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Antiproliferation of Hepatoma Cell and Progression of Cell Cycle as Affected by Isoflavone Extracts from Soybean Cake

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Abstract: The objectives of this study were to isolate various isoflavone fractions and extracts from soybean cake by preparative column chromatography and compare them with isoflavone standards with regards to inhibition of HepG2 cancer cell proliferation. Four fractions, including malonylglucoside, glucoside, acetylglucoside and aglycone, and two isoflavone extracts, ISO-1 and ISO-2, were collected for evaluation. MTT test results showed that most treatments were slightly protective against HepG2 cell growth at a low dose of isoflavone (5 and 10 μg/mL). However, at elevated concentration of isoflavone (20-50 μg/mL), both aglycone and acetylglucoside fractions as well as a mixture of isoflavone standards were the most effective in inhibition, demonstrating a possible synergistic phenomenon. Genistein showed a better retardation effect than daidzein. For cell cycle analysis, both aglycone and acetylglucoside fractions and a mixture of isoflavone standards exhibited a high G2/M ratio, correlating well with the result of MTT test. The presence of some other functional components in soybean cake like saponins and phenolic compounds may also play a vital role in inhibiting HepG2 cell growth.

Keywords: Soybean cake; isoflavone extracts; MTT test; cell cycle analysis.

1. Introduction

According to a report by the American Institute for Cancer Research [1], Western people are more likely to be exposed to cancer risks such as breast, colon and prostate cancers than Oriental people, which may be associated with lifestyle and diet habits. Adlercreutz [2] further pointed out that Oriental

men consumed more vegetable foods, while Western men consumed more animal products rich in protein and fat, which may affect hormone synthesis and metabolism leading to difference in incidence of cancers. Epidemiological studies have demonstrated that the intake of more vegetables and fruits in diets can reduce cancer risk, which can be attributed to the presence of functional components like flavonoids [3,4].

Isoflavones, a major class of flavonoids, are a class of phytoestrogen mainly present in soybean foods and has been shown to be present at a high concentration in blood and urine of men in regions with a low occurrence of cancer risk [5]. Isoflavones have been shown to be effective in preventing breast cancer through binding with estrogen receptor and sex hormone binding globulin for reduction of estrogen activity [6]. In addition, isoflavones may inhibit cancer cell proliferation and phosphorylation by retarding activities of tyrosine kinase and DNA topoisomerase, as well as induce cancer cell differentiation and apoptosis through anti-angiogenesis [6].

Over the past decade numerous reports have been published regarding cancer inhibition by isoflavones, but most studies have focused on the aglycone isoflavones daidzein and genistein. It has been well documented the inhibition efficiency of growth of various types of cancer cells can be dependent upon isoflavone concentration. Peterson and Barnes [7] reported that both daidzein and genistein may suppress growth of breast cancer cell lines MCF-7 and MDA-468. In a similar study Scholar and Toews [8] found that a 50% inhibition of breast cancer cell occurred for 1-2 μM genistein, however, a complete retardation was attained at 100 μM. Likewise, the genistein dose to achieve a 50% inhibition of breast cancer cell line MCF-10F was 19-22 μM (IC_{50}), whereas a 90% retardation was noted at 45 μM [9]. It was also observed that breast cancer cell failed to proliferate after genistein treatment because the cell cycle was terminated at G2/M phase, but in a study dealing with inhibition of prostate cancer cell lines PC3 and LNCaP as affected by genistein alone and a mixture of genistein and β-lapachone, Kumi-Diaka [10] revealed that both provided an inhibitory effect and increased cell apoptosis rate following a rise in genistein concentration, but a level of 70 μM was necessary to attain a similar effect. In a later study Chang *et al*. [11] reported that liver cancer cell HepG2 can be terminated at G2/M phase by genistein because of activation of ataxia-telangiectasia-mutated (ATM) gene through decrease of cdc 2 activity and enhancement of checkpoint kinase (chk 2) activity. Likewise, the dose in retarding 50% proliferation of Chinese hamster lung fibroblast cell line (V 79) by genistein was 75 μM (20.3 μg/mL) [12]. Oki *et al*. [13] also reported a complete inhibition (100%) of human prostate cancer cell line (DU 145) in the presence of 50, 75 or 100 μM of genistein.

All the studies mentioned above clearly indicated that isoflavones like daidzein and genistein were efficient in inhibiting cancer cell growth. However, there is still a paucity of data regarding the tumor cell growth as affected by isoflavone extracts or a mixture of isoflavone standards. Soybean cake, a defatted soybean meal product obtained during processing of soybean oil, has been shown to be a rich source of isoflavones and other functional components such as saponin and phenolic compounds [14]. The objectives of this study were to isolate four fractions of isoflavones, malonylglucoside, acetylglucoside, glucoside and aglycone, as well as two isoflavone extracts from soybean cake by preparative column chromatography and compared with various isoflavone standards in terms of antiproliferation of HepG2 tumor cell and progression of cell cycle.

2. Results and Discussion

The concentration of isoflavone standards and extracts used in this experiment were based on those reported in the literature and the results of our preliminary studies. Table 1 shows the contents of various isoflavones in each fraction and standard solutions [14].

