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Effect of Black Soybean Extract on the Suppression of the Proliferation of Human AGS Gastric Cancer Cells via the Induction of Apoptosis

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ABSTRACT: Black soybean is known to have a health-promoting effect because of its high content of polyphenolic compounds and antioxidant activities. The objective of the present study was to investigate the chemopreventive effects of black soybean extract against human AGS gastric cancer cells and its possible mechanism in inducing apoptosis. Black soybean extract was obtained by extracting black soybean with acidified aqueous acetone, and its phytochemical constituents, as determined by HPLC-DAD methods, were demonstrated to contain various phenolics. The black soybean extract inhibited AGS cell growth in a dose-dependent manner with an IC_{50} of 3.69 mg/mL as measured by the MTT assay. This growth inhibition effect was further confirmed by the CFDA-SE assay. Flow cytometry analysis showed that black soybean extract dose-dependently induced apoptosis of AGS cells. Moreover, the involvement of black soybean extract in inducing apoptosis was confirmed by the expression of Bax, Bcl-2, caspase-3, and PARP. The results of the present study indicated that black soybean extract could be used as an apoptosis inducer in AGS cells and a natural chemopreventive agent in the treatment of human gastric cancer

KEYWORDS: black soybean extract, AGS cells, phenolics, antiproliferative, apoptosis, gastric cancer

INTRODUCTION

Even though the incidence and mortality have steadily declined worldwide in recent decades, gastric cancer is still a global health problem and remains the fourth most common cancer and the second leading cause of cancer mortality behind lung cancer.[1,2] Dietary factors play an important role in the incidence of gastric cancer. Studies show that the incidence of gastric cancer increases with a high intake of smoked, salted, nitrated foods and carbohydrates, but a low intake of vegetables, fruits, and milks.[2] Accumulating evidence from several fields, including epidemiology, human medicine, and nutrition, has outlined the inverse association between the risk of gastric cancer and the dietary intake of fruits and vegetables.[3] It is believed that phytochemicals such as polyphenolic compounds, ascorbic acid, carotenoid, folate, and vitamins in fruits and vegetables are primary contributors to the lowering of gastric cancers.[4,5]

Black soybean [*Glycine max* (L.) Merr.], which is characterized by a black seed coat and yellow or green cotyledon, has long been consumed worldwide, especially in Asian countries. It not only is a nutrition-rich foodstuff, which affords macronutrients such as protein, fatty acids, fibers, and carbohydrates, but also contains isoflavones, anthocyanins, soysaponins, and vitamin E, which have been documented to exhibit various biological activities.[6] In China, black soybean is also used as a medicinal food for the treatment of kidney disease, improving blood circulation and water passage and counteracting toxicity, and it has anti-inflammation and antiaging effects. The beneficial effects of black soybean have been recorded since the 16th century in the *Ben-Cao-Gang-Mu*, an ancient Chinese Botanical Encyclopedia.[7]

Recently, many studies have demonstrated the health benefits of black soybean. In vitro antioxidant studies showed that black soybean possessed strong free radical scavenging activities against DPPH, peroxyl, and hydroxyl radicals[8] and inhibited low-density lipoprotein (LDL) oxidation.[9] Various components of black soybean exhibited anticancer activities in in vitro and in vivo studies. Anthocyanins from black soybean suppressed the proliferation of human HT-29 colon cancer cells and human leukemia Molt 4B cells.[10,11] Tryptophol, an active compound isolated from vinegar, which was produced from boiled extract (a waste product from the natto-making process) of black soybean, induced apoptosis of human U937 lymphoblast cells by involving caspase-8 and -3 activiation and PARP cleavage.[12] A high molecular weight of polysaccharide of black soybean provided antitumor effect in U937 cells, promoted myelopoiesis, and reconstituted bone marrow cells.[13] A trypsin−chymotrypsin inhibitor from black soybean suppressed proliferation of MCF-7 breast cancer cells and HepG2 hepatoma cells.[14] In addition, a case-control study reported that a high consumption of black soybean reduced the risk of breast cancer in Korean women.[15] Other bioactivities of black soybean including antidiabetes, antiobesity, hypolipidemic, anti-inflammation, antimutagen, and antiviral activities have been extensively reported.[16,17] All of the above studies suggested black soybean exerted a broad range of bioactives and thus could be used as a functional food for disease prevention and health promotion.

Although some studies have been conducted on the antiproliferative effects of black soybean against several cancer cell lines, to our best knowledge, studies on the antiproliferative effect of polyphenolics from black soybean are rare, except the antiproliferative effect of anthocyanins conducted by Kim et al.[10] and Katsuzaki et al.[11] Moreover, no information is available on the effects of black soybean in human gastric cancer cells to date. Our previous study showed that extracts from raw and processed soy milk made from yellow soybeans dose-dependently inhibited the proliferation of several cell lines including human gastric AGS

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cells, although the antiproliferative potential decreased after raw soy milk was processed.[18] The antigastric cancer effect of the extract from the whole black soybean has not been studied. Therefore, the objectives of this study were to study the proliferation inhibitory activity of the phytochemical extract of the whole black soybeans and to investigate the possible mechanisms of antiproliferation by cell apoptosis, including the expression of Bax and Bcl-2 and the activation of the caspase cascade.

