EFFECTS OF PROCESSING ON HEALTH-PROMOTING ATTRIBUTES OF SOYMILK PHENOLICS

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ABSTRACT

Tan, Yingying, M.S., Department of Biological Sciences, College of Science & Mathematics, North Dakota State University, September 2010. Effects of Processing on Health-Promoting Attributes of Soymilk Phenolics. Major Advisors: Dr. Sam Chang and Dr. Katie Reindl.

This study was designed to investigate the effects of different processing methods on the antioxidant and anti-cancer properties of soymilk crude phenolic extract (CPE) using the human prostate cancer cell line DU 145 as a model system. Four grinding methods and two ultra-high temperature (UHT) processing methods were investigated. Briefly, the four grinding methods included grinding with tap water (Method 1), okara-washed water (Method 2), soaked water (Method 3), as well as both okara-washed water and soaked water (Method 4); the two UHT processing conditions included one-phase UHT (143 ^{0}C, 60 s) and two-phase UHT (120 ^{0}C, 80 s + 140 ^{0}C, 4 s) methods. The antioxidant capability of CPE was measured by oxygen radical absorbance capacity (ORAC) and/or ferric reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging activity (DPPH) assays. The DU 145 human prostate cancer cell line was sub-cultured in the presence of CPE at various concentrations (0, 1, 2, 4, 8 mg/mL) for 48 h and the percentage of cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and/or flow cytometric analysis with carboxyfluorescein succinimidyl ester (CFSE) staining. An induction of

apoptosis was detected by flow cytometric analysis with annexin V/ propidium iodide (PI) double staining, morphological change observation with acridine orange/ethidium bromide (AO/EB) double staining, and Western blot assay of apoptotic proteins.

The results showed that grinding Method 4 and the two-phase UHT method exhibited greater total phenolic content and antioxidant capability than the other three grinding methods and one-phase UHT, respectively. In addition, soymilk CPE inhibited cell proliferation in a dose-dependent manner and upon purification. The anti-proliferation effects observed in soymilk CPE were associated with the apoptosis induction in DU 145 cell line as evidenced by morphological changes and the expression of apoptotic proteins. These results suggest that the soymilk CPE plays an important role in anti-proliferation, and apoptotic properties of soy in DU 145 cells. Furthermore, these health-promoting properties were affected by processing conditions, such as grinding and thermal conditions. The results of this study benefit the soy product industry to select appropriate processing conditions to retain more health-promotion phytochemicals during soy food's processing.

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LIST OF ABBREVIATIONS

AAPH 2, 2'-azobis (2-methylpropionamide) dihydrochloride

ANOVA Analysis of variance

AO/EB Acridine orange/ethidium bromide

CE Catechin equivalent

CFSE Carboxyfluorescein succinimidyl ester

CPE Crude phenolic extract

CTC Condensed tannin content

DMSO Dimethyl sulfoxide

DPPH 2, 2-diphenyl-1-picrylhydrazyl

FBS Fetal bovine serum

FRAP Ferric reducing antioxidant power assay

GAE Gallic acid equivalent

HPLC High performance liquid chromatography

IC_{50} The half maximal inhibitory concentration

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

One-phase UHT 143 ^{0}C, 60 s

ORAC Oxygen radical absorbance capacity

PBS Phosphate buffered saline

PI Propidium iodide

SPPE Semi-purified phenolic extract

TE Trolox equivalents

TFC Total flavonoid content

THB 2,4,4- trihydroxydeoxybenzoin

TPC Total phenolic content

Two-phase UHT 120 ^{0}C, 80 s + 140 ^{0}C, 4 s

UHT ultra-high temperature

CHAPTER 1. GENERAL INTRODUCTION

1.1. Soy Foods Background

Soy foods are consumed worldwide because of their nutrition and health benefits. The nutritional value of soy foods comes from a full range of amino acids and high protein content. In the past twenty years, scientists have paid more attention to soy foods' health-promoting properties than their basic nutritional value. Recently, even more evidence suggests that soy-food consumption has health-promoting benefits. Consumption of soy foods is considered to lower the risk of aging-associated diseases, including cancer and cardiovascular disease, through various mechanisms. The National Cancer Institute has supported the investigation of the anti-cancer properties of soybeans (*1*). The protective effects of soy have been demonstrated on breast, intestine, liver, colon, bladder, prostate, skin and stomach cancers (*2-4*). Soy saponins may prevent colon cancer advancement by inhibiting cell growth and enzymes involved in cell proliferation, as well as affecting cell morphology (*5*). Genistein in soy is suggested to inhibit tumor growth by affecting the activities of cell cycle regulators, pro-apoptotic and anti-apoptotic proteins, and the Akt/NF-κB family (*6*).

Soymilk is made from soybean, and is the basic product from which all blended soy-dairy foods are made. Other soy food products, such as tofu and soy yogurt, are also derived from soymilk. Raw soymilk must be cooked for human consumption, since it contains antinutrients such as trypsin inhibitor and

lectins. Accumulating evidence suggests that the processing conditions, such as temperature and time of heating affect the nutritional quality of soy (*7-9*). However, how food processing conditions affect the health-promoting propertiesof soy is not well understood. The Compendium of Material Medica has long recognized black soybean for its medicinal value and has credited black soybean with anti-aging and detoxification effects. However, the advantages of black over yellow soybean have not been fully understood.

1.2. Phytochemicals in Soy

Individual soy components have been studied for their health benefits (*10-13*). Soybeans, in particular, contain an abundance of phytochemicals that may confer important health benefits. Phenolics, including isoflavones, are the largest category of phytochemicals found in soy that are thought to contribute to the reduction of prostate cancer incidence in humans (*14*).

Phenolic compounds are characterized by the presence of one or more aromatic benzene rings with one or more hydroxyl group. Polyphenols, phenolic acids, and flavonoids are the three major groups of phenolics that are classified based on their chemical structures (*15*). Phenolic polymers, commonly known as tannins, are grouped into hydrolyzable and condensed tannins. Phenolic acids mainly include benzoic and cinnamic acid derivatives. Flavonoids, the largest group of phenolics, are divided into anthocyanins and anthoxanthins, which include flavonols, flavones, flavanols, and isoflavones (*15*). It is widely

considered that the beneficial health effects of soy are attributed, at least in part, to the presence of phenolics (*16, 17*). However, *in vitro* or *in vivo* studies have seldom been conducted on soy crude phenolics extract (CPE).

Isoflavones, a major group of phenolic compounds found in soybean (*18*), have attracted considerable attention for their potential health benefits. Purified isoflavones have been shown to inhibit cell growth *in vitro* and prevent tumor development in animal models (*19, 20*). Moreover, a great number of biological studies have been carried out to examine the anti-cancer activities of isoflavones on prostate cancer (*21-23*).

However, studies comparing the soy crude isoflavone extract to the purified commercial isoflavone standards suggest that the crude isoflavone extracts have greater anti-cancer and antioxidant capacities than the purified commercial standards (*24, 25*). A reasonable explanation for this phenomenon might be that some other phenolic compounds, either acting alone or in combination with isoflavones, may be involved in the anti-cancer effect of soy (*26*). Moreover, Hsu *et al.* suggest that soy foods, which have a combination of natural compounds, may be more effective and safer than individual compounds for inhibiting prostate cancer growth (*24*). Another study has indicated that soy extract has a greater ability to inhibit tumor growth than purified genistin (*36*). Another study showed that crude soy flour did not affect the tumor size, and purified isoflavones actually stimulated tumor growth (*37*). These observations

indicate that the purified isoflavones do not appear to have stronger anti-cancer effects than the crude extracts. To date, the health-promoting activities of soy CPE, such as the antioxidant and anti-cancer properties, have not been systematically studied.

Besides phenolics, other types of phytochemicals in soy have been investigated for their health benefits. Bowman-Birk inhibitor (BBI), a peptide found in many soy foods, is recognized as a health-promoting compound because of its ability to inhibit angiogenesis and promote anti-cancer activities (*27, 28*). Phytosterols are also components of soy with health benefits. Phytosterols inhibit the absorption of cholesterol from the small intestine, which leads to decreased blood LDL-cholesterol levels, and lowered cardiovascular disease risk. Phytosterols also have inhibitory effects on lung, stomach, ovarian, and breast cancers (*4, 29*). Saponin, another component of soy that enhances immune function, binds to cholesterol, and limits its absorption in the intestine, has anti-cancer effects (*30*). Phytic acid has long been considered an anti-nutritional component because it binds to minerals, proteins, and starch, and lowers the absorption of these elements. However, phytic acid has been shown to inhibit the growth of human gastric cancer SGC-7901 cells (*31*). Phytic acid also has anti-photocarcinogenic effects and might protect against UVB-induced tumor formation (*32*).

1.3. The Effect of Processing on Soy Components

Soybeans are mostly consumed as soymilk, tofu, and fermented products. Soymilk and tofu are the two major soy foods consumed worldwide, particularly in several Asian countries. Soymilk can be easily made into tofu by coagulating the proteins in soymilk. Soymilk is made by grinding soybeans in water or by homogenizing soy isolate with lipids. Soymilk contains similar protein content to cow's milk, but has very little saturated fat and no cholesterol. These healthy and nutritional characteristics make soymilk an important source of phenolics in human diets (*33*).

Because specific processing conditions are needed for improving the yield and quality of food products, the impacts of the grinding methods and processing protocols on the health benefits of soy food have been under investigation. Studies suggest that the degree of soy processing inversely affects the metabolism of daidzein to equol (*34*). Daidzein is a component of isoflavones that can be absorbed *in vivo*. A study has indicated that soy extract has a greater ability to inhibit tumor growth than purified genistin (*35*). Another study showed that crude soy flour did not affect the tumor size, and purified isoflavones actually stimulated tumor growth (*36*). These observations indicate that the purified isoflavones do not appear to have stronger anti-cancer effects than the crude extracts. Recent studies on soy fermentation indicate that the antioxidant activity of soy increases with increasing fermentation time, while the total isoflavone content does not change (*37*). To date, the health-promoting

activities of soy CPE, such as the antioxidant and anti-cancer properties, as affected by grinding and high temperature heating have not been systematically studied.

While soy foods contain significant health-promoting components, they also contain components that have negative effects on health. For example, high levels of trypsin inhibitor activity (TIA) can lead to pancreatic disease (*38*). Thermal processing is used to inactivate these health-impairing components, such as TIA and lipoxygenases (*39*). A recent publication from our lab has suggested that the TIA of soymilk decreases with the increase of temperature and time of ultra-high temperature (UHT) heating treatment. The maximal trypsin inhibitor inactivation is achieved by UHT methods with residual activities of approximately 10% (*39*). Therefore, thermal treatment is required for soymilk produced during and/or after grinding. Our laboratory, as well as other researchers, has reported that the phenolic contents and their biological activities in soybean and soymilk may be altered by the thermal processing (*40-44*).

Soymilk is usually heated in two different ways, traditional and UHT methods. The traditional cooking method is widely used in Asian countries, as it is the simplest and least expensive method. Traditional cooking is characterized by cooking the soymilk on a stove top for about 20 min, while regularly stirring the soymilk to avoid burning. Because the traditional cooking method takes a

long time and often results in the partial loss of food quality and beany flavor, two UHT methods have been designed according to literature and industry practice (*39, 45-47*). In this study, soymilk was processed by two UHT methods in order to evaluate the thermal processing effects on the anti-proliferative and apoptotic activities of soymilk. The rationale for this approach is that adequate heat is needed to inactivate the TIA and lipoxygenase activity in soymilk before it is consumed.

Previous work from our lab demonstrated that thermal processing significantly increased the antioxidant capacities of soymilk, while decreased the anti-proliferative capacities on leukemia, gastric, prostate, and colorectal cancer cells (*48*). Thermal processing significantly reduced total phenolic content (TPC) values, but increased total flavonoid content (TFC) values. UHT processing tends to increase antioxidant activities more than the traditional and steam processing methods (*41, 48*). Indirect UHT (steaming) processing transforms more isoflavones from malonyl glucosides into 7-O-β-glucosides than the direct UHT does (*49*). The steaming method retained much more antioxidants and phenolic components than the boiling method, while the boiling method reduces much more saponin and phytic acid contents than the steaming method (*43*).

Food processing studies suggest that the isoflavone profile of soymilk shifts from malonylglucosides toward beta-glucosides, and the isoflavone content changes under different pressure and thermal treatments (4.32 to 6.06

μmol/g) (*50)*. Furthermore, beta-glycosidase is used to convert isoflavone glycosides to isoflavone aglycone during tofu processing. This conversion may enhance the health benefits of isoflavones because aglycone is the isoflavone form that is absorbed (*34*) better than their glucosides. Although the literature provides invaluable information regarding the processing effects on the phytochemical content of soy foods, the processing effect of soymilk on the anti-cancer activities of phenolic compounds has not been well studied. Therefore, one aim for this study was to examine the anti-cancer effects of soymilk crude pheolic extract (CPE) on DU 145 prostate tumor cells after processing by specific thermal and grinding methods.

1.4. Soy Consumption and Prostate Cancer

Prostate cancer is the most commonly diagnosed type of cancer and also the third leading cause of death due to cancer among American males (*51*). Epidemiological studies on prostate cancer indicate that the incidence of prostate cancer, however, is much lower in Asian males than in Western males (*52-55*). Interestingly, studies involving Asian men that have migrated to Western countries show an increased incidence of prostate cancer in those men than those living in their homelands (*56, 57*). These observations suggest that environmental factors and changes in life styles, such as dietary habits, may play a pivotal role in the development of prostate cancer. Evidence shows that higher soy food consumption in Asian populations has relevance to the lower

prevalence of prostate cancer (*58*). Furthermore, an inverse association between soy consumption and the risk of prostate cancer in Asian and Western males has been observed (*59*).

Epidemiological studies have investigated the effects of soy consumption on the prevalence of prostate disease. These studies have identified an inverse relationship between soy consumption and the risk of developing prostate cancer in men (*60-62*). Animal studies suggest that commercial isoflavones inhibit chemically-induced prostate cancer in rats without any adverse effects. This anti-cancer effect is related to inhibiting progression of the prostatic intra-epithelial neoplasia to carcinoma, and down-regulation of ornithine decarboxylase and prothymosin alpha (*63*).

The protective effect of soy on prostate cancer is related to the type and quantity of soy food intake. Studies demonstrate that consumption of non-fermented soy foods (i.e. soymilk and tofu) reduces prostate cancer risk by 30%, whereas consumption of fermented soy foods (i.e. miso and natto) is not associated with prostate cancer risk (*64*). Miso, as a fermented soy ingredient, has been studied in breast, stomach, and colon cancers (*65-67*), but not in a prostate cancer model.

It has been reported that the isoflavone intake in the United States and Europe is very low (<3 mg/d/person) (*68, 69*), whereas it is much higher in Asian countries (about 10% of the Asian population consumes100 mg of isoflavones

per day) (*70*). In addition, Japanese men consuming soy foods tend to have higher concentrations of plasma isoflavones (a sub-type of phenolics) than Western men who do not consume soy foods (*71, 72*). The prostate cancer incidence of Japanese males is 12.6 per 100,000, while the incidence in Chinese males is lower at 1.6 per 100,000 (*73*). This difference may be partly explained by the different types of soy foods consumed. Chinese people mainly consume tofu and soymilk, which are non-fermented soy foods, while Japanese people also consume miso and natto, which are fermented soy foods. This phenomenon is consistent with the previous research, showing that the consumption of non-fermented soy foods results in a lower incidence of prostate cancer while fermented soy foods does not (*64*).

The effects of genistein on cellular proliferation, extracellular signal-regulated kinase (ERK1/2) activity and apoptosis have been studied in a nontumorigenic human prostate epithelial cell line (RWPE-1) (*74*). In this study, genistein increased cell proliferation and signal transduction at low concentrations (0-12.5 μmol/L), whereas it decreased cell proliferation and signal transduction at higher concentrations (50 and 100 μmol/L). The alterations in cell proliferation were mediated through activation of ERK1/2 and estrogen-dependent signaling pathways.

Another study on isoflavones suggests that glycitein might reduce the risk of prostate cancer by inducing basal cell differentiation in the non-cancerous

prostate cell line, RWPE-1. The responses of luminal epithelial cell marker p63, cytokeratins 18 and 5, as well as prostate specific antigen (PSA) to the glycitein treatment were determined (*75*) in three cell lines, RWPE-1, RWPE-2 and WPE1-NB14. Expression of cytokeratin 18 is not affected by glycitein treatment in WPE1-NB14 and RWPE-2 cell lines. However, the expression of cytokeratin 18 and PSA decreases in the RWPE-1 cell line in response to glycitein treatment, whereas the expression of basal epithelial cell markers p63 and cytokeratin 5 remained unchanged. This suggests that glycitein promotes cell differentiation in non-cancerous prostate cells, and provides a mechanism by which glycitein may reduce prostate tumorigenesis.

Other *in vitro* studies also have been carried out to examine the anti-cancer effects of soy isoflavones on human prostate cancer cell lines. The methylated isoflavones (glycitein, biochanin A, formononetin) are considered to have greater anti-cancer potential than nonmethylated isoflavones (genistein, daidzein, equol) (*71*). It has been reported that the proliferation of prostate cancer cell lines (LNCaP and PC-3) is inhibited by genistein treatment in a dose-dependent manner. This inhibition may be obtained through the up-regulation of glutathione peroxidase (GPx)-1 and down-regulation of the apoptosis inhibitor (survivin), DNA topoisomerase II, cell division cycle 6 (CDC6) and mitogen-activated protein kinase 6 (MAPK 6) (*76*). The molecular mechanisms by which genistein inhibits cell growth and induces apoptosis have

been investigated in androgen-dependent (LNCaP) and androgen-independent (PC3) prostate cancer cell lines (*77*). The observations suggest that down-regulation of cyclin B and up-regulation of the growth-inhibitory protein, p21, may be the main mechanisms of growth inhibition by genistein. Genistein, at concentrations of 15–120 mM, has been reported to inhibit proliferation and induce apoptosis in DU 145 cells (*78*). Additionally, genistein-combined polysaccharide (GCP) inhibits proliferation and promotes apoptosis through molecular mimicry of androgen ablation. GCP down-regulates the androgen receptor and provides an androgen receptor-independent, pro-apoptotic signal through mammalian target of rapamycin (mTOR) inhibition (*79*).

1.5. Cancer and Oxidative Stress

Oxygen free radicals have been identified as potential carcinogens (*80*), and antioxidants can provide protection against their carcinogenic potential (*81*). Many phenolic compounds have antioxidant properties. Oxidative stress is thought to play a role in prostate cancer (*82*), and therefore, the anti-cancer effects of soymilk on prostate cancer may be due in part to soy-food's antioxidant activity. One animal study has suggested that soymilk consumption interferes with the aging process through antioxidant capability (*83*). Research has demonstrated that soy phenolics have a high antioxidant capacity and are powerful antioxidants (*48*). While some studies suggest that reactive oxygen species play an important role in cancer development (*84*) and the antioxidant

activity of tissue can be enhanced by soy consumption in animals (*85*), the relationship between the anti-cancer effects and antioxidant properties of phenolics has not been fully studied. Hence, this project aimed to investigate the anti-cancer effects of soymilk CPE, and to relate those effects to their antioxidant properties.