2.1 MTT test

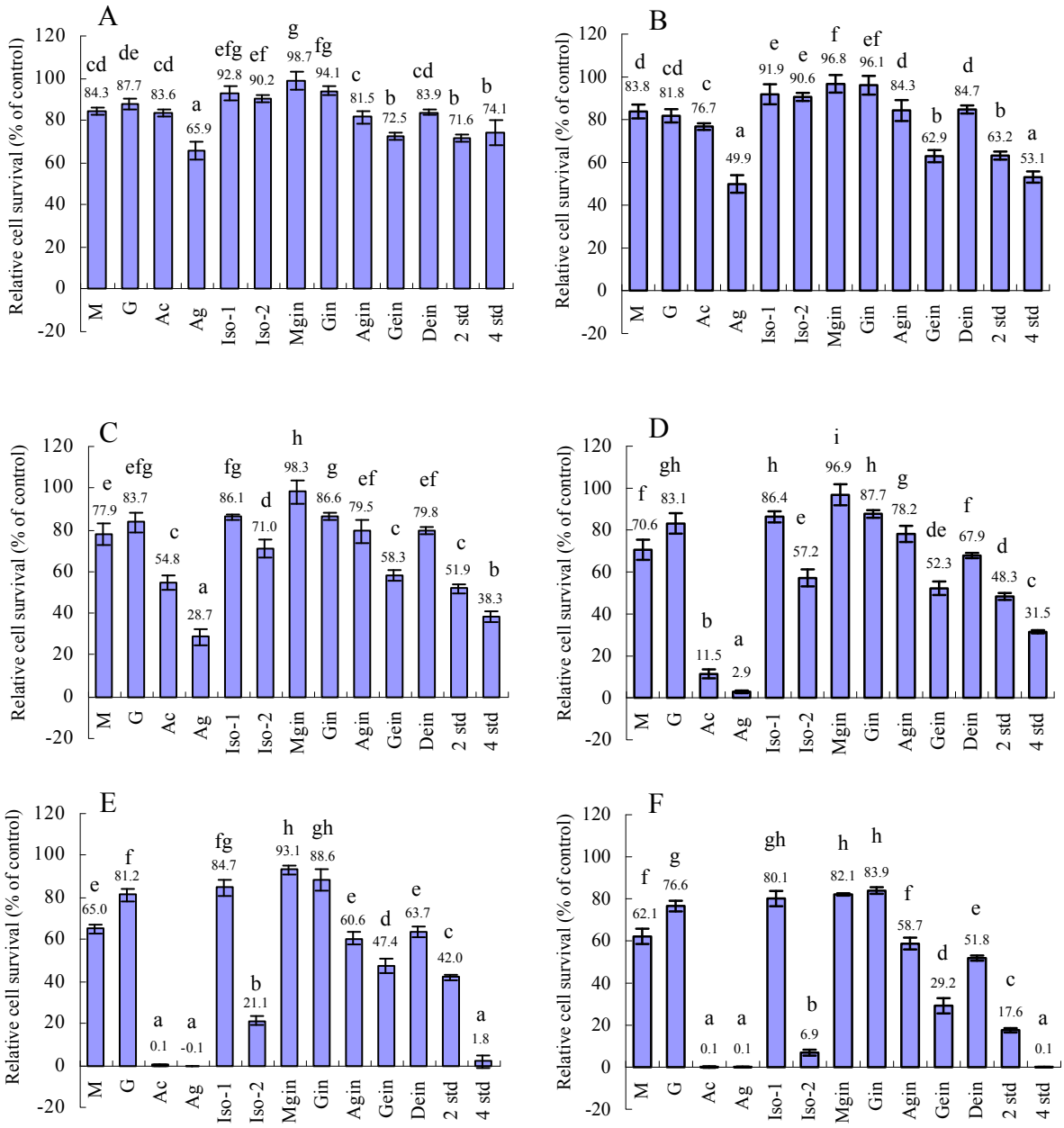
Figure 1 shows the effect of various concentrations of isoflavone fractions and extracts as well as standards on relative cell survival percentage of HepG2 cell after 72 h. The aglycone fraction showed a 65.9% cell survival at a low dose of 5 μg/mL, which was substantially lower than the other four isoflavone fractions and two extracts (ISO-1 and ISO-2) as well as standards at the same dose, with the survival rate ranging from 83.6-92.8%. This outcome indicated that a low concentration of 5 μg/mL is inadequate to suppress HepG2 cell growth. However, with concentration at 10 μg/mL, a slight improvement in HepG2 cell growth inhibition was observed for all the isoflavone fractions and extracts, especially for the aglycone fraction, as the cell survival rate decreased to 49.9%. Following a rise in both concentrations of isoflavone fractions and extracts, the aglycone fraction possessed the most pronounced inhibition (71.3%) at 20 μg/mL, followed by acetylglucoside (45.2%), ISO-2 (29.0%), malonylglucoside (22.1%), glucoside (16.3%) and ISO-1 (13.9%).

Table 1. Contents (μg/mL) of isoflavones in each fraction and standard solutions.

Isoflavones	Isoflavone fractions						Isoflavone standard solutions						
	M	G	Ac	Ag	ISO-1	ISO-2	Mgin	Gin	Agin	Gein	Dein	2 std	4 std
Malonyldaidzin	48.8±0.3	-	-	-	22.9±0.0	12.0±0.0	-	-	-	-	-	-	-
Malonylglycitin	16.0±0.2	-	-	-	8.1±0.2	4.0±0.0	-	-	-	-	-	-	-
Malonylgenistin	35.1±1.2	-	-	-	17.0±0.1	8.8±0.1	100.0	-	-	-	-	-	100.0
Daidzin	-	25.3±0.1	-	-	9.1±0.1	6.3±0.1	-	-	-	-	-	-	-
Glycitin	-	29.2±0.2	-	-	9.0±0.1	7.3±0.1	-	-	-	-	-	-	-
Genistin	-	45.9±1.6	-	-	26.3±0.1	11.5±0.2	-	100.0	-	-	-	-	100.0
Acetyldaidzin	-	-	19.7±0.9	-	1.3±0.0	4.9±0.1	-	-	-	-	-	-	-
Acetylglycitin	-	-	12.8±0.7	-	1.0±0.0	3.2±0.2	-	-	-	-	-	-	-
Acetylgenistin	-	-	69.0±1.6	-	2.5±0.0	17.3±0.3	-	-	100.0	-	-		100.0
Daidzein	-	-	-	47.3±0.5	1.4±0.1	11.9±0.1	-	-	-	-	100.0	100.0	-
Glycitein	-	-	-	16.3±0.2	0.6±0.0	4.1±0.0	-	-	-	-	-	-	-
Genistein	-	-	-	36.4±0.1	1.0±0.0	9.2±0.1	-	-	-	100.0	-	100.0	100.0
Total	99.9±1.7	100.4±0.8	101.5±2.1	100.0±0.6	100.2±0.0	100.5±0.2	100.0	100.0	100.0	100.0	100.0	200.0	400.0

M: malonylglucoside fraction; G: glucoside fraction; Ac: acetylglucoside fraction; Ag: aglycone fraction; ISO-1: soybean cake extract containing 12 isoflavones; ISO-2: a mixture of 4 fractions of isoflavone extracts. Mgin: malonylgenistin; Gin: genistin; Agin: acetylgenistin; Gein: genistein; Dein: daidzein; 2 std: a mixture of daidzein and genistein standards; 4 std: a mixture of malonylgenistin, genistin, acetylgenistin and genistein standards. Based on a previous report by Kao and Chen [14].

Figure 1. Inhibition effect of isoflavones on Hep G2 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: aglycone fraction; ISO-1: soybean cake extract containing 12 isoflavones; ISO-2: a combination of four groups of isoflavone fractions; Mgin: malonylgenistin; Gin: genistin; Agin: acetylgenistin; Gein: genistein; Dein: daidzein; 2 std: a combination of daidzein and genistein; 4 std: a combination of malonylgenistin, genistin, acetylgenistin and genistein.



Likewise, a distinct suppression occurred for both aglycone and acetylglucoside fractions at 30 μg/mL (97.1 and 88.5%, respectively), whereas a complete inhibition (100%) was attained at 40 or 50 μg/mL. For ISO-2 at 40 and 50 μg/mL, a high inhibition of 78.9 and 93.1% was also shown, respectively. Like isoflavone fractions and extracts at low dose (5 μg/mL), all isoflavone standards

only provided a slightly protective effect against tumor cell growth. Nevertheless, a mixture of isoflavone standards (2 std and 4 std) did show a better inhibition than single isoflavone standard, implying a synergistic effect may occur. Of the five isoflavone standards, genistein was the most effective against cancer cell proliferation, which may be due to the presence of one more hydroxyl group. Comparatively, a marked inhibition was achieved for 2 std and 4 std at 50 μg/mL, which amounted to 82.4 and 99.9%, respectively.

In the MTT test, all the treatments at low dose (5 and 10 μg/mL) showed poor inhibition of HepG2 cell growth, with the exception of acetylglucoside fraction and a mixture of isoflavone standards as well as genistein. However, at elevated concentrations (20-50 μg/mL), a marked inhibition occurred for both aglycone and acetylglucoside fractions compared to isoflavone standards, which may be due to the presence of some other functional components like saponins, flavonoids and phenolic compounds [14]. In a previous study Kao and Chen [14] reported that both aglycone and acetylglucoside fractions exhibited higher antioxidant activity than isoflavone standards, which may be also responsible for retardation of HepG2 cell growth. It is worth pointing out that a combination of four isoflavone fractions (ISO-2) did possess a high inhibition at a level of 40 or 50 μg/mL.