MATERIALS AND METHODS

Materials and Chemicals. Black soybean (*G. max* L.) with green cotyledons, cropped in 2005, was purchased from a local grocery in Taiwan. All of the solvents used for quantification of phenolic compounds were of HPLC-grade and purchased from EMD Chemicals Inc. (Gibbstown, NJ). Acetone for the extraction was a product of VWR (West Chester, PA). Sixteen phenolic acid standards (gallic, protocatechuic, 2,3,4-trihydroxybenzoic, *p*-hydroxybenzoic, gentistic, vanillic, caffeic, chlorogenic, syringic, *p*-coumaric, *m*-coumaric, *o*-coumaric, ferulic, salicylic, sinapic, and *trans*-cinamic acid), three aldehydes (vanillin, syringealdehyde, and protocatechualdehyde), (+)-catechin, (−)-epicatechin, epigallocatechin, epicatechin gallate, myricetin, luteolin, quercetin, kaempferol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl (MTT), dimethyl sulfoxide (DMSO), and Hoechst 33342 were obtained from Sigma-Aldrich Inc. (St. Louis, MO). F-12 nutrient mixture medium, fetal bovine serum (FBS), penicillin−streptomycin mixed antibiotics, phosphate-buffered solution (PBS), and 0.25% trypsin−EDTA were from Hyclone Laboratories Inc. (Logan, UT). The primary antibodies against Bax, Bcl-2, caspase-3, PARP, and β-actin and the horseradish peroxidase (HPR)-conjugated goat anti-rabbit secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Other chemicals were of the highest quality commercially available.

Preparation of Black Soybean Extract. Black soybeans were ground with an IKA all basic mill (IKA Works Inc., Wilmington, NC) and passed through a 60-mesh seive. During the grinding, dry ice was mixed with black soybeans to prevent the thermal decomposition of active compounds by heat produced by high-speed milling. Two hundred grams of powders was extracted with 1 L of acetone/water/acetic acid (70:29.5:0.5, v/v/v) at room temperature for 12 h. The extract was filtered through Whatman no. 1 filter paper in a Büchner funnel. The residues were re-extracted two times, and all of the extracts were combined and concentrated to a small volume at 40 °C using a rotary evaporator (Labconco Co., Kansas City, MO) under vacuum. Then the concentrate was lyophilized and stored at −20 °C until further use.

Determination of Total Phenolic Content (TPC). Total phenolics in the extracts were determined with the Folin−Ciocalteu assay[19] with minor modifications using gallic acid as a standard phenolic compound. Sample preparation and detail of the procedure have been reported in our previous publication.[18] The TPC in the black soybean extract was expressed as milligram gallic acid equivalents (mg GAE/g).

Determination of Crude Protein Content. Crude protein content was analyzed according to the Kjeldahl method.[20] A conversion factor of 6.25 was used.

Determination of Total Sugar Content. Total polysaccharide content was determined by using the phenol−sulfuric acid assay with slight modifications.[21] One milliliter of sample solution was vortex-mixed with 0.5 mL of 5% phenol in water. Then, 2.5 mL of concentrated sulfuric acid was rapidly added and mixed with an Analog Vortex Mixer (VWR International, LLC). After 30 min of standing at room temperature, the absorbance of the sample solution was measured at 490 nm against the blank, which was prepared by substituting distilled water for the sample solution. Different concentrations of glucose (5, 10, 20, 40, 80, 160, and 320 μg/mL) were used for the standards. Results were expressed as grams of glucose per 100 g of black soybean extract.

Determination of Lipid Content. Lipid content was measured according to the rapid acid hydrolysis method, and the detailed procedure was conducted according to our previous study.[22]

Quantification of Phytochemicals by HPLC Analysis. *Phenolic Acids, Isoflavones, and Anthocyanins.* The quantitative analysis of phenolic acids, isoflavones, and anthocyanins was performed by HPLC according to our recent publication.[8] The phenolic acid contents were expressed as micrograms of phenolic acid per gram of black soybean extract (μg/g). 2,4,4-Trihydroxydeoxybenzoin (THB) was used as internal standard to quantify isoflavones, and the unimolar mixture of 3-*O*-β-glucoside of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Polyphenols Laboratories, Sandnes, Norway) was used as external standard to quantify the content of anthocyanins in black soybean extract.

Flavanols. The identification and quantification of flavanols were performed on an Agilent 1200 series HPLC system equipped with a G13798 degasser, a G1312A binary pump, a G1329A autosampler, and a G1315D diode array detector (Agilent Technologies, Santa Clara, CA). HPLC separation was achieved using an YMC-Pack ODS-AM-303 C_{18} (250 × 4.6 mm, 5 μm) at 35 °C. Elution was performed using mobile phase A (0.1% acetic acid in water) and mobile phase B (0.1% acetic acid in acetonitrile), and 20 μL samples (5 mg/mL) were eluted at a flow rate of 1 mL/min. The UV−vis spectra were scanned from 220 to 600 nm on a DAD with a detection wavelength of 270 nm. The solvent gradient in volumetric ratios was as follows: 0−36 min, 10−29% B; 36−44 min, 29−35% B; 44−46 min, 35−50% B. Then the solvent gradient was held at 50% B for an additional 10 min and decreased to 10% B at 58 min. The column was re-equilibrated by the initial conditions for 5 min before the next run.

Cell Line and Cell Culture. AGS cells were obtained from the American Type Culture Collection (ATCC; CRL-1739). AGS cells were cultured in F-12 nutrient mixture medium supplemented with 10% heated-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, and the medium was changed every other day. When the AGS cells were about 80% confluent, cells were washed with PBS, detached with 0.25% trypsin−EDTA, resuspended, and subcultured onto 96- or 6-well plates at an appropriate density according to each experimental scale. All experiments were carried out 24 h after cells were plated in triplicate.

Cell Viability Assay. Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Briefly, AGS cells were plated at density of 5×10^3 cells/well in 96-well plates. After 24 h of incubation, black soybean extract at different concentrations (1−5 mg/mL) were added to the wells and incubated for 48 h, and then the culture medium was replaced by 200 μL of a solution of MTT (5 mg/mL stock solution in PBS, diluted with culture medium to the final concentration 0.5 mg/mL). After 4 h of incubation at 37 °C, this solution was removed, and the produced formazan was solubilized in 150 μL of DMSO. Absorbance was measured at 540 nm using a Multiskan Spectrum (Thermo Electron Corp., Asheville, NC). Cell viability was expressed as a percentage of the control culture value, which considered as 100% viable.

