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Antiproliferation Effect and Mechanism of Prostate Cancer Cell Lines as Affected by Isoflavones from Soybean Cake

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The objectives of this study were to determine the antiproliferation effect of prostate cancer cell lines LNCaP and PC-3 as affected by 4 isoflavone fractions prepared from soybean cake and isoflavone standards genistein and daidzein. With the MTT test, most treatments were effective in inhibiting prostate cancer cell growth at a low dose of 5 and 10 μg/mL. In cell cycle analysis, the fractions of aglycon, a mixture of acetylglucoside and aglycon, as well as genistein and a combination of genistein and daidzein standards exhibited a high G2/M ratio for LNCaP, as did the acetylglucoside, genistein and a combination of genistein and daidzein standards for PC-3. Results of Western blotting revealed an increase in p53 protein expression of LNCaP following treatments of the aglycon fraction, genistein and a combination of genistein and daidzein standards. However, all the treatments did not affect Bcl-2 protein expression significantly in both LNCaP and PC-3 cells. A decline in cyclin B1 expression of LNCaP was observed for all the treatments, with the mixture of acetylglucoside and aglycon possessing the most pronounced effect. But for PC-3, a decrease in cyclin B1 expression was shown for all the isoflavones, with the exception of malonylglucoside, glucoside and acetylglucoside fractions. The outcome of this study may provide a basis for possible production of functional food in the future with soybean cake as raw material.

KEYWORDS: Soybean cake; isoflavone; human prostate cancer cell line; cell cycle analysis; Western blotting

INTRODUCTION

Isoflavones, a kind of plant isoflavonoids, are mainly distributed in soybean and its products. Twelve isoflavones have been reported to be present in soybeans and are divided into 4 groups, namely, aglycon, glucoside, acetylglucoside and malonylglucoside, with each group containing 3 isoflavones: daidzein, genistein and glycitein for aglycon; daidzin, genistin and glycitin for glucoside; 6″-*O*-acetyldaidzin, 6″-*O*-acetylgenistin and 6″-*O*-acetylglycitin for acetylglucoside; and 6″-*O*-malonyldaidzin, 6″-*O*-malonylgenistin and 6″-*O*-malonylglycitin for malonylglucoside (*1*−*3*). Most isoflavones are present in glycosidic form in nature and can be converted to their corresponding aglycon under the condition of acid, base or the presence of glucosidase (*4*, *5*).

Soybean cake is a byproduct obtained during soybean oil processing and is often used as feed. However, soybean cake has been shown to contain a significant amount of isoflavones (*1*−*4*, *6*). Wang and Murphy (*4*) reported the presence of malonylglucoside, glucoside, acetylglucoside and aglycon at 1341, 595, 33 and 45 μg/g, respectively, in defatted soybean flour, whereas in a later report, a level of 779, 463, 93 and 26 μg/g, respectively, was shown (*6*). In a similar study, the defatted soybean was found to contain malonylglucoside at 1340 μg/g, glucoside at 249 μg/g, acetylglucoside at 207 μg/g and aglycon at 11 μg/g (*7*). Obviously the difference in the level of four groups of isoflavones can be accounted for by the variation in soybean variety, processing conditions, geographic location and time of harvest (*2*, *5*). In a recent report soybean cake was shown to contain high amounts of malonylglucoside (2411 μg/g), glucoside (2184 μg/g), acetylglucoside (256 μg/g) and aglycon (159 μg/g) (*5*). Therefore, it would be a great advantage to the functional food industry if the isoflavones in soybean cake can be proven to be anticarcinogenic. In addition to isoflavones, several other functional components like flavonoids and saponins are also present in soybean cake and have been demonstrated

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to possess antioxidant, anticarcinogenic and anti-inflammation effects (*2*, *8*, *9*).

In the past decade many studies have indicated that soybean isoflavones are effective in antiproliferation of breast and liver tumor cells (*8*, *10*). Steiner et al. (*10*) summarized the mechanisms of prevention of prostate and breast cancer by isoflavone, including arresting the cell cycle to G1 and G2/M phases, regulating cell-cycle-related protein by lowering expression of cyclin B, and inducing apoptosis by lowering expressions of antiapoptotic Bcl-2, Bcl-x and promoting expressions of Bax, Bak and Bok. However, the arrest of G1 or G2/M phase and the lowering of cyclin B expression in LNCaP cells were only observed in vitro at high genistein concentration (20−60 μM). But the expressions of Bcl-2, Bcl-x, Bax, Bak and Bok were unaffected in the presence of genistein concentrations less than 40 μM.

The results shown above clearly demonstrated the potential of isoflavones in inhibiting cancer cell growth. However, most reports in the literature dealt with the effect of isoflavone standards such as genistein or daidzein on antiproliferation of cancer cell. There is a paucity of data regarding the anticancer effect as affected by a mixture of isoflavone standards and isoflavone extracts from soybean cake. The objectives of this study were thus undertaken to use soybean cake as raw material to isolate four groups of isoflavones by preparative column chromatography and study their effects on the antiproliferation mechanism of human prostate cancer cell lines LNCaP and PC-3, with an isoflavone extract and isoflavone standards being used for comparison.

MATERIALS AND METHODS

Materials. A total of 50 kg of soybean cake was procured from Chung-Lian Oil Co. (Taichung, Taiwan). Isoflavone standards including genistein and daidzein were purchased from LC Laboratories (Woburn, MA). Both RPMI 1640 and F-12K culture media, phosphate buffered saline (PBS), fetal bovine serum (FBS), trypan blue stain (0.4%), trypsin (2.5%), Hanks' balanced salt solution (HBSS) and penicillin-streptomycin were purchased from Gibco Co. (CA, USA). Fungizone (amphotericin B solution), 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT), RNase A, propidium iodide were obtained from Sigma (St. Louis, MO). Alcohol (95%) was from Taiwan Tabacco and Wine Co (Tainan, Taiwan). Deionized water was made using a Milli-Q purification system (Millipore Co, Bedford, MA). Human prostate cancer cell lines LNCaP and PC-3 were from Taiwan Food Industry Development Research Institute/National Health Research Institute of Taiwan (Hsinchu, Taiwan).

Preparation of Isoflavone Fractions and Extracts from Soybean Cake. The various isoflavone fractions and extracts were prepared from soybean cake using a method described in our previous study (*2*) and modified. Briefly, soybean cake (50 g) was ground into powder and mixed with 150 mL of 50% ethanolic solution, followed by shaking at room temperature for 2 h and centrifuging at 6000 rpm (25 °C) for 20 min. The supernatant was collected and filtered through a glass fiber filter paper (pore size 0.6 μm) to obtain the isoflavone extract (ISO). For preparative chromatography, a 200 g adsorbent Diaion HP-20 was poured into a glass column (375 × 45 mm i.d., particle size 200−600 μm, Mitsubishi Chemical Co., Tokyo, Japan), after which 1 L of ethanol was added, followed by 1 L of deionized water passing through the column for activation. Then 80 mL of isoflavone extract was poured into the column, and the high-polarity impurities were removed with 400 mL of deionized water, followed by elution of malonylglucoside with 900 mL of 15% ethanolic solution and glucoside with 3300 mL of 27% ethanolic solution with flow rate at 10 mL/min. Next, the acetylglucoside fraction was eluted with 200 mL of 70% ethanolic solution and 400 mL of 95% ethanolic solution. However, because of overlapping, both acetylglucoside and aglycon fractions were combined and evaporated to dryness to dissolve in 20 mL of isopropanol, in which a portion (10 mL) was collected and poured into a Yamazen Hi-Flash silica gel column (170 × 48 mm i.d., particle size 40 μm, Yamazen Co., Osaka, Japan). Both acetylglucoside and aglycon fractions were eluted separately with a solvent system of hexane−isopropanol−ethanol (8:9:1, v/v/v) with flow rate at 20 mL/min. All four fractions of isoflavones were then evaporated to dryness under vacuum and dissolved in 20 mL of 50% ethanolic solution for HPLC analysis. A Vydac 201TP54 C18 column (250 × 4.6 mm i.d., particle size 5 μm, GRACE Co., Deerfield, IL) and a gradient mobile phase of acetonitrile and water was used to separate 12 isoflavones with flow rate at 2 mL/min, column temperature at 35 °C and detection wavelength at 262 nm (*3*, *11*).

Preparation of Isoflavone Extracts and Fractions for Cell Antiproliferation Study. After HPLC analysis, the malonylglucoside, glucoside, acetylglucoside and aglycon fractions were found to contain isoflavone at 3115, 2738, 2661 and 2528 μg/mL, respectively. Then each fraction was adjusted to a final concentration of 2500 μg/mL with water−ethanol (1:1, v/v). By mixing 1.5 mL of acetylglucoside and aglycon fractions each, a total concentration of 2500 μg/mL was obtained. For isoflavone extract (ISO), 20 mL was collected and evaporated to dryness to dissolve in 2 mL of water−ethanol (1:1, v/v) for HPLC analysis, and the isoflavone concentration was shown to be 15844.8 μg/mL. Likewise, the final concentration of ISO was adjusted to 2500 μg/mL with water−ethanol (1:1, v/v).

For isoflavone standards, by dissolving 25 mg of daidzein and genistein each in water−ethanol (1:1, v/v) and diluted to 10 mL for a final concentration at 2500 μg/mL. Similarly, a combination of daidzein and genistein at 2500 μg/mL could be obtained by using the same method.

All the isoflavone fractions, extracts and standards were further diluted to six concentrations of 2500, 2000, 1500, 1000, 500 and 250 μg/mL with water−ethanol (1:1, v/v) for use. Prior to cell culture experiment, the six concentrations were further diluted to 50, 40, 30, 20, 10 and 5 μg/mL with RPMI 1640 or F-12K culture medium.