1.6. Cancer and Apoptosis

In a healthy individual, cell proliferation and cell death remain in balance and are strictly controlled. Apoptosis is defined as a controlled cell death with the possibilities of being initiated or inhibited by environmental factors (*86*). It is well known that failure to undergo apoptosis in cells might partly contribute to the pathogenesis of cancer. This apoptotic process results in a series of morphological changes which involve swelling of cytoplasm and its organelles, rupture of the plasma membrane, and fragmentation of the nucleus (*87*). Since human cancers arise from an imbalance of cell growth and cell death, inducing apoptosis in tumor cells is a mark of anti-cancer activity.

Several apoptotic proteins are known to govern the balance between cell proliferation and death (*88-90*), including caspase-3, bcl-2, and PARP-1. The expression of these apoptosis-related proteins provide mechanistic evidence of apoptosis, and could be determined using a Western blot assay.

Caspase-3 is an enzyme that plays a key role in cancer cell apoptosis, and is extensively studied among the caspase family members. Caspase-3 is

synthesized as an inactive pro-enzyme. It can be activated both by extrinsic and intrinsic pathways during apoptosis (*91*), through self proteolysis, and/or other upstream proteases, such as cleavage of caspase-8, 9, and 10. The activation of caspase-3 is considered a central molecular event, which leads to apoptosis (*92*).

Bcl-2 is a protein family, which can regulate apoptosis through the mitochondrial pathway. The Bcl-2 family includes either anti-apoptotic (including Bcl-2, Bcl-xl, and Bcl-w) or pro-apoptotic (including Bax, BAD, Bak, and Bok) proteins. Bcl-2 is an anti-apoptotic protein that functions to inhibit apoptosis (*93*). Under healthy conditions, bcl-2 is displayed on the surface of the outer mitochondrial membrane, where it protects mitochondria from being destroyed. Intrinsic stimuli cause bax (a pro-apoptotic protein) to migrate to the surface of the mitochondria to punch holes in the mitochondrial membrane causing cytochrome c to leak out. This triggers an apoptotic signaling cascade to result in cell death. The formation of a pore in the mitochondrial membrane is a key step towards cell death (*94*).

Poly ADP-ribose polymerase-1 (PARP-1) is a marker for apoptosis, and exists as a 116 kDa nuclear protein. PARP-1 is cleaved by many caspases during apoptosis (*95*). Evidence shows that PARP-1 activity triggers the release of a mitochondrial pro-apoptotic protein called apoptosis-inducing factor (AIF)

that promotes programmed cell death through a caspase-independent pathway (*96*).

The goals of this project were to detect how different processing conditions affect the amount of phenolic compounds in soymilk, to use soymilk as raw material to extract phenolic compounds, and to study their anti-cancer effects on human prostate cancer DU 145 cells. Since tumor growth is a result of increased cell division (proliferation) and/or decreased cell death (apoptosis), the ability of soy CPE to reduce cell proliferation and induce apoptosis was studied.

The overall objectives of this study were to determine the effects of processing conditions on the phenolic content, antioxidant and anti-proliferative properties of soymilk CPE on prostate cancer cells, and to detect the mechanisms involved in the anti-cancer effects using *in vitro* studies.

The following hypotheses and specific aims were proposed.

1.7. Hypotheses

1.7.1. Grinding effects on health-promoting attributes of soymilk

We hypothesize that grinding with or without okara-washed water and/or soaked water will produce soymilk with different phytochemical contents that will induce different anti-proliferative effects in cancer cells.

1.7.2. UHT effects on health-promoting attributes of soymilk

In our lab, the effect of different UHT processing conditions with different heat powers on the antioxidant levels of soymilk has been carried out. The UHT

processing retains higher antioxidant capability than other types of thermal processing. Therefore, we hypothesize that UHT will improve the anti-proliferative capacity of soymilk on human prostate cancer cells due to the enhanced antioxidant activity.

1.8. Specific Aims

1.8.1. Comparison of grinding methods on the phenolic contents of soymilk

Our first aim was to study the effects of four grinding methods on the total phenolic content (TPC), total flavonoid content (TFC), condensed tannin content (CTC), oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), and DPPH free radical scavenging activities of soymilk CPE. High performance liquid chromatography (HPLC) was performed to analyze the phenolic compounds in the soymilk CPE. In addition, this study investigated the effects of these grinding methods on the anti-proliferative activity of soymilk CPE using DU 145 cells.

1.8.2. Comparison of the two UHT methods on anti-cancer properties of soymilk

Two UHT processing methods (120 ^{0}C, 80 s + 140 ^{0}C, 4 s and 143 ^{0}C, 60 s) with different heat powers were selected to study the effect of high-temperature thermal processing on the anti-cancer properties of soymilk. This was accomplished by monitoring the anti-proliferative and apoptosis-inducing activities of soymilk exposed to UHT on DU 145 cells. HPLC was performed to analyze the phenolic compounds in UHT processed soymilk extract.

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CHAPTER 2. EFFECTS OF GRINDING METHODS ON SOYMILK ATTRIBUTES

2.1. Abstract

The anti-cancer benefits of soy are well recognized, but it is unclear which specific components demonstrate the anti-cancer effects. Phenolic components of soy are thought to have such anti-cancer attributes. In this study, we applied four grinding methods to soybean in order to detect which one results in greater anti-cancer properties. Briefly, soybeans were ground with tap water (Method 1), okara-washed water (Method 2), soaked water (Method 3), and both okara-washed water and soaked water (Method 4), respectively. The soymilk was cooked at 100 ^{0}C for 20 min. We found that grinding Method 4 produced the greatest anti-cancer effects by inhibiting proliferation and promoting apoptosis of DU 145 prostate cancer cells. Grinding Method 4 also showed higher oxygen species scavenging capabilities. Therefore, our studies demonstrate that grinding methods do affect soymilk components and can affect the anti-cancer properties of soymilk crude phenolic extract (CPE).

2.2. Introduction

Prostate cancer is one of the most common cancers in men in the United States and is the third cause of male cancer death worldwide. The number of new prostate cancer cases reported worldwide in 2000 was approximately 550,200 with an approximated 286,500 disease-related deaths (*1*). Epidemiological studies have shown that prostate cancer mortality is commonly

lower in Asian males than in Western males, which is thought to be due to the high consumption of soy products (*2*).

Soy products have received much attention for their potential health benefits. The health benefits of soy foods have been partly attributed to phenolics. Among the soy phenolics, isoflavones have been recognized as phytoestrogens, and are suggested to act as estrogen antagonists in a high estrogen environment or estrogen agonists in a low estrogen environment (*3*). Although isoflavones contribute health benefits for diseases such as cancer (*4*), bone loss (*5*), hypertension (*6*), and endocrine diseases (*7*), there are many other phytochemicals in soy that may have such potential bioactivities. Recently, athymic nude mice (Balb/c), which were implanted with estrogen-independent human breast cancer cells, were used to detect the anti-cancer capabilities of purified genistein, one component of isoflavones, and soy extract. The results showed that the soy extract has more potential capabilities to inhibit tumor growth than purified genistein (*8*). Another study has indicated that soy extract improves the tibial trabecular bone quality in ovariectomized mice more effectively than purified genistein (*9*). The reasonable explanation for these findings may due to the synergistic effects of the various bioactive components in the soy extract. Therefore, soy CPE should be studied for its anti-cancer properties.

Soymilk is one of the traditional soy foods, and is produced using the

following steps: 1) soaking, 2) grinding, 3) filtrating, and 4) heating. Grinding is an important step, and the grinding conditions of soy have effects on the protein content, bacteria counts, soymilk color, viscosity, and solid yield (*10*). In the soymilk and tofu manufacturing industry, the soaked water and okara which come out from the filtrating step are commonly discarded. In this study, we ground soybeans with soaked water and/or okara-washed water to recover more phytochemicals, especially the phenolics, during soymilk processing and assessed the impact of different grinding methods on the anticancer properties of soybeans.

2.3. Materials and Methods

2.3.1. Materials

Dry matured Prosoy (harvested in 2008) and Black (harvested in 2006) soybeans (*Glycine max*) were obtained from Sinner Brothers & Bresnahan (Casselton, ND). Prosoy is a type of yellow soybean.

Dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), (+)-catechin, fluorescein disodium, Folin-Ciocalteu reagent, sodium carbonate, 3-(4,5-dimenthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), vanillin, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman -2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Analytical

grade acetic acid and acetone used for extraction were purchased from VWR International (West Chester, PA).

The human prostate cancer cell line DU 145 was 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 mixed antibiotics, fetal bovine serum (FBS), and all cell culture media (Eagle's Minimum Essential Medium) were purchased from Mediatech, Inc. (Herndon, VA).

2.3.2. Methods

2.3.2.1. <u>*Soymilk making*</u> Soymilk was produced by the following processing steps: 1) soaking 20 g of soybeans in cold water overnight (soybean/water, w/w, 1:10); 2) grinding the soaked soybeans at room temperature to form a slurry using an electric blender at high speed for 3 min (soybean/water, w/w, 1:10); 3) separating the soy slurry into soymilk and okara using a filtering cloth; 4) cooking soymilk using oven at 100 ^{0}C for 20 min.

2.3.2.2. <u>*Grinding methods*</u> With the goal to improve the quality of soymilk by recovering a greater amount of phenolic contents, four different grinding methods were employed and compared. These four grinding methods are summarized in Table 1. Briefly, soybeans were ground with tap water,

okara-washed water, soaked water, and both okara-washed water and soaked water. The soymilk was cooked at 100 ^{0}C for 20 min.

Table 1. Four Grinding Methods for Making Soymilk

Grinding	Method 1	Method 2	Method 3	Method 4
Vol. of soaked water	600 mL	600 mL	1000 mL	600 mL
First grinding (3 min)	1000 mL of tap water	200 mL of tap water plus 800 mL of okara -washed water	1000 mL of soaked water	600 mL of soaked water
Re-grinding (3 min)	No re-grinding	No re-grinding	No re-grinding	400 mL of okara-washed water
Weight of soybean	100 g for all methods			

Note: All grinding methods were carried out at room temperature.

2.3.2.3. *Extraction* The phenolic compounds in soymilk were extracted using the following protocol (*11*). After cooling, all soymilk samples were immediately frozen and then freeze-dried. The freeze-dried soymilk samples were accurately weighed (1 g) and put into centrifuge tubes Ten mL of the extraction solvent (acetone/water, 50:50 (v/v) for yellow soymilk; acetone/water/acitic acid, 70/29.5/0.5 (v/v/v) for black soymilk) were added into the tubes. The mixture was shaken at 300 rpm at room temperature on an orbital shaker (model no. 3520, Lab-Line Instruments, Inc, Melrose Park, IL.) for 3 h.

The mixture was centrifuged by using an Allegra 21R Centrifuge (Beckman Coulter Ltd, Palo Alto, Calif., USA) at 3,000 rpm for 10 min, and the supernatant was placed into a new set of tubes. The residues were extracted with 10 mL of the solvent for an additional 12 h in the dark overnight with shaking. Both the extracts were combined and stored at 4 ^{0}C in dark. Two mL of the extract solution were reserved for phytochemical analysis. The rest of the solution was evaporated at 38 ^{0}C on a rotary evaporator under vacuum. After evaporation, the extract was freeze-dried to obtain the crude phenolic extract, which was kept at -20 ^{0}C until use.

2.3.2.4. Determination of phytochemicals

2.3.2.4.1. TPC determination The total phenolic content (TPC) of soymilk CPE was determined by a Folin-Ciocalteu assay (*12, 13*) using gallic acid (GA) as the standard. A mixture of the sample solution (50 µL), distilled water (3 mL), 250 µL of Folin-Ciocalteu's reagents solution, and 7% $NaCO_3$ (750 µL) was vortexed and incubated for 8 min at room temperature. The mixture was diluted with 950 µL of distilled water and allowed to stand for 2 h at room temperature. The absorbance was measured at 765 nm against distilled water as a blank. The TPC, expressed as gallic acid equivalents (mg of GAE/g of sample), was determined using the calibration curve of gallic acid.

2.3.2.4.2. TFC determination Total flavonoid content (TFC) of soymilk CPE was determined using a slightly modified colorimetric method

described previously (*14*). A 30 µL aliquot of appropriately diluted sample solution was mixed with 180 µL of distilled water in a well of 96-well plate, and subsequently 10 µL of a 5% $NaNO_2$ solution were added. After 6 min, 20 µL of 10% $AlCl_3$ solution were added and allowed to stand for 6 min, then 60 µL of 4% NaOH solution were added to the mixture and incubated 15 min. The absorbance of the mixture was determined at 510 nm versus a prepared water blank using a Multiskan Spectrum microplate reader. (+)-Catechin was used as a standard compound for the quantification of total flavonoids. All values were expressed as milligrams of catechin equivalents per gram of sample (mg of CE/g of sample).

2.3.2.4.3. CTC determination The condensed tannin content (CTC) of soymilk CPE was determined using the modified vanillin assay (*15*). Ten µL of the sample solution was mixed with 200 µL of 4% vanillin solution (in methanol) in a well of a 96-well plate, and then 100 µL of concentrated HCl were added and mixed. After 15 min, the absorbance of the mixture was determined at 500 nm against a blank solution, which was prepared by the same procedure described above except that the extract solution was substituted with 10 µL of water. Different concentrations of (+)-catechin ranging from 31.25 to 1000 µg/mL were used as a standard compound for the qualification of total condensed tannins. All values were expressed as milligrams of catechin equivalents per gram sample (mg of CE/g of sample).

2.3.2.5. *Quantification of phytochemicals by HPLC analysis* Twenty milligrams of freeze-dried soymilk extract was dissolved in 1 mL of distilled water to obtain a concentration of 20 mg/mL, which was used to inject into the HPLC detector. Phenolic acids were analyzed on an Agilent 1200 series HPLC system equipped with a G13798 degasser, G1312A binary pump, G1329A autosampler, and G1315D diode array detector (Agilent Technologies, Santa Clara, CA). HPLC separation was achieved using a Zorbax Stablebond Analytical SB-C18 column (250×4.6 mm, 5 μm, Agilent Technologies, Santa Clara, CA) at 40 ^{0}C. Elution was performed using mobile phase A (0.1% trifluoroacetic acid aqueous solution) and mobile phase B (100% methanol); samples (20 μL) were eluted at a flow rate of 0.7 mL/min. The UV-Vis spectra were scanned from 220 to 600 nm on a DAD with a detection wavelength of 270 nm. The solvent gradient in volumetric ratios was as follows: 5-30% B over 50 min. The solvent gradient was held at 30% B for an additional 15 min and increased to 100% B for 66 min. The solvent gradient was held at 100% B for an additional 10 min to clean up the column, followed by re-equilibration of the column for 5 min with 95% A and 5% B before the next run. Identification of phenolic compounds was made by comparison of their retention time and UV spectra with those of the authentic standards. The phenolic acid contents were expressed as micrograms of phenolic acid per gram of soymilk CPE (μg/g).

Isoflavones were analyzed using the same HPLC systems for phenolic acids according to Hou and Chang (*16*) with a slight modification. A YMC-Pack ODS-AM-303 C18 column (250×4.6 mm, 5 μm) was used. Mobile phase A (0.1% acetic acid aqueous solution) and mobile phase B (0.1% acetic acid in acetonitrile) were used to elute isoflavones. The system was eluted with 15% of solvent B for 5 min at the flow rate of 1.0 mL/min, increased to 29% for 31 min at the flow rate up to 1.5 mL/min, and then increased to 35% for 8 min at the same flow rate of 1.5 mL/min. Next, the gradient was equilibrated to 50% of solvent B for 2 min and the system was eluted with 50% of solvent B for 10 min at the flow rate of 1.5 mL/min. The solvent was recycled back to 15% B at the flow rate of 1.0 mL/min for 2 min, and then the column was equilibrated with initial solvent for 2 min prior to running the next sample. Identification of isoflavones was made by comparison of their retention time and UV spectra with those of the authentic standards.

2.3.2.6. Chemical antioxidant assays Three methods were used to quantify the effects of processing on the antioxidant capabilities of soymilk.

2.3.2.6.1. ORAC assay The oxygen radical absorbance capacity (ORAC) was determined using a method similar to that developed by Prior and Wu (*17, 18*). This protocol uses a BMG Fluostar Optima Microplate Reader (BMG Labtech GmbH, Offenburg, Germany), which is equipped with two autoinjectors, an incubator, and wavelength-adjustable fluorescence filters. The

temperature of the incubator was set to 37 ^{0}C, and fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used. Briefly, AAPH [2, 2'-azobis (2-methylpropionamide) dihydrochloride] was used as the peroxyl generator and Trolox as a standard. Twenty microliters of suitable diluted soymilk CPE samples, blank, and Trolox calibration solutions were loaded on a clear polystyrene 96-well microplate (flat bottom, Nalge Nunc Intl., Denmark) in duplicate based on a randomized layout. The plate reader was programmed to record the fluorescence of fluorescein on every cycle. Kinetic readings were recorded for 60 cycles at 40 s per cycle. The hydrophilic soymilk CPEs were diluted with phosphate buffer saline (75 mM, pH 7.0) to the proper concentration range in order to fit the linearity range of the standard curve. Trolox standards were prepared with phosphate buffer saline (75 mM, pH 7.0), which was used as blank. After loading 20 µL of sample, standard, and blank, and 200 µL of the fluorescein solution into each well according to layout, the microplate (covered with a piece of film) was incubated for at least 30 min in plate reader. Next, the film was removed and 20 µL of peroxyl generator AAPH (3.2 µM) was added to initiate the oxidation reaction. The kinetics of the fluorescence changes were recorded immediately by SoftMax Pro software (Molecular Devices). The final ORAC values were calculated using a linear equation between the Trolox standards or sample concentration and the net area under the fluorescence decay curve. The data were analyzed using

Microsoft Excel (Microsoft, Roselle, Ill., U.S.A.). The area under curve (AUC) was calculated as AUC = 0.5 + (R2 + R3 + R3 +.... + Rn)/R1, where R1 is the fluorescence reading at the initiation of the reaction and Rn is the last measurement.

The net AUC was obtained by subtracting the AUC of the blank from that of a sample or standard. The ORAC value was calculated and expressed as micromoles of Trolox equivalent per gram sample (μmol of TE/g of sample) using the calibration curve of Trolox. For each specific sample, triplicate extractions were performed.