Cell Proliferation Using the CFDA-SE Assay. Cell proliferation was further determined by dilution of 5-(and -6)carboxyfluorescein diacetate, succinimidyl ester probe (CFDA-SE, Invitrogen, Camarillo, CA) according to the experimental protocol. Briefly, isolated AGS cells were resuspended in prewarmed PBS/0.1% BSA at a concentration of 1×10^6 cells/mL and stained with CFDA-SE solution (final concentration of 10 μM) for 10 min at 37 °C. Cells were then washed with PBS three times to remove unbound CFDA-SE and resuspended in complete medium, and then 3×10^5 cells were seeded in 6-well plates. After

incubation for 24 h, different concentrations of black soybean extract were added and incubated for 48 h. At the end of incubation, cells were collected, washed with PBS, and resuspended in PBS, and then 1×10^4 cells were analyzed with an Accuri C6 Cytometer (Accuri Cytometers, Inc., Ann Arbor, MI).

Morphological Evaluation. AGS cells were incubated in a 6-well plate for 24 h at a density of 3×10^5 cells/well. After treatment with various doses of black soybean extract for 48 h, the medium was vacuum aspirated, and the cells were washed with PBS. The morphology of cells was observed under phase contrast microscopy (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

Nuclei staining with Hoechst 33342 was performed to investigate morphological changes. Approximately 3×10^5 cells/well AGS cells were plated in 6-well plate. After incubation for 24 h, cells were treated with various doses of black soybean extract for 48 h. The cells were subsequently washed with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature and then washed again with PBS. The fixed cells were incubated with Hoechst (100 μg/mL) for 20 min at room temperature in the dark. Stained solution was washed out. The cells were visualized with a fluorescence microscope (Leica Microsystems Wetzlar GmbH) for determination of nuclear morphological changes.

Apoptosis Detection by Flow Cytometry. An Apoptosis Detection kit (Assign Designs, Ann Arbor, MI) was used to detect apoptosis by flow cytometry. The procedure was performed according to the instructions of the manufacturer. Briefly, after treatment with different concentrations (1, 3, and 5 mg/mL) of black soybean extract for 48 h, the cells were harvested and collected by centrifugation. The cells were washed with ice-cold PBS and were suspended in ice-cold 1× binding buffer at a concentration of 1×10^6 cells/mL. Ninety-six microliters of cell suspension was mixed with 1 μL of annexin V−FITC and 2.5 μL of propidium iodide (PI) solutions. The mixtures were incubated for 10 min on ice in the dark and diluted with 1× binding buffer to 250 μL. Then 1×10^4 cells were analyzed by flow cytometry.

Western Blot Analysis. AGS cells (5×10^5) were plated in a T-25 flask. After incubating for 24 h, the cells were treated with different concentrations (1, 3, and 5 mg/mL) of black soybean extract for 48 h. Then the cells were collected and lysed with 0.1 mL of cold lysis buffer [150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 1% Triton X-100, 0.5% SDS, 0.01% Protease Inhibitor Cocktail]. The cell lysate was centrifuged at 10000*g* for 20 min at 4 °C. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as the standard. Equal amounts of cell lysate proteins (60 μg) were mixed with a one-fourth volume of electrophoresis loading buffer and boiled for 5 min, followed by electrophoresis using SDS-PAGE for 90 min at 120 V. The separated proteins were transferred to polyvinylidene difluoride membrane for 120 min at 360 mA for assessment of the levels of Bax, Bcl-2, PARP, caspase-3, and β-actin. After nonspecific sites had been blocked with 5% nonfat dry milk in PBS for 30 min, the transferred membrane was incubated at 4 °C overnight with primary antibodies at the following concentrations: rabbit anti-Bax [1:1000]; rabbit anti-Bcl-2 [1:1000]; rabbit anti-PARP [1:1000]; rabbit anti-caspase-3 [1:1000]; rabbit anti-β-actin [1:1000]. Then the membrane was extensively washed by PBST (0.1% Tween-20 in PBS buffer), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody [1:3000] for 2 h. Proteins of interest were incubated with ECL detection reagent (Pierce) and developed by exposure to X-ray films. The protein expression was quantified densitometrically using ImageJ software (version 1.43).

Statistical Analysis. Data were expressed as the mean ± SD. The data were statistically analyzed using statistical software, SAS version 9.1 (SAS institute Inc., Cary, NC). One-way analysis of variance (ANOVA) followed by Dunnett's test was conducted, and significant difference was set at $p < 0.05$.

Table 1. Composition of Black Soybean Extract

composition	content
total polyphenolics (mg GAE/g)	32.5 ± 0.9
protein (%, w/w)	5.7 ± 0.1
total sugar (g glucose/100 g)	80.4 ± 7.7
lipid (%, w/w)	5.1 ± 0.5

RESULTS

Yield, Phenolic and Other Chemical Components. The yield of black soybean extract, which was extracted with aqueous acidified acetone, was 15.3%. The major component of black soybean extract is shown in Table 1. It mainly included total phenolics (3.25%, gallic acid as standard), polysaccharide (80.4%, glucose as standard), protein (5.7%), and lipid (5.1%). The profiles of phenolic compounds in black soybean extract are shown in Table 2. Four categories of phenolics were found in black soybean extract. Total phenolic acids content was 6625.2 μg/g, including gallic acid, protocatechuic acid, caffeic acid, chlorogenic acid, *m*-coumaric acid, ferulic acid, and sinapic acid, of which the ferulic acid and sinapic acid were the main phenolic acids. All 12 forms of isoflavones including daidzein, genistein, glycitein, daidzin, genistin, glycitin, acetyldaidzin, acetylgenistin, acetylglycitin, malonyldaidzin, malonylgenistin, and malonylglycitin existed in black soybean extract. The malonyl glycoside (7736.7 μg/g) was the main form of isoflavone, which accounted for 65.8% of the total isoflavones, followed by glucoside (3350.4 μg/g) and acetyl glycoside (438.2 μg/g), whereas the aglycone (251.2 μg/g) was the lowest form. In the case of individual isoflavones, malonylgenistin (4014.4 μg/g) and malonyldaidzin (3178.5 μg/g) were the pronounced forms, followed by genistein (1548.7 μg/g) and daidzin (1302.0 μg/g). The most abundant anthocyanin in black soybean extract was cyanidin-3-*O*-glucoside (921.4 μg/g), followed by peonidin-3-*O*-glucoside (113.6 μg/g), delphinidin-3-*O*-glucoside (50.9 μg/g), petunidin-3-*O*-glucoside (40.7 μg/g), and pelargonidin-3-*O*-glucoside (38.8 μg/g). Flavanols included epigallocatechin (3003.8 μg/g), epicatechin (635.8 μg/g), and epicatechin gallate (738.5 μg/g). These data confirmed that various kinds of phenolics including phenolic acids, isoflavones, anthocyanins, and flavanols were indeed present in black soybean extract.

