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Black Soybean Promotes the Formation of Active Components with Antihepatoma Activity in the Fermentation Product of *Agaricus blazei*

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The antihepatoma activity and related active components in the fermentation products of *Agaricus blazei* (AB) cultured in the medium containing soybean (S) or black soybean (BS) were investigated. AB(BS)-pE and AB(S)-pE were the ethanolic extracts from the fermentation products of AB(BS) and AB(S), respectively. According to the IC_{50} values, AB(BS)-pE (161.1 and 24.0 μg/mL for Hep 3B and Hep G2 cells, respectively) exhibited stronger cytotoxicities against hepatoma cells than AB(S)-pE (>200 and 99.9 μg/mL for Hep 3B and Hep G2 cells, respectively). AB(BS)-pE was separated by silica gel column chromatography and eluted with *n*-hexane/ethyl acetate/methanol gradient solvent system into 21 fractions. Fraction 3 [AB(BS)-pE-F3], eluted with *n*-hexane/ethyl acetate (97:3 and 19:1, v/v), was the most active fraction having inhibitory activity on the proliferation of Hep 3B and Hep G2 cells (IC_{50} of 3.6 and 1.9 μg/mL, respectively). Three major compounds, compounds **1**−**3**, were further isolated from the AB(BS)-pE-F3 fraction by reversed-phase semipreparative high-performance liquid chromatography. Compounds **2** and **3** gave better antihepatoma activity than that of compound **1**. The IC_{50} values of compounds **2** and **3** were 2.8 and 4.5 μg/mL for Hep 3B cells and 1.4 and 2.0 μg/mL for Hep G2 cells, respectively. The structures of compounds **2** and **3** were identified by UV, IR, electron impact mass spectrometry, and ^{1}H and ^{13}C NMR to be blazeispirols A and C, respectively. Blazeispirols A and C existed in the mycelia but not in the broth and were more in AB(BS)-pE (49.9 ± 8.9 and 14.2 ± 2.4 mg/g, respectively) than AB(S)-pE (15.9 ± 1.7 and 3.9 ± 0.6 mg/g, respectively). Additionally, the result shows that the production of blazeispirols A and C was increased after cultivation in the medium containing black soybean on day 6 and reached the maximum on day 12, and the contents of blazeispirols A and C were negatively correlated with Hep 3B and Hep G2 cell viabilities ($r = -0.84$ to -0.93, $P < 0.01$). It suggests that blazeispirols A and C could be used as biomarkers to produce the fermentation product of *A. blazei* with antihepatoma activity.

KEYWORDS: ***Agaricus blazei*; soybean; black soybean; fermentation product; fraction; antihepatoma activity; Hep 3B cells; Hep G2 cells; blazeiperol A; blazeiperol C; mycelia**

INTRODUCTION

Agaricus blazei Murill (Agaricaceae) (AB), known as the "sun mushroom" or "himematsutake", is an edible and medicinal mushroom. The fruiting body of AB has been reported to have many biological activities, including immunomodulation (*1*), liver protection (*2*), antitumor and antiangiogenesis (*3*), antigenotoxicity (*4*), antioxidation (*5*), and antidiabetes (*6*).

Some chemical compositions with medicinal properties in AB have also been reported. Four steroids that were cytotoxic against HeLa cells have been identified from the fruiting body of AB (*7*). Machado et al. tested hexane extracts (one fraction and its four subfractions) of the fruiting body of AB for cytotoxicity in cultured mammalian cells (CHO-K1). The results indicated that the greater cytotoxicity of the fraction as compared

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to the subfractions could be due to the fraction that contained several compounds resulting in combinational or synergistic effects in cytotoxicity than the semipurified subfractions (*8*). It has also been demonstrated that a highly branched β-1,3-glucan isolated from AB has antitumor activity through immunomodulation (*9*).

Because cultivation of the fruiting body of AB may take several months, it may also be difficult to control the quality of the product. Many researchers are interested in utilizing the fermentation process to produce a larger amount of fungal products with biological activity. Currently, the fermentation products of AB have been reported to have immunomodulation, antitumor, antioxidation, and antivirus effects (*10−13*). Additionally, an active polysaccharide against Sarcoma 180 was isolated from the hot water soluble fraction of the mycelia of liquid-cultured AB, and its structure was different from β-1,6-glucan, which is from the fruiting body of AB (*14*). Hirotani et al. have identified many des-*A*-ergostane derivatives such as blazeispirane and protoblazeispirane from the cultured mycilia of AB, but these compounds were not detectable from the AB fruiting body (*15*). Therefore, some unique constituents were biosynthesized from the fermentation products of AB, but the compounds with antihepatoma activity have not been clearly studied.

Soybean (*Glycine max*) is a nutritional and functional food with many valuable components such as isoflavones, proteins, and polysaccharides (*16*). Its biological functions include antioxidant capacity, anticancer activity, and prevention of atherosclerosis (*17−19*). Black soybean is a soybean cultivar with a black seed coat, but some constituents, anthocyanins and phenolic compounds, for example, are different between two strains (*17, 20*). On the basis of different compositions, some studies demonstrated that black soybeans have better biological activities including antioxidant activities against the oxidation of low-density lipoproteins and radical scavenging activities (*17, 21*). A few reports showed that some compounds and bioactivities were changed in fungal-fermented soybean or black soybean (*22, 23*). However, the interactions for antihepatoma activity between AB and soybean or black soybean have not yet been elucidated.

The objective of the present study is to investigate the antihepatoma activity of the submerged fermentation products of AB cultured in the medium containing soybean or black soybean. The active components with antihepatoma activity were elucidated as important markers for the fermentation products of AB.

MATERIALS AND METHODS

Materials and Chemicals. Ethanol (95%), which was used for the extraction of the fermentation product, was purchased from Echo Chemical (Taipei, Taiwan). Ethyl acetate and methanol (Merck, Darmstadt, Germany) and *n*-hexane (95%) (Seedchem, Melbourne, Australia) were used for silica gel column chromatography and thin-layer chromatography (TLC). Methanol and acetic acid used for the preparation and analysis of the compounds were obtained from Tedia (OH). Dulbecco's modified Eagle medium (DMEM), antibiotic−antimycotic solution, minimal essential medium (MEM) nonessential amino acid solution, and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY). *N*,*N*-Dimethylfluoramide (DMF) was purchased from the Laboratory-Scan (Dublin, Ireland). Trypsin-EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and sodium dodecyl sulfate (SDS) from Sigma Chemical (St. Louis, MO) were used.

