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Effect of Soyasapogenol A and Soyasapogenol B Concentrated Extracts on Hep-G2 Cell Proliferation and Apoptosis

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The growth inhibition and the induction of apoptosis brought about by soyasaponins extracted from soy flour (*Glycine max* (L.)) and concentrated for soyasapogenols A and B formed by hydrolysis were tested for cytoactivity in the human hepatocellular carcinoma cell line Hep-G2. Concentrated soyasapogenol A (SG-A) and soyasapogenol B (SG-B) extracts contained approximately 69.3% and 46.2% of their respective aglycones (soyasapogenols) assessed by HPLC and ESI-MS, while the soyasaponin extract (TS), derived from crude methanol extraction, did not contain any detectable amounts of SG-A or SG-B. An MTT viability assay showed that all three extracts had an effect on Hep-G2 proliferation in a dose-response manner with 72 h LC50 values of 0.594 ± 0.021 mg/mL for TS, 0.052 ± 0.011 mg/mL for SG-A, and 0.128 ± 0.005 mg/mL for SG-B. Apoptotic cells were determined by flow cytometry cell cycle analysis and confocal laser scanning microscopy (CLSM). Cell cycle analysis indicated a significant ($P < 0.05$) greater sub-G1 buildup of apoptotic cells at 24 h (25.63 ± 2.1%) and 72 h (47.1 ± 3.5%) for the SG-A extract compared to SG-B, whereas the TS extract produced only a minor buildup of sub-G1 cells. CLSM confirmed a morphological change of all treatments after 24 h, at the respective LC50 concentrations. These results show that the samples that contained mainly soyasapogenols A and B showed a greater ability to inhibit proliferation of cultured Hep-G2 when compared to a total soyasaponin extract that did not contain any soyasapogenols.

KEYWORDS: Soyasaponins; soyasapogenol; apoptosis; Hep-G2

INTRODUCTION

There has been a considerable focus on soy constituents, for their potential health-promoting functions such as the estrogen-like activity of phytoestrogens and the cholesterol lowering properties of soy protein. A third potential bioactive group of compounds are soyasaponins which are oleanane-triterpenes based on two main sapogenol (aglycones) structure classifications (A, B) and are found in various glycoside forms along with DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) forms conjugated at position 22 of the structure (*1*). Soyasaponins A and soyasaponins B are the most abundant type of saponins found in soybean (*Glycine max* (L.)) and related products. The saponin content is generally between 1.83% and 4.35%, depending on the variety and cultivation conditions (*2*). The structures of soyasapogenols A and B are very similar but differ slightly by the addition of a hydroxyl group on soyasapogenol A compared to soyasapogenol B (**Figure 1**). Cytoactivity studies have recently reported that a concentrated soyasaponin extract can inhibit proliferation and induce apoptosis in Hela cells (*3, 4*) and induce macroautophagic cell death in cultured human colon cells (HCT-15) (*5, 6*). Soyasaponins have also been reported to protect against *tert*-butyl hydroperoside damage in liver cells (Hep-G2) (*7*) and rat hepatocytes (primary culture) (*8*). Soyasapogenols A and B have been shown to possess greater cytoactivity compared to their respective soyasaponin glycosides (*9*). Rowlands et al. (*10*) reported soyasapogenols A and B inhibited cell proliferation of estrogen-insensitive breast cancer cells and soyasapogenol B inhibited estrogen-sensitive breast cancer cells (MCF-7), while soyasapogenols A were found to be estrogenic and stimulated the growth of these cells. In this study, we have refined an extraction methodology (*11*) to concentrate soyapogenols derived from soy flour for cytoactive testing. The purpose of this study was to test the hypothesis that the cytoactive response varies between extracts of soyasaponins (TS), derived from methanol extraction, and ones concentrated for either soyasapogenol A (SG-A) or B (SG-B) formed by hydrolysis, on cell proliferation and apoptosis in human hepatocarcinoma cells (Hep-G2), a common model of carcinoma, liver metabolism, and cholesterol regulation (*12*).

