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ARTICLE

Comparative Studies on the Chemical and Cell-Based Antioxidant Activities and Antitumor Cell Proliferation Properties of Soy Milk Manufactured by Conventional and Commercial UHT Methods

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The aims of this work were to compare antiproliferation, antioxidant activities and total phytochemicals and individual isoflavone profiles in soy milk processed by various methods including traditional stove cooking, direct steam injection, direct ultrahigh temperature (UHT), indirect UHT, and a two-stage simulated industry method, and a selected commercial soy milk product. Various processing methods significantly affected total saponin, phytic acid, and total phenolic content and individual isoflavone distribution. The laboratory UHT and the two-stage processed soy milk exhibited relatively higher total phenolic content, total flavonoid content, saponin and phytic acid than those processed by the traditional and steam processed methods. Thermal processing caused obvious intertransformation but did not cause severe degradation except for breaking down of aglycons. Thermal processing significantly increased antioxidant capacities of soy milk determined by chemical analyses, but decreased cellular antioxidant capacities as compared to the raw soy milk. The raw and all processed soy milk exhibited antipoliferative activities against human HL-60 leukemia cells, AGS gastric tumor cells, and DU145 prostate cancer cells in a dose-dependent manner. The raw soy milk, but not the processed soy milk, exhibited a dose-dependent antiproliferative effect against colorectal adenocarcinoma Caco-2 cells. Taken together, these results indicate that various thermal processing methods change not only phytochemcials but also potential health-promoting effects of soy milk.

KEYWORDS: Soy milk; thermal processing; UHT; saponin; phytic acid; phenolics; isoflavones; antioxidants; antitumor cell proliferation; CAA; HPLC

INTRODUCTION

Studies have shown that the typical Asian diet results in lower rates of breast and prostate cancers, compared to the typical Western diet (*1*, *2*). A particular difference between Asian and Western diet is that soy (especially tofu and soy milk) is a staple in Asian diets, but is not common in Western diets. A case-control study showed that frequent intake of soy milk (more than once a day) was associated with a 70% reduction in the risk of prostate cancer (*3*).

Soy milk is one of the most popular soy foods in East and Southeast-Asian countries. Soy milk was introduced to North America in recent years as an alternative dietary protein source for common consumers, vegetarians, and people with lactose-intolerance and milk allergy and is now available in major supermarket chains. Despite the increased consumption trend, it has not yet been extensively accepted by Americans due to its beany flavor (*4*). Therefore, processing techniques become extremely important to maximize the retention of desired health-promoting components (like proteins, essential amino acids, vitamins, antioxidants and antitumor properties), and to maximize the elimination or reduction of unwanted components (such as beany flavor, trypsin inhibitors and lipoxygenases) for improving soy milk quality to benefit the consumers (*5*, *6*). To achieve a balance between maximizing desired and minimizing undesired components by thermal processing is a major challenge since heat can inactivate the undesirable antinutrients, and also can degrade desirable constituents in soy milk (*5*, *6*).

A variety of heating methods and time−temperature conditions are being used by soy milk manufacturers. Traditional methods, which have been used in East Asia for a long time, include heating freshly prepared soy milk to boiling in an open kettle for 20−30 min. As compared to the traditional cooking, the ultrahigh-temperature UHT systems for soy milk processing are

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Table 1. Processing Conditions and Properties of Soy Milk[a]

soy milk ID	processing methods	processing conditions	sterilization power[b]
raw			
traditional	stove cooking	100 °C, 20 min	<0.2
steam	direct steam injection	100 °C, 20 min	<0.2
direct UHT	direct UHT	143 °C, 60 s	158.5
indirect UHT	indirect UHT	143 °C, 60 s	158.5
industry	two-stage indirect UHT	120 °C, 80 s +140 °C, 4 s	6.35
commercial	unknown	unknown	unknown

[a] All soy milk except the commercial soy milk product was made from the Proto soybean variety. The soybean source for the commercial soy milk product is unknown. [b] Sterilization power is calculated by the formula $F_0 = 10^{(T-121)/10}$ for the inactivation of PA3679 spores.

relatively new. Both traditional and UHT methods can use direct-steam injection or indirect heat exchange systems. The UHT system can heat up quickly to a high temperature (up to 150 °C) in a short period of time (in a few seconds), and can be easily automated for aseptic packaging of the products. It has been reported that, for inactivating 90% of the trypsin inhibitors in soy milk, the conditions would require as high as 143 °C for 60 s (*5*, *7*) or 150 °C for 50 s (*7*). Recently, a two-stage UHT processing method with two different temperature and time conditions (120 °C, 80 s, + 140 °C, 4 s) has been widely practiced in modern soy milk industries (*8*). This two-stage method is able to inactivate approximately 80% of the trypsin inhibitor activity in the raw soy milk. However, these two processes differed by more than 20-fold in sterilization power even though they differ only by 10% in trypsin inactivation (**Table 1**). Little information is available regarding the phenolic compositions and their biological activities as affected by these two drastically different thermal processing methods.

Furthermore, it remains largely unknown how various thermal processing methods affect the health-promoting effect of soy milk with respect to the prevention of chronic disease such as cardiovascular disease and cancer prevention. Phenolic compositions, including isoflavones, as affected by selected UHT processing conditions have been investigated by others (*8*) and our laboratory (*9*). A UHT processing method has been reported to eliminate the cholesterol lowering effect of soy protein (*10*). However, the mechanism for the reduction is unknown. Our previous report (*9*) on antioxidant properties did not include soy milk processed by the two-stage industry method, and did not compare to commercial soy milk product. Thus far, no research has reported how different thermal processing methods with different sterilization power affect cellular antioxidant capacity and antitumor proliferation characteristics of soy milk. Therefore, to address these questions, our objective was directed to compare chemical and cellular antioxidant activities and antitumor cell proliferation properties of various soy milk products manufactured by four selected methods, which include two traditional methods (traditional stove cooking and steam injection), a laboratory UHT method of 143 °C for 60 s, and a two-stage industry processing method (120 °C, 80 s, + 140 °C, 4 s).

MATERIALS AND METHODS

Chemicals and Materials. Dimethyl sulfoxide (DMSO), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), (+)-catechin, fluorescein disodium, Folin−Ciocalteu reagent, sodium carbonate, soyasaponin, phytic acid, sulfosalicylic acid, 3-(4,5-dimenthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), vanillin, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Nine authentic isoflavone standards were purchased from LC Laboratories (Woburn, MA). 2,4,4′-Trihydroxybenzoin (THB, one of the internal standards for isoflavone quantification), was synthesized and purified in our lab. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). HPLC-grade solvents, analytical grade acetic acid and other analytical grade solvents using for extraction were purchased from VWR international (West Chester, PA). Nine human cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Hanks balanced salt solution (HBSS) and 0.4% trypan blue stain solution were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Phosphate-buffered saline (PBS), trypsin-EDTA solution, penicillin−streptomycin, fetal bovine serum (FBS), and all cell culture media (Eagle's MEM, DMEM, McCoy's 5a, Leibovitz's L-15, F-12K, Iscove's MDM) were purchased from Mediatech, Inc. (Herndon, VA).