Liquid Fermentation of AB. The fermentation products [AB(S) and AB(BS)] were obtained from Professor Chin-Hang Shu (Department of Chemical and Materials Engineering, National Central University, Taiwan). An industrial AB strain (NCUC AB001) was from Brazil, and soybean and black soybean were purchased from Hseija (Tainan, Taiwan). The cultivation of the microorganism involved three stages: activation of the stock culture, growth of an inoculum, and batch cultivation for 6 days. Details of the inoculum preparation were described elsewhere (*24*). On day 6, the inoculum was transferred into a 500 L stirred tank bioreactor with a working volume of 350 L. The medium contained 5 g/L glucose, 1.5 g/L malt extract, 1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L vitamin B_1, and 10 g/L soybean for AB(S) or black soybean for AB(BS). The fermentation conditions were as follows: inoculum ratio, 10%; agitation, 50 rpm; culture temperature, 28 °C; air flow rate, 100 L/min; and culture pH, 5. The total fermentation time for AB(S) or AB(BS) was 17 days.

Table 1. Effect of Ethanolic Extracts of the Fermentation Products of AB on the Growth of Hep 3B and Hep G2 Cells

concentration (μg/mL)	cell viability (% of control)[a−c] Hep 3B AB(S)-pE	Hep 3B AB(BS)-pE	Hep G2 AB(S)-pE	Hep G2 AB(BS)-pE
12.5	77.8 ± 2.9 a	81.6 ± 3.8 a	80.6 ± 8.6 a	57.4 ± 3.6 a
25	68.7 ± 2.3 b	83.5 ± 1.1 a	66.8 ± 9.3 ab	48.7 ± 1.2 b
50	60.7 ± 2.1 c	83.0 ± 5.3 a	56.8 ± 4.8 bc	34.4 ± 2.1 c
100	63.1 ± 2.9 c	66.6 ± 1.6 b	50.6 ± 6.3 c	16.5 ± 1.0 d
200	55.3 ± 2.9 d	39.8 ± 6.6 c	35.2 ± 8.3 d	9.2 ± 0.8 e
IC_{50} (μg/mL)[d]	>200	161.1	99.9	24.0

[a] AB(S) and AB(BS) represent the fermentation products of AB cultivated in the medium containing soybean (S) and black soybean (BS), respectively. [b] Cells (5×10^3/well) were seeded in complete DMEM on a 96 well plate for 24 h. Subsequently, cells were incubated in serum-free DMEM and treated with 12.5, 25, 50, 100, or 200 μg/mL of each sample for another 48 h. The cell viability was determined by MTT assay. [c] Each data presents mean ± SD ($n = 3$). Data with different letters in the column were significantly different with each other ($P < 0.05$). [d] IC_{50} was the concentration of each sample required to inhibit cell growth by 50%.

Preparation of Sample. The fermentation product of AB [AB(S) or AB (BS)] was lyophilized and ground into powder (p). One gram of dry powder was shaken at 100 rpm with 95% ethanol (20 mL) at 30 °C for 24 h. The extracts [AB(S)-pE and AB(BS)-pE] were evaporated to dryness under vacuum, and their yields were 7.7 and 9.0 g/100 g dry weight of fermentation product, respectively. Additionally, AB(S) or AB(BS) was separated into broth (liquid part) [AB(S)-b or AB(BS)-b] and mycelia (solid part) [AB(S)-m or AB(BS)-m]. Broth was sterilized at 121 °C for 30 min and then filtered with 0.22 μm filiter. One gram of dry mycelium powder was shaken at 100 rpm with 95% ethanol (20 mL) at 30 °C for 24 h. The extracts [AB(S)-mE or AB(BS)-mE] were evaporated to dryness under vacuum.

Fractionation of AB(BS)-pE. *Silica Gel Column Chromatography.* The method used was based on that reported by Ahmed et al. (*25*), Chang et al. (*26*), and Machado et al. (*8*). AB(BS)-pE (40.9 g) was applied to a silica gel column chromatography (Si gel 60, 40−63 μm, Merck, Darmstadt, Germany) (9.5 cm × 40 cm) and eluted with a stepwise gradient of *n*-hexane/ethyl acetate and ethyl acetate/methanol to afford 149 collections. The flow rate of eluting solvent was 1 L/h, and 1 L of eluting solution was one collection (**Table 2**).

TLC. Collections were checked by TLC using different percentages of *n*-hexane/ethyl acetate or ethyl acetate/methanol for the development. The 20 cm × 20 cm silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany) were developed in *n*-hexane/ethyl acetate (19:1, 9:1, 8:2, 17:3, 5:5, and 3:7, v/v), ethyl acetate, ethyl acetate/methanol (9:1, 7:3, 5:5, and 3:7, v/v), and methanol, after spotting each collection. Sulfuric acid (10%) was used in the detection of possible compounds. According to the weight distribution after silica gel column chromatography and the results of TLC, the collections from the above separation were combined to give 21 fractions for further antihepatoma experiment. The fractions were evaporated to dryness under vacuum.

Hepatoma Cell Culture. Human hepatoma Hep 3B and Hep G2 cells were obtained from the Department of Medical Research and

Table 2. Effects of Fractions Eluted by Silica Gel Column Chromatography from AB(BS)-pE on the Growth of Hep 3B and Hep G2 Cells

