ANTICANCER EFFECTS OF DAIDZEIN, GENISTEIN AND SOY EXTRACTS ON HUMAN PROSTATE CANCER CELLS

by

Xin Dong

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AND SOY EXTRACTS ON HUMAN PROSTATE CANCER CELLS

by

Xin Dong

Approved: _____

Changqing Wu, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Jack Gelb, Ph.D.
Chair of the Department of Animal and Food Sciences

Approved: _____

Robin Morgan, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____

Charles G. Riordan, Ph.D.
Vice Provost for Graduate and Professional Education

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LIST OF ABBREVIATIONS

AAPH	2,2'-Azobis (2-methylpropionamidine) Dihydrochloride
Com	Combination
Daid	Daidzein
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Gen	Genistein
HPLC	High performance liquid chromatography
LDH	Lactate dehydrogenase
ORAC	Oxygen radical absorbance capacity
PDA	Photodiode array
PI	Propidium iodide
PCa	Prostate cancer
RT	Retention time
SD	Standard deviation
TFA	Trifluoroacetic acid

TPC	Total soluble phenolic contents
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TE	Trolox equivalent

ABSTRACT

Prostate cancer is one of the most common cancers in men. However, the incidence of clinical prostate cancer varies widely between ethnic populations and countries. Epidemiological studies have shown that higher intake of soy foods can lower risk of prostate cancer. Soy isoflavones, such as daidzein, genistein, and their sugar conjugates, daidzin and genistin, are considered to be major bioactive compounds in soy and provide chemoprotection of prostate cancer.

The majority of studies to assess the anticancer efficacy of soy products for prostate cancer (PCa) have been performed using purified bioactive compounds such as daidzein and genistein at pharmacological concentrations for short periods using androgen independent PCa cells such as PC-3 or androgen sensitive PCa cells such as LNCaP. However, attention has been drawn to the safety of using high levels of soy isoflavones in humans and the limited effect of individual soy isoflavones and these studies cannot reflect the real effects that soy may induce through diets. Currently, little is known about the bioavailability of isoflavones from whole soy foods and their bioactivities after cooking and digestion.

The objectives of this study include: 1) examine the antiproliferative and apoptotic effects of high-dose (25 to 200 μM) individual soy isoflavones, daidzein and genistein, using two human prostate cancer cell lines, LNCaP and C4-2B, from the

LNCaP progression model and explore the possible synergistic effects of the two isoflavones; 2) study the effects of low-dose (serum level), long-term exposure to daidzein and genistein on proliferation, apoptosis and cell cycle of LNCaP prostate cancer cells; 3) determine the antioxidant activities, isoflavone contents, antiproliferative and apoptotic effects on LNCaP and C4-2B human prostate cancer cells of extracts from whole soybeans and investigate the effects of heating and in vitro digestion on their bioactivities.

In the study of acute effects of high-dose isoflavones, significant apoptotic and antiproliferative effects were observed on both LNCaP and C4-2B cells. Daidzein and genistein showed a synergistic effect on inhibiting prostate cancer cell proliferation and inducing apoptosis. The low-dose long-term treatment did not change the proliferation and apoptosis of LNCaP cells significantly. However, the treatment with 250 nM daidzein reduced the effectiveness of docetaxel, a clinical chemotherapy drug, and all the three treatments had significant impact on LNCaP cell cycle progression. To evaluate the anticancer effects of soy food intake, we stimulated food intake by performing cooking and in vitro digestion process on whole soy. It turned out that after cooking and in vitro digestion, antioxidant activities of soy extracts increased while no consistent increase of the contents of four soy isoflavones was found. Better apoptotic effects on both LNCaP and C4-2B cells were found in cooked and digested soy extracts when compared with purified individual isoflavones, which indicated synergistic interactions between various bioactive compounds in whole soy foods.

Our study is the first study that investigated the effects of low-dose, long-term treatment of soy isoflavones on prostate cancer cells, evaluated the impact of low-dose, long-term exposure to soy isoflavones on the potency of docetaxel. Compared to other studies, we first used cooking and in vitro digestion to prepare soy extracts and examined the effects of cooking and digestion on the bioactivities of the whole soy exacts, thus it is more comparable to real ingestion of whole food by humans, and can provide more and better information for the preventive and therapeutic effects of soy food consumptions on prostate cancers.

Chapter 1

LITERATURE REVIEW

1.1 Prostate cancer risk

Prostate cancer (PCa) is third to lung and colon cancer as the cause of cancer-related deaths in American men and is the most common cause of death from cancer in men over age 75. The estimated new cases of PCa in United States were over one fifth million, and the deaths were over thirty thousand in year 2010 (National Cancer Institute, 2011). PCa is generally detected in men older than 50, and is rarely found in men younger than 40. The incidence of clinical prostate cancer varies widely between ethnic populations and countries. The Asian countries are considered to be a low-risk region, while the USA, Canada, New Zealand, Australia, the Northwestern European countries and Caribbean region are considered to be the high-risk countries or regions (Jian, 2009). However, after migrating to Western countries or after adopting a westernized lifestyle, the risk in Asian men increased (Cook et al., 1999; Shimizu et al., 1991; Sim and Cheng, 2005). This indicated that certain substances in the Asian diet, which are not rich in the high-fat, high-protein and low-fiber western diet may protect against the development of prostate cancer (Lee et al., 2007; Chan et al., 1998).

1.2 Soy food consumption and prostate cancer

Several epidemiologic studies suggested that the high consumption of soy products in Asian diet may be responsible for the lower incidence of prostate cancer (Yan and Spitznagel, 2005; Dalais et al., 2004; Lee et al., 2003). In a case-control study in China between 1989 and 1992 (Lee et al., 2003), 133 cases and 265 age- and residential community-matched controls, from 50 to 89 years old, were interviewed in person. Usual consumption of soy foods and isoflavones was assessed using a food frequency questionnaire developed in China and a nutrient database developed and validated in Asian-American populations. The results showed that, the age- and total calorie-adjusted odds ratio of prostate cancer risk decreased more than 40%, comparing the highest tertile of tofu intake to the lowest tertile. A significant dose-response trend for the reduction in risk of each level of tofu consumption was also observed. The highest quartiles of combined soy food, genistein, and daidzein were also associated with a 50% decrease in risk compared with the lowest quartile, although the dose-response trends were not statistically significant (Lee et al., 2003).

One case-control study performed in Japan assessed the hypothesis that the traditional Japanese diet, including soybean products, reduces the risk of prostate cancer. Four geographical areas of Japan were selected for the survey. Average daily intake of food from 5 years before the diagnosis was measured by means of a semi-quantitative food frequency questionnaire. The researchers studied 140 cases and 140 individually age (+/- 5 years)-matched hospital controls for analysis. The results showed consumption of all soybean products, tofu (bean curds), and natto (fermented

soybeans) was associated with decreased risk. Odds ratio of the fourth vs. first quartile and 95% confidence intervals (95% CIs) were 0.53 (0.24-1.14) for all soybean products, 0.47 (0.20-1.08) for tofu, and 0.25 (0.05-1.24) for natto. Consumption of natto showed significantly decreasing linear trends for risk ($P < 0.05$) (Sonoda et al., 2004).

In a population-based prospective study conducted in Japan, 43,509 Japanese men ages 45 to 74 years who generally have a high intake of isoflavones and low incidence of prostate cancer were asked to respond to a validated questionnaire, which included 147 food items. During follow-up from 1995 through 2004, 307 men were newly diagnosed with prostate cancer, of which 74 cases were advanced, 220 cases were organ localized, and 13 cases were of an undetermined stage. The results indicated that intakes of genistein, daidzein, miso soup, and soy foods were not associated with total prostate cancer, but decreased the risk of localized prostate cancer (Kurahashi et al., 2007). In another prospective study with 225 incident cases of prostate cancer in 12,395 California Seventh-Day Adventist men in 1976 who stated how often they drank soy milk, a 70 percent reduction of the risk of prostate cancer (relative risk = 0.3, 95 percent confidence interval 0.1-1.0, p-value for linear trend = 0.03) was associated with frequent consumption (more than once a day) compared no use of soy milk (Jacobsen et al., 1998).

Meanwhile, some studies did not find any significant relationship between isoflavones in soy food and prostate cancer risk. In a multiethnic cohort study of the relationship between legume, soy and isoflavone intake and prostate cancer risk

carried out in Hawaii and Los Angeles, 82,483 men were involved by completing a detailed quantitative food frequency questionnaire in 1993-1996. In the average follow-up period of 8 years, a total of 4,404 prostate cancer cases including 1,278 nonlocalized or high-grade cases were recorded. It is found that there was an 11% reduction for total prostate cancer risk and 26% reduction for nonlocalized or high-grade cancer in men who have the highest intake of legumes compared to those with lowest intake. Similar risk reductions were also observed for soy products and for legumes excluding soy products in separate analysis. However, no significant relations between prostate risk reduction and intake of total or specific isoflavones were found. Although legume intake can moderately decrease prostate cancer risk, the isoflavones in soy products are probably not responsible for the effects (Park et al., 2008).

1.3 Isoflavones in soy food

In the past two decades, bioactive compounds from plants have received considerable attention with particular interest of isoflavones. Isoflavones are diphenolic compounds present mainly in plants of the Leguminosae family. Highest concentrations of isoflavones occur in soybeans, red clover, and kudzu root (Mortensen et al., 2009). Isoflavones possess both hormonal and nonhormonal properties. As one of the three major classes of phytoestrogens, isoflavones have a spatial configuration similar to that of mammalian estrogens, and can bind to estrogen receptors and exert estrogenic effects (Usui, 2006). The nonhormonal properties, such as antioxidant activities, have also been related to the potential chemopreventive

properties of isoflavones and have led to the considerable speculation that isoflavones reduce the risk of both hormone-dependent and independent cancers (Sarker and Li, 2003).

Unprocessed soybeans contain 1.2-4.2 mg of total isoflavones/g of soybean (dry weight), mainly genistein, daidzein and their conjugates, with large variation due to variety, crop year, and growth location (Wang and Murphy, 1994a). The isoflavones in soybeans are predominantly genistin, daidzin and glycitin, and their respective aglycones, genistein, daidzein and glycitein (Figure 1.1). Among which, genistin and genistein are of the highest concentrations in soybeans and soy foods, and glycitin and glycitein are the lowest, which comprise only less than 10% of the total isoflavones in soybean (Murphy et al., 1999).

Soybeans are consumed only after being processed. These processes include but not limit to extraction, fermentation and cooking. During processing, losses and modifications of isoflavones may occur (Wang and Murphy, 1994b; Wang and Murphy, 1996). Heating can cause changes in the conjugation profile of the isoflavones in soy products and moist heat can increase the content of β-glucoside conjugates (Coward et al., 1998). However, a decrease of total isoflavone content would be caused by excessive heating (Grün et al., 2001). Fermentation usually includes aerobic and anaerobic fermentation. During aerobic fermentation, total isoflavone content may decrease, but the aglycone content would increase. In contrast, there may be only a slight change of total isoflavone content during anaerobic fermentation (Watanabe et al., 2007). Thus, the content of isoflavones varies a lot in

different types of soy foods. According to USDA-Iowa State University Database on the Isoflavone (2002) Content of Food, among the most consumed soy foods, soy flour contains the highest amount of isoflavones (daidzein, genistein and glycitein) with concentration range from 59.80 to 264.84 mg/100 g. Tofu contains 5.09 to 63.50 mg/100 g total isoflavones, soy milk contains 1.26 to 21.13 mg/100 g, and in soy sauce, isoflavone contents are generally low, with a concentration range from 0.10 to 2.30 mg/100 g.

In general, after the ingestion of soy foods, the serum isoflavone levels increase within 1-2 h and the peak value can be achieved after 4-6 h. After 24 h, almost all isoflavones are excreted (Rowland et al., 2003). Isoflavones in serum increase in a dose-dependent manner in response to soy food intake (Xu et al., 1994; Xu et al., 1995; Karr et al., 1997) and the efficiency of isoflavone absorption decreases as doses rise above 50mg (Setchell et al., 2001; Setchell et al., 2003).

There are significant variations in the absorption and metabolism of isoflavones among individuals, and the possible reasons for this include gender and age (Ilvine et al., 1998; Rowland et al., 2003; Cassidy, 2006; Lu et al., 1998; Wiseman et al., 2004), the degree of food processing (Kim et al., 2004; Allred et al., 2004; Luijten et al., 2004), the background diet, the food matrix, the chemical composition of the isoflavones (Cassidy, 2006) as well as the composition of intestinal mircro flora (Kirjavainen et al., 1999; Rowland et al., 1999; Kano et al., 2006).

1.4 Isoflavones and prostate cancer

There is evidence that isoflavones may have effects on various cancers, particularly breast cancer, however, researchers have a particular interest in prostate cancer recently. Evidences from in vitro and in vivo studies indicated a positive effect of isoflavones on prostate cancer.

Effects of isoflavones and soy extracts have been widely studied using cell lines and animal models. Recent studies have revealed that the inhibition of human prostate cancer cells induced by genistein and daidzein are related to the modulation of genes that are related to the control of cell cycle and apoptosis (Rabiau et al., 2010).

Hsu et al. (2010) showed that high doses of genistein and daidzein (>100 μmol/L) have ability to induce apoptotic effect and cell cycle arrest in benign prostate hyperplasia (BPH-1) cells, malignant androgen-independent prostate cancer epithelial cells (PC3) and early-stage androgen-dependent prostate cancer cells (LNCaP). They also found that soy extract induced more apoptosis on PCa cells (PC-3 and LNCaP cells) as compared to purified genistein or daidzein. In addition, soy extract induced insignificant apoptosis in non-cancerous BPH-1 cells while both genistein and daidzein induced apoptosis in BPH-1 cells, suggesting that individual isoflavones may have higher cytotoxicity in non-cancerous cells.

Genistein, one of the major isoflavones in soybeans and soy products, can act over multiple targets in cells. Genistein is known as an inhibitor of protein-tyrosine kinase (PTK), which can attenuate the growth of cancer cells by inhibiting PTK mediated signaling pathway (Akiyama et al., 1987). Genistein can also inhibit

topoisomerase I and II (Okura et al., 1988) and protein histidine kinase (Huang et al., 1992), which may contribute to the antiproliferative or pro-apoptotic effects. It also has been found that genistein has an antioxidant effect and protects cells against reactive oxygen species by scavenging free radicals, inhibiting the expression of stress response related genes, and reducing their contribution to the progression of carcinogenesis (Ruiz-Larrea et al., 1997; Zhou and Lee, 1998). Recently, it is also found that genistein is an inhibitor of NF-κB and Akt signaling pathway, both of which are important for cell survival (Wu et al., 1996; Van Antwerp et al., 1996; Cardone et al., 1998; Brunet et al., 1999). These effects of genistein are believed to be involved in the induction of apoptotic processes in genistein treated cells. Other studies have shown genistein and daidzein can both influence expression of genes implicated in cell cycle and angiogenesis, such as the up-regulation of CDKN1A in LNCaP cells, which encodes the p21CIP1 protein, an important cdk inhibitor involved in the regulation of cell cycle at both the G0/G1 and G2/M phases (Rabiau et al., 2010; el-Deiry et al., 2002; Gartel and Tyner, 2002) and down-regulation of CHEK2 and TP53 in LNCaP cells, which are two key regulators in the DNA damage signaling pathway (Rabiau et al., 2010).

Although the chemopreventive and therapeutic effects of genistein have been well established from in vitro studies, contradictory results from animal studies were reported. Thus the therapeutic actions of genistein in vivo have been questioned (Nakamura et al., 2011). Wang et al. (2009) observed dietary genistein reduces the incidence of advanced prostate cancer induced by N-methylnitrosourea in male

Lobund-Wistar rats during adult and life-time exposure. However, results from El Touny and Banerjee (2009) suggested the presence of a biphasic regulation of prostate cancer growth and metastasis by genistein in TRAMP-FVB mice. Raffoul et al. (2007) reported one year later that pure genistein could contribute to increased metastasis in PC-3 orthotopic metastatic mouse model after they found genistein can inhibit radiation-induced activation of NF-κB in prostate cancer cells and promote apoptosis and G2/M cell cycle arrest (Raffoul et al., 2006). Nakamura et al. (2011) examined the effects of genistein in a patient-derived prostate cancer xenograft model, in which a clinical prostatectomy sample was grafted into immune deficient mice. The results showed an increased lymph node and secondary organ metastases in genistein-treated mice compared to untreated controls. Invasive malignant cells aggregated to form islands/micrometastasis only in the secondary organs of the genistein-treated groups, not in the untreated control group. Tumors of genistein-treated groups have more proliferating and fewer apoptotic cancer cells than those of the untreated group, and the increased proliferation and metastasis are linked to enhanced activities of tyrosine kinases, EGFR and its downstream Src (Nakamura et al., 2011).

1.5 Conclusions

The current treatments of PCa, including surgery, radiation therapy and hormone therapy, are usually associated with unsatisfactory outcomes. Besides, there is still no curative and effective long-term treatment for bone metastatic PCa. Therefore, to develop novel preventive approaches to control this disease is necessary

and urgent. Chemoprevention using natural dietary substance is one of the approaches that have been widely studied recently (Syed et al., 2007).

Isoflavones and soy products have chemopreventive potential in prostate cancers. However, the controversy in epidemiological studies suggests more proper study designs and larger-scale of studies are demanded and the inconsistent evidence from laboratory research indicates the need for more research on the mechanisms, as well as carefully and well-designed clinical trials before giving isoflavone supplements to people, especially prostate cancer patients. More studies are also required to evaluate the effects of taking the whole food as source of isoflavones, rather than the individual purified compounds on cancer prevention.