Soybean and Soy Milk Materials. The dry matured soybeans (*Glycine max*) of the variety of Proto (harvested in 2006) were obtained from Sinner Brothers & Bresnahan (Casselton, ND). One commercial soy milk product (Silk, plain style, WhiteWave, Boulder, Co) was purchased from a local grocery store (Fargo, ND). Phenolic quantification and antioxidant activities were expressed on a dry weight basis.

Preparation of Raw Soy Milk. For each batch of soy milk, 2 kg of soybeans was soaked overnight in 20 L of tap water at room temperature (solid:liquid ratio 1:10, w/v). The hydrated beans were drained, rinsed and ground with tap water [the ratio of water to dry bean was 9:1 (w/w)]. In the traditional stove cooking treatment, the soaked soybeans were ground for 3 min at the high speed using a Hamilton Beach blender (model: 585-1, Peabody, MA). The soy slurry was filtered through a muslin cloth to separate the okara from the soy milk. For the traditional direct steam injection and direct/indirect UHT treatments, the soaked soybeans were ground using an automated soy milk grinder/extractor (Chang-Seng Mech. Co., Taoyuan, Taiwan), which was equipped with a centrifugal 120-mesh screen to separate raw soy milk automatically from the residues. Approximately 100 mL of raw soy milk from each batch processing was sampled in duplicate after grinding and filtration, subsequently freeze-dried and stored at −20 °C until further analyses.

Thermal Processing of Soy Milk. A total of five processing methods that produce high inactivation of trypsin inhibitor activity were used (*5*). One traditional stove cooking method (100 °C for 20 min), which is the general method for preparing homemade soy milk, and a direct steam injection method (100 °C for 20 min), which is one of the popular methods for making soy milk and tofu in Japan, were employed. Three UHT methods were also designed according to literature and industry practice (*5*, *7*, *8*). Briefly, the raw soy milk that came out of the continuous grinder was processed by a Microthermics Direct/Indirect Steam Injection Processor (DIP, Microthermics, Inc., Raleigh, NC). The processing conditions were chosen from earlier optimization work based on mathematical models and computer program (*5*, *7*, *8*). These three UHT methods included (1) 143 °C, 60 s by direct heating; (2) 143 °C, 60 s by indirect heating; and (3) a two-stage method involving 120 °C for 80 s in the first stage, following by 140 °C for 4 s in the second stage. Although the two-stage method also involved milder UHT heating conditions, for the convenience of the comparison, we will refer to this method as the two-stage industry method or simply the industry method to avoid confusion with the first two higher power UHT methods at 143 °C for 60 s. The processing conditions and properties of soy milk are summarized in **Table 1**. The details for processing soy milk by each method were as follows.

(*1*) *Traditional Stove Cooking and* (*2*) *Traditional Direct Steam Injection*. The detailed procedures for the two traditional soy milk processing methods were described in our latest publication (*9*).

(*3*) *Direct UHT Processing*. The direct UHT process was carried out at a temperature of 143 °C for 60 s. Food-grade steam was injected into the product, and soy milk was in direct contact with the heating medium at 143 °C. The heated soy milk was pumped through a holding tube (60 s) so that the processing could be continued in a continuous manner. The direct method included a vacuum chamber for cooling, evaporating the added water from the steam injected and removing odors. After vacuum cooling, the product was further cooled by circulating cold tap water in a tubular heat exchanger. The soy milk at the final product outlet was approximately 25 °C and freeze-dried.

(*4*) *Indirect UHT Processing*. The indirect system was based on tubular heat exchangers whereby heat was transferred from the heating medium to

the product through a heat exchanger. The indirect UHT process was also carried out at a temperature of 143 °C for 60 s. The heated soy milk that came out of the holding tube was cooled by a tubular heat exchanger using cold tap water. The soy milk at the final product outlet was approximately 25 °C and collected. Approximately 100 mL of UHT soy milk from each batch processing was sampled in duplicate. The UHT soy milk was freeze-dried and stored at −20 °C for further analyses.

(5) *The Two-Stage Industry Method*. The first stage was 120 °C for a longer period of time (80 s) to inactive most of the trypsin inhibitor, and the second stage was 140 °C for a short period of time (4 s) to take advantage of the high power of sterilization before aseptic packaging (*8*). Therefore, a two-stage process using the small pilot scale of the Microthermics DIP Processor was carried out to mimic this commercial practice to investigate its effect on phenolic substances and their bioactivities. The raw soy milk that came out of the continuous grinder was then processed at 120 °C for 80 s, cooled to room temperature, and followed by direct heating as at 140 °C for 4 s. The soy milk obtained was freeze-dried and stored at −20 °C for further analyses.

Extraction and Determination of Total Phenolics. After the traditional and UHT processing, all soy milk samples were immediately frozen and then freeze-dried. Moisture content was determined by drying the sample after 24 h at 105 °C in an air oven until a constant weight was obtained (*11*). The freeze-dried soy milk samples (0.5 g in triplicate) were accurately weighed into a set of centrifuge tubes. Extraction procedures were carried out according to our earlier communication (*12*). Freeze-dried soy milk was extracted twice each with 5 mL of acetone/water (50:50, v/v). The extracts were used for the analyses of total phenolics, antioxidant activities and antiproliferation activities. The total phenolic content (TPC) and total flavonoid content (TFC) were determined according to our earlier publication (*12*).

Determination of Total Saponin and Phytic Acid. Saponin and phytic acid were quantified according to our latest publications (*13*, *14*).

HPLC Analysis of Isoflavone Content. Isoflavones were extracted and quantified by following our previous publication (*14*). Briefly, the quantitative analysis of soy milk isoflavones was performed on a Waters Associates chromatography system (Milford, MA) coupled with a YMC-Pack ODS-AM-303 C_{18} reversed-phase column (250 mm × 4.6 mm). Calibration curves were established for each of nine external standards by plotting response factor (RF) of each standard against concentration. The response factors are the ratios of peak area external to internal standards. For the other isoflavones without commercial standards, concentrations were calculated from the standard curves that were adjusted appropriately from the standard curves of each respective aglycon isoflavone form based on the differences in the molecular weight and molar extinction coefficients of the compounds. Isoflavone contents were expressed as micrograms of isoflavone per gram of dry soy milk (μg/g).