MATERIALS AND METHODS

Extraction and Isolation of Sample Material. *Extraction of Total Soyasaponins (TS) from Soy Flour.* Defatted soy flour was obtained from Archer Daniels Midland Co. (Decatur, IL, USA). The flour (100 g) was extracted in methanol (500 mL) for 4 h at 60 °C as described by Gurfinkel et al. (*11*). After filtration, the liquid extract was evaporated to 100 mL and mixed with 100 mL of 0.4 M ammonium sulfate. The

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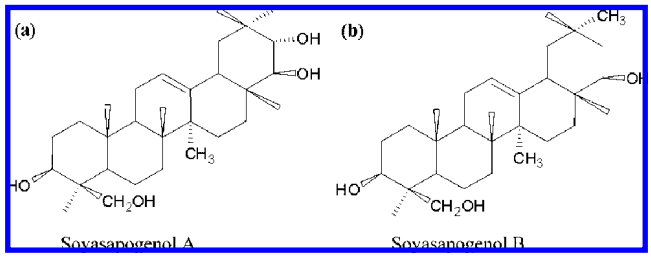

Figure 1. Chemical structure of soysapogenol A **(a)** and Soyasopgenol B **(b)**, respectively.

mixture was kept at 4 °C overnight. After removing the precipitate by filtration which contained nonsoyasaponin components and protein, the liquid extraction was evaporated to less than 100 mL to remove methanol, and ammonia sulfate (3 M) was added and refrigerated. The precipitate was recovered by centrifugation and lyophilized and stored at 4 °C and is referred to herein as the total soyasaponins extract (TS).

Preparation of Concentrated Soyasapogenol A (SG-A) and B (SG-B) Extracts. The total soyasaponin extract (560 mg) was dissolved in 200 mL of methanol and subsequently diluted to a methanol concentration of 30% prior to solid phase extraction (SPE). The sample was loaded onto a 30% methanol preconditioned 10 g C-18 Sep-Pak cartridge (Phenomenex, USA). The Sep-Pak was flushed with 150 mL of water and then 150 mL of 30% methanol to remove any remaining isoflavone glucosides. The Sep-Pak was washed with 50% methanol (100 mL) to remove the group A soyasaponins. A wash with 70% methanol (100 mL) removed the group B soyasaponins. The group A soyasaponins containing 50% methanol solution (100 mL) were diluted to 30% methanol with water (total volume of 333 mL), and group B soyasaponins with 70% methanol were diluted to 50%. Both were loaded again onto a 10 g C-18 Sep-Pak, respectively, and eluted by 100% methanol (each 50 mL).

To produce a concentrated soyasapogenol A and soyasapogenol B extract, samples were hydrolyzed using a modified method of Gurfinkel et al. (*11*). Briefly, group A soyasaponins were hydrolyzed in 200 mL of methanol and 25 g of anhydrous sodium sulfate and 6 mL of concentrated sulfuric acid (3% of the total volume) and refluxed for 5 h. The methanol was decanted from the sodium sulfate and diluted to 30% with water and loaded onto a preconditioned 10 g C-18 Sep-Pak cartridge (Phenomenex, USA). The Sep-Pak was flushed with 200 mL of water and then 150 mL of 30% methanol to remove isoflavone glucosides. Soyasapogenol A was removed by 100% methanol and lyophilized, yielding SG-A extract (105 mg), and a similar method yielded SG-B extract (112 mg).

HPLC and ESI-MS Analysis. A Waters Symmetry column (C18, 4.6 mm × 250 mm, 5 μm particle size, Waters, Milford, MA, USA) was used for the separation, and the sample injection volume was 50 μL. Solvent A consisted of 0.025% (v/v) acetic acid in water. Solvent B was 0.025% (v/v) acetic acid in acetonitrile, and the column temperature was held constant at 35 °C. The flow rate was 1.0 mL/min, and the elution program was originally described by Jin et al. (*13*). The solvent program was as listed: Time 0 (13% B), 12.5 min (30% B), 17.5 min (40% B), 23.5 min (40% B), 27.5 min (60% B), 30.0–35 min (100% B), 40.0 min (13% B). The detection wavelength was 250 nm.

Authentic standards of soyasapogenols A and B (APIN Chemicals LTD, UK) were used to establish a calibration curve. The calibration curves were linear over the amount with square correlation coefficients of $r^2 > 0.995$. The calibration curves were $y = 1 \times 10^9 x + 511\,175$ and $y = 7 \times 10^9 x + 1 \times 10^7$ for soyasapogenols A and B, respectively.