Inhibition of Cell Proliferation in Black Soybean Extract-Treated AGS Cells. First, the effect of black soybean extract on the growth of AGS cells was investigated using the MTT assay. As shown in Figure 1, black soybean extract inhibited the growth of AGS cells in a dose-dependent manner. When the concentration of black soybean extract was above 3 mg/mL, the cell viability was significantly decreased compared with that of control cells ($p < 0.05$), only 65.7, 38.8, and 22.5% cells survived after treatment with 3, 4, and 5 mg/mL of black soybean extract for 48 h, respectively. On the other hand, no cytotoxicity of black soybean extract in the rat normal fibroblast cells was observed under the above concentrations (data not shown). The value of IC_{50}, defined as the concentration at which the cell proliferation was inhibited by 50% of control cells, was 3.69 mg/mL.

To further confirm the proliferation inhibitory effect of black soybean extract on AGS cells, CFDA-SE was used to determine the effect of black soybean extract on cell division. The effect of black soybean extract on AGS cells division is illustrated in Figure 2. After labeling with CFDA-SE and treatment with black

Table 2. Phytochemical Content (μg/g) of Black Soybean Extract[a]

phenolic acids		isoflavones		anthocyanins		flavanols	
phytochemical	content	phytochemical	content	phytochemical	content	phytochemical	content
GA	259.5 ± 7.2	daidzein	117.1 ± 5.8	Dp-3-glc	50.9 ± 2.1	epigallocatechin	3003.8 ± 34.9
PCA	533.9 ± 8.7	genistein	71.2 ± 7.7	Cy-3-glc	921.4 ± 15.4	epicatechin	635.8 ± 10.7
CA	459.2 ± 9.5	glycitein	62.9 ± 2.1	Pt-3-glc	40.7 ± 1.3	epicatechin gallate	738.5 ± 15.6
CLA	955.9 ± 16.3	daidzin	1302.0 ± 21.0	Pg-3-glc	38.8 ± 1.7		
m-CMA	305.9 ± 9.1	genistin	1548.7 ± 14.5	Pn-3-glc	113.6 ± 4.6		
FA	1259.4 ± 30.6	glycitin	499.7 ± 4.5				
SPA	2851.4 ± 51.2	acetyldaidzin	69.5 ± 11.3				
		acetylgenistin	47.1 ± 3.7				
		acetylglycitin	321.6 ± 25.3				
		malonyldaidzin	3178.5 ± 26.5				
		malonylgenistin	4014.4 ± 208.4				
		malonylglycitin	543.8 ± 65.8				
totals	6625.2 ± 78.4		11776.5 ± 90.4		1165.4 ± 20.8		4378.1 ± 65.1

[a] GA, gallic acid; PCA, protocatechuic acid; CA, caffeic acid; CLA, chlorogenic acid; *m*-CMA, *m*-coumaric acid; FA, ferulic acid; SPA, sinapic acid; Dp-3-glc, delphinidin-3-*O*-glucoside; Cy-3-glc, cyaniding-3-*O*-glucoside; Pt-3-glc, petunidin-3-*O*-glucoside; Pg-3-glc, pelargonidin-3-*O*-glucoside; Pn-3-glc, peonidin-3-*O*-glucoside.

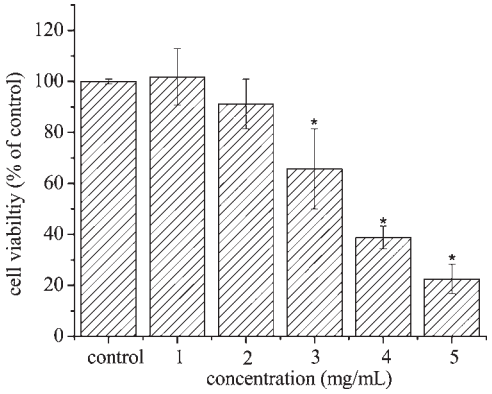

Figure 1. MTT assay of the effect of black soybean extract on AGS cells viability. AGS cells were treated with different concentrations of black soybean extract for 48 h. Cell viability was analyzed by MTT assay. The results are expressed as the mean ± SD, $n = 3$. *, $p < 0.05$ as compared with the control group.

soybean extract, the intensity of fluorescence increased with increasing concentration. The intensity of fluorescence of AGS cells treated with 3, 4, and 5 mg/mL was 1.27-, 1.35-, and 1.51-fold of control cells, respectively. These facts imply that black soybean extract dose-dependently inhibited cell proliferation because CFDA-SE was equally inherited by daughter cells in each cell division cycle.

Morphology Changes Induced by Black Soybean Extract. As illustrated in Figure 3, after treatment with 3 and 5 mg/mL of black soybean extract, the morphology of the cells showed great changes. Observed under a phase contrast microscope, the shape of the normal cells was regular, whereas cells treated with black soybean extract exhibited morphological alterations such as cell shrinkage and membrane blebbings; the cells became irregular, disorganized, and elongated. Moreover, some of them lost their ability to adhere to the plate surface, detached from the bottom, aggregated, and floated in the medium. Thus, the density of black soybean extract treated cells was less than that of the control cells. The extent of the changes in cell morphology and density depended on the concentration of black soybean extract.