For isoflavone standards used alone, genistein provided the most distinct inhibition, followed by daidzein and acetylgenistin, while both malonylgenistin and genistin showed a low suppression effect. From the structural point of view, genistein contains one more hydroxyl group than daidzein and should exhibit a higher antioxidant activity, which may lead to a greater inhibition of HepG2 tumor cell growth. Similar outcome was reported by several other authors. Hewitt and Singletary [15] studied the effect of various levels of soybean extracts and genistein as well as daidzein in feed on breast cancer cell line in BALB/c rats, and found the most efficient inhibition (90%) to be 0.6% soybean extract, with daidzein being less effective in decreasing tumor weight than genistein. A report by Constantinou *et al*. [16] also revealed soybean extract containing 750 ppm genistein to be more effective in inhibiting cancer cell growth than isoflavone standards, probably caused by the combination effect of genistein and some other functional components like saponins and phenolic compounds [14]. Di Virgilio *et al*. [12] compared the effect of different levels of genistein, daidzein and equol on inhibition of tumor cell V79 and reported the retardation concentration of genistein to be 5-25 μM. However, a level of 25 or 100 μM was necessary for daidzein to achieve the same inhibition effect. Sakamoto [17] studied the effect of thearubigin and genistein on inhibition of human prostate cancer cell line (PC-3) and found the former to be ineffective at a dose of 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 μg/mL. But, for genistein, a level of 5 μg/mL was adequate for inhibition, and a synergistic effect did occur for thearubigin and genistein when combined. In a similar study dealing with inhibition of prostate cancer cell line PC3 and LNCaP as affected by β-lapachone (topoisomerase inhibitor) and genistein, Kumi-Diaka [10] demonstrated that a combination of both showed a greater inhibition effect than when used alone. All these results strongly suggested that a mixture of isoflavone standards should perform better than single isoflavone standard in tumor cell antiproliferation.

According to a report by Wei *et al*. [18,19], genistein could inhibit tumor promoter-induced hydrogen peroxide formation and UV-induced 8-hydroxy-2′-deoxyguanosine in mice skin. The antioxidant activity of isoflavones may lead to diminishing peritoneal tumor recurrence, inhibiting cancer cell metastasis and lowering occurrence of prostatic intraepithelial neoplasia [20-22]. In our previous study we also demonstrated that with concentration at 100 μg/mL, both isoflavone fractions

and standards were effective in scavenging DPPH free radicals, chelating ferrous ion and inhibiting conjugated diene formation as well as liposome oxidation [14]. It was also observed that the isoflavone fractions exhibited a larger antioxidant activity than isoflavone standards, especially for the aglycone and acetylglucoside, which may be due to the presence of some other functional components such as saponins and phenolic compounds [14]. This phenomenon correlated well with the result of MTT test, implying the antioxidant activity of isoflavone fractions should play a vital role in retarding HepG2 cell proliferation. Nonetheless, it is worth pointing out that for antioxidant activity test, acetylgenistin was less efficient than genistein or daidzein, but for MTT test, the difference between acetylgenistin and daidzein or genistein was minor. This outcome suggested that the presence of acetyl group in acetylgenistin may be important in inhibiting HepG2 cell growth. A similar phenomenon was reported by Popiolkiewicz *et al*. [23], who studied the effect of genistein and its glucoside on toxicity of tumor cell Balb/c 3T3 and found that genistein glucoside containing an acetyl moiety possessed a higher biological activity. Also, the more the acetyl group in the isoflavone structure, the better the antiproliferation of cancer cells. It can be postulated that the acetyl group-containing isoflavone glucosides may be more selective to motality of tumor cells.

Table 2. The IC_{50} value of isoflavone fractions and extracts from soybean cake as well as isoflavone standards.

Isoflavone	M	G	Ac	Ag	Iso-1	Iso-2	Mgin	Gin	Agin	Gein	Dein	2 std	4 std
IC_{50} (μg/mL)	69.4	186.8	19.9	8.8	163.7	28.7	171.3	196.8	65.3	29.9	53.4	23.8	15.1

M: malonylglucoside fraction; G: glucoside fraction; Ac: acetylglucoside fraction; Ag: aglycone fraction; ISO-1: soybean cake extract containing 12 isoflavones; ISO-2: a combination of four groups of isoflavone fractions; Mgin: malonylgenistin; Gin: genistin; Agin: acetylgenistin; Gein: genistein; Dein: daidzein; 2 std: a combination of daidzein and genistein; 4 std: a combination of malonylgenistin, genistin, acetylgenistin and genistein.

Table 2 shows the IC_{50} value of isoflavone fractions and extracts as well as standards. The lowest IC_{50} value was shown for the aglycone fraction, implying a low concentration (8.8 μg/mL) is adequate to inhibit 50% HepG2 cell growth. The mixture of four isoflavone standards (4 std) also showed a low IC_{50} value (15.1 μg/mL), followed by acetylglucoside fraction (19.9 μg/mL), 2 std (23.8 μg/mL), ISO-2 (28.7 μg/mL), genistein (29.9 μg/mL), daidzein (53.4 μg/mL), acetylgenistin (65.3 μg/mL), malonylglucoside (69.4 μg/mL), ISO-1 (163.7 μg/mL), malonylgenistin (171.3 μg/mL), glucoside (186.8 μg/mL) and genistin (196.8 μg/mL). As there is no information available regarding the IC_{50} of HepG2 cell as affected by isoflavone fractions and extracts, our study demonstrated that isoflavone and its glucoside derivatives may possess different inhibition effect on tumor cell growth, with a mixture of isoflavone standards being more effective than single isoflavone standard. A similar outcome was observed by several other authors. Frey et al. [9] reported that a level of 45 μM (12 μg/mL) genistein was adequate to inhibit growth of 90% human mammary cancer cell MCF-10F.

Kumi-Diaka [10] depicted that the human prostate cancer cell (PC-3 and LNCaP) could be retarded by genistein at 10, 30, 50 and 70 μg/mL, with a high inhibition (90-100%) attained at 70 μg/mL. Likewise, the IC_{50} of genistein in inhibiting Chinese hamster liver fibroblast cancer cell (V79) was 75 μM (20.3μg/mL) [12]. However, a complete inhibition (100%) of human prostate cancer cell (DU 145) occurred for genistein at 50, 75 and 100 μM [13]. All these results further proved that the inhibition efficiency of various carcinoma cells can be dependent upon the level of genistein.

2.2 Cell cycle analysis

Figure 2 and Table 3 show the cell cycle and proportion of cells at sub-G0/G1, G0/G1, S and G2/M phases as affected by isoflavone fractions and extracts as well as standards. The ratio of sub-G0/G1 increased with increasing concentrations of isoflavone fractions and extracts as well as standards, indicating a large proportion of cell may undergo apoptosis. A higher ratio of sub-G0/G1 was shown for ISO-2, 2 std, 4 std and genistein at a dose of 10 μg/mL. Likewise, the 4 std treatment showed the highest sub-G0/G1 ratio at 30 μg/mL, followed by ISO-2, acetylglucoside, aglycone, 2 std, genistein and acetylgenistin, whereas the other treatments only showed minor change. With concentration at 50 μg/mL, a similar trend occurred in the ratio of sub-G0/G1 for 2 std, 4 std, genistein, acetylgenistin, ISO-2 and aglycone, but there were no significant difference between any of these treatments.