2.3.2.6.2. FRAP assay The ferric reducing antioxidant power (FRAP) assay was performed as previously described by Benzie and Strain (*19*). The FRAP reagent was prepared by mixing 10 volumes of 250 mM acetate buffer (pH 3.6), with 1 volume of 10 mM TPTZ in 40 mM HCl, and with 1 volume of 20 mM $FeCl_3 \cdot 6\ H_2O$. A total of 10 μL of properly diluted sample and 30 μL of distilled water were added to 260 μL of freshly prepared FRAP reagent in a well of 96-well plate. The mixture was incubated at 37 ^{0}C throughout the reaction. After 8 min, the absorbance was read using a Multiskan Spectrum microplate reader at 593 nm against a water blank. The FRAP value was calculated and expressed as millimoles of Fe^{2+} equivalents per 100 g of sample (mmol Fe^{2+} equivalents/100 g of sample) based on a calibration curve plotted using $FeSO_4 \cdot 7H_2O$ as standard at concentrations ranging from 0.125 to 2 mM.

2.3.2.6.3. DPPH assay The DPPH (2, 2-diphenyl-1-picrylhydrazyl) antioxidant capacity assay is an electron-donating assay. The radical scavenging activity of soymilk CPE against DPPH free radicals was measured using the method of Brand-Williams (*20*), slightly modified as follows: 10 μL of the tested samples or Trolox solutions (31.25, 62.5, 125, 250, 500, 750, and 1000 μM) were added to 190 μL of DPPH solution (final concentration was 0.1 mM in methanol) in a well of 96-well plate. The mixture was shaken gently and left to stand at room temperature in the dark for 30 min. Thereafter, the absorbance at 517 nm was measured against methanol using a Multiskan Spectrum microplate reader. The DPPH radical scavenging activity of CPE was calculated from the standard curve of Trolox and expressed as micromoles of Trolox equivalents (TE) per gram of sample (μmol TE/g of sample).

2.3.2.7. Anti-proliferative assays

2.3.2.7.1. Cell culture The DU 145 human prostate cancer cell line was used for studying the biological activity of soymilk CPE. Cancer cells were maintained and sub-cultured in Eagle's Minimum Essential Medium supplemented with 10% (v/v) FBS and 1% antibiotics (penicillin/streptomycin) in 75 cm^2 flasks at 37 ^{0}C under a constant humidified atmosphere of 5% carbon dioxide.

2.3.2.7.2. MTT assay The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to test the viability of tumor cells

after treating with soymilk CPE (*21*). DU 145 cells were grown to 80-90% confluency in 75 cm^2 flasks, harvested using trypsinization and diluted to an appropriate cell concentration with growth medium. The cells were seeded into 96-well plates at a density of 5×10^3 cells/well in 180 μL of medium. After 24 h of incubation, the cells were treated with 20 μL of 10, 20, 40 and 80 mg/mL stock soymilk CPE solution, respectively, to reach final treated concentrations of 1, 2, 4 and 8 mg/mL. Each treatment was conducted in triplicate. After 48 h of incubation, 20 μL of 5 mg/mL MTT solution was added to each well. The plates were incubated for 4 h at 37 ^{0}C with 5% CO_2. The solution in each well was removed carefully after incubation, and then 150 μL/well of dimethyl sulfoxide (DMSO) was added. The plates were shaken for 15 min, and the absorbance was measured at 570 nm in a microplate reader (Bio-Tech Instruments). The viability was obtained by telling the difference in the absorbance values between the treated and control wells divided by the absorbance value of the control.

2.3.3. Statistical analysis

All experiments were repeated at least twice. For the *in vitro* experiments, triplicate culture wells were used. One-way ANOVA and an appropriate Post-hoc comparing (Tukey) test were used when more than two groups were compared. The significance level for all tests was set at a 95% confidence limit. Various software packages (e.g. SigmaStat, Sigmaplot) were used to perform statistical analyses and to assess the significance of the data ($P < 0.05$).

2.4. Results

2.4.1. Phytochemicals determination

2.4.1.1. Total phenolic content (TPC) determination Four different grinding methods were applied to produce raw and cooked soymilk, as well as okara from Prosoy and black soybeans. The TPC values of Prosoy soymilk CPE are presented in Table 2. For the Prosoy raw soymilk, the grinding Method 4 resulted in a significantly (P < 0.05) greater TPC value than the other three grinding methods, and the TPC values of the soymilk produced by the grinding Method 3 were higher than that of grinding Method 1. For the cooked soymilk, the TPC value of soymilk produced by grinding Method 4 was significantly (P < 0.05) higher than that of the grinding Methods 1 and 2, but there was no significant differences between the soymilk produced by the grinding Methods 3 and 4. There were no significant differences in TPC values for the okara produced by the four grinding methods.

The TPC values of the black soymilk CPE are presented in Table 3. For the raw soymilk, grinding Method 4 resulted in a significantly (P < 0.05) greater TPC value than the other three grinding methods, and the TPC values of the black soymilk produced by grinding Methods 2 and 3 were higher than that of grinding Method 1. For the cooked black soymilk, the TPC value of the soymilk produced by grinding Method 4 was significantly (P < 0.05) higher than those of Methods 1, 2 and 3, but no significant differences were observed between the

black soymilk produced by grinding Methods 1 and 2. There were no significant differences observed among the TPC values of the okara processed by the four grinding methods. Black soymilk had significant higher TPC than Prosoy soymilk ($P < 0.05$) produced by methods 2, 3 and 4, but no significant difference in soymilk produced by traditional method.

Table 2. TPC Values of Crude Phenolic Extracts from Prosoy Soymilk

	TPC values (mg of GAE/g of soymilk CPE)		
Grinding	Raw	Cooked	Okara
Method 1	1.94±0.04cC	1.68±0.14bB	1.09±0.17aA
Method 2	2.09±0.03bcC	1.74±0.04bB	0.97±0.03aA
Method 3	2.11±0.04bB	1.84±0.09abB	1.02±0.22aA
Method 4	2.61±0.11aC	2.17±0.25aB	1.30±0.03aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

Table 3. TPC Values of Crude Phenolic Extracts from Black Soymilk

	TPC values (mg of GAE/g of soymilk CPE)		
Grinding	Raw	Cooked	Okara
Method 1	2.49±0.04cC	1.90±0.07cB	2.77±0.05aA
Method 2	3.26±0.19bC	2.03±0.01cB	2.88±0.04aA
Method 3	3.44±0.02bC	2.25±0.05bB	3.00±0.11aA
Method 4	4.43±0.13aB	2.84±0.17aA	3.00±0.13aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

The distribution of total phenolics in Prosoy and black soymilk are presented in Tables 4 and 5, respectively. With either the Prosoy or black soymilk, grinding Method 4 reserved many more phenolics than the other three grinding methods ($P < 0.05$). Additionally, a greater content of phenolic was observed in raw soymilk than cooked soymilk ($P < 0.05$), indicating that heat

treatment decreased the total phenolic content. This reduction of phenolics may be partly due to the destruction of phenolic compounds by the heat. These findings are consistent with the results from the proliferation experiments.

Table 4. Distribution of TPC (%) of Crude Phenolic Extracts from Prosoy Soymilk

Grinding	Raw	Cooked	Okara	Total	Loss
Method 1	43.47±1.32cA	37.14±3.39bB	12.49±1.67aC	55.95±2.86b	44.05±2.86b
Method 2	54.03±1.20bA	44.45±1.21abB	10.90±0.67aC	64.94±1.62ab	35.06±1.62ab
Method 3	48.06±1.92cA	41.63±1.16bB	11.45±2.46aC	59.51±3.15ab	40.49±3.15ab
Method 4	62.90±2.81aA	52.19±6.86aB	12.47±0.48aC	75.37±2.56a	24.63±2.56a

This Table shows the percentage of total phenolic content obtained for Prosoy raw and cooked soymilk and okara processed with four different grinding methods. The "Total" column represents the phenolic content from the raw soymilk and okara combined. The loss represents the difference in total phenolic content between the raw soymilk and the Prosoy soybean powder. Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

Table 5. Distribution of TPC (%) of Crude Phenolic Extracts from Black Soymilk

Grinding	Raw	Cooked	Okara	Total	Loss
Method 1	32.80±0.21dA	25.28±1.83cB	18.77±0.42aC	51.57±0.60c	48.43±0.60c
Method 2	48.73±1.57bA	29.99±1.35bB	19.76±1.39aC	68.49±2.44b	31.51±2.44b
Method 3	45.83±1.01cA	29.63±1.17bcB	20.85±1.07aC	66.68±2.02b	33.32±2.02b
Method 4	57.78±0.82aA	36.76±2.50aB	20.47±1.51aC	78.25±2.07a	21.75±2.07a

This Table shows the percentage of total phenolic content obtained for Black raw and cooked soymilk, and okara processed with four different grinding methods. The "Total" column represents the phenolic content from the raw soymilk and okara combined. The loss represents the difference in total phenolic content between the raw soymilk and the Black soybean powder. Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

The distribution of TPC was compared between Prosoy and black soymilk, for raw and cooked soymilk, Prosoy had higher TPC distribution than black

soymilk produced by four methods, as a result, the TPC distribution in black okara was higher than Prosoy okara ($P < 0.05$). The higher TPC distribution of Prosoy soymilk may be due to the low TPC content in Prosoy powder, which is used to divide the TPC value in soymilk.

2.4.1.2. *Total Flavonoid Content (TFC) Determination* The TFC values of Prosoy soymilk CPE are presented in Table 6. For the raw Prosoy soymilk, grinding Method 4 caused a significantly ($P < 0.05$) greater TFC value than the other three grinding methods. However, no significant differences were observed among raw soymilk produced by grinding Methods 1, 2 and 3. For the cooked soymilk, no significant differences were observed among all soymilk produced by the four grinding methods. The TFC value of the okara produced by the grinding Method 4 was higher ($P < 0.05$) than that of the other three grinding methods.

The TFC values of the Black soymilk CPE are presented in Table 7. For the raw Black soymilk, grinding Methods 3 and 4 caused a significantly ($P < 0.05$) greater increase in TFC values than grinding Methods 1 and 2. The TFC value of the Black soymilk produced by the grinding Method 2 was greater than that of grinding Method 1 ($P < 0.05$). For the cooked soymilk, grinding Methods 3 and 4 resulted in significantly ($P < 0.05$) greater TFC values than grinding Methods 1 and 2. In addition, grinding Method 4 produced higher TFC value than grinding Method 3 ($P < 0.05$). The TFC values of the okara produced by grinding Methods 3 and 4 were higher than that by grinding Methods 1 and 2 ($P < 0.05$). A comparison of TFC was conducted between Prosoy and black soymilk and okara. For raw soymilk, the TFC values of black soymilk were higher than

Prosoy soymilk ($P < 0.05$) in all four methods. For cooked soymilk, only the TFC values of black soymilk produced by methods 3 and 4 were higher Prosoy ($P < 0.05$). All the TFC values of black okara were significantly higher than Prosoy ($P < 0.05$).

Table 6. TFC Values of Crude Phenolic Extracts from Prosoy Soymilk

	TFC values (mg of CE/g of soymilk CPE)		
Grinding	Raw	Cooked	Okara
Method 1	0.25±0.03bC	0.38±0.03aB	0.63±0.06bA
Method 2	0.35±0.03bB	0.42±0.04aB	0.65±0.10bA
Method 3	0.26±0.05bB	0.38±0.03aB	0.76±0.10bA
Method 4	0.37±0.04aB	0.43±0.03aB	0.89±0.05aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

Table 7. TFC Values of Crude Phenolic Extracts from Black Soymilk

	TFC values (mg of CE/g of soymilk CPE)		
Grinding	Raw	Cooked	Okara
Method 1	0.36±0.03cB	0.37±0.02cB	2.38±0.01bA
Method 2	0.57±0.06bB	0.43±0.03cB	2.43±0.18bA
Method 3	0.71±0.03aB	0.66±0.02bB	2.77±0.10aA
Method 4	0.79±0.01aB	0.76±0.06aB	2.73±0.15aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

2.4.1.3. Condensed tannin content (CTC) determination The CTC values of Prosoy soymilk CPE are presented in Table 8. For the raw soymilk, grinding Method 4 produced the highest ($P < 0.05$) CTC value among all the grinding methods. The CTC values of the soymilk produced by grinding Methods 2 and 3 were greater ($P < 0.05$) than that of grinding Method 1. The CTC values of the cooked soymilk produced by grinding Methods 2 and 4 were greater ($P <$

0.05) than those of grinding Methods 1 and 3. The CTC value of the okara produced by grinding Method 4 was greater ($P < 0.05$) than those of the other three grinding methods. Okara produced by grinding Method 3 had a greater ($P < 0.05$) CTC value than that of grinding Methods 1 and 2.

The CTC values of Black soymilk CPE are presented in Table 9. For the raw soymilk, the grinding Method 4 produced a significantly ($P < 0.05$) greater CTC value than the other grinding methods. For cooked Black soymilk, the grinding Method 4 caused a significantly ($P < 0.05$) greater increase in CTC values than grinding Method 1, but no significant differences were observed among the Black soymilk produced by the grinding Methods 1, 2, and 3. The CTC values of the okara produced by grinding Methods 3 and 4 were greater ($P < 0.05$) than that of grinding Methods 1 and 2. All the CTC values of black soymilk and okara produced by four methods were greatly higher than those of Prosoy ($P < 0.05$).

Table 8. CTC Values of Crude Phenolic Extracts from Prosoy Soymilk

	CTC values (mg of CE/g of soymilk CPE)		
Grinding	Raw	Cooked	Okara
Method 1	0.21±0.02aBA	0.14±0.02aB	0.46±0.05aA
Method 2	0.37±0.05bC	0.27±0.03bB	0.49±0.01aA
Method 3	0.29±0.05cC	0.13±0.01aB	0.60±0.03bA
Method 4	0.48±0.02dC	0.32±0.03bB	0.70±0.07cA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

Table 9. CTC Values of Crude Phenolic Extracts from Black Soymilk

	CTC values (mg of CE/g of soymilk CPE)		
Grinding	Raw	Cooked	Okara
Method 1	2.19±0.06bC	1.85±0.20bB	4.27±0.14cA
Method 2	2.47±0.09bB	2.19±0.18abB	5.07±0.30bA
Method 3	2.54±0.23bC	2.06±0.17abB	5.70±0.04aA
Method 4	3.03±0.28aC	2.38±0.09aB	6.19±0.44aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

2.4.2. Quantification of phytochemicals by HPLC analysis

2.4.2.1. Effect of grinding methods on isoflavone compositions The isoflavone contents of the Prosoy and Black soymilk produced by the four different grinding methods are presented in Tables 10 (parts I, II and III) and 11 (parts I, II and III). In this study, the Black soymilk exhibited greater ($P < 0.05$) total isoflavone content than the Prosoy soymilk. In general, the cooked soymilk (part II of Tables 10 and 11) displayed lower isoflavone content than the raw soymilk (part I of Tables 10 and 11) for both Prosoy and Black soymilk. This comparative study showed that grinding Method 4 retained more isoflavones than the other three grinding methods in most of cases. Additionally, the raw soymilk had the highest isoflavone content among all the samples, followed by the cooked soymilk. These results are consistent with the findings of the TPC, TFC, and CTC assays.

Table 10-part I. Isoflavone Content (μg/g) of Crude Phenolic Extracts from Prosoy Soymilk Produced by Different Grinding Methods

Raw	Method 1	Method 2	Method 3	Method 4
Din	274.7± 2.3c	260.5± 1.9d	289.1± 3.8b	333.1± 1.9a
Gly	43.9± 4.1a	42.8± 1.1a	48.3± 3.9a	48.9± 0.5a
Gin	314.7± 17.1b	317.0± 3.6b	346.9± 6.9ab	372.3± 15.5a
MDin	625.4± 5.1d	790.3± 52.8c	974.8± 3.7ab	985.9± 22.0a
MGly	113.5±7.7a	110.7±0.2a	128.3±1.2a	134.5±13.0a

Table 10-part I-continued.

MGin	1743.4± 49.5a	1705.9± 33.0a	1818.8± 16.5a	1812.5± 16.5a
AGly	119.5± 2.2b	134.9± 6.7b	130.3± 8.8b	180.8± 5.3a
Dein	49.7± 4.9a	53.6± 4.6a	57.3± 0.6a	62.9± 4.3a
Gein	62.0± 2.5a	65.0± 3.3a	63.7± 7.1a	67.8± 5.9a
Total	3346.8±90.3b	3480.9±34.1b	3857.6±37.4a	3998.8±84.9a

Table 10-part II. Isoflavone Content (μg/g) of Crude Phenolic Extracts from Prosoy Soymilk Produced by Different Grinding Methods

Cook	Method 1	Method 2	Method 3	Method 4
Din	540.5± 5.7b	572.6± 24.5ab	610.0± 20.0ab	641.9± 12.6a
Gly	69.6± 6.9a	62.7± 6.9a	79.2± 1.4a	78.1± 5.7a
Gin	648.5± 12.1b	636.5± 17.5b	672.0± 9.0ab	716.0± 3.1a
MDin	653.3± 22.0b	677.6± 22.4ab	715.9± 21.2ab	754.5± 4.4a
MGly	111.9±5.0a	108.7±2.5a	120.8±5.0a	123.6±2.5a
MGin	1351.3± 18.1ab	1337.9± 19.8b	1384.2± 19.7ab	1429.4± 28.0a
AGly	157.2± 4.8a	162.0± 5.0a	173.3± 7.0a	183.9±10.1a
Dein	39.9± 2.6b	44.7± 4.3ab	50.0± 3.9ab	56.6± 2.9a
Gein	52.3± 2.2a	58.4± 3.3a	60.7± 2.2a	58.5± 7.7a
Total	3624.7±65.4b	3666.1±42.7b	3866.0±29.5a	4042.5±46.7a

Table 10-part III. Isoflavone Content (μg/g) of Crude Phenolic Extracts from Prosoy Soymilk Produced by Different Grinding Methods

Okara	Method 1	Method 2	Method 3	Method 4
Din	49.7± 0.6b	73.9± 3.7a	71.7± 3.8a	76.5±3 .8a
Gly	22.1± 1.2a	24.6± 1.2a	25.1± 2.5a	22.0± 2.2a
Gin	103.7± 5.9a	103.6± 3.1a	112.7± 5.8a	78.5± 6.6b
MDin	353.1± 22.0b	352.8± 20.2b	338.1± 12.0b	239.1± 19.8
MGly	62.5±2.5a	58.6±3.5a	59.3±1.5a	47.8±0.7b
MGin	749.8± 18.5a	661.4± 16.5b	714.3± 11.4ab	477.3± 26.4c
AGly	67.2± 6.6a	59.1± 5.3a	62.6± 3.2a	36.9± 5.4b
Dein	124.3± 5.7a	106.9± 5.0ab	105.2± 1.4ab	101.5± 7.1b
Gein	172.2± 12.0a	152.3± 4.3ab	138.2± 7.6b	149.2± 4.3ab
Total	1704.6±71.9a	1593.4±12.1a	1627.6±14.9a	1229.1±39.5b

The three parts of Table 10 represent the isoflavone components detected after HPLC analysis of Prosoy products (raw soymilk, cooked soymilk, and okara) following the application of four different grinding methods. The data were calculated on a dry weight basis and are expressed as the mean ± standard deviation (n = 2). Din, daidzin; Gin, genistin; Gly, glycitin; MDin, malonyldaidzin; MGin, malonylgenistin; MGly, malonylglycitin; AGly, acetylglycitin; Dein, daidzein; Gein, genistein; nd, not detectable. The "Total" represents the mass (μg/g) sum of the nine different isoflavones detected by HPLC. Values marked by the different lowercase letters within four methods are significantly different ($P < 0.05$).