To elucidate apoptotic cell death due to the exposure of AGS cells to black soybean extract, a DNA binding dye, Hoechest 33342, was also used for nuclear staining (Figure 4). Under a fluorescence microscope, nuclear staining with Hoechst 33342 showed that control cells exhibited intact and round-shape nuclei characterized by a diffused chromatin structure and, therefore, slight staining (A). In contrast, exposure to 3 and 5 mg/mL black soybean extract treatment for 48 h dramatically altered AGS cell morphology; the nucleus exhibited condensed or fragmentation chromatin (B and C), which is characteristic of apoptosis cells.

Induction of Apoptosis by Black Soybean Extract. To evaluate the effect of black soybean extract on the induction of apoptosis, the AGS cells were incubated with different concentrations of black soybean extract for 48 h, and then the cells were double stained with annexin V−FITC and PI, followed by quantitative flow cytometry analysis. The apoptotic cells induced by black soybean extract are illustrated in Figure 5. The results confirmed that black soybean extract induced apoptosis of AGS cells in a concentration-dependent manner. The control cells demonstrated normal cell viability without significant cell apoptosis. In contrast, at concentrations of 3 and 5 mg/mL, black soybean extract caused 7.9 and 23.8%, respectively, of the cells to undergo apoptosis within 48 h. However, at the lowest concentration (1 mg/mL), only 2.4% cells underwent apoptosis, which was not significantly different from the control cells.

Western Blot. The underlying mechanism of black soybean extract in the induction of apoptosis of AGS cells was delineated by Western blotting. As shown in Figure 6, examination of Bax and Bcl-2 expression during apoptosis indicated that treatments with 3 and 5 mg/mL black soybean extract dose-dependently up-regulated the expression of pro-apoptotic protein Bax (up to 1.43- and 1.64-fold as compared to the control cells, respectively; Figure 6C), whereas the expression of anti-apoptotic protein Bcl-2 was down-regulated with increasing concentration of black

soybean extract (up to 0.50- and 0.31-fold as compared with the control cells, respectively; Figure 6C). The ratios of Bax/Bcl-2 with 3 and 5 mg/mL black soybean extract treatment were also significantly elevated compared to the control cells (up to 2.85- and 5.33-fold as compared to the control cells, respectively; Figure 6B). Next, we examined whether caspase-3 was activated during the induction of apoptosis by black soybean extract. The level of caspase-3 was significantly increased in 3 and 5 mg/mL black soybean extract treated cells compared with the control cells (up to 1.39- and 1.57-fold as compared with the control cells, respectively; Figure 6C), which suggested the black soybean extract treatment activated the expression of caspase-3. Because poly(ADP-ribose) polymerase (PARP) protein is known to be a major substrate for executing caspases and a hallmark of apoptosis, Western blotting analysis of PARP expression was performed. The data showed that the intensity of the intact 116 kDa PARP was decreased in cells treated with 3 and 5 mg/mL black soybean extract (up to 0.61- and 0.52-fold as compared with the control cells, respectively; Figure 6C), and the cleavage form of PARP (83 kDa) appeared (Figure 6).

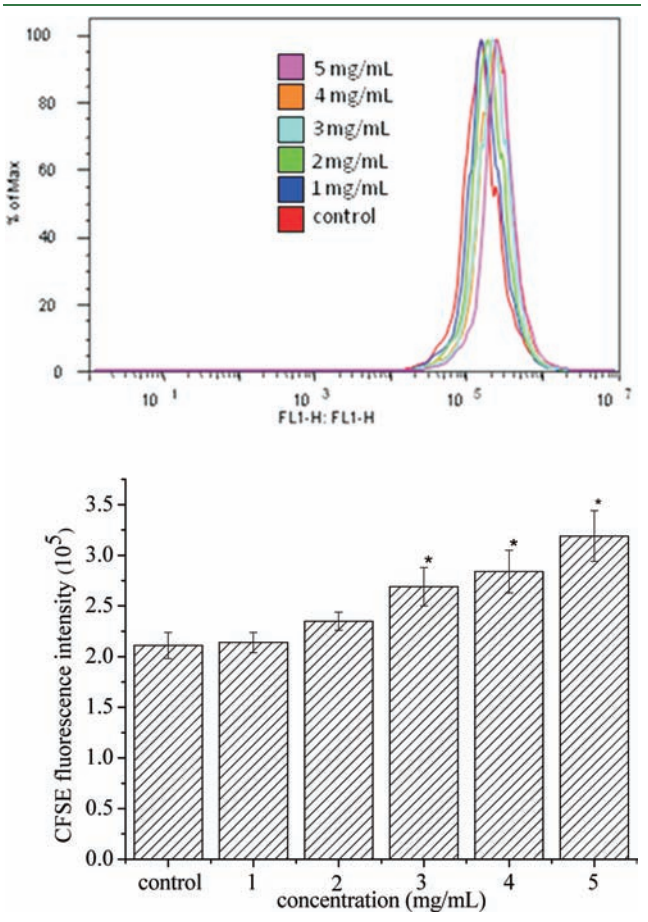

Figure 2. CFDA-SE assay of the effect of black soybean extract on AGS cells proliferation: (top) representative histogram of three independent experiments; (bottom) black soybean extract dose-dependently inhibited the proliferation of AGS cells. AGS cells were labeled with CFDA-SE and incubated with different concentrations of black soybean extract for 48 h. AGS cells were harvested, washed, and resuspended in PBS, and then 10000 cells were analyzed by flow cytometry. ∗, $p < 0.05$ as compared with the control group.