In contrast to sub-G0/G1, the ratio of G0/G1 declined following a rise in concentrations of isoflavone fractions and extracts as well as standards, indicating the G0/G1 phase of cell cycle regulation was not perturbated. A low dose of 10 μg/mL resulted in the G0/G1 ratio of all the treatments being significantly lower than control treatment, but with a slight difference among these treatments. The lowest G0/G1 ratio was shown for 4 std, followed by ISO-2, aglycone, genistein, genistin, 2 std, ISO-1, malonylglucoside, daidzein, acetylglucoside, malonygenistin, acetylgenistin and glucoside. A similar tendency occurred for both doses at 30 and 50 μg/mL, with a low G0/G1 ratio for 4 std, 2 std, aglycone, ISO-2, acetylglucoside and genistein.

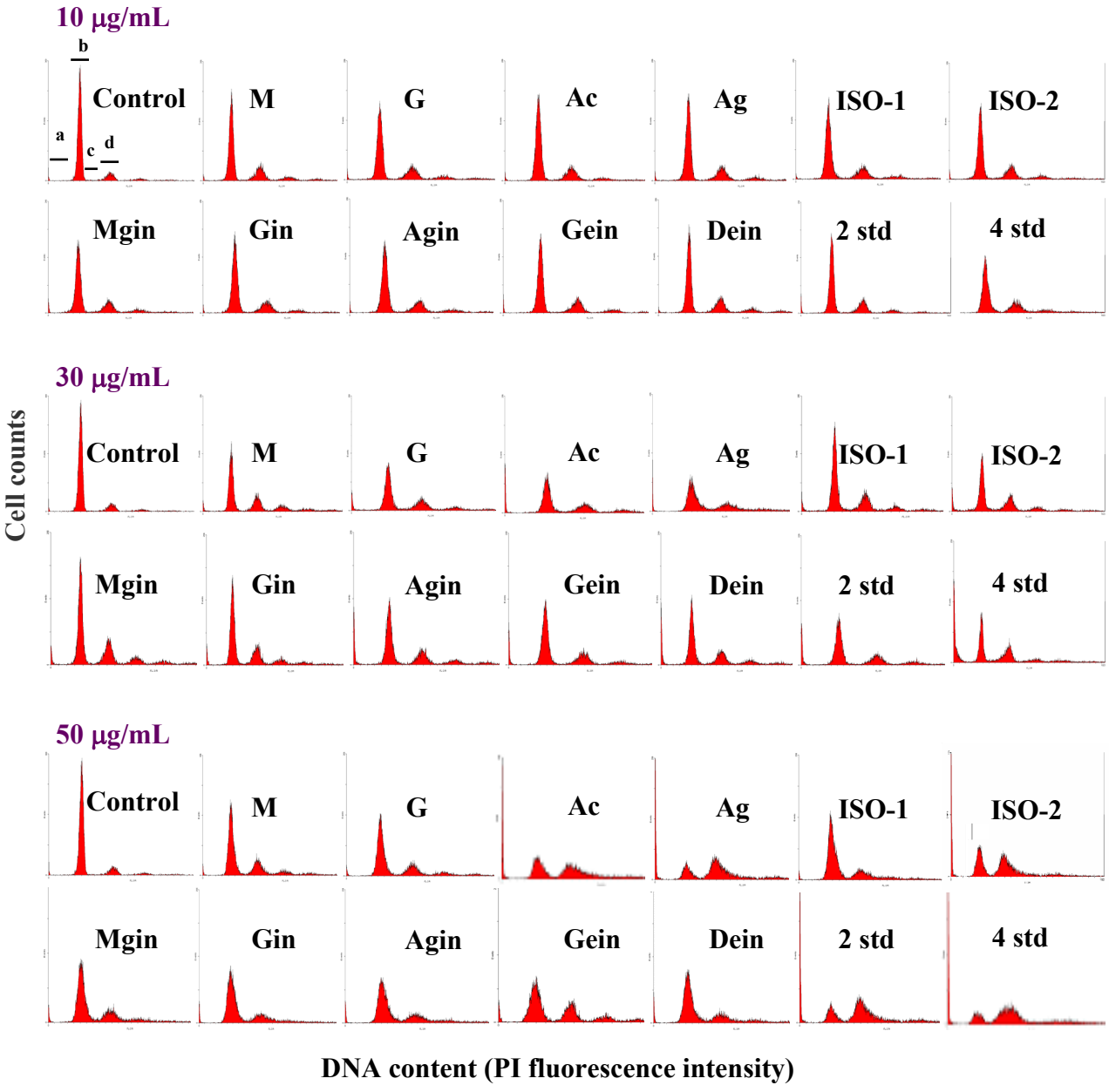
Like G0/G1, the ratio of S phase decreased along with increasing concentrations of isoflavone fractions and extracts as well as standards, revealing the stage of DNA synthesis was retarded. Only a slight change in S phase ratio was observed at 10 μg/mL. However, with doses at 30 and 50 μg/mL, most treatments showed a lower S phase ratio than the control treatment.

In contrast to S phase, the ratio of G2/M phase rose following an increase in concentrations of isoflavone fractions and extracts as well as standards, implying the cell mitosis stage was inhibited at concentrations of 10, 30 and 50 μg/mL. The difference in G2/M ratio was minor at low dose (10 μg/mL) between isoflavone fractions or extracts and standards. However, a large difference occurred at 30 and 50 μg/mL, especially for the latter, with 4 std showing the highest G2/M ratio (64.2%), followed by aglycone (57.3%), 2 std (52.4%), ISO-2 (52.0%), acetylglucoside (45.5%) and genistein (37.4%), and the other treatments showed a low G2/M ratio ranging from 24.1-27.4%.

The cell cycle study results clearly demonstrated that both aglycone and acetylglucoside fractions as well as a mixture of isoflavone standards were more effective in inhibiting HepG2 cell growth than the other treatments. Many studies also indicated that the G2/M ratio could be increased greatly in the presence of isoflavone standards such as genistein and daidzein, with the former being more effective [9,11,17,24-27]. The mechanism in inhibiting tumor cell growth by genistein has been attributed to

retardation of activities of protein tyrosine kinase, topoisomerase I and II, 5α-reductase, focal adhesion kinase and protein histidine kinase [28,29], promotion of tumor suppressor gene expression like P53 and P21 [9,13] and formation of cyclin-dependent kinase inhibitor [9,11], regulation of Bax and Bcl-2 gene expression [27] and transcription factor NF-KB binding activity [29], inhibition of Akt and androgen receptor [29] as well as angiogenesis and metastasis [30,31], enhancement of connexin 43 expression [32] and antioxidant activity [18, 20-22].