Determination of Functional Components in Isoflavone Fractions and Extracts. The various functional components in isoflavone fractions and extracts were determined based on a previous report by Kao and Chen (*2*). However, no gallic acid, caffeic acid, chlorogenic acid, ascorbic acid and α-tocopherol were detected. Instead, only the total flavonoids, total phenolic compounds and saponins were determined.

Total Flavonoids. Five catechin standard solutions of 1, 5, 10, 25 and 50 μg/mL were prepared with water−ethanol (1:1, v/v) and 500 μL each was collected and mixed with 75 μL of 5% sodium nitrite solution. After standing at room temperature for 5 min, 150 μL of 10% aluminum chloride solution was added and reacted for 5 min, followed by adding 500 μL of 1 M sodium hydroxide solution, and the absorbance was measured at 510 nm. Then the standard curve was prepared by plotting concentration against absorbance. Similarly, 500 μL each of isoflavone fraction and extract was collected and the absorbance measured using the same method shown above. The total flavonoid in each isoflavone fraction and extract was obtained based on the standard curve of catechin and was expressed as μg/mL of catechin equivalents.

Total Phenolic Compounds. Five gallic acid standard concentrations of 10, 50, 100, 200 and 250 μg/mL were prepared with water−ethanol (1:1, v/v), and 50 μL each was collected and mixed with 200 μL of Folin−Ciocalteu reagent. The solution was then homogenized and stood at room temperature for 5 min, followed by adding 1000 μL of 15% sodium carbonate solution. The mixture was allowed to react at room temperature for 60 min, and the absorbance was measured at 750 nm. The standard curve of gallic acid was prepared by plotting concentration against absorbance. Likewise, 50 μL each of isoflavone fractions and extracts was collected and the absorbance measured using the same method shown above. The total phenolic compound was determined based on the gallic acid standard curve and was expressed as μg/mL of gallic acid equivalents.

Saponins. Ten milliliters of isoflavone extract or fraction was collected and evaporated to dryness, after which the residue was dissolved in 5 mL of 1 N methanolic hydrochloric acid solution and shaken at 75 °C (150 rpm) for 2.5 h for hydrolysis. After filtration through a 0.22 μm membrane filter, 1 mL was collected and poured

Table 1. Contents (μg/mL) of Isoflavones in Various Isoflavone Fractions and Extracts Based on Total Isoflavone Concentration at 50 μg/mL[a]

	isoflavone fractions						isoflavone standard solutions		
isoflavones	M	G	Ac	Ag	Ac+Ag	ISO	Dein	Gein	2 std
malonyldaidzin	20.4 ± 0.1	−	−	−		11.2 ± 0.3	−	−	−
malonylglycitin	11.3 ± 0.1	−	−	−		4.2 ± 1.7	−	−	−
malonylgenistin	18.3 ± 0.5	−	−	−		8.9 ± 0.3	−	−	−
daidzin	−	16.9 ± 0.3	−	−		4.2 ± 0.2	−	−	−
glycitin	−	6.3 ± 0.2	−	−		5.2 ± 0.5	−	−	−
genistin	−	26.8 ± 0.2	−	−		12.3 ± 0.4	−	−	−
acetyldaidzin	−	−	14.2 ± 0.5	−	7.1 ± 0.2	0.7 ± 0.0	−	−	−
acetylglycitin	−	−	5.2 ± 0.3	−	2.6 ± 0.1	0.8 ± 0.1	−	−	−
acetylgenistin	−	−	31.3 ± 0.3	−	15.6 ± 0.1	1.2 ± 0.0	−	−	
daidzein	−	−	−	24.4 ± 0.7	12.3 ± 0.4	0.8 ± 0.2	50.0		50.0
glycitein	−	−	−	5.6 ± 0.1	2.7 ± 0.1	0.1 ± 0.0	−	−	−
genistein	−	−	−	20.0 ± 0.2	10.0 ± 0.1	0.5 ± 0.0		50.0	50.0
total	50.0 ± 0.5	50.0 ± 0.4	50.7 ± 0.5	50.0 ± 0.8	50.4 ± 0.1	50.1 ± 3.0	50.0	50.0	100

[a] M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycon fraction; Ac+Ag, a mixture of fraction Ac and fraction Ag; ISO, soybean cake extract containing 12 isoflavones; Gein, genistein; Dein, daidzein; 2 std, a mixture of daidzein and genistein standards.

Table 2. Contents (μg/mL) of Functional Components in Various Isoflavone Fractions and Extracts Based on Total Isoflavone Concentration at 50 μg/mL[a]

			saponin	
isoflavones	total flavonoid[b]	total phenolic compound[c]	soyasapogenol A	soyasapogenol B
M	2.5 ± 0.1 e	93.0 ± 0.6 c	nd[d]	nd
G	0.7 ± 0.2 f	69.3 ± 0.7 e	10.5 ± 0.0 c	12.6 ± 0.2 d
Ac	6.8 ± 0.1 c	97.4 ± 3.7 c	36.9 ± 1.9 a	198.8 ± 8.8 a
Ag	14.3 ± 0.5 a	147.0 ± 0.9 a	nd	11.4 ± 0.1 e
Ac+Ag	10.3 ± 0.1 b	112.0 ± 3.7 b	26.8 ± 0.3 b	91.5 ± 2.0 b
ISO	6.1 ± 0.1 d	81.7 ± 0.4 d	27.0 ± 0.2 b	30.6 ± 0.0 c

[a] Average of duplicate analyses ± standard deviation. Symbols bearing different letters (a−f) in the same column are significantly different ($P < 0.05$). M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycon fraction; Ac+Ag, a mixture of fraction Ac and fraction Ag; ISO, soybean cake extract containing 12 isoflavones. [b] Data expressed as μg/mL of catechin equivalents. [c] Data expressed as μg/mL of gallic acid equivalents. [d] nd: not detected.

into a Phenomenex Strata C18-E (500 mg/3 mL) cartridge, which was previously activated with 10 mL of methanol. The high-polarity impurities were first removed with 2 mL of deionized water, followed by elution of saponins with 1 mL of methanol. Saponins including soyasapogenol A and soyasapogenol B (Chromadex Co., Santa Ana, CA) were separated by a Gemini C18 column (250 × 4.6 mm i.d., particle size 5 μm, Phenomenex Co., Torrance, CA) and a mobile phase of 100% acetonitrile with flow rate at 1 mL/min and ELSD detection with temperature at 70 °C, pressure at 3 bar and gain value at 8. The various saponins were quantified based the standard curves of saponins and the formula as described in a previous study (*2*).

Cell Culture. Human prostate cancer cell lines LNCaP and PC-3 were cultured in RPMI 1640 and F-12K, respectively, both of which contained 10% fetal bovine serum (FBS) and were added with 10 mL of fungizone and 10 mL of penicillin-streptomycin. Both cell lines were incubated at 37 °C under 5% CO_2 and humidified atmosphere (96% humidity), and the medium was replaced every two days to maintain normal cell growth.

Both LNCaP and PC-3 cell lines were grown to the desired density at approximately 80% confluence, followed by washing with 5 mL of PBS, adding 0.5 mL of 2.5% trypsin and shaking to cover cell surface uniformly. After reaction for 1−2 min, 2 mL of RPMI 1640 or F-12K was added for neutralization for harvesting cells. Next, cells were transferred to a 15 mL tube for centrifugation at 2000 rpm for 5 min. The supernatant was discarded, and 3 mL of RPMI 1640 and F-12K was added separately to suspend cells. Ten milliliters of culture medium was added to 1 mL of cell suspension for cultivation. For LNCaP cells, the culture medium could be replaced only after 48 h incubation to allow complete cell attachment. But for PC-3 cells, the culture medium was replaced after 24 h.

Endurability Test of Sample Solvent. In this experiment sample solvent water−ethanol (1:1, v/v) was used for isoflavone extraction, and the toxicity effect of sample solvent on prostate cancer lines LNCaP and PC-3 has to be investigated. Both RPMI 1640 and F-12K media containing water−ethanol (1:1, v/v) at 3, 2, 1, 0.5 and 0% were prepared separately. 0.2 mL of LNCaP and PC-3 was seeded separately at a cell number of 2.0 × 10^4/well for the former and 2.5 × 10^4/well for the latter to a 96-well plate. Both LNCaP and PC-3 were cultured for 48 and 24 h respectively at 37 °C under 5% CO_2 to allow cells attaching to the bottom. Then the culture media were sucked and replaced with new media containing 3, 2, 1, 0.5 and 0% of water−ethanol (1:1, v/v). Triplicate experiments were carried out for each concentration. After incubation for 72 h, the residual culture media were washed with PBS twice, and then 0.2 mL of MTT solution (0.5 mg/ mL in HBSS) was added and cultured at 37 °C for 3 h in the dark. After suction of MTT solution, 0.2 mL of dimethyl sulfoxide was added to dissolve purple crystal, and the absorbance was measured at 570 nm with an ELISA reader after 15 min.

Cell Viability Test. This test is aimed to elucidate if the viability decrease of both LNCaP and PC-3 prostate cancer cell lines are mainly due to sample treatment, not overgrowth of cells themselves during 72 h incubation. Likewise, 2.0 × 10^4 cells per well for LNCaP and 2.5 × 10^4 cells per well for PC-3 were seeded to 96-well plates. Both LNCaP and PC-3 were incubated for 48 and 24 h respectively to allow cells attaching to the bottom, followed by replacing with fresh culture medium and incubating for 72 h. Cells were collected every 24 h and stained with trypan blue for determination of cell survival rate.