			Hep 3B			Hep G2		
fraction	eluting solvent (v/v, eluting volume)[a]	weight (g)	IC_{50} (μg/mL)[b,c]	activity unit[d]	percentage in total activity (%)[e]	IC_{50} (μg/mL)	activity unit	percentage in total activity (%)
AB(BS)-pE		39.75	161.1			24.0		
AB(BS)-pE-F1	H/EA (1:0, 11 L; 49:1, 5 L)	0.10	–	–	–	–	–	–
AB(BS)-pE-F2	H/EA (49:1, 5 L)	0.81	–	–	–	–	–	–
AB(BS)-pE-F3	H/EA (97:3, 4 L; 19:1, 4 L)	2.91	3.6	8083333	84.01	1.9	15315789	85.29
AB(BS)-pE-F4	H/EA (19:1, 2 L; 9:1, 4 L; 8:2, 1 L)	0.34	78.8	43147	0.45	73.5	46259	0.26
AB(BS)-pE-F5	H/EA (8:2, 7 L)	0.91	–	–	–	–	–	–
AB(BS)-pE-F6	H/EA (7:3, 3 L)	0.36	20.6	174757	1.82	27.9	129032	0.72
AB(BS)-pE-F7	H/EA (7:3, 4 L)	0.36	29.2	123288	1.28	23.5	153191	0.85
AB(BS)-pE-F8	H/EA (7:3, 1 L; 5:5, 2 L)	0.44	40.1	109726	1.14	40.9	107579	0.60
AB(BS)-pE-F9	H/EA (5:5, 3 L)	0.69	32.8	210366	2.19	39.8	173367	0.97
AB(BS)-pE-F10	H/EA (5:5, 5 L)	0.72	32.1	224299	2.33	32.1	224299	1.25
AB(BS)-pE-F11	H/EA (3:7, 5 L; 0:1, 1 L)	1.11	51.2	216797	2.25	61.3	181077	1.01
AB(BS)-pE-F12	H/EA (0:1, 6 L)	2.53	–	–	–	79.7	317440	1.77
AB(BS)-pE-F13	H/EA (0:1, 6 L)	1.35	68.7	196507	2.04	61.8	218447	1.22
AB(BS)-pE-F14	EA/M (1:0, 1 L; 9:1, 4 L)	5.33	–	–	–	92.4	576840	3.21
AB(BS)-pE-F15	EA/M (9:1, 6 L)	3.20	133.8	239163	2.49	112.4	284698	1.59
AB(BS)-pE-F16	EA/M (9:1, 5 L; 7:3, 1 L)	1.25	–	–	–	–	–	–
AB(BS)-pE-F17	EA/M (7:3, 7 L)	8.81	–	–	–	–	–	–
AB(BS)-pE-F18	EA/M (7:3, 15 L)	4.13	–	–	–	–	–	–
AB(BS)-pE-F19	EA/M (5:5, 13 L)	2.73	–	–	–	118.7	229992	1.28
AB(BS)-pE-F20	EA/M (3:7, 5 L)	0.64	–	–	–	–	–	–
AB(BS)-pE-F21	EA/M (3:7, 1 L; 0:1, 12 L)	1.04	–	–	–	–	–	–

[a] H, EA, and M represent *n*-hexane, ethyl acetate, and methanol, respectively. [b] Cells (5×10^3/well) were seeded in complete DMEM on a 96 well plate for 24 h. Subsequently, cells were incubated in serum-free DMEM and treated with fractions of AB(BS)-pE for another 48 h. Cell viability was determined by MTT assay. IC_{50} was the concentration of each sample required to inhibit cell growth by 50%. [c] "–" means IC_{50} > 200 μg/mL. [d] An activity unit was defined as the amount of material causing 50% inhibition in a cell viability assay using Hep 3B or Hep G2 cell models. The value of the activity unit for each fraction was expressed as the weight of fraction of AB(BS)-pE (g)/[IC_{50} (μg/mL) × 100 μL]. [e] The percentage in total activity was defined as the activity unit of each fraction/total activity unit of fractions of AB(BS)-pE × 100%.

Education, Taipei Veterans General Hospital (Taipei, Taiwan). Cells were cultured in complete DMEM (cDMEM) (pH 7.0) at 37 °C, 5% CO_2, and 90% relative humidity. The cDMEM containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 100 μM nonessential amino acid was used. To remove the cells from the culture dish, they were trypsinized using 1 mL of trypsin-EDTA solution for 3 min at 37 °C.

Antihepatoma Activity Assay. Hep 3B or Hep G2 cells were cultured in 96 well plates at a density of 5×10^3 cells/100 μL cDMEM/well. After 24 h of incubation, the medium in the 96 well plate was replaced by 100 μL of serum-free DMEM containing different concentrations of samples for 48 h of treatment. The sample was dissolved in DMSO, and the final concentration of DMSO in the medium was 0.5%. Then, the medium was discarded, and 25 μL of MTT solution (5 mg/mL PBS) and 100 μL of serum-free DMEM were added to every well and reincubated for an additional 4 h. One hundred microliters of MTT lysis buffer (20 g of SDS in 50 mL of DMF and 50 mL of water) was added to dissolve the formazan crystals formed for 14−16 h. Then, the plates were read at 570 nm in a microplate reader (Anthos 2001, Salzburg). The cells that were not exposed to sample served as a control of 100% survival. All analyses were carried out in triplicate, and the mean values were determined. The concentration of each sample required to inhibit cell growth by 50% (IC_{50}) was determined by interpolation from dose−response.

Preparation of Major Compounds from AB(BS)-pE-F3. The fraction with the best antihepatoma activity from AB(BS)-pE after silica gel column chromatography was purified via revered-phase semipreparative high-performance liquid chromatography (RP-HPLC). A model L-7100 solvent delivery system (Hitachi, Tokyo, Japan) equipped with a model L-2200 autosampler (Hitachi), a model L-7420 UV−vis detector (Hitachi), and a Peak-ABC chromatography data handling system (JiTeng, Singapore) was used in the HPLC analysis. AB(BS)-pE-F3 was separated by a reversed-phase semipreparative HPLC column (Cosmosil $5C_{18}$-AR-II, 5 μm, 250 mm × 10 mm i.d.; Nacalai Tesque, Kyoto, Japan) at a column temperature of 25 °C. The mobile phase composed of methanol/water/acetic acid (89:11:0.5, v/v/v) was isocratically eluted at a flow rate of 2 mL/min, and the injection volume was 90 μL (40 mg/mL for [AB(BS)-pE-F3]. The effluent was monitored at 243 nm wavelength, and three major compounds, compounds **1** (t_R = 28.3 min), **2** (t_R = 35.8 min), and **3** (t_R = 39.5 min), were collected according to the elution profile.

Identification of Compounds 2 and 3. Purified compounds **2** and **3** were determined by UV, IR, MS, and NMR. The UV spectra were measured in methanol on a spectrophotometer. IR spectra were recorded on a Perkin-Elmer 983G spectrophotometer. ^1H and ^{13}C NMR spectra were recorded using a Varia Unity Plus 400 spectrometer, operating in $CDCl_3$ at 400 and 100 MHz, respectively. EI-MS spectra were obtained on a Finnigan MAT-95S mass spectrometer. UV, IR, MS, and ^1H and ^{13}C NMR data were consistent with the literature (*27*).