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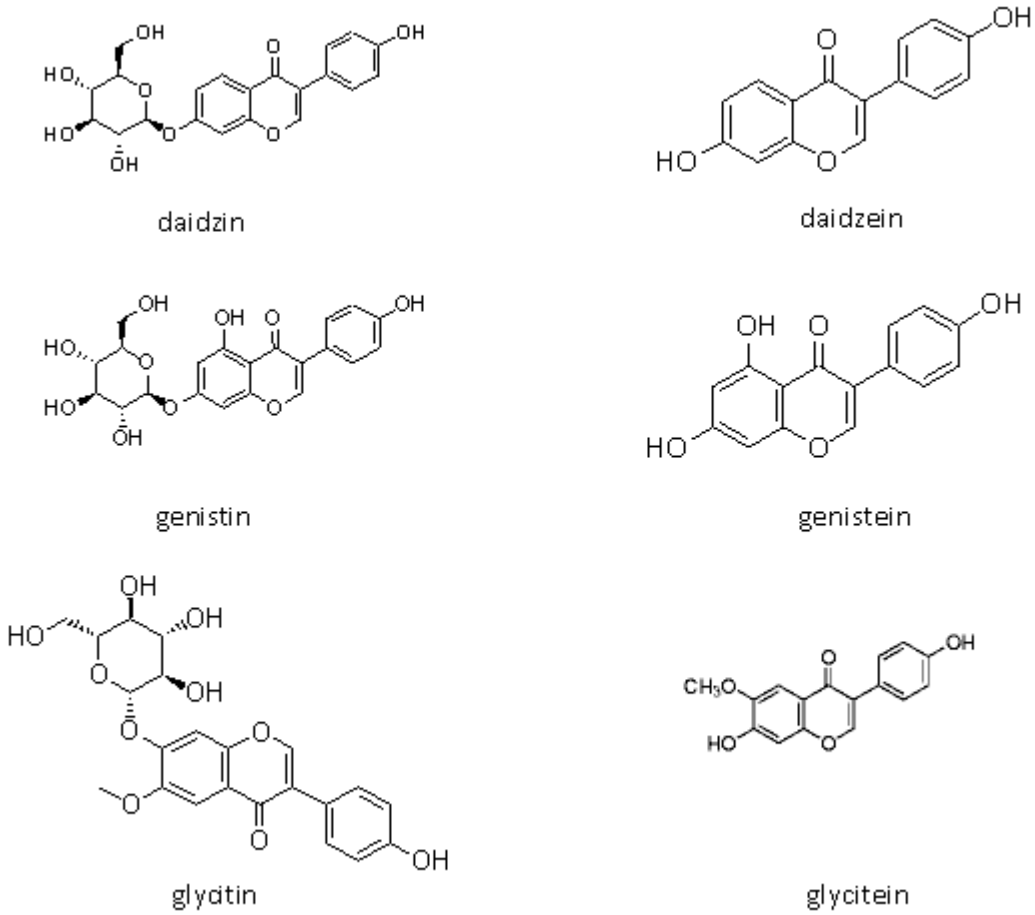

Figure 1.1 Chemical structures of major isoflavones in soybeans

Chapter 2

ANTIPROLIFERATIVE AND APOPTOTIC EFFECTS OF DAIDZEIN AND GENISTEIN ON HUMAN PROSTATE CANCER CELLS

ABSTRACT

The majority of anticancer efficacy of soy products for prostate cancer (PCa) has been performed using purified bioactive compounds such as daidzein and genistein at pharmacological concentrations for short time (≤ 72 h) using androgen independent PCa cells such as PC-3 or androgen sensitive PCa cells such as LNCaP. In this study, the antiproliferative and apoptotic effects of individual soy isoflavones, daidzein and genistein, and their combinations on prostate cancer cells were compared. The human prostate cancer cell line LNCaP, and metastatic subline of LNCaP, C4-2B, two cell lines from LNCaP PCa progression model, were used in this study. LNCaP and C4-2B cells were treated with varying concentrations of daidzein (25 to 200 μM), genistein (25 to 200 μM) or their combinations (25 or 50 μM) and analyzed for inhibition of proliferation and induction of apoptosis after a 48-hour treatment. All the treatments, except treatment with 25 μM daidzein, had significant antiproliferative effects on both LNCaP and C4-2B cells. At concentrations below 100 μM, no significant apoptotic effect on LNCaP or C4-2B cells had been found when the isoflavone was used individually. At concentrations of 100 μM or above, while

daidzein still had no effect, genistein showed a significant apoptotic effect on both LNCaP and C4-2B cells. Significant apoptotic effect was observed on C4-2B cells with combined treatments of daidzein and genistein when the concentration of genistein reached 50 μM but not on LNCaP cells. In conclusion, genistein is more effective than daidzein on inducing apoptosis of LNCaP and C4-2B cells. Daidzein and genistein had a synergistic effect on inhibiting prostate cancer cell proliferation and inducing apoptosis.

2.1 INTRODUCTION

Prostate cancer (PCa) research has become one of the most active areas of research since it has become a common malignancy in men, especially in United States and other western countries. The estimated new cases of PCa in United States were over one fifth million, and the deaths were over thirty thousand in year 2010 (National Cancer Institute, 2011). PCa is generally detected in men >50 years of age, usually at an advanced stage of the disease (also called metastatic prostate cancer), in which cancer has spread beyond the prostate gland and the area around the prostate (eg. bone, lymph nodes and rectum). For PCa patients with cancers that have spread to distant lymph nodes, bone, or other organs, five year survival rate is about 31%, while five year survival rate for patients with local and regional cancers approaches 100% (American Cancer Society, 2011).

The current treatments of PCa include surgery, radiation therapy and hormone therapy; however, unsatisfactory outcomes are associated. Besides, there is

no curative and effective long-term treatment for bone metastatic PCa. Therefore, to develop novel preventive approaches to control this disease is necessary and urgent. Chemoprevention using natural dietary substance is one of the approaches that have been widely studied recently (Syed et al., 2007). It is suggested that the lower incidence of PCa in Asian men compared to men in western countries might be attributable to the dietary difference. The typical Asian diet is more soy-rich and increased soy consumption has been associated with a lower risk of prostate cancer (Yan and Spitznagel, 2005; Dalais et al., 2004). However, the exact mechanism is still unknown.

Soy isoflavones, such as daidzein and genistein, are believed to have estrogen-like effects in the body, thus have an important role in reducing the incidence of prostate cancer. High doses of daidzein and genistein (>100 μmol/L) have the ability to induce apoptosis and cell cycle arrest in benign prostate hyperplasia (BPH-1) cells, malignant androgen-independent prostate cancer epithelial cells (PC3) and early-stage androgen-dependent prostate cancer cells (LNCaP) (Hsu et al., 2010). It also has been found that soy extract induced more apoptosis on PCa cells (PC-3 and LNCaP cells) as compared to purified daidzein or genistein. In addition, soy extract did not induce significant apoptosis in non-cancerous BPH-1 cells while both daidzein and genistein induced apoptosis in BPH-1 cells, suggesting that individual isoflavones may have higher cytotoxicity in non-cancerous cells (Hsu et al., 2010). In vivo studies showed that dietary genistein reduces the incidence of advanced prostate cancer induced by N-methylnitrosourea in male Lobund-Wistar rats during adult and life-time

exposure (Wang et al., 2009). Recent studies have revealed that the inhibition of human prostate cancer cells induced by daidzein and genistein are related to the modulation of genes that are related to the control of cell cycle and apoptosis (Rabiau et al., 2010).

Nevertheless, attention has been drawn to the safety of using high levels of soy isoflavones in humans and the limited effect of individual soy isoflavones (Matsukawa et al., 1993; Perabo et al., 2008). Due to some reports on genistein immunosuppressive properties and negative impacts on thymic function (Divi et al., 1997), concerns have been raised about the overconsumption of this soy isoflavone. Both in vitro and in vivo experiments have shown controversial evidence as to whether these ingredients of soy, such as genistein, promote or inhibit tumor growth (Messina and Loprinzi, 2001). Although lots of work has been done on the effects of these isoflavones on PCa, limited information is available about their effects on metastasis of PCa. Interactions between various bioactive phytochemicals may work synergistically to provide additional benefits. Zhou et al. (2003) verified the synergistic inhibitory effect of soy phytochemicals and tea bioactive components on androgen-sensitive human prostate tumors in mice. Kim et al. (2006) showed that soy extract is more potent than genistein in inhibition of tumor growth. However, the interactions may also be negative and undesirable (Lila and Raskin, 2006).

The LNCaP progression model consisting of LNCaP, C4, C4-2 and C4-2B represents four stages of PCa progression (Thalmann et al., 2000). LNCaP is poorly metastatic and androgen sensitive, C4 is highly tumorigenic but poorly metastatic, C4-

2 is castrate independent, aggressively tumorigenic and metastatic while C4-2B is castrate resistant and bone adapted. The LNCaP progression model shares remarkable similarities with human PCa (Thalmann et al., 2000), thus it can be an excellent model to study the potential activities of soy foods during PCa progression. In this study, we evaluated the effects of daidzein, genistein and the combined effects of daidzein and genistein on the proliferation and apoptosis of early-stage androgen-dependent prostate cancer cells (LNCaP) and bone metastatic LNCaP-derivative prostate cancer cells (C4-2B).

2.2 MATERIALS AND METHODS

2.2.1 Materials

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), daidzein, genistein, docetaxel, resazurin, trifluoroacetic acid, acetonitrile, Triton X-100, propidium iodide and RNase A were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Daidzein, genistein and docetaxel were made up as stock solutions in DMSO and stored at -20 ℃. On the day of the experiments they were diluted with DMSO.

2.2.2 Cell culture

LNCaP and C4-2B cells were maintained in T-medium supplemented with 5% (v/v) fetal bovine serum (FBS) and 100 U/ml penicillin G sodium and 100 mg/ml streptomycin sulfate in 0.085% (w/v) saline (PS) in 5.0% CO^2 atmosphere at 37 ℃.

Medium was changed every two days. Cells were routinely passaged using 0.25% (w/v) trypsin with ethylenediaminetetraacetic acid (EDTA) 4 Na when 90-95% confluence was reached.

2.2.3 Treatment of cells

Cells were seeded in 24-well plates (Corning, Lowell, MA, USA) at a density of 1×10^5 cells per well. Cells were grown to 50-70% confluence and then treated with daidzein (25 to 200 μM), genistein (25 to 200 μM) or their combinations (25 or 50 μM of each, 4 combinations in total). The final concentration of DMSO used was 0.1% (v/v) for each treatment. Control cells treated with 0.1% DMSO served as the vehicle group. Fifty nM docetaxel was used as a positive control. After 48 h of treatment, cells were harvested and pelleted by centrifugation at 200 × g for 10 min. Pelleted cells were used for apoptosis assay immediately and the supernatant was kept at 4 ℃ for measurement of cytotoxicity.

2.2.4 Apoptosis assay

DNA fragmentation was measured using the Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Indianapolis, IN, USA) following the protocol of the manufacturer. Cells were lysed with 200 μL lysis buffer and incubated at room temperature for 30 min. The lysate was then centrifuged at 200 × g for 10 min. Twenty μL supernatant was transferred into the streptavidin coated microplate and 80 μL immunoreagent was added to each well. The plate was covered with an adhesive foil cover and incubated on a shaker at 300 rpm for 2 h at room temperature. The solution

was thoroughly removed and each well was washed three times with 300 μL incubation buffer. One hundred μL ABTS solution was added to each plate and the plate was incubated on a shaker at 250 rpm. When the color development was sufficient for a photometric analysis, 100 μL ABTS stop solution was added to each well. Colorimetric detection was carried out according to manufacturer's instructions using MRX microplate reader (Dynex Technologies, Chantilly, VA, USA) at 405 nm excitation/490 nm emission.

2.2.5 Cytotoxicity analysis

Cytotoxicity to cells was determined by measuring the amount of lactate dehydrogenase (LDH) enzyme leaked from the cytosol of damaged cells into the medium after exposure of cells to the chemicals for 48 h. The LDH release represents necrosis as opposed to apoptosis. LDH in the supernatant was measured using the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Indianapolis, IN, USA) following the protocol of the manufacturer. A total of 50 μL of the supernatant from the cells was placed in a 96-well plate, and 100 μL of LDH assay solution (mixture of catalyst lyophilizate and dye solution) was added to each well and incubated for 5 min in the dark at room temperature. Absorbance of the mixture was read with MRX microplate reader at 490 nm.

2.2.6 Measurement of proliferation

Antiproliferative activity was measured by a resazurin assay. Cells were seeded in a 96-well plate (Corning, Lowell, MA, USA) at a density of 1×10^4 cells per

well. Cells were grown to 50-70% confluence and then treated with chemicals prepared in DMSO. Twenty μL 1 mg/mL resazurin solution (10% of medium, v/v) was then added into each well. After 48 h of treatment, the absorbance was measured at 570 nm and 600 nm using a Synergy™ 2 multi-mode microplate reader (BioTek Instruments, Inc, Winooski, VT, USA). Data was processed according to the following equation provided by the manufacturer: $\frac{117216 \times A_{570} - 80586 \times A_{600}}{117216 \times A^{\circ}_{570} - 80586 \times A^{\circ}_{600}}$ (A_{570} and A_{600} are the absorbance of test wells, while A°_{570} and A°_{600} are the absorbance of control wells.)

2.2.7 High-performance liquid chromatographic analysis of spent medium

Shimadzu LC-20A automated liquid chromatographic system (Shimadzu Scientific Instruments, Columbia, MD, USA), which consisted of a photodiode array (PDA) detector, a CBM system controller, a LC-20AT pumps, a SIL-20AC autosampler with cooler and a reverse-phase C18 (5 μm, 150×4.6 mm, 110Å) column with a matched guard column was used for the quantification of daidzein and genistein in spent medium. Spent medium was collected and filtered with a Costar Spin-X centrifuge tube filter (0.45 μm, nylon) (Corning, Lowell, MA, USA), then 20 μL spent medium was injected into and analyzed with Shimadzu LC-20A automated liquid chromatographic system. The mobile phase was composed of 0.1% trifluoroacetic acid (TFA) (v/v) (A) and acetonitrile (B) with a gradient elution program of 40-60% (B) from 0-15.00 min, 60-40% (B) from 15.01-17.00 min. A 0.5 min re-equilibration time was used between HPLC runs. Flow rate was 0.5 mL/min at 35 ℃. The detection

wavelength was 254 nm. The stock solution of daidzein and genistein was prepared in DMSO at concentration of 200 mM. Standard solutions were prepared at concentrations of 12.5 - 200 μM by diluting the stock solution in DMSO. All measurements were performed in triplicate.

2.2.8 Cell cycle analysis using flow cytometry

Cells were seeded in 6-well plates (Corning, Lowell, MA, USA) at a density of 2×10^5 cells per well. When 50-70% confluence was reached, cells were treated with chemicals as stated previously. After a 48-h treatment with daidzein, genistein or their combinations, both floating and adherent cells were collected. The cells were washed with PBS and fixed in ice cold 70% ethanol at a density of 1×10^5 cells/mL. Cells were stored at -20 ℃ before staining. On the day of analysis, cells were centrifuged, washed with PBS and then resuspended and incubated at room temperature in dark for 30 min in DNA staining solution: 0.1% (v/v) Triton X-100, 10 μg/mL propidium iodide, and 100 μg/mL DNase-free RNase A in PBS. Cell cycle analysis was performed using a FACSAria II flow cytometer (BD Biosciences, Sparks, MD, USA). A minimum of 10,000 cells per sample were counted and histograms were constructed using FlowJo 7.6.4 software (Tree Star, Inc., Ashland, OR, USA).

2.2.9 Statistical analysis

Results of each treatment were expressed as mean ± standard deviation for three treatment replicates in two independent tests. Treatment means were compared using Dunnett's test (DMSO treated group as control) ($p<0.05$). All statistical analyses

were performed using JMP 8 (SAS Institute Inc., Cary, NC, USA). In addition, expected values of combination were calculated based on the following equation (Zhou et al., 2003): Expected value of combination = [(observed value of daidzein)/ (control value)] *[(observed value of genistein)/ (control value)] * (control value). Ratio= (observed value/expected value). For apoptosis assays, a ratio of >1 indicates a synergistic effect, and a ratio of <1 indicates a less than additive effect. While for proliferation measurement, a ratio of <1 indicates a synergistic antiproliferative effect, and a ratio of >1 indicates a less than additive effect.

2.3 RESULTS

2.3.1 Effects of individual soy isoflavones and their combinations on apoptosis of prostate cancer cells

Fifty nM docetaxel showed higher apoptotic effect than all the concentrations of daidzein, genistein or their combinations used in this study. The apoptotic effects of daidzein and genistein on both LNCaP and C4-2B cells were in a dose-dependent manner. One hundred μM and 200 μM genistein showed similar apoptotic effects on LNCaP cells (Figure 2.1a). One hundred μM and 200 μM genistein also showed significant apoptotic effects on C4-2B cells; yet, the effect of 100 μM genistein was lower than 200 μM genistein (Figure 2.1b). It is worth mentioning that combined daidzein and genistein had a synergistic effect on cell apoptosis on both LNCaP cells and C4-2B cells (Table 2.1), although the effect on

LNCaP cells was not significant. Twenty-five μM daidzein/50 μM genistein and 50 μM daidzein/50 μM genistein significantly increased the apoptotic effects on C4-2B cells although they did not show any effect when used individually. The observed effects of the 25 μM daidzein/25 μM genistein, 25 μM daidzein/50 μM genistein, 50 μM daidzein/25 μM genistein and 50 μM daidzein/50 μM genistein combinations on LNCaP cell apoptosis were greater than the expected combination effects with a ratio of 1.51, 1.47, 1.40 and 1.47, respectively (Table 2.1). The observed effects of the 25 μM daidzein/25 μM genistein, 25 μM daidzein/50 μM genistein, 50 μM daidzein/25 μM genistein and 50 μM daidzein/50 μM genistein combinations on C4-2B cell apoptosis were greater than the expected combination effects with a ratio of 1.42, 1.58, 1.06 and 1.79, respectively (Table 2.1).

2.3.2 Cytotoxicity of individual soy isoflavones and their combinations on prostate cancer cells

One hundred μM and 200 μM genistein, and 50 μM daidzein/50 μM genistein caused slightly but not significantly more LDH leakage from LNCaP cells than DMSO control. Except for treatments of 100 μM and 200 μM genistein and 50 μM daidzein/50 μM genistein, all other treatments decreased LDH released from LNCaP cells compared to that of the DMSO control, however, no significant difference was observed (Figure 2.2a).

All genistein treatments (25, 50, 100 and 200 μM) and 50 μM daidzein/25 μM genistein, 50 μM daidzein/50 μM genistein caused significantly more LDH

leakage from C4-2B cells than DMSO control, while 25 μM and 50 μM daidzein showed significantly lower LDH leakage compared to DMSO control (Figure 2.2b).

2.3.3 Effects of individual soy isoflavones and their combinations on proliferation of prostate cancer cells

Daidzein and genistein exhibited significant antiproliferative activities on LNCaP and C4-2B cells (Figure 2.3a and 2.3b). The antiproliferative activities of daidzein and genistein on both LNCaP and C4-2B cells were in a dose-dependent manner. Proliferations of both LNCaP and C4-2B cells decreased with increased concentrations of daidzein or genistein. Comparing the antiproliferative effects of daidzein and genistein, genistein was more effective. LNCaP cell proliferations were 82.44%, 49.47 %, 41.63% and 42.60% of the control (DMSO) after 48 h of cell incubation with genistein of 25, 50, 100 and 200 μM, respectively. LNCaP cell proliferations were 89.88%, 89.45%, 74.72% and 68.80% of the control (DMSO) after 48 h of cell incubation with daidzein of 25, 50, 100 and 200 μM, respectively. C4-2B cells were more responsive to daidzein than LNCaP cells. The cell proliferations of C4-2B cells were 83.39%, 68.05%, 76.94% and 46.44% of the control (DMSO) after 48 h of cell incubation with daidzein of 25, 50, 100 and 200 μM, respectively. When incubated with genistein of 25, 50, 100 and 200 μM for 48 h, C4-2B cell proliferation were 72.19%, 52.34%, 46.74% and 46.79% of the control (DMSO).