DISCUSSION

Gastric cancer has been a worldwide concern. It has been reported that dietary patterns play an important role in the incidence and mortality of gastric cancer. Consumption of fruits and vegetables has an inverse association with the incidence of gastric cancer.[2] Phenolics are a large group of secondary metabolites derived from the plant kingdom, and more than 8000 polyphenolics are identified. Extracts of phenolic substances from various sources, including fruits, vegetables, tea, and medicinal plants, have been reported to suppress the proliferation of several gastric cancer cell lines in vitro and to inhibit the growth of tumor xenograft in vivo.[1,23] Black soybean has long been used as a foodstuff and medicine in Asian countries, and it exhibited antioxidant activity, anti-inflammation activity, and antiproliferative activity against human HT-29 colon cancer cells, human leukemia Molt 4B cells, and human U937 lymphoblast cells.[10−12] In this study, the antiproliferative effect of black soybean extract in gastric cancer AGS cells and its underlying mechanism for the induction of apoptosis were demonstrated.

The percentage of surviving cells is an indicator of the cell proliferative activity of the extracts. In this study, the proliferation inhibitory effect of black soybean extract on AGS cells was first determined by the MTT assay. MTT is a yellow dye. When passing into mitochondria of living cells, the MTT is reduced to an insoluble, purple formazan product by mitochondrial succinate dehydrogenase. The formazan can be dissolved with an organic solvent, and this colored solution can be quantified by measurement at a certain wavelength with a spectrophotometer. Because reduction of MTT can only occur in viable cells, the level of activity is a measurement of the viability of the cells. The viable cells decreased by black soybean extract in a dose-dependent manner. Treatment with 3 mg/mL black soybean extract resulted in a 34.3% reduction of viable cells, whereas treatment with 5 mg/mL black soybean extract resulted in a 77.5% reduction of

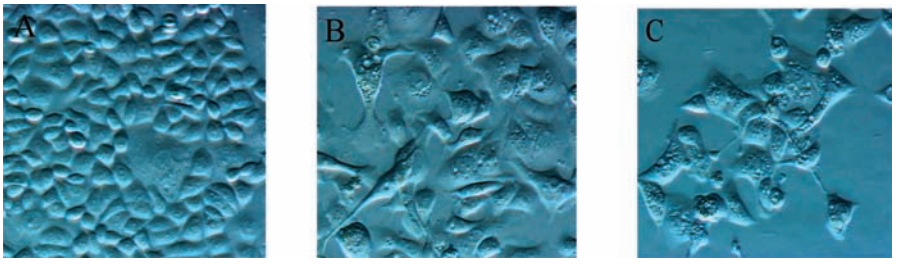

Figure 3. Effect of black soybean extract treatment on the morphological changes of AGS cells: (A) control cells; (B) cells treated with 3 mg/mL black soybean extract; (C) cells treated with 5 mg/mL black soybean extract. AGS cells were grown and treated with or without black soybean extract for 48 h. The medium was removed, and the cells were washed with PBS. The cells were observed under phase contrast microscopy (200×).

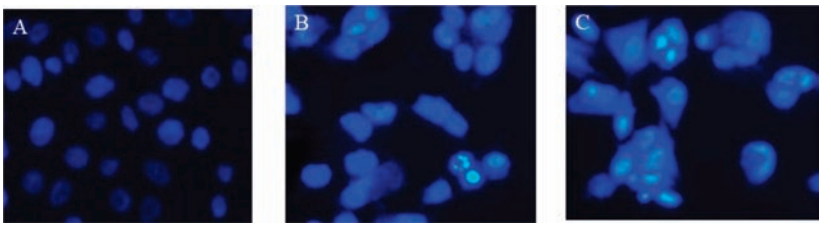

Figure 4. Effect of black soybean extract treatment on the nuclear morphological changes of AGS cells: (A) control cells; (B) cells treated with 3 mg/mL black soybean extract; (C) cells treated with 5 mg/mL black soybean extract. AGS cells were grown and treated with or without black soybean extract for 48 h. The medium was removed, and the cells were fixed with 4% paraformaldehyde and incubated with 100 μg/mL Hoechst for 20 min at room temperature in the dark. Stained solution was washed out, and cells were visualized under fluorescence microscopy (200×).