Antiproliferation of Prostate Cancer Cell Lines by Isoflavones. 0.2 mL of cell suspension was seeded to 96-well plates with each well containing 2.0 × 10^4 for LNCaP and 2.5 × 10^4 for PC-3, both of which were subjected to cultivation for 48 and 24 h respectively for attachment to the bottom. Next, the culture media were sucked and replaced with six concentrations of 5, 10, 20, 30, 40 and 50 μg/mL of isoflavone fractions and extracts as well as standards. Triplicate experiments were conducted for each concentration. Then 0.2 mL of MTT solution (0.5 mg/mL in HBSS) was added after 72 h, followed by continued cultivation of LNCaP for 1.5 h and PC-3 for 3 h in the dark. After suction of MTT solution, 0.2 mL of dimethyl sulfoxide was added to dissolve purple crystal, and the absorbance was measured at 570 nm with an ELISA reader after 15 min. The inhibition effect of prostate cancer cells was expressed as relative cell survival rate using the following formula: relative cell survival rate (%) = [(absorbance of the isoflavone treatment)/(absorbance of control treatment)] × 100%.

Cell Cycle Analysis. A method as described by Kao et al. (*8*) was modified to study cell cycle analysis. Both LNCaP and PC-3 cells were seeded at approximately 1 × 10^6 to culture plate, and subjected to cultivation for 48 h for the former and 24 h for the latter, followed by adding three concentrations (10, 30 and 50 μg/mL) of isoflavone fractions, extracts and standards. After 24 h, cells were washed with PBS and trypsin was added for detachment. Cells were collected and

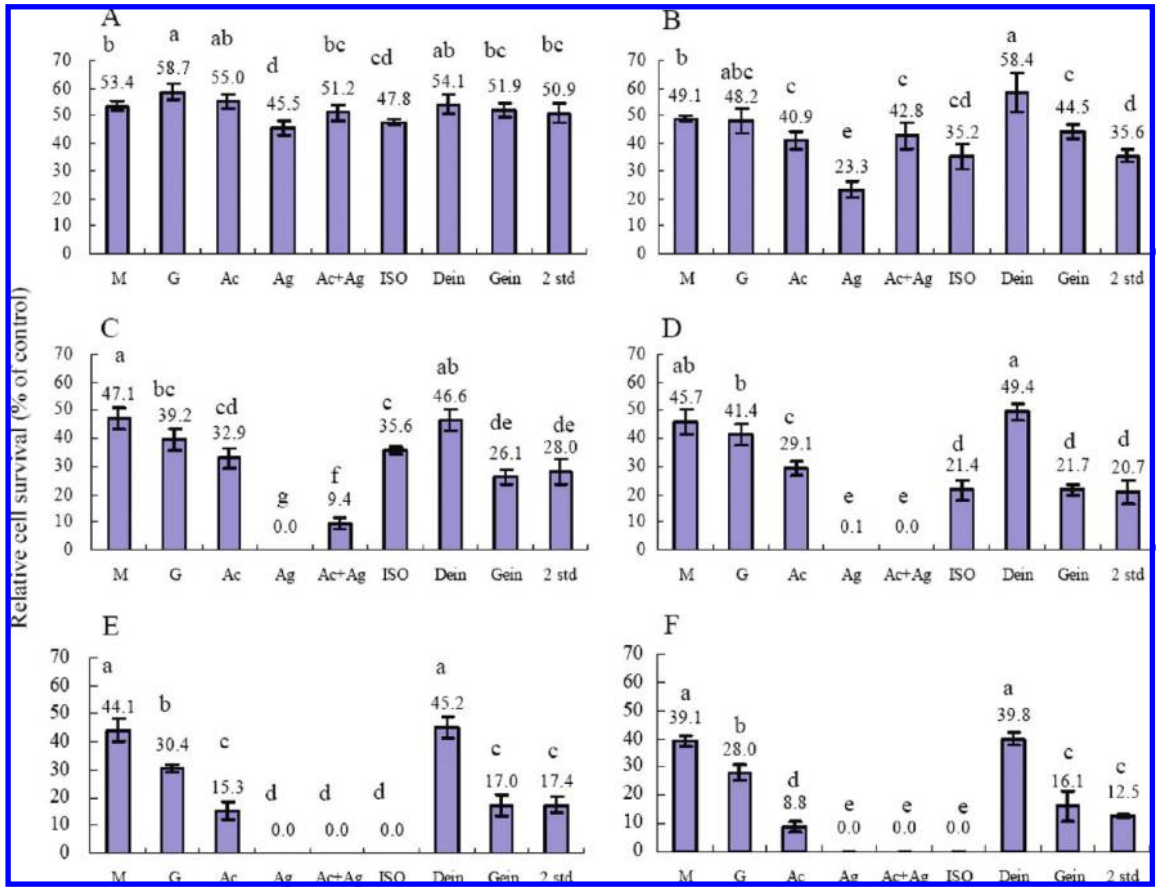

Figure 1. Inhibition effect of isoflavones on LNCaP cell growth as determined by MTT: (**A**) 5 μg/mL; (**B**) 10 μg/mL; (**C**) 20 μg/mL; (**D**) 30 μg/mL; (**E**) 40 μg/mL; (**F**) 50 μg/mL. M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycon fraction; Ac+Ag, a mixture of acetylglucoside and aglycon fractions; ISO, soybean cake extract containing 12 isoflavones; Dein, daidzein; Gein, genistein; 2 std, a combination of daidzein and genistein.

centrifuged at 2000 rpm for 5 min, and the supernatant was removed. Then cells were washed with PBS, and PBS containing 70% ethanol was added slowly to fix cells at 4 °C overnight. Next, ethanol was removed by centrifugation and cells were washed twice with PBS, followed by adding 0.2 mL of PBS and 0.1 mL of RNase A (100 μg/mL) in the dark. After reacting at 37 °C for 30 min, 0.1 mL of 10 μg/mL propidium iodide was added for cell cycle analysis by using a flow cytometer (Coulter EPICS XL-MCL, Beckman Co., Fullerton, CA).

Determination of Expression of Proteins Associated with Apoptosis and Cell Cycle. LNCaP and PC-3 cells were seeded at about 1 $\times$ 10^6 to culture plate for cultivation for 48 and 24 h, respectively, after which 20 μg/mL of the aglycon fraction and 50 μg/mL of the other isoflavone fractions, extract and standards were added for 24 h cultivation. Cells were washed with PBS, and trypsin was added for cell detachment. Cells were collected and centrifuged at 2000 rpm for 5 min, followed by adding 100 μL of lysis solution to each plate to disperse cells, and protein was extracted by reacting for 5 min on ice, and centrifuged at 14000 rpm (4 °C) for 10 min. The supernatant containing protein was collected, and the residue was mixed with 50 μL of lysis solution, followed by shaking the mixture for 10 s and centrifugation again at 14000 rpm (4 °C) for 10 min. Both supernatants were combined and stored at −80 °C for use. Different concentrations of bovine serum albumin (BSA) were prepared, and the absorbance was measured at 590 nm with an ELISA reader. The standard curve of BSA was obtained by plotting concentration against absorbance, and the amounts of cell proteins were calculated based on the standard curve. Cell lysates were then collected for analyses of p53, Bcl-2 and cyclin B1.

For Western blotting, the premade electrophoretic gel (8.5−10% SDS−polyacrylamide) was placed in a tank. Standards with known MW and cell lysates were added to the well in order, followed by separation of cell protein for 2−3 h under 80 V at room temperature, and transferring of protein to PVDF membrane, which was then collected to immerse in blocking buffer (5% skim milk in Tris buffered saline with 0.1% Tween 20) with shaking for 1 h. Again the PVDF membrane was washed with TBS-T (Tris buffered saline containing 0.1% Tween 20) for 10 min three times to remove unattached protein. Diluted antibodies, including anti-p53 2000x, anti-Bcl-2 1000x, anti-cyclin B1 2000x and anti-β actin 1000x were added separately and reacted with PVDF membrane at room temperature for 2 h, followed by washing with TBS-T for 10 min three times, adding with secondary antibody bonded with HRP to react for 1 h and washing again with TBS-T three times. Next, the ECL detection kits was added to catalyze oxidation of luminol to emit chemiluminescence, and X-ray film was used to press for image expression by film developer.