Figure 2. Flow cytometric analysis of Hep G2 cells treated with isoflavone fractions and extracts as well as standards.M: malonylglucoside fraction; G: glucoside fraction; Ac: acetylglucoside fraction; Ag: aglycone fraction; ISO-1: soybean cake extract containing 12 isoflavones; ISO-2: a combination of four groups of isoflavone fractions; Mgin: malonylgenistin; Gin: genistin; Agin: acetylgenistin; Gein: genistein; Dein: daidzein; 2 std: a combination of daidzein and genistein; 4 std: a combination of malonylgenistin, genistin, acetylgenistin and genistein. a: sub-G0/G1; b: G0/G1; c: S; d: G2/M.



In addition to isoflavone, the presence of saponin in acetylglucoside fraction may also play an important role in inhibiting cancer cell growth. Kim *et al*. [33] reported that soy saponins may inhibit colon cancer cell growth through retardation of IkBα degradation and decrease of cyclooxygenase-2 and protein kinase C expression. A similar outcome was also observed by Ellington *et al*. [34,35], who found that the colon cancer cell HTC-15 was accumulated at S phase of cell cycle in the presence of soy saponins.

Table 3. Effect of isoflavone fractions and extracts as well as standards on cell cycle distribution of Hep G2 cells[A].

Isoflavone	sub-G0/G1 (%)			G0/G1 (%)			S (%)			G2/M (%)		
	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	2.2±0.1eA	2.3±0.3hA	2.2±0.3eA	80.4±1.7aA	80.7±2.0aA	80.5±1.3aA	6.6±0.6aA	6.8±0.4aA	6.8±0.9aA	10.9±0.6cA	10.2±1.3gA	10.5±2.3fA
M	3.1±0.1dB	4.9±0.2gA	5.7±0.5dA	73.0±1.6bA	67.1±3.4bAB	63.8±2.6bC	6.1±0.3aA	5.6±0.6bA	5.1±0.2bAB	17.8±1.8abB	22.4±2.6defAB	25.4±2.5eA
G	3.3±0.3cdB	6.1±0.2deA	6.0±0.1cdA	74.5±0.8bA	67.5±0.9bB	65.0±1.6bB	5.4±0.1bA	5.3±0.7bAB	4.9±0.1bB	16.8±1.2abB	21.1±1.4efA	24.1±1.4eA
Ac	3.2±0.4cdC	7.4±0.8cB	10.1±1.2aA	73.1±1.6bA	61.0±3.6cdB	39.5±1.2dC	6.7±0.7aA	5.1±1.0bAB	5.0±0.3bB	17.0±1.5abC	26.5±1.8bcdB	45.5±3.1cA
Ag	2.7±0.4deC	7.2±0.3cdB	9.8±1.2aA	71.4±0.0bcA	58.4±2.0dB	27.9±4.0eC	6.1±1.2abAB	6.1±0.3aA	5.0±0.4bB	19.8±0.8abC	28.3±2.0bcB	57.3±4.0bA
ISO-1	3.4±0.2cdC	5.0±0.1fgB	6.3±0.7cdA	72.7±2.9bcA	69.0±2.8bA	61.7±1.4bB	6.0±0.7abA	4.6±0.9bAB	4.6±0.5bB	18.0±2.6abB	21.4±1.9efAB	27.4±1.5eA
ISO-2	5.3±0.6aC	9.1±0.2bB	10.0±0.6aA	70.9±1.2bcA	61.1±2.3cdB	33.5±3.0eC	6.1±0.4abA	4.8±0.6bB	4.5±1.0bB	17.6±1.7abB	25.0±2.7cdeB	52.0±3.3bA
Mgin	4.3±0.4bC	5.5±0.5efgB	6.8±0.1bcdA	73.2±1.7bA	67.5±1.6bB	62.5±1.6bC	6.3±1.1abA	6.3±0.5aA	5.0±0.5abA	16.2±1.7bC	20.7±0.6efB	25.7±0.6eA
Gin	4.5±0.4abC	5.3±0.2efgB	6.6±0.4cdA	71.9±1.8bcA	69.2±3.6bB	63.9±0.1bC	6.5±1.7abA	6.1±1.2aA	5.1±0.2bA	17.1±1.2abB	19.5±2.2fAB	24.5±2.2eA
Agin	4.4±0.5bC	6.3±0.5cdeB	8.2±0.6bA	73.4±0.3bA	64.4±1.2bcB	60.4±1.1bC	5.7±0.7abA	5.4±0.7bA	4.4±0.8bA	16.5±1.6abB	24.0±1.4defA	27.0±2.4eA
Gein	4.5±0.3abC	6.5±0.7cdeB	10.6±0.9aA	71.6±1.4bcA	57.5±1.0dB	47.9±1.4cC	6.3±0.9abA	6.7±0.8aA	4.1±0.3bB	17.6±0.8abC	29.4±2.4bB	37.4±3.2dA
Dein	4.0±0.1bcC	6.1±0.3defB	7.4±0.8bcA	73.1±2.7bcA	69.5±0.2bB	64.2±3.9bC	6.2±0.9abA	5.0±0.1bA	4.9±0.1bB	16.8±2.5abB	19.5±0.2fAB	23.5±2.2eA
2 std	4.7±0.3abC	6.8±0.7cdB	11.1±0.8aA	71.9±0.2bA	57.5±1.9dB	32.3±4.2eC	6.0±0.7abA	5.2±0.6bA	4.2±0.8bB	17.4±0.3abC	30.4±0.6bB	52.4±2.8bA
4 std	4.6±0.5abC	11.0±1.1aA	11.1±0.2aA	69.3±1.9cA	48.8±2.1eB	19.9±3.5fC	6.1±0.3aA	5.1±1.6bAB	4.8±0.2bB	20.0±1.1aC	35.2±2.6aB	64.2±3.6aA

[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: aglycone fraction; ISO-1: soybean cake extract containing 12 isoflavones; ISO-2: a combination of four groups of isoflavone fractions; Mgin: malonylgenistin; Gin: genistin; Agin: acetylgenistin; Gein: genistein; Dein: daidzein; 2 std: a combination of daidzein and genistein; 4 std: a combination of malonylgenistin, genistin, acetylgenistin and genistein.

Most human studies of isoflavones dealt with inhibition of osteoporosis, alleviation of menopause syndrome and reduction of blood triacylglycerol. Dalais *et al*. [36] reported that a daily supply of 117 mg isoflavone could lower the level of prostate-specific antigen in blood of male with prostate cancer. Similarly, a supply of isoflavone at 160 mg/day for one week prior to prostate surgery could lead to mortality of prostate cancer cell without any side effect [37]. In a recent human study the maximum isoflavone concentration in blood was determined to be 0.64 μM when a dose of 1 μmole/kg BW genistein was provided. Of the various isoflavone fractions in our experiment, the IC_{50} for the most effective acetylglucoside and aglycone were 19.9 and 8.8 μg/mL, respectively, which is equivalent to a dose of genistein at 31.6 and 14.0 mg/kg BW. Although this level was higher than those reported in the literature, it should not be toxic to human. But for cancer inhibition by isoflavones, both animal and cell models were frequently conducted. No systemic toxicity occurred in rat for genistein and daidzein at a dose of 140 and 250 mg/kg BW, respectively [38]. Likewise, a higher level of genistein at 500 or

2000 mg/kg was shown not to cause embryo teratogenesis for the former and genetic toxicity for the latter [39,40]. Thus, it is apparent that the doses of acetylglucoside (31.6 mg/kg BW/day) and aglycone (14.0 mg/kg BW/day) used in our study should not induce toxicity to both animal and human. In conclusion, both aglycone and acetylglucoside fractions prepared from soybean cake were more effective in HepG2 cancer cell antiproliferation than the other isoflavone fractions and extracts. A mixture of isoflavone standards showed a better inhibition than single isoflavone standard. For cell cycle study, both aglycone and acetylglucoside fractions as well as a mixture of isoflavone standards possessed a high G2/M ratio, correlating well with the result of MTT test.

