EFFECT OF PHYTOESTROGENS ON THE MCF-7 BREAST CANCER CELL LINE

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BY

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To the Dean of the Graduate School:

I am submitting herewith a thesis written by Rebecca Johnson entitled "Effect of Phytoestrogens on the MCF-7 Breast Cancer Cell Line." I have examined this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Biology.

Camelia Maier, Major Professor

We have read this thesis and recommend its acceptance:

Department Chair

Accepted:

Dean of the Graduate School

DEDICATION

This thesis is dedicated to the memory of my grandmother, Anna Bell Teal King, who passed away before I began working on my Ph.D. She was the driving force, motivating me to finish my MS. I carry my memories of her and her convictions to persevere and always be grateful to God. So, I say to her 'Thank You, I could not have done it without you'.

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ABSTRACT

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MAY 2012

Breast cancer is the second leading cause of cancer death among women. Phytoestrogens exert anti-estrogenic effects in some tissues, including breast tissue, by interfering with estrogen receptors (ERs). In this study, solid phase extraction (SPE) fractions of Alfalfa, *Medicago sativa* L. and White clover, *Trifolium repens* L. (*Fabaceae*), were analyzed for their effects on the ER positive breast cancer cell line MCF-7. Treatment with Alfalfa SPE at 1 µg estradiol (E) equivalents/ml for 72 h decreased MCF-7 cell viability by 90%. Western blot analyses of cell cycle arrest marker, p21, and apoptosis markers, p53, Bax, Cytochrome c, poly-(ADP-ribose)-polymerase and caspase-3, revealed an up-regulated expression correlating with Alfalfa treatment at 1 µg E equivalents. These results suggest that Alfalfa phytoestrogens may have potential anti-cancer benefits. Further fractionation and chemical characterization of Alfalfa phytoestrogens may result in the development of potential anticancer agents.

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LIST OF ABBREVIATIONS

AA SPE –Alfalfa Solid Phase Extraction Fraction

AF - Activation Function

AI - Aromatase Inhibitors

c-fos - Cellular Fos

DBD - DNA Binding Domain

E - Estrogen

ER - Estrogen Receptor

ERE - Estrogen Response Element

HER - Human Epidermal Growth Factor

HRT - Hormone Replacement Therapy

LBD - Ligand Binding Domain

LDL - Low-Density Lipoprotein

O-DMA - O-Desmethylangolensin

PTK – Protein Tyrosine Kinase

SERM - Selective Estrogen Receptor Modulator

TAM - Tamoxifen

TGF-β- Transforming Growth Factor

VLDL - Very Low-Density Lipoprotein

CHAPTER I

INTRODUCTION

What are Phytoestrogens?

The steroid sex hormone, estradiol, regulates the expression patterns of specific genes, thus influencing the development and function of a diverse variety of vertebrate-targeted tissues (1,2). These tissues include female and male mammalian reproductive tissues such as the mammary gland, uterus, vagina, ovary, testes, epididymus, prostrate, but also bone, lung, and brain tissue (1,3,4).

Plant secondary metabolites, such as genistein, daidzein, and coumestrol are a few of the many compounds that resemble the mammalian sex hormone 17β-estradiol (Figure 1). Collectively, these compounds are called plant estrogens, or phytoestrogens, and are known as several classes of phenylpropanoid compounds such as lignans, isoflavones, flavonols, flavones, flavanones, and coumestans (1). Phytoestrogens are able to elicit biological responses in mammals by acting as agonists or antagonists at estrogen receptors (ERs) (2,5,6). Estrogenic activity in mammals is dependent on the binding of ligands to ERs. The binding of 17β-estradiol is determined by the presence of hydroxyl groups on its aromatic rings. Based on their similar chemical structure to estradiol, phytoestrogens can compete with the female sex hormone for binding to ERs. The hydroxyl groups on the phenolic rings of the phytoestrogens correspond to the hydroxyl groups on the aromatic rings of 17β-estradiol, thus enabling binding of the phytoestrogen

to ERs (1,2). Once formed, the ER-ligand complex binds to estrogen response elements (ERE) found in the DNA sequence of targeted genes, which regulates transcription (7-10).

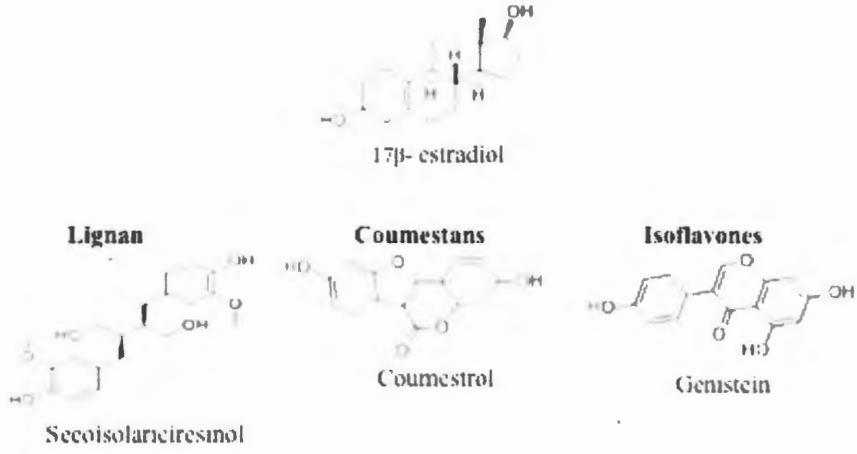

Figure 1. Structural comparison of the phytoestrogens secoisolariciresinol, coumestrol, and genistein to 17β-estradiol, the female sex hormone and ligand for the estrogen receptors [adapted from (54)].

Some phytoestrogens like genistein and coumestrol have been characterized as selective estrogen receptor modulators (SERMs) (11). SERMs are a class of compounds that act upon ERs and modulate their functions according to the specific tissue in which they are found. There are three forms of the ER, namely ERα, ERβ, and the transmembrane form. It is the ability of the phytoestrogens to trigger ERβ-mediated pathways that have also earned them the name of natural selective estrogen receptor modulators (SERMs) (9,11,12). Although phytoestrogens can bind to ERs, they do not

bind with the same high affinity as 17β-estradiol. The isoflavones, genistein and daidzein, bind to the ER with 100 and 1000 times lower affinity, respectively, than estradiol (7).

The discovery of phytoestrogens dates back to 1926 when Dohrn *et al.* isolated a plant substance that caused estrus in female mice. Dohrn called the substance tokokinin (13). In 1933, Butenandt and Jacobi, while working with palm kernel residue, found a substance that closely resembled the structure of the mammalian sex hormone, estradiol, and they called it ketohydroxy-oestrin (14). Also, Skarzynski, working with female willow catkins, isolated a crystalline substance whose physical properties match those of 17β-estradiol and named it trihydroxy-oestrin (15). Phytoestrogens drew attention from those outside of plant sciences when it was found that sheep grazing on clover in Western Australia became sterile (16). It was discovered that the clover contained the phytoestrogen, coumestrol, that caused a condition known as cystic endometrium, which resulted in infertility in the affected sheep or the occurrence of stillborn offspring (16).