Chemical Antioxidant Assays. Free radical DPPH scavenging activity (DPPH), ferric reducing antioxidant power (FRAP) and oxygen radical absorbing capacity (ORAC) were determined according to one of our earlier publications (*12*). Peroxyl radical scavenging capacity (PRSC) was determined according to our latest publication (*13*) without modifications.

Cellular Antioxidant Activity (CAA) Assay. *Cell Culture*. Human gastric adenocarcinoma cell line AGS (CRL-1739) were grown in complete growth medium F-12K (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin−streptomycin (v/v), and was maintained at 37 °C in a humidified 5% CO_2 incubator. Cells used in this study were between passages 47 and 51.

CAA Assay. The CAA assay was performed by modifying the methods of Eberhardt et al. (*15*) and Wolfe and Liu (*16*) by using gastric AGS tumor cells. The detailed assay procedures were described in our latest publication (*13*). The median effective concentration (IC_{50}) was defined as the dose required for a 50% inhibition for sample extract or standard compound, and calculated by a CurveExpert (Version 1.3) software.

Antiproliferation Assay. *Cell Lines and Cell Cultivation*. Nine human cancer cell lines were used for antiproliferation assays: (1) Acute promyelocytic leukemia cell line HL-60 was maintained in the Iscove's MDM medium; (2) ovary adenocarcinoma cell line SK-OV-3 was maintained in the McCoy's 5a medium; (3) gastric adenocarcinoma cell line AGS was maintained in the F-12K medium; (4) tongue squamous carcinoma cell line CAL 27 was maintained in the DMEM medium; (5) colorectal adenocarcinoma cell line SW480 was maintained in the L-15 medium; and (6) breast adenocarcinoma cell line MCF-7, (7) prostate carcinoma cell line DU145, (8) hepatocellular carcinoma cell line HepG2, and (9) colorectal adenocarcinoma cell line Caco-2 were maintained in the Eagle's MEM medium. All media were supplemented with 10% FBS (except for Caco-2 and HL-60 with 15% FBS) and 1% penicillin/streptomycin (v/v). Cells were maintained at 37 °C and 5% CO_2 (except for cell line SW480 without CO_2). Routine observation for cell viability was performed under phase contrast inverted microscopy. Cell viability was determined by trypan blue exclusion and counting in a hemocytometer.

MTT Assay. The solvent-free freeze-dried extract (10 mg) dissolved in cell culture medium was the stock sample solution, which was diluted to final working solutions with medium (0.125, 0.25, 0.5, 1, 2, 5, and 10 mg/mL). The antiproliferation assay was performed as a well-established MTT method (*17*). The detailed procedures were described in our latest publication (*13*). The absorbance was measured on the BMG microplate reader at 540 nm. The percent viability of treated cells was calculated as follows: $A_{sample}/A_{control} \times 100$. The 50% growth inhibitory concentration (IC_{50}) was defined as the dose required (soy milk extract concentration mg/mL) for causing a 50% inhibition for sample extract and was used as the basis for comparing antiproliferation activities of different samples.

Statistical Analysis. The data were expressed as mean ± standard deviation. Statistical analysis was performed using 2005 SAS (Version 9.1, SAS institute Inc. Cary, NC). Analysis of variance (ANOVA) was conducted. Duncan's multiple range tests were used to determine the significant differences between group means. Significant levels were defined as probabilities of 0.05 or less. Pearson correlation test was conducted to determine the correlation coefficients between variables.

RESULTS AND DISCUSSION

Total Phenolic Substances, Phytic Acid, and Saponins of Soy Milk Processed by Different Methods. Total phenolic content (TPC) and total flavonoid content (TFC) of the soy milk extracts are presented in **Figures 1A** and **1B**. All soy milk processing methods, including the two-stage industry method, decreased ($P < 0.05$) approximately around 5−10% of the TPC in the raw soy milk. In the cases of TFC assay, as compared to the raw soy milk, all processing treatments caused significant ($P < 0.05$) increases in TFC values. Significant differences ($P < 0.05$) in TFC values were found among most processing treatments. Especially, the industry (the two-stage) method caused significantly ($P < 0.05$) greater increases in TFC values than the other four methods. The extent of increase in the TFC as affected by the direct and indirect UHT conditions (143 °C, 60 s) was not as high as that observed (90−110% increase) in our previous report (*9*). The commercial Silk plain soy milk product had significantly lower TPC and TFC values than those of soy milk processed by the selected methods, including the conventional and UHT methods and the two-stage industry method. The differences between our laboratory processed soy milk and the commercial soy milk may be partly due to differences in the raw bean materials and the processing methods used.

Phytic acid has long been considered to interfere with the absorption of minerals, especially zinc. Several earlier works suggested that phytic acid might have anticarcinogenic properties (*18*). Phytic acid is also considered to be an antioxidant agent, because it is a potent inhibitor of the iron-catalyzed hydroxyl radical formation by chelating the free iron and then blocking its coordination site (*19*). Most listings of soybean antinutritional factors in the past included saponins, although with little or no justification. In recent years, saponins attracted considerable interest as a result of their diverse properties. Clinical studies suggested that saponins affected the immune system in ways that helped protect the human body against cancers, and also helped lower cholesterol levels, decrease blood lipids, lower cancer risks, and lower blood glucose response (*20*). Consequently, there is a renewed interest in phytic acid and saponin compounds.