Analysis of Blazeispirols A (2) and C (3) in Samples. The samples, AB(BS)-pE, AB(BS)-mE, AB(BS)-b, and AB(BS)-pE-F3 and -F4, were analyzed by RP-HPLC. The column was Cosmosil $5C_{18}$-AR-II (5 μm, 250 mm × 4.6 mm i.d.) (Nacalai Tesque, Kyoto, Japan) at a column temperature of 25 °C. The mobile phases were solvents A (methanol/water/acetic acid, 80:20:0.5, v/v/v) and B (methanol/acetic acid, 100:0.5, v/v). Gradient elution began at 80% methanol, increased linearly to 84% in 15 min, to 86% in a further 15 min, and finally to 88% in 10 min. The flow rate was 0.7 mL/min, and effluent was monitored at 230 nm. The retention times (t_R) of blazeispirols A (**2**) and C (**3**) were 35 and 38 min, respectively. Quantification was performed by standard curves. All analyses were carried out in triplicate, and the mean values were determined.

Statistical Analysis. Correlation and regression analyses between the antihepatoma activity and the contents of blazeispirols A (**2**) and C (**3**) were performed using SigmaPlot8.0 scientific graph system. Statistical analyses were performed using one-way analysis of variance and Duncan's multiple comparison test (SAS Institute Inc., Cary, NC) to determine significant differences among means ($P < 0.05$).

RESULTS

Effect of Fermentation Products on Hepatoma Cells. The effects of ethanolic extracts of AB fermentation products on Hep 3B and Hep G2 cells are shown in **Table 1**. Both AB(S)-pE and AB(BS)-pE decreased cell viabilities with increasing

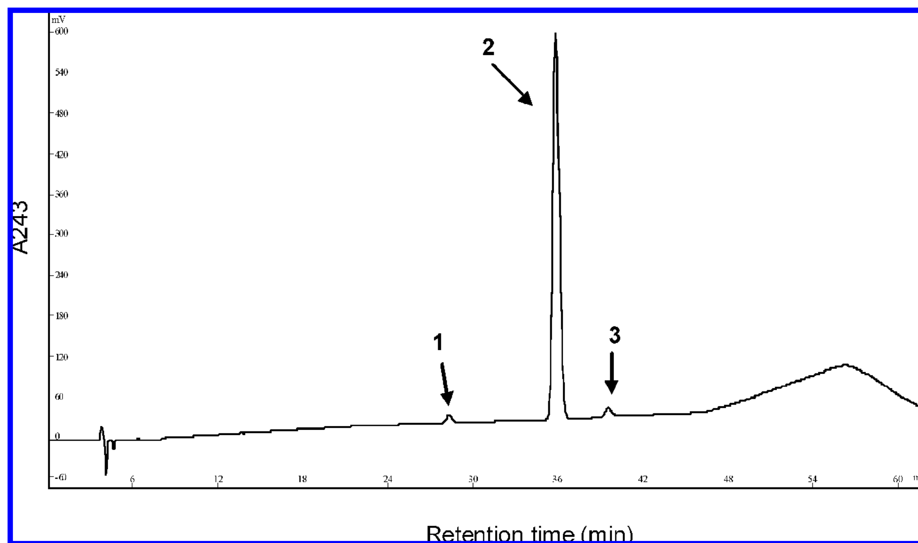

Figure 1. HPLC chromatograms of AB(BS)-pE-F3. Conditions: column, reversed-phase Cosmosil 5C18-AR-II (250 mm × 10 mm i.d., 5 μm); mobile phase, methanol/water/acetic acid (89:11:0.5, v/v/v); flow rate, 2.0 mL/min; and UV detection at 243 nm. The number of each major peak represents compounds **1**, **2**, and **3**, respectively.

concentration in both cell lines significantly ($P < 0.05$). IC_{50} values against Hep 3B cells for AB(S)-pE and AB(BS)-pE were >200 and 161.1 μg/mL, respectively. Besides, IC_{50} values against Hep G2 cells for AB(S)-pE and AB(BS)-pE were 99.9 and 24.0 μg/mL, respectively. It suggested that AB(BS)-pE exhibited better cytotoxic effects than AB(S)-pE on both hepatoma cells.

Antihepatoma Activities of Fractionation from AB(BS)-pE. To search the components with antihepatoma activity, AB(BS)-pE was fractionated by chromatography on a silica gel column and separated into 149 collections. The collections were combined to give 21 fractions on the basis of the weight distribution of the collections after silica gel column chromatography and the results of TLC, including R_f values, colors, and shapes of blots (data not shown). The yields of the fractions from AB(BS)-pE after silica gel column chromatography are shown in **Table 2**. The total yield of the fractions from 39.75 g of AB(BS)-pE was 97.3%. Among all fractions, yields of AB(BS)-pE-F17 (8.81 g), -F14 (5.33 g), and -F18 (4.13 g) were higher and followed by AB-pE-F15 (3.20 g), -F3 (2.91 g), -F19 (2.73 g), and -F12 (2.53 g). The results suggested that a large proportion of AB(BS)-pE is composed of higher polar constituents, which were eluted with higher polar-eluting solvents containing methanol.

The effects of the fractions of AB(BS)-pE on the growth of Hep 3B and Hep G2 cells for 48 h of treatment are also shown in **Table 2**. When Hep 3B and Hep G2 cells were treated with various concentrations (10−200 μg/mL) of AB(BS)-pE for 48 h, the IC_{50} values were 161.1 and 24.0 μg/mL, respectively. Among 21 fractions from AB(BS)-pE, AB(BS)-pE-F3 gave the lowest IC_{50} (3.6 and 1.9 μg/mL) against Hep 3B and Hep G2 cells. Furthermore, an activity unit is defined as the amount of sample causing 50% inhibition in a cell viability assay, and the percentage in total activity is defined as the percentage of the activity unit of each fraction in total activity unit of fractions of AB(BS)-pE. It shows that the percentages in total activity of AB(BS)-pE-F3 against Hep 3B and Hep G2 cells were 84.0 and 85.3%, respectively. Additionally, AB(BS)-pE-F6, -F7, -F8, -F9, and -F10 also effectively decreased the cell viability in Hep 3B and Hep G2 cells (IC_{50} values from 20.6 to 40.9 μg/mL), but the yields of these fractions were low (<2%) and their percentages in total activity were lower than 3% in both cell lines. Other fractions displayed weaker antihepatoma activities because their IC_{50} values are higher than 50 μg/mL. These results suggested that AB(BS)-pE-F3 might be the major contributor in AB(BS)-pE to give the antihepatoma activity against Hep 3B and Hep G2 cells. Moreover, the components with antihepatoma activity in AB(BS)-pE-F3 were purified in this study.