Proposed Functions of Phytoestrogens in Plants

In recent years, new light has been shed on the role of phytoestrogens in plants. Studies on 128 plant species using radioimmunoassay (RIA) techniques for the occurrence of mammalian steroids revealed that 70% of plants examined contained androgens (testosterone and dihydrotestosterone) and 50% contained estrone and 17β-estradiol (17). In 1991, Zhang *et al.* collected pollen, styles and pistils from *Ginko biloba, Zea mays* and *Brassica campestris* and, using RIA, revealed that the concentrations of phytoestrogens

and 17β-estradiol *in planta* changed during flower development (18). Khaleel *et al.* (2003), while working with *Populus tremuloides,* observed a higher estrogen content in vegetative and reproductive buds in spring than in winter (19). However, little is known about the functions of phytoestrogens *in planta.*

Isoflavone phytoestrogens are known as phytoalexins (antibacterial and antifungal) or phytoanticipins in plants (20). When subjected to environmental chemical toxins or ultraviolet radiation, plants produce phytoalexins (21). In contrast, phytoanticipins are constitutively present within the plant in advance of any attempted pathogenic attacks or harsh abiotic treatments (20). Daidzein protects plants from stress and acts as part of the plant's defense mechanism (20,21). Daidzein has been shown to inhibit the growth of *Fusarium culmorum,* a pathogenic fungus (21). Biochanin A and genistein have antifungal activity against *Rhizoctonia solani, Sclerotium rolfsii, Cercospora beticola,* and *Monilinnia fructicola* (21). In addition, genistein and daidzein also serve as attractants, in a concentration-dependent manner, to some nitrogen-fixing bacteria during establishment of symbioses (22). It has been shown that genistein and daidzein isolated from soybean root extract induce *nod* gene expression in *Bradyrhizobium japonicam* and *Rhizobium fendii* involved in the early processes of nodulation in *Fabaceae* plants (22). According to work done by Wang *et al.* (23), gènistein, biochanin A, and formononetin bioassays, at concentrations between 0.01% and 0.05%, deter redlegged earth mites from *Trifolium subterraneum.* However, at lower concentrations, the isoflavonoids acted as attractants to mites (23).

Other implied functions were suggested by experiments with applied mammalian sex hormones on plants. Estrone and 17β-estradiol applied to *Cichorium intybus* and *Salvia splendens* led to a 55% and 85% stimulation of flowering, respectively, while the controls remained vegetative (24,25). Two members of the *Moraceae* family, *Morus microphylla* and *Maclura pomifera,* were found to contain phytoestrogens (26). A correlation between the high levels of estrogenic activity and the development of vestigial gynoecium in male flowers of *Morus rubra* and functional gynoecium in female flowers of *Morus* and *Maclura* has been found (27). Estrogenic activities induced by phytoestrogens in the estrogen-responsive transcriptional system in *S. cerevisiae* strain BJ3505 were shown to increase prior to and during flowering for both male and female plant extracts of *Morus rubra* and *Maclura pomifera,* leading to the premise that phytoestrogen may be necessary for gynoecium development (27). Milanesi *et al.* (2001, 2004) published results in favor of the existence of estrogen-binding proteins in *Solanum glaucophyllum.* Using 0.1 mg protein of *Solanum glaucophyllum* callus cultures and 10 nM [^{3}H] 17β-estradiol, a 4 h radioligand binding assay revealed several binding sites for 17β-estradiol giving credence to the possibility of estrogen receptor-like transcription factors in plant species (28,29).

Phytoestrogens and Breast Cancer

Cancer is the second leading cause of death in the United States accounting for one in four deaths each year (30). Breast cancer is the second leading cause of cancer death in women after lung cancer (30). There is approximately a one in eight chance that a

woman will develop an invasive form of breast cancer sometime in her lifetime. Though breast cancer strikes woman predominately, men can get the disease as well (30). Michigan Cancer Foundation (MCF-7) human breast adenocarcinoma cell line has been widely used for the study of breast cancer. The cells originated from the breast ductal tumors of a 69-year old Caucasian female and were collected for the Michigan Cancer Foundation -7 in 1970 (31). MCF-7 cells maintain the characteristics of differentiated mammary epithelial cells that have the ability to process 17β-estradiol and are classified as estrogen and progesterone receptor positive (31,32).

The SERM, tamoxifen (TAM), has been a drug of choice for hormone responsive breast cancer treatment since 1971 (9). Tamoxifen blocks the expression of epidermal growth factor receptor (EGFR), which is involved in cell proliferation, as well as of transforming growth factor receptor β (TGF-β), which, among other functions, is involved in death signaling to downstream targets and is expressed on the cell surface, leading to growth arrest of ER-positive cancer cells. Raloxifen, a second generation SERM, along with TAM, has been approved for treatment of breast cancer in post-menopausal women. However, these drugs can lead to the risk of developing other types of cancer as well as fractures, ischemic heart disease and stroke (9,11,12). Recipients of TAM have a distinct incidence of reoccurrence of breast cancer with a poor prognosis (9,11). The alternative to SERM drugs has been aromatase inhibitors (AI), which block the synthesis of estrogen. However, these drugs come with the added risk of loss of bone density leading to brittle bones and osteoporosis (9,11). The challenge is to find effective

and long lasting treatments to defeat breast cancer with as little harm to the patient as possible.

The isoflavone, genistein, has dual effects depending on the concentration used for treatment. In previously published studies on the effects of genistein on breast cancer cell growth, it was reported that at concentrations less than 10 μmol/L (average level recorded in humans), genistein has actually stimulated the growth of the estrogen receptor positive tumors. However, at concentrations greater than 10 μmol/L, genistein was inhibitory and a prolonged exposure of greater than 10 days did not reverse or alter the inhibitory effects of genistein (33), despite the fact that concentrations greater than 10 μmol/L have not been reported through dietary intake of phytoestrogens (34).

Pythoestrogens can exert an effect on cancer cells through the ERs as well as through non-ER mechanisms of action. As stated above, phytoestrogens bind preferentially to ERβ. It is through the ERβ that phytoestrogen may act to inhibit cell growth and the stimulatory effects of ERα in breast cancer (35,36). It has also been reported that phytoestrogens can function as AIs, inhibiting the conversion of androstenedione and testosterone to estradiol, thus limiting the level of estradiol in tissues (36,37). Thompson *et al.* (2005) found that 25 g/day of flaxseed reduced cancer cell proliferation, increased apoptosis and reduced HER-2 expression in postmenopausal breast cancer patients before surgery (38).

Phytoestrogens have other non-ERβ effects. They have been shown to decrease proliferation of ER negative breast cancer cells (38,39) mostly through inhibition or

down-regulation of protein tyrosine kinases (PTK) (40-43), which are involved in growth signaling pathways. Genistein has been shown to inhibit the autophosphorylation and activation of EGFR, a PTK that is important in regulating apoptosis and cell proliferation (44). Pharmacologic doses of genistein inhibited the PTK-dependent transcription of c-fos and subsequent cellular proliferation in estrogen receptor negative human breast cancer cell lines (44,45). Others reported that possible mechanisms include the antioxidant activity, stimulatory effect on the immune system and inhibitory effects on angiogenesis by phytoestrogens (30,41,46,47).