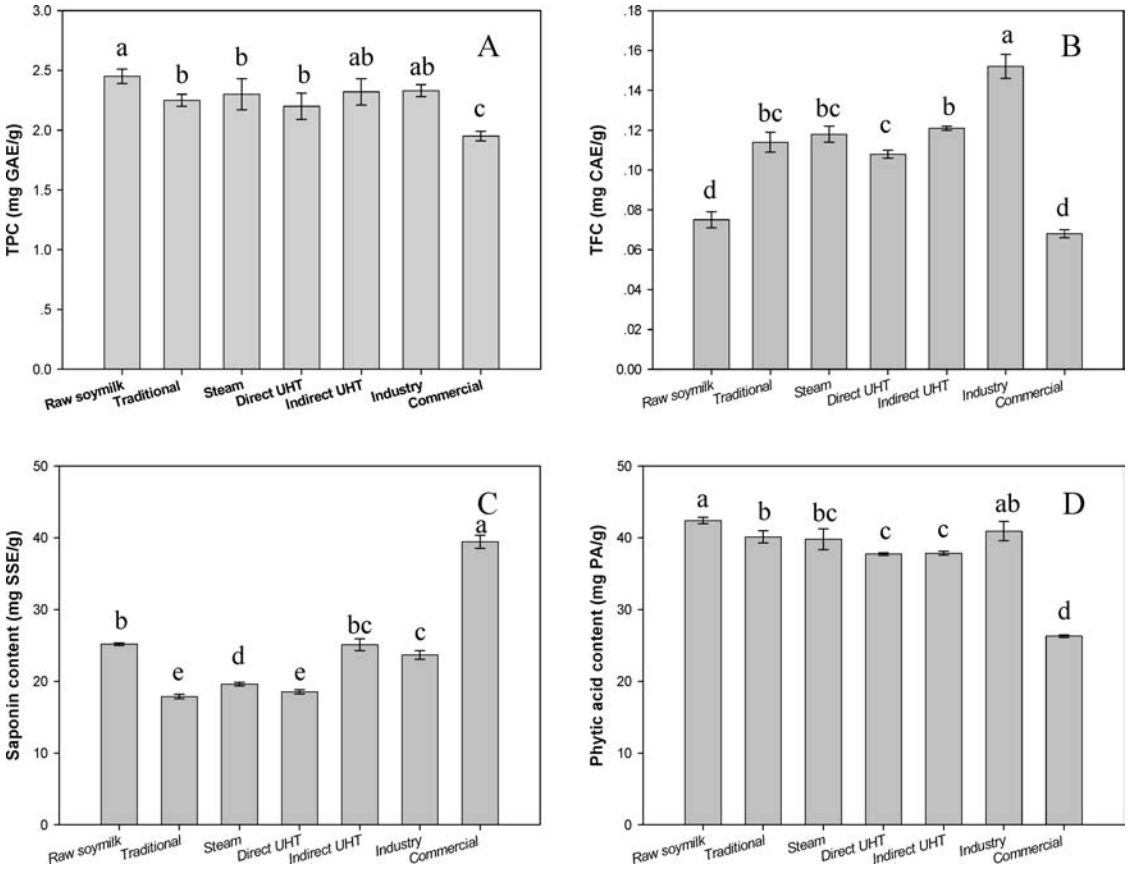

Figure 1. Comparison of phytochemicals of soy milk processed by various methods: **A**, total phenolic content; **B**, total flavonoid content; **C**, saponin content; **D**, phytic acid content. Bar data are expressed as mean ± standard deviation ($n = 3$) on dry weight basis. Values marked above the bars with the same letter are not significantly different ($p < 0.05$).

Although certain attention had been paid to the bioactivities of these two classes of compounds in soybean, their chemical profiles and potential contribution to the health benefits of soy milk are not clear. As shown in **Figures 1C** and **1D**, most processing treatments caused significant ($P < 0.05$) decreases in total saponin content (TSC) as compared to the raw soy milk. However, the indirect UHT and the two-stage industry methods retained significantly ($P < 0.05$) higher TSC values than traditional stove cooking, steam injection and direct UHT. Except the two-stage industry method, all processing methods decreased phytic acid as compared to the raw soy milk. However, the changes among all methods were within 10% of the raw soy milk. The commercial product exhibited the highest TSC, but the lowest phytic acid content among all soy milk samples. The discrepancies between the commercial and our laboratory-processed soy milk might be due to differences in soybean materials and processing methods used.

In this comparative study, we found that the commercial soy milk exhibited the lowest TPC, TFC, and phytic acid, while the highest saponin content among all processed soy milk. Our results also indicated that processing might cause complex changes in chemical compositions through the degradation of polyphenols and/or release of bound phenolic components.

Isoflavone Profile Comparison of Soy Milk Processed by Different Methods. Isoflavones have been the center of research in the prevention of cancer and cardiovascular disease by soy. Isoflavone profiles in soybean and soy milk had been investigated (*8*, *9*, *21*−*24*). However, no studies had been conducted to characterize isoflavone profiles in soy milk processed by different thermal processing methods that produced wide differences in sterilization values for achieving high trypsin inhibitor inactivation. Our current study on isoflavone composition went beyond the previously studied UHT conditions (*9*) by including a two-stage industry process and a commercial soy milk product for comparison. The isoflavone contents are presented in **Tables 2** and **3** in three ways: (i) Individual isoflavone contents (**Table 2**) were directly measured from HPLC chromatograms for all 12 forms. (ii) Subtotal isoflavone contents of aglycons (aglycon equivalents) for each of the three types of isoflavones (**Table 2**) were calculated by converting the malonylglucoside, acetylglucoside, and 7-*O*-*β*-glucoside weight into the aglycon weight using the respective molecular weight factors prior to summation. Total isoflavone (TIF) contents (**Table 2**) were the sum of the adjusted sums of subtotal genistein + subtotal daidzein + subtotal glycitein according to Murphy et al. (*21*). Therefore, the TIF values were not the simple addition of the mean individual values. (iii) The percentage content of subtotal individual aglycons (**Table 3**) was obtained by dividing subtotal individual aglycon contents by TIF.

As shown in **Table 2**, all 12 individual isoflavone compounds were detected in both raw and all processed soy milk. The TIF contained in the raw and processed soy milk was about 1500−1800 μg/g. Similar to what has been reported in the literature (*9*, *21*−*23*), most isoflavones in soy milk existed as glucosides (including 7-*O*-*β*-glucosides, malonylglucosides and acetylglucosides), while aglycons occurred in only very small proportions. There was a significant impact on the transformation of isoflavones as a result of different processing methods.

As compared to the raw soy milk, all thermal processing significantly ($p < 0.05$) increased the content of 7-*O*-*β*-glucosides

Table 2. Isoflavone Profiles and Contents (μg/g) of Soy Milk Processed by Different Methods[a]

	7-*O*-β-glucosides			malonylglucosides			acetylglucosides		
soy milk ID	Din	Gin	Gly	MDin	MGin	MGly	ADin	AGin	AGly
raw	111.1 ± 2.22 e	250.4 ± 18.09 e	64.81 ± 0.20 d	540.8 ± 47.21 a	1209.1 ± 82.82 a	58.86 ± 6.26 ab	26.47 ± 0.69 d	83.48 ± 3.48 e	2.01 ± 0.11 d
traditional	266.3 ± 25.68 d	420.3 ± 29.06 d	122.3 ± 7.65 a	267.5 ± 20.09 d	1054.8 ± 55.63 b	51.94 ± 7.32 ab	52.01 ± 4.69 b	22.04 ± 1.33 f	47.51 ± 4.52 c
steam	256.4 ± 0.29 d	429.0 ± 11.00 d	56.08 ± 1.15 de	371.1 ± 1.43 b	1199.9 ± 28.75 a	58.19 ± 3.90 ab	62.20 ± 1.76 a	36.54 ± 8.90 f	54.86 ± 1.31 b
direct UHT	353.7 ± 4.63 c	546.6 ± 33.46 c	52.96 ± 0.14 e	380.2 ± 62.84 b	1219.9 ± 95.64 a	48.23 ± 8.42 b	61.73 ± 0.48 a	264.8 ± 18.28 b	50.50 ± 3.75 bc
indirect UHT	382.6 ± 0.79 b	637.9 ± 8.62 b	131.3 ± 8.13 a	324.7 ± 0.22 c	928.7 ± 21.90 dc	18.99 ± 1.29 c	58.78 ± 0.55 ab	365.9 ± 5.66 a	129.00 ± 0.26 a
industry	394.9 ± 9.50 b	573.6 ± 37.71 c	109.5 ± 2.44 b	294.3 ± 8.81 d	1122.0 ± 93.71 a	64.92 ± 6.31 a	66.02 ± 6.49 a	201.9 ± 19.76 c	55.12 ± 3.95 b
commercial	477.1 ± 12.14 a	893.9 ± 57.12 a	79.91 ± 2.00 c	232.42 ± 23.60 e	804.2 ± 59.10 c	10.93 ± 1.08 c	37.43 ± 1.51 c	143.2 ± 4.25 d	57.50 ± 3.23 b

