mycelia from a 500 L fermentor, we followed the HPLC analytical methodology published by Chyr and Shiao (1991) to analyze ethanolic extracts of AC mycelia from a 500 L fermentor. Their result indicated that the major activity components appeared in the retention time of HPLC profile between 15 and 52 min. In this study, the results showed that the HPLC profile of ethanolic extract of 6 wk fermented AC mycelia possessed more peak areas than those of 3 wk fermented AC mycelia (Figure 5). Besides, the HPLC profile of ethanolic extract of 1 wk fermented AC mycelia possessed fewer peaks in the retention time between 15 and 50 min. This result indicated that there were many possible active components in the ethanolic extract of 6 wk fermented AC mycelia from 500 L fermentor. Moreover, these possible active components could be related to that the ethanolic extract of the 6-wk fermented AC mycelia possessed high antihepatoma activity.

Conclusions

In this study, the antihepatoma activities of AC products fermented with different time and scales were evaluated. The result showed that the treatment with ethanolic extracts of AC mycelia from 5 and 500 L fermentation could significantly decrease the cell viability of HepG2 and Hep3B cells, and the 100 μg/mL of treatment with the same samples did not have any adverse effect on normal primary rat hepatocytes. It indicated that the cultivation time of 5 and 500 L fermentation required only 4 wk to produce AC mycelia which had significant antihepatoma activity. These results could be used as a guide to produce AC products possessing antihepatoma activity in large-scale fermentation processes. Moreover, the high antihepatoma activity ethanolic extracts of AC mycelia contain

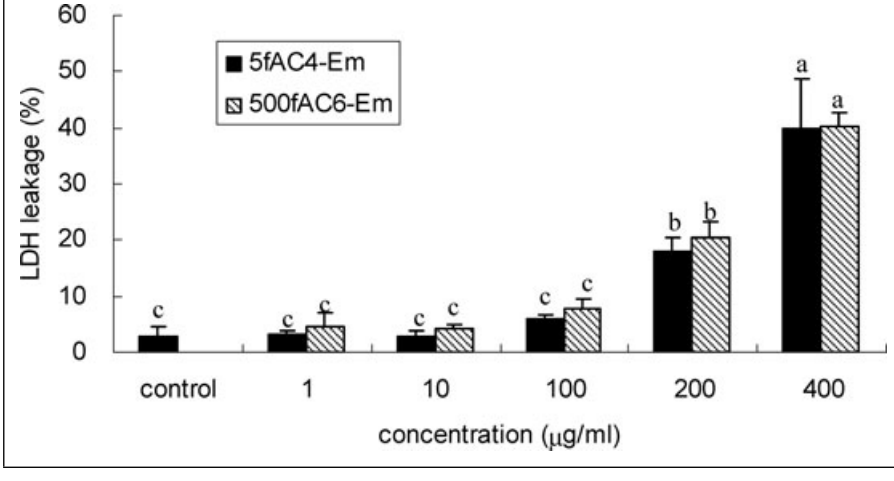

Figure 3—The effect of various concentrations of ethanolic extracts of AC mycelia from 5 and 500 L fermentors on the cell viability of primary rat hepatocytes at 24 h treated. Values bearing different letter are significantly different ($P < 0.05$) by 1-way ANOVA and Tukey's multiple comparison test ($n = 6$).