Detection and molecular weight confirmations of the soyasapogenols were established by a Finnigan LCQ quadrupole ion trap MS with MS^n capabilities in negative mode. With an ion spray voltage of 4.5 kV and with a capillary temperature of 350 °C and capillary voltage of 40 eV, 0.4 mL/min was delivered to ESI-MS, and the rest diverted to waste. The scan mass spectra were focused on the *m/z* range of 200–500 u.

Cell Culture. The human hepatocellular carcinoma cell line (Hep-G2) was obtained from ATCC (Manassas, VA). Cells were maintained in DMEM medium (Dulbecco's modified Eagle's medium, Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum (Sigma, Steinheim, Germany) and penicillin/streptomycin (100 units/mL) (GIBCO; Invitrogen; Canada). Cultures were maintained at a cell concentration between 2×10^5 and 1×10^6 cells/mL. Cells were subcultured by total medium replacement using 0.25% (w/v) trypsin−0.53 mM EDTA solution (GIBCO; Invitrogen; Canada) every 3 days, depending on cell number, and incubated at 37 °C in a 5% CO_2 humidified incubator. Viable cells and cell count were assessed by 0.04% trypan blue (MP Biomedicals; Ohio; USA) exclusion dye using a hemocytometer. Viable cell numbers were assessed in quadruplicate.

Cell Viability MTT Assay Dose-Response. A cell viability assay, based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Steinheim, Germany) reduction by viable cells, was used to establish an LC50 (e.g., concentration to inhibit 50% of cells) of the prepared extracts. Hep-G2 cells were seeded in 96-well plates to a final concentration of 1×10^5 cells/mL. Controls contained test model cells and culture medium, but no test compounds. Concentrations of TS extract (dissolved in medium) were between 0.1 and 1.0 mg/mL. Concentrations of SG-A and SG-B extracts (dissolved in medium with less than 5% DMSO) were between 0.05 and 0.5 mg/mL. Cells were incubated for 3 days before MTT was added to a concentration of 0.5 mg/mL and incubated in the dark for 4 h (*14*). To solubilize the formazan crystal, 100 μL of SDS (10%) (National University of Medical Institution, Singapore) in HCl (0.1 M) was added to each well and incubated overnight. The optical density was read at 550 nm absorbance and 650 nm (reference absorbance) in a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, USA).

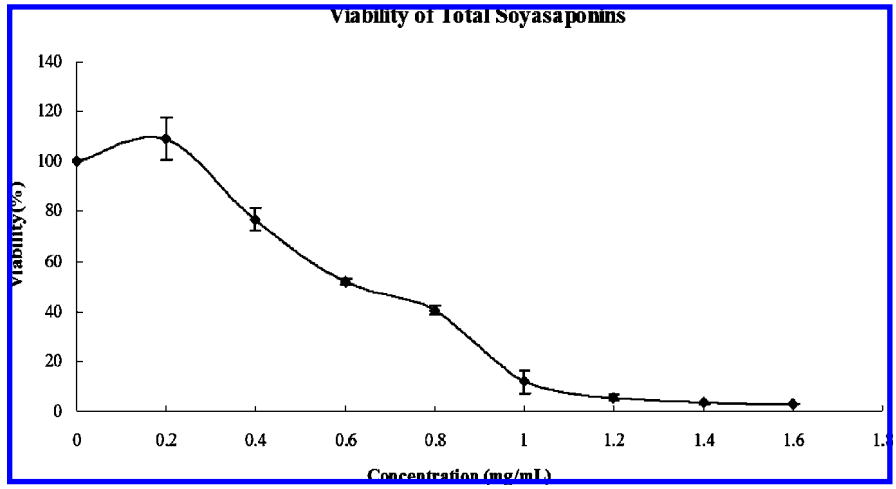

Figure 2. Dose-response relationship of a total saponin (TS) extract after 3 day incubations with Hep-G2 cells ($n = 8$, triplicate) by an MTT viability assay as outlined in the Materials and Methods. Values are expressed as a percentage of untreated cells (mean ± SD).