	aglycons			subtotal individuals[b]		total[c]	
soy milk ID	Dein	Gein	Glein	T-Dein	T-Gein	T-Glein	isoflavones
raw	146.8 ± 8.11 a	332.5 ± 20.67 a	5.71 ± 0.01 d	515.9 ± 23.76 a	1177.2 ± 77.13 a	79.67 ± 4.41 e	1772.7 ± 45.32 a
traditional	59.72 ± 3.47 b	140.1 ± 7.25 b	20.92 ± 1.59 b	389.5 ± 4.64 b	965.2 ± 82.83 c	154.3 ± 4.57 b	1506.0 ± 2.89 c
steam	12.56 ± 0.62 c	33.80 ± 2.27 c	20.07 ± 1.64 b	391.4 ± 1.05 b	937.9 ± 37.54 c	176.3 ± 10.07 a	1505.6 ± 26.42 c
direct UHT	14.26 ± 0.79 c	35.44 ± 1.87 c	56.13 ± 2.84 a	456.8 ± 28.45 b	1137.8 ± 16.46 a	145.12 ± 9.44 b	1739.7 ± 21.44 a
indirect UHT	13.05 ± 0.45 c	32.12 ± 1.30 c	19.41 ± 0.33 b	443.6 ± 1.12 b	1123.4 ± 14.88 a	188.4 ± 5.69 a	1755.4 ± 21.69 a
industry	10.88 ± 0.08 c	28.32 ± 2.36 c	9.68 ± 0.37 c	437.5 ± 2.33 b	1086.7 ± 86.03 b	146.4 ± 4.49 b	1670.5 ± 92.86 b
commercial	14.59 ± 0.73 c	28.05 ± 1.66 c	8.74 ± 0.30 cd	444.2 ± 7.16 b	1087.5 ± 70.58 b	99.05 ± 0.89 d	1630.7 ± 108.6 b

[a] Data are expressed as mean ± standard deviation (n = 3) on dry weight basis. Values marked by the same letter within a column are not significantly different (P < 0.05). Din, daidzin; Gin, genistin; Gly, glycitin; MDin, malonyldaidzin; MGin, malonylgenistin; MGly, malonylglycitin, ADin, acetyldaidzin; AGin, acetylgenistin; AGly, acetylglycitin; Dein, daidzein; Gein, genistein; Glein, glycitein. T-Dein, subtotal daidzein; T-Gein, subtotal genistein; T-Glein, subtotal glycitein. [b] Subtotal individuals = moles of isoflavone × molecular weight of aglycon form isoflavone. [c] Total isoflavone content = sum of subtotal individuals of aglycons.

Table 3. Normalized Isoflavone Content and Ratios of Malonylglucosides to 7-*O*-β-Glucosides of Soy Milk[a]

soy milk ID	total isoflavone (μg/mL)	total daidzein[a] (%)	total genistein[a] (%)	total glycitein[a] (%)	malonyldaidzin/daidzin[b]	malonylgenistin/genistin[b]
raw	1772.7 ± 45.32	29.09 ± 0.59	66.41 ± 0.67	4.49 ± 0.08	5.10 ± 0.15	4.94 ± 0.48
traditional	1506.0 ± 2.89	25.66 ± 0.26	64.09 ± 0.06	10.24 ± 0.32	1.01 ± 0.17	2.52 ± 0.31
steam	1505.6 ± 26.42	25.99 ± 0.53	62.28 ± 1.40	11.72 ± 0.87	1.45 ± 0.01	2.53 ± 0.04
direct UHT	1739.7 ± 21.44	26.25 ± 1.31	65.41 ± 1.75	8.34 ± 0.43	1.08 ± 0.19	2.15 ± 0.18
indirect UHT	1755.4 ± 21.69	25.27 ± 0.25	63.99 ± 0.06	10.73 ± 0.19	0.85 ± 0.00	1.46 ± 0.01
industry	1670.5 ± 92.86	26.23 ± 1.32	65.01 ± 1.54	8.77 ± 0.22	0.75 ± 0.04	1.95 ± 0.03
commercial	1630.7 ± 108.6	27.24 ± 0.60	66.68 ± 0.69	6.07 ± 0.09	0.49 ± 0.01	0.91 ± 0.15

[a] The percentage content of subtotal individual aglycons was normalized by dividing the subtotal individual aglycon content by the total isoflavone content. [b] The ratios equal the malonylglucoside content divided by the 7-*O*-β-glucoside content.

(daidzin and genistin) and 6″-*O*-acetyl-β-glucosides (acetyldaidzin and acetylglycitin). Generally, thermal processing tended to decrease the content of 6″-*O*-malonyl-β-glucosides (malonyldaidzin and malonylgenistin) and aglycons (daidzein and genistein). In addition, the soy milk processed by both direct and indirect UHT processing methods and the industry UHT method as well as commercial soy milk exhibited significantly higher 6″-*O*-acetyl-β-glucosides (acetyldaidzin, acetylgenistin and acetylglycitin) as compared to the raw soy milk. In terms of the contents of subtotal individuals, as compared to the raw soy milk, all thermal processing significantly ($p < 0.05$) decreased the subtotal daidzein and subtotal genistein (except for the two UHT methods). In terms of TIF contents (sum of total individuals of aglycons), as compared to the raw soy milk, the traditional stove cooking, steam injection method and the industry method significantly ($p < 0.05$) decreased the TIF contents, while the two UHT methods did not.

When comparing the two UHT methods, significantly ($p < 0.05$) higher 7-*O*-β-glucosides (daidzin, genistin and glycitin) and higher 6″-*O*-acetyl-β-glucosides (acetyldaidzin and acetylglycitin) and significantly ($p < 0.05$) lower 6″-*O*-malonyl-β-glucosides were found in the indirect UHT processed soy milk as compared to the direct UHT processed soy milk. There were no significant differences in TIF contents between traditional stove cooking and steam injection, between direct and indirect UHT, and between industry and commercial soy milk.