Significant reductions in proliferation of LNCaP and C4-2B cells by combined treatment of daidzein and genistein were also observed (Figure 2.3a and

2.3b), and a synergistic effect between daidzein and genistein was found (Table 2.2). The observed proliferations of the 25 μM daidzein/25 μM genistein, 25 μM daidzein/50 μM genistein, 50 μM daidzein/25 μM genistein and 50 μM daidzein/50 μM genistein combinations on LNCaP cells were less than the expected combination effects with a ratio of 0.86, 0.87, 0.75 and 0.98, respectively (Table 2.2). And the observed proliferations of the 25 μM daidzein/25 μM genistein, 25 μM daidzein/50 μM genistein, 50 μM daidzein/25 μM genistein and 50 μM daidzein/50 μM genistein combinations on C4-2B cells were less than the expected combination effects with a ratio of 0.96, 0.85, 0.84 and 0.79, respectively (Table 2.2).

2.3.4 High-performance liquid chromatographic analysis of spent medium

To determine the amount of daidzein and genistein taken up by the cells, we measured the content in the spent medium after 48 h of the daidzein and genistein being introduced. HPLC analysis of freshly prepared standard solution revealed a distinct peak at retention time (RT) of 5.2 min for daidzein and 7.2 min for genistein at a UV absorption wavelength of 254 nm. A linear calibration curve was constructed using stock solutions with daidzein or genistein concentrations of 12.5-200 μM. The concentrations of daidzein and genistein within the spent medium were calculated according to the curves obtained. Our results showed that as the initial concentration of daidzein or genistein increased, the higher percentage and higher amount of daidzein or genistein was taken up by LNCaP and C4-2B cells (Figure 4a and 4b). In the groups treated with 25 μM, 50 μM and 100 μM of individual soy isoflavones,

genistein can be better taken up by both LNCaP and C4-2B cells than daidzein, while in the groups treated with 200 μM of individual soy isoflavones, daidzein was better taken up than genistein. In the groups treated with both soy isoflavones, genistein was always better taken up by both LNCaP and C4-2B cells than daidzein.

2.3.5 Cell cycle analysis

To study the potential mechanisms by which daidzein and genistein inhibit LNCaP and C4-2B cell proliferation, the effects of daidzein and genistein on the cell cycle progression were evaluated by flow cytometry (Figure 2.5 and Figure 2.6). We observed 50 nM docetaxel was able to induce cell cycle arrest in G2/M phase in both LNCaP and C4-2B cells after a 48 h treatment and reduced the cell numbers in G1 phase. For LNCaP cells, genistein (≥100 μM) decreased the percentage of cells in G1 and G2/M phase significantly and resulted in a significant increase in sub-G1 phase. A significant increase of cells in sub-G1 phase was observed after a treatment with 50 μM daidzein/50 μM genistein (Table 2.3). For C4-2B cells, genistein (≥100 μM) also decreased the percentage of cells in G1 phase significantly while a significant increase in sub-G1 phase and a significant decrease in G2/M phase were observed after cells being treated with 200 μM genistein. An increase of cells in sub-G1 phase was also found in the group treated with 100 μM genistein, however, it is not statistically significant (Table 2.4).

2.4 DISCUSSION

Our study is one of the first to compare the effects of individual and combined soy isoflavones (daidzein and genistein) in inhibiting prostate cancer cell proliferation and apoptosis using a prostate cancer progression model. Results from the present study suggested that combination of daidzein and genistein was more effective on inducing apoptosis and inhibiting proliferation in prostate cancer cells than individual soy isoflavones at equivalent concentrations. Genistein induced apoptosis in both LNCaP and C4-2B cells, whereas daidzein did not show any significant effect. However, at lower concentrations, combinations of daidzein and genistein induced apoptosis on C4-2B cells. More interestingly, when the concentration of genistein reached 50 μM in combined treatments of daidzein and genistein (comb2 and comb4), significant apoptotic effect was observed on C4-2B cells but not on LNCaP cells. This indicated that the combination of soy isoflavones may be more efficacious and safe for prostate cancer treatment, especially on metastatic prostate cancer cells. Our results are consistent with the study of Hsu et al. (2010), who found that soy extracts, containing a mixture of soy isoflavones and other bioactive components, induced a significantly higher percentage of cells undergoing apoptosis than daidzein or genistein, thus would be a more potent chemopreventive agent than individual soy isoflavones.

The HPLC analysis of spent medium revealed that not all the soy isoflavones introduced can be taken up by the cells, and the amounts that can be taken up by the cells depend on the initial concentrations of soy isoflavones added. In our

study, the higher concentrations of the soy isoflavones added, the higher percentage of the soy isoflavones can be taken up by the cells. These results were consistent with the apoptotic and antiproliferative effects we have observed.

Although we didn't observe significant cell cycle arrest using flow cytometry induced by daidzein, genistein or their combinations, we found a significant increase of cell numbers in sub-G1 in several groups, namely 100 μM, 200 μM genistein and 50 μM daidzein/50 μM genistein for LNCaP cells, and 200 μM genistein for C4-2B cells. This was consistent with the results of apoptosis assay, which tested the content of DNA fragments. According to other studies, greater than or equal to 100 μM genistein or daidzein can induce cell cycle arrest in LNCaP cells (Hsu et al., 2010), however, no cell cycle arrest was found in our study.

In previous studies, high levels of daidzein and genistein (>10 μM) were required to induce antiproliferative effects in prostate cancer cells (Magee and Rowland, 2004). In our study, daidzein or genistein, as low as 25 μM, was effective in inducing antiproliferative effects in LNCaP and C4-2B prostate cancer cells. This finding was consistent with previous studies, which demonstrated that relatively high doses of soy isoflavones (50-100 μM) were necessary to inhibit cell growth in breast and prostate cancer cells (Valachovicova et al., 2004; Davis et al., 1999, Perabo et al., 2008; Gong et al., 2003). In our study, genistein showed better apoptotic and antiproliferative effects on LNCaP and C4-2B cells, this also agreed with a previous study carried out by Rabiau et al. (2010). However, daidzein did not show any significant apoptotic effect in this study up to 200 μM.

Genistein is known as an inhibitor of protein-tyrosine kinase (PTK), which can attenuate the growth of cancer cells by inhibiting PTK mediated signaling pathway. The half maximal effect was observed at 0.7 μg/mL of genistein in A431 cells (Akiyama et al., 1987). Genistein can also inhibit topoisomerase I and II (Okura et al., 1988) and protein histidine kinase (Huang et al., 1992), which may contribute to the antiproliferative or pro-apoptotic effects. It also has been found that genistein has an antioxidant effect and protects cells against reactive oxygen species by scavenging free radicals, inhibiting the expression of stress response related genes, and reducing their contribution to the progression of carcinogenesis (Ruiz-Larrea et al., 1997; Zhou and Lee, 1998). Recently, it is also found that genistein is an inhibitor of NF-κB and Akt signaling pathway, both of which are important for cell survival (Wu et al., 1996; Van Antwerp et al., 1996; Cardone et al., 1998; Brunet et al., 1999). These effects of genistein are believed to be involved in the induction of apoptotic processes in genistein treated cells. Other studies have shown daidzein and genistein can both influence expression of genes implicated in cell cycle and angiogenesis, such as the up-regulation of CDKN1A in LNCaP cells, which encodes the p21CIP1 protein, an important cdk inhibitor involved in the regulation of the cell cycle at both the G0/G1 and G2/M phases (Rabiau et al., 2010; el-Deiry et al., 2002; Gartel and Tyner, 2002) and down-regulation of CHEK2 and TP53 in LNCaP cells, which are two key regulators in the DNA damage signaling pathway (Rabiau et al., 2010).

There is growing evidence both in vivo and in vitro demonstrating the chemoprotective effects of soy isoflavones, such as daidzein and genistein on cancer

prevention and treatment. Meanwhile, there are still questions as to whether using high levels of soy isoflavones in humans is feasible and safe. Our study suggested that combination of daidzein and genistein can lower the concentrations of single isoflavones and applying a combination of isoflavones may be safer and more feasible.

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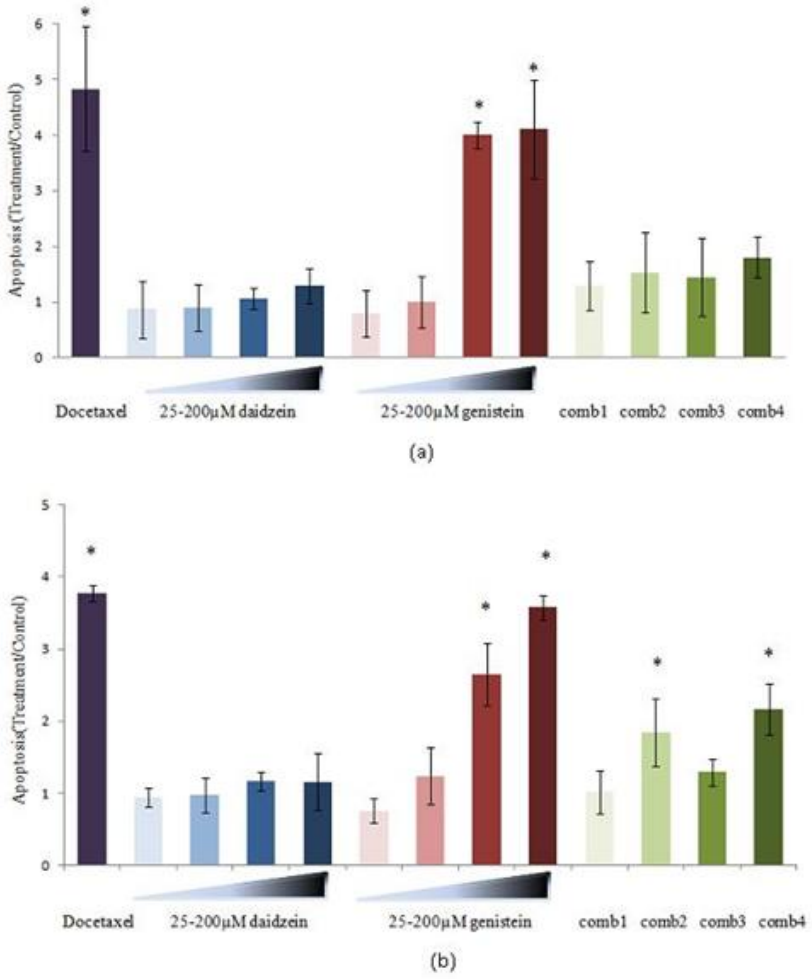

Figure 2.1 **Apoptotic effects of daidzein, genistein and their combinations on LNCaP (a) and C4-2B (b) cells.** LNCaP and C4-2B cells were treated with varying concentrations (25, 50, 100 and 200 μM) of daidzein or (25, 50, 100 and 200 μM) genistein or their combinations (comb1: 25 μM daidzein/25 μM genistein, comb2: 25 μM daidzein/50 μM genistein, comb3: 50 μM daidzein/25 μM genistein, comb4: 50 μM daidzein/50 μM genistein) for 48 h, and apoptosis was detected using a Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Indianapolis, IN, USA). Fifty nM docetaxel was used as a positive control. Data were expressed in the average of treatment/control reading (405 nm excitation/490 nm emission) ratio of two trials, three replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with control. Control treatment consisted of medium with 0.1% dimethyl sulfoxide.

Table 2.1 Synergistic effects of daidzein and genistein on cell apoptosis on LNCaP and C4-2B cells

	LNCaP cells			C4-2B cells		
	observed	expected	ratio	observed	expected	ratio
DMS	0.48			0.59		
Daid 25 μM	0.50			0.56		
Daid 50 μM	0.50			0.58		
Gen 25 μM	0.44			0.45		
Gen 50 μM	0.56			0.74		
Daid 25 μM Gen 25 μM	0.69	0.46	1.51	0.60	0.42	1.42
Daid 25 μM Gen 50 μM	0.85	0.58	1.47	1.09	0.69	1.58
Daid 50 μM Gen 25 μM	0.64	0.46	1.40	0.76	0.72	1.06
Daid 50 μM Gen 50 μM	0.85	0.58	1.47	1.29	0.72	1.79

a. Expected value of combination = [(observed value of daidzein)/(control value)] *[(observed value of genistein)/(control value)]*(control value).
b. Ratio=(observed value/expected value). A ratio of <1 indicates a synergistic effect, and a ratio of >1 indicates a less than additive effect.
c. Calculated in 570 and 600 nm reading.
d. Means of each treatment were used.

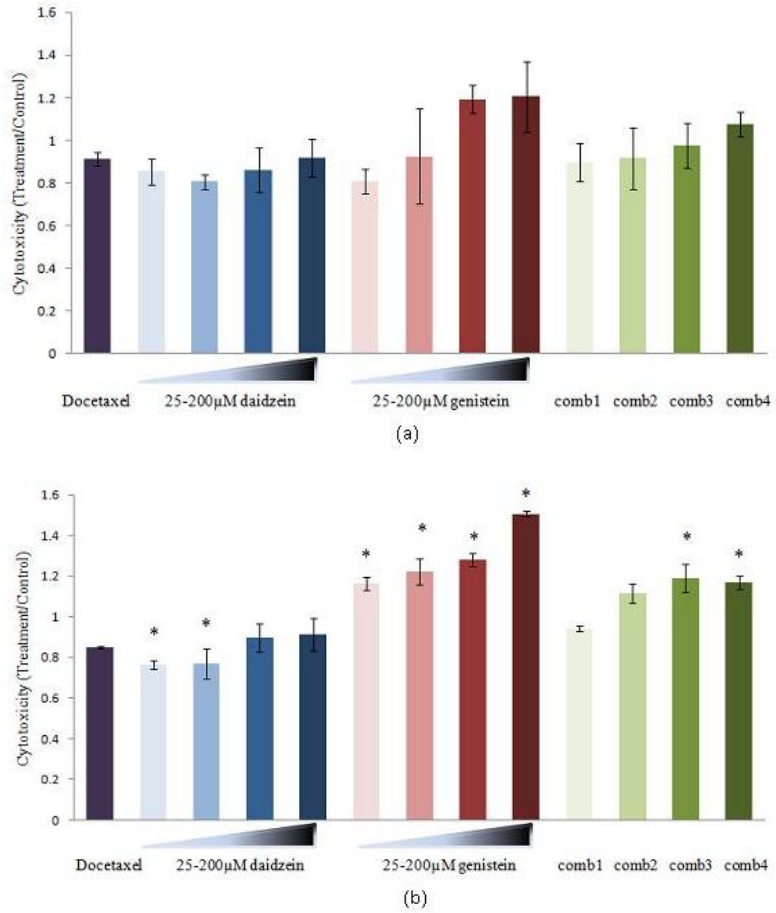

Figure 2.2 **Cytotoxicity of daidzein, genistein and their combinations on LNCaP (a) and C4-2B (b) cells.** LNCaP and C4-2B cells were treated with varying concentrations (25, 50, 100 and 200 μM) of daidzein or (25, 50, 100 and 200 μM) genistein or their combinations (comb1: 25 μM daidzein/25 μM genistein, comb2: 25 μM daidzein/50 μM genistein, comb3: 50 μM daidzein/25 μM genistein, comb4: 50 μM daidzein/50 μM genistein) for 48 h, and cytotoxicity was detected using a Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Indianapolis, IN, USA). Fifty nM docetaxel was used as a positive control. Data were expressed in the average of treatment/control reading (490 nm) ratio of two trials, three replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with control. Control treatment consisted of medium with 0.1% dimethyl sulfoxide.

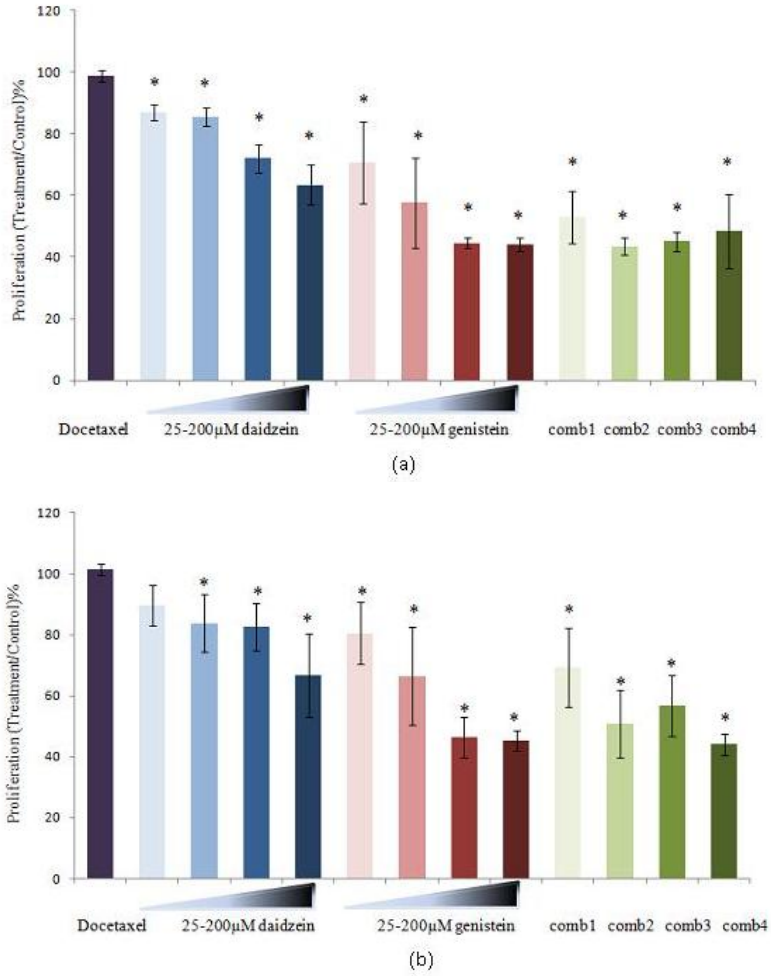

Figure 2.3 **Antiproliferative effects of daidzein, genistein and their combinations on LNCaP (a) and C4-2B (b) cells.** LNCaP and C4-2B cells were treated with varying concentrations (25, 50, 100 and 200 μM) of daidzein or (25, 50, 100 and 200 μM) genistein or their combinations (comb1: 25 μM daidzein/25 μM genistein, comb2: 25 μM daidzein/50 μM genistein, comb3: 50 μM daidzein/25 μM genistein, comb4: 50 μM daidzein/50 μM genistein) for 48 h, and proliferation was detected using a resazurin assay. Fifty nM docetaxel was used as a positive control. Data were expressed in the average of treatment/control reading (570 and 600 nm) ratio of two trials, three replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with control. Control treatment consisted of medium with 0.1% dimethyl sulfoxide

Table 2.2 Synergistic effects of daidzein and genistein on cell proliferation on LNCaP and C4-2B cells

	LNCaP cells			C4-2B cells		
	observed	expected	ratio	observed	expected	ratio
DMSO	100.00			100.00		
Daid 25 μM	86.92			89.56		
Daid 50 μM	85.38			83.78		
Gen 25 μM	70.68			80.51		
Gen 50 μM	57.59			66.50		
Daid 25 μM Gen 25 μM	52.89	61.44	0.86	69.37	72.10	0.96
Daid 25 μM Gen 50 μM	43.51	50.06	0.87	50.85	59.56	0.85
Daid 50 μM Gen 25 μM	45.04	60.35	0.75	56.75	67.45	0.84
Daid 50 μM Gen 50 μM	48.36	49.17	0.98	44.07	55.72	0.79

a. Expected value of combination = [(observed value of daidzein)/(control value)] *[(observed value of genistein)/(control value)]*(control value).
b. Ratio=(observed value/expected value). A ratio of <1 indicates a synergistic effect, and a ratio of >1 indicates a less than additive effect.
c. Calculated in 570 and 600 nm reading.
d. Means of each treatment were used.