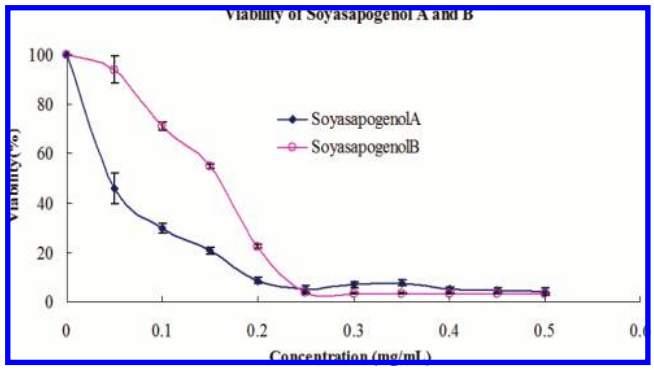

Figure 3. Dose-response relationship of concentrated soyasapogenol A (SG-A) and soyasapogenol B (SG-B) extracts after 3 day incubations with Hep-G2 cells ($n = 8$, triplicate) by an MTT viability assay as outlined in the Materials and Methods. Values are expressed as a percentage of untreated cells (mean ± SD).

Flow Cytometry Cell Cycle Analysis. Three distinct extracts, TS, SG-A, and SG-B, were added to Hep-G2 cells (1×10^6 cells/mL) at their respective LC50 concentrations as described below. Cells were incubated at 37 °C in a 5% CO_2 humidified incubator for 24, 48, and 72 h with untreated cells acting as a control. After treatment, cells in suspension were centrifuged for 10 min (80*g*). The supernatant was discarded, and cells were further washed twice in PBS and centrifuged to remove the cell pellet. The pellet was vortexed vigorously, and 1 mL of ice-cold 70% ethanol was added slowly to fix the cells and was stored overnight at 4 °C. Ethanol was removed by centrifugation (5 min, 750*g*) and gently vortexed followed by the addition of 1 mL of PBS containing propidium iodide (50 μg/mL) (Sigma, Steinheim, Germany) and RNase A (100 U/mL) (Applichem Inc., Cheshire, USA) as described by Noguch (*15*). Samples were incubated at room temperature for 30 min and analyzed by Dako Cytomation Cyan LX flow cytometry (Beckman Coulter, Fullerton, Calif, USA) and a software package.

Confocal Observation and Examination. Hep-G2 cells were seeded in a 4-well Laboratory-Tek Chamber Slide System (Electron Microscopy Science, Hatfield, PA) at a concentration of 2×10^5 cells/mL. Three extracts, TS, SG-A, and SG-B, were added to the chamber slide at their respective LC50 concentrations, and untreated cells acted as control. Untreated and treated cells were incubated at 37 °C in a 5% CO_2 humidified incubator for 24 h. After treatment, the supernatant was discarded and cells were washed by PBS. Cells were treated in 4% formalin in PBS for 20 min and washed by PBS for 5 min. Absolute cold methanol was added to cells for 5 min to permeate the cell membrane. Cells were further washed in PBS and stained with 30 μg/mL of propidium iodide in PBS and incubated for 1 h. Cells were washed by PBS three times after incubation and air-dried. The cell samples were scanned with a confocal laser scanning microscope (CLSM) (Carl Zeiss Lsm510 META, Thornwood, NY). CLSM was equipped with an argon laser providing 25 mW of argon ions at 458, 488, and 515 nm and 1 mW HeNe Green 543 nm wavelengths and a