Mammalian Estrogen Receptor Structure and Function

Estrogen receptor alpha is a product of the ESR1 gene found on chromosome 6 and ERβ is a product of the ESR2 gene found on chromosome 14 in humans (7). Splice variants of ERα transcript make up the several known transmembrane ERs (7). The ERα and ERβ are members of the nuclear receptor (NR) superfamily, which consists of 48 structurally similar transcription factors (8). A typical ER contains an N-terminus region, which consists of a context-specific activation domain (AF-1), a DNA binding domain (DBD) in the C region, and a ligand-binding domain (LBD) in the E region (Figure 2) (36). The hormone-dependent activation domain (AF-2) is closer to the C terminus. ERα and ERβ share a 97% degree of conservation in the DNA binding domain and a 55-59% degree of conservation in the ligand-binding domain (12). The ER is part of the ligand inducible transcription activation group along with the progesterone, androgen and retinol

receptors. In the absence of ligand, the receptor remains inactive. Binding of 17β-estradiol causes homodimerization of the ER and subsequent binding of the complex to EREs, which are *cis*-acting enhancers within the promoter regions of targeted genes (12,48,49). Once bound to the ERE, the ligand-ER complex will recruit coactivators and corepressors through its AF-2 region (3,10). It is the balance of four coactivators and corepressors that dictate transcriptional activity (6,12). There is abundant proof available that the nuclear receptors, specifically the ERβ, interact with phytoestrogens.

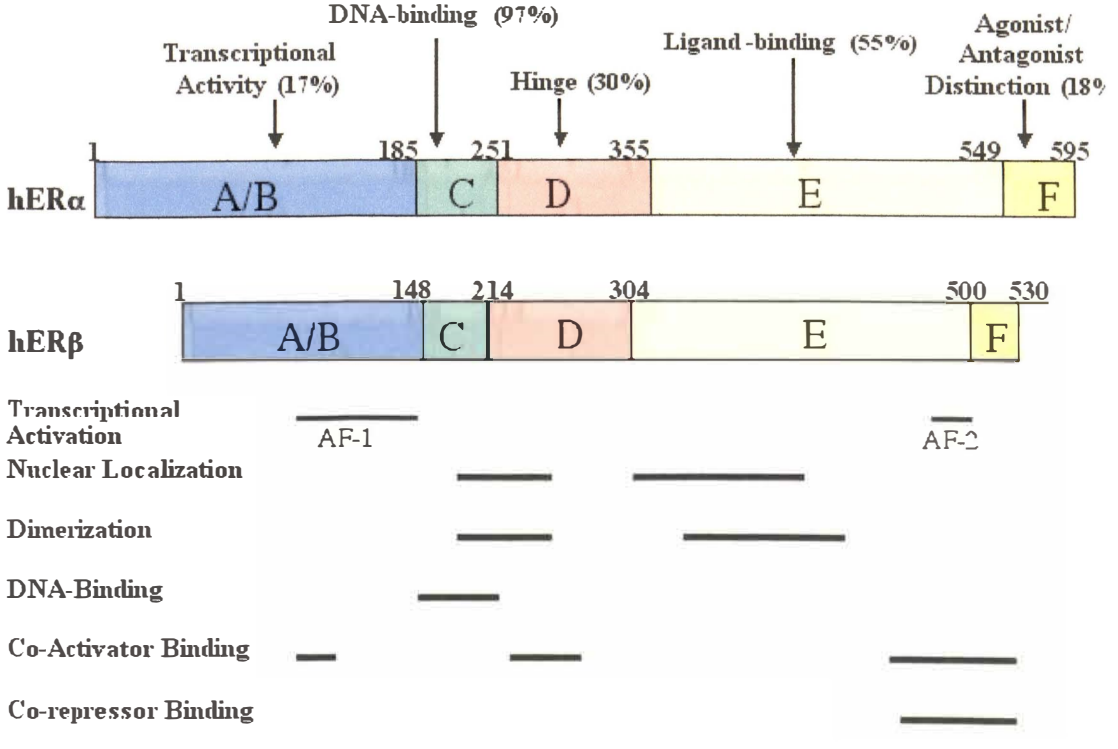

Figure 2. Domain comparison of the human ERs α and β. Domain A/B contains the activation function 1 which is responsible for gene modulation; the C domain is the DNA binding domain, which contains two zinc fingers; domain D is the hinge region; domain E is the ligand-binding domain; and F is the N-terminal activation domain containing AF-2 where coactivators and corepressors are recruited. The percent homology between the two forms of the ER is shown above the hERα [Adapted from (36)].

Dietary Consumption of Phytoestrogens

The major source of dietary phytoestrogens is from plants of the legume (*Fabaceae)* family, such as soy and bean (1,5,9). In the past, much of the scientific research on phytoestrogens has focused on the isoflavones, genistein and daidzein, contained in soy and on biochanin A, formononetin, and coumestans, contained in red clover, and their possible beneficial effects on preventing human diseases such as endometrial hyperplasia, hypertriglyceridia, and tumorigenesis (1,3,9). To date, preclinical studies have shown that phytoestrogens may help to lower pathogenic angiogenesis, atherosclerotic lesions, and very low density lipoprotein (VLDL), thereby lowering low density lipoprotein (LDL) and cholesterol, as well as increasing antioxidant enzymes, and bone mineral density (1,9). The stronger affinity of phytoestrogens for ERβ compared to ERα may account for some of their beneficial effects on tissue expressing ERβ such as bone, blood vessels and brain (1,9,12). The antagonistic effects of genistein and coumestrol on ERβ may account for the lower incidence of breast cancer among Asian populations who take in large amounts of the phytoestrogen through their diet (1,9,50,51). Because of the harmful risks of estrogen therapy, the medical community has explored the use of phytoestrogens as an alternative form of estrogen for hormone replacement therapy (HRT) (1,9,52).

Isoflavones, a major class of phytoestrogens, are abundant in soy, chickpeas, and green peas (53-56). Another major class of phytoestrogens is represented by lignans (36,46,57,58). Flaxseed is the single most abundant dietary source of lignans; however,

whole grains, tea, and vegetables are also significant sources and are more abundant in the average American diet (57). In general, phytoestrogens are found as glycosides in plant cells and tissues. A glycoside is a molecule containing a sugar bound to a non-sugar moiety known as aglycone. Many plants species store and transport chemicals in the form of inactive glycosides, which are consumed by humans through their diet. The isoflavones genistein and daidzein are present in soy primarily as β-D glycosides, genistin and dadzin, respectively (46). These glycosides are metabolized by enteric bacteria in the human digestive system releasing active aglycones (34,59,60-62). Though the aglycone form of a phytoestrogen is biologically active the compound is further metabolized in the liver and intestines to active glucuronide conjugates (48). Daidzein may be metabolized to equol or O-desmethylangolensin (O-DMA) and genistein to p-ethyl phenol (34,46,48). These three compounds are the major isoflavone derivatives that can be detected in human serum and urine (48,51,60).