Table 11-part II. Isoflavone Content (µg/g) of Crude Phenolic Extracts from Black Soymilk Produced by Different Grinding Methods

Raw	Method 1	Method 2	Method 3	Method 4
Din	316.3±13.2b	281.8±1.8c	336.5±5.6b	383.8±3.7a
Gly	68.5±5.1b	70.2±0.6b	83.9±3.4ab	87.6±5.3a
Gin	361.5±2.7b	362.1±22.0b	381.1±2.7ab	429.5±11.6a
MDin	623.7±8.8d	747.3±4.4c	837.3±8.8b	915.5±22.0a
MGly	169.8±0.0a	172.8±3.2a	176.0±7.7a	178.1±27.2a
MGin	1907.4±15.8b	2106.9±8.2a	2129.7±19.7a	2176.6±29.6a
AGly	684.0±30.6b	712.5±52.5b	801.3±10.9ab	859.2±17.5a
Dein	86.9±2.8b	117.6±7.1a	119.9±6.1a	127.1±5.7a
Gein	93.8±6.5a	93.8±7.6a	97.8±6.5a	100.3±8.5a
Total	4312.2±49.3c	4665.4±1.5b	4964.0±66.4ab	5258.2±131.6a

Table 11-partII. Isoflavone Content (µg/g) of Crude Phenolic Extracts from Black Soymilk Produced by Different Grinding Methods

Cooked	Method 1	Method 2	Method 3	Method 4
Din	267.7±7.5b	279.7±3.7b	365.4±4.7a	387.3±5.6a
Gly	107.8±2.7a	103.3±5.5a	114.8±7.5a	119.7±5.1a
Gin	482.8±7.9b	503.7±2.1b	566.2±8.5a	569.8±8.3a
MDin	463.9±45.3a	444.0±22.0a	449.3±27.2a	481.7±17.6a
MGly	155.0±2.5a	152.5±4.0a	152.0±5.2a	154.7±5.0a
MGin	684.7±1.6a	722.9±28.0a	693.6±26.3a	731.3±8.2a
AGly	464.9±50.3b	511.3±17.5ab	550.0±8.7ab	613.7±13.5a
Dein	70.2±2.8a	70.3±4.7a	77.2±6.1a	83.3±6.7a
Gein	87.9±3.2a	138.4±4.3a	71.3±4.3a	79.5±6.5a
Total	2775.3±129.3a	2926.6±5.5a	3040.1±4.6a	3221.5±14.3a

Table 11-part III. Isoflavone Content (µg/g) of Crude Phenolic Extracts from Black Soymilk Produced by Different Grinding Methods

Okara	Method 1	Method 2	Method 3	Method 4
Din	67.5±1.1a	19.3±0.5b	71.5±5.6a	69.1±7.6a
Gly	37.8±4.1a	21.0±2.7b	39.0±3.5a	42.0±4.1a
Gin	141.8±5.6a	32.7±5.3b	141.7±1.1a	142.9±6.5a
MDin	253.1±6.6a	39.1±4.4b	254.9±22.0a	220.4±8.8a
MGly	94.3±5.0a	42.7±2.5b	93.6±5.0a	89.3±2.5a
MGin	471.2±16.4a	109.4±8.2b	502.5±16.4a	455.8±16.4a
AGly	215.1±6.5b	37.4±4.3c	246.6±8.7a	227.5±6.5ab
Dein	127.3±8.6b	174.5±5.7a	160.8±14.3ab	156.6±2.2ab
Gein	161.6±4.1c	274.5±13.1a	229.5±3.2b	211.5±9.8b
Total	1570.0±14.0b	751.0±8.2c	1742.9±59.2a	1615.4±9.6b

The three parts of Table 11 represent the isoflavone components detected after HPLC analysis of Black products following the application of four grinding methods. The data were calculated on a dry weight basis and are expressed as the mean ± standard deviation (n = 2). Din, daidzin; Gin, genistin; Gly, glycitin; MDin, malonyldaidzin; MGin, malonylgenistin; MGly, malonylglycitin; AGly, acetylglycitin; Dein, daidzein; Gein, genistein; nd, not detectable. The "Total" represents the mass (µg/g) sum of the nine different isoflavones detected by HPLC. Values marked by the different lowercase letters within four methods are significantly different ($P < 0.05$).

2.4.2.2. <u>Effect of grinding methods on phenolic acid compositions</u>

The phenolic acid contents of the extracts from the four grinding methods produced from Prosoy or Black soymilk are presented in Tables 12 and 13. Five phenolic acid components and four phenolic acid components were detected in Prosoy and Black soymilk, respectively. The results demonstrated that grinding Method 4 retained more phenolic acids than the other three grinding methods. The total phenolic acid content was higher ($P < 0.05$) in Black soymilk than in Prosoy soymilk. Moreover, the raw soymilk exhibited more phenolic acids than cooked soymilk and okara ($P < 0.05$).

Table 12. Phenolic Acid Content (μg/g) of Crude Phenolic Extracts from Prosoy Soymilk Produced by Different Grinding Methods

	GA	VA	CA	CLA	PHA	Total
Raw-M1	29.5±1.4b	17.3±1.6ab	1.5±0.1a	35.9±4.0a	nd	84.2±7.0a
M2	31.4±1.5b	15.7±1.1ab	nd	37.4±3.4a	nd	84.5±6.0a
M3	37.1±1.2a	20.2±1.3a	1.3±0.1a	42.9±5.5a	nd	101.4±8.2a
M4	41.1±0.9a	14.0±1.2b	1.4±0.2a	48.5±4.3a	1.5±0.2	106.5±6.8a
Cooked-M1	22.0±2.0b	12.7±0.6b	0.9±0.1c	nd	nd	35.7±2.7b
M2	23.3±1.1b	12.3±1.3b	nd	nd	nd	35.6±2.3b
M3	29.4±1.5a	21.8±1.3a	2.5±0.1a	nd	2.1±0.1	55.8±3.0a
M4	30.4±1.1a	24.6±1.3a	1.4±0.1b	nd	nd	56.4±2.5a
Okara-M1	18.0±0.9a	nd	nd	nd	nd	18.0±0.9a
M2	17.2±1.3a	nd	nd	nd	nd	17.2±1.3a
M3	16.9±1.3a	nd	nd	nd	nd	16.9±1.3a
M4	13.5±1.4a	nd	nd	nd	nd	13.5±1.4a

This Table represents the phenolic acid content observed after HPLC analysis of Prosoy products (raw soymilk, cooked soymilk, and okara) following the application of four different grinding methods. The data were calculated on a dry weight basis and are expressed as the mean ± the standard deviation (n = 2). The phenolic acids detected by HPLC included: GA, gallic acid; VA, vanillic acid; CA, caffeic acid; CLA, chlorogenic acid; PHA, p- hydroxybenoinic acid. nd, not detectable. The "Total" represents the sum of the five phenolic acids listed in this table ± the standard deviation. Values marked by the different lowercase letters within four methods are significantly different ($P < 0.05$).

Table 13. Phenolic Acid Content (μg/g) of Crude Phenolic Extracts from Black Soymilk Produced by Different Grinding Methods

	GA	PCA	MCA	SPA	Total
Raw-M1	20.1±0.5c	nd	3.8±0.4b	nd	24.0±0.9b
M2	24.5±0.2b	1.7±0.3a	3.1±0.2b	nd	29.2±0.7b
M3	27.2±0.8a	1.4±0.1a	3.9±0.1b	14.5±0.0	47.0±0.9a
M4	29.5±0.6a	1.7±0.4a	5.8±0.4a	16.9±5.2	53.9±6.7a
Cooked-M1	12.3±0.2c	nd	2.7±0.2b	nd	15.0±0.5c
M2	22.0±0.2a	nd	3.2±0.1b	nd	25.2±0.2ab
M3	15.6±0.6b	1.3±0.1	5.8±0.4a	nd	22.7±1.1b
M4	20.8±1.1a	1.4±0.4	6.0±0.7a	nd	28.3±2.2a
Okara-M1	13.1±0.7b	nd	nd	nd	13.1±0.7b
M2	7.9±0.1c	nd	nd	nd	7.9±0.1c
M3	17.8±0.6a	nd	nd	nd	17.8±0.6a
M4	17.5±0.7a	nd	nd	nd	17.5±0.7a

This table represents the phenolic acid content observed after HPLC analysis of Black products (raw soymilk, cooked soymilk, and okara) following the application of four different grinding methods. The data were calculated on a dry weight basis and are expressed as the mean ± the standard deviation (n = 2). The phenolic acids detected by HPLC included: GA, gallic acid; PCA, protocatechuic acid; MCA, *m*-coumaric acid; SPA, sinapic acid. nd, not detectable. The "Total" represents the sum of the four phenolic acids listed in this table ± the standard deviation. Values marked by the different lowercase letters within four methods are significantly different ($P < 0.05$).

2.4.3. Determination of antioxidant activity

The antioxidant activity of soymilk produced as a result of the four grinding methods was determined by three chemical approaches: ORAC, FRAP, and DPPH assays. The results for Prosoy soymilk are presented in Tables 14, 16, and 18, respectively. Significant ($P < 0.05$) differences in ORAC, FRAP, and DPPH values were found among most samples. For the raw soymilk, grinding Methods 2, 3, and 4, significantly ($P < 0.05$) increased ORAC, FRAP, and DPPH values compared to grinding Method 1 (Table 14, 16, and 18). The soymilk produced by grinding Method 4 especially exhibited high ($P < 0.05$) antioxidant activity (FRAP and DPPH values) than the other three grinding methods (Tables 16 and 18). For the cooked soymilk, grinding Methods 3 and 4 produced

significantly ($P < 0.05$) higher antioxidant activities (ORAC and FRAP values) than grinding Methods 1 and 2 (Tables 14 and 16). The soymilk produced by grinding Method 4 exhibited the highest DPPH values among all soymilk samples. For the okara, no significant differences were found in the antioxidant activities (ORAC and FRAP values) of the soymilk produced by all grinding methods; however, the DPPH value of the grinding Method 3 was significantly ($P < 0.05$) greater than that of the other three methods (Table 18).

The antioxidant activities of the Black soymilk produced by the four grinding methods are presented in Tables 15, 17, and 19. Significant differences ($P < 0.05$) in ORAC, FRAP, and DPPH values were observed among most samples. For the raw soymilk, grinding Methods 2, 3, and 4 significantly ($P < 0.05$) increased ORAC, FRAP, and DPPH values of soymilk compared to grinding Method 1 (Tables 15, 17, and 19). The soymilk produced by grinding Method 4 exhibited significantly ($P < 0.05$) higher antioxidant activities (FRAP and DPPH values) than that of the other three grinding methods (Tables 17 and 19). The cooked soymilk produced by grinding Methods 3 and 4 had significantly ($P < 0.05$) higher antioxidant activities (ORAC and FRAP values) than that of grinding Methods 1 and 2 (Tables 15 and 17). The soymilk produced by grinding Method 4 exhibited the highest DPPH value among all soymilk samples. No significant differences were detected in the FRAP values of the okara produced by all grinding methods. The DPPH value of the okara produced by grinding Method 2 was significantly ($P < 0.05$) higher than that of the other three grinding methods. Okara produced by grinding Method 1 exhibited the lowest ORAC values among all Black okara samples.

2.4.3.1. Oxygen radical absorbance capacity (ORAC) determination

Table 14. ORAC Values of Crude Phenolic Extracts from Prosoy Soymilk

ORAC values (μmol TE/g of soymilk CPE)			
Grinding	Raw	Cooked	Okara
Method 1	71.49±4.26bB	89.89±1.68cA	67.50±6.13aB
Method 2	75.36±3.92abC	101.80±4.67bcB	59.77±2.14aA
Method 3	88.25±7.96aC	109.65±9.54bB	56.40±3.31aA
Method 4	86.45±6.20abC	132.56±4.26aB	59.94±5.00aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

Table 15. ORAC Values of Crude Phenolic Extracts from Black Soymilk

ORAC values (μmol TE/g of soymilk CPE)			
Grinding	Raw	Cooked	Okara
Method 1	87.54±4.50dB	111.36±5.13bA	108.89±7.37bA
Method 2	106.55±9.74acC	122.10±3.81bB	88.02±4.78aA
Method 3	105.09±4.67abC	134.64±7.41aB	76.94±6.98aA
Method 4	120.85±5.12aC	140.11±2.86aB	68.41±4.43aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

2.4.3.2. Ferric reducing antioxidant power (FRAP) determination

Table 16. FRAP Values of Crude Phenolic Extracts from Prosoy Soymilk

FRAP Values (mmol Fe^{2+} equivalents/100g of soymilk CPE)			
Grinding	Raw	Cooked	Okara
Method 1	0.92±0.02dC	1.08±0.04bB	0.78±0.03aA
Method 2	1.07±0.04cB	1.10±0.03bB	0.79±0.02aA
Method 3	1.01±0.01bC	1.25±0.06aB	0.77±0.04aA
Method 4	1.13±0.04aC	1.30±0.09aB	0.71±0.03aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

Table 17. FRAP Values of Crude Phenolic Extracts from Black Soymilk

FRAP Values (mmol Fe^{2+} equivalents/100g of soymilk CPE)			
Grinding	Raw	Cooked	Okara
Method 1	1.31±0.07dB	1.39±0.03bBA	5.98±0.20aA
Method 2	1.44±0.07cB	1.42±0.05bB	5.87±0.41aA
Method 3	2.21±0.06bB	2.17±0.12aB	6.51±0.43aA
Method 4	2.37±0.05aB	2.29±0.09aB	6.70±0.34aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

2.4.3.3. *DPPH free radical scavenging activity determination*

Table 18. DPPH Values of Crude Phenolic Extracts from Prosoy Soymilk

DPPH Assay (µmol TE/g of soymilk CPE)			
Grinding	Raw	Cooked	Okara
Method 1	0.58±0.09cB	1.13±0.04cA	0.43±0.09bB
Method 2	0.83±0.03bC	1.78±0.07baB	0.37±0.04bA
Method 3	0.77±0.08bB	1.57±0.05bA	0.73±0.09aB
Method 4	1.45±0.12aC	1.96±0.21aB	0.47±0.07bA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

Table 19. DPPH Values of Crude Phenolic Extracts from Black Soymilk

DPPH Assay (µmol TE/g of soymilk CPE)			
Grinding	Raw	Cooked	Okara
Method 1	3.95±0.17cC	4.72±0.14cB	5.51±0.07bA
Method 2	4.58±0.09bB	5.14±0.16bA	5.11±0.11aA
Method 3	4.37±0.14bC	4.87±0.08cB	5.35±0.09bA
Method 4	6.02±0.16aC	7.07±0.14aB	5.39±0.11bA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

2.4.4. Anti-proliferative properties of soymilk against prostate cancer cell line

The anti-proliferative properties of the Prosoy soymilk produced by the four grinding methods against prostate cancer cells are summarized in Table 20.

Among Prosoy soymilk and okara tested, the raw soymilk exhibited the highest anti-proliferative capacities than the cooked soymilk and okara (Table 20). In most cases the okara exhibited the lowest anti-proliferative capacity among all samples as indicated by the highest IC_{50} values. The raw Prosoy soymilk produced by the grinding Method 4 possessed the strongest ($P < 0.05$) anti-proliferative capacity against prostate cancer cells (lowest IC_{50}) (Table 20), followed by the soymilk produced by grinding Methods 2 and 3. For the cooked soymilk, the IC_{50} value of grinding Method 4 was the lowest one, followed by the soymilk produced by grinding Method 2. The IC_{50} values among all okara produced by the four grinding methods were not significantly different.

Table 20. IC_{50} Values (mg/mL) of Crude Phenolic Extracts from Prosoy Soymilk

IC_{50} values (mg/mL) of MTT Assay			
Grinding	Raw	Cooked	Okara
Method 1	8.5±0.7cA	8.1±0.6bA	9.6±0.7aA
Method 2	7.7±0.7bcA	7.7±0.9bA	8.4±0.3aA
Method 3	7.0±0.6bB	10.1±0.6aA	9.5±1.2aA
Method 4	4.9±0.2aB	6.8±0.1bBA	9.4±0.7aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

Among all Black soymilk and okara tested, the raw soymilk exhibited greater anti-proliferative capacity than the cooked soymilk and okara (Table 21). The okara exhibited the lowest anti-proliferative capacities among all samples as indicated by the highest IC_{50} values. For the raw soymilk, the soymilk produced by grinding Methods 3 and 4 possessed stronger anti-proliferative capacities against prostate cancer cells by comparing their IC_{50} values with that

of Methods 1 and 2 (Table 21). For the cooked soymilk, the IC_{50} value of grinding Method 3 was the highest one. The IC_{50} values for the okara that was produced by the four different grinding methods were not significantly different.

Table 21. IC_{50} Values (mg/mL) of Crude Phenolic Extracts from Black Soymilk

Grinding	Raw	Cooked	Okara
Method 1	4.3±0.1bC	5.4±0.3bB	9.0±0.8aA
Method 2	4.2±0.2bB	5.2±1.2bBA	9.3±1.3aA
Method 3	3.9±0.3abC	6.8±0.4aB	8.3±0.9aA
Method 4	3.5±0.2aB	4.4±0.5bB	8.1±0.7aA

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$). Values marked by the same uppercase letter within same row are not significant different ($P < 0.05$).

Table 22 shows the composition of isoflavone compounds in the crude soymilk phenolic extract that were calculated according to the molecular weight of each isoflavone and expressed in micro mol per liter. For Prosoy raw soymilk produced by Method 1, we calculated the IC_{50} for daidzin (Din) as follows. The treatment volume in each well was 0.2 mL. Therefore, the 1 mg/mL treatment contained 0.0002 g of crude extract. From Table 10, we knew that Din content of crude soymilk extract was 274.7 µg/g crude extract. Therefore, 274.7 µg/g crude extract * 0.0002 g crude extract was equal to 0.055 µg of Din for this treatment. The molecular weight of Din is 416. Therefore, 0.055 µg was divided by 416 to get 0.00013205 µmol of Din. Then, 0.00013205 µmol was divided by 0.0002 L to get 0.66 µmol/L of Din for this treatment. From Table 20, the IC_{50} for raw soymilk produced by Method 1 was 8.5 mg/mL, therefore, we can finally got the IC_{50} value by calculating (0.66 µmol/L * 8.5 mg/mL)/1 mg/mL= 5.61µmol/L.