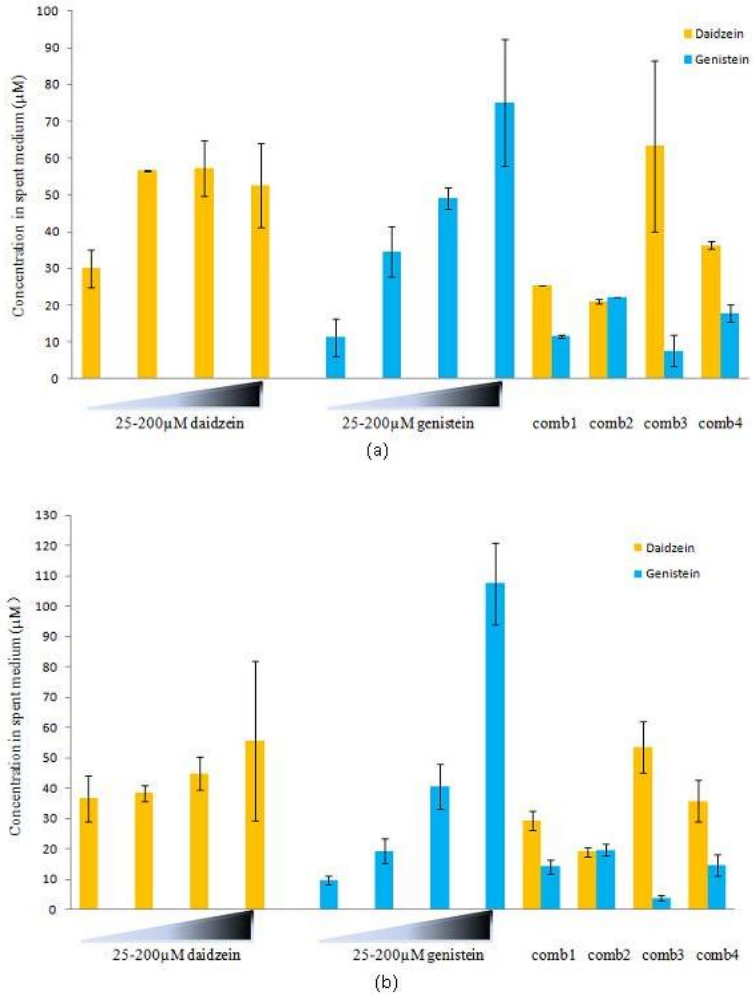

Figure 2.4 **HPLC analysis of daidzein and genistein in LNCaP (a) and C4-2B (b) cells spent medium.** LNCaP and C4-2B cells were treated with varying concentrations (25, 50, 100 and 200 μM) of daidzein or (25, 50, 100 and 200 μM) genistein or their combinations (comb1: 25 μM daidzein/25 μM genistein, comb2: 25 μM daidzein/50 μM genistein, comb3: 50 μM daidzein/25 μM genistein, comb4: 50 μM daidzein/50 μM genistein) for 48 h, and spent medium was collected, filtered and analyzed by Shimazu HPLC. Values represent means ±SD.

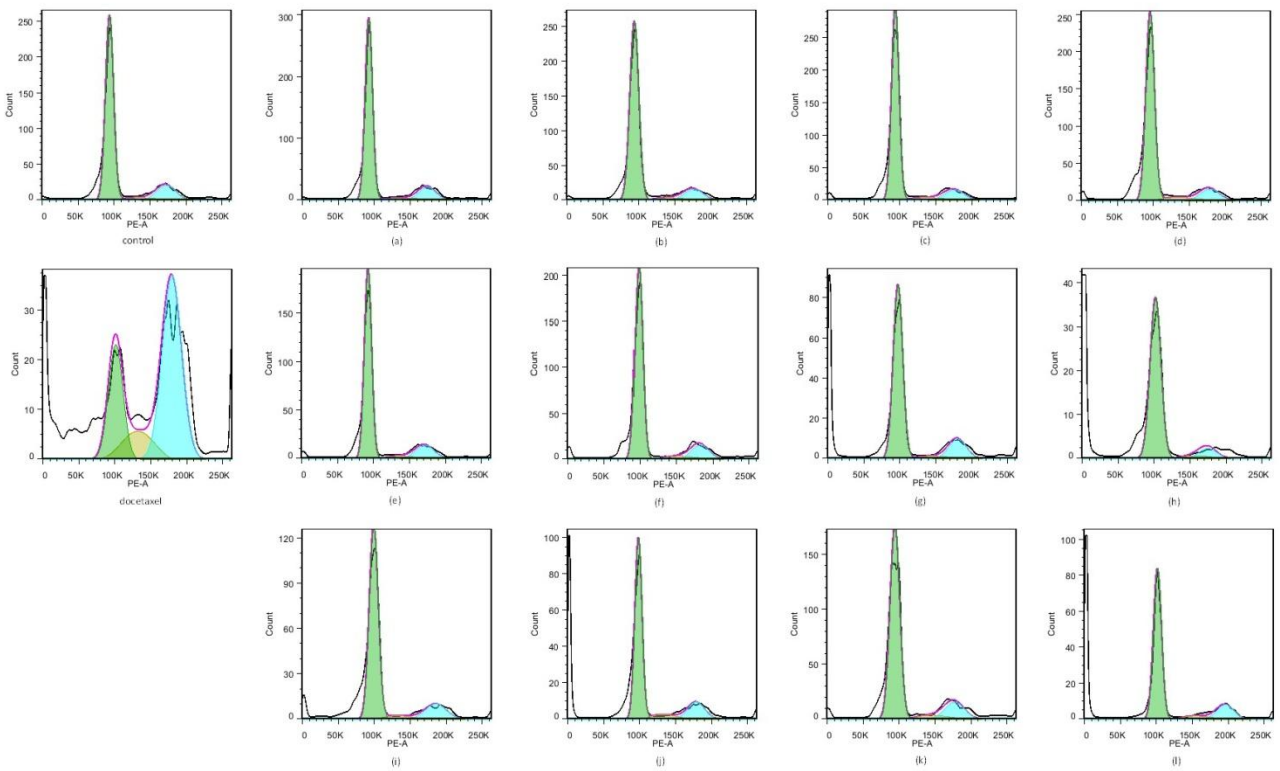

Figure 2.5 **Effects of daidzein and genistein on cell cycle progression of LNCaP cells.** LNCaP cells were treated with varying concentrations (25, 50, 100 and 200 µM) of daidzein (a to d) or (25, 50, 100 and 200 µM) genistein (e to h)or their combinations (comb1: 25 µM daidzein/25 µM genistein (i), comb2: 25 µM daidzein/50 µM genistein (j), comb3: 50 µM daidzein/25 µM genistein (k), comb4: 50 µM daidzein/50 µM genistein (l)) for 48 h, and distribution of cells in different phases of cell cycle was analyzed by propidium iodide (PI) staining followed by flow cytometry. Control treatment consisted of medium with 0.1% dimethyl sulfoxide. Fifty nM docetaxel was used as a positive control.

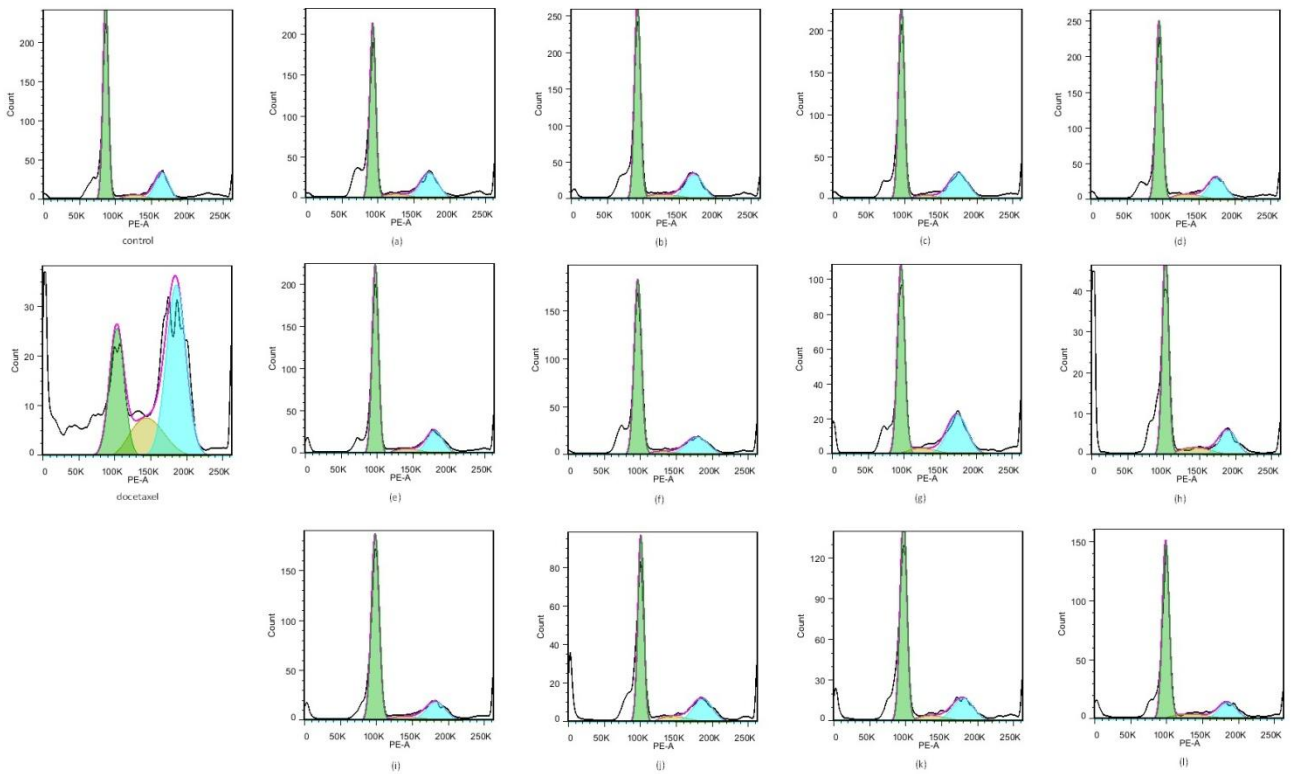

Figure 2.6 **Effects of daidzein and genistein on cell cycle progression of C4-2B cells.** C4-2B cells were treated with varying concentrations (25, 50, 100 and 200 µM) of daidzein (a to d) or (25, 50, 100 and 200 µM) genistein (e to h)or their combinations (comb1: 25 µM daidzein/25 µM genistein (i), comb2: 25 µM daidzein/50 µM genistein (j), comb3: 50 µM daidzein/25 µM genistein (k), comb4: 50 µM daidzein/50 µM genistein (l)) for 48 h, and distribution of cells in different phases of cell cycle was analyzed by propidium iodide (PI) staining followed by flow cytometry. Control treatment consisted of medium with 0.1% dimethyl sulfoxide. Fifty nM docetaxel was used as a positive control.

Table 2.3 Flow cytometric analysis of effects of daidzein and genistein on LNCaP cell cycle

	G1	S	G2/M	sub G1
DMSO	71.08 ±1.44	5.21 ±1.25	13.56 ±1.52	6.90 ±1.69
Docetaxel 50 nM	37.74 ±4.19*	5.31 ±1.12	36.35 ±1.29*	18.28 ±1.50
Daid 25 μM	67.12 ±8.62	5.47 ±0.87	11.70 ±2.71	13.54 ±12.04
Daid 50 μM	70.85 ±5.24	5.72 ±2.91	11.31 ±1.81	9.62 ±2.52
Daid 100 μM	67.99 ±2.65	5.32 ±1.17	10.34 ±1.39	14.33 ±1.84
Daid 200 μM	67.90 ±3.22	4.59 ±1.98	11.60 ±1.18	14.01 ±3.57
Gen 25 μM	67.99 ±4.92	4.33 ±1.53	12.85 ±1.56	13.56 ±4.52
Gen 50 μM	63.16 ±7.48	2.69 ±1.11	14.68 ±2.83	16.91 ±5.16
Gen 100 μM	30.42 ±26.04*	2.12 ±0.72*	6.76 ±5.44*	59.67 ±31.79*
Gen 200 μM	20.53 ±9.21*	7.68 ±3.91	5.29 ±3.07*	65.10 ±7.71*
Daid 25 μM Gen 25 μM	68.88 ±4.31	3.47 ±0.87	11.29 ±1.22	14.63 ±4.52
Daid 25 μM Gen 50 μM	62.29 ±11.06	2.37 ±1..54	12.98 ±2.04	20.56 ±8.99
Daid 50 μM Gen 25 μM	64.17 ±8.35	2.87 ±0.91	11.90 ±1.54	19.25 ±8.86
Daid 50 μM Gen 50 μM	54.68 ±15.06	4.02 ±1.94	10.49 ±2.63	29.85 ±17.62*

a. Values are means ± SD.
b. Statistical significance is as follows: *$p < 0.05$ comparing with control.

Table 2.4 Flow cytometric analysis of effects of daidzein and genistein on C4-2B cell cycle

	G1	S	G2/M	sub G1
DMSO	51.52 ±7.34	4.15 ±0.96	17.80 ±1.73	18.86 ±6.23
Docetaxel 50 nM	18.28 ±3.01*	14.97 ±1.69*	35.23 ±2.48*	26.38 ±2.74
Daid 25 μM	55.40 ±6.81	3.97 ±0.76	17.85 ±2.92	16.19 ±2.06
Daid 50 μM	55.92 ±7.00	4.89 ±1.68	18.40 ±2.35	15.52 ±4.60
Daid 100 μM	57.05 ±6.29	6.65 ±2.54	17.03 ±4.14	13.23 ±2.81
Daid 200 μM	56.39 ±3.43	5.83 ±1.40	16.53 ±3.65	16.43 ±3.63
Gen 25 μM	55.41 ±4.92	5.56 ±2.00	16.02 ±2.90	17.54 ±2.37
Gen 50 μM	43.77 ±21.20	6.05 ±3.82	14.06 ±6.88	31.57 ±31.38
Gen 100 μM	25.36 ±24.10*	4.20 ±1.68	13.53 ±13.19	42.42 ±38.33
Gen 200 μM	30.73 ±19.30*	7.59 ±3.08	8.81 ±4.54*	49.89 ±25.12*
Daid 25 μM Gen 25 μM	55.46 ±4.84	5.31 ±2.28	15.35 ±3.16	19.12 ±5.59
Daid 25 μM Gen 50 μM	51.35 ±4.92	4.99 ±2.45	17.18 ±2.66	21.18 ±6.26
Daid 50 μM Gen 25 μM	54.46 ±4.01	5.99 ±1.76	15.60 ±2.99	18.68 ±4.02
Daid 50 μM Gen 50 μM	51.44 ±7.75	5.99 ±2.29	17.78 ±3.45	20.87 ±7.44

a. Values are means ± SD.
b. Statistical significance is as follows: *$p < 0.05$ comparing with control.

Chapter 3

EFFECTS OF LOW-DOSE, LONG-TERM EXPOSURE TO DAIDZEIN AND GENISTEIN ON HUMAN PROSTATE CANCER CELLS

ABSTRACT

The reduced incidence of prostate cancer in some countries has been attributed to high soy diets and it is believed that the most effective compounds are soy isoflavones, among which daidzein and genistein are the most important ones. However, most of previous research on daidzein and genistein were conducted at pharmacological doses and for short periods of time (≤ 72 h). To determine the effects of low-dose, long-term daidzein and genistein exposure, we have cultured LNCaP prostate cancer cells in 250 nM daidzein, 500 nM genistein or their combinations for 60 days and investigated whether or not this long-term treatment altered the proliferation, apoptosis and cell cycle of LNCaP cells. In addition, we tested if the low-dose long-term exposure would affect the potency of docetaxel on LNCaP cells. The long-term treatment did not significantly change the proliferation and apoptosis of LNCaP cells. However, the treatment with 250 nM daidzein reduced the effectiveness of docetaxel, a clinical chemotherapy drug, and all the three treatments had significant impact on LNCaP cell cycle progression.

3.1 INTRODUCTION

It was noticed that the incidence of prostate cancer in Asian countries was much lower when compared to North-America or Europe (Hsing et al., 2000), but after migrating to Western countries or after adopting a westernized lifestyle, the risk increased (Cook et al., 1999; Shimizu et al., 1991; Sim and Cheng, 2005). Epidemiological studies have indicated that diets rich in soy products protects against the incidence of prostate cancer (Magee and Rowland, 2004; Munro et al., 2003). The putative cancer-preventing effect of soy products is believed to be associated with its bioactive compounds, particularly daidzein and genistein.

Although many studies have shown that daidzein and genistein can inhibit human prostate cancer (Wang et al., 2009, Rabiau et al., 2010, Hsu et al., 2010), high level of single isoflavone is required to achieve such effects (>10 μM). However, it is not possible to achieve such high concentrations of isoflavones in our bodies. What's worse, studies showed that at lower concentrations, genistein can stimulate the growth of cancer cells (Liu et al., 2005). Thus it is very important and necessary to investigate the impact of low concentration of isoflavones, which can be achieved through diet, on cancer development.

Few studies have investigated the long-term effects of daidzein and genistein on cell lines, although lots of studies have been done on the acute effects of daidzein and genistein on prostate cancer cell lines, such as malignant androgen-independent prostate cancer epithelial cells (PC3) and early-stage androgen-dependent prostate cancer cells (LNCaP) (Hsu et al., 2010). Even though good inhibitory effects

can be achieved, it cannot reflect the long-term effects that isoflavones in diet may induce. In addition, it is necessary to determine if the soy isoflavones interfere the potency of PCa drugs such as docetaxel. Thus, in our study, we used a long-term exposure model to serum level daidzein and genistein (Fanti et al., 1999) in LNCaP cells and investigated whether or not this treatment affect the proliferation and apoptosis, and the effectiveness of docetaxel on LNCaP cells.