3. Experimental Section

3.1 Materials

A total of 50 Kg of soybean cake was procured from Chong-Liang Oil Co. (Taichung, Taiwan). Isoflavone standards, malonylgenistin, genistin, acetylgenistin, genistein and daidzein were obtained from LC laboratories (Woburn, MA, USA). Reagents, including phosphate buffered saline (PBS), fetal bovine serum (FBS), trypsin blue stain (0.4%), 2.5% trypsin-EDTA, Hank's balanced salt solution (HBSS) and penicillin-streptomycin were from Gibco Co. (California, USA). Minimum essential medium alpha modification (α-MEM), dimethyl sulfoxide (DMSO), hypoxanthin, thymidine, sodium bicarbonate, fungizone (amphotericin B solution), 3-(4,5-dimethylthiazole)-2,5-diphenyltetrazolium bromide (MTT), RNase A and propidium iodide were from Sigma Co. (St. Louis, MO, USA). Ethanol (95%) was from Taiwan Tobacco Co. (Tainan, Taiwan). Deionized water was made using a Milli-Q water purification system from Millipore Co. (Bedford, MA, USA). Human hepatoma cell line (HepG2) was from Taiwan Food Industry Development Institute/National Health Research Institute (Hsinchu, Taiwan).

3.2 Preparation of isoflavone extracts and fractions from soybean cake

A method based on Kao *et al*. (41) was used to prepare isoflavone extracts from soybean cake. Briefly, soybean cake powder (50 g) was mixed with aqueous ethanol solution (50%, 150 mL). Then the mixture was shaken at room temperature for 2 h and centrifuged at 6000 rpm (25°C) for 20 min, followed by collecting the supernatant and filtering through a glass filter paper to obtain the isoflavone extract (ISO-1). For preparation of four isoflavone fractions, Diaion HP-20 resin (200 g) was poured into a glass column (375 x 45 mm I.D.) and pre-activated with ethanol (1 L) and deionized water (1 L). Then, an isoflavone extract (80 mL) was poured into the column and high-polar impurities were eluted with deionized water (400 mL), followed by subsequent elution of malonylglucoside and glucoside fractions with 15% aqueous ethanol solution (900 mL) and 27% aqueous ethanol solution (3300 mL), respectively, at a flow rate of 10 mL/min. Both acetylglucoside and aglycone fractions were eluted with 70% aqueous ethanol solution (200 mL) and 95% aqueous ethanol solution (400 mL), respectively. Because of overlapping by HPLC analysis, both acetylglucoside and aglycone fractions were combined and evaporated to dryness, dissolved in isopropanol (20 mL) and a portion (10 mL) was poured onto a Yamazen Hi-Flash^{TM} silica gel column (170 x 48 mm I.D.) for elution of these two fractions separately with hexane-isopropanol-ethanol (8:9:1, v/v/v) and flow rate at 20 mL/min. All the

four isoflavone fractions were also combined (ISO-2) for subsequent cell proliferation study. The variety and amount of isoflavones in each fraction was analyzed by HPLC using a method described by Hsieh *et al*. [42]. In addition, some other functional components like saponins, phenolic compounds and total flavonoids in each fraction were determined based on a procedure as described by Kao and Chen [14]. Total phenolic compounds were present at a level ranged from 142.3-319.2 μg/mL in all the isoflavone fractions and extracts, while total flavonoids were 1.82-26.44 μg/mL. With the exception of malonylglucoside fraction, saponins (soyasapogenol A and soyasapogenol B) were also present at 14.75-372.23 μg/mL in the other isoflavone fractions and extracts.

3.3 Preparation of isoflavone extracts and fractions for cell antiproliferation study

A sample of isoflavone extract (ISO-1), malonylglucoside and glucoside fractions (20 mL each) was collected separately, evaporated to dryness and dissolved in water-ethanol (1:1, v/v, 2 mL). A sample (20 μL) was mixed with internal standard formononetin (200 μg/mL, 200 μL) and water-ethanol (1:1, v/v, 1780 μL), followed by filtering the mixture through a 0.2-μm membrane filter and 20 μL was injected for HPLC analysis. After quantitation, ISO-1 was found to contain isoflavone at 15844.8 μg/mL, whereas malonylglucoside fraction contained isoflavone at 12471.3 μg/mL and glucoside fraction at 9884.0 μg/mL. Next, a volume of ISO-1 (632 μL), malonylglucoside fraction (802 μL) and glucoside fraction (1012 μL) was collected separately and mixed with 1368, 1198 and 988 μL of water-ethanol (1:1, v/v), with the total volume of 2 mL each and the final isoflavone concentrations being 5007, 5001 and 5001 μg/mL, respectively.

Both acetylglucoside and aglycone fractions were collected five times from a preparative column as described above separately, pooled and evaporated to dryness. The residue was dissolved in water-ethanol (1:1, v/v, 2 mL), and a portion (20 μL) was mixed with internal standard formononetin (200 μg/mL, 200 μL) and water-ethanol (1:1, v/v, 1780 μL), after which the mixture was filtered through a 0.2-μm membrane filter and 20 μL was injected for HPLC analysis. After quantitation, both acetylglucoside and aglycone fractions were shown to contain isoflavones at 5530.7 and 6004.8 μg/mL, respectively. Again, a volume of acetylglucoside (1810 μL) and aglycone (1660 μL) was mixed with water-ethanol (1:1, v/v, 190 and 340 μL, respectively), for a total volume of 2 mL each with isoflavone levels being 5005 and 5002 μg/mL. For preparation of the other isoflavone extract (ISO-2), a volume of 500 μL malonylglucoside, glucoside, acetylglucoside and aglycone was collected separately and combined for a total volume of 2 mL containing isoflavone at 5002 μg/mL.

3.4 Preparation of isoflavone standards for cell antiproliferation study

A standard (10 mg) of daidzein, malonylgenistin, genistin, acetylgenistin and genistein was mixed with water-ethanol (1:1, v/v) separately for a total volume of 2 mL containing isoflavone at 5000 μg/mL each. These standards were used alone or combined to 2 std (daidzein and genistein) and 4 std (malonylgenistin, genistin, acetylgenistin and genistein) for subsequent cell antiproliferation study.

3.5 Preparation of final isoflavone concentration

A volume of 1.5 mL was collected from four isoflavone fractions, two isoflavone extracts (ISO-1 and ISO-2), malonylgenistin, genistin, acetylgenistin, genistein, 2 std and 4 std separately, followed by mixing with α-MEM medium (148.5 mL) and filtering through a 0.2-μm membrane filter for an isoflavone level at 50 μg/mL each. Again, a volume of 40, 30, 20, 10 and 5 mL medium was collected and mixed with 10, 20, 30, 40 and 45 mL α-MEM medium for a final isoflavone concentration at 40, 30, 20, 10 and 5 μg/mL, respectively.