In this study, two locally available plants, White clover, *Trifolium repens* L. and Alfalfa, *Medicago sativa* L. (*Fabaceae*), were tested for their effect on MCF-7 breast cancer cell line. Both plants are perennial and known forage crop for livestock (63,64). Alfalfa is widely used as hay for farm animals (63). White clover, widely distributed within grassy areas in North America, is a herbaceous perennial plant with whitish flower heads and trifoliate leaves which may or may not have a "crescent" or "water mark" on the upper surface (USDA Plants Database). Humans use White clover powder on their salads and rice (64). Alfalfa looks similar to White clover except for its purple flower

heads and a smaller pinnately-compound leaf (63). Alfalfa is consumed by humans mostly as sprouts in salads and sandwiches (63).

Both Alfalfa and White clover contain phytoestrogens. Alfalfa contains apigenin, alpha-spinasterol, coumestrol, and formononetin among others (65). The main phytoestrogens found in White clover are coumestrol, formononetin, kaempferol, and quercetin (66,67). Some of these phytoestrogens, individually as pure compounds at concentrations from 30μM to 90μM have been shown to inhibit cancer cell growth and in some cases induce cell death (68,69). The recent interest in the estrogenic activity of plant chemical compounds has been spurred by the popularity of diets and herbal supplements containing phytoestrogens, mostly soybean and grape phytoestrogens. Other legumes besides soybeans are known to contain phytoestrogens, but not many research projects have employed them. For this study, two known legume plants, White clover and Alfalfa, were chosen to study their effects on MCF-7 breast cancer cells. Very few research publications on the effect of Alfalfa and even less on the effect of White clover plant extracts and phytoestrogens on the fate of cancer cell lines were found in the databases. Also phytoestrogens in Alfalfa sprouts but not in the mature plant were shown to reduce cell viability and induce macroautophagy in MCF-7 cells at concentrations higher than dietary consumption concentrations (70). For this study mature plants in bloom were used for obtaining phytoestrogen fractions.

CHAPTER II

PROJECT GOAL AND HYPOTHESIS

The goal of the project was to determine the effect of phytoestrogens in White clover (*Trifolium repens*, *Fabaceae*) and Alfalfa (*Medicago sativa*, *Fabaceae*) solid phase extraction (SPE) fractions on the viability of the MCF-7 cancer cell line.

It was hypothesized that phytoestrogens in White clover and Alfalfa SPE fractions (F80) would have a deleterious effect (decrease cell viability and induce cell death) on the ER positive MCF-7 breast cancer cell line.

The objectives of the study were to:

1. Determine cell viability of MCF-7 cells treated with SPE fractions of White clover and Alfalfa relative to vehicle, E_2 and genistein controls;
2. Determine if expression of appropriate cell cycle arrest and apoptosis markers are modified by treatment with Alfalfa SPE fraction.

CHAPTER III

MATERIALS AND METHODS

Materials

Genistein and 17β-estradiol were purchased from Sigma-Aldrich, USA.

Preparation of Plant Extracts

Samples of White clover, *Trifolium repens* and Alfalfa, *Medicago sativa* (*Fabaceae*) were collected from the Texas Woman's University campus in May 2010. Aerial parts of the plants were washed with a mild detergent solution, rinsed thoroughly with tap water followed by deionized water, and dried in paper towels. The fresh samples were extracted with 80% methanol (1:4 fresh w/v) by homogenizing plant tissues in a Warring blender on low and high settings for 5 min. Homogenates were extracted at room temperature for 48-72 h prior to centrifugation at 3,000 rpm for 30 min. Supernatants were collected, filtered through Whatman 54 filter paper and stored at -20°C.

Solid Phase Extraction

Reverse-phase Sep-pak C18 cartridges were used to perform solid phase extraction (SPE). The crude plant extracts (each 3 ml of concentrated extract) were dried down completely under N_2 gas and resuspended in 1 ml 80% methanol and 3 ml of H_2O, then loaded on the column previously activated with 5 ml of methanol and 5 ml of water.

Solid matrix fraction (SM) was obtained first and discarded (Figure 3). Retained phytoestrogens in the column were successively eluted first with 2 ml of 20% methanol and then with 2 ml of 80% methanol to obtain F20 (discarded) and F80 fractions, respectively. Fractions F80 were dried under nitrogen gas and resuspended in DMSO. The SPE F80 fractions were estimated spectrophotometrically for estrogen equivalents content based on an estradiol standard curve before treatment of MCF-7 cell cultures.

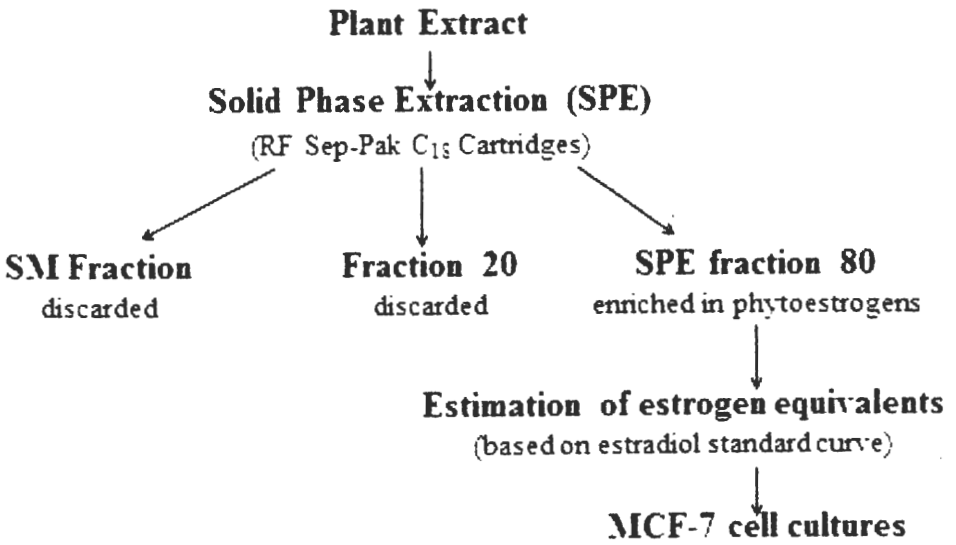

Figure 3. General diagram of plant extract preparation for treatment of MCF-7 cultures. SM, solid matrix

MCF-7 Cell Cultures

Michigan Cancer Foundation (MCF-7) cells were used in this study. The cells were maintained in 75 cm^2 flasks with RPMI 1640 media with phenol red (Invitrogen Life Technologies), supplemented with 10% charcoal-inactivated fetal bovine serum (FBS), and 1% streptomycin (10,000 µg/ml)/penicillin (10,000 units/ml) at 37°C in a humidified

incubator with 5% CO_2. Cells were subcultured once a week at a 1:2 ratio.