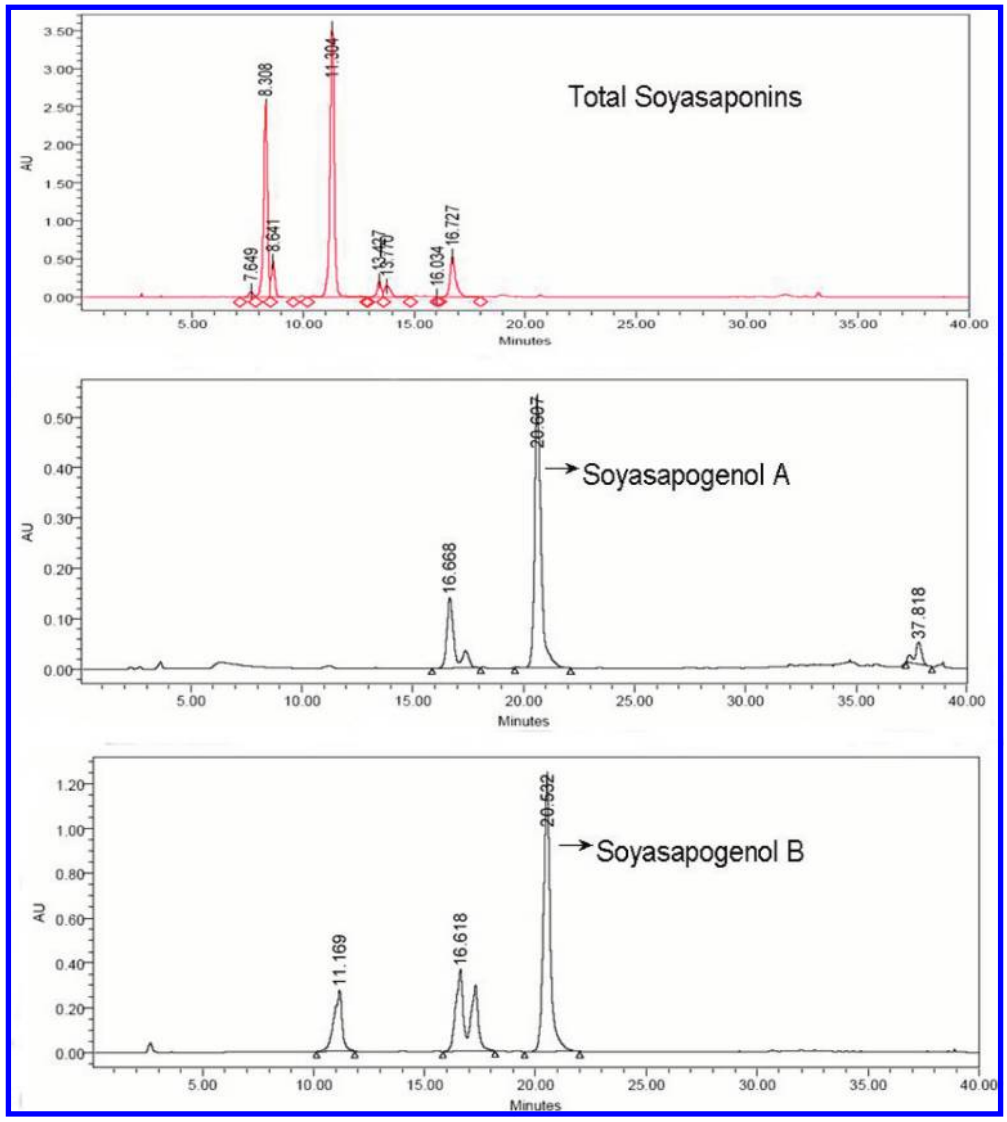

Figure 4. High-performance liquid chromatographic trace of total soyasaponin and soyasapogenol A and B extracts.

Table 1. ESI-MS Ion Fragments of Concentrated Soyasapogenol A and B Extracts

soyasapogenol	empirical formula	calculated mass	main ion fragments $[M - H]^-$
A	$C_{30}H_{50}O_4$	474.4	473.3
			269.3
			249.0
			325.2
B	$C_{30}H_{50}O_3$	458.4	457.4
			311.3
			339.4
			325.2

Table 2. Cell Cycle Distribution of Hep-G2 Cells Treated with TS, SG-A, and SG-B extracts for 24, 48, and 72 h, and Untreated Cells Acted as Control[a]

time	control	TS	SG-A	SG-B
		Sub-G1 (%)		
24 h	1.17 ± 0.6^a	7.38 ± 1.6^{ab}	25.63 ± 2.1^c	12.16 ± 7.0^b
48 h	2.03 ± 0.9^a	6.1 ± 0.4^b	22.36 ± 1.0^c	23.17 ± 2.4^{cd}
72 h	2.57 ± 1.4^a	9.32 ± 0.2^b	47.1 ± 3.5^d	15.32 ± 4.2^{bc}
		G 0/G 1 (%)		
24 h	74.90 ± 2.3^a	62.64 ± 0.2^a	41.36 ± 1.3^b	58.85 ± 15.6^a
48 h	81.17 ± 2.7^a	67.48 ± 1.0^b	52.19 ± 0.3^c	62.09 ± 13.5^a
72 h	85.62 ± 1.3^a	64.44 ± 0.3^b	41.43 ± 2.3^c	77.31 ± 6.5^a
		S (%)		
24 h	10.8 ± 1.8^a	6.83 ± 0.3^a	6.12 ± 0.4^a	11.41 ± 4.3^a
48 h	7.50 ± 1.5^a	3.15 ± 0.3^b	3.89 ± 0.3^b	3.99 ± 0.4^b
72 h	3.82 ± 0.4^{ab}	3.27 ± 0.1^{ab}	4.26 ± 0.2^a	3.15 ± 1.1^b
		G 2/M (%)		
24 h	14.13 ± 1.4^a	23.87 ± 1.4^b	27.62 ± 0.6^b	16.03 ± 8.9^a
48 h	10.16 ± 1.4^a	23.59 ± 1.0^b	22.18 ± 0.5^b	12.22 ± 14.4^a
72 h	8.58 ± 0.5^a	23.41 ± 0.4^b	8.57 ± 1.1^a	5.85 ± 3.8^a