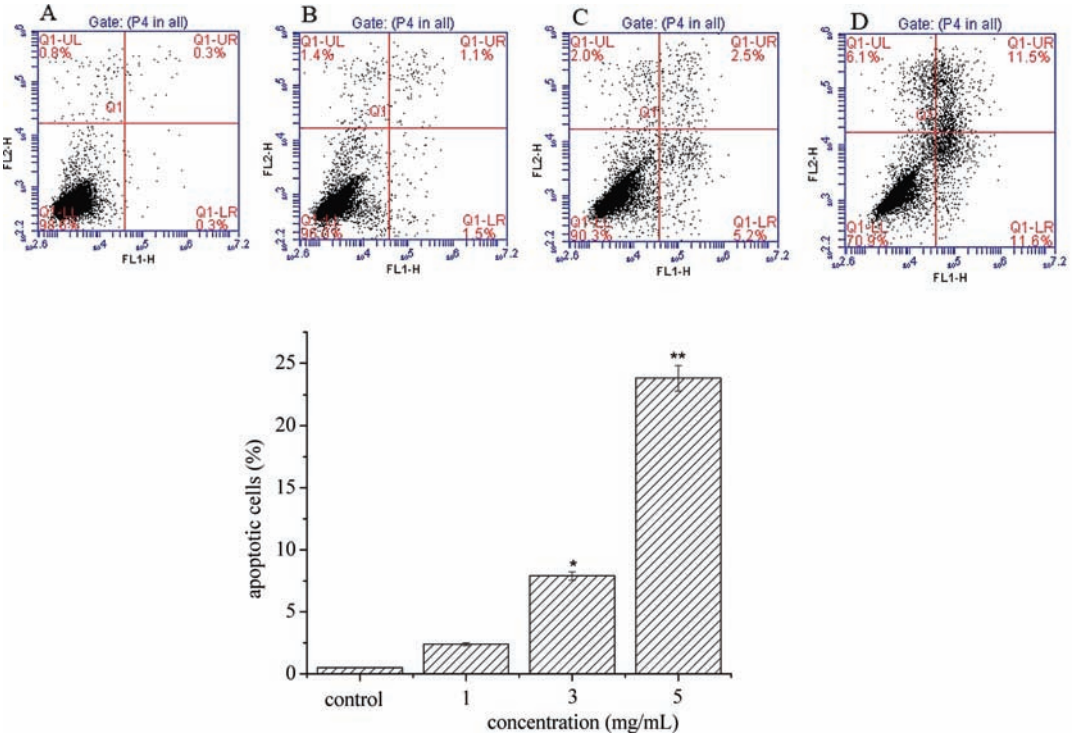

Figure 5. Quantitative analysis of apoptotic cells induced by black soybean extract using annexin V−PI double-staining assay: (top) representative histograms of three independent experiments [criteria were set to distinguish between viable (bottom left), early apoptotic (bottom right), late apoptotic (top right), and necrotic (top left) cells]; (bottom) black soybean extract dose-dependently induced the apoptosis of AGS cells. AGS cells were treated with different concentrations of black soybean extract (A, control; B, 1 mg/mL; C, 3 mg/mL; D, 5 mg/mL) for 48 h. After harvesting, AGS cells were double-stained with annexin V−FITC and PI, and then 10000 cells were analyzed by flow cytometry. *, $p < 0.05$, and **, $p < 0.01$, as compared with the control group.

viable cells (Figure 1). Next, the inhibitory effect of black soybean extract was confirmed by the CFDA-SE assay. CFDA-SE is a fluorescein probe and can passively diffuse into cells. It is colorless and nonfluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are stably retained in cells and equally distributed between daughter cell populations after every cell division. The greater the intensity of the fluorescence, the fewer cells undergo division. With the increase of the concentration of black soybean extract, the intensity of fluorescence increased (Figure 2), which suggested that when the AGS cells were treated with black soybean extract, fewer cells underwent division. Therefore, AGS cell proliferation was inhibited by black soybean extract.

Morphological alterations occurred in the cells treated with black soybean extract under observation with a phase contrast microscope. The cells treated with black soybean extract showed irregular and elongated shape, and sparse cells were adhered to the plate surface (Figure 3). Necrosis and apoptosis are two patterns of cell death in multicellular organisms. Apoptosis is a process of programmed cell death, which is characterized by various biochemical and morphological changes, including cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and formation of "apoptotic bodies".[24] To evaluate the effect of black soybean extract on the nuclear morphology, Hoechest 33342 staining was performed. As shown in Figure 4, the nuclei of cells treated with 3 and 5 mg/mL black soybean extract were darkly stained, and thus fluoresced brightly, which indicated the condensation of chromatin.

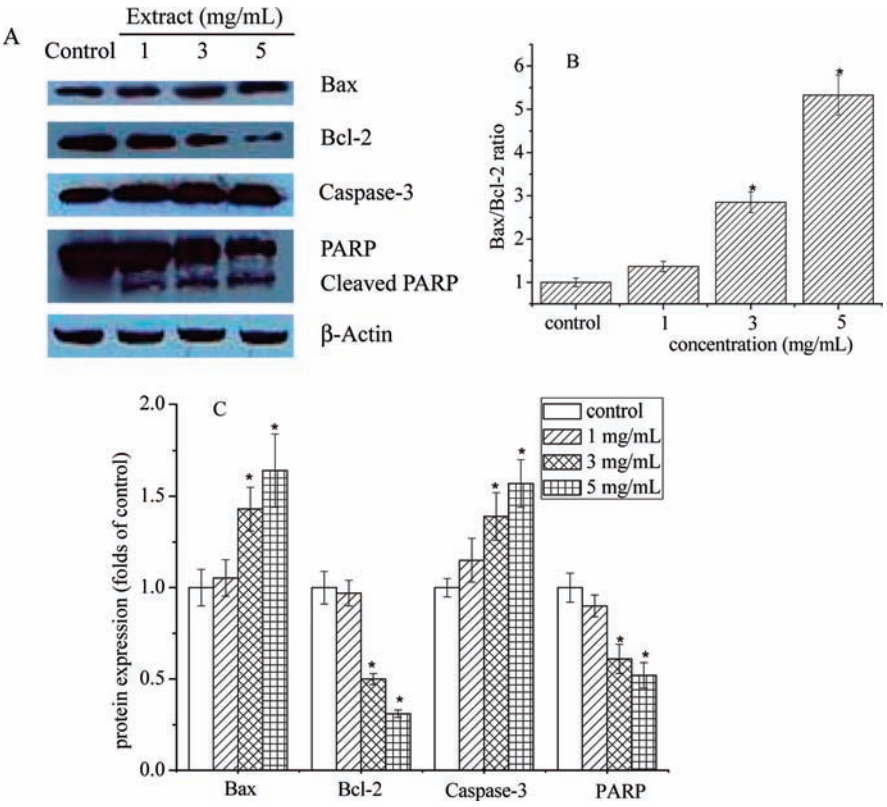

Figure 6. Effect of black soybean extract on the expression of apoptosis-associated proteins in AGS cells (A), the Bax/Bcl-2 ratio (B), and the folds of relative expression of proteins as compared to the control cells (C). AGS cells were treated with different concentrations of black soybean extract for 48 h. After cell culture protocol, Western blot analysis was performed as described under Materials and Methods. The relative expression of protein was quantified densitometrically using ImageJ version 1.43, and the β-actin was used as reference control. *, $p < 0.05$ as compared with the control group ($n = 3$).

Similar apoptotic effects of black soybean extract on AGS cells were also observed by the annexin V and PI double-staining assays. Upon apoptosis, the cell changes the structure of its plasma membrane, leading to the exposure of phosphatidylserine (PS) on the cell surface. In the presence of calcium, high-affinity binding of annexin V to PS occurs. A cell population with annexin V positive and PI negative is considered as an early apoptotic population, whereas a cell population with both annexin V and PI positive is considered as a late apoptotic/necrotic population.[25] The dose-dependent induction of apoptosis is shown in Figure 5. Totals of 7.9 and 23.8% of cells were induced to apoptosis by the treatment of AGS cells with 3 and 5 mg/mL black soybean extract, respectively. However, the necrotic cells were concomitantly increased with the increased concentration of black soybean extract.