Cell Culture and Treatments

MCF-7 cells were seeded into 96-well cell culture plates at 5000 cells/well with up to 200 µl of RPMI 1640 media containing phenol red, supplemented with charcoal-inactivated 10% FBS and 1% streptomycin (10,000 µg/ml)/penicillin (10,000 units/ml). The cells were then incubated overnight in a humidified incubator at 37°C with 5% CO_2 overnight. When cells reached 60% confluency, they were treated with White clover or Alfalfa SPE F80 fractions at concentrations ranging from 100 ng, 500 ng, to 1 µg E equivalents for 24 and 72 h.

Cell Viability Assay

Cell viability was quantified using a Calcein AM assay. In brief, the media was removed from the 96-well plate and the cells were washed three times with 200 µl PBS. The cells were then incubated with 100 µl Calcein AM dye (1mg/ml) diluted 1:1000 at 37°C for 15 min in a plate wrapped in foil to protect from light. As a result of cleavage of the dye molecule in the mitochondria, Calcein AM fluoresces in live cells only. Calcein AM fluorescence was determined at an excitation of 485 nm and an emission of 530 nm using an Infinite m200 multimode microplate reader (Tegan AG, Mannedorf, Switzerland). There were three independent experiments each with 8 replicates each.

Western Blot Analysis

Cells were lysed in RIPA (Radioimmunoprecipitation assay) buffer (50 nM Tris-HCl, 150 mM Nacl, 0.1% SDS (sodium dodecyl sulfate), 0.5% sodium deoxycholate, 1% Triton X-100, 1mM (EDTA) ethylenediaminetetraacetic acid, with protease inhibitor and phosphatase inhibitor cocktail (Abcam, USA) added at 1:100 dilution)) and protein concentration of the lysates were measured with a Commassie blue reagent (Bio-Rad) at 595 nm using the Spectra Max 340 microplate reader (Molecular Devices, CA). 1X Sodium dodecyl sulfate (SDS) 1X sample buffer containing 1.5 mM Tris–HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol, was added to each protein sample. The protein samples (20 μg each) were resolved by SDS-polyacrylamide gel electrophoresis at a constant 100V at 4°C for 1 h 15 min using a mini 1-D electrophoresis system (Biorad, CA). After electrophoresis, the gel was washed with 0.05% Tween-Tris-HCl buffer solution (TTBS) for 5 min. Proteins were transferred onto an Immunoblot nitrocellulose transfer membrane (Protran, Germany), which was also pre-washed in 0.05% TTBS, using a Mini Trans-Blot Cell (Biorad, CA). The transfer was run at a constant 35V at 4°C overnight. Ponceau staining for 1 min confirmed protein transfer. Membranes were washed for 20 min with TTBS and blocked with 5% milk in TBS or (PBS) phosphate buffered saline for 45 min. The membranes were washed again for 20 min with TTBS and probed overnight at 4°C with primary antibodies at 1:1000 dilutions. Membranes were probed with anti-human caspase-3, anti-PARP antibodies (Cell Signaling, MA), and anti-ERβ (Santacruz Biotechnology, CA). The loading control used

was β-actin (Santacruz Biotechnology, CA). Once the primary antibody was removed, the membrane was washed with TTBS for 20 min followed by the addition of the appropriate secondary antibody at a 1:10,000 dilution in milk. The secondary antibodies were allowed to react at room temperature for 45 min to 1 h. Secondary antibodies used were anti-rabbit IgG and anti-mouse IgG (HRP) horseradish peroxidase (Cell Signaling, MA). Western blots were developed using the femto-enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, NJ) and given dynamic exposure for 1-2 min using the Biospectrum Imaging System 810 (UVP, CA).

Densitometry Analysis

Densitometry analysis using spot analysis of Western blots was performed with a Biospectrum Imaging System 810 (UVP, CA). A rectangular box was drawn around the visible band at the desired position of one sample. Background was subtracted and density of the band was measured within the area of the rectangle drawn. The exact same rectangular box with the same dimensions/area was used to determine density of all other visible bands being compared. The mean density of each compared visible band was graphed.

Statistical Analyses

Statistical analysis was performed on cell viability data using a three - way analysis of variance (ANOVA) with plant, dose, and time as the three fixed independent variables was performed with SPSS software v.15. Post–hoc evaluation was done using the

conservative Tukey Test at 16 degrees of freedom. All data were normalized to 0 count control, data were presented as the mean $\pm$ SE. $P \leq .05$ was considered statistically significant.

CHAPTER IV

RESULTS

Effects of White Clover and Alfalfa SPE Fractions on the Growth of MCF-7 Cancer Cells

To determine the effect of the phytoestrogens contained within Alfalfa and White clover SPE fractions on the MCF-7 cell line, the cells were treated with 10, 100, 500, or 1000 ng E equivalents/ml of each plant fractions for 24 h and 72 h. The extent of cell death was measured by the Calcein AM assay (Figure 4).

Type of plant, incubation time, and SPE concentration interacted to influence the measure of cell viability (ANOVA for 3-way interaction: $F3, 32 = 4.12$, $p \leq 0.05$). There were only minimal effects of the SPE fractions in White Clover while in Alfalfa, the highest SPE concentration essentially eliminated fluorescence. As a consequence, the only significant main effect was for f80 concentration ($F3, 32 = 12.65$, $p \leq 0.001$). However, type of plant ($F3, 32 = 7.40$, $p \leq 0.001$) and incubation time ($F3, 32 = 3.41$, $p \leq 0.05$) interacted significantly with f80 concentration. Group differences were attributed primarily to the low fluorescence of Alfalfa cells treated for 72 h with 1 μg SPE; this was the only point where White Clover and Alfalfa differed significantly (Tukey $q32,16 = 7.53$, $p \leq 0.05$).

Interestingly, for Alfalfa, however, 10 and 100 ng SPE appeared to increase cell viability over the control values. However, in each case, this reflected one exceptionally

high fluorescence value. In the absence of these extreme values, the averages for 10 and 100 ng SPE, respectively were 112.5 and 102, suggesting that the only effect of the SPE was for the 1 μg SPE in Alfalfa.

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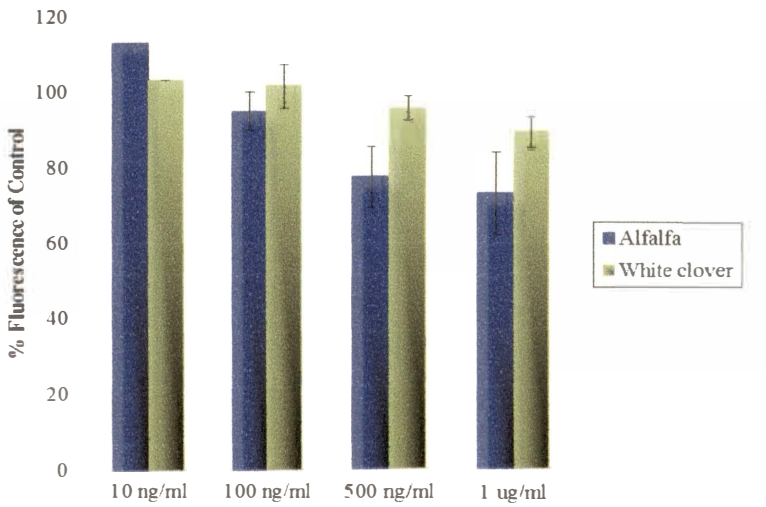