3.6 Cell culture

HepG2 human hepatoma cells were cultured in α-MEM medium (pH 7.4) supplemented with 10% fetal bovine serum (FBS), 36 μM hypoxanthin, 36 μM thymidine, 0.5% fungizone and 1% penicillin-streptomycin. Cells were incubated at 37°C under 5% CO_2 humidified atmosphere and replaced every two days to maintain normal growth. Prior to MTT and cell cycle experiments, cells have to undergo solvent endurability and growth stability test and our result indicated that a culture medium containing 0.5% ethanol would not affect normal cell growth as the activity still maintained even after 72 h incubation.

3.7 MTT test

HepG2 cells were grown to the desired density at about 80% confluence and washed with 2 mL PBS twice, followed by adding 0.5 mL 2.5% trypsin-EDTA. The solution was shaken gently to cover cell surface and placed into an incubator for 1-2 min until floating, after which 1 mL α-MEM medium was added for neutralization and cells were harvested, then transferred to a 15 mL tube for centrifugation at 2000 rpm for 5 min. The supernatant was collected and 2 mL α-MEM medium added to suspend cells, and an appropriate amount of cell medium was collected and diluted for calculation. For MTT test, a 0.2 mL HepG2 cell suspension was seeded at a cell density of about 4 x 10^4 cell/plate to 96-well plates (100 μL medium/well) and cultured for 24 h at 37 °C under 5% CO_2 so that cells were attached to the bottom. The culture medium was sucked and replaced with isoflavone extracts and fractions as well as standards at six concentrations of 5, 10, 20, 30, 40 and 50 μg/mL. Triplicate wells were used for each concentration. After incubation for 72 h, 0.2-mL of MTT (0.5 mg/mL in HBSS) was added and further cultured for 3 h at 37 °C in the dark. After removal of MTT solution, 0.2 mL dimethyl sulfoxide was added to dissolve purple crystal for 15 min, after which the absorbance of the solution was measured at 570 nm with an ELISA reader. The inhibition effect of HepG2 cell growth was expressed as relative cell survival percentage:

$$\text{Relative cell survival rate (\%)} = \frac{\text{Absorbance of the solution after reaction with isoflavone}}{\text{Absorbance of the solution before reaction with isoflavone}} \times 100$$

The IC_{50} value was also calculated based on the regression equation of the curve obtained by plotting concentration against cell survival rate.

3.8 Cell cycle analysis

A method based on Chang *et al*. [11] was slightly modified to study cell cycle analysis. Cells were seeded at a density of about 1 x 10^6 cell to culture plate. After incubation for 24 h, all the isoflavone extracts and fractions as well as standards were added at a concentration of 0, 10, 30 and 50 μg/mL and cultured for another 24 h. The cells were then subjected to trypsinization with trypsin-EDTA and washed with PBS, followed by centrifuging at 2000 rpm for 5 min. The supernatant was collected and 1-mL PBS solution containing 70% ethanol was added for cell fixation at 4 °C overnight. The mixture was centrifuged to remove PBS solution and cells were washed again with PBS, after which 0.1 mL RNase A PBS solution (100 μg/mL) was added. The mixture was reacted in a water bath at 37 °C for 30 min and 0.1 mL propidium iodide (10 μg/mL) was added for cell cycle analysis by using a flow cytometer (Beckman Coulter EPICS XL-MCL).

3.9 Statistical analysis

Triplicate analyses were performed for MTT test while duplicate experiments were carried out for flow cytometry. All the data were subjected to analysis of variance using ANOVA and Duncan's multiple range test for significant difference comparison (α=0.05) by using SAS [43].

References and Notes

1. World Cancer Research Fund, American Institute for Cancer Research. *Food, nutrition and the prevention of cancer: a global perspective*; American Institute for Cancer Research: Washington, **1997**.
2. Adlercreutz, H. Phyto-oestrogens and cancer. *Lancet Oncol*. **2002**, *3*, 364-372.
3. Block, G.; Patterson, B.; Subar, A. Fruits, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer.* **1992**, *18*, 1-29.
4. Messina, M.; Deshcheemaker, K.; Erdman, J.W. The role of soy in preventing and treating chronic disease. *Am. J. Clin. Nutr.* **1998**, *68*, 1329-1544.
5. Adlercreutz, H.; Honjo, H.; Higash, A. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming traditional Japanese diet. *Am. J. Clin. Nutr.* **1991**, *54*, 1093-1100.
6. Dixon, R.A.; Ferreira, D. Genistein. *Phytochemistry* **2002**, *60*, 205-211.
7. Peterson, G.; Barnes, S. Genistein inhibition of the growth of human breast cancer cells: Independence from estrogen receptors and the multi-drug resistance gene. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 661-667.
8. Scholar, E.M.; Toews, M.L. Inhibition of invasion of murine mammary carcinoma cells by the tyrosine kinase inhibitor genistein. *Cancer Lett.* **1994**, *87*, 159-162.