Statistical Analysis. All the experiments were conducted in duplicate or triplicate, and the data were analyzed using SAS software system (version 9.1, SAS Institute, Cary, NC) (*12*). The data were also subjected to analysis of variance (ANOVA) and Duncan's multiple range test for mean comparison of significant difference ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Contents of Isoflavones and Functional Components in Isoflavone Fractions and Extracts. Table 1 shows the contents of isoflavones in isoflavone fractions and extracts based on a total concentration of 50 μg/mL each. Similar to a report by Kao and Chen (*2*), malonyldaidzin, genistin, acetylgenistin and daidzein were present in the largest amounts in fractions of malonylglucoside, glucoside, acetylglucoside and aglycon, respectively, with a high level of malonylgenistin and genistin in isoflavone extract (ISO). **Table 2** shows the contents of

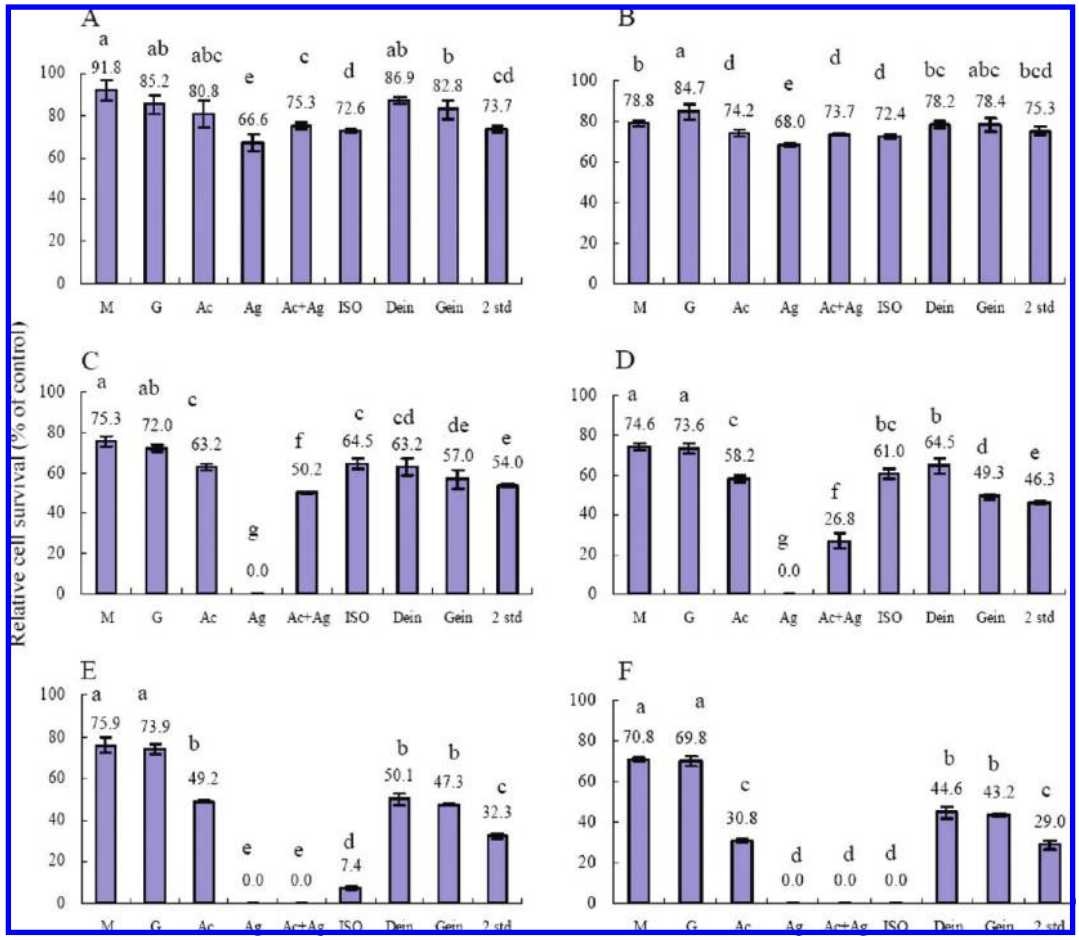

Figure 2. Inhibition effect of isoflavones on PC-3 cell growth as determined by MTT: (**A**) 5 μg/mL; (**B**) 10 μg/mL; (**C**) 20 μg/mL; (**D**) 30 μg/mL; (**E**) 40 μg/mL; (**F**) 50 μg/mL. M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycon fraction; Ac+Ag, a mixture of acetylglucoside and aglycon fractions; ISO, soybean cake extract containing 12 isoflavones; Dein, daidzein; Gein, genistein; 2 std, a combination of daidzein and genistein.

Table 3. The IC50 Value (μg/mL) for Both LNCaP and PC-3 Cells As Affected by Isoflavones from Soybean Cake[a]

isoflavone	M	G	Ac	Ag	Ac+Ag	Iso	Dein	Gein	2 std
IC50 for LNCaP cell	12.3	11.2	5.04	2.43	5.23	2.88	22.6	8.96	5.29
IC50 for PC-3 cell	160	139	43.0	10.6	22.1	28.1	52.4	44.6	32.9

[a] M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycon fraction; Ac+Ag, a mixture of fraction Ac and fraction Ag; ISO, soybean cake extract containing 12 isoflavones; Dein, daidzein; Gein, genistein; 2 std, a combination of daidzein and genistein.

functional components in isoflavone fractions and extracts based on a total concentration of 50 μg/mL each. No gallic acid, caffeic acid, chlorogenic acid, ascorbic acid and α-tocopherol were detected, all of which may undergo considerable loss during soybean oil processing (*2*). A maximum level of flavonoids was found in the aglycon fraction (14.3 μg/mL), followed by a mixture of acetylglucoside and aglycon (10.3 μg/mL), acetylglucoside (6.8 μg/mL), ISO (6.1 μg/mL), malonylglucoside (2.5 μg/mL) and glucoside (0.7 μg/mL). The amounts of total phenolic compounds were substantially higher than total flavonoids, with the former ranging from 69.3 μg/mL in the glucoside fraction to 147 μg/mL in the aglycon fraction. For saponins, the acetylglucoside fraction contained the highest concentration of soyasapogenol A (36.9 μg/mL), followed by ISO (27.0 μg/mL), a mixture of acetylglucoside and aglycon (26.8 μg/mL) and the glucoside fraction (10.5 μg/mL), whereas a peak level of soyasapogenol B (198.8 μg/mL) was shown in the acetylglucoside fraction and a low level (11.4 μg/mL) in the aglycon fraction.

Inhibiton of Prostate Cancer Cell Proliferation by Isoflavones. After cultivation of both LNCaP and PC-3 cell lines in culture media containing 3, 2, 1, 0.5 and 0% of water−ethanol (1:1, v/v), the normal cell growth was not affected and a level of 2% water−ethanol (1:1, v/v) was thus chosen for subsequent study. After cultivation of both LNCaP and PC-3 cell lines in a culture medium containing 2.0% water−ethanol (1:1, v/v) for 24−72 h separately, the cell viability was determined with a blood cell counter and a cell survival rate of 97 and 98% was shown for LNCaP (2×10^4 cells/well) and PC-3 (2.5×10^4 cells/well), respectively, implying the normal growth of both cell lines after 72 h incubation. **Figure 1** shows the effect of different concentrations of isoflavones on inhibition of LNCaP as determined by the MTT test. Both the aglycon fraction and ISO exhibited the most pronounced inhibition at a dose of 5 μg/mL, but the other treatments only showed a slight difference. Similarly, at a dose of 10 μg/mL, the aglycon fraction was the most effective in inhibition, while the daidzein standard was the least. A complete inhibition was attained for the aglycon

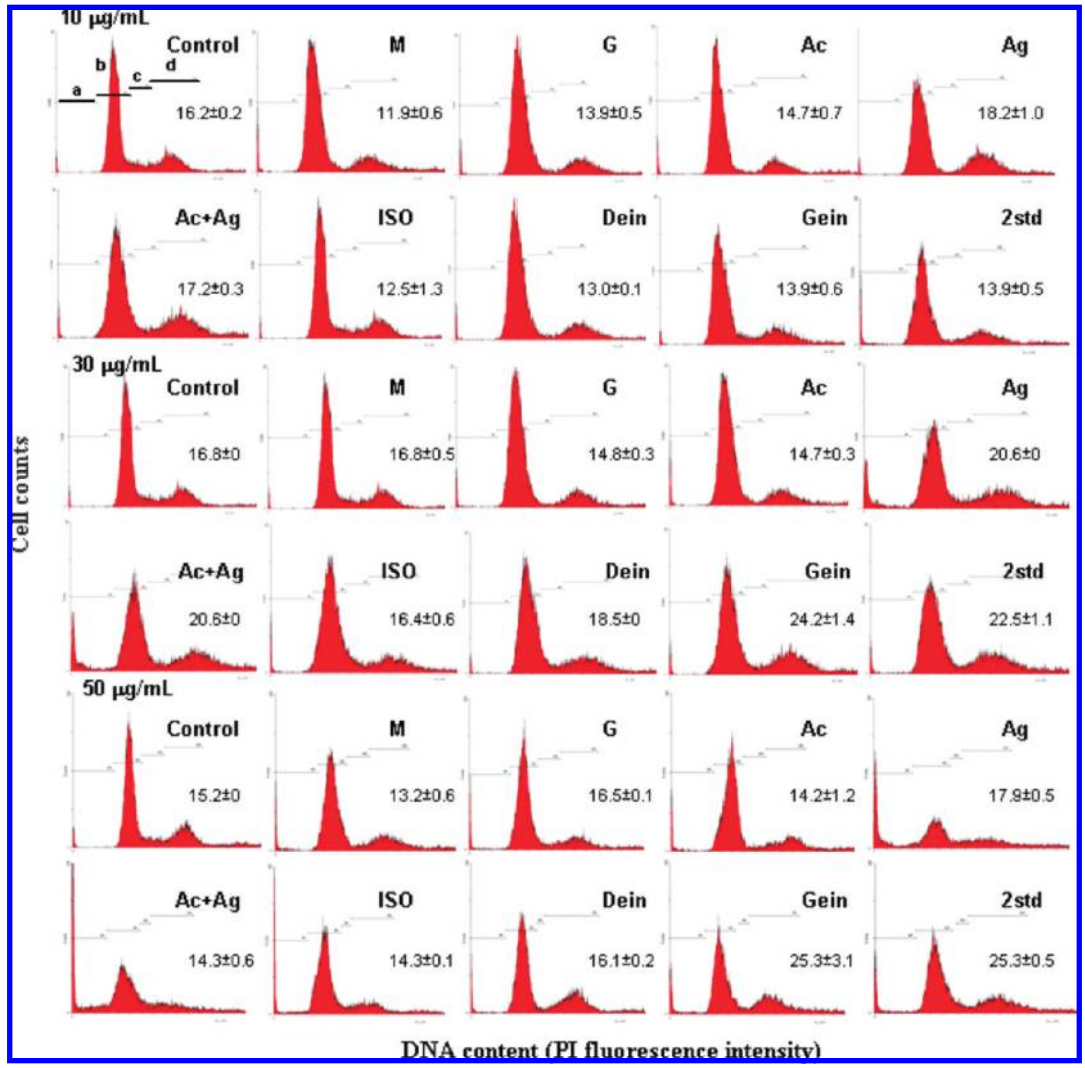

Figure 3. Flow cytometric analysis and G2/M phase ratio of LNCaP cells treated with isoflavone fractions and extracts as well as standards: M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycon fraction; Ac+Ag, a mixture of acetylglucoside and aglycon fractions; ISO, soybean cake extract containing 12 isoflavones; Dein, daidzein; Gein, genistein; 2 std, a combination of daidzein and genistein. a: sub-G0/G1. b: G0/G1. c: S. d: G2/M.