As the results were normalized into percentages of the total isoflavones (**Table 3**), we found that the ratios (5.1 and 4.9, respectively) of malonyldaizin to daidzin and malonylgenistin to genistin in the raw soy milk were dramatically decreased in the processed soy milk. These analyses also showed that thermal processing significantly affected individual isoflavone content. Thermal processing caused obviously the intertransformation but did not cause severe degradations except for breaking down of some aglycons (**Table 2**). Huang et al. (*22*) also found that aglycon forms (daidzein and glycitein) decreased during the early stages of heating.

Isoflavones contained in soybeans or soy milk are mainly 6″-*O*-malonyl-β-glucosides, which are partly transformed to 7-*O*-β-glucosides and 6″-*O*-acetyl-β-glucosides upon thermal processing (*21*, *22*) or storage (*24*). The bioavailability of aglycons versus glucosides of isoflavones in humans have been a controversial subject since some reported aglycons were more bioavailable (*25*) but others observed that glucosides were more available (*26*). Furthermore, some researchers found that no differences in bioavailability existed between the aglycons and glucosides (*27*, *28*). However, processing-induced changes in the individual isoflavones (free and conjugated) and their ratios is still important to their health-promoting effects upon absorption since individual isoflavones do not have the same absorption and body retention (*28*). In this current study, we found that UHT processed soy milk lost significantly higher aglycon contents when compared to the raw soy milk and traditional stove cooked soy milk. The losses may due to ultrahigh temperatures, which caused ring skeleton structures of aglycons to open up, and finally caused the degradation of aglycons. This degradation phenomenon might contribute to the reduction in cellular antioxidant activity or

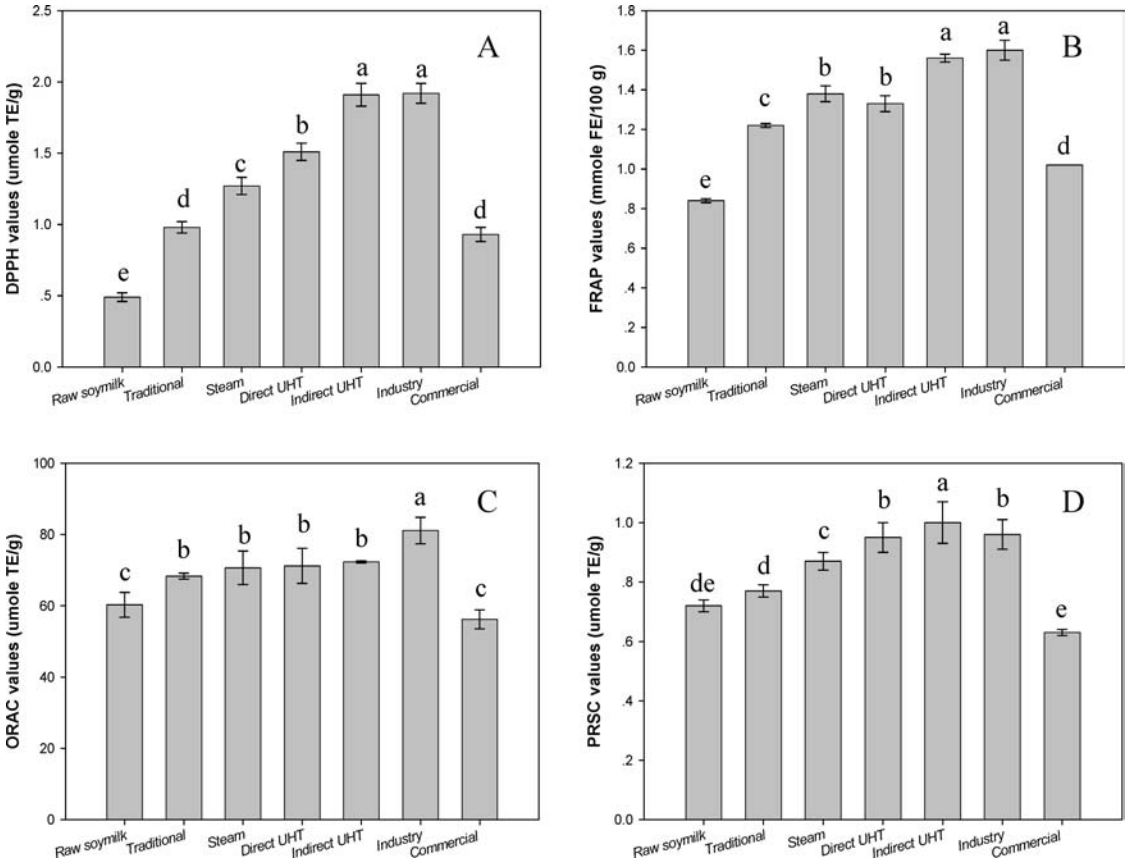

Figure 2. Comparison of chemical antioxidant capacities (**A**, DPPH; **B**, FRAP; **C**, ORAC; **D**, PRSC) of soy milk processed by various methods. Bar data are expressed as mean ± standard deviation (n = 3) on dry weight basis. Values marked above the bars with the same letter are not significantly different ($p < 0.05$).

antiproliferation effect since aglycons are more hydrophobic molecules and may move across cell membranes better than more hydrophilic glucosides and conjugated glucosides. However, this hypothesis remains to be elucidated. In addition, we found that indirect UHT processing transformed more 6″-*O*-malonyl-β-glucosides into 7-*O*-β-glucosides and 6″-*O*-acetyl-β-glucosides than direct UHT. This result is different from the findings of Probhakaran and Perera (*8*), who found that both direct and indirect UHT (143 °C, 10 s) methods produced similar compositions of isoflavones. The discrepancies may be due to the different processing times used in our study (60 s). Interestingly, the commercial soy milk exhibited significantly higher 7-*O*-β-glucosides (daidzin, genistin) than any soy milk processed in our laboratory. The reason for the higher 7-*O*-β-glucosides contents may be due to more complex pretreatment and thermal processes that may be involved in the commercial practice.