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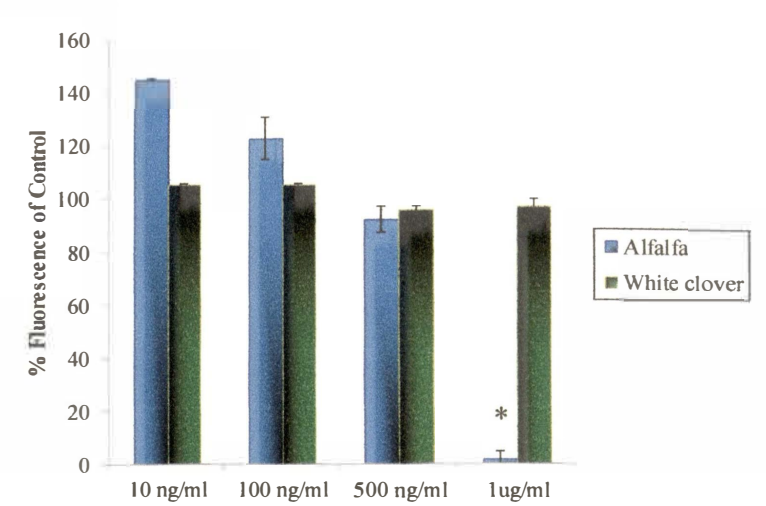

Figure 4. Calcein AM assay for MCF-7 cultures treated with Alfalfa or White clover SPE for (**A**) 24 h and (**B**) 72 h. MCF-7 cells in a 96-well plate were treated with Alfalfa or White clover SPE fraction. The data are normalized to the untreated control. For each experiment N=3. Bars shown are standard errors. Statistical analysis was done by a three-way ANOVA and post-hoc with Tukey test. * = $P \leq .02$

Phytoestrogens in Alfalfa SPE Fraction May Work Through the Estrogen Receptor β in MCF-7 Cells

Phytoestrogens have a stronger affinity for ERβ than ERα (1). To check the expression levels of ERβ in cells treated with AA SPE, Western blot analyses were performed. MCF-7 cells were plated at 500,000 cells/well in a six-well plate. The cells were treated with the AA SPE F80 fraction at 100 ng, 500 ng, and 1 μg E equivalents. Cell cultures treated with 17β-estradiol and genistein (10 ng each) were used as controls. The proteins were extracted and the H150 polyclonal ERβ antibody was used as the primary antibody for the Western blots (Santa Cruz Biotechnologies, CA).

In one representative experiment of this study, there was an increase in ERβ 1 and ER β isoform expressions in all treatments in comparison to the control at the 24 h time point (Figure 5). The 100 ng E equivalents concentration of the AA SPE fraction induced the highest expression levels in both the ERβ 1 and the ERβ isoforms (Figure 5B). The ratio of the densitometry data shown in Figure 4C gives a better perspective on the expression levels of the ERβ relative to other isoforms. The graph shows that AA SPE at 100 ng E equivalents produced similar expression levels as 10 μM of 17 β-estradiol, reflected in the ratios of ERβ 1 and ER β isoform expression levels as a result of 17 β-estradiol treatment (1.434) and AA SPE (100 ng) treatment (1.443) (Fig. 5C). The Western blots in Figures 5 and 6 reveals that, despite the overall decrease in all protein expression levels blotted from the 72 h treatment, the ERβ/ERβ isoform ratio has increased as compared to

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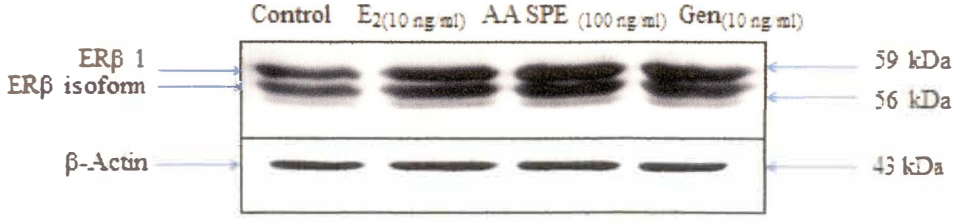

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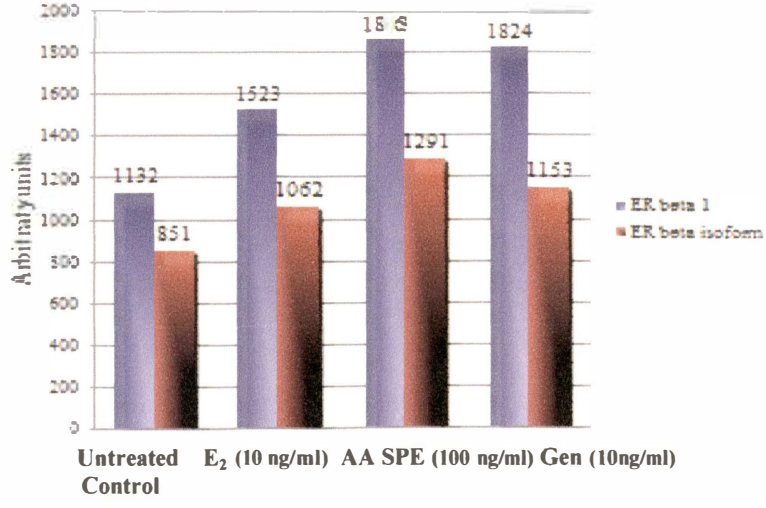

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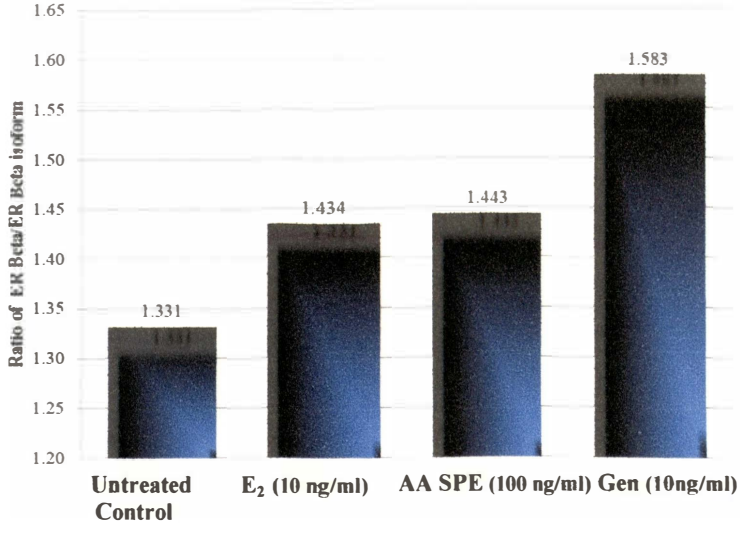

Figure 5. The effect of AA SPE on the ERβ expression level after 24 h of treatment (10% SDS-PAGE gels used, N=1). (**A**) Western blot analysis of ERβ expression after 24 h treatment with Alfalfa SPE ER β 1 at 53kDa, β–Actin at 46kDa. (**B**) Densitometry data of ER β and ER β isoform after 24 h treatment. (**C**) Ratio of the ERβ/ERβ isoform densitometry data.

the 24 h treatment to at least a 2:1 ratio, with AA SPE at 100 ng E equivalents inducing the highest increase in expression level.