3.2 MATERIALS AND METHODS

3.2.1 Materials

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), daidzein, genistein, docetaxel, resazurin, Triton X-100, propidium iodide and RNase A were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Daidzein and genistein stock solutions were prepared in DMSO and stored at -20 ℃. On the day of the experiments they were appropriately diluted with DMSO such that 10 µl of the solution was added to each flask.

3.2.2 Cell culture

LNCaP cells were cultured in T-medium supplemented with 5% (v/v) fetal bovine serum (FBS) and 100 U/ml penicillin G sodium and 100 mg/ml streptomycin sulfate in 0.085% (w/v) saline (PS) in 5.0% CO_2 atmosphere at 37 ℃. The initial stock of cells was of passage 11. Stock cells were then divided into 4 groups: control (0.1% DMSO), 250 nM daidzein, 500 nM genistein and 250 nM daidzein with 500 nM

genistein. Medium was changed every two days. Cells were passaged using 0.25% (w/v) trypsin with ethylenediaminetetraacetic acid (EDTA) 4 Na when 90% confluence was reached. Experiments were performed after cells had been grown in daidzein, genistein or both daidzein and genistein for 60 days although daidzein and genistein were not present in the experimental protocols outlined below.

3.2.3 Cell growth

Cell growth was measured using a resazurin assay (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Experiments were carried out to determine the optimal plating density of LNCaP cells and the period of linear growth. In these experiments, cells were seeded in 24-well plates at a density of 5×10^3 cells per well on day 1. On each day from day 2 to day 7, 100 μL 1 mg/mL resazurin solution (10% of medium, v/v) was added to three wells of each treatment and after 24 h, fluorescence was measured at 530 nm excitation/590 nm emission using a Synergy™ 2 multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Medium was replaced every two days. For other experiments, resazurin assay was performed on 4 control wells on day 2. On day 5, growth of the cells was measured by the resazurin assay. Growth was quantified by taking the ratio of the optical densities measured on day 5 to the mean optical density measured on day 2 and all data were normalized to control values. (Anastasius et al., 2009)

To test whether or not the long-term exposure to serum level daidzein and genistein affect the effectiveness of docetaxel on LNCaP cell proliferation, cells were

seeded in 24-well plates at a density of 1×10^5 cells per well. Cells were grown to 50-70% confluence, medium was changed and appropriate amount of docetaxel was added to the cells so the final concentration was 50 nM, and then 100 μL 1 mg/mL resazurin solution (10% of medium, v/v) was added to each well. Cells were allowed to grow for another 48 h, and fluorescence was then measured at 530 nm excitation/590 nm emission using a Synergy™ 2 multi-mode microplate reader.

3.2.4 Apoptosis assay

Cells were seeded in 24-well plates at a density of 1×10^5 cells per well. Cells were grown to 50-70% confluence, medium was changed and cells were allowed to grow for another 48 h with or without 50 nM docetaxel. After that, cells were harvested and pelleted by centrifugation at 200 × g for 10 min. Pelleted cells were used for apoptosis assay immediately and the supernatant was kept at 4 ℃ for measurement of cytotoxicity.

DNA fragmentation was measured using the Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Indianapolis, IN, USA) following the protocol of the manufacturer. Cells were lysed with 200 μL lysis buffer and incubated at room temperature for 30 min. The lysate was then centrifuged at 200 × g for 10 min. Twenty μL supernatant was transferred into the streptavidin coated microplate and 80 μL immunoreagent was added to each well. The plate was covered with an adhesive foil cover and incubated on a shaker at 300 rpm for 2 h at room temperature. The solution was thoroughly removed and each well was washed three times with 300 μL

incubation buffer. One hundred μL ABTS solution was added to each plate and the plate was incubated on a shaker at 250 rpm. When the color development was sufficient for a photometric analysis, 100 μL ABTS stop solution was added to each well. Colorimetric detection was carried out according to manufacturer's instructions using MRX microplate reader (Dynex Technologies, Chantilly, VA, USA) at 405 nm excitation/490 nm emission.

3.2.5 Cytotoxicity analysis

Cytotoxicity to cells was determined by measuring the amount of lactate dehydrogenase (LDH) enzyme leaked from the cytosol of damaged cells into the medium after exposure of cells to the chemicals for 48 h. The LDH release represents necrosis as opposed to apoptosis. LDH in the supernatant was measured using the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Indianapolis, IN, USA) following the protocol of the manufacturer. A total of 50 μL of the supernatant from the cells was placed in a 96-well plate, and 100 μL of LDH assay solution (mixture of catalyst lyophilizate and dye solution) was added to each well and incubated for 5 min in the dark at room temperature. Absorbance of the mixture was read with MRX microplate reader (Dynex Technologies, Chantilly, VA, USA) at 490 nm.

3.2.6 Cell cycle analysis using flow cytometry

Cells were seeded in 6-well plates (Corning, Lowell, MA, USA) at a density of 2×10^5 cells per well. When 50-70% confluence was reached, medium was changed and cells were allowed to grow for another 48 h. After that, adherent and

floating cells were collected, washed with PBS and fixed in ice cold 70% ethanol at 1×10^5 cells/mL. Cells were stored at -20 ℃ before staining. On the day of analysis, cells were centrifuged, washed with PBS and then resuspended and incubated at room temperature for 30 min in dark in DNA staining solution: 0.1% (v/v) Triton X-100, 10 μg/mL propidium iodide, and 100 μg/mL DNase-free RNase A in PBS. Cell cycle analysis was performed using a FACSAria II flow cytometer (BD Biosciences, Sparks, MD, USA). A minimum of 10,000 cells per sample were counted and histograms were constructed using FlowJo 7.6.4 software (Tree Star, Inc., Ashland, OR, USA).

3.2.7 Statistical analysis

Results of each treatment were expressed as mean ± standard deviation for four treatment replicates in two independent tests. Treatment means were compared using Dunnett's test (DMSO treated group as control) ($p<0.05$). All statistical analyses were performed using JMP 8 (SAS Institute Inc., Cary, NC, USA).

3.3 RESULTS

3.3.1 Effect of long-term exposure to daidzein and genistein on cell growth

The result showed that after a 60-day exposure to 250 nM daidzein, 500 nM genistein or their combination, no significant growth promoting or inhibiting effect was found. However, cell proliferation increased about 2% with treatment of single isoflavones and 4% with treatment of daidzein and genistein combination (Figure 3.1b).

To test whether or not the long-term exposure to serum level daidzein and genistein affect the effectiveness of docetaxel on LNCaP cell proliferation, we also compared the effect of docetaxel on control cells and the long-term treated cells. As the results showed in Figure 3.1b, we did not find any significant change of effectiveness of docetaxel, that is to say, the long-term exposure to serum level daidzein, genistein or their combination would not affect the activity of docetaxel on LNCaP cell proliferation.

3.3.2 Effect of long-term exposure to daidzein and genistein on apoptosis

From the results of cell death detection ELISA, which measured the content of DNA fragments, we did not find any impact of 60-day exposure to 250 nM daidzein on LNCaP cell apoptosis. The 60-day exposure to 500 nM genistein increased apoptosis of LNCaP cells about 29.3%, and the combination of 250 nM daidzein and 500 nM genistein decreased it by about 16.4% (Figure 3.2).

It is showed that a 60-day exposure to 250 nM daidzein may compromise the effectiveness of docetaxel on apoptosis. After a 48 h treatment of 50 nM docetaxel on long-term treated LNCaP cells, the apoptosis significantly decreased by about 22.8% in the cells treated with daidzein, compared to DMSO treated cells with a 48-h treatment of 50 nM docetaxel. The treatment of genistein increased the apoptotic effect of docetaxel by about 5.0% while treatment of daidzein and genistein combination decreased it by about 9.9% (Figure 3.2); however, these changes were not statistically significant.

3.3.3 Cytotoxicity of long-term exposure to daidzein and genistein

Long-term exposure to 250 nM daidzein caused slightly but not significantly more LDH leakage from LNCaP cells than DMSO control, and long-term exposure to 500 nM genistein or 250 nM daidzein and 500 nM genistein combination significantly decreased LDH released from LNCaP cells compared to that of the DMSO control (Figure 3.3)

After a 48-h treatment of 50 nM docetaxel on the long-term treated cells, we measured the LDH leakage from LNCaP cells. As shown in Figure 3.3, only the cells that had been treated with combination of 250 nM daidzein and 500 nM genistein had significantly less LDH leakage than cells treated with DMSO, while cells treated with 500 nM genistein had more but not significantly different LDH leakage and cells treated with 250 nM daidzein had almost the same LDH leakage compared to cells treated with DMSO (Figure 3.3).

3.3.4 Effect of long-term exposure to daidzein and genistein on cell cycle

To study the effect of long-term exposure to daidzein and genistein on LNCaP cell cycle progression, flow cytometry was used to determine cell cycle distribution (Figure 3.4). After being treated for 60 days with 250 nM daidzein, 500 nM genistein or their combination, LNCaP cells were allowed to grow in 6-well plates and cell cycle analysis was carried out. As shown in Table 3.1, cells treated with 500 nM genistein decreased cells in the G1 phase and resulted in a marked increase of cells in the S phase. Cells in the G2/M phase also increased; however, the difference was

not statistically significant compared to the controls. Cells treated with 250 nM daidzein or combination of 250 nM daidzein and 500 nM genistein resulted in a significant accumulation of cells in the G2/M phase in comparison to the controls.

3.4 DISCUSSION

Although studies reported that high doses of daidzein and genistein (>10 μM) can induce antiproliferative effects in prostate cancer cells (Magee and Rowland, 2004), it is not quite possible to achieve such high level in vivo. Besides, the safety of using high levels of soy isoflavones in humans, the limited effect of individual soy isoflavones and overconsumption of soy isoflavones also become concerns (Matsukawa et al., 1993; Perabo et al., 2008; Divi et al., 1997). Our study is the first to investigate the effects of long-term exposure to low-dose daidzein and genistein in LNCaP cells. Normal serum levels of daidzein and genistein were used in this study to ensure the amount can be achieved by daily diets.

From our results, no statistically significant difference was found in cell proliferation compared to the control cells after a 60-day exposure to serum level of soy isoflavones. The reasons might be the limitations of the resazurin assay in determination of cell growth, since it is influenced by the physiologic state of cells and by the variance in mitochondrial metabolic activity in different cell types. However, the resazurin assay is much easier and safer to use for the user compared to reference viability assays such as formazan-based assays (MTT/XTT) and tritiated thymidine

based techniques, which also have certain limitations, without compromising the accuracy (Xiao et al., 2010).

No significant increase in apoptosis was found after the long-term treatment; however, results from the present study suggested that a long-term exposure to serum level of daidzein may reduce the effectiveness of the clinical drug, docetaxel, by decreasing cell apoptosis. And the long-term exposure to the combination of daidzein and genistein decreased LDH leakage from LNCaP cells after being treated with docetaxel. This indicated that long-term exposure to serum level of daidzein or both daidzein and genistein may cause docetaxel resistant in prostate cancer cells.

In addition, we also revealed that genistein treatment caused accumulation of cells in both S and G2/M phase, and a significant decrease in G1 phase, while daidzein and combination of daidzein and genistein induced accumulation of cells in G2/M phase only, which was different from previous work demonstrating a cessation of the cell cycle in G2/M after genistein treatment and in G0/G1 with daidzein on prostate cancer cells (Hedlund et al., 2003). This difference might be caused by the differences in concentrations and duration of exposure.

Recent studies have shown that a low-dose isoflavone enriched diet abrogated tamoxifen-associated mammary tumor prevention on both human and mouse mammary tumor cell lines (Liu et al., 2005). And it is known that dietary phytoestrogens may influence signal transduction of the steroid receptor and the receptor tyrosine kinase (erbB-1, erbB-2, erbB-3, and erbB-4) signaling pathways as well as other biological phenomena. ErbB-2 encodes a transmembrane glycoprotein,

p185, with intrinsic tyrosine kinase activity (Akiyama T et al., 1986). Although most of the studies aimed at understanding the molecular basis of erbB-2 overexpression have been performed in breast cancers, erbB-2 gene is also overexpressed in other cancers such as prostate cancers, and this alteration has also been considered as a negative prognosis marker (Klapper et al., 2000).

Even though we did not find any cancer preventive effects of low-dose, long-term exposure to isoflavones, and studies on cell line cannot be directly translatable to the intake of variable or intermittent soy isoflavones by humans, the results of this study still provided important insights into soy food consumption, especially for prostate cancer patients.

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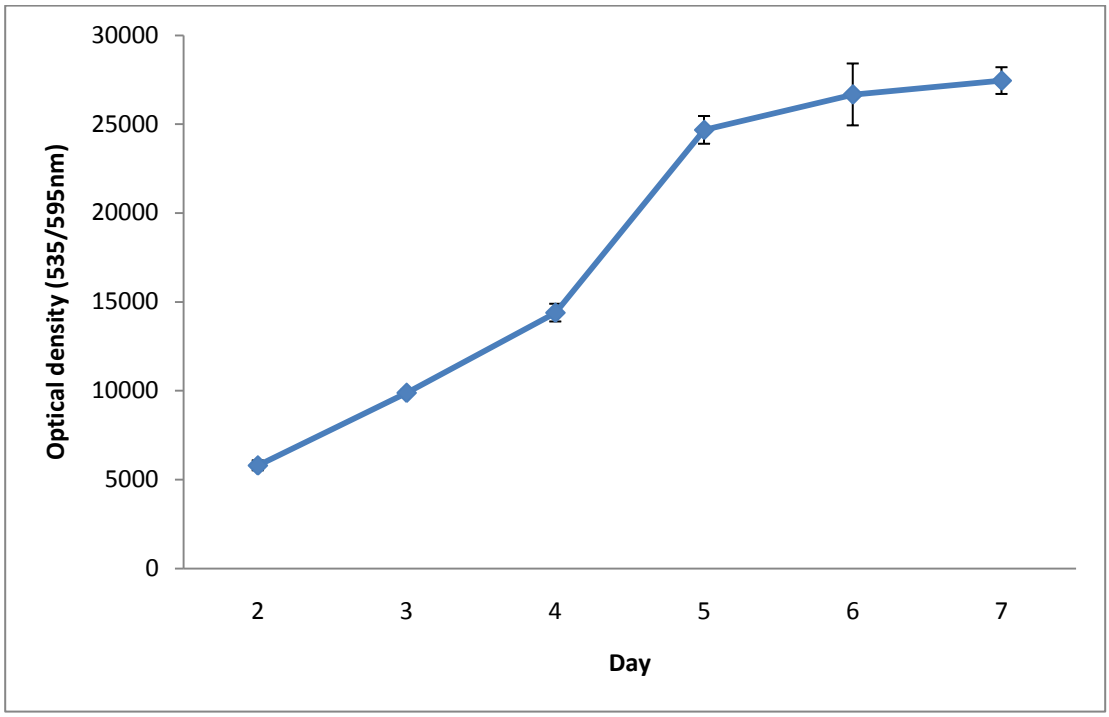

Figure 3.1a **Basal growth rate of LNCaP cells.** Cells were seeded on day 1 and cell growth, measured by the resazurin assay, was assessed on days 2, 3, 4, 5, 6 and 7. Data represent the means ±SD of triplicate observations.

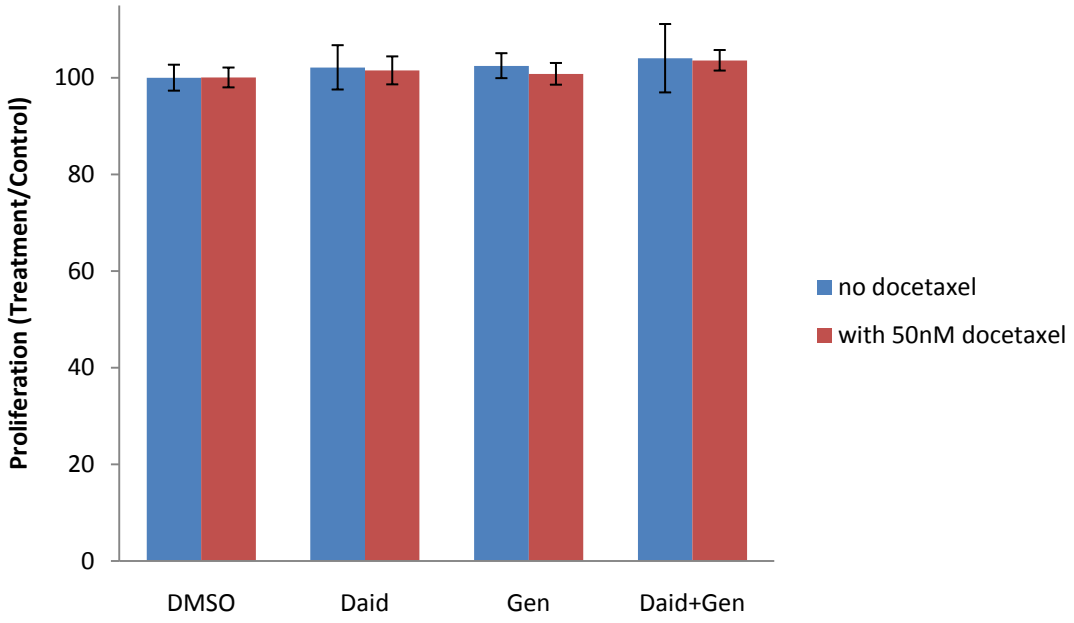

Figure 3.1b **Effects of long-term exposure to daidzein, genistein and their combinations on LNCaP cell proliferation and antiproliferative effects of docetaxel on the long-term treated LNCaP cells.** LNCaP cells were treated with 250 nM daidzein, 500 nM of genistein or their combination for 60 days, and proliferation was detected with or without a 48 h treatment of 50 nM docetaxel using a resazurin assay. Data were expressed in the average of treatment /control reading (530 nm excitation/590 nm emission) ratio of two trials, four replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with DMSO group (or DMSO with docetaxel).