To confirm which apoptosis-associated proteins were involved in the induction of apoptosis of AGS cells, we performed Western blotting assays on the expression of Bax, Bcl-2, caspase-3, and PARP. The Bcl-2 family of proteins is important in regulating apoptosis. It consists of both pro-apoptotic proteins (Bax, Bak, Bid, Bad, Bim, Bik, Blk, and Bcl-10) and anti-apoptotic proteins (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, and Bcl-w), which share one or more of the four characteristic domains of homology entitled the Bcl-2 homology domains. The function of Bax is to promote cell apoptosis by translocation of the mitochondrial voltage-dependent anion channel membrane and disruption of the integrity of mitochondria, resulting in the release of cytochrome *c* and other pro-apoptotic factors into the cytosol, leading to the activation of caspase. In contrast, anti-apoptotic protein Bcl-2 inhibits apoptosis by inhibiting the release of cytochrome *c* and apoptosis-inducing factor from mitochondria to the cytosol and by limiting the activation of caspase.[26,27] Therefore, the ratio of Bax to Bcl-2, rather than the increase or decrease in Bax or Bcl-2 alone, is crucial to cell apoptosis in the mitochondria-mediated apoptosis pathway. The ratio of Bax to Bcl-2 decides the fate of the cells, and the change in the ratio of Bax to Bcl-2 initiates caspase-cascade signaling.[26] As shown in Figure 6, the total protein concentration of Bax was dose-dependently increased, and the Bcl-2 protein was dose-dependently decreased; thus, the ratio of Bax to Bcl-2 was significantly changed. Our results suggested that black soybean extract induced apoptosis by up-regulation of Bax expression and down-regulation of Bcl-2 expression in AGS cells. The increased ratio of Bax to Bcl-2 might be a key factor in inducing apoptosis of AGS cells.

The caspases, which are cysteine proteases, play crucial roles in cell apoptosis. Particularly, caspase-3 has been shown to play a central role in the terminal and execution phase of apoptosis induced by diverse stimuli.[28] Caspase-3 is an apoptosis executioner and activated by other activated caspases, such as caspase-8 and -9, in response to pro-apoptotic signals. This enzyme has substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves PARP into 83 and 24 kDa fragments, which respond to DNA fragmentation and eventually lead to apoptosis.[29] Here, we demonstrated that black soybean extract treatment increased the ratio of Bax to Bcl-2, which resulted in the subsequent activation of caspase-3 as manifested by the

dose-dependently up-regulated expression of caspase-3. Subsequently, PARP, a well-known substrate of caspase-3, was cleaved by the activated caspase-3 (Figure 6). Our investigation indicated that the molecular mechanisms involved in black soybean extract-induced apoptosis might be attributed to both extrinsic and intrinsic pathways, as shown by the shifting ratio of Bax to Bcl-2, as well as the activation of caspase-3.

In the present study, the treatment of AGS cells with black soybean extract induced apoptosis in a dose-dependent manner. To some extent, it also induced necrosis. Black soybean extract was a rich source of phenolics, in which the TPC was 32.5 mg GAE/g. Black soybean extract contained as high as 80% of sugars. However, after removal of the sugar fractions by adsorption of black soybean extract with XAD-7 and then desorption by water and 80% MeOH successively, the resultant water eluate (mainly including sugars) had almost no effect on the growth of AGS cells (data not shown), whereas the IC_{50} of the 80% MeOH eluate (mainly including polyphenolics) decreased to 0.67 mg/mL. These facts suggested that polyphenolics in black soybean extract were effective in inhibiting the growth of AGS cells. Phenolics in black soybean extract could be categorized into, but not limited to, four groups, namely, phenolic acids (6625.2 μg/g), isoflavones (11776.5 μg/g), anthocyanins (1165.4 μg/g), and flavanols (4378.1 μg/g) (Table 2), which were within the range of our previous study[8] and other literature.[30] These components from different sources have shown antiproliferative effects on stomach cancer cells in previous studies.[31−33] A polyphenol-rich extract, in which the main constituents were protocatechuic acid, catechin, epigallocatechin, epigallocatechin gallate, and caffeic acid, from a medicinal plant *Hibiscus sabdariffa* L., induced apoptosis of AGS cells through the activation of p38/Jun/Fasl singaling and stabilization of p53, which led to up-regulation of the expression of Bax, the release of cytochrome *c*, and the activation of caspase-3.[31] The antiproliferative effects of soybean isoflavones on SGC-7901 stomach cancer cells were investigated. The authors pointed out that genistein had the prominent antiproliferative effects in a dose- and time-dependent manner.[32] The anthocyanin fraction from red wine had a greater antiproliferative effect in AGS cells than other non-anthocyanin fractions, and TLC analysis revealed delphinidin was the most abundant compound in the anthocyanin fraction.[33] In another study, Shih et al. reported that malvidin exhibited the most potent antiproliferative effect on AGS cells. Malvidin induced apoptosis of AGS cells via interference with membrane permeability, which resulted in elevation of the Bax/Bcl-2 ratio, increased the p38 kinase expression, and inhibited the ERK activity.[34] Because most of the reported active compounds were included in black soybean extract, which compound or which group of polyphenolics was prominent or whether these polyphenolics played a synergistic role in inducing apoptosis should be investigated in future studies.

In summary, the present study reported that black soybean extract induced apoptosis in human AGS gastric cancer cells through altering the ratio of Bax to Bcl-2 and activation of caspase-3, followed by cleavage of PARP. However, further identifications of which components or which group of phytochemicals is the most pronounced in inducing apoptosis of AGS cells should be considered. On the basis of the long history of its use as a food and traditional herbal medicine and its antiproliferative effects against human AGS gastric cancer cells, black soybean extract might be a potential therapeutic candidate for the prevention or treatment of human gastric cancer.

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