[a] Values are expressed as mean ± SD. Numbers of the same period and phase with different letters are significantly different ($P < 0.05$).

CCD camera for real color imaging by a halogen lamp or epifluorescence with a mercury lamp. The cell surface was confirmed by prescanning. Then, the specimen from top to bottom was scanned layer by layer to obtain optical sections, and each image was recorded. The optical sections were reconstructed to stereoscopic images with the CLSM reconstruction program.

Statistical Methods. A one-way ANOVA (SPSS 12.0) was used to analyze the experimental data at 24, 48, and 72 h time periods. Significance was judged at $P < 0.05$ using the Duncan post hoc multiple comparisons of observed means.

RESULTS

Dose-Response LC50 Determination of Total Soyasaponins, Soyasapogenols A and B. Dose-response curves of TS, SG-A, and SG-B extracts are shown in **Figures 2** and **3**, respectively. The LC50 was calculated from a plot of viability (%) versus log concentration (graph not shown) which yielded a linear equation of $y = -132.97x + 418.96$ ($r^2 = 0.9808$) for TS and $y = -66.502x + 164.01$ ($r^2 = 0.9871$) and $y = -126.89x + 317.61$ ($r^2 = 0.9238$) for SG-A and SG-B, respectively. The LC50's were determined from three separate experiments with at least eight replicates per experiment and were found to be 0.594 + 0.021 mg/mL for TS, 0.052 ± 0.011 mg/mL for SG-A, and 0.128 + 0.005 mg/mL for SG-B.

HPLC-MS. Figure 4 shows the HPLC chromatograph of three extracts, a total soyasaponin extract and both of the concentrated soyasapogenol A and B extracts. The soyasapogenol A content of the SG-A extract was calculated to be 69.3 mg/100 mg of dry weight (69.3%), and soyasapogenol B content of the SG-B extract was found to be 46.2 mg/100 mg of dry weight (46.2%). Neither soyasapogenol A nor B was detectable in the TS extract. Confirmation of the soyasapogenol extracts was needed due to the similar HPLC retention time, and this was achieved by ESI-MS analysis. Each soyasapogenol extract gives different EI mass spectra and exact mass measurement of the molecular ions as shown in **Table 1**. The measurement of soyasapogenols A and B $[M - H]^-$ were 473.3 and 457.4, respectively, corresponding to their individual molecule weights (**Table 1**).

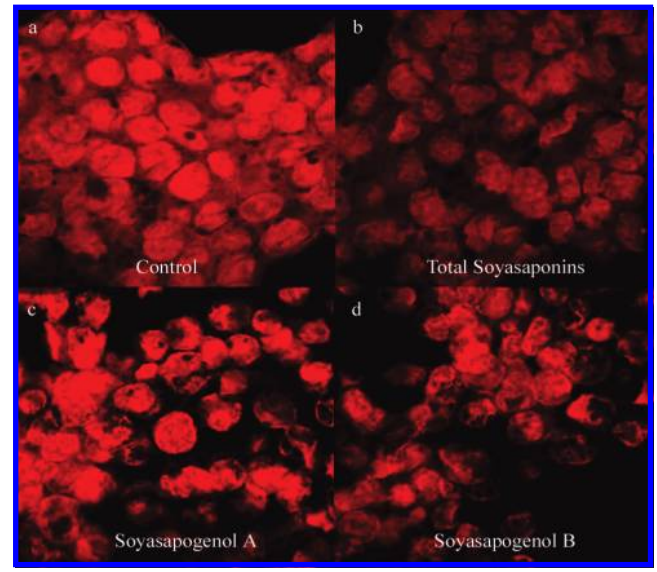

Figure 5. Confocal laser scanning microscopic image after 24 h exposure to three extracts at their respective LC50 concentrations and stained with propidium iodide as described in the Materials and Methods. Panel **(a)** is untreated control cells, **(b)** represents total soyasaponins, and panels **(c)** and **(d)** are soyasapogenol A and B extracts, respectively.