Antihepatoma Activities of Major Compounds from AB(BS)-pE-F3. The fraction, AB(BS)-pE-F3, with the best antihepatoma activity was further separated and purified by RP-HPLC. As shown in **Figure 1**, AB(BS)-pE-F3 could be resolved into three major peaks (compounds **1**−**3**) by isocratic elution with methanol/water/acetic acid (89:11:0.5, v/v/v). Three isolated compounds (compounds **1**−**3**) were evaluated for their antihepatoma activity against Hep 3B or Hep G2 cells by MTT assay, and the results are shown in **Table 3**. As compared to cisplatin (IC_{50} of 17.2 μg/mL), an anticancer drug (*28*), as a positive control, AB(BS)-pE-F3 and compounds **1**−**3** gave 79, 17, 84, and 74% difference in IC_{50} values against Hep 3B cells, respectively. In terms of Hep G2 cells, AB(BS)-pE-F3 and compounds **1**−**3** exhibited 84, 40, 88, and 83% difference in IC_{50} values as compared to cisplatin (IC_{50} of 12.0 μg/mL), respectively. Because their IC_{50} values of compounds **2** and **3** against both Hep 3B and Hep G2 cells were lower than that of compound **1** and similar to that of AB(BS)-pE-F3, it indicates that these two compounds are the major active compounds with antihepatoma activity in AB(BS)-pE and AB(BS)-pE-F3. Therefore, the structures of compounds **2** and **3** were subsequently elucidated.

Identification of Compounds 2 and 3. Figure 2 shows the structures of compounds **2** and **3** isolated from AB(BS)-pE-F3. Compounds **2** and **3** were identified as follows.

Compound **2**, blazeispirol A, $C_{25}H_{34}O_4$, (20*S*,22*S*,23*R*,24*S*)-14β,22:22,25-diepoxy-5-methoxy-des-*A*-ergosta-5,7,9,11-tetraen-23-ol. Colorless powder. UV λ_{max} (methanol) nm: 231, 268, 309. IR ν_{max} (KBr) cm^{-1}: 3511, 2977, 2926, 1575, 1470, 1256, 1093, 973. EI-MS *m/z* (rel int %): 398 $[M]^+$ (75), 380 $[M - H_2O]^+$ (99), 299 (30), 225 (61), 216 (100), 199 (35), 97 (13). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (3H, *s*, H-18), 2.19 (3H, *s*, H-19), 1.02 (3H, *d*, $J = 7$, H-28), 1.14 (3H, *d*, $J = 7$, H-21), 1.15 (3H, *s*, H-26), 1.43 (3H, *s*, H-27), 1.47 (1H, *m*, H-16), 1.81 (1H, *ddd*, $J = 13.5, 12.5, 3.5$, H-15), 1.93 (1H, *dd*, $J = 6,$

Table 3. Effects of Cisplatin, AB(BS)-pE-F3, and Compounds **1**−**3** on the Growth of Hep 3B and Hep G2 Cells

	cell viability (% of control)[a–c]									
	Hep 3B					Hep G2				
			compound					compound		
concentration (μg/mL)	cisplatin	AB(BS)-pE-F3	**1**	**2**	**3**	cisplatin	AB(BS)-pE-F3	**1**	**2**	**3**
0.5	84.3 ± 2.6 a	77.5 ± 3.9 a	83.9 ± 4.7 a	83.5 ± 2.3 a	84.1 ± 4.9 a	86.0 ± 6.8 a	59.9 ± 9.3 a	89.8 ± 13.8 a	96.2 ± 9.3 a	86.7 ± 8.2 a
5	65.9 ± 4.0 b	7.5 ± 3.4 b	86.0 ± 4.5 a	2.4 ± 0.5 b	30.4 ± 9.6 b	62.8 ± 9.9 b	3.4 ± 1.4 b	65.0 ± 7.1 b	2.7 ± 1.8 b	1.8 ± 0.3 b
20	47.3 ± 1.4 c	1.8 ± 1.2 c	2.3 ± 0.7 b	2.0 ± 0.5 b	2.8 ± 0.9 c	32.6 ± 6.2 c	2.3 ± 1.7 b	0.7 ± 0.7 c	2.3 ± 0.5 b	2.0 ± 0.4 b
IC_{50} (μg/mL)[d]	17.2	3.6	14.2	2.8	4.5	12.0	1.9	7.2	1.4	2.0

[a] Cells (5 × 10^3/well) were seeded in complete DMEM on a 96 well plate for 24 h. Subsequently, cells were incubated in serum-free DMEM and treated with 0.5, 5, or 20 μg/mL of each sample for another 48 h. Cell viability was determined by MTT assay. [b] Cisplatin is a positive control. [c] Each data represents mean ± SD ($n = 3$). Data with different letters in the column were significantly different with each other ($P < 0.05$). [d] IC_{50} was the concentration of each sample required to inhibit cell growth by 50%.

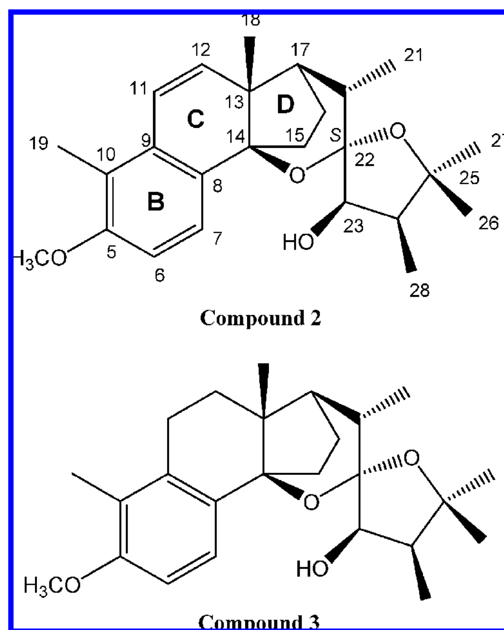

Figure 2. Chemical structures of blazeispirols A (**2**) and C (**3**).