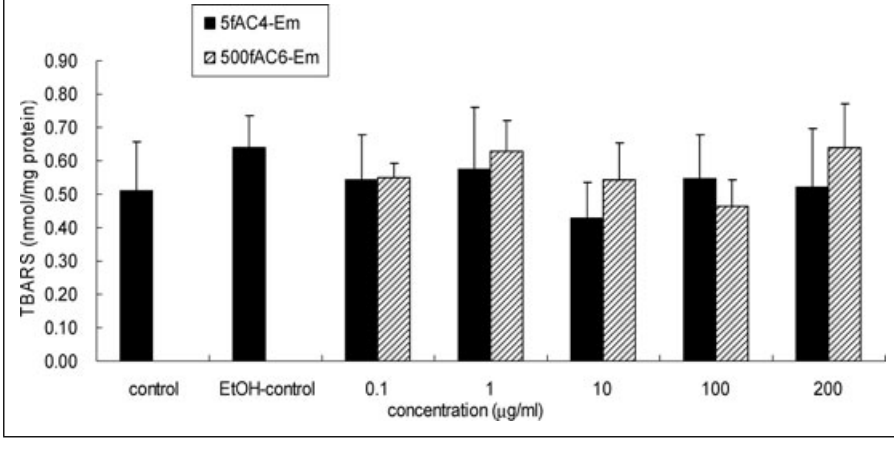

Figure 4—The effect of various concentrations of ethanolic extracts of AC mycelia from 5 and 500 L fermentors on the TBARS of primary rat hepatocytes at 24 h treated.

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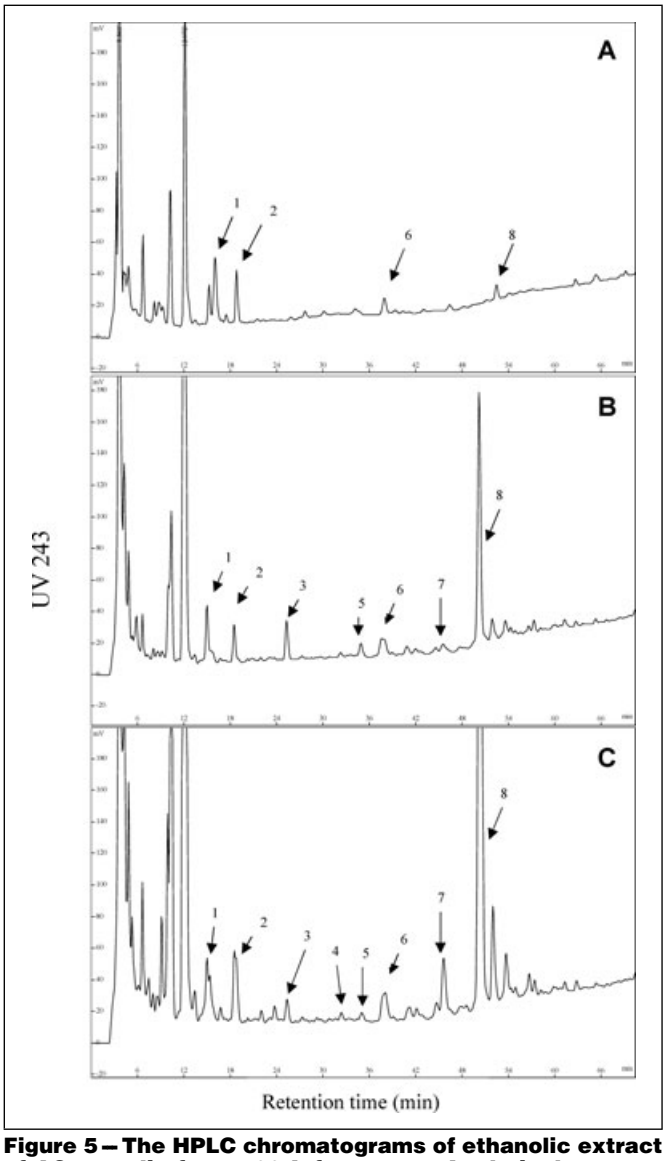

Figure 5—The HPLC chromatograms of ethanolic extract of AC mycelia from 500 L fermentor. Analytical concentration is 20000 ppm. The captions, (A), (B), and (C), indicate that the HPLC profile of ethanolic extract of AC mycelia fermented 1, 3, and 6 wk in 500 L fermentor, respectively. The retention time of peak 1 is 15.9 min, peak 2 is 18.8 min, peak 3 is 25.8 min, peak 4 is 32.2 min, peak 5 is 34.1, peak 6 is 40.3 min, peak 7 is 46.3, and peak 8 is 51 min.

many potential active components. The study on the identification of active compounds from AC products with antihepatoma activity is in progress.

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