Cell Cycle Distribution. The cell cycle of the control and treated Hep-G2 cells was measured by flow cytometry, and representative DNA histograms of Hep-G2 cells treated by test compounds at 24, 48, and 72 h are shown in **Figure 6**. Soyasapogenol A treatment produced significantly ($P < 0.05$) greater sub-G1 apoptotic cells at 24 h (25.63 + 2.1%) and 72 h (47.1 + 3.5%) compared to SG-B, TS, and control (**Table 2**). At 48 h, all extracts produced a significantly greater percentage of sub-G1 cells compared to control.

Cell Morphology. CLSM images of PI stained Hep-G2 cells treated with three extracts are shown in **Figure 5**. All treated cells displayed some morphological traits such as nuclear condensing (pyknosis) and fragmentation (karyorrhexis) consistent with the apoptotic program cell death and serve as visual confirmation of the cell cycle analysis.

DISCUSSION

In this study, we have shown the relative effects of three distinct extracts derived from soy flour on cultured human hepatocellular carcinoma cell (Hep-G2) proliferation and apoptosis. Extracts that contained mainly soyasapogenols A and B showed a greater ability to inhibit proliferation of cultured Hep-G2 when compared to a TS extract that did not contain any detectable soyasapogenols. These results are consistent with other reports on potential cytoactive saponins which show that the aglycones derived from ginseng triterpenes have a lower LC50 than their corresponding glycosides (ginsenosides) (*16*). The LC50 value of the total soyasaponin extract prepared from soy flour without acid hydrolysis was approximately 4.5 times greater than SG-A and slightly 11 times greater than that for