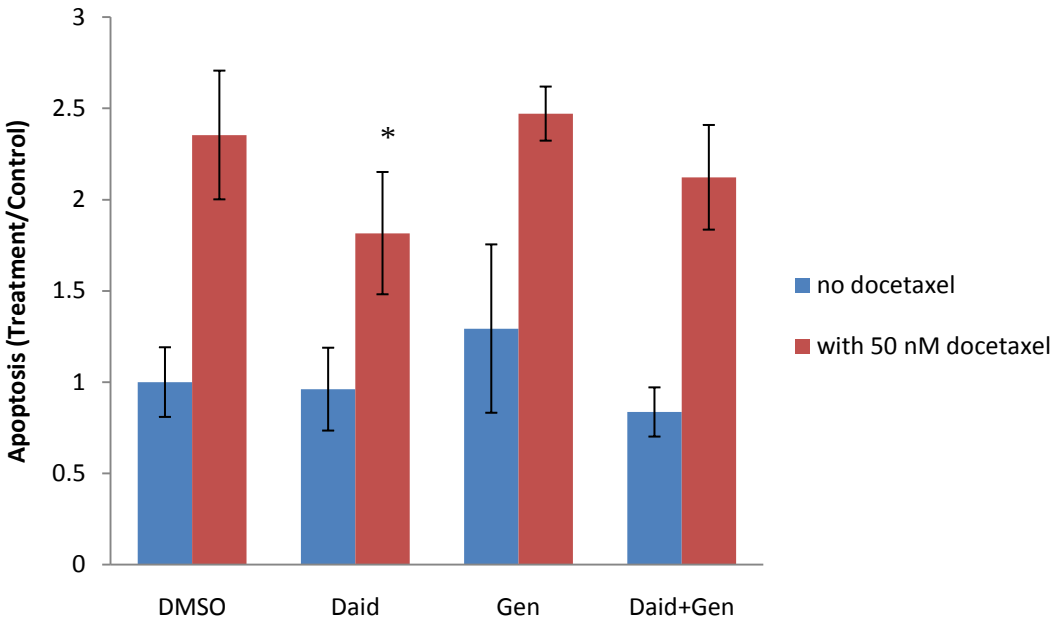

Figure 3.2 **Effects of long-term exposure to daidzein, genistein and their combinations on LNCaP cell apoptosis and apoptotic effects of docetaxel on the long-term treated LNCaP cells.** LNCaP cells were treated with 250 nM daidzein, 500 nM of genistein or their combination for 60 days, and apoptosis was detected with or without a 48 h treatment of 50 nM docetaxel using a Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Indianapolis, IN, USA). Data were expressed in the average of treatment /control reading (405 nm excitation/490 nm emission) ratio of two trials, four replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with DMSO group (or DMSO with docetaxel).

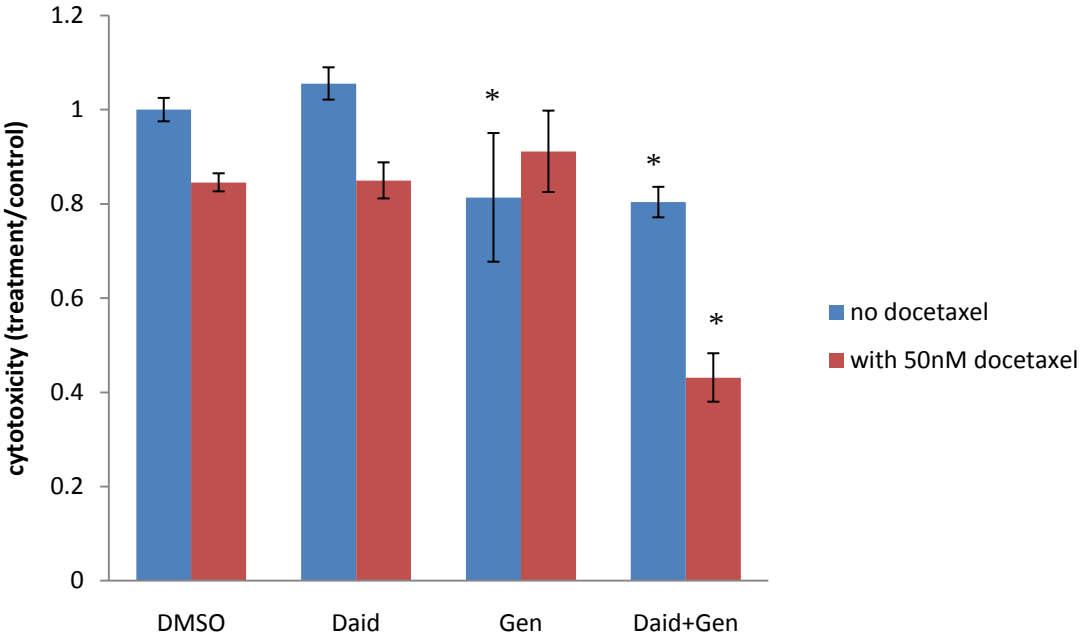

Figure 3.3 **Cytotoxicity of long-term exposure to daidzein, genistein and their combinations on LNCaP cells and cytotoxicity of docetaxel on the long-term treated LNCaP cells.** LNCaP cells were treated with 250 nM daidzein, 500 nM of genistein or their combination for 60 days, and cytotoxicity was detected with or without a 48 h treatment of 50 nM docetaxel using a Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Indianapolis, IN, USA). Data were expressed in the average of treatment /control reading (490 nm) ratio of two trials, four replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with DMSO group (or DMSO with docetaxel).

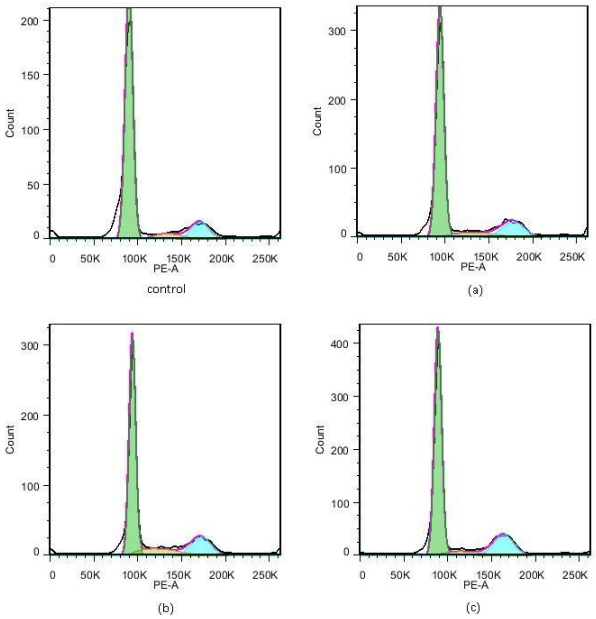

Figure 3.4 **Effects of long-term exposure to daidzein and genistein on LNCaP cell cycle.** After LNCaP cells had been treated with 250 nM daidzein (a), 500 nM genistein (b) or their combination (c) for 60 days, cells were grown in 6-well plates and distribution of cells in different phases of cell cycle was analyzed by propidium iodide (PI) staining followed by flow cytometry. Control treatment consisted of medium with 0.1% dimethyl sulfoxide.

Table 3.1 Flow cytometric analysis of effects of long-term exposure to daidzein and genistein on LNCaP cell cycle

	G1	S	G2/M	sub G1
DMSO	73.45 ±1.63	4.47 ±1.69	11.60 ±0.40	8.48 ±2.86
Daid	70.37 ±0.65	6.20 ±0.68	15.89 ±1.18*	5.2 ±0.22
Gen	67.17 ±2.66*	8.79 ±2.40*	15.11 ±2.54	6.35 ±1.91
Daid+Gen	71.01 ±1.38	5.33 ±2.09	16.21 ±1.70*	5.84 ±1.63

a. Values are means ±SD.
b. Statistical significance is as follows: *$p < 0.05$ comparing with control.

Chapter 4

APOPTOTIC EFFECTS OF COOKED AND IN VITRO DIGESTED SOY EXTRACTS ON HUMAN PROSTATE CANCER CELLS

ABSTRACT

Most previous laboratory and animal studies reported that soy isoflavones, such as daidzein and genistein, were major bioactive compounds in soy to exert chemoprotection of prostate cancer. However, these studies cannot reflect the realistic effects that soy may induce through diets, and little is known about the bioavailability of isoflavones from whole soy food and their bioactivities after cooking and digestion. We hypothesized that cooked and digested soy foods have better bioactivities such as antioxidant and anticarcinogenic effects, compared to soy foods without digestion and cooking. In this study, we determined the antioxidant activities, isoflavone contents, antiproliferative and apoptotic effects on LNCaP and C4-2B human prostate cancer cells of extracts from whole soybeans, and investigated the effects of heating and in vitro digestion on their isoflavone contents and bioactivities. Antioxidant activities of soy extracts increased after cooking and in vitro digestion, while no consistent increase of the four soy isoflavones was determined. The cooking procedure generally increased the amount of daidzin, genistin and daidzein, but decreased that of genistein. Digestion process significantly lowered contents of daidzin and genistin in 60 min

cooked sample, while increased the contents of daidzin and daidzein and decreased the content of genistein in the uncooked sample. The apoptotic effects of soy extracts were generally in a dose-dependent manner. And similar apoptotic effects on both LNCaP and C4-2B cells were found in soy extracts, especially the cooked and digested ones, compared to purified single isoflavones at higher concentrations, which indicated synergistic interactions between various bioactive compounds in whole soy. However, there was no direct correlation between the total contents of soy isoflavones and the bioactivities of soy extracts. In this study, we first used cooking and in vitro digestion to prepare soy extracts and examined the effects of cooking and digestion on the bioactivities of the whole soy extracts, thus it is more comparable to real ingestion of whole food by humans, and can provide more and better information for the prevention of prostate cancer by soy food consumptions.

4.1 INTRODUCTION

Prostate cancer is one of the most common cancers in men (Jemal et al., 2008). However, the incidence of clinical prostate cancer varies widely between ethnic populations and countries. The Asian countries are considered to be a low-risk region, while the USA, Canada, New Zealand, Australia, the Northwestern European countries and Caribbean region are considered to be the high-risk countries or regions (Jian, 2009). Although genomic factors are important in the aetiology of prostate cancer, environmental factors including diet have been presumed to play a key role in prostate carcinogenesis (Carter et al., 1990; Tominaga and Kuroishi, 1997). Over the

past decades, epidemiological studies have shown that diets rich in fruits and vegetables have preventive effects on chronic diseases including prostate cancer (Veluri et al., 2006; Yi et al., 2005; Matito et al., 2003). Adlercreutz (1995) hypothesized that the high intake of soybean products may be partly responsible for the lower incidence of prostate cancer in Japanese men. Yan and Spitznagel (2005) and Dalais et al. (2004) also suggested that a lower risk of prostate cancer has been associated with more soy-rich Asian diet and increased soy consumption.

In the past two decades, most soy research has focused on isoflavones and soy protein. Soy isoflavones, such as genistein, showed potential protective mechanisms against cancer development in both in vitro and in vivo studies (Magee and Rowland, 2004; Park and Surh, 2004; Sarkar and Li, 2003). However, there are still questions as to whether using high levels of soy isoflavones in humans is feasible and safe (Matsukawa et al., 1993; Perabo et al., 2008). Besides, not only may other soybean components exert important health effects, but there is also the potential for interactions among the various components to result in outcomes difficult to predict from knowledge of the individual soybean components (Messina et al., 2001). Lila and Raskin (2005) postulated that the interactions between various bioactive compounds in whole foods may work synergistically to provide additional benefits, meanwhile, these interactions may also interfere their biological activities.

Soybeans contain three types of isoflavones, as four chemical forms: aglycons, glycosides, acetylglucosides, and malonylglucosides. The major isoflavones

in soybeans are the aglycons, daidzein, genistein, and glycitein, and the glycosides, genistin, daidzin, and glycitin (Vacek et al., 2008; Kudou et al., 1998). Unprocessed soybeans contain 1.2-4.2 mg of total isoflavones/g of soybean (dry weight), mainly genistein, daidzein and their conjugates, with large variation due to variety, crop year, and growth location (Wang and Murphy, 1994a). However, soybeans are consumed only after being processed. During processing, losses of isoflavones may occur (Wang and Murphy, 1994b; Wang and Murphy, 1996). Heating can cause changes in the conjugation profile of the isoflavones in soy products and moist heat can increase the content of β-glucoside conjugates (Coward et al., 1998). However, a decrease of total isoflavone content would be caused by excessive heating (Grün et al., 2001).

In vitro digestion models that simulate the human digestion tract are developed and more widely used in recent years due to its advantages, which include, time saving, low-cost, allowing the measurement of bioaccessibility of nutrients (Hedrén et al., 2002), and certain levels of reproducibility in comparison with in vivo studies (Kong and Singh, 2008a; Kong and Singh, 2008b). Numerous studies have been conducted using these in vitro enzymatic digestion models and concluded that in vitro digestion had positive impacts on bioactivities, such as antioxidant activities, of purified compounds or whole foods (Liu et al., 2004).

We hypothesized that cooked and digested soy foods have better bioactivities such as antioxidant and anticarcinogenic effects, compared to soy foods without cooking and digestion. In this study, we determined the antioxidant activities,

isoflavone contents, antiproliferative and apoptotic effects on LNCaP and C4-2B human prostate cancer cells of extracts from whole soybeans, and investigated the effects of heating and in vitro digestion on their isoflavone contents and bioactivities.

4.2 MATERIALS AND METHODS

4.2.1 Materials

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), daidzein, genistein, daidzin, genistin, docetaxel, resazurin, bile extract (from porcine), pepsin (from porcine stomach mucosa), pancreatin (from porcine pancreas), Folin-Ciocalteu reagent, gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), AAPH (2,2'-Azobis (2-methylpropionamidine) Dihydrochloride) and fluorescein sodium salt were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

4.2.2 Sample preparation

Dry soybeans purchased from local Farmer's Market (Newark, DE, USA), were first soaked in deionized (DI) water for 12 hours. After soaking, fresh DI water was added into the expanded soybeans at a ratio of 10:1 (v: w). Soybeans were boiled for 0 (uncooked), 30 or 60 minutes and soybeans and soup were separated after cooking. Separated soybeans were then ground with mortar in order to stimulate oral chewing process while the soup was cooled to room temperature and DI water was added to reach a final volume of 30 mL to make up the loss during cooking. Ground

beans as well as the soup were transferred to amber bottle for further in vitro digestion process.

4.2.3 In vitro digestion

The in vitro digestion was processed according to the method reported by Miller et al. (2004) with modification. The mixture of ground soybeans and the soup was acidified to pH 2.0 with 0.1M HCl, mixed with 1mL 40mg/mL pepsin solution prepared in 0.1 M HCl and incubated in a shaking water bath for 1 h at 37 ℃ to simulate gastric digestion. After that, pH was adjusted to 6.9 with 0.1M $NaHCO_3$. Intestine digestion was performed by adding 5mL of bile extract-pancreatin solution (0.45 g of bile extract and 0.075 g of pancreatin in 37.5 mL of 0.1 M $NaHCO_3$) and incubating in a shaking water bath at 37 ℃ for 2 h. Samples were then centrifuged for 10 min at 5000 rpm and the supernatants was considered to be bioaccessible soy extract to humans. The supernatant was then collected and directly used for antioxidant and analysis of soy isoflavone contents, and then the supernatants were freeze dried and stored at -20 ℃ for further experiments.

4.2.4 Analysis of antioxidant activities

The hydrophilic oxygen radical absorbance capacity (ORAC) assay modified based on Wu et al. (2008) and modified Folin-Ciocalteu test (Xu et al., 2010) were used to determine the antioxidant activities.

In ORAC assay, an aliquot of 25 μL of the diluted samples, or Trolox calibration solutions were added to 96-well bottom reading microplate (BioTek

Instruments, Inc., Winooski, VT, USA). One-hundred-fifty μL of fluorescein solution at 4.0×10^{-6} mM was added to each well, and the microplate was incubated at 37 ℃ for 30 min before 25 μL 153 mM AAPH solutions was added to each well as peroxyl generator to start the reaction. The microplate reader was then programmed to record the fluorescence reading with an excitation wavelength of 485 nm and an emission wavelength of 520 nm at 1 min intervals for 1 h using Synergy™ 2 multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The standard curve was linear between 0 (phosphate buffer, pH 7.4) and 100 μM Trolox. Results were expressed as μmol Trolox equivalent (TE)/g sample.

Total soluble phenolic contents were determined by Folin-Ciocalteu test. A solution (30μL) of sample was mixed with 60 μL of 1×10^{-4} M Folin-Ciocalteu reagent and allowed to react at room temperature for 5 min. Then 120 μL of saturated Na_2CO_3 was added and incubated at 30 ℃ for 30 min in dark. The absorbance was measured at 765 nm using Synergy™ 2 multi-mode microplate reader. A calibration curve using gallic acid (ranging from 0.0025 to 0.5 mg/mL) was used to derive the gallic acid equivalent (GAE) concentrations for samples. The results were expressed as milligrams of gallic acid equivalents (GAEs) per gram dry soybean.

4.2.5 Analysis of soy isoflavone contents by high-performance liquid chromatography

Soy isoflavone contents in soy extracts were analyzed using a Shimadzu LC-20A automated liquid chromatographic system (Shimadzu Scientific Instruments,

Columbia, MD, USA), which consisted of a photodiode array (PDA) detector, a CBM system controller, a LC-20AT pumps, a SIL-20AC autosampler. The separation was completed on a reverse-phase C18 (5 μm, 150×4.6 mm, 110Å) column with a matched guard column. Supernatant collected before or after in vitro digestion was filtered with a Costar Spin-X centrifuge tube filter (0.45 μm, nylon) (Corning, Lowell, MA, USA), then 20 μL supernatant was injected into and analyzed with Shimadzu LC-20A automated liquid chromatographic system. The mobile phase was composed of 0.1% trifluoroacetic acid (A) and acetonitrile (B). Elution was performed with the linear gradient as follows: 0-40 min, 12-18% (B); 40-55 min, 18-20% (B); 55-60 min, 20% (B); 60-70 min, 20-40% (B) and 70-75 min, 40-12% (B). Flow rate was 0.5 mL/ min at 35 ℃. The detection wavelength was 254 nm. The stock solution of daidzein and genistein was prepared in DMSO at concentration of 200 mM and stock solution of daidzin and genistin was prepared in DMSO at concentration of 10 mM. Standard solutions were prepared at concentrations of 5 – 100 μM by diluting the stock solution in DMSO. All measurements were performed in duplicates.

4.2.6 Cell culture

LNCaP and C4-2B cells were maintained in T-medium supplemented with 5% (v/v) fetal bovine serum (FBS) and 100 U/ml penicillin G sodium and 100 mg/ml streptomycin sulfate in 0.085% (w/v) saline (PS) in 5.0% CO_2 atmosphere at 37 ℃. Medium was changed every two days. Cells were routinely passaged using 0.25%

(w/v) trypsin with ethylenediaminetetraacetic acid (EDTA) 4 Na when 90-95% confluence was reached.

4.2.7 Treatment of cells

Cells were seeded in 24-well plates (Corning, Lowell, MA, USA) at a density of 1×10^5 cells per well. Cells were grown to 50-70% confluence and then treated with six soy extracts (three different cooking times with/without digestion) of concentrations between 100 and 2000 μg/mL dissolved in T-medium. Cells without any treatment served as the control group. Fifty nM docetaxel was used as positive control. After 48 h of treatment, cells were harvested and pelleted by centrifugation at 200 × g for 10 min. Pelleted cells were used for apoptosis assay immediately and the supernatant was kept at 4 ℃ for measurement of cytotoxicity.