Table 22. Estimated IC_{50} Values (μmol/L) of Crude Phenolic Extracts from Prosoy and Black Soymilk

IC_{50}	Din	Gin	Gein	Mdin	Mgin	Mgein	Agin	Dein	Gein	Total
P1R	5.61	0.84	6.19	10.59	1.81	28.61	2.08	1.66	1.95	59.35
P2R	4.82	0.74	5.65	12.12	1.60	25.36	2.13	1.62	1.85	55.90
P3R	4.86	0.76	5.62	13.59	1.69	24.58	1.87	1.58	1.65	56.20
P4R	3.92	0.54	4.22	9.62	1.30	17.15	1.82	1.21	1.23	41.02
P1C	10.53	1.26	12.16	10.54	1.70	21.13	2.61	1.27	1.57	62.78
P2C	10.60	1.17	11.34	10.39	1.57	19.89	2.56	1.35	1.66	60.54
P3C	14.81	1.79	15.71	14.40	2.29	26.99	3.59	1.99	2.27	83.85
P4C	10.49	1.19	11.27	10.22	1.58	18.76	2.56	1.52	1.47	59.07
P1O	1.15	0.48	2.30	6.75	1.13	13.90	1.32	4.70	6.12	37.85
P2O	1.49	0.46	2.01	5.90	0.93	10.73	1.02	3.54	4.74	30.82
P3O	1.64	0.53	2.48	6.40	1.06	13.10	1.22	3.94	4.87	35.23
P4O	1.73	0.46	1.71	4.48	0.85	8.66	0.71	3.76	5.20	27.55
B1R	3.27	0.66	3.60	5.34	1.36	15.83	6.03	1.47	1.49	39.06
B2R	2.85	0.66	3.52	6.25	1.36	17.08	6.13	1.94	1.46	41.27
B3R	3.16	0.73	3.44	6.51	1.32	16.04	6.40	1.84	1.41	40.85
B4R	3.23	0.69	3.48	6.38	1.29	14.71	6.16	1.75	1.30	38.99
B1C	3.48	1.31	6.04	4.88	1.57	7.14	5.14	1.49	1.76	32.81
B2C	3.50	1.21	6.06	4.60	1.49	7.26	5.45	1.44	2.67	33.67
B3C	5.97	1.75	8.91	6.09	1.94	9.11	7.66	2.07	1.80	45.30
B4C	4.10	1.18	5.80	4.22	1.28	6.21	5.53	1.44	1.30	31.07
B1O	1.46	0.76	2.96	4.54	1.59	8.19	3.97	4.51	5.39	33.37
B2O	0.43	0.44	0.71	0.73	0.75	1.96	0.71	6.39	9.46	21.57
B3O	1.43	0.77	2.72	4.22	1.46	8.05	4.20	5.26	7.06	35.16
B4O	1.35	0.76	2.68	3.56	1.36	7.13	3.78	4.99	6.35	31.95

Data are calculated on a dry weight basis. P, Proto; B, Black; 1, Method 1; 2, Method 2; 3, Method 3; 4, Method 4; R, Raw soymilk; C, Cooked soymilk; O, Okara; Din, Daidzin; Gin, Glycitin; Gein, Genistin; Mdin, Malonyl daidzin; Mgin, Malonyl glycitin; Mgein; Agin, Acetyl glycitin; Dein, Daidzein; Gein, Genistein.

2.5. Discussion

2.5.1. Effects of processing on phytochemicals of soymilk

Soybeans are considered a functional food because they have many positive effects on health due to components such as phytochemicals. Phytochemicals are bioactive non-nutrient compounds found in plants, including

soybeans (*22*). Phenolic compounds are important phytochemicals in soy. They have attracted considerable attention due to their positive effects on diseases, such as, cardiovascular disease and cancers. Although the antioxidant properties of soy phenolics have been reported (*23, 24*), the effects of processing methods on the phytochemical contents and antioxidant activities have not been well characterized.

The soy grinding methods and the effects of grinding on soy's anti-cancer properties had not been previously investigated. In our study, four grinding methods were studied on two soybean types, Prosoy and Black soybean. Grinding Method 1 is the traditional method which is used by most soymilk and tofu producers in Asia. Grinding Methods 2 and 3 are designed to retain more phytochemicals than Method 1 by grinding with okara-washed water and soybean-soaked water, respectively. Grinding Method 4 is expected to retain more phytochemicals than Methods 2 and 3 due to grinding with both okara-washed water and soybean-soaked water.

Based on our experimental results, significant ($P < 0.05$) differences in TPC, TFC, and CTC values of soymilk extract were observed among most of grinding methods. Methods 3 and 4 tended to increase the TPC, TFC, and CTC values of the raw soymilk and cooked soymilk even though in some cases, the increases were not statistically different. However, Method 4 resulted in higher

phenolic compositions than the other three methods, particularly in Black soymilk production.

Interestingly, grinding Method 4 did significantly increase the TPC, TFC, and CTC values in okara compared to the other three methods (refer to Tables 2 and 3). Therefore, grinding soaked soybean with soaked water, and grinding soaked soybean in two steps (first soybean-soaked water and then okara-washed water) would enhance the retention of phenolics in the soymilk.

However, cooking tended to have a negative impact on phenolic retention by reducing the amount in the raw soymilk by 10% on average (Tables 2 and 3). With respect to variety differences, Black soymilk contained higher TPC, TFC and CTC than the Prosoy soymilk. This was consistent with our earlier reports (*25, 26*) and mostly due to the high anthocyanin content in the Black soybean seed hulls (*27*). Most isoflavones in soybean are present in the crude phenolic extract. Malonyl glyceitin seems to be much greater than that reported in the soymilk (*25, 26*). However, only four to five phenolic acids were detected in the crude phenolic extracts, as compared to 12-13 phenolic acids reported in the soymilk and soybean (*25, 27*).

The effect of heating on soymilk phytochemicals has been studied. A previous study on the effect of thermal processing on phytochemicals in soymilk indicated that all TPC, TFC and CTC values decreased after heat treatment (*28*). In this study, we found that about 13-17% of TPC was reduced by heating in

Prosoy soymilk, about 23-36% of TPC was reduced by heating in Black soymilk. Heat treatment increased about 16-52% of TFC in Prosoy soymilk, increased 2.8% of TFC in Black soymilk produced by Method 1, whereas decreased about 3-25% of TFC in other Black soymilk products. The CTC values were reduced about 27-55% by heating in Prosoy soymilk, about 11-21% of CTC was reduced by heating in Black soymilk.

Our results demonstrated that TFC values increased by the heat treatment in all the Prosoy soymilk and one of the Black soymilk products. This might be related to the release of free phenolic substances from polymerized structural substances (such as lignin) in cell walls upon thermal processing (*28*). These results indicated that processing caused complex changes on soy chemical compositions. Thermal processing might cause the degradation of polyphenols and release of bound phenolic substances.

As there are no data available on the effect of grinding method on the phytochemicals of soymilk in the literature. Overall, a comparison can be done between Method 4 and Method 1 in our study. Grinding Method 4 increased about 34% of TPC for raw Prosoy soymilk, 29% for cooked soymilk, 78% for raw Black soymilk, and 50% for cooked Black soymilk. Grinding Method 4 increased about 48% of TFC for raw Prosoy soymilk, 13.2% for cooked Prosoy soymilk, 119.4% for raw Black soymilk, 105.4% for cooked Black soymilk. Grinding Method 4 increased 128.6% of CTC for both raw and cooked Prosoy soymilk,

38.4% for raw Black soymilk, and 28.6% for cooked Black soymilk.

2.5.2. Effects of processing on antioxidant activity of soymilk

Oxidative stress is considered a major contributor to cancer (*29*), because oxidants have the ability to induce DNA damage and to stimulate cell division. Antioxidants help to protect cells by scavenging free radicals and by preventing uncontrolled cell division. Soybeans have been widely recognized for their health benefits for some time, while the function of phenolics in soy have only more recently come under investigation for their potential health benefits. Phenolic compounds, such as flavonoids, are becoming increasingly known for their anti-cancer effects and other health-promoting properties. Soy phenolics act as natural antioxidants to promote health, but the exact mechanisms of the health benefits of soy phenolics is unknown.

Antioxidant activity determination is reaction-mechanism dependent because antioxidants exist in a wide variety of functional structures. Some phenolic antioxidants are able to scavenge free-radicals, whereas some are able to donate a hydrogen atom in the reaction. Therefore, three analytical methodologies (ORAC, FRAP, and DPPH assays) were selected to quantify the antioxidant capability of soymilk CPE. Significant ($P < 0.05$) differences in FRAP and DPPH values were found among most grinding methods. Compared to Method 1, the other three grinding methods caused significant ($P < 0.05$) increases in the ORAC, FRAP, and DPPH values of the raw soymilk, and

cooked soymilk, and the extent of increases was consistent with the trend of increases of the phenolic compositions, with Methods 3 and 4 yielding higher antioxidant capabilities.

The soymilk produced by grinding Method 4 exhibited the highest antioxidant capability than those of the three other grinding methods in both Prosoy and Black soymilk. In addition, when raw soymilk was heated to produce the final products, the antioxidant capacities of the yellow Prosoy soybean were increased ($P < 0.05$). The ORAC of the raw Black soymilk was also increased when it was cooked. On the other hand, the FRAP and DPPH values of the Black soymilk were not enhanced by cooking. The differences may be due that some anthocyanins in Black soymilk were degraded by heating to compounds and/or the generation of new compounds that were responsive to the ORAC assay, but not to the FRAP and DPPH assays.

Previous reports on antioxidant activities of raw or processed soymilk mainly focused on the effect of fermentation (*30-32*). Although their findings have indicated that fermented soymilk possessed significantly higher antioxidant properties than unfermented soymilk, the antioxidant properties of unfermented soymilk have only been reported by our lab (*25*). Compared to the previous findings of increase of all FRAP values, a slightly decrease of FRAP values by heat treatment was found in Black soymilk samples in this study. This might be explained by the different soybean sources. Our results were consistent with the

finding that thermal treatments could induce the formation of compounds with new antioxidant properties (*33*). Aside from soymilk, the increase of antioxidant activity by thermal treatment had been found in the pasteurization of tea extracts (*34*).

It is impossible to compare current data with the literature since there are no available data on grinding method effect on the antioxidant activity. However, compared to grinding Method 1, the increases of antioxidant activities of soymilk processed by grinding Method 4 may be attributed to, in part, by the increased retention of phenolics.

2.5.3. Proliferation inhibition of DU 145 cells

In this study, we investigated the anti-proliferative effects of soymilk CPE on the DU 145 cell line. We found that soymilk CPE significantly inhibited the proliferation of DU 145 cells in a dose-dependent manner. All soymilk produced by the four grinding methods resulted in IC_{50}'s in the range of about 2 to 8 mg/mL of CPE after an incubation period of 48 h (Tables 20 and 21).

Genistein inhibited the growth of DU 145 cells in a concentration-dependent manner. According to our HPLC results (Table 22), genistein inhibits the growth of DU 145 cells at concentrations of 1.23 to 9.46 μmol/L. In a similar study, Li reported that genistein (50 μmol/L) inhibited the growth of PC3 cells (*35*). Oki et al. reported a complete inhibition of DU 145 cell growth by genistein at 50, 75 and 100 μmol/L (*36*) Kumi et al. demonstrated that genistein inhibited

the growth of LNCaP and PC3 prostate cancer cells (IC_{50}'s around 40 μg/mL) (*37*). Yu et al. further reported that genistein completely inhibited the expression of prostate androgen-regulated transcript-1 at 50 mmol/L (*38*). Our results suggest that genistein can inhibit growth of DU 145 prostate cancer cells and support previous studies that demonstrate the growth-inhibitory effects of genistein on other prostate cancer cell lines.

Treatment of DU 145 cells with CPE resulted in a strong anti-proliferative effect that was concentration-dependent. Compared to grinding Method 1 (control method), the raw Prosoy soymilk produced by grinding Method 4 inhibited the proliferation more than that of the other three grinding methods. However, the raw Black soymilk produced by Methods 3 and 4 had higher anti-proliferative activity than Methods 1 and 2. Cooking reduced the anti-proliferative abilities of soymilk (about 37% for Prosoy and 25% for black soymilk), and this phenomenon is consistent with the cooking effect on the phenolic compositions. Therefore, the increases in antioxidant capacities of soymilk by cooking did not result in higher anti-proliferative effects. No significant differences in IC_{50} were observed in Prosoy and Black okara. Interestingly, we found Black soymilk always had a higher anti-proliferative capability than Prosoy soymilk ($P < 0.05$). Compared to the literature, the raw Prosoy soymilk had lower anti-proliferative power as compared to the Proto raw soymilk (*26*). However, the effects of cooked soymilk on cancer cell proliferation

were consistent with our previous finding (*26*).

In summary, grinding methods significantly affected the total phenolic contents, compositions, and antioxidant capabilities of soymilk. Furthermore, powerful anti-proliferative effects were observed in DU 145 cells after CPE treatment. These effects depended upon the grinding methods. Overall, grinding Method 4 significantly increased the total phenolic contents, antioxidant, and anti-proliferative properties of raw soymilk when compared with the other three grinding methods, but the increases in the anti-proliferative were attenuated by cooking. Meanwhile, Black soymilk exhibited higher total phenolic contents, antioxidant, and anti-proliferative capabilities than Prosoy soymilk.

The precise mechanisms of the anti-cancer effects of soymilk CPE on prostate cancer are not clear. It is known that isoflavones can lower the risk of cancer by binding to the alpha estrogen receptor isoform (*39*). This results in decreased binding of estrogen to this receptor and limits signaling to stimulate cell proliferation (*40, 41*). Isoflavones also bind to androgen receptors where they could inhibit growth stimuli in prostate cancer cells (*42*).

It is well known that isoflavones (daidzein and genistein) bind to estrogen and androgen receptors and donate a hydrogen ion to the free radicals to act as powerful antioxidants (*43*). However, the potential relationship between the antioxidant and anti-cancer effects of soymilk extract needs further investigation. The antioxidants found in soymilk may inhibit the growth of human prostate

cancer cells by binding to estrogen or androgen receptors on the surface of prostate cancer cells. Similar effects of antioxidants have been observed in other cancers (*44*). Additionally, the antioxidants found in soymilk may inhibit the growth of prostate cancer cells by eliminating reactive oxygen species. The mechanisms by which antioxidants from crude soybean extract inhibit growth need further study. Based on our results, we hypothesize that the combined effect of phenolic compounds with other phytochemicals are more effective at inhibiting prostate cancer cell growth than individual compounds.

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CHAPTER 3. EFFECTS OF ULTRA-HIGH TEMPERATURE THERMAL PROCESSING ON SOYMILK ATTRIBUTES

3.1. Abstract

The aims of this study were to evaluate and compare the phenolic contents and antioxidant activities of soymilk processed by the one-phase UHT and two-phase UHT methods. These two UHT methods have very different heat sterilization power. Additionally, the effects of the two UHT methods on the growth inhibition and apoptosis induction by soymilk CPE were determined in DU 145 cells. Our results showed that the two-phase UHT significantly increased total phenolic contents and antioxidant capacities of soymilk CPE compared to the one-phase UHT. The soymilk processed by either UHT method inhibited the proliferation of DU 145 cells. However, soymilk processed by two-phase UHT exhibited lower IC_{50} value than soymilk processed by one-phase UHT. The mechanism by which soymilk inhibited DU 145 cell proliferation was through induction of apoptosis. Soymilk CPE enhanced the expression of caspase-3, Bcl-2, and PARP-1 proteins of DU 145 cells. These findings indicated that soymilk had anti-cancer attributes that are affected by thermal processing conditions.

3.2. Introduction

The incidence rate of prostate cancer is historically much lower in Asian countries than in the United States and European countries (*1*). One possible explanation for this is that Asian populations consume more soy foods than

Western populations (*2*). However, migration studies have shown an increased prostate cancer incidence in Asian men after emigration to the United States (*3*). These observations suggest that environmental factors may partly play a role in the formation and development of prostate cancer.

Soy foods are rich in phenolics which include isoflavones. The crude protein extract (CPE) of soy, however, has received less attention than purified isoflavones, and its potential has not been investigated. The health benefits of soy and isoflavones have been studied, and include: anti-cancer effects (*4*), increased epithelial thickness and blood vessel elasticity of postmenopausal women (*5*), increased bone mineral density of postmenopausal women (*6*), decreased post-menopausal hot flushes of women (*7*), and lowered plasma cholesterol (*8*).

Heat treatment is necessary to produce soymilk, and the heating conditions are critical variables in soymilk processing. The effects of heat treatment on the elimination of off-flavors (*9*), inactivation of anti-nutritional factors such as trypsin inhibitors (*10, 11*), and the recovery of nutrients (*12, 13*) in soymilk have been well studied. However, the effects of heat treatment on the anti-cancer properties of soymilk have not been well characterized. A variety of heating conditions are used by various soymilk manufacturers. The most widely used UHT treatment involves a two-phase (120 ^{0}C, 80s + 140 ^{0}C, 4s) UHT heating in the soy industry to inactivate approximately 80-90% of trypsin inhibitor

activity (*14*). A one-phase UHT (143 ^{0}C, 60s) has also been reported to reduce approximately 90% of the trypsin inhibitor activity in soymilk. However, these two UHT processes generated very different thermal power in terms of pathogenic spore sterilization values, with one being 6 and the other 120. Therefore, we selected these two thermal conditions [one-phase UHT (143 ^{0}C, 60s) and two-phase UHT (120 ^{0}C, 80s + 140 ^{0}C, 4s)] to process soymilk for investigating thermal effects on anti-proliferative abilities of the soymilk products.

3.3. Materials and Methods

3.3.1. Materials

Dry matured Proto (harvested in 2006) and Black (harvested in 2006) soybeans (*Glycine max*) were obtained from Sinner Brothers & Bresnahan (Casselton, ND). Proto is a type of yellow soybean.

All of the solvents used for quantification of phenolic compounds were HPLC-grade and purchased from EMD Chemicals Inc. (Gibbstown, NJ). Sixteen phenolic acid standards (gallic, protocatechuic, 2, 3, 4-trihydroxybenzoic, *p*-hydroxybenzoic, gentistic, vanillic, caffeic, chlorogenic, syringic, *p*-coumaric, *m*-coumaric, *o*-coumaric, ferullic, salicylic, sinapic, and trans-cinamic acid), dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), (+)-catechin, fluorescein disodium, Folin-Ciocalteu reagent, sodium carbonate, 3-(4,5-dimenthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,4,4-trihydroxydeoxybenzoin (THB) and 6-hydroxy-2,5,7,8-tetramethylchroman-

2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Analytical grade acetic acid, bovine serum albumin (BSA), and acetone using for extraction were purchased from VWR International (West Chester, PA).