Alfalfa SPE Fraction May Induce Cell Cycle Arrest and Apoptotic Death in MCF-7 Cells

MCF-7 cells were treated with AA SPE to confirm apoptosis. Cells were treated with 100 ng E equivalents of AA SPE for 24 h followed by protein extraction for Western blot analysis. An antibody against Poly (ADP-ribose) polymerase (PARP) (Cell Signaling, MA), which aids in DNA repair (72,73), was used. Though many of the caspases involved in apoptosis can cleave PARP, caspase-3 is the most common caspase that does the cleavage (73), and thus PARP cleavage is an indicator of cells undergoing apoptosis. Western blot analysis indicated that no cleavage of PARP occurred after a 24 h treatment with the AA SPE (Figure 7A); however cleavage was seen after 72 h of treatment with the SPE fraction (Figure 7B).

MCF-7 cells were treated with AA SPE at the higher concentrations of 500 ng and 1 μg E equivalents to see the effect of the AA SPE fraction on PARP (Figure 8). Western blot analysis clearly indicated PARP cleavage after 24 h treatment when the cells were treated with 500 ng and 1 μg E equivalents of AA SPE (Figure 8).

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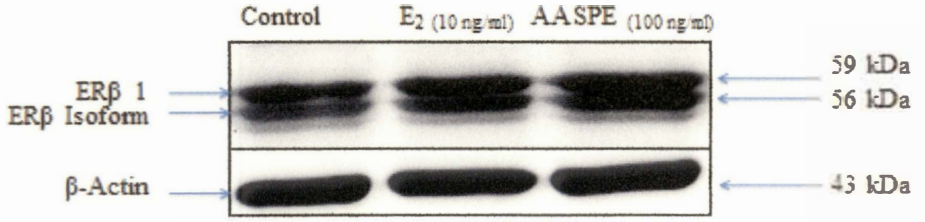

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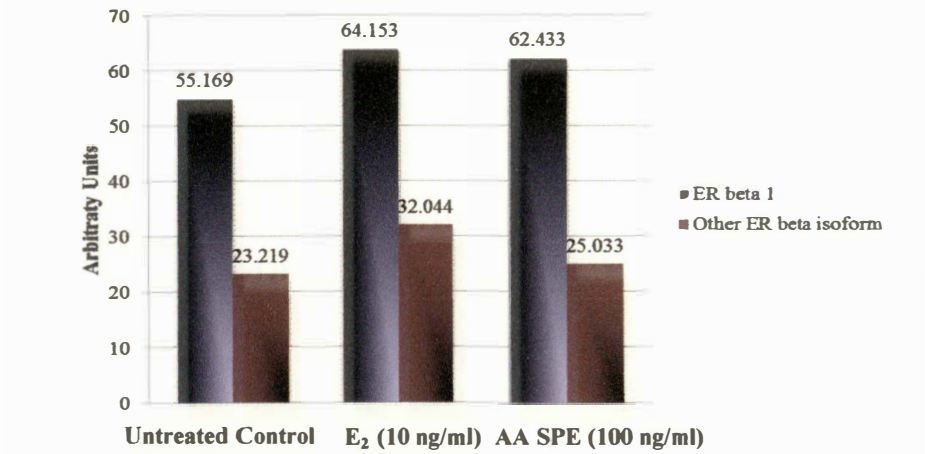

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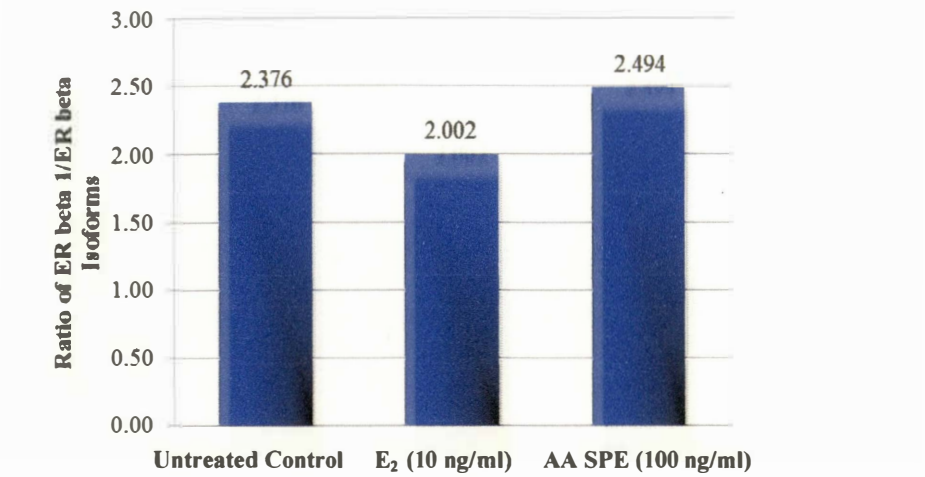

Figure 6. The effect of AA SPE on the ERβ expression level after 72 h of treatment; N=1, ER β 1 at 53kDa, β–Actin at 46kDa (**A**) Western blot analysis of ERβ expression with AA SPE. (**B**) Densitometry data of the expression levels of ER β1 and other ER β isoform. (**C**) Ratio of the densitometry data of ERβ expression levels, treated with the 500 ng and 1 µg E equivalents AA SPE. Exposure time for control and ERβ was 1 min.

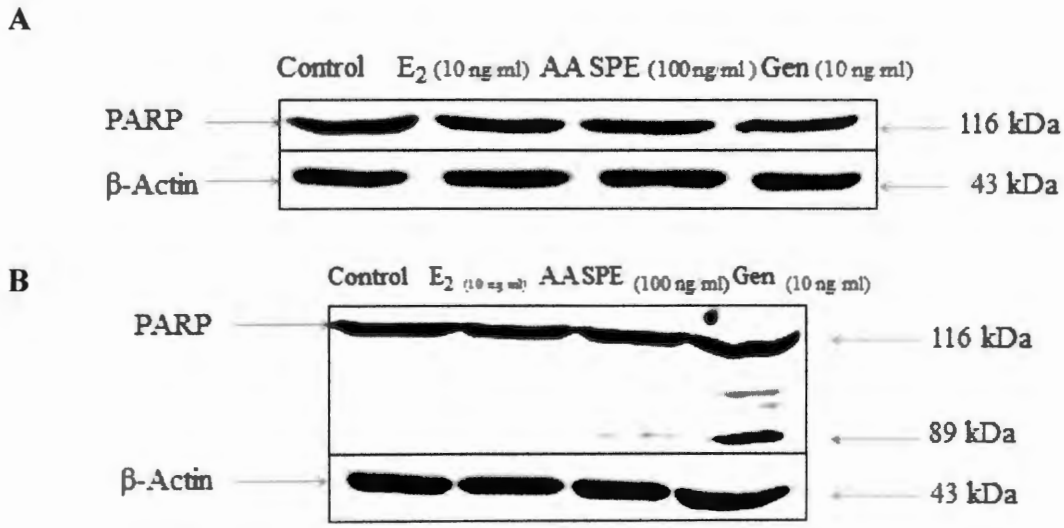

Figure 7. The effect of AA SPE (100 ng) on PARP expression, N=1. **(A)** Western blot analysis of PARP after 24 h treatment cleavage shows no cleavage of PARP at 116 kDa. **(B)** Western blot analysis of PARP after 72 h treatment with alfalfa SPE, showed PARP cleavage PARP cleavage at 89 kDa. Exposure time for the actin control and PARP was 1 min.