3.5, H-17), 2.05 (1H, *ddd*, J = 13, 9.5, 3.5, H-16), 2.52 (1H, *ddd*, J = 13.5, 9.5, 5.5, H-15), 2.53 (1H, *qdd*, J = 7, 3.5, 1, H-20), 2.63 (1H, *qd*, J = 7, 4.5, H-24), 3.79 (3H, *s*, OCH_3), 3.93 (1H, *dd*, J = 4.5, 4.5, H-23), 5.88 (1H, *d*, J = 10, H-12), 6.52 (1H, *d*, J = 10, H-11), 6.71 (1H, *d*, J = 8.5, H-6), 7.22 (1H, *d*, J = 8.5, H-7). ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.2 (C-28), 11.3 (C-19), 16.1 (C-18), 16.8 (C-21), 25.3 (C-16), 26.1 (C-26), 31.0 (C-27), 33.8 (C-20), 37.4 (C-15), 44.3 (C-24), 47.1 (C-13), 50.7 (C-17), 55.7 (OCH_3), 83.9 (C-14), 84.0 (C-25), 84.8 (C-23), 107.1 (C-22), 108.3 (C-6), 121.0 (C-7), 122.0 (C-11), 122.1 (C-10), 129.9 (C-9), 131.5 (C-8), 138.6 (C-12), 155.7 (C-5).

Compound **3**, blazeispirol C, $C_{25}H_{36}O_4$, (20*S*,22*S*,23*R*,24*S*)-14β,22:22,25-diepoxy-5-methoxy-des-*A*-ergosta-5,7,9-trien-23-ol. Colorless powder. UV λ_{max} (methanol) nm: 208, 277. IR ν_{max} (KBr) cm^{-1}: 3511, 2977, 2926, 1575, 1470, 1256, 1093, 973. EI-MS *m/z* (rel int %): 400 $[M]^+$ (9), 382 $[M - H_2O]^+$ (37), 301 (32), 204 (100). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.91 (3H, *s*, H-18), 1.01 (3H, *d*, J = 7, H-28), 1.11 (3H, *s*, H-26), 1.13 (3H, *d*, J = 7, H-21), 1.49 (3H, *s*, H-27), 1.54 (1H, *ddd*, J = 13.5, 7, 1.5, H-12), 1.72 (1H, *dd*, J = 6, 3, H-17), 1.74 (1H, *m*, H-15), 1.74 (1H, *m*, H-16), 1.91 (1H, *ddd*, J = 13.5,11, 8, H-12), 2.01 (1H, *dd*, J = 9, 9, H-16), 2.08 (3H, *s*, H-19), 2.52 (1H, *dd*, J = 9, 9, H-15), 2.59 (1H, *qd*, J = 7, 4, H-24), 2.63 (1H, *qd*, J = 7, 3, H-20), 2.70 (2H, *m*, H-11), 3.78 (3H, *s*, OCH_3), 3.89 (1H, *dd*, J = 5.5, 4, H-23), 6.73 (1H, *d*, J = 7.5, H-6), 7.28 (1H, *d*, J = 7.5, H-7). ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.1 (C-28), 11.6 (C-19), 15.1 (C-18), 17.1 (C-21), 21.3 (C-16), 24.2 (C-11), 26.1 (C-26), 29.6 (C-12), 31.1 (C-27), 34.2 (C-20), 39.0 (C-15), 43.0 (C-13), 44.1 (C-24), 50.5 (C-17), 55.6 (OCH_3), 83.3 (C-14), 83.9 (C-25), 84.9 (C-23), 107.5 (C-6), 107.9 (C-22), 123.0 (C-7), 123.7 (C-10), 132.2 (C-8), 133.0 (C-9), 155.1 (C-5).

Table 4. Contents of Blazeispirols A (**2**) and C (**3**) in AB(S)-pE, AB(BS)-pE, AB(BS)-mE, AB(BS)-b, and AB(BS)-pE-F3 and -F4

	content (mg/g)[c]	
sample[a]	blazeispirol A (**2**)	blazeispirol C (**3**)
AB(S)-pE	15.9 ± 1.7 f	3.9 ± 0.6 f
AB(BS)-pE	49.9 ± 8.9 c	14.2 ± 2.4 e
AB(S)-mE	41.2 ± 0.3 d	20.7 ± 0.4 d
AB(BS)-mE	97.2 ± 1.4 b	29.8 ± 1.0 c
AB(S)-b	ND[b]	ND
AB(BS)-b	ND	ND
AB(BS)-pE-F3	457.9 ± 3.1 a	96.7 ± 5.5 b
AB(BS)-pE-F4	26.4 ± 0.1 e	157.8 ± 2.9 a

[a] AB(S) and AB(BS) represent the fermentation products of AB cultivated in the medium containing soybean (S) or balck soybean (B), respectively; p represents whole fermentation product containing mycelia and broth; m and b represent mycelia and broth from fermentation product, respectively; and E represents ethanolic extract. [b] ND means "not detectable". [c] Each data presents mean ± SD ($n = 3$). Data with different letters in the column were significantly different with each other ($P < 0.05$).

They belong to des-*A*-ergostane type compounds and have been previously established (*27*).

Quantification of Blazeispirols A (2) and C (3). As shown in **Table 4**, blazeispirol A (**2**) and C (**3**) in AB(S)-pE, AB(BS)-pE, AB(S)-mE (the ethanolic extract of mycelia), AB(BS)-mE, AB(S)-b (broth only, not including mycelia), AB(BS)-b, and the fractions of AB(BS)-pE were detected by RP-HPLC. The contents of blazeispirols A and C in AB(S)-pE were 15.9 ± 1.7 and 3.9 ± 0.6 mg/g, respectively, and significantly less than those in AB(BS)-pE (49.9 ± 8.9 and 14.2 ± 2.4 mg/g, respectively) ($P < 0.05$). As compared to AB(S)-mE, AB(BS)-mE contained significantly higher amounts of blazeispirol A (97.2 ± 1.4 mg/g) and blazeispirol C (29.8 ± 1.0 mg/g) ($P < 0.05$), but these two compounds were not detected in AB(S)-b or AB(BS)-b. In terms of the fractions of AB(BS)-pE after silica gel chromatography, the contents of blazeispirols A and C that existed in AB(BS)-pE-F3 and -F4 were higher than other fractions. AB(BS)-pE-F3 contained a significantly higher amount of blazeispirol A (457.9 ± 3.1 mg/g) than that in AB(BS)-pE-F4 (26.4 ± 0.1 mg/g) ($P < 0.05$). On the contrary, the content of blazeispirol C in AB(BS)-pE-F4 (157.8 ± 2.9 mg/g) was significantly higher than that in AB(BS)-pE-F3 (96.7 ± 5.5 mg/g) ($P < 0.05$).