fraction at 20 μg/mL, followed by a mixture of acetylglucoside and aglycon, the genistein standard and a combination of daidzein and genistein standards, with the other treatments ranging from 32.9 to 47.1%. With concentration at 30 μg/mL, both the aglycon fraction and a mixture of acetylglucoside and aglycon showed a complete inhibition as well. Likewise, a complete retardation occurred for the aglycon fraction, a mixture of acetylglucoside and aglycon, and ISO at an elevated dose of 40 and 50 μg/mL.

Figure 2 shows the effect of different concentrations on inhibition of PC-3 cells by the MTT test. Both doses of 5 and 10 μg/mL showed a similar tendency in inhibition, with the cell survival rates being 66.6−91.8% for the former and 68.0−84.7% for the latter. A complete inhibition of PC-3 was observed for both levels at 20 and 30 μg/mL for the aglycon fraction, and the cell survival rates ranged from 50.2 to 75.3% and 26.8 to 74.6% for the other treatments, respectively. At an elevated concentration of 40 μg/mL, both the aglycon and a mixture of acetylglucoside and aglycon fractions also showed a complete retardation, with the other treatments ranging from 7.4% for ISO to 75.9% for the malonylglucoside fraction. Similarly, a complete retardation occurred for the aglycon fraction, a mixture of acetylglucoside and aglycon and ISO at 50 μg/mL, with a high cell survival rate of 70.8% being found for the malonylglucoside fraction and a low cell survival rate of 29.0% for a mixture of two standards. This outcome clearly indicated the aglycon fraction was the most effective in inhibiting PC-3 growth, followed by a mixture of acetylglucoside and aglycon, ISO, a combination of two standards, genistein, daidzein, acetylglucoside, glucoside and malonylglucoside.

Table 3 shows the IC50 value of prostate cancer cell lines of LNCaP and PC-3 as affected by isoflavones from soybean cake. The aglycon fraction showed the lowest IC50 (2.43 μg/mL) for LNCaP cells, followed by ISO (2.88 μg/mL), acetylglucoside (5.04 μg/mL), a mixture of acetylglucoside and aglycon (5.23 μg/mL), a combination of two standards (5.29 μg/mL), glucoside (11.2 μg/mL), malonylglucoside (12.3 μg/mL) and daidzein (22.6 μg/mL). Compared to LNCaP cells, a significantly higher IC50 ($P < 0.05$) was observed for PC-3 cells, which ranged from 10.6 μg/mL for the aglycon fraction to 160 μg/mL for the malonylglucoside fraction.

In comparison, all the isoflavone fractions and extracts as well as isoflavone standards were effective in retarding growth of both LNCaP and PC-3 prostate cancer cells, and the higher the concentration, the better the inhibition. A synergistic effect was also observed for the various isoflavone fractions and standards. In addition, both isoflavone fractions and standards were more efficient in antiproliferation for LNCaP cells than

Table 4. Effect of Isoflavone Fractions and Extracts as Well as Standards on Cell Cycle Distribution of LNCaP Cells[a]

	sub-G0/G1 (%)			G0/G1 (%)			S (%)			G2/M (%)		
isoflavone	10 μg/mL	30 μg/mL	50 μg/mL	10 μg/mL	30 μg/mL	50 μg/mL	10 μg/mL	30 μg/mL	50 μg/mL	10 μg/mL	30 μg/mL	50 μg/mL
control	1.0 ± 0.0 fC	1.2 ± 0.0 efB	1.6 ± 0.1 fA	70.4 ± 1.6 abA	65.2 ± 0.2 bB	61.9 ± 0.2 abC	7.6 ± 0.4 aB	8.5 ± 0.3 bA	7.3 ± 0.1 aB	16.0 ± 1.2 bcAB	15.0 ± 0.2 deB	16.8 ± 0.1 cdA
M	1.0 ± 0.0 fC	1.7 ± 0.1 deB	5.7 ± 1.0 dA	71.7 ± 0.7 aA	53.3 ± 1.4 fC	63.9 ± 1.5 aB	5.3 ± 0.1 bcdC	16 ± 0.7 aA	5.7 ± 0.1 abcB	11.9 ± 0.6 fB	13.2 ± 0.6 fB	16.8 ± 0.5 cdA
G	1.2 ± 0.3 efC	1.9 ± 0.3 cdB	4.5 ± 0.1 deA	69.8 ± 0.8 abA	68.5 ± 0.9 aA	60.0 ± 1.1 abcB	5.1 ± 0.1 bcdeB	6.5 ± 1.3 bcA	5.4 ± 0.0 abcA	13.9 ± 0.5 deC	14.8 ± 0.3 deB	16.5 ± 0.2 cdA
Ac	1.4 ± 0.1 defC	2.5 ± 0.1 cB	5.9 ± 1.9 dA	69.7 ± 0.7 abA	69.7 ± 0.3 aA	59.4 ± 0.6 bcB	4.3 ± 0.1 eA	3.5 ± 0.9 dA	4.7 ± 0.7 abcA	14.7 ± 0.7 cdA	14.7 ± 0.3 deA	14.2 ± 1.2 dA
Ag	6.6 ± 0.2 aC	8.5 ± 0.1 aB	18 ± 0.5 bA	59.9 ± 0.3 dA	45.2 ± 0.5 gB	29.9 ± 0.0 fC	5.3 ± 0.1 bcdB	6.4 ± 0.3 bcA	3.8 ± 0.3 bcdC	18.2 ± 0.4 aB	17.9 ± 0.5 cB	19.8 ± 1.1 bA
Ac+Ag	2.1 ± 0.1 cC	8.8 ± 0.2 aB	21 ± 1.9 aA	65.5 ± 1.7 cA	45.4 ± 0.6 gB	39.1 ± 1.8 eC	5.3 ± 0.8 bcdA	6.0 ± 0.0 bcdA	5.8 ± 1.4 abcA	17.1 ± 1.1 abB	14.3 ± 0.6 efB	20.8 ± 0.3 bA
ISO	2.6 ± 0.1 bC	3.2 ± 0.1 bB	14 ± 0.1 cA	69.4 ± 1.7 abA	61.7 ± 0.4 cB	57.7 ± 0.1 bcC	5.5 ± 0.3 bcA	6.6 ± 1.8 bcA	3.3 ± 0.1 cdB	12.5 ± 1.3 efC	14.3 ± 0.0 efB	16.4 ± 0.6 cdA
Dein	1.1 ± 0.0 fB	1.1 ± 0.0 fB	3.0 ± 0.1 efA	71.6 ± 0.4 aA	64.5 ± 1.3 bB	61.3 ± 4.9 abC	4.4 ± 0.3 deB	7.0 ± 0.6 bcA	6.3 ± 2.9 abAB	13.0 ± 0.1 defC	16.1 ± 0.2 dB	18.5 ± 0.0 bcA
Gein	1.7 ± 0.2 cdB	1.7 ± 0.7 defB	3.4 ± 0.2 efA	69.4 ± 1.4 abA	58.2 ± 0.0 dB	56.9 ± 0.4 cC	5.0 ± 0.6 cdeA	5.0 ± 1.8 cdA	1.9 ± 0.8 dB	13.9 ± 0.6 deB	24.2 ± 1.4 aA	25.3 ± 3.1 aA
2 std	1.6 ± 0.5 deB	1.9 ± 0.2 cdB	5.7 ± 0.2 dA	68.6 ± 1.4 bA	55.7 ± 2.0 eB	48.3 ± 1.2 dC	6.0 ± 0.5 bA	6.0 ± 1.4 bcdA	3.1 ± 0.8 cdB	13.9 ± 0.5 deB	22.5 ± 1.1 bA	23.5 ± 0.5 aA

[a] Average of duplicate analyses ± standard deviation. Symbols bearing different letters (a−g) in the same column are significantly different ($P < 0.05$). Symbols bearing different letters (A−C) in the same row within each period of cell cycle are significantly different ($P < 0.05$). M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycon fraction; Ac+Ag, a mixture of fraction Ac and fraction Ag; ISO, soybean cake extract containing 12 isoflavones; Dein, daidzein; Gein, genistein; 2 std, a combination of daidzein and genistein.

for PC-3 cells. In a similar study Kumi-Diaka (*13*) reported the inhibition of both PC-3 and LNCaP cell growth by genistein at 10, 30, 50 and 70 μg/mL, with a higher concentration showing a better inhibition effect. An inhibition of 69% PC-3 cell growth after 72 h incubation by 50 μM of genistein was reported by Li and Sarkar (*14*). In a later study Oki et al. (*15*) demonstrated a complete inhibition of prostate cancer cell growth of DU145 by genistein at 50, 75 and 100 μM after 72 h incubation. Similarly, the inhibition of LNCaP growth by 50 μM of genistein was 49, 70 and 76% after 24, 48 and 72 h incubation, respectively (*16*). Kumi-Diaka et al. (*17*) further reported the IC50 of LNCaP and PC-3 cells as affected by genistein were approximately 40 μg/mL, and a lower IC50 of LNCaP should be associated with the presence of androgen receptor. Xiang et al. (*18*) compared the effect of genistein and daidzein on growth of LNCaP cells with androgen receptor and DU145 cells without androgen receptor, with the IC50 of the former and the latter being 26 and 45 μM respectively for genistein, 111.7 and >157.4 μM for daidzein, indicating the prostate cancer cells without androgen receptor were less sensitive to isoflavone. In another study Suzuki et al. (*19*) further demonstrated the cell survival rate of LNCaP to be higher than PC-3 in the presence of 50−200 μM genistein after 92 h incubation.