The human prostate cancer cell line DU 145 was 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, fetal bovine serum (FBS), penicillin-streptomycin mixed antibiotics, and all cell culture media (Eagle's Minimum Essential Medium) were purchased from Hyclone Laboratories Inc. (Logan, Utah).

The ApoDETECT Annexin V-FITC Kit and CFSE Cell Proliferation Kit were purchased from Invitrogen, Inc (Carlsbad, CA). Acridine orange (AO) and ethidium bromide (EB) were purchased from Sigma-Aldrich. The primary antibodies against caspase-3, bcl-2, and PARP-1, β-actin, and the horseradish peroxidase (HPR)-conjugated goat anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

3.3.2. Methods

3.3.2.1. Soymilk making Soymilk was produced by the following processing steps: 1) soaking the soybeans in cold water overnight at room temperature (soybean/water, w/w, 1:5); 2) wet grinding the soaked soybeans (water to bean ratio, 10:1, w/w) at room temperature to form a slurry using a continuous grinder, which was equipped with a centrifugal filter of 120 mesh size (Chang-Seng Mech. Co., Taoyuan, Taiwan), and 3) cooking soymilk under specified methods.

3.3.2.2. Thermal processing UHT is a relatively new method for manufacturing soymilk. A wide range of UHT temperatures is used to heat liquid foods in order to retain high food quality characteristics; in this case, to eliminate or reduce the problem beany flavor and optimize yield and functionality. In this study, the raw soymilk was processed by a Microthermics Direct Steam Injection Processor (DIP, Microthermics, Inc., Raleigh, NC). The two UHT methods includes: (1) two-phase method: 120 ^{0}C for 80 s in the first phase followed by 140 ^{0}C for 4 s in the second phase; (2) one-phase method: 143 ^{0}C, 60 s. The details of the method have been described by Yuan *et al.* (*10*).

3.3.2.3. Crude extraction of soymilk phenolics The extraction of soymilk phenolic compounds was performed as follows. After thermal processing, all soymilk samples were immediately frozen and then freeze-dried. The freeze-dried soymilk samples were accurately weighed (1 g) and put into a set of centrifuge tubes. Ten mL of the extraction solvent (acetone/water, 50:50,

v/v for yellow soymilk; acetone/water/acitic acid, 70/29.5/0.5, v/v/v for Black soymilk) were added into the tubes. The mixture was shaken at 300 rpm at room temperature on an orbital shaker (Lab-Line Instruments, Inc, Melrose Park, IL) for 3 h. The mixture was centrifuged by using an Allegra 21R Centrifuge (Beckman Coulter Ltd, Palo Alto, CA) at 3, 000 rpm for 10 min, and the supernatant was placed into a new set of tubes. The residues were extracted with 10 mL of the solvent for an additional 12 h in the dark overnight with shaking at room temperature. After the 12 h of extraction, both the extracts were combined and stored at 4 ^{0}C in dark. Two mL of the extract solution was reserved for phytochemical analysis. The rest of the solution was evaporated at 38 ^{0}C on a rotary evaporator (Labconco Co., Kansas City, MO) under vaccum. After evaporation, the extract was freeze-dried to obtain crude phenolic extract, which was kept at -20 ^{0}C until use.

3.3.2.4. Semi-purification of soymilk phenolics Four grams of Proto CPE was suspended in 20 mL of water by vortexing vigorously. The suspension was centrifuged to remove the insoluble part, and the supernatant was filtered to get a clear solution. The residue was suspended in water twice, and all the supernatants were combined. The clear solution was poured in a column previously packed with a macroporous resin XAD-7 (column of 20 × 1.6 cm, i. d., bed volume (BV) = 33.5 mL). The solution was pumped down through the column at a speed of 1.8 bed volumes/h (BV/h). The resin was washed with 2

BV of distilled water to remove the sugars, organic acids, and other water-soluble non-binding compounds (water eluate). Methanol (80%) was used to elute the phenolic compounds at a speed of 3.6 BV/h. The eluate was rotary-evaporated under vacuum to remove solvents, and then freeze-dried to yield semi-purified phenolic extract (SPPE).

3.3.2.5. Determination of Soymilk TPC In this experiment, the TPC of soymilk CPE was determined by a Folin-Ciocalteu assay (*15, 16*) using gallic acid (GA) as the standard. The mixture of the sample solution (50 μL), distilled water (3 mL), Folin-Ciocalteu's reagents solution (250 μL), and 7% $NaCO_3$ (750 μL) was vortexed and incubated for 8 min at room temperature. Then, a dose of 950 μL of distilled water was added. The mixture was allowed to stand for 2 h at room temperature. The absorbance was measured at 765 nm against distilled water as a blank. The TPC was expressed as gallic acid equivalents (mg of GAE/g of sample) by comparing to the gallic acid standard curve.

3.3.2.6. ORAC assay The ORAC was determined according to our lab's procedure which was initially developed by Prior and Wu (*17, 18*). This procedure is accomplished using a BMG Fluostar Optima Microplate Reader (BMG Labtech GmbH, Offenburg, Germany), which is equipped with two autoinjectors, an incubator, and wavelength adjustable fluorescence filters. The temperature of the incubator was set to 37 ^{0}C, and fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were

used. Briefly, AAPH (2, 2'-azobis (2-methylpropionamide) dihydrochloride) was used as the peroxyl free radical generator and Trolox as a standard. Twenty microliters of suitable diluted soymilk CPE samples, blank, and Trolox calibration solutions were loaded on a clear polystyrene 96-well microplate (flat bottom, Nalge Nunc Intl., Denmark) in duplicate based on a randomized layout. The plate reader was programmed to record the fluorescence of fluorescein on every cycle. The kinetic reading was recorded for 60 cycles at 40 s per cycle. The hydrophilic soymilk CPEs were diluted with phosphate buffer saline (75 mM, pH 7.0) to the proper concentration range for fitting the linearity range of the standard curve. Trolox standards were prepared with phosphate buffer saline (75 mM, pH 7.0), which was used as blank. After loading 20 μL of sample, standard, and blank, and 200 μL of the fluorescein solution into appointed wells, the microplate (sealed with film) was incubated for at least 30 min in the plate reader, the film was removed, and 20 μL of peroxyl free radical generator AAPH (3.2 μM) was added to initiate the oxidation reaction. The kinetics of the fluorescence changes were immediately recorded using SoftMax Pro software (Molecular Devices). The final ORAC values were calculated using a linear equation between the Trolox standards or sample concentration and net area under the fluorescence decay curve. The data were analyzed using Microsoft Excel (Microsoft, Roselle, IL). The area under curve (AUC) was calculated as

AUC = 0.5 + (R2 + R3 + R3 +.... + Rn)/R1, where R1 is the fluorescence reading at the initiation of the reaction and Rn is the last measurement.

The net AUC was obtained by subtracting the AUC of the blank from that of a sample or standard. The ORAC value was calculated and expressed as micromoles of Trolox equivalent per gram sample (μmol of TE/g) using the calibration curve of Trolox. For each specific sample, triplicate extractions were performed.

3.3.2.7. Quantification of phytochemicals by HPLC analysis Phenolic acids were analyzed on an Agilent 1200 series HPLC systems equipped with a G13798 degasser, G1312A binary pump, G1329A autosampler, and G1315D diode array detector (Agilent Technologies, Santa Clara, CA). HPLC separation was achieved using a Zorbax Stablebond Analytical SB-C18 column (250×4.6 mm, 5 μm, Agilent Technologies, Santa Clara, CA) at 40 ^{0}C. Elution was performed using mobile phase A (0.1% trifluoroacetic acid aqueous solution) and mobile phase B (100% methanol); samples (20 μL) were eluted at a flow rate of 0.7 mL/min. The UV-vis spectra were scanned from 220 to 600 nm on a DAD with a detection wavelength of 270 nm. The solvent gradient in volumetric ratios was as follows: 5-30% B over 50 min. The solvent gradient was held at 30% B for an additional 15 min and increased to 100% B for 66 min. The solvent gradient was held at 100% B for an additional 10 min to clean up the column, followed by re-equilibration of the column for 5 min with 95% A and 5% B before

the next run. Identification of phenolic compounds was made by comparison of their retention time and UV spectra with those of the authentic standards. The phenolic acid contents were expressed as micrograms of phenolic acid per gram of soymilk CPE (μg/g).

Isoflavones were analyzed using the same HPLC systems for phenolic acids according to Hou and Chang (*19*) with a slight modification. A YMC-Pack ODS-AM-303 C18 column (250×4.6 mm, 5 μm) was used. Mobile phase A (0.1% acetic acid aqueous solution) and mobile phase B (0.1% acetic acid in acetonitrile) were used to elute isoflavones. The system was eluted with 15% of solvent B for 5 min at the flow rate of 1.0 mL/min, increased to 29% for 31 min at the flow rate up to 1.5 mL/min, and then increased to 35% for 8 min at the same flow rate of 1.5 mL/min. Next, the gradient was equilibrated to 50% of solvent B for 2 min and the system was eluted with 50% of solvent B for 10 min at the flow rate of 1.5 mL/min. The solvent was recycled back to 15% B at the flow rate of 1.0 mL/min for 2 min, and then the column was equilibrated with initial solvent for 2 min prior to running the next sample. Identification of isoflavones was made by comparison of their retention time and UV spectra with those of the authentic standards.

3.3.2.8. Anti-proliferative assays

3.3.2.8.1. Cell Culture The DU 145 human prostate tumor cell line was used for studying the biological activities of soymilk CPE. Tumor cells

were maintained and sub-cultured in Eagle's Minimum Essential Medium supplemented with 10% (v/v) FBS and 1% antibiotics in 75 cm^2 flasks at 37 ^0C under a constant humidified atmosphere of 5% carbon dioxide.

3.3.2.8.2. <u>MTT assay</u> MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetra- zolium bromide) was used to test the proliferation rate of tumor cells after treating with soymilk CPE in order to detect the anti-proliferative capabilities of these extracts (*20*). The growing cells were harvested when they reached 80-90% confluence in 75 cm^2 flasks, and were diluted to an appropriate cell concentration with culture medium. The cells were seeded into 96-well plates at a density of 5×10^3 cells/well in 180 μL of medium. After 24 h of incubation, the cells were treated with 20 μL of 1, 2, 4 and 8 mg/mL soymilk CPE, respectively. Each treatment was conducted in triplicates. After 48 h incubation, 20 μL of 5 mg/mL MTT solution was added to each well. The plates were incubated for 4 h under the same culture conditions. The solution in each well was removed carefully after incubation, and then 150 μL/well of DMSO was added. The plates were shaken for 15 min, and the absorbance was measured at 570 nm in a microplate reader (Bio-Tech Instruments). The proliferation rate was determined by calculating the difference in absorbance values between the treated and control wells divided by the control absorbance.

3.3.2.8.3. <u>Flow cytometric analysis with CFSE staining</u> CFSE is a fluorescent dye used to measure cell proliferation via a flow cytometer. During

incubation CFSE is transported into the cell and binds covalently to cytoplasmic proteins, without adversely affecting cellular function. The intensity of CFSE staining is indicative of the degree of cell division occurring in a cell population (*21*). Analysis of cell division can be determined through its intensity when measured by flow cytometry. With each cell division, the CFSE fluorescent intensity is reduced by 50%, thus providing a read-out of the mitotic activity within a specific population of cells.

Cells (1×10^{6}) were harvested and suspended in 2 mL of medium. The cell suspension was put into a 15 mL conical tube. The cells were pelleted by centrifuging at $200\times g$ for 5 min (VWR, Parkway West Chester, PA). The cells were resuspended in 1 mL of pre-warmed PBS/0.1% bovine serum albumin (BSA), and then 2 µL of 5 mM stock CFSE solution was added into the cell suspension. The cells were incubated at 37 ^{0}C for 10 min, then 5 mL of ice-cold medium was added to the cells, and the tubes were incubated 5 min on ice. The cells were pelleted and washed three times with fresh medium. The CFSE stained cells were seeded into 6-well plates. The cells were treated with 1, 2, 4, and 8 mg/mL soymilk CPE, respectively. After 48 h of incubation, the cells were collected and analyzed on a flow cytometer (Accuri's C6 Flow Cytometer® System, Accuri Cytometers, Inc. Ann Arbor, MI).

3.3.2.9. <u>Apoptosis detection</u>

3.3.2.9.1. Flow cytometry analysis with Annexin V/PI staining

Apoptosis was assessed by flow cytometry using Annexin V and PI double staining. PI is a fluorescent dye that stains DNA. It does not cross the plasma membrane of cells that are viable or in the early stages of apoptosis because they maintain plasma membrane integrity. In contrast, cells that are in the late stages of apoptosis or are already dead have lost their plasma membrane integrity and are permeable to PI. Annexin V binds to cells that are early in apoptosis and continues to be bound throughout cell death. Early stage apoptosis is Annexin V positive but PI negative, and late apoptotic/necrotic cells are both Annexin V positive and PI positive (*22*).

Tumor cells were treated with 1, 2, 4, and 8 mg/mL soymilk CPE, respectively. After 48 h of incubation, the cells (1×10^6) were harvested and washed twice by PBS, Annexin V was diluted at a concentration of 1 mg/mL in binding buffer, the cells were resuspended in 1 mL of this solution (prepared freshly each time), and incubated for 10 min in dark at room temperature. PI solution (0.1 mL) was added to the cell suspension prior to analysis to give a final concentration of 1 mg/mL. The stained cells were analyzed by a flow cytometer.

3.3.2.9.2. AO/EB staining AO (acridin orange) and EB (ethidium bromide) staining method were used to detect the morphology of the apoptotic cells (23). The cells were treated with 1, 2, 4, and 8 mg/mL soymilk CPE,

respectively. After 48 h of incubation, the cells were collected and washed with cold PBS. Cell pellets were resuspended and diluted with PBS to a concentration of 5×10^5 cells/mL. One µL of AO (100 µg/mL)/EB (100 µg/mL) aqueous solution was mixed with 9 µL of cell suspension. The mixture was loaded on a clean microscope slide, and the slide was immediately examined under a fluorescent microscope (IX70, SIF2 Olympus, Tokyo, Japan).

3.3.2.9.3. Western blot analysis of apoptotic proteins The Western blots were performed as described by Liu and others (24, 25). DU 145 cells were seeded into 6-well plates at a concentration of 1×10^5 cells / well. After 24 h of adhesion, the cells were treated with 1, 2, 4, and 8 mg/mL soymilk CPE, respectively. After another 48 h culture, the medium was removed from each well, and each well was washed with 2 mL of PBS. The cells were collected using trypsinization and pelleted, then resuspended in 200 µL of cell lysis buffer containing 20 mM Tris·HCl (pH 6.8), 150 mM NaCl, 10% glycerol, 1% NP-40, and 8 µl/ml inhibitor cocktail (125 mM PMSF, 2.5 mg/ml aprotinin, 2.5 mg/ml leupeptin, 2.5 mg/ml antipain, and 2.5 mg/ml chymostatin). The cell suspension was homogenized on a Sonifier (500 Watt Model, Sonics & Materials Inc., USA) and centrifuged at 10,000×g for 15 min at 4 ^{0}C. The protein in the supernatant was collected into a new tube. The protein concentration of each sample was determined using the Pierce BCA protein assay kit (26). The samples were loaded on an 8-12% SDS-PAGE gel (8-10 µg of protein, 20 µL per well) for

electrophoresis using a Bio-Rad minigel apparatus at 100 V for 60 min. The separated protein on the gel was electrophoretically transferred onto the nitrocellulose membranes at 350 mA for 90 min. The membrane was blocked with PBS containing 5% nonfat milk at 4 ^{0}C for 1 h, probed with primary antibodies at 4 ^{0}C for 12 h, and incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. After incubation, the membrane was treated with enhanced chemiluminescence substrate (ECL Western blotting detection kit, Amersham Pharmacia Biotechnology) for 5 min at room temperature. The bands in the membrane were scanned and evaluated by using a ChemiImager 4000 densitometer (Alpha Innotech). The experimental values were normalized to β-actin reactivity.

3.3.3. Statistical analysis

All experiments were repeated at least twice. For the *in vitro* experiments, triplicate culture wells were used. One-way ANOVA and an appropriate Post-hoc comparison (Tukey) test were used when more than two groups were compared ($P < 0.05$). The significance level for all tests was set at 95% confidence limit. Various software packages (e.g. SigmaStat, Sigmaplot) were used to perform statistical analyses and assess significance of the data.

3.4. Results

3.4.1. Total phenolic content (TPC) determination

The TPC values of soymilk CPE are presented in Table 23. For Proto samples, the TPC value of the two-phase UHT produced soymilk was significantly ($P < 0.05$) higher than that of the one-phase UHT processed soymilk. For Black soymilk samples, the TPC value of the two-phase UHT soymilk was not significantly higher than that of one-phase UHT processed soymilk. For both one-phase and two-phase heated conditions, Black soymilk had significantly ($P < 0.05$) higher TPC values than Proto soymilk.

3.4.2. Oxygen radical absorbance capacity (ORAC) determination

The ORAC values of soymilk CPE are presented in Table 24. For Proto soymilk samples, the ORAC value of the two-phase UHT processed soymilk was not significantly ($P > 0.05$) higher than that of one-phase UHT processed soymilk. For Black soymilk samples, the ORAC value of two-phase UHT processed soymilk was significantly ($P < 0.05$) higher than that of one-phase UHT processed soymilk. The ORAC values of the UHT processed soymilk were significantly ($P < 0.05$) lower than that processed by the traditionally cooked soymilk (Tables 14, 15, and 24).

Table 23. TPC Values of Crude Phenolic Extracts from Proto and Black Soymilk Processed by UHT Methods

Processing	TPC (mg of GAE/g of CPE)
Proto,120^{0}C, 80s+140^{0}C, 4s	2.28±0.02d
Proto,143^{0}C, 60s	2.02±0.04c
Black,120^{0}C, 80s+140^{0}C, 4s	2.51±0.06a
Black,143^{0}C, 60s	2.43±0.07ab

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$).

Table 24. ORAC Values of Crude Phenolic Extracts from Proto and Black Soymilk Processed by UHT Methods

Processing	ORAC (μmol TE/g of CPE)
Proto,120^{0}C, 80s+140^{0}C, 4s	62.49±2.23c
Proto,143^{0}C, 60s	59.80±5.40c
Black,120^{0}C, 80s+140^{0}C, 4s	99.25±8.76a
Black,143^{0}C, 60s	82.28±10.25b

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$).

3.4.3. Quantification of phytochemicals by HPLC analysis

3.4.3.1. *Effect of thermal processing on isoflavone compositions* The isoflavone contents of the Proto and Black soymilk processed by the two UHT methods are presented in Table 25. Soy isoflavones exist in the form of glucosides, which means that the isoflavones are sugar conjugated.