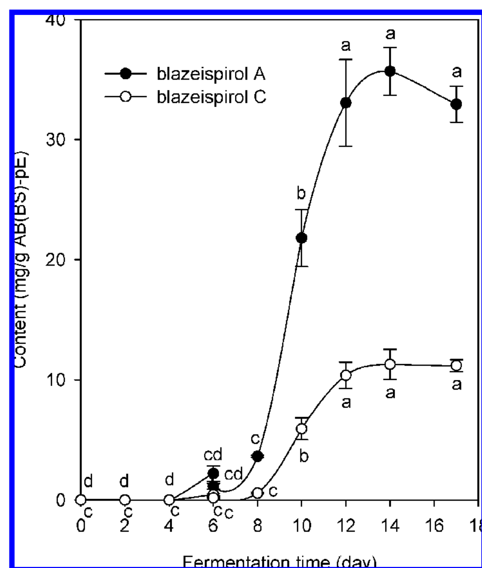

Figure 3. Contents of blazeispirols A and C in ethanolic extracts of the fermentation product of AB [AB(BS)-pE] with different fermentation times. Numbers within each sample ($n = 3$) not sharing the same letter are significantly different from one another ($P < 0.05$).

Figure 3 shows the contents of blazeispirols A and C in ethanolic extracts of fermentation product of AB [AB(BS)] with continuous fermentation times. The contents of blazeispirols A and C were not produced significantly before cultivation in the medium containing black soybean on day 6. These two compounds increased significantly from day 8 to day 12 ($P < 0.05$). On day 12, the contents of blazeispirols A and C reached a maximum of 33.1 ± 3.6 and 10.4 ± 1.1 mg/g in AB(BS)-pE, respectively. The contents of blazeispriols A and C were not detected in black soybean (data not shown); therefore, the result shows that black beans can promote the biosynthesis of blazeispirols A and C during fermentation of AB.

Relationship between Blazeispirols A (2) and C (3) Contents and Antihepatoma Activity. Hepatoma Hep 3B and Hep G2 cells were treated with AB(BS)-pE with different fermentation days, and the relations between cell viabilities and the contents of blazeispirols A and C in AB(BS)-pE are shown in **Figure 4**. The correlation coefficients were observed between the influence of blazeispirols A ($r = -0.84$, $P < 0.01$) and C ($r = -0.87$, $P < 0.01$) on the cell viability of Hep 3B cells in **Figure 4A**,**B**. In terms of correlation between cell viability of Hep G2 cells and blazeispirols A or C, the correlation coefficients (r) were −0.93 ($P < 0.01$) and −0.92 ($P < 0.01$) in **Figure 4C**,**D**, respectively. These results suggest that the contents of blazeispirols A and C in the ethanolic extract of AB(BS) are highly negatively correlated to the proliferation of Hep 3B and Hep G2 cells.

DISCUSSION

The aims of this study were to compare antihepatoma activity in the fermentation product of AB cultured in the medium containing soybean or black soybean and to find the active components. We found that the fermentation product of AB cultured without soybean or black soybean did not exhibit antihepatoma activity against Hep 3B and Hep G2 cells (data not shown). However, addition of black bean during formentaion of AB improved the antihepatoma activity of fermented product (**Table 1**). It is suggested that black soybean might change the constituents in the fermentation product.

In a previous study, Machado et al. reported that 200 μg/mL of fraction (dichloromethane/methanol, 90:10, v/v) from hexane extract of AB fruiting body could cause Chinese hamster ovary (CHO-K1) cells death (*8*). Takaku et al. proved that tumor growth in sarcoma 180-bearing mice was retarded by the oral administration of the extract from AB with a chloroform/methanol (1:1, v/v) mixture, because ergosterol in the fraction exhibited antitumor activity via inhibition of angiogenesis (*3*). In the present study, to isolate the compounds with antihepatoma activity from the fermentation product of AB, the ethanolic extract from the fermentation product of AB (AB(BS)-pE) was fractionated by silica gel column chromatography. **Table 2** shows that among 21 fractions obtained, fraction 3 from AB(BS)-pE [AB(BS)-pE-F3], which was eluted with *n*-hexane/ethyl acetate (97:3 and 19:1, v/v), exhibited the strongest cytotoxicity against Hep 3B and Hep G2 cells (IC_{50} of 3.6 and 1.9 μg/mL, respectively). It also suggested that AB(BS)-pE-F3 was the major fraction responsible for the antihepatoma activity in AB(BS)-pE based on the values of percentage in total activity. As shown by HPLC analysis (**Figure 1**), there are three major compounds in the fraction of AB(BS)-pE-F3. Among these three major compounds, compounds **2** and **3** showed more potent cytotoxic efficacy against Hep 3B and Hep G2 cells (IC_{50} from 1.4 to 4.5 μg/mL) than that of compound **1** as shown in **Table 3**. The spectrometric data of compounds **2** and **3** were consistent with the published data of blazeispirols A and C (**Figure 2**), respectively (*27*). Total amounts of blazeispirols A and C were 55.5 and 18.4% in AB(BS)-pE-F3 and -F4, respectively (**Table 4**). It suggested that blazeispirols A and C were the major components to contribute antihepatoma activity in AB(BS)-pE-F3 but not in AB(BS)-pE-F4, even though it contained the highest amount of blazeispirol C.

"Blazeispirane" derivatives such as blazeispirol A−G and I are a group of naturally occurring steroids built on a des-*A*-ergostane type skeleton in which C-14, C-22, and C-25 are appropriately oxidized to form a 14,22:22,25-diepoxy structure (*27*). Blazeispirol A (**2**) ($C_{25}H_{34}O_4$) is a ring *A*-lost steroid with an aromatic ring system and with a tetrahydropyran-2-spiro-20-tetrahydrofuran moiety as a side chain. It was reported that blazeispirol A is the first steroid discovered with des-*A*-ergostane skeleton in a living organism (*29−31*). Blazeispirol C (**3**) was very similar to that of blazeispirol A (**2**) except for double bond at C-11/C-12 in blazeispirol A (**2**) (*27*). By the way, compound **1** also exhibited good antihepatoma activity according to the low IC_{50} values against Hep 3B and Hep G2 cells (14.2 and 7.2 μg/mL, respectively), but the quantity of compound **1** was not sufficiently enough for complete structural determination.

Currently, it has been published that agariblazeispirols A−C existed in the mycelia of AB. Unlike blazeispirane derivatives, the occurrence of the 18-methyl group at C-14 is these novel des-*A*-ergostane type compounds is unprecedented. The experimental results supported that agariblazeispirols A−C should be biosynthesized from blazeispirol A (*32, 33*). It has also been shown that 2.4 and 4.7 μg/mL of agariblazeispirols A and B, respectively, inhibited the growth of P388/VCR mouse leukemia cells with multidrug resistance cells in the presence of 13 ng/mL of vincristine, which alone did not possess the growth inhibitory activity on P388/VCR cells (*32, 34*). However, the biological activities of blazeispirol A or C have not been investigated so far. This study is the first of its kind to demonstrate that these two compounds exhibited antihepatoma activity.