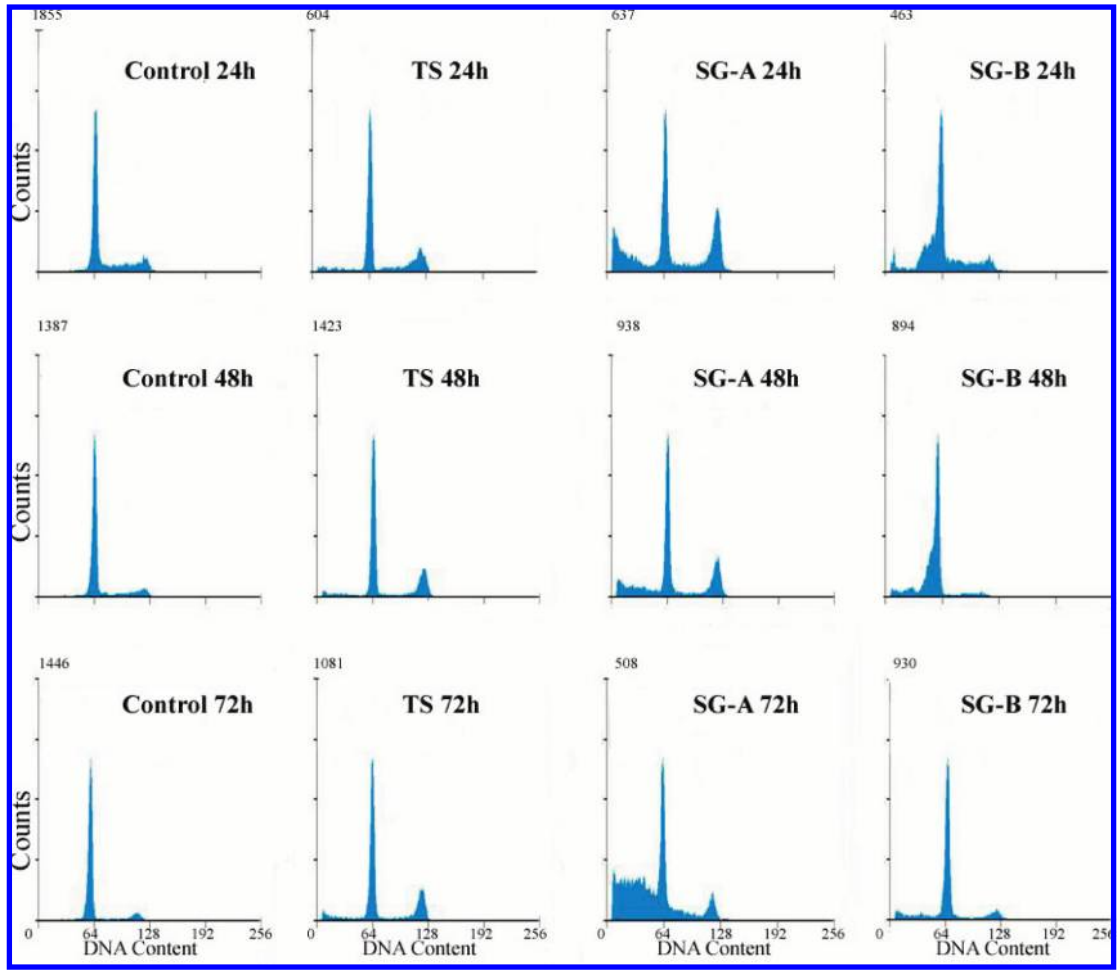

Figure 6. DNA cell-cycle histograms of control (untreated) cells and TS-, SG-A-, and SG-B-treated cells for 24, 48, and 72 h, respectively. Cells were fixed in 70% ethanol and stained with PI as described in the Materials and Methods. DNA histograms shown are representative histograms of three separate experiments.

the SG-B extract. These results demonstrate that soyasapogenols have a stronger affinity to inhibit cell proliferation than samples containing soyasaponin glycosides alone. Aglycones formed by hydrolysis of saponins have been reported to have biological activity which is absent or present in a lesser degree in their corresponding saponins (*17*). However, this is in contrast to the antiprotozoan activity of saponin glycosides in the rumen compared to the aglycones reviewed by Cheeke (*18*). Gurfinkel and Rao (*9*) reported a relationship between soyasaponin structure based on the aglycones lipophilicity and a cytotoxic effect on colon cancer cells (HT-29) compared to glycosidic soyasaponins A1, A2, and I and both deacetylated and acetylated groups. They reported that soysaponin III and B monoglucoronide had only marginal activity (*9*). The nonpolar relationship of ginsenoside aglycones compared to the glycosides has also been observed (*16, 19*), and this influenced cytoactivity. In soy and other saponin-containing plants, the saponin and glycoside content varies considerably and depends on the plant part being extracted, plant species, environmental storage, and processing conditions (*17*) and can be activity influenced by in vivo intestinal biotransformation (*20*). It is noteworthy that all three extracts produced were able to influence sub-G1 buildup of apoptotic cells which is a characteristic of apoptosis (*21*). SG-A achieved nearly 50% apoptotic cells at 72 h of exposure, whereas TS and SG-B did not at the respective LC50 concentrations. It is possible that soyasapogenol A can influence cell death by a different cellular mechanism compared to soyasapogenol B and a total soyasaponin extract. Soyasaponin B has been reported to influence macroautophagic cell death, a type of programmed cell death in cultured breast (*6*) and intestinal cancer cells (*5*). Saponins have long been observed to interact with cholesterol effectively (*18*) and have been shown to interact and alter cellular membranes in cell culture models (*22*). Soyasaponin I, a group B soyasaponin, has been reported to interact with the cell surface and decease the migration and metastatic potential through decreasing expression of α 2,3-linked sialic acid in cultured murine cells (*23*).

In this study, concentrated extracts of soyasapogenols A and B extracted from soy flour were shown to influence cultured hepatocarcinoma cell viability and apoptosis to a greater degree than a soyasaponin extract. It is noteworthy that all prepared extracts of soyasaponins were found to be cytoactive and triggered apoptotic cell death, whereas an extract without any detectable soyasapogenols only marginally influenced apoptosis. Further studies are planned to determine the precise mode of cell death and cytoactive mechanism attributed to individual soyasaponins and soyasapogenols.

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