In addition to prostate cancer cells, our previous study (*8*) also demonstrated the inhibition effect of isoflavones on HepG2 cell, with the aglycon fraction and a mixture of 4 isoflavone standards as well as the acetylglucoside fraction showing the most distinct effect. This tendency correlated well to the outcome of our present study, revealing the possibility of isoflavone extract from soybean cake to be effective in antiproliferation of some other types of cancer cell lines.

By summarizing the results in **Table 2**, the most effective aglycon fraction was also found to contain a high level of total flavonoid and phenolic compound, as did a mixture of acetylglucoside and aglycon. But for saponin, both fractions of the acetylglucoside and a mixture of acetylglucoside and aglycon contained the largest content. As all these three isoflavone fractions showed a marked effect in retarding prostate cancer cell growth as well, the presence of functional components like flavonoids, phenolic compounds and saponins in each fraction should play a vital role in antiproliferation of tumor cells. Several authors have also demonstrated the protective effect against cancer cell growth by phenolic compounds and saponins (*20*), and the former may play a more important role in antiproliferation of cancer cells than the latter. In our previous study we proved that the isoflavone fractions possessing high antioxidant activity are more efficient in retarding cancer cell growth (*2, 8*), which is correlated well with the outcome in this study.

Effects of Isoflavones on Cell Cycle of LNCaP and PC-3. **Figure 3** shows the cell cycle of LNCaP as affected by isoflavone from soybean cake at 3 doses of 10, 30 and 50 μg/mL, which was characterized by an increase in the G2/M phase ratio following a rise in isoflavone concentration, implying that most isoflavone fractions and all the isoflavone standards were effective in inhibiting cancer cell mitosis. This phenomenon is in agreement with a previous finding by Oki et al. (*15*), who demonstrated the induction of G2/M cell cycle arrest in the DU145 human prostate cancer cell line by genistein standard. It is worth pointing out that peaks were present in the sub-G0/G1 phase for the fractions of aglycon and a mixture of acetylglucoside and aglycon as well as ISO, indicating that cell apoptosis at high dose may occur (**Table 4**). Meanwhile, the sub-G0/G1 ratio of LNCaP cells increased along with increasing

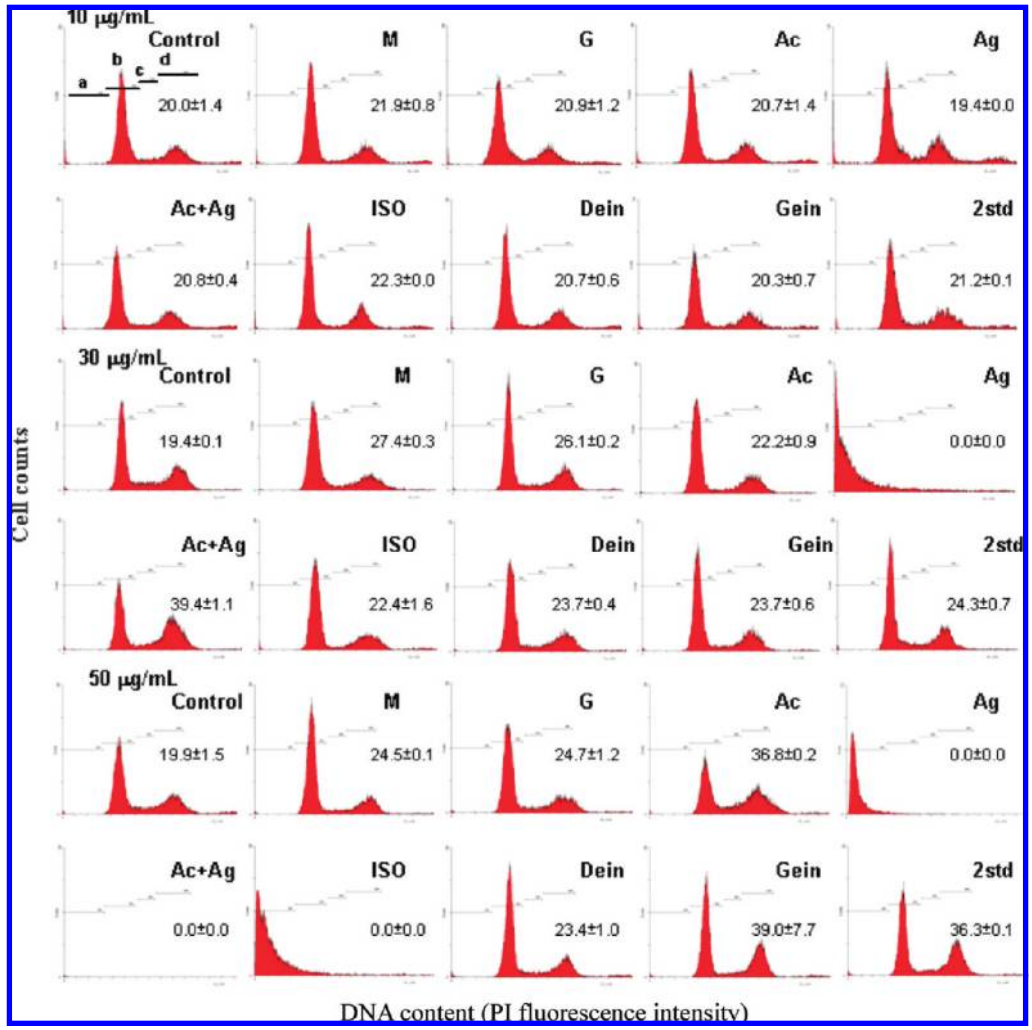

Figure 4. Flow cytometric analysis and G2/M phase ratio of PC-3 cells treated with isoflavone fractions and extracts as well as standards. M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycon fraction; Ac+Ag, a mixture of acetylglucoside and aglycon fractions; ISO, soybean cake extract containing 12 isoflavones; Dein, daidzein; Gein, genistein; 2 std, a combination of daidzein and genistein. a: sub-G0/G1. b: G0/G1. c: S. d: G2/M.

isoflavone concentration for all the treatments, raising the possibility of isoflavones to result in a rise in proportion of cell death or apoptosis. Compared to the control treatment, the sub-G0/G1 ratio of LNCaP cells for the fractions of aglycon and a mixture of acetylglucoside and aglycon, ISO, genistein and a combination of two isoflavone standards was significantly higher ($P < 0.05$), with the aglycon fraction showing the largest ratio. A similar tendency was followed at 30 μg/mL, with a high sub-G0/G1 ratio being shown for fractions of aglycon and a mixture of acetylglucoside and aglycon, and the other treatments ranged from 1.1 to 3.2%. The sub-G0/G1 ratio at 50 μg/mL was significantly higher than at 10 or 30 μg/mL, with a large ratio being observed for the fractions of aglycon and a mixture of acetylglucoside and aglycon as well as ISO, whereas the other treatments showed a low ratio ranging from 1.6 to 5.9%.

With the exception of the malonylglucoside fraction at 50 μg/mL, the G0/G1 ratio declined following a rise in isoflavone concentration, which is mainly caused by an increase in G2/M ratio. This result revealed the cell cycle of LNCaP cells failed to be arrested at G0/G1 phase in the presence of isoflavones. At a dose of 10 μg/mL, there was a slight difference in G0/G1 ratio between most isoflavone treatments and the control treatment. Likewise, at an elevated level of 30 and 50 μg/mL, the G0/G1 ratio of LNCaP cells for most treatments was lower than the control treatment, except the fractions of malonylglucoside, glucoside and acetylglucoside. A low G0/G1 ratio was shown for the fractions of aglycon and a mixture of acetylglucoside and aglycon, which equaled 30 and 39% at 50 μg/mL, respectively, indicating that these treatments did not block the cell cycle at G0/G1 phase.

There was only a slight change in S phase ratio of LNCaP cells for all the isoflavone concentrations. However, the S ratios of LNCaP cells for all the isoflavone treatments were lower than the control treatment except the malonylglucoside fraction at 30 μg/mL. Comparatively, the S ratios of most treatments at 50 μg/mL were less than at 10 or 30 μg/mL, with an S ratio of 1.9, 3.8, 3.3 and 3.1 being observed for genistein, the aglycon fraction, ISO and a combination of two isoflavone standards, respectively, implying these isoflavones could prevent LNCaP cells from entering into the DNA synthesis stage.