Antioxidant Capacities Comparison of Soy Milk Processed by Different Methods. Chemical antioxidant activities of the raw and processed soy milk, including DPPH free radical scavenging capacity (DPPH), ferric reducing antioxidant power (FRAP), oxygen radical absorbing capacity (ORAC), and peroxyl radical scavenging activity (PRSC), are presented in **Figure 2**. Significant differences ($P < 0.05$) in DPPH, FRAP, ORAC and PRSC values were found among most samples. As compared to the raw soy milk, all processing significantly ($P < 0.05$) increased DPPH, FRAP, ORAC and PRSC values. The industry method and indirect UHT processed soy milk exhibited significantly ($P < 0.05$) higher antioxidant activities (DPPH and FRAP values) than those processed by direct UHT and conventional methods. The two-stage industry method processed soy milk exhibited the highest ORAC values among all soy milk. Both direct and indirect UHT methods and the two-stage industry method processed soy milk exhibited higher PRSC values than traditional stove cooked and steam injection processed soy milk. The commercial soy milk product exhibited the lowest antioxidant capacities (DPPH, FRAP, ORAC and PRSC values) among all processed soy milk. Our previous study also showed that the selected UHT processing conditions could increase antioxidant abilities of soy milk. However, the degrees of the increases in the current study were higher in DPPH and FRAP values, but lower in the ORAC values as compared to that in the previous study. This is understandable since food processing at the pilot plant scale at different times may produce slightly different results.

The probe DCFH-DA, which is cell permeable, had been used to monitor the intracellular level of oxidative stress (*16*). Thermal degradation of 2,2′-azobis(amidinopropane) produces peroxyl radicals (ROO•), which oxidizes nonfluorescent dichlorofluorescein (DCFH) to highly fluorescent dichlorofluorescein (DCF). The degree of inhibition of DCFH oxidation, by antioxidants that scavenge peroxyl radicals, was used as the basis for calculating cellular antioxidant activity (CAA), which takes into account cellular bioavailability. The kinetics of DCFH oxidation by peroxyl radicals recorded as fluorescence generation is shown in **Figure 3A** for the raw soy milk. The results indicated that peroxyl radicals generated from thermal degradation of AAPH oxidized DCFH into fluorescent products over time and that the raw soy milk could scavenge peroxyl radicals and inhibit the oxidation reaction in a dose-dependent manner (**Figure 3B**). The CAA values of the raw and processed soy milk are listed in **Table 2**. Only raw soy milk exhibited dose-dependent CAA with IC_{50} value at 0.79 mg/mL. The other soy milk did not show dose-dependent CAA.

Although previous findings have demonstrated that fermented soy milk possessed significantly higher antioxidant properties than unfermented soy milk, the antioxidant properties of the raw and heat-processed unfermented natural soy milk have not been systematically investigated. It is well-known that natural antioxidants in foods can be lost significantly during thermal processing. Nevertheless, it had been demonstrated that thermal treatments could induce the formation of new compounds with antioxidant activities (*29*). In the present study, we found that thermal processing, including conventional processes and UHT, had increased antioxidant activities of soy milk as determined by four chemically antioxidant assays when compared to the raw soy milk. Similar positive heat effects were found in steamed yellow soybeans (*14*) and steamed broccoli (*30*). In addition, previous research on fruits and vegetables indicated that processing increased antioxidant potential due to improvement of antioxidant properties of naturally occurring compounds or the formation of novel compounds, such as the Maillard reaction products having antioxidant activity (*31*). The increases in antioxidant activities of processed soy milk may be attributed to, in part, the formation of new compounds with antioxidant properties or the transformation of existing compounds into compounds with higher antioxidant properties. Besides natural phenolic phytochemicals, soybean protein derived peptides also had been reported to exhibit radical-scavenging activities. Chen et al. (*32*) reported that a total 22 peptides that derived from a soybean major storage protein exhibited certain DPPH radical-scavenging activities. In our current study, the processed soy milk demonstrated higher antioxidant activities than the raw soy milk, suggesting that the increased activities may be attributable, in part, to the active peptides (with antioxidant activities) released from soybean storage proteins during soy milk processing. Further studies are needed to confirm that peptides are released by thermal processing to support this hypothesis.

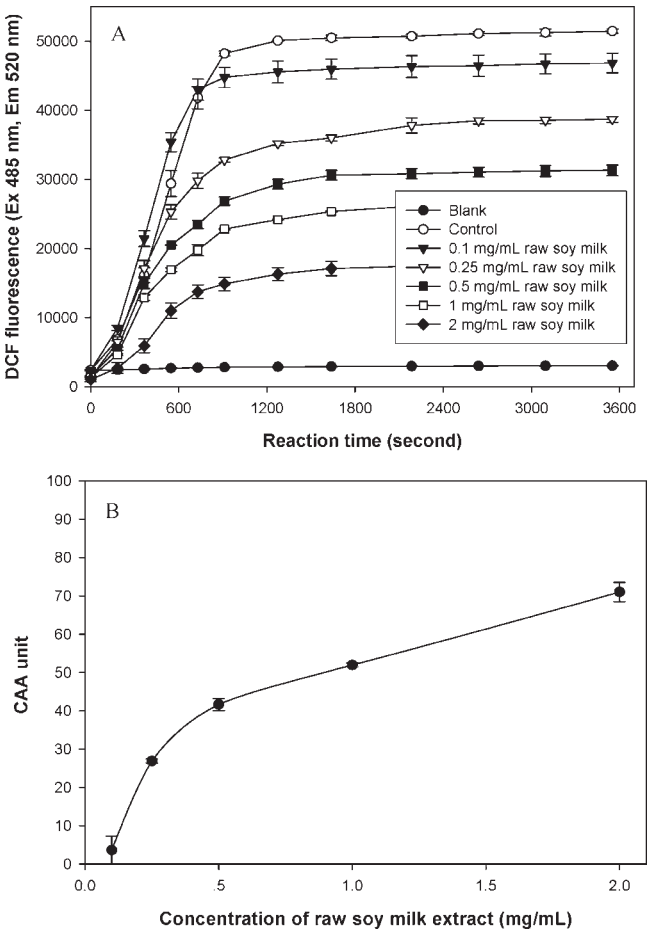

Figure 3. Time kinetics and dose−response curves for inhibition of peroxyl radical-induced DCFH oxidation in cell-based antioxidant (CAA) assay: **A**, time kinetics of raw soy milk against DCFH oxidation. **B**, dose−response plots of raw soy milk against DCFH oxidation.

In contrast with the previous finding that steamed broccoli possessed a higher CAA (*27*), we found that all processed soy milk lost their original CAA in the raw soy milk. Namely, dose-dependent CAA in the raw soy milk became non-dose-dependent after thermal processing. This was also in contrast with the increased antioxidant activities determined by chemical antioxidant assays (DPPH, FRAP, ORAC and PRSC). Further investigations need to be carried out to elucidate the reasons why processed soy milk exerted the antioxidant capacity when determined chemically, but did not exert antioxidant capacity when determined in a biological system with *in vitro* cultured cells.