Table 25. Isoflavone Content (μg/g) of Crude Phenolic Extracts from Soymilk Processed by UHT Methods

	Proto, one-phase	Proto two-phase	Black one-phase	Black, two-phase
Din	274.9±26.7a	222.9±21.3a	186.3±14.7a	170.8±13.3a
Gly	22.3±12.4a	27.6±11.0a	28.4±12.4a	25.3±8.4a
Gin	337.1±44.3 ab	272.4±22.7 b	470.5±20.3 a	480.3±46.14 a
AGly	101.5±4.4b	140.5±4.3b	585.6±43.8a	655.3±65.7a
MDin	279.6±7.0a	364.9±37.4a	311.2±16.5a	350.7±31.9a
MGin	609.5±50.9b	810.6±50.3a	551.9±16.5b	614.5±33.0b
MGly	75.7±10.7a	81.0±23.2a	89.7±5.0a	96.0±7.5a
Dein	39.9±4.5b	44.7±1.4b	60.7±1.4a	65.5±0.7a
Gein	52.5±11.0a	54.8±3.3a	104.9±20.6a	116.8±32.7a
total	1793.0±129.5c	2019.4±128.1bc	2389.4±109.2ab	2575.3±172.2a

This table represents the isoflavone components detected after HPLC analysis of Proto and Black soymilk following the application of two different UHT methods. The data were calculated on a dry weight basis and are expressed as the mean ± standard deviation (n = 2). Din, daidzin; Gin, genistin; Gly, glycitin; MDin, malonyldaidzin; MGin, malonylgenistin; MGly, malonylglycitin; AGly, acetylglycitin; Dein, daidzein; Gein, genistein; nd, not detectable. Values marked by the different lowercase letters within each row are significantly different ($P < 0.05$).

These water soluble glycosides can be partially hydrolyzed by bacterial ß-glucosidases in the large intestine to release the aglycones (daidzein, genistein, and glycitein), which are bioactive and absorbed by body. However, ß-glucosidases are not available in some human individuals. In this study, the aglycone content of Black soymilk was 1.6 to 2-fold of Proto soymilk. In either Proto or Black soymilk, the two-phase UHT method retained higher aglycone content than the one-phase UHT method ($P < 0.05$).

3.4.3.2. Effect of thermal processing on phenolic acid compositions

Phenolic acid content in soy has been rarely studied. In this study, we compared the total phenolic acids of the CPE of both Proto and Black soymilk processed by two UHT methods. Seven to nine phenolic acids were detected and quantified in soymilk samples using HPLC with authentic standards (Table 26).

Table 26. Phenolic Acid Content (µg/g) of Crude Phenolic Extracts from Soymilk Processed by UHT Methods

Phenolic Acids	Proto, one-phase	Proto, two-phase	Black, one-phase	Black, two-phase
GA	284.5±17.0c	612.5±21.2b	468.3±28.5c	664.6±25.6a
PA	208.7±3.0a	213.5±7.8a	229.2±11.2a	229.2±37.6a
PCD	188.1±11.1ab	170.5±17.8b	253.1±18.1a	224.9±21.2ab
CLA	1424.9±14.5a	1388.0±85.6a	1611.5±107.0a	1557.6±56.0a
FA	32.8±1.6a	32.5±3.1a	23.5±3.4ab	17.7±1.4b
SPA	79.5±0.7a	78.0±5.8a	90.8±13.8a	97.5±16.0a
OCA	151.1±2.2b	154.0±5.8b	258.1±17.0a	282.8±27.8a
PCA	nd	nd	nd	22.1±2.6
MCA	nd	nd	nd	14.7±3.0
total	2218.5±9.7b	2649.1±197.4ab	2676.3±273.0ab	2713.1±219.0a

This table represents the phenolic acid components detected after HPLC analysis of Proto and Black soymilk following the application of two different UHT methods. The data were calculated on a dry weight basis and are expressed as the mean ± standard deviation (n = 2). GA, gallic acid; PA, protocatechuic acid; PCD, protocatechuicdehyde; CLA, chlorogenic acid; FA, ferulic acid; SPA, sinapic acid; OCA, o-coumaric acid; PCA, protocatechuic acid; MCA, *m*-coumaric acid; nd, not detectable. Values marked by the different lowercase letters within each row are significantly different ($P < 0.05$).

The results showed that the total phenolic acid content was higher in Black soymilk than in Proto soymilk. The two-phase UHT method retained more phenolic acids than the one-phase UHT method for both Proto and Black soymilk.

3.4.4. Anti-proliferative properties of UHT soymilk against the DU 145 cell line

3.4.4.1. MTT assay of CPE The anti-proliferative properties of soymilk against prostate cancer cells are summarized in Table 27. Among all soymilk products tested, the Black soymilk exhibited higher anti-proliferative capacities than Proto soymilk as indicated by the lower IC_{50} values (Table 27). For the Proto soymilk samples, the soymilk processed by two-phase UHT had higher anti-proliferative capacities than soymilk processed by one-phase UHT (Table 27) ($P < 0.05$). For Black soymilk samples, the two-phase UHT soymilk exhibited significantly ($P < 0.05$) higher anti-proliferative capacities than the one-phase UHT soymilk. The growth inhibitory abilities of the soymilk produced by the two UHT methods were consistent with their TPC values.

Table 27. IC_{50} Values (mg/mL) of Crude Phenolic Extracts from Soymilk Processed by UHT methods against Proliferation of DU 145 Cells

Processing	IC_{50} (mg/mL)
Proto,120^{0}C, 80s+140^{0}C, 4s	4.88±0.29a
Proto,143^{0}C, 60s	5.07±0.43a
Black,120^{0}C, 80s+140^{0}C, 4s	3.68±0.18c
Black,143^{0}C, 60s	4.52±0.25b

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$).

The IC_{50} values of each isoflavone compound of crude soymilk phenolic extract were calculated according to the molecular weight of each isoflavone and expressed in micro mol per liter (Table 28). See Chapter 2 (page 52) for details on the calculations.

Table 28. Estimated IC_{50} Values (μmol/L) of isoflavones Crude Phenolic Extracts from Soymilk Processed by UHT Method

IC_{50} (μmol/L)	Proto, one-phase	Proto, two-phase	Black, one-phase	Black, two-phase
Daidzin	3.23	2.72	1.65	1.86
Glycitin	0.24	0.31	0.23	0.26
Genistin	3.81	3.20	4.01	5.03
Malonyl daidzin	2.72	3.69	2.28	3.16
Malonyl glycitin	0.69	0.77	0.62	0.82
Malonyl genistin	5.74	7.93	3.92	5.36
Acetyl glycitin	1.02	1.46	4.42	6.07
Daidzein	0.77	0.89	0.88	1.17
Genistein	0.95	1.03	1.43	1.96
sum(μmol/L)	19.16	22.00	19.44	25.66

Data are calculated on a dry weight basis.

3.4.4.2. MTT assay of semi-purified phenolic extract The anti-proliferative properties of SPPE against DU 145 cells are summarized in Table 29. Raw soymilk exhibited a higher anti-proliferative capacity than traditional cooked soymilk. The soymilk produced by two-phase UHT method exhibited significantly ($P < 0.05$) higher inhibition of cell growth than traditional cooked soymilk. Compared to the crude extract, the semi-purified extract showed much higher anti-proliferative capabilities. Semi-purification by the XAD-7 column concentrated phenolics approximately 10 folds. After semi-purification, the anti-proliferative capacity was improved more than 10 folds (Table 27 vs. Table

29), indicating a strong relation- ship between the purity of the phenolics with the anti-proliferative capacity of the soymilk extracts.

Table 29. IC_{50} Values (mg/mL) of Semi-Purified Phenolic Extracts from Soymilk Processed by UHT methods against Proliferation of DU 145 Cells

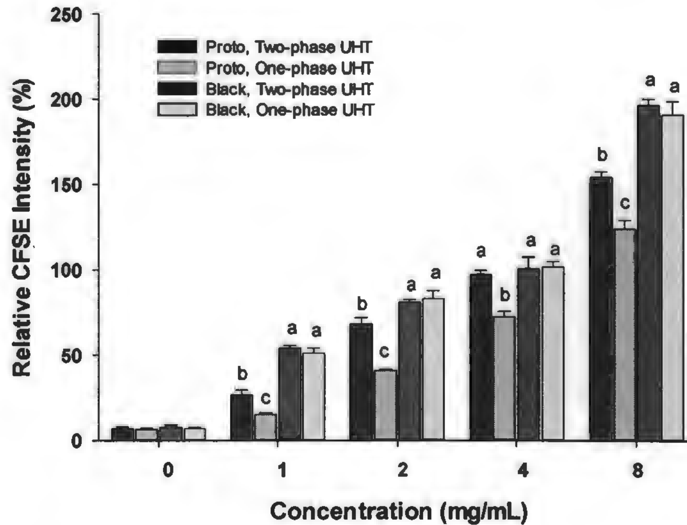
Processing	IC_{50} (mg/mL)
Proto, raw	0.41±0.01b
Proto, traditional cooked	0.64±0.12 a
Proto,120^{0}C,80s +140^{0}C,4s	0.26±0.03d
Proto,143^{0}C,60s	0.36±0.02c

Data are calculated on a dry weight basis and expressed as mean ± standard deviation (n = 3). Values marked by the different lowercase letters within each column are significantly different ($P < 0.05$).

3.4.4.3. Flow cytometry with CFSE staining The anti-proliferative properties of soymilk against DU 145 cells are summarized in Figure 1. The proliferation of prostate cancer cells was inhibited by soymilk CPE in a dose-dependent manner. Among all soymilk tested, Black soymilk exhibited a higher anti-proliferative capacity than Proto soymilk as indicated by the higher CFSE intensities (Figure 1). For both Proto and Black soymilk samples, there was a pattern that the two-phase UHT soymilk seemed to have higher anti-proliferative capacity than the one-phase UHT soymilk (Figure 1). However, no significant differences were observed.

DU 145 cells were labeled with CFSE and incubated with different concentrations of soymilk CPE for 48 h. DU 145 cells were harvested, washed, and resuspended in PBS, and then 10,000 cells were analyzed by flow cytometry.

Figure 1. CFSE fluorescence intensity of DU 145 cells.



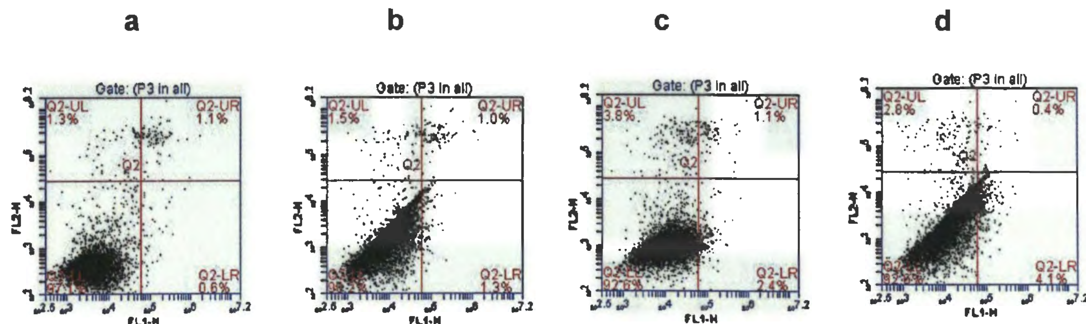
3.4.5. Detection of apoptosis-inducing characteristics in DU 145 cells

3.4.5.1. *Flow cytometry with annexin V/PI double staining* DU 145 cells (10,000 cells) treated with with annexin V-FITC and PI were analyzed by flow cytometry. The cells that were annexin V-positive and PI-negative are in an early stage of apoptosis and are still viable. The cells that stained with both PI and Annexin V are in the late stage of apoptosis. The results showed that a higher CPE (two-phase UHT processed Proto soymilk) concentration induced greater number of cells to undergo spontaneous apoptosis than the control cells (Fig. 2.a.1.7%, b. 2.3%, c. 3.5%, d. 4.5%).

DU 145 cells were treated with Proto soymilk CPE processed by two-phase UHT at concentrations of 0 mg/mL (**a**), 2 mg/mL (**b**), 4 mg/mL (**c**), and 8 mg/mL (**d**), respectively, for 48 h. The left upper quadrant represents the percentage of dead cells, the left lower quadrant represents the percentage of

live cells, the right upper quadrant represents the percentage of late apoptotic cells, the right lower quadrant represents the percentage of early apoptotic cells. The percentage of apoptotic cells is expressed as the percentage sum of early and late apoptotic cells.

Figure 2. Flow Cytometric Analysis of Apoptotic Rate of DU 145 cells Treated with Proto Soymilk Crude Phenolic Extract using Annexin V/PI Double Staining.

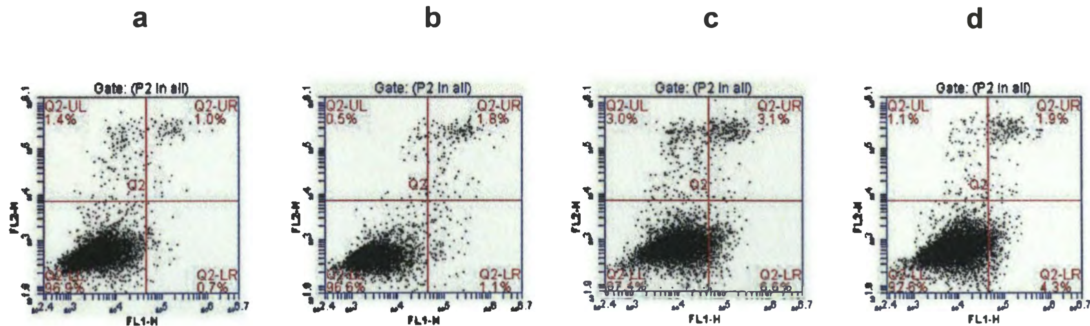


After 48 h of CPE (two-phase UHT processed Black soymilk) treatment, the results showed that higher CPE concentrations induced larger numbers of cells undergoing spontaneous apoptosis than the control cells (Fig. 3.a.1.7%; b. 2.9%; c. 6.2%; d.9.7%). Therefore, on an average, Black soymilk induced higher apoptosis properties than the Proto soymilk.

DU 145 cells were treated with two-phase UHT processed Black soymilk CPE at concentrations of 0 mg/mL (**a**), 2 mg/mL (**b**), 4 mg/mL (**c**), and 8 mg/mL (**d**), respectively, for 48 h. The left upper quadrant represents the percentage of dead cells, the left lower quadrant represents the percentage of live cells, the right upper quadrant represents the percentage of late apoptotic cells, the right lower quadrant represents the percentage of early apoptotic cells. The

percentage of apoptotic cells is expressed as the percentage sum of early and late apoptotic cells.

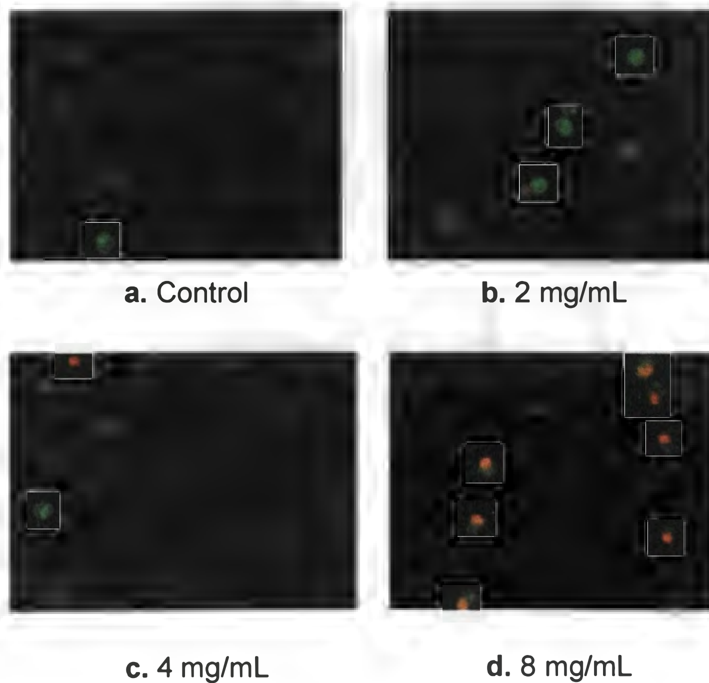
Figure 3. Flow Cytometric Analysis of Apoptotic Rate of DU 145 cells Treated with Black Soymilk Crude Phenolic Extract using Annexin V/PI Double Staining.



3.4.5.2. AO/EB Staining DU 145 cells were treated with different concentrations of two-phase UHT- processed Proto soymilk CPE for 48 h. DU 145 cells pre-treated with soymilk CPE were collected, about 5000 cells were stained with acridine orange/ ethidium bromide, then detected by a fluorescent microscope (200X) to determine the number of cells undergoing apoptosis. a, PBS-treated cells showed a normal green colored nucleus with virtually few signs of apoptosis (9.1%); b, exposure to 2 mg/mL of soymilk CPE revealed signs of early apoptosis (22.2%) with bright green nuclear dots and condensed chromatin; c, exposure to 4 mg/mL of soymilk CPE resulted in mostly late apoptotic events featuring a high number of cells (27.3%) with a condensed nucleus; and d, exposure to 8 mg/mL of soymilk CPE resulted in 100% cells undergoing late apoptotic events, featuring condensed orange chromatin (Figure 4). These images provide evidence that the soymilk crude phenolic extracts induce apoptosis in a dose-dependent manner.

DU 145 cells were treated (a) without or (b) with two-phase UHT processed Proto soymilk CPE 2mg/mL, (c) 4mg/mL and (d) 8mg/mL for 48 h. Photographs were taken under identical conditions. Each experiment was performed in triplicate (n = 3) and generated similar morphological features. Apoptotic rate of cells: a. 7.63%±1.29%, b. 19.93%±2.05%, c. 26.63%±2.37%, d. 91.67%±14.43%.

Figure 4. Apoptosis Detection with AO/EB Staining in DU 145 Cells.