4.2.8 Apoptosis assay

DNA fragmentation was measured using the Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Indianapolis, IN, USA) following the protocol of the manufacturer. Cells were lysed with 200 μL lysis buffer and incubated at room temperature for 30 min. The lysate was then centrifuged at 200 × g for 10 min. Twenty μL supernatant was transferred into the streptavidin coated microplate and 80 μL immunoreagent was added to each well. The plate was covered with an adhesive foil cover and incubated on a shaker at 300 rpm for 2 h at room temperature. The solution was thoroughly removed and each well was washed three times with 300 μL incubation buffer. One hundred μL ABTS solution was added to each plate and the

plate was incubated on a shaker at 250 rpm. When the color development was sufficient for a photometric analysis, 100 μL ABTS stop solution was added to each well. Colorimetric detection was carried out according to manufacturer's instructions using MRX microplate reader (Dynex Technologies, Chantilly, VA, USA) at 405 nm excitation/490 nm emission.

4.2.9 Cytotoxicity analysis

Cytotoxicity to cells was determined by measuring the amount of lactate dehydrogenase (LDH) enzyme leaked from the cytosol of damaged cells into the medium after exposure of cells to the chemicals for 48 h. The LDH release represents necrosis as opposed to apoptosis. LDH in the supernatant was measured using the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Indianapolis, IN, USA) following the protocol of the manufacturer. A total of 50 μL of the supernatant from the cells was placed in a 96-well plate, and 100 μL of LDH assay solution (mixture of catalyst lyophilizate and dye solution) was added to each well and incubated for 5 min in the dark at room temperature. Absorbance of the mixture was read with MRX microplate reader at 490 nm.

4.2.10 Measurement of proliferation

Antiproliferative activity was measured by a resazurin assay. Cells were seeded in a 96-well plate (Corning, Lowell, MA, USA) at a density of 1×10^4 cells per well. Cells were grown to 50-70% confluence and then treated with soy extracts of concentrations between 100 and 2000 μg/mL dissolved in T-medium. Twenty μL 1

mg/mL resazurin solution (10% of medium, v/v) was then added into each well. After 48 h of treatment, fluorescence was measured at 530 nm excitation/590 nm emission using a Synergy™ 2 multi-mode microplate reader.

4.2.11 Statistical analysis

Student t-test ($p<0.05$) was performed to determine the antioxidant activities and soy isoflavone contents. Results were expressed as mean ± standard deviation for two replicates in two independent tests (4 replicates in total). In apoptosis, cytotoxicity and proliferation assays, results of each treatment were expressed as mean ± standard deviation for three treatment replicates in two independent tests. Treatment means were compared using Dunnett's test ($p<0.05$). All statistical analyses were performed using JMP 8 (SAS Institute Inc., Cary, NC, USA).

4.3 RESULTS

4.3.1 Antioxidant activities of soy extracts

As shown in Table 4.1, no significant change of total phenolic contents (TPC) was found in the three indigested soy extracts. However, significant increase of total phenolic contents has been obtained after in vitro digestion. The 60 min cooked digested extract had the highest total phenolic contents and other two digested ones, namely 30 min cooked digested and uncooked digested soy extracts, also had significantly higher total phenolic contents than all the three indigested ones.

In addition, cooking process as well as the in vitro digestion process can significantly increase ORAC value of soy extracts, as shown in Table 4.1. However, when cooking time prolonged from 30 min to 60 min, there was no significant increase in ORAC value for both digested and indigested soy extracts. The cooked (30 or 60 min) and digested soy samples had highest ORAC value among all test 6 samples.

4.3.2 Soy isoflavone contents in soy extracts

High performance liquid chromatographic analysis was used to characterize and quantify the contents of four soy isoflavones: daidzein, genistein and the β-glycoside conjugates of daidzein and genistein, daidzin and genistin. As shown in Table 4.2, the cooking procedure could generally increase the amount of daidzin, genistin and daidzein, but decrease that of genistein. Once heating was applied, genistein content decreased and remained at a very low level regardless of the cooking time, while daidzin and genistin content increased significantly through whole cooking process and 60 min cooked sample provided peak contents of these two compounds. Daidzein, which behaved in a more complicated manner, increased during first 30 min of cooking, and decreased significantly when cooking time was prolonged. However, the content of daidzein in 60 min cooked soy extract was still significantly higher than that in the uncooked one.

The effect of in vitro digestion process on the tested isoflavone contents was more complicated. Digestion process lowered contents of daidzein, genistein and

the two sugar conjugates, daidzin and genistin in 30 min and 60 min cooked sample, but the decreases were only significant in daidzin and genistin contents in the 60 min cooked sample. In vitro digestion showed a different effect on the uncooked sample. Daidzin and daidzein contents in uncooked sample increased significantly after in vitro digestion. Genistin content also increased; however, the change is not statistically significant. Digestion process markedly decreased the content of genistein in the uncooked sample (Table 4.2).

Comparing the three digested samples, the 30 min and 60 min cooked ones contained approximately twice content of daidzin, and significantly more genistin compared to the amounts in uncooked one. However, the content of daidzein decreased approximately half and the content of genistein was not significantly affected (Table 4.2).

4.3.3 Apoptotic effects of soy extracts on LNCaP and C4-2B cells

The apoptotic effects of soy extracts on prostate cancer cells were investigated using a Cell Death Detection ELISA^{PLUS} kit. At concentrations equal to or higher than 500 μg/mL, the 30 min cooked indigested and all three digested extracts induced significantly more apoptosis in both cell lines compared with untreated cells (Figure 4.2 and 4.3). At concentration of 200 μg/mL, only the uncooked digested soy extract showed a marked apoptotic effect on LNCaP cells, but not on C4-2B cells. At concentrations of 500 and 1000 μg/mL, both the uncooked indigested and 60 min cooked indigested samples had significant apoptotic effects on C4-2B cells but not

LNCaP cells (Figure 4.2 and 4.3). No significant apoptotic effect on any of the two cell lines was observed for all six extracts when sample was added to a concentration of 100 μg/mL (Figure 4.2 and 4.3). Furthermore, uncooked indigested samples had no apoptotic effect on LNCaP cells regardless of the test level (Figure 4.2).

4.3.4 Cytotoxicity of soy extracts

One-hundred μg/mL of 60 min cooked digested soy extract caused slightly but significantly more LDH leakage from LNCaP cells than the DMSO control. Even at higher concentrations, none of other soy extracts were cytotoxic to LNCaP cells. LDH released by LNCaP cells with all other treatments were neither significantly higher nor lower than that of the control (DMSO) (Figure 4.4).

Two-hundred and 2000 μg/mL of uncooked indigested soy extract caused significantly more LDH leakage than the control in C4-2B cells (Figure 4.5). LDH released by C4-2B cells after treatment with 2000 μg/mL of 60 min cooked indigested soy extract, 100, 200, 500, 1000 and 2000 μg/mL of uncooked digested soy extract, 1000 μg/mL of 30 min cooked digested soy extract and 500 μg/mL of 60-min cooked digested soy extract were significantly higher than that of the DMSO control. The 30 min cooked indigested soy extract was not cytotoxic to C4-2B cells in the concentration range of 100 and 2000 μg/mL.

4.3.5 Effects of soy extracts on cell proliferation

The statistical analysis results of effects of soy extracts on LNCaP and C4-2B cell proliferation are presented in Figures 4.6 and 4.7, respectively. No significant

antiproliferative effects were observed. Instead, significant promotion of proliferation was detected in the group treated with 2000 μg/mL uncooked digested soy extract for LNCaP cells, and the group treated with 2000 μg/mL uncooked digested soy extract and the group treated with 2000 μg/mL 60 min cooked digested soy extract for C4-2B cells.

4.4 DISCUSSION

Numerous studies have theorized possible protective mechanisms of soy isoflavones against cancer development. Despite their estrogen-like effects in the body, they also possess antioxidant activities (Barnes and Peterson, 1995; Satih et al., 2008) and may act as an inhibitor of angiogenesis (Fotsis et al., 1993). Although researchers had proven that high doses of single isoflavones, such as daidzein and genistein, can inhibit human prostate cancer (Wang et al., 2009; Rabiau et al., 2010; Hsu et al., 2010), bioaccessibility and safety are always concerns. Besides, Dinnella et al. (2007) indicated that phytochemicals showed satisfying in vitro antioxidant abilities may not perform same potential in vivo, unless they meet two requirements: bioaccessible and stable during human digestion process. Since most of previous in vitro research was done with purified soy isoflavones or crude soy extracts, the results cannot reflect the actual effects that soy foods may induce through diets. In our study, we stimulated the situation of dietary intake of soy foods by cooking soybeans and then using in vitro enzymatic digestion. Phytochemicals, specifically soy isoflavones, were detected and the bioactivities of soy extracts persisted after cooking and enzymatic digestion.

Although it has been widely recognized that heating process would lower nutritional value of food because of nutrient degradation, we found that cooking process increased the antioxidant capacities of soy extracts according to the results of ORAC assay and it may due to release of phytochemicals during cooking or structure modifications of some bioactive compounds. Liu et al. (2004) reported that the cooking process increased the bioavailability of carotenoids from corn. Pellegrini et al. (2010) studied the effect of different cooking methods on phytochemical concentration and antioxidant capacity of raw and frozen brassica vegetables and the results showed that among cooking procedures, boiling increased carotenoids in fresh broccoli and polyphenols in fresh Brussels sprout, whereas decreased the amount of almost all other phytochemicals in fresh and frozen samples. Stahl et al. (1992) also found that lycopene concentrations in human serum increased only when processed tomato juice was consumed, when they studied the uptake of lycopene from processed (boiled for 1 h) and unprocessed tomato juice in humans. Meanwhile, Adefegha et al. (2011) concluded that cooking decreases the vitamin C contents in some tropical leafy green vegetables in Africa, but it increased antioxidant activities measured by free radical scavenge abilities, reducing property and Fe^{2+} chelating ability.

In vitro enzymatic digestion models had been proved to have positive impacts on bioactivities, such as antioxidant abilities, of purified compounds or whole foods. In our study, total phenolic contents and ORAC value increased significantly after digestion. There are two possible reasons for this: 1) in vitro digestion applied in

our study may release some bioactive compounds from the food matrix and improve the bioaccessibility of bioactive compounds; 2) the digesting enzymes together with pH adjusting may affect chemical structures of some phytochemicals and provide higher bioactivities. Similar results which were consistent with our hypothesis have been published by Walsh et al. (2003). They found in vitro digestion enhanced the bioaccessibility of isoflavonoid aglycones from soy bread. Rufian-Henares et al. (2007) found that low molecular weight compounds released from melanoidin after gastrointestinal digestion exerted the highest antioxidant activity, even higher than compounds bound ionically to melanoidins. Toor et al. (2009) investigated release of antioxidant components from tomatoes determined by an in vitro digestion method and the results showed that a higher amount of total phenolics and total flavonoids were released from tomatoes during digestion. Besides releasing bioactive compounds from food matrix, in vitro digestion may affect antioxidant activities by convert these phytochemicals to those with higher bioactivities. Dinnella et al. (2007) suggested that differences in the potential bioactivities of extra-virgin olive oil samples were related to different phenolic profiles since antioxidant activity of olive oil phenols is related to their chemical structures. However, no direct correlation between the total contents of soy isoflavones and the antioxidant activities of soy extracts was found in our study.

Based on these facts, we tested the anticancer activities of soy extracts on two cell lines from the LNCaP progression model of human prostate cancer, LNCaP and C4-2B prostate cancer cell lines. Although no significant antiproliferative effects

were found, the soy extracts exhibited good apoptotic effects on both cell lines. Generally, digested soy extracts were more effective than the indigested ones and both digested and indigested ones were more effective on C4-2B cells. Researchers suggested that the anticancer activities of soy have been attributed to isoflavones, especially daidzein and genistein. However, according to our results from HPLC analysis, no consistent increase of the four soy isoflavones was found after both cooking and digestion, although three of the four main isoflavones increased after cooking only. It suggested that the increase of biological activities, both antioxidant and anticancer activities, may be caused by newly released bioactive compounds during cooking and digestion. In addition, it could be caused by synergistic interactions between various bioactive compounds, including isoflavones, other phytochemicals such as soy proteins/peptides and oligosaccharides, and minerals in whole soy (Lila and Raskin, 2005).

Comparing our previous data using purified daidzein and genistein (data shown in Chapter 2), we found soy extracts, especially cooked and digested ones, were more effective on cell apoptosis, that is to say, same effects can be achieved by lower concentrations. For example, all three digested soy extracts at concentration of 1000 μg/mL (equals to 4-7 mg of raw soybean/mL) can achieve the same apoptotic effect as 100 μM purified genistein on C4-2B cells. Similar results were published by Hsu et al. (2010), who found that soy extracts, containing a mixture of soy isoflavones and other bioactive components, induced a significantly higher percentage of cells

undergoing apoptosis than daidzein or genistein, thus would be a more potent chemopreventive agent than individual soy isoflavones. Compared to other studies, in which crude soy extract contains high concentrations of isoflavones was used directly by incubating with cells, our study, which involved cooking and in vitro digestion process in preparing soy extracts, is more comparable to real ingestion of whole foods by humans, thus can provide more and better information for the preventive and therapeutic effects of soy food consumptions on prostate cancers.

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Table 4.1 Antioxidant capacities of digested/indigested soy extracts

	cooking time (min)	TPC (mg GAE/g dry soybean)	ORAC (μmol TE/g dry soybean)
Indigested	0	1.35 ± 0.01^{d}	31.33 ± 6.39^{d}
	30	1.36 ± 0.01^{d}	52.91 ± 0.62^{c}
	60	1.37 ± 0.02^{d}	55.37 ± 0.23^{c}
Digested	0	1.73 ± 0.07^{bc}	101.94 ± 8.53^{b}
	30	1.68 ± 0.13^{bc}	140.85 ± 0.29^{a}
	60	1.91 ± 0.02^{a}	146.39 ± 4.36^{a}

a. ORAC and total soluble phenolic contents (TPC) are means ±SD. Means in a column without a common letter have significant difference, $p < 0.05$.

Table 4.2 Soy isoflavone contents in soy extracts determined by HPLC

	cooking time (min)	daidzin (nmol/g dry soybean)	genistin (nmol/g dry soybean)	daidzein (nmol/g dry soybean)	genistein (nmol/g dry soybean)
Indigested	0	59.33 ± 12.21^{e}	73.22 ± 15.79^{e}	164.99 ± 11.02^{f}	332.12 ± 75.31^{a}
	30	380.25 ± 27.20^{bc}	200.57 ± 40.46^{b}	504.37 ± 14.16^{bc}	33.50 ± 4.62^{b}
	60	537.17 ± 48.70^{a}	352.96 ± 28.04^{a}	359.56 ± 24.16^{d}	31.24 ± 1.98^{b}
Digested	0	167.73 ± 35.10^{d}	114.78 ± 27.47^{de}	828.70 ± 87.18^{a}	26.06 ± 11.91^{b}
	30	324.09 ± 13.02^{c}	173.14 ± 6.43^{bc}	395.69 ± 9.53^{cd}	20.46 ± 1.19^{b}
	60	350.13 ± 6.87^{c}	199.28 ± 2.41^{b}	284.07 ± 5.18^{de}	20.49 ± 1.51^{b}

a. Soy isoflavone contents are means ±SD. Means in a column without a common letter have significant difference, $p < 0.05$.

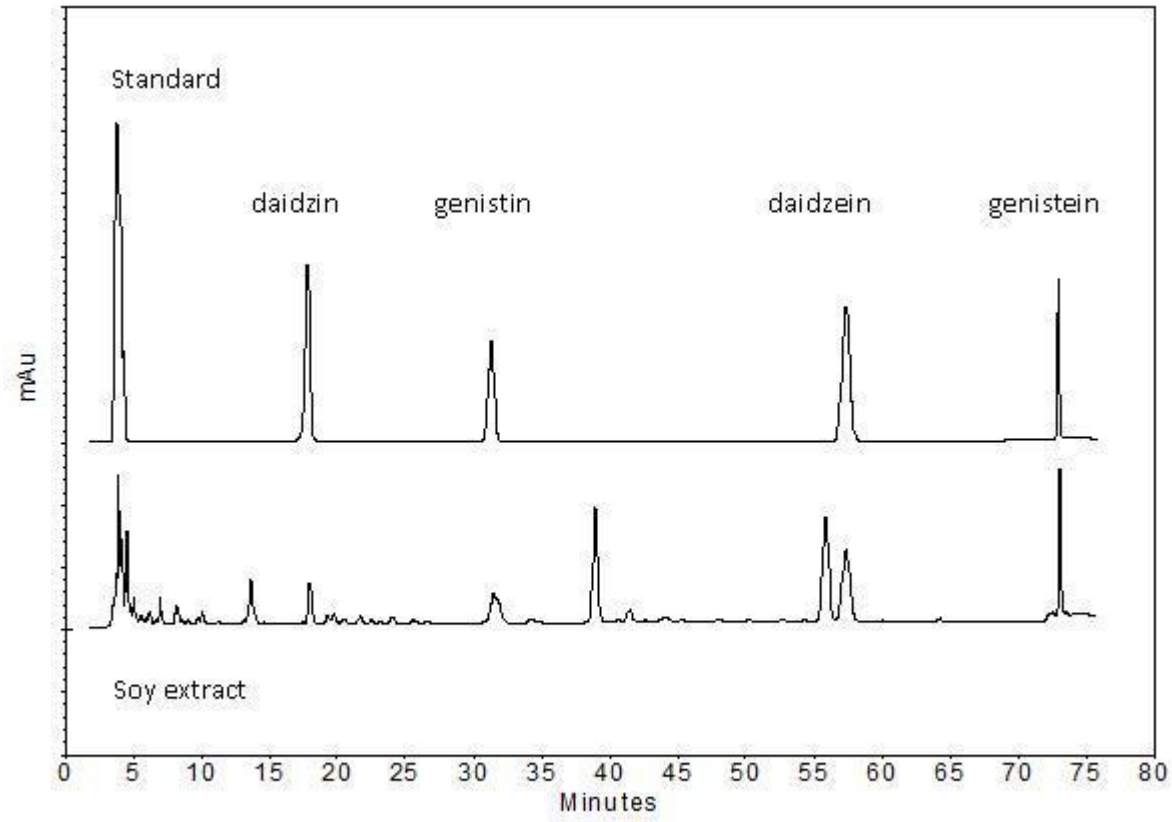

Figure 4.1 HPLC analysis of soy extract sample and mixture of isoflavone standards.