9. Frey, R.S.; Li, J.; Singletary, K.W. Effects of genistein on cell proliferation and cell cycle arrest in nonneoplastic human mammary epithelial cells involvement of Cdc2, $p21^{waf/cip1}$, $p27^{kip1}$, and Cdc25C expression. *Biochem. Pharmacol.* **2001**, *61*, 979-989.
10. Kumi-Diaka, J. Chemosensitivity of human prostate cancer cells PC3 and LNCaP to genistein isoflavone and β-lapachone. *Bio. Cell.* **2002**, *94*, 37-44.
11. Chang, K.L.; Kung, M.L.; Chow, N.H.; Su, S.J. Genistein arrests hepatoma cells at G2/M phase: involvement of ATM activation and upregulationof $p21^{waf1/cip1}$ and Weel. *Biochem. Pharmacol.* **2004**, *67*, 717-726.
12. Di Virgilio, A.L.; Iwami, K.; Wätjjen, W.; Kahl, R.; Gegen, G.H. Genotoxicity of the isoflavones genistein, daidzein and equol in V79 cells. *Toxicol. Lett.* **2004**, *151*, 151-162.
13. Oki, T.; Sowa, Y.; Hirose, T.; Takagaki, N.; Horinaka, M.; Nakanishi, R.; Yasuda, C.; Yoshida, T.; Kanazawa, M.; Satomi, Y.; Nishino, H.; Miki, T.; Sakai, T. Genistein induces Gadd45 gene and G2/M cell cycle arrest in the DU145 human prostate cancer cell line. *FEBS Lett.* **2004**, *577*, 55-59.
14. Kao, T.H.; Chen, B.H. Functional components in soybean cake and their effects on antioxidant activity. *J. Agric. Food Chem.* **2006**, *54*, 7544-7555.
15. Hewitt, A.L.; Singletary, K.W. Soy extract inhibits mammary adenocarcinoma growth in a syngeneic mouse model. *Cancer Lett.* **2003**, *192*, 133-143.
16. Constantinou, A.I.; Mehta, R.G.; Vaughan, A. Inhibition of N-methyl-N-nitrosourea-induced mammary tumors in rats by the soybean isoflavones. *Anticancer Res.* **1996**, *16*, 3293-3298.
17. Sakamoto, K. Synergistic effects of thearubigin and genistein on human prostate tumor cell (PC-3) growth via cell cycle arrest. *Cancer Lett.* **2000**, *151*, 103-109.
18. Wei, H.; Wei, L.; Frenkel, K.; Bowen, R.; Barnes, S. Inhibition of tumor promoter-induced hydrogen peroxide formation *in vitro* and *in vivo* by genistein. *Nutr. Cancer* **1993**, *20*, 1-12.
19. Wei, H.; Bower, R.; Zhang, X.; Lebwohl, M. Isoflavone genistein inhibits the initiation and promotion of two-stage skin carcinogenesis in mice. *Carcinogenesis* **1998**, *19*, 1509-1514.
20. van Rossen, M.E.; Sluiter, W.; Bonthuis, F.; Jeekel, H.; Marquet, R.L.; van Eijck, C.H. Scavenging of reactive oxygen species leads to diminished peritoneal tumor recurrence. *Cancer Res.* **2000**, *60*, 5625-5629.
21. Bostwick, D.G.; Alexander, E.E.; Singh, R.; Shan A.; Qian J.; Santella, R.M.; Oberley, L.W.; Yan, T.; Zhong, W.; Jiang, X.; Oberley, T.D. Antioxidant enzyme expression and reactive oxygen species damage in prostatic intraepithelial neoplasia and cancer. *Cancer* **2000**, *89*, 123-134.
22. Dudek, E.J.; Shang, F.; Taylor, A. H_2O_2-mediated oxidative stress activates NF-kappaB in lens epithelial cells. *Free Radic. Biol. Med.* **2001**, *31*, 651-658.
23. Popiolkiewicz, J.; Polkowski, K.; Skierski, S.; Mazurek, A.P. In vitro toxicity evaluation in the development of new anticancer drugs-genistein glycosides. *Cancer Lett.* **2005**, *229*, 67-75.
24. Papazisis, K.T.; Zambouli, D.; Kimoundri, O.T.; Papadakis, E.S.; Vala, V.; Deromichalos, D.; Voyatzi, S.; Markala, D.; Destouni, E.; Boutis, L.; Kortsaris, A.H. Protein tyrosine kinase inhibitor, genistein, enhances apoptosis and cell cycle arrest in K562 cells treated with γ-irradiation. *Cancer Lett.* **2000**, *160*, 107-113.
25. Casagrande, F.; Darbon, J.M. $p21^{CIP1}$ is dispensable for the G2 arrest caused by genistein in human melanoma cells. *Exp. Cell Res.* **2000**, *258*, 101-108.

26. Ying, C.; Hsu, J.T.; Hung, H.C.; Lin, D.H.; Chen, L.F.O.; Wang, L.K. Growth and cell cycle regulation by isoflavones in human breast carcinoma cells. *Reprod. Nutr. Dev.* **2002**, *42*, 55-64.
27. Ismail, I.A.; Kang, K.S.; Kim, J.W.; Sohn, Y.K. Genistein induces G2/M cell cycle arrest and apoptosis in rat neuroblastoma B35 cells; involvement of $p21^{waf1/cip1}$, Bax and Bcl-2. *Korea J. Pathol.* **2006**, *40*, 39-347.
28. Katdare, M.; Osborne, M.; Telang, N.T. Soy isoflavone genistein modulates cell cycle progression and induces apoptosis in HER-2/neu oncogene expressing human breast epithelial cells. *Int. J. Oncol.* **2002**, *21*, 809-815.
29. Sarkar, F.H.; Li, Y. Mechanisms of cancer chemoprevention by soy isoflavone genistein. *Cancer Metastasis Rev.* **2002**, *21*, 265-280.
30. Li, Y.; Bhuiyan, M.; Alhasan, S.; Senderowicz, A.M.; Sarkar, F.H. Induction of apoptosis and inhibition of c-erbB-2 in breast cancer cells by flavopiridol. *Clin. Cancer Res.* **2000**, *6*, 223-229.
31. Su, S.J.; Yeh, T.M.; Chuang, W.J.; Ho, C.L.; Chang, K.L.; Cheng, H.L.; Liu, H.S.; Cheng, H.L.; Hsu, P.Y.; Chow, N.H. The novel targets for anti-angiogenesis of genistein on human cancer cells. *Biochem. Pharmacol*. **2005**, *69*, 307-318.
32. Conklin, C.M.J.; Bechberger, J.F.; MacFabe, D.; Guthrie, N.; Kurowska, E.M.; Naus, C.C. Genistein and quercetin increase connexin 43 and suppress growth of breast cancer cells. *Carcinogenesis* **2007**, *28*, 93-100.
33. Kim, H.Y.; Yu, R.; Kim, J.S.; Kim, Y.K.; Sung, M.K. Antiproliferative crude soy saponin extract modulates the expression of IκBα, protein kinase C, and cyclooxygenase-2 in human colon cancer cells. *Cancer Lett*. **2004**, *210*, 1-6.
34. Ellington, A.A.; Berhow, M.; Singletary, K.W. Induction of macroautophagy in human colon cancer cells by soybean B-group triterpenoid saponins. *Carcinogenesis* **2005**, *26*, 159-167.
35. Ellington, A.A.; Berhow, M.; Singletary, K.W. Inhibitionof Akt signaling and enhanced ERK1/2 activity are involved in induction of marcoautophagy by triterpenoid B-group soyasaponins in colon cancer cells. *Carcinogenesis* **2006**, *27*, 298-306.
36. Dalais, F.S.D.; Meliala, A.; Wattanapenpaiboon, N.; Frydenberg, M.; Siter, D.A.I.S; Thomson, W.K.; Wahlqvist, M.L. Effects of a diet rich in phytoestrogens on prostate-specific antigen and sex hormones in men diagnosed with prostate cancer. *Urology.* **2004**, *64*, 510-515.
37. Stephens, F.O. Phytoestrogens and prostate cancer: possible preventive role. *Med. J. Aust.* **1997**, *167*, 138-140.
38. Galati, G.; O'Brien, P.J. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radic. Biol. Med.* **2004**, *37*, 287-303.
39. McClain R.M.; Wolz, E.; Davidovich, A.; Bausch, J. Genetic toxicity studies with genistein. *Food Chem. Toxicol*. **2006**, *44*, 42-55.
40. McClain R.M.; Wolz, E.; Davidovich, A.; Edwards, J.; Bausch, J. Reproductive safety studies with genistein in rats. *Food Chem. Toxicol*. **2007**, *45*, 1319-1332.
41. Kao, T.H.; Lu, Y.F.; Chen, B.H. Preparative chromatography of four groups of isoflavones from soybean cake. *Eur. Food Res. Technol.* **2005**, *221*, 459-465.
42. Hsieh, H.C.; Kao, T.H.; Chen, B.H. A fast HPLC method for analysis of isoflavones in soybean. *J. Liquid Chrom. Rel. Technol.* **2004**, *27*, 315-324.

43. SAS. *SAS Procedures and SAS/Graph User's Guide, version 6, Software Release 8.2 (TS2MO) of the SAS System for Microsoft Windows*; SAS Institute, Inc.: Cary, NC, **2004**.