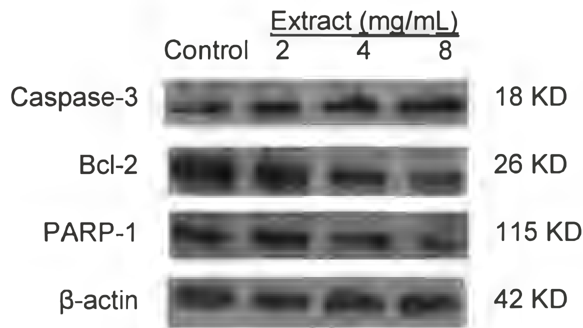
a. Control **b.** 2 mg/mL

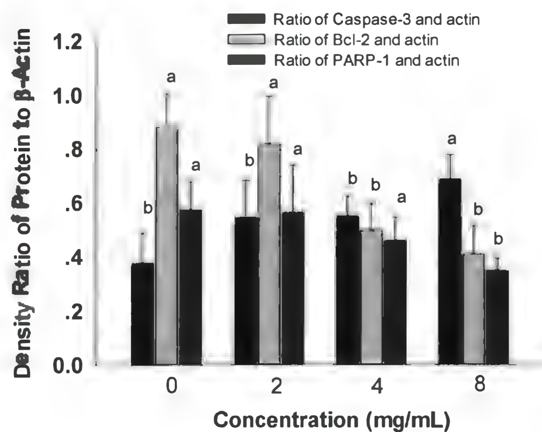
c. 4 mg/mL **d.** 8 mg/mL

3.4.5.3. Western blot The expression of caspase-3 (18 kDa), bcl-2 (26 kDa), and PARP-1 (115 kDa) in DU 145 cells was detected after treatment with 2, 4, and 8 mg/mL of CPE (two-phase UHT processed Proto soymilk) for 48 h, respectively (Figure 5). Soymilk CPE increased caspase-3 expression and decreased bcl-2 and PARP-1 expression in a dose-dependent manner.

The expression of caspase-3, bcl-2, and PARP-1 were determined by Western blotting, and the bands in the film were visualized and analyzed using Quantity One Software (Bio-Rad). The relative density is given as the density of caspase-3, bcl-2, or PARP-1 band divided by the density of β-actin band. Bars with different letters for among different concentrations within each protein indicate significant differences ($P < 0.05$).

Figure 5. Effects of Soymilk Phenolic Extract on the Expression of Apoptotic Proteins.





3.5. Discussion

3.5.1. Effects of processing on phytochemicals of soymilk

Soybeans are considered a functional food because they have many positive effects on health due to substances such as phytochemicals. Phytochemicals are bioactive non-nutrient compounds found in plants, including soybeans (*27*). Phenolic compounds are important phytochemicals in soy. They have attracted considerable attention due to their positive effects on diseases such as cardiovascular disease and cancers. Although the antioxidant properties of soy phenolic compounds have been reported (*28, 29*), the effects of processing methods on the phytochemical contents and the resulting antioxidant activities have not been well characterized.

This study investigated the effects of two UHT thermal processing methods: one-phase UHT and two-phase UHT on the phenolic compounds of soymilk. Different from the grinding experiment, the phenolic acid extracted were equivalent to that of the isoflavones, even though the soymilk produced by the two-phase UHT method had higher phenolic content than the soymilk produced by the one-phase UHT method for both Proto soymilk and Black soymilk. Therefore, the higher temperature used in the one-phase UHT method tended to degrade more phenolic compounds during heating than the two-phase UHT method. In addition, Black soymilk exhibited significantly ($P < 0.05$) higher TPC values compared to Proto soymilk; and this was due to the higher TPC value in

the raw Black soymilk than the yellow (Prosoy) soymilk (Tables 2 and 3). The TPC values of soymilk processed by the UHT methods were similar to the TPC values of soymilk processed by the traditional method (Tables 2, 3, and 20). The two-phase UHT processing produced higher retention of both isoflavone and phenolic acid components in both Proto and Black soymilk. These results suggest the two-phase UHT method should be selected for thermal processing in order to retain higher amounts of phenolic substances in soy.

In this study, we found that the TPC value of soymilk produced by the two-phase UHT method was 12.9% higher than the TPC value of soymilk produced by the one-phase UHT method for Prosoy soymilk, and 3.3% higher for Black soymilk. These results were verified by our HPLC findings that demonstrated the two-phase UHT method produced higher (isoflavones: 12.6% and 7.8% for yellow and black soymilk, respectively; phenolic acids: 19.4% and 1.4% for yellow and black soymilk, respectively.) phenolic substances than the one-phase UHT. Our results are consistent with a previous report from our lab, which indicated that the two-phase UHT method produced higher TPC than the one-phase UHT method (*30*). This may be partly due to the fact that the two-phase UHT method protects the soymilk phenolic compounds from higher temperature degradation during thermal processing.

3.5.2. Effects of processing on antioxidant activity of soymilk

Oxidative stress is considered a major contributor to cancer development

(*31*), because oxidants have the ability to damage DNA and to stimulate abnormal cell division. Antioxidants exert their protective effects by decreasing oxidative damage to DNA and minimizing abnormal cell division.

Soybeans have long been recognized for their health benefits, although the specific soy components and the mechanisms through with their health benefits have not been fully identified. The phenolics present in soy were once considered non-nutritive substances, since they can inhibit digestion of food components in the intestines. However, phenolics have recently gained attention as they have the ability to act as antioxidants. Research is ongoing to determine the antioxidant activities and the exact mechanisms of the health benefits of soy phenolics.

The antioxidant capability of soymilk was determined using the ORAC assay in this study. The effects of thermal processing on the antioxidant activities of soymilk were evaluated for the two UHT methods. It is known that thermal processing may destroy the natural antioxidants in foods, while it has been demonstrated recently that thermal treatment could induce the formation of new antioxidant compounds during processing (*32*). In the present study, we compared the effects of two thermal processing methods on the antioxidant activity of soymilk. Our results demonstrate that the soymilk produced by two-phase UHT had a higher antioxidant capability than the soymilk produced by one-phase UHT for Black soymilk, but not Proto soymilk. Black soymilk exhibited

a significantly ($P < 0.05$) higher antioxidant capability as compared to Proto soymilk.

The higher antioxidant activity of soymilk produced by the two-phase UHT method compared to the one-phase UHT method may be attributable, in part, to the higher recovery of TPC by two-phase UHT during thermal processing. This observation is consistent with the findings that two-phase UHT method increased the TPC values in soymilk.

3.5.3. Anti-cancer effects of soymilk phenolic extracts

The present study provides the first evidence that 1) crude soymilk phenolic extracts inhibit the growth of prostate cancer DU 145 cells, 2) the inhibition of DU 145 cell growth is a result of decreased proliferation and induction of apoptosis, 3) thermal processing of soymilk impact the overall anti-cancer attributes of the phenolic extracts. The anti-cancer effects of soy extracts and purified soy compounds have been reported as mentioned above (*33*).

It is known that thermal processing affects the content of phytochemicals obtained from soy. However, the effects of thermal processing on the anti-cancer attributes of soymilk have never been characterized. The present study fills this gap in knowledge by identifying the effects of thermal processing of soymilk on prostate cancer cell proliferation and apoptosis.

The concentration (IC_{50}) of isoflavones that inhibit cell proliferation has been reported in a large range. For example, glycitein exerts its growth inhibitory effect at concentrations ranging from 0.4 to 50 µmol/L (*34*). HPLC results from the present study suggest that the specific concentration (IC_{50}) effects of genistin, daidzin, and glycitin from soymilk extract (genistin: daidzin: glycitin, 1:0.8:0.1) on prostate cancer cells range from 0.63 to 8.89 µmol/L, 0.41 to 5.29 µmol/L, and 0.05 to 0.51 µmol/L, respectively. Dr. Helferich's group has reported that the concentration effects of pure and whole soy extract (genistin: daidzin: glycitin, 1:5.5:3.5) on prostate cancer are 100 µmol/L and 25 µmol/L, respectively (*33*), which could induce cell cycle arrest and caspase activation in LNCaP and PC3 cells. The isoflavone concentration differences between our data and literature may come from the use of different soy sources, chemical reagents, extraction methods, and processing conditions. However, our results are within the range of the literature.

3.5.3.1. Inhibiting proliferation in DU 145 cells This study investigated the anti-proliferative and apoptosis-inducing effects of soymilk CPE on the DU 145 cell line. In this study, we found that CPE significantly inhibited the growth of DU 145 cells in a dose-dependent manner. The semi-purified phenolic extracts from soymilk processed by UHT displayed a stronger anti-proliferative activity on prostate cancer cells, indicating the involvement of soymilk phenolics in inhibiting cancer cell proliferation. In conclusion, the results demonstrate that

the soymilk produced by two-phase UHT had higher anti-proliferative capabilities than the soymile produced by one-phase UHT. For the same processing conditions, Black soymilk exhibited significantly ($P < 0.05$) higher anti-proliferative capabilities than Proto soymilk.

The results of the present study show that the inhibitory effects of soymilk extract on the growth of prostate cancer cells were consistent with the report of an epidemiological study (*35*) and our previous publication data (*36*). Further, it has been reported that genistein, one compound of isoflavones, acts as chemopreventive agent by suppressing poorly differentiated cancerous lesions in androgen-dependent and independent prostate cancer animal models (*37*).

The effective growth inhibitory concentrations of polyphenols on prostate cancer cells have been studied in different cell lines. There is evidence that tea polyphenol inhibits the proliferation of the PC3 prostate cancer cell line in a concentration-dependent manner (with an IC_{50} value of 39.0 μM), but does not inhibit the proliferation of the non-tumorigenic prostate epithelial cell line (RWPE-1). A MEK-independent and PI3K (phosphoinositide-3-kinase)-dependent signaling pathway is suggested to be partially responsible for this inhibition (*38*). As previously reported in the literature, the IC_{50} values of genistein on the proliferation of human tumor cell lines ranged from 2.6 to 79 μM (*39*). These IC_{50} values of purified genistein are high compared with the IC_{50} values of genistein-containing soymilk crude extract on prostate cancer cells in

our study, which ranged from 0.95 to 1.96 μM. The better growth inhibition obtained with the crude extract may be due to the synergistic action of other phenolic compounds with genistein in the extract.

In this study, the growth inhibitory effects of the soymilk extract on DU 145 cells were observed; however, the mechanism for the anti-cancer effects of soymilk is still largely unknown. We investigated the effect of thermal processing on the profiles of phytochemicals and antioxidant activity of soymilk. The findings of higher TPC values and ORAC values of soymilk extract produced by the two-phase UHT method were consistent with a higher growth-inhibitory capability of the same method. However, the results from our grinding methods indicated that an increase in antioxidant capacities by cooking did not always significantly enhance the anti-proliferative power.

Therefore, the mechanism of growth inhibition seems to be beyond the antioxidant effects. The anti-cancer effect of the soymilk extract is dependent upon the thermal processing conditions and the properties of the different types of soy.

3.5.3.2. <u>Inducing apoptosis by soymilk CPE</u> To investigate the degree to which apoptosis is involved in the cell death caused by CPE treatment in DU 145 cells, we assessed apoptotic rate, morphological changes, and the expression of apoptotic proteins. We selected the Black and/or Proto soymilk produced by the two-phase UHT method to investigate the apoptosis-inducing

effect on DU 145 cells.

Prostate cancer cells treated with CPE for 48 h showed a concentration-dependent induction of apoptosis as determined by flow cytometric analysis of cells double stained with Annexin V/PI. Moreover, morphological changes, such as cell shrinkage, chromatin condensation, and cell membrane blebbing were also observed using a phase contrast microscope with AO/EB double staining. More apoptotic cells were observed with increasing concentrations of CPE.

In addition, the results from Western blotting experiments conducted to measure the expression of apoptotic proteins showed increased expression of caspase-3 as well as decreased expression of bcl-2 and PARP-1 after treatment with CPE for 48h. These results further support our observation that soymilk extract induces apoptosis in prostate cancer cells. These hallmark changes in indicators of apoptosis (annexin V/PI staining), morphology, and the expression of apoptotic proteins suggest that soymilk CPE causes induction of apoptosis in DU 145 cells.

Previous studies have shown in several human cell lines, especially breast and prostate cancer cell lines, that soy extracts and soy isoflavones induce apoptosis (*40-42*). Our results with soy polyphenols in prostate cancer cells are consistent with those of Xiang (*43*) and Zhou et al. (*44*), who reported that soy polyphenol inhibited the growth of human prostate cancer LNCaP and DU 145 cells and reduced the tumor volumes in a prostate cancer animal model.

To elaborate on the physiological relevance, the concentration of genistein- containing soymilk extract is compared with others. In Zhou's report (*45*), an IC_{50} of 50 μM was observed for genistein, whereas an IC_{50} of only 1.23 to 9.46 was observed for genistein- containing soymilk crude extract. Obviously, crude soymilk extract is more effective at inhibiting cancer cell growth than purified genistein. Hence, our results indicate that soymilk CPE may have more powerful ability to decrease cell proliferation and to induce apoptosis in human prostate cancer cells.

This study suggests that the IC_{50} value of genistein for DU 145 cells ranges from 1.23 to 9.46 μmol/L. This finding is different from a study done by Dr.Bomser's group (*45*), where they reported that genistein treatment increased cell proliferation in non-tumorigenic human prostate epithelial cells (RWPE-1) at a concentration of 0-12.5 μmol/L and inhibited cell proliferation at a concentration of 50 and 100 μmol/L. Furthermore, Dr.Bomser's group has reported that soy isoflavones (genistein, daidzein, glycitein and equol) could activate the prostate cells (RWPE-1 cell line) by increasing the activity of extracellular signal- regulated kinase (ERK1/2) at a concentration of 10 μmol/L (*46*). The reason for the different effects of genistein on DU 145 cells compared to RWPE-1 cells may be due to the fact that DU 145 cells are highly proliferative cancer cells and RWPE-1 cells are more slow-growing non-tumorigenic prostate cells. Further, crude soymilk extract was used in our experiment, while purified

genistein or the mixture of purified isoflavones were used in Dr.Bomser's group. The processing degree may explain these different findings. Moreover, soy source, chemical reagents, and extraction methods used in the experiments may contribute to the difference.The two processing methods employed in this study significantly affected the phenolic substances and antioxidant capabilities of soymilk. Furthermore, powerful anti-proliferative effects were obtained when soymilk CPE induced apoptosis in the human prostate cancer cells. These effects depended upon the processing conditions. There are limited data on the effects of thermal processing conditions on anti-cancer contributes; therefore, we are only able to compare the two thermal processing methods used in our study.

In general, the two-phase UHT method increased the total phenolic compositions, antioxidant capacity, and anti-proliferative effects of soymilk as compared to the one-phase UHT method. Meanwhile, Black soymilk exhibited higher total phenolic compositions, antioxidant, and anti-proliferative capabilities than yellow Proto soymilk.

This study aimed to identify the apoptotic mechanisms underlying the growth-inhibitory effects of soymilk extract on DU 145 prostate cancer cells. The mechanism by which soymilk CPE exhibits anti-proliferative effects involved the up-regulation of caspase-3 protein expression, and the down-regulation of bcl-2 and PARP-1. However, further research is needed in order to identify the

contributions of the individual components that are responsible for the anti-proliferative effects. In future studies, greater emphasis should be given to the effect of soymilk phenolic extract on the growth of normal prostate cell lines. Similarly, the effects of thermal processing on the anti-cancer attributes of soymilk need to be examined using animal models.

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CHAPTER 4. OVERALL CONCLUSIONS AND RECOMMENDATIONS

4.1. Processing Effects on the Total Phenolic Contents of Soymilk

Four grinding methods and two UHT processing methods significantly affected the total phenolic contents of soymilk. Among these processing conditions, grinding Method 4 significantly increased the total phenolic contents of soymilk because this grinding method retained more phenolics from both okara-washed water and soaked water; also, the two-phase UHT method exhibited higher total phenolic contents than the one-phase UHT method. The reasonable explanation for this may be partly due to lower thermal power of the two-phase method, which degraded less phenolics.

4.2. Processing Effects on the Antioxidant Capabilities of Soymilk

Consistent with the increased total phenolic contents, soymilk produced by grinding Method 4 and the two-phase UHT method exhibited higher antioxidant capacities than the other three grinding methods and the one-phase UHT method, respectively. Traditional cooking at 100 ^{0}C for 20 min tended to decrease phenolic contents, but increased antioxidant capacities. Grinding methods affected the retention of phenolic substances, antioxidant capacities, and their anti-proliferative capacities. Grinding in soaked water and re-grinding with okara-washed water produced better results.

4.3. Processing Effects on the Anti-proliferative Activity of Soymilk

Again, the anti-proliferative activities of soymilk CPE were affected by

processing conditions. Soymilk produced by grinding Method 4 and the two-phase UHT method inhibited cell growth more than the other grinding methods and the one-phase UHT method, respectively. As mentioned in Chapter 1, oxidative stress is considered as a major contributor to cancer development by stimulating cell division. Removing oxidants from cancer cells would have anti-proliferative effects. Soymilk phenolic compounds are known as natural antioxidants and this may be one reason why the soymilk with the higher phenolic contents caused a greater reduction in cancer cell proliferation. Furthermore, the SPPE exhibited greater anti-proliferative properties than CPE because the IC_{50} values of SPPE was about 1/10 of CPE. The semi-purification removed sugar, organic acids, and other water-soluble components from the extract in order to obtain more purified phenolic extracts. The higher phenolic contents of SPPE may be responsible for the greater growth inhibition of the prostate cancer cells. However, the effects may not be totally due to their antioxidative actions. These results are significant because they help guide the soy food industry regarding which processing techniques result in the greatest health benefit due to phytochemicals.

4.4. The Apoptosis-induced Effects by Soymilk CPE

A flow cytometric assay, morphological assay, and Western blot assays were conducted to investigate the induction of apoptosis in DU 145 cells. The Proto and Black soymilk produced by the two-phase UHT method were selected

for the apoptotic studies because of their high phenolic contents and antioxidant capacities. These studies were important in order to determine the potential mechanism by which soymilk affects the proliferation of prostate cancer cells. Moreover, the anti-cancer effects of soymilk on DU 145 prostate cancer cells addresses a new way to treat or prevent prostate cancer since DU 145 is a hormone-insensitive cell line. This study provided useful information for future *in vivo* studies for using soy to treat or prevent hormone-resistant prostate cancer.

4.5. Broader Impact on Soy Food Processing

The broader impacts of these results on the soy food industry are as follows. Most soymilk producers currently use the traditional grinding method (grinding Method 1) followed by two-phase UHT to process yellow soybeans. The results presented in this thesis provide evidence that black soybeans provide the greatest total phenolic content, antioxidant capability, and anti-cancer properties. Additionally, grinding Method 4 was far superior on all these attributes for both black and yellow soybeans. Therefore, the preferred method for obtaining the greatest health benefits from soybean would be to use grinding Method 4 followed by two-phase UHT method on black soybeans.

4.6. Limitations and Recommendations

Some limitations of this study include the fact that the phenolics in the CPE may have interacted with other phytochemicals in soy. This prevents us from attributing the anti-cancer effects observed soy solely to soy phenolic. Secondly,

the research was carried out only on tumor cells (*in vitro*). The same studies could be applied to hormone-sensitive prostate cancer cells, normal prostate cells, and even applied to animal models (*in vivo*) studies. Therefore, further fractionation, identification, and characterization of all potential phytochemicals and their interactions are necessary for understanding the precise mechanisms involved in the anti-proliferative effects of soymilk extracts on prostate cancer.