For the G2/M phase, the ratios of all the treatments rose following an increase in isoflavone concentration except the acetylglucoside fraction, revealing that most isoflavones were effective in inhibiting LNCaP cell mitosis. However, only a slight difference in G2/M ratio was shown for most treatments at 10 μg/mL, with the exception of the fractions of aglycon and a mixture of acetylglucoside and aglycon, which amounted to 18 and 17%, respectively, and were not significantly different ($P > 0.05$). At a dose of 30 μg/mL, the aglycon fraction, genistein and a combination of two isoflavone standards

Table 5. Effect of Isoflavone Fractions and Extracts as Well as Standards on Cell Cycle Distribution of PC3 Cells[a]

	sub-G0/G1 (%)			G0/G1 (%)			S (%)			G2/M (%)		
isoflavone	10 μg/mL	30 μg/mL	50 μg/mL	10 μg/mL	30 μg/mL	50 μg/mL	10 μg/mL	30 μg/mL	50 μg/mL	10 μg/mL	30 μg/mL	50 μg/mL
control	0.4 ± 0.2 bA	0.6 ± 0.2 bA	0.5 ± 0.1 cA	60.6 ± 2.5 abC	64.7 ± 0.0 aB	66.6 ± 0.5 aA	4.5 ± 0.0 aB	4.8 ± 0.0 cA	4.1 ± 0.1 eC	20.0 ± 1.4 bcA	19.4 ± 0.1 eA	19.9 ± 1.5 eA
M	0.3 ± 0.1 bB	0.4 ± 0.0 bA	0.5 ± 0.1 cA	62.5 ± 0.6 aA	52.1 ± 0.4 dB	53.3 ± 0.5 cB	3.2 ± 0.1 aC	7.7 ± 0.1 aA	6.7 ± 0.1 bB	21.9 ± 0.8 abC	27.4 ± 0.3 bA	24.5 ± 0.1 cB
G	0.5 ± 0.1 bA	0.4 ± 0.1 bA	0.4 ± 0.0 cA	60.4 ± 0.5 abB	60.4 ± 0.3 cB	61.7 ± 1.0 bA	4.7 ± 2.6 aA	5.2 ± 0.0 cA	4.8 ± 0.0 cdA	20.9 ± 1.2 abcB	26.1 ± 0.2 bA	21.6 ± 1.7 deB
Ac	0.6 ± 0.2 bAB	0.5 ± 0.1 bB	0.7 ± 0.1 cA	56.7 ± 1.2 cdB	64.8 ± 0.9 aA	41.7 ± 1.1 eC	3.6 ± 0.4 aB	3.8 ± 0.1 dB	9.1 ± 0.4 aA	20.7 ± 1.4 bcB	22.2 ± 0.9 dB	36.8 ± 0.2 aA
Ag	5.2 ± 0.7 aC	81.0 ± 6.9 aB	89.1 ± 0.1 aA	46.1 ± 1.4 eA	7.1 ± 1.8 fB	3.8 ± 0.1 gC	4.2 ± 0.1 aA	2.1 ± 0.7eB	1.0 ± 0.0 fC	19.3 ± 0.2 cA	2.3 ± 1.0 fB	1.1 ± 0.0 fB
Ac+Ag	0.4 ± 0.0 bB	0.8 ± 0.2 bA	0.0 ± 0.0 dC	55.6 ± 1.6 dA	42.4 ± 0.5 eB	0.0 ± 0.0 hC	4.2 ± 0.2 aB	6.2 ± 0.1 bA	0.0 ± 0.0 gC	20.8 ± 0.4 abcB	39.4 ± 1.1 aA	0.0 ± 0.0 fC
ISO	0.5 ± 0.1 bC	0.8 ± 0.1 bB	83.9 ± 1.2 bA	61.3 ± 0.1 abA	61.9 ± 0.9 bcA	6.5 ± 1.2 fB	3.1 ± 0.1 aA	3.4 ± 0.4 dA	1.3 ± 0.1 B	22.9 ± 0.9 aA	22.4 ± 1.6 dA	1.8 ± 0.4 fB
Dein	0.1 ± 0.0 bC	0.4 ± 0.0 bB	0.7 ± 0.2 cA	58.8 ± 1.1 bcB	61.2 ± 0.5 cA	61.8 ± 0.4 bA	3.4 ± 0.2 aC	5.4 ± 0.0 cA	4.5 ± 0.2 deB	20.7 ± 0.6 abcB	23.7 ± 0.4 cdA	23.4 ± 1.0 cdA
Gein	0.6 ± 0.1 bAB	0.6 ± 0.1 bA	0.8 ± 0.2 cA	56.1 ± 0.5 cdB	63.4 ± 1.2 abA	54.7 ± 2.7 cB	4.4 ± 1.4 aA	4.9 ± 0.2 cA	4.2 ± 0.1 eA	20.3 ± 0.7 bcC	23.2 ± 0.1 cdB	33.4 ± 0.2 bA
2 std	0.2 ± 0.1 bC	0.6 ± 0.0 bB	0.8 ± 0.0 cA	55.4 ± 0.0 dB	62.3 ± 0.9 bcA	48.0 ± 1.7 dC	4.0 ± 0.5 aB	5.4 ± 0.2 cA	5.1 ± 0.4 cA	21.2 ± 0.1 abcC	24.3 ± 0.7 cB	36.3 ± 0.1 aA

[a] Average of duplicate analyses ± standard deviation. Symbols bearing different letters (a−h) in the same column are significantly different ($P < 0.05$). Symbols bearing different letters (A−C) in the same row within each period of cell cycle are significantly different ($P < 0.05$). M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag, aglycon fraction; Ac+Ag, a mixture of fraction Ac and fraction Ag; ISO, soybean cake extract containing 12 isoflavones; Dein, daidzein; Gein, genistein; 2 std, a combination of daidzein and genistein.

exhibited a higher G2/M phase ratio than the other treatments, which ranged from 13 to 16%. A similar trend occurred at 50 μg/mL as well, with a high G2/M phase ratio of 20, 21, 25 and 24% for the fractions of aglycon and a mixture of acetylglucoside and aglycon, genistein and a combination of two isoflavone standards, respectively, implying that the cell cycle was arrested at the G2 check point and mitosis stage.

The tendency in cell cycle distribution of PC-3 cells was similar to that of LNCaP cells (**Figure 4** and **Table 5**). The sub-G0/G1 ratio of PC-3 cells for most treatments also followed an increased trend for a rise in concentration, depicting that a high dose of isoflavones could facilitate cell death or apoptosis. At a low level of 10 μg/mL, only the aglycon fraction showed a higher sub-G0/G1 ratio than the control treatment, indicating cell apoptosis could be induced by the former. The same phenomenon was shown at 30 and 50 μg/mL, with the sub-G0/G1 ratio of the aglycon fraction being 127 and 198 times higher than the control treatment, respectively. Interestingly, the sub-G0/G1 ratio of ISO climbed sharply to 83.9% at 50 μg/mL, which was 186 fold larger than the control treatment. Surprisingly, no cell cycle was detected for a mixture of acetylglucoside and aglycon fractions at 50 μg/mL, probably caused by cell death under high concentration.

Unlike LNCaP cells, the changes in ratios of G0/G1 of PC-3 cells were inconsistent. Most isoflavone treatments showed a lower G0/G1 ratio than the control treatment at 10 μg/mL, with the exception of the fractions of acetylglucoside, aglycon, and a mixture of acetylglucoside and aglycon, daidzein, genistein and a combination of two isoflavone standards. However, at 30 μg/mL, the G0/G1 ratios of all the treatments were lower than the control treatment except the acetylglucoside fraction. Similarly, the G0/G1 ratios of PC-3 cells for all the treatments were significantly lower than the control treatment at 50 μg/mL, especially for the aglycon fraction and ISO. Like the sub-Go/G1 phase, the cell cycle was not detected for a mixture of acetylglucoside and aglycon fractions, which can be attributed to cell death under high concentration (50 μg/mL). Similar to LNCaP cells, the S phase ratio change of PC-3 cells was inconsistent, which should be due to presence of a small quantity of cells at S phase.

For the G2/M phase ratio, it increased along with increasing isoflavone concentration for most treatments, but a slight difference in G2/M phase ratio was shown at 10 μg/mL. However, at 30 μg/mL, all the treatments possessed a higher G2/M phase ratio than the control treatment except the aglycon fraction, which should be caused by apoptosis of most cells. The G2/M phase ratio further climbed to 37, 33 and 36% at 50 μg/mL for the acetylglucoside fraction, genistein and a combination of two isoflavone standards, respectively, suggesting that the cell cycle be arrested at the G2/M phase. Meanwhile, both the aglycon fraction and ISO may induce cell apoptosis amid a large decline in G2/M ratio and a rise in sub-G0/G1 ratio. Like sub-G0/G1 and G0/G1 phases, a mixture of acetylglucoside and aglycon fractions could cause cell death as no cell cycle was detected under high concentration (50 μg/mL). Comparatively, the cell cycle analysis of both LNCaP and PC-3 cells showed a similar trend in that the cell mitosis stage was arrested, and the cell death could occur for aglycon fraction, a mixture of acetylglucoside and aglycon fraction and ISO at high concentration.