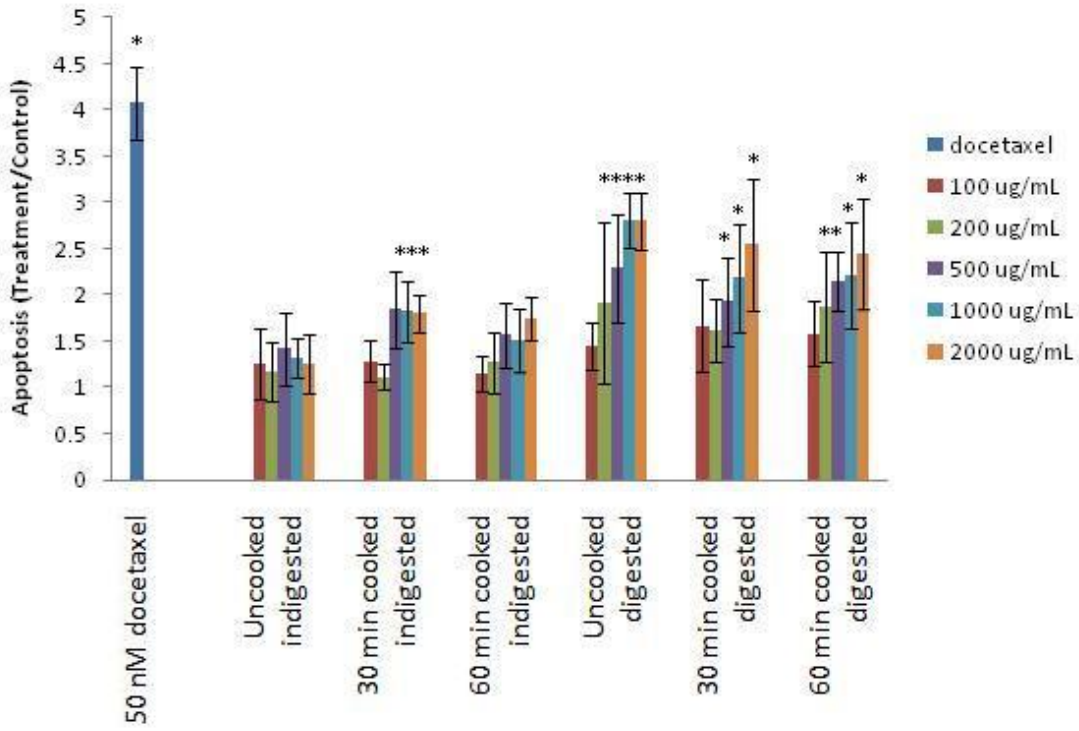

Figure 4.2 **Apoptotic effects of soy extracts on LNCaP cells.** LNCaP cells were treated with varying concentrations (100 to 2000 μg/mL) of 6 different soy extracts for 48 h, and apoptosis was detected using a Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Indianapolis, IN, USA). Fifty nM docetaxel was used as a positive control. Data were expressed in the average of treatment/control reading (405 nm excitation/490 nm emission) ratio of two trials, three replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with control (no treatment).

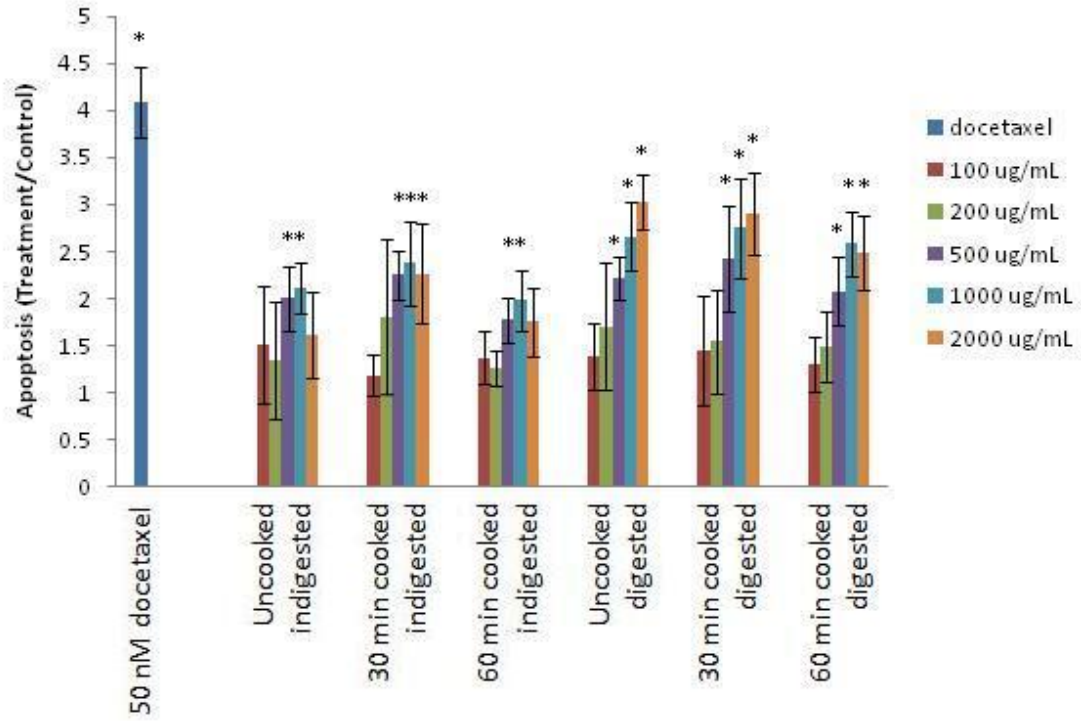

Figure 4.3 **Apoptotic effects of soy extracts on C4-2B cells.** C4-2B cells were treated with varying concentrations (100 to 2000 μg/mL) of 6 different soy extracts for 48 h, and apoptosis was detected using a Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Indianapolis, IN, USA). Fifty nM docetaxel was used as a positive control. Data were expressed in the average of treatment/control reading (405 nm excitation/490 nm emission) ratio of two trials, three replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with control (no treatment).

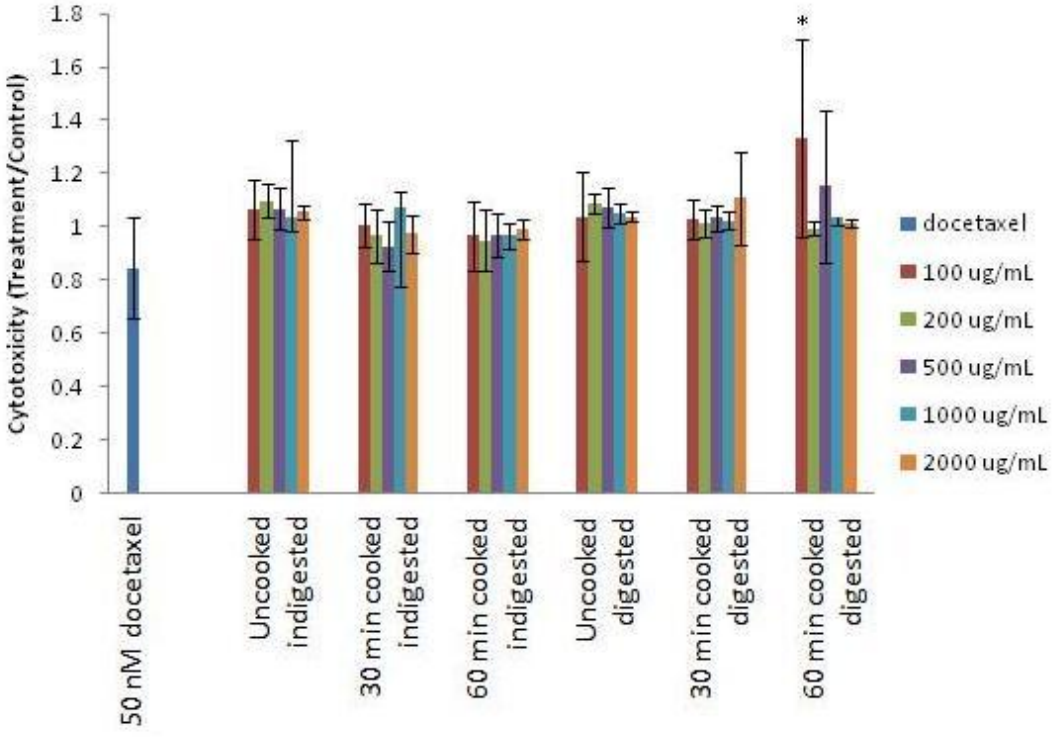

Figure 4.4 **Cytotoxicity of soy extracts on LNCaP cells.** LNCaP cells were treated with varying concentrations (100 to 2000 μg/mL) of 6 different soy extracts for 48 h, and cytotoxicity was detected using a Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Indianapolis, IN, USA). Fifty nM docetaxel was used as a positive control. Data were expressed in the average of treatment/control reading (490 nm) ratio of two trials, three replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with control (no treatment).

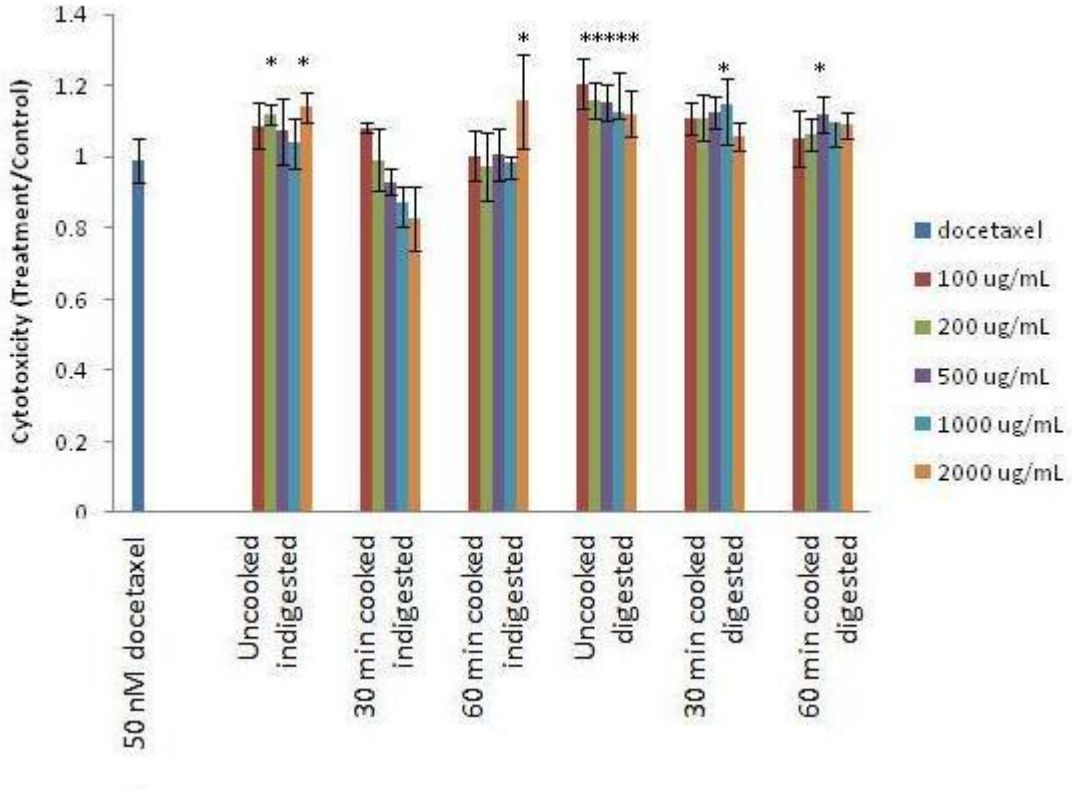

Figure 4.5 **Cytotoxicity of soy extracts on C4-2B cells.** C4-2B cells were treated with varying concentrations (100 to 2000 μg/mL) of 6 different soy extracts for 48 h, and cytotoxicity was detected using a Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Indianapolis, IN, USA). Fifty nM docetaxel was used as a positive control. Data were expressed in the average of treatment/control reading (490 nm) ratio of two trials, three replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with control (no treatment).

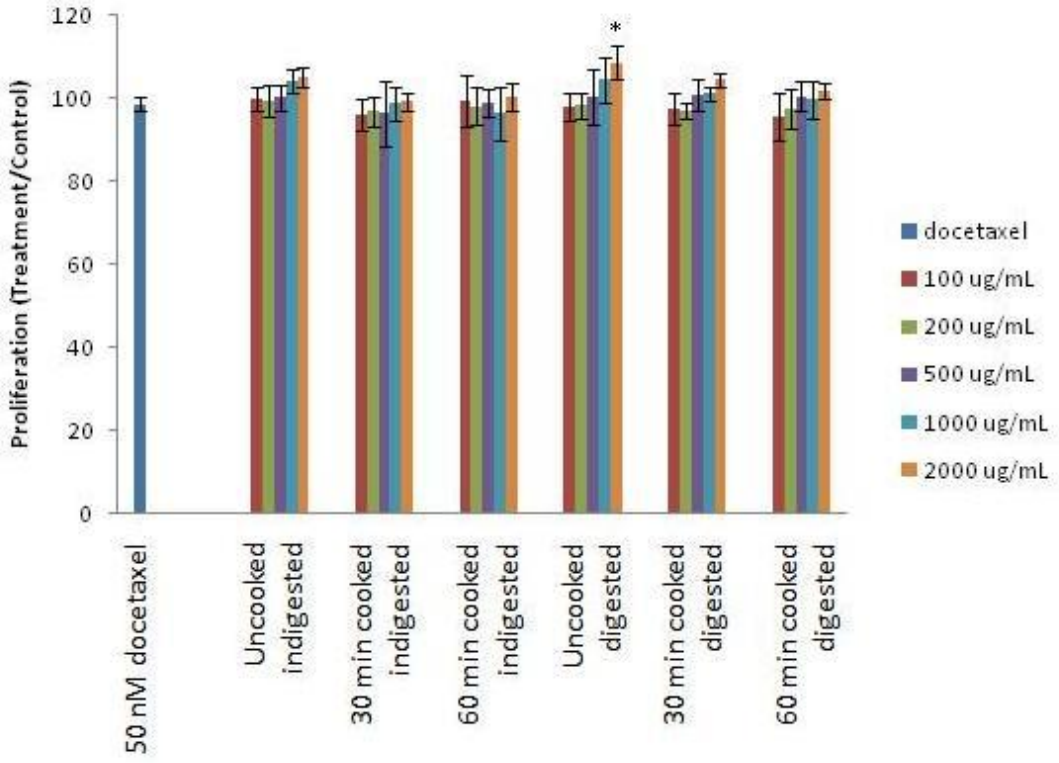

Figure 4.6 **Effects of soy extracts on LNCaP cell proliferation.** LNCaP cells were treated with varying concentrations (100 to 2000 μg/mL) of 6 different soy extracts for 48 h, and proliferation was detected using resazurin assay. Fifty nM docetaxel was used as a positive control. Data were expressed in the average of treatment/control reading (530 nm excitation/590 nm emission) ratio of two trials, three replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with control (no treatment).

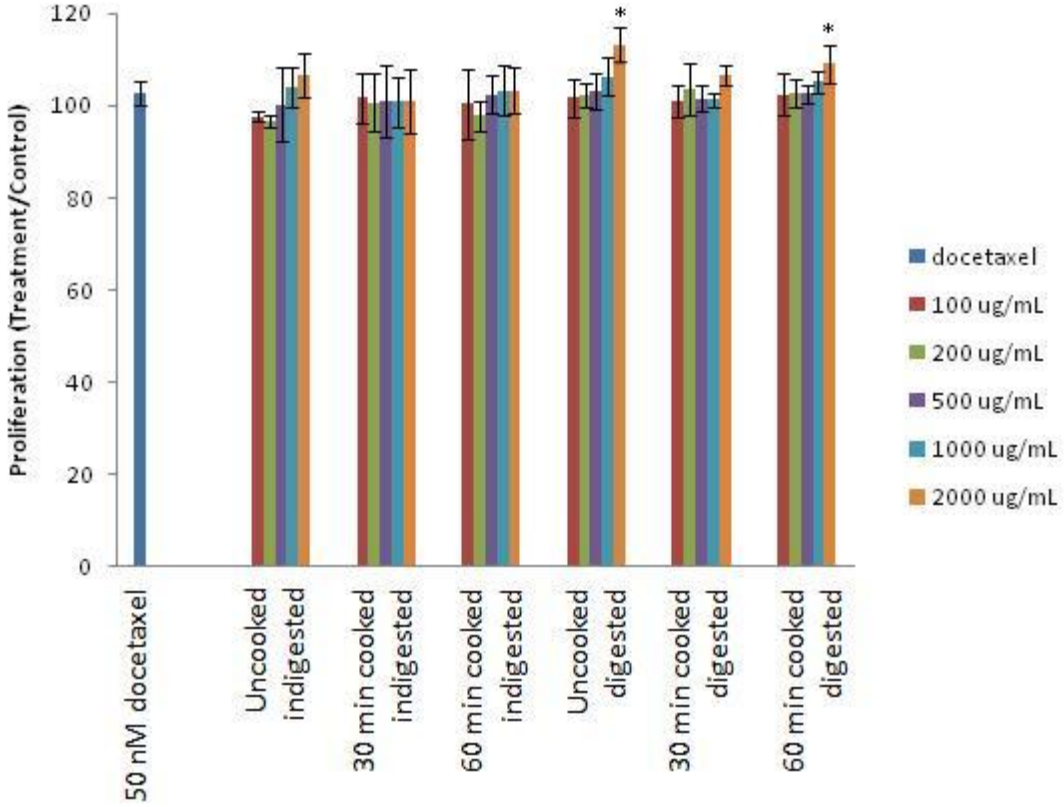

Figure 4.7 **Effects of soy extracts on C4-2B cell proliferation.** C4-2B cells were treated with varying concentrations (100 to 2000 μg/mL) of 6 different soy extracts for 48 h, and proliferation was detected using resazurin assay. Fifty nM docetaxel was used as a positive control. Data were expressed in the average of treatment/control reading (530 nm excitation/590 nm emission) ratio of two trials, three replicates in each trial. Values represent means ±SD. *$p < 0.05$ comparing with control (no treatment).

Chapter 5

FUTURE WORK

Although most in vitro and in vivo studies carried out in laboratory showed isoflavones, especially genistein, have chemopreventive and therapeutic effects against prostate cancer, epidemiological and human intervention studies showed inconsistent results. The reasons might be different biomarkers detected, small subject numbers involved in the studies (Mortensen et al., 2009), and more importantly, the bioavailability of isoflavones to humans, which can be affected by many factors as stated previously. Recently, it was also found that single nucleotide polymorphism in certain genes can affect the risk of PCa, for example, men with the CC genotype for the ESRI PvuII polymorphism had significantly higher risk for prostate cancer compared with men with the TT genotype (Low et al., 2006). In order to better assess the effects of isoflavones, carefully designed, larger scale and long-term epidemiological and human intervention studies, considering both genetic and epigenetic factors are necessary.

Compared to isoflavone supplements, soy extracts showed better anticancer effects in our studies as well as other studies (Hsu et al., 2010). It is suggested that the possible interactions among isoflavones and other phytochemicals

may be responsible for the increase of bioactivities and consumption of whole soy foods might be more effective than taking isoflavone supplements. However, since processing of soy foods has a great impact on the bioactive compounds, it is very essential to carry out studies to evaluate the bioactivities of different soy foods.

Besides, using high-doses of isoflavones in humans have been questioned about the safety. Divi et al. (1997) reported that high-dose genistein has immunosuppressive properties and negative impacts on thymic function. Thus, for people who need to take isoflavone supplements, recommendations of feasible dose cannot be made without more research and profound evidence.

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