Antiproliferation Properties Comparison of Soy Milk Processed by Different Methods. The antiproliferation properties of the raw and processed soy milk against cancer cell lines are summarized in **Table 4**. Among nine different human cancer cell lines tested, dose-dependent antiproliferation effects of both raw and processed soy milk were found only in three cell lines (leukemia cell line HL-60, stomach AGS and prostate DU145). For the antiproliferation assay using Caco-2 cells, only the raw soy milk exhibited dose-dependent effects with an IC_{50} of 1.09 mg/mL. All processed soy milk did not exhibit dose-dependent antiproliferation effects in this Caco-2 cell line. The antiproliferative effects of both the raw and processed soy milk against the other five cell lines tested were non-dose-dependent. For this reason, the non-dose-dependent data of these cell lines were not included in **Table 4**.

Among all soy milk tested, the raw soy milk possessed the strongest antiproliferation capacities against gastric adenocarcinoma cells by comparing their IC_{50} values (**Table 4**), followed by

Table 4. Cellular Antioxidant Activities and Antiproliferation Properties of Soy Milk against Human Cancer Cell Lines[a]

	cellular antioxidant act.		antiproliferation					
	gastric adenocarcinoma cells AGS		gastric adenocarcinoma cells AGS		leukemia cells HL-60		prostate carcinoma cells DU-145	
soy milk ID	act.	IC_{50} (mg/mL)	act.	IC_{50} (mg/mL)	act.	IC_{50} (mg/mL)	act.	IC_{50} (mg/mL)
raw	DDI[b]	0.79	DDI	2.43 ± 0.06 d	DDI	4.70 ± 0.27 d	DDI	2.12 ± 1.02 c
traditional	NDD[c]	NA	DDI	5.91 ± 0.76 bc	DDI	6.25 ± 0.07 bc	DDI	6.67 ± 2.48 ab
steam	NDD	NA	DDI	6.42 ± 0.09 b	DDI	5.90 ± 0.17 c	DDI	7.50 ± 1.15 a
direct UHT	NDD	NA	DDI	5.64 ± 0.08 bc	DDI	5.44 ± 0.18 cd	DDI	7.29 ± 1.11 ab
indirect UHT	NDD	NA	DDI	5.64 ± 0.39 bc	DDI	7.12 ± 0.28 ab	DDI	3.31 ± 1.04 bc
industry	NDD	NA	DDI	4.81 ± 0.67 c	DDI	3.34 ± 0.31 e	DDI	1.88 ± 0.11 c
commercial	NDD	NA	DDI	8.71 ± 1.02 a	DDI	7.28 ± 0.77 a	DDI	7.79 ± 2.14 a

[a] Data were obtained from triplicate wells for each sample and duplicate running on 96-well plates. Values with different letters within a column are significantly different ($P < 0.05$). [b] DDI, dose-dependent inhibition. [c] NDD, non-dose-dependent inhibition. NA, not available.

soy milk processed by the two-stage industry method. The IC_{50} value against HL-60 cells by the industry method was the lowest among all processing methods, followed by raw soy milk, and the direct UHT method (**Table 4**). The IC_{50} value of raw soy milk against the DU145 prostate cancer cells was the lowest, followed by soy milk processed by the two-stage industry method, and then soy milk processed by the indirect UHT method (**Table 4**). The inhibitory ability of soy milk extract against prostate cancer cells DU145 was consistent with the findings of an epidemiological study (*3*). The commercial soy milk exhibited the lowest antiproliferation capacity against all three tumor cell lines among all soy milk as indicated by its highest IC_{50} values. In summary, the antiproliferation capacities of soy milk depended upon cell lines and was influenced by processing methods though there is a tendency that raw soy milk and the two-stage industry method had higher antiproliferation capacities than others.

In general, five classes of compounds in soybeans have been identified as anticarcinogens that include isoflavones, saponins, phytates, protease inhibitors (protein) and phytosterols (*1*, *33*). The anticancer effects of soy extract containing isoflavones and purified genistein have been extensively investigated. However, the interactions among the five anticarcinogen factors in processed soy products and their relative contributions to the prevention of tumors are not known. The effect of dietary isoflavones against estrogen-dependent MCF-7 breast cancer cells was affected by the degree of food processing (*34*). Another study showed that a commercial soy extract (with a mixture of isoflavones, proteins, and saponins) has better effect than purified genistein on the inhibition of an estrogen-independent breast tumor growth in mice (*35*). In fact, in this case, purified genistein was found to stimulate the tumor growth. However, in another study, purified genistein was found to be as effective as the crude extract of soybean cake in inhibiting two prostate tumor cell lines (*36*). Therefore, the effect of bioactive components in soy on tumor prevention is dependent upon the sources of the bioactive components and the properties of different types of cancer cell lines.

The anticancer potential of soy milk is still largely unknown. In examining the effect of thermal processing on the content of isoflavones, saponins, phytates and total phenolic and flavonoid contents in our current study, it did not appear that these contents have any consistent relationships with the effect of antiproliferation against the selected cancer cells. A case control epidemiological study showed that the daily consumption of soy milk was associated with a 70% reduction in the risk of prostate cancer (*3*). However, the anticancer mechanism is not clear. An animal study using hairless mice with high risk of developing skin tumors found that topical application of non-denatured soy milk but not heat-denatured soy milk once a day for a week to these high risk mice inhibited the formation and growth of UVB-induced skin tumors (*37*). Trypsin inhibitor in the raw soy milk was found to contribute to this effect. Our current study at least verified that raw soy milk (non-denatured) is more powerful than most processed soy milk in inhibiting selected cancer cell growth. However, raw soy milk is not drinkable since it has a high beany flavor and a high trypsin inhibitor content that may damage the pancreas. Therefore, solvent extracts of raw soy milk may be used to treat certain cancers by using special administration. The current *in vitro* preliminary screening on anticancer potential of soy milk using nine different cell lines verified the anticancer potential of denatured soy milk.

In summary, thermal processing significantly affected both chemical and cellular antioxidant activities, anticancer activities and phytochemical profiles in terms of the content, retention and distribution of saponins, phytic acid, total phenolics and individual isoflavones. The effects depended upon processing conditions. Overall, thermal processing increased chemical antioxidant capacities of soy milk but lost cellular antioxidant capacities, and decreased the antiproliferation capacities of the raw soy milk in most cases. When compared with the traditional processing method under atmospheric pressure, the UHT methods and the two-stage industry method showed advantages in the retention of total phenolics, total isoflavones and chemical antioxidant capacities, but disadvantages in the degradation of aglycons.

ABBREVIATION USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; CAA, cellular antioxidant activity; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; FBS, fetal bovine serum; FRAP, ferric reducing antioxidant power; HBSS, Hanks balanced salt solution; MTT, 3-(4,5-dimenthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ORAC, oxygen radical absorbing capacity; PBS, phosphate buffered saline; PRSC, peroxyl radical scavenging activity; TPC, total phenolic content; TFC, total flavonoid content; THB, 2,4,4′-trihydroxybenzoin; TSC, total saponin content; UHT, ultrahigh temperature.

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