Most studies in the literature showed that genistein could induce G2/M phase arrest (*15*). Some authors also suggested the cell cycle to be arrested at the G0/G1 phase by isoflavones (*21*). Our outcome indicated the soybean cake isoflavones could

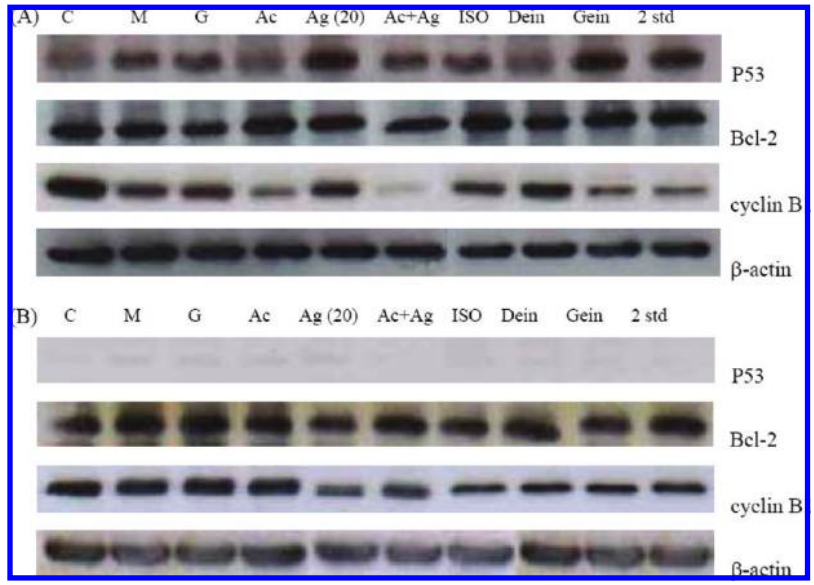

Figure 5. The p53, Bcl-2, cyclin B1 and β-actin protein expression of LNCaP (**A**) and PC-3 (**B**) cells treated with isoflavone fractions and extracts as well as standards. C, control treatment; M, malonylglucoside fraction; G, glucoside fraction; Ac, acetylglucoside fraction; Ag (20), aglycon fraction with a concentration of 20 μg/mL; Ac+Ag, a mixture of acetylglucoside and aglycon fractions; ISO, soybean cake extract containing 12 isoflavones; Dein, daidzein; Gein, genistein; 2 std, a combination of daidzein and genistein.

elevate the ratios in sub-G0/G1 and G2/M phases, but lower the ratios in S and G0/G1 phases of both LNCaP and PC-3 cells, resulting in an arrest of the cell cycle of cancer cells at G2/M phase with the cell mitosis being diminished to inhibit DNA synthesis to cause cell apoptosis or death. Sakamoto (*22*) reported a level of 5−20 μg/mL of genistein to raise the G2/M ratio from 14.7 to 28.1% for PC-3 cells. An increment in G2/M ratio of LNCaP from 25.5% to 39.9% and 43.5% in the presence of 37 and 74 μM genistein, respectively, was observed by Xiang et al. (*18*). In another study Oki et al. (*15*) illustrated the presence of 75 μM genistein to induce a decline in G1 and S ratios from 39 and 36% to 4 and 15%, respectively, accompanied by a rise in G2/M ratio from 25 to 81%. In a similar study Shenouda et al. (*23*) demonstrated that the G2/M ratios of LNCaP and PC-3 cells in the presence of genistein were raised from 16.7 to 38.8% for the former and 14.0 to 28.4% for the latter. Bemis et al. (*24*) reported an increase in G2/M ratio for both LNCaP and PC-3 cells after treatment of 25 μM of genistein in combination with polysaccharide for 24 h. In a recent study Kao et al. (*8*) proved the presence of 10−50 μg/mL of isoflavone fractions and extracts from soybean cake to raise the ratios in sub-G0/G1 and G2/M phases of HepG2 cells. The result in our experiment was similar to that reported above, but because of difference in cell line and reaction time, the ratios of different phases in cell cycle may be varied.

Of the various isoflavone fractions and extracts, both aglycon and a mixture of acetylglucoside and aglycon were the most effective in inhibiting prostate cancer cell growth, which may be accounted for by the difference in isoflavone structure and the presence of functional components like phenolics and flavonoids as mentioned in the previous section. Furthermore, the presence of acetyl group attached to the glucose moiety may be vital in inhibiting cancer cell growth (*25*). In addition, the antioxidant activity of isoflavones should play a key role in inhibiting growth of both LNCaP and PC-3 prostate cancer cell lines.

Effect of Isoflavones on Expression of Proteins Associated with Apoptosis and Cell Cycle. As described before, isoflavones can induce arrest of the G2/M phase during cell cycle and cell apoptosis. Thus, the expressions of proteins associated with G2/M phase regulation such as cyclin B1 and that with cell apoptosis like p53 and Bcl-2 have to be examined. Since a high dose (50 μg/mL) of the aglycon fraction can cause cell death after 24 h cultivation, a level of 20 μg/mL was chosen to study the protein expression for the aglycon fraction, whereas a concentration of 50 μg/mL was used for the other treatments.

Figure 5 shows the expressions of p53, Bcl-2 and cyclin B1 of both LNCaP and PC-3 prostate cancer cell lines following treatments of isoflavones from soybean cake. β-Actin was not discussed in the text because it was regarded as an internal loading control. With the exception of the acetylglucoside fraction and daidzein, the other treatments showed a higher expression of p53 than the control treatment for LNCaP cells, especially for the aglycon fraction, genistein and a combination of two standards (**Figure 5A**). However, no p53 expression was observed for PC-3 cells (**Figure 5B**). The Bcl-2 expression was less affected by isoflavone treatments in both LNCaP and PC-3 cell lines. Only the aglycon fraction showed a slightly lower expression of Bcl-2 than the control treatment in PC-3 cells. For inhibition of cyclin B1 expression of LNCaP cells, a mixture of acetylglucoside and aglycon fractions exhibited the most pronounced effect, followed by acetylglucoside fraction, genistein and a combination of two standards. The other treatments only showed a slight difference, but were significantly lower than the control treatment. Likewise, there was only a slight difference in cyclin B1 expression of PC-3 cells between the control treatment and fractions of malonylglucoside, glucoside and acetylglucoside. But for the fractions of aglycon and a mixture of acetylglucoside and aglycon, the cyclin B1 expression of PC-3 cells was substantially lower than the other treatments.

p53 is a tumor suppressor gene, which can bind with DNA to regulate genes of $p21^{WAF1}$, *GADD45* and *14-3-3σ*. Among them, $p21^{WAF1}$ can bind with cdks2 (cell division-stimulating protein) leading to arrest of cell cycle at G1 phase, while both *GADD45* and *14-3-3σ* genes were closely related to G2/M transition. But, if DNA was seriously damaged, cells could undergo apoptosis through regulation of *Bax*, *CD95* (*Fas/APO-1*), *Killer/DR5*, *Ei24/PIG8*, *Noxa*, *PERP*, *Pidd*, *p53AIP1* and *PUMA* genes by p53 (*26*). The Bcl-2-family proteins are important regulators of apoptosis and consist of both antiapo-

ptotic proteins (Bcl-xL, Bcl-2, KSHV-Bcl-2, Bcl-w) and proapoptotic proteins (Bax, Bid), which share sequence homology within conserved regions known as BCL-2 homology domains (*27*). The main function of Bcl-2 is to inhibit cell apoptosis, yet the functional p53 can down-regulate Bcl-2 expression to induce apoptosis (*28*). In our study cell apoptosis was more pronounced for the aglycon fraction, genistein and a combination of two standards, which was probably caused by a higher protein expression of p53. Bemis et al. (*24*) also found the p53 expression of LNCaP could be enhanced by 10−40 μM genistein, with the effect being proportional to concentration. Despite the effect that p53 can lower Bcl-2 expression, our study did show the Bcl-2 expression to be unaffected by p53, indicating that isoflavone fractions and ISO may only have a minor influence on Bcl-2 expression. Instead, these isoflavones may possess a greater effect on proteins such as Bax and Caspase-3.

In addition to cell apoptosis, isoflavones have been proved to be capable of inhibiting cancer cell growth through cell cycle regulation (*10, 28*). The complex formation of cyclin D/Cdk4 (or cyclin D/cdk6) is necessary for cells to enter into the G1 phase, followed by proceeding to the S phase in the presence of cyclin E/cdk2 complex. Cyclin A was not formed until the final period of G1 phase, and then accumulated at the S and G2 phases (*29*). Cyclin B is mainly responsible for regulation of the G2 and M phases, and the former could be transferred to the latter at elevated concentration of cyclin B (*29*). As discussed previously, both LNCaP and PC-3 cell lines could be arrested at the G2/M phase by isoflavones from soybean cake, resulting in a decrease of cyclin B1 expression, especially for LNCaP cells. Of the various isoflavones, the fractions of acetylglucoside, aglycon, and a mixture of acetylglucoside and aglycon, genistein and a combination of two standards showed the most pronounced effect in lowering cyclin B1 expression. A similar outcome was observed by Choi et al. (*29*), who reported that the presence of 100 μM of genistein could diminish cyclin B1 expression of PC-3. The same phenomenon was also demonstrated in several other studies (*10, 29*).

In conclusion, the fractions of aglycon, a mixture of acetylglucoside and aglycon, and ISO were more effective in antiproliferation of LNCaP and PC-3 cancer cells than the other isoflavone fractions. The p53 protein expression of LNCaP increased following treatments of aglycon, genistein and a combination of genistein and daidzein standards, however, all the treatments did not affect Bcl-2 protein expression significantly in both LNCaP and PC-3 cell lines. The mixture of acetylglucoside and aglycon fractions showed the highest inhibition in cyclin B1 expression of LNCaP cells, but not for PC-3 cells. The outcome of this study clearly demonstrated the potential of using soybean cake as raw material for production of isoflavone-based functional food. Moreover, this is the first report to prove the antiproliferation effect of prostate cancer cell lines by various isoflavone fractions prepared from soybean cake. The production cost can thus be reduced as soybean cake is a byproduct obtained during soybean oil processing and often used in the feed industry.

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