Blazeispirols A and C were not detected in both soybean and black soybean (data not shown), because they are the secondary

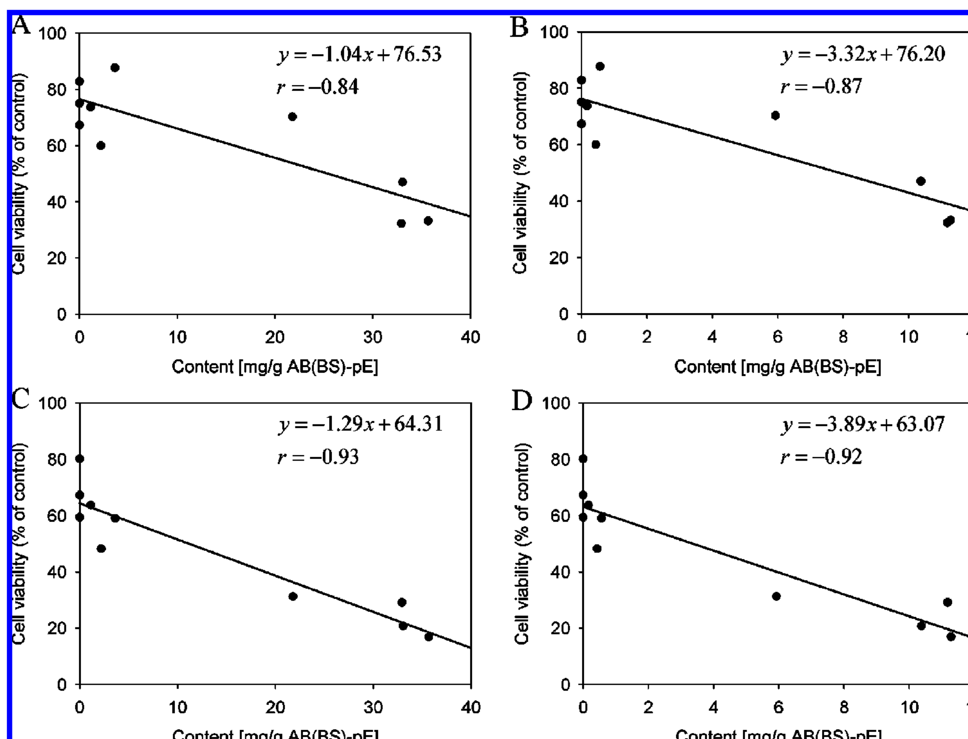

Figure 4. Correlation between the concentrations of blazeispirol A or C in ethanolic extracts of the fermentation products of AB [AB(BS)-pE] with different fermentation times and the cell viabilities of Hep 3B or Hep G2 cells. Hep 3B or Hep G2 cells were cultured with serum-free DMEM containing 100 μg/mL of ethanolic extracts of fermentation products (pE) of AB for 48 h ($n = 3$). Cell viability was determined by MTT assay. The correlations were between Hep 3B cells and blazeisperol A (**A**) or C (**B**) and Hep G2 cells and blazeisperol A (**C**) or C (**D**). The correlation coefficient (r) was determined between cell viability and treated concentrations of blazeispirols A or C.

metabolites in the fermentation production of AB. The formations of blazeispirols A and C in the fermentation products of AB might be due to some ingredients in soybean or black soybean. Such as fatty acids, squalene and ergosterol are precursors or intermediates of blazeispirols A or C (*30*), and soybean contains fatty acids, squalene, and phytosterol (*35*), which could be the materials to biosynthesize blazeispirols A or C. In addition, other components such as trace elements, phenolic compounds, isoflavones, or saponins existing in soybean or black soybean (*16, 17, 20*) could affect enzymatic activities in the biosynthesis pathway of blazeispirols A or C. According to the results in **Table 4**, black soybean can promote the formation of blazeispirols A and C in the fermentation products of AB more effectively than soybean. Black soybean is another soybean cultivar with different constituents, especially anthocyanins and phenolic compounds (*17, 20*).

The concentration of blazeispirols A or C in AB(BS) fermentation product increased constantly after black soybean was added and reached its maximum on day 12 (**Figure 3**). **Figure 4** shows the correlation coefficients of cell viabilities in Hep 3B or Hep G2 cells treated with 100 μg/mL of AB(BS)-pE harvested at different fermentation days in relation to the contents of blazeispirols A or C are highly negative correlations ($r = -0.84$ to -0.93, $P < 0.01$). It indicated that the increased contents of blazeispirol A or C with increased fermentation days affected antihepatoma activities of fermentation products of AB. On the other hand, Hep G2 cells with higher negative correlation coefficients were more sensitive to blazeispirols A or C than Hep 3B cells. It suggested different effects of blazespirols A or C on these two cells with different characteristics. Whether with tumor suppressor p53 gene or not in liver cancer cells might be one of the factors affecting the sensitivity of liver cancer cells to blazespirols A or C. For some chemotherapeutic drug such as cisplatin or bleomycin, Hep G2 cells with wild-type p53 gene were susceptible to apoptosis, but Hep 3B cells without p53 gene were more resistant to apoptosis (*28, 36*).

In conclusion, these findings stated above imply that black soybean can promote antihepatoma activity due to improvement of the formation of blazeispirols A and C. Therefore, it is suggested that the contents of blazeispirols A and C could be used as biomarkers to produce the fermentation product of AB with antihepatom activity.

ABBREVIATIONS USED

AB, *Agaricus blazei*; S, soybean; BS, black soybean; b and m, broth and mycelia of fermentation product, respectively; pE, ethanolic extract of fermentation product containing broth and mycelia; mE, ethanolic extract of mycelia from fermentation product; AB(S)-pE and AB(BS)-pE, ethanolic extract of fermentation product containing broth and mycelia of *A. blazei* cultured in the medium containing soybean and black soybean, respectively; AB(BS)-pE-F1−F21, fractions of AB(BS)-pE after silica gel column chromatography; IC_{50}, concentration of sample required to inhibit cell growth by 50%; AB(BS)-b, broth of fermentation product of *A. blazei*; AB(BS)-mE, ethanolic extract of mycelia of fermentation product of *A. blazei*.

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