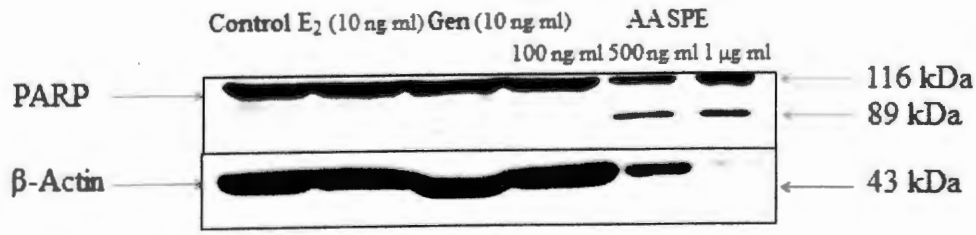

Figure 8. Western blot analysis revealing PARP cleavage in MCF-7 cells after 24 h treatment with AA SPE, N=1. Exposure times for the actin control and PARP were 1 min and 2 min, respectively.

Antibodies against caspase-3, the tumor suppressor p53 and its downstream target p21 (Cell Signaling, MA) were also used. There was evidence of cleavage of caspase-3 after 24 h treatment of MCF-7 cells with 1 μg E equivalents of AA SPE (Figure 8).

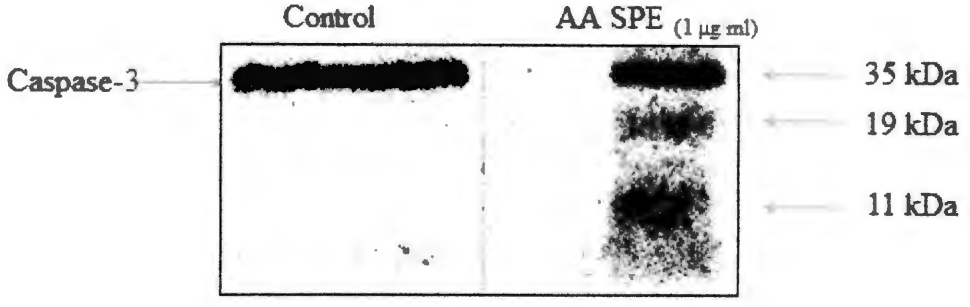

Figure 9. Western blot analysis revealing Caspase-3 cleavage in MCF-7 cells after 24 h treatment with 1μg E equivalents of AA SPE; Capsase-3 at 35 kDa, cleaved caspase-3 at 19 kDa, and 11kDa, N=1. Exposure time for caspase-3 was 1 min.

Western blot analyses of the p21 and p53 proteins after a 24 h treatment with AA SPE at 1 μg concentration E equivalents revealed an increase in expression in the p53 tumor suppressor level, and two of its downstream targets, the CDK inhibitor p21, and the pro-apoptotic protein, Bax, as compared to the actin control. Cytochrome c expression levels were also increased as compared to the actin control (Figure 10).

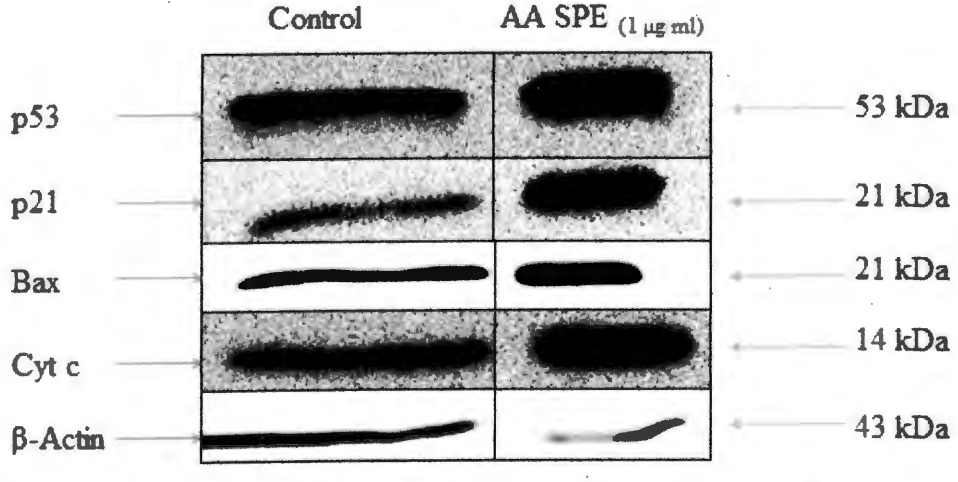

Figure 10. Western blot analysis of protein cell cycle arrest markers expression level in MCF-7 cells after 24 h treatment with AA SPE, N=1. An additional control has been removed from the middle lane. Exposure time for all blots was 1 min.

CHAPTER V

DISCUSSION

Given the significant interest in the estrogenic activity of phytoestrogens, in this study, a complex mixture of phytoestrogens as SPE fractions of White clover and Alfalfa were used to determine their effect on the proliferation of MCF-7 ER-positive breast cancer cells. Both White clover, *Trifolium pretense,* and Alfalfa, *Medicago sativa*, have also gained popularity due to research into their use for the treatment for menopausal symptoms (74,75).

Treatment with 1 μg E equivalents/ml AA SPE decreased MCF-7 cell viability by 90% as measured by Calcein AM assay. However, the same concentration of White clover SPE had only minimal effects on MCF-7 cell viability. Of the known phytoestrogens contained in White clover, (kaempferol, coumestrol, formonetin and quercetin), quercetin is the most abundant (76). In a study by Klappan *et al.,* cell inhibition began at about 45 μM of pure quercetin in MCF-7 cell cultures (71). However, the combination of phytoestrogens in the White clover SPE fraction had a minimal effect on MCF-7 cell viability. Therefore, for the rest of the experiments, treatments of MCF-7 cells were employed only with AA SPE fraction.

Phytoestrogens interact with ERs in a ligand-binding manner (7) although with significantly lower binding affinities than estradiol and show both agonistic and

antagonistic activities. Therefore, it has been shown that phytoestrogens, such as genistein, can have dual effects on ER-positive breast cancer cell proliferation depending on the concentration used. At concentrations less than 10 μmol/L (average level recorded in humans), genistein has actually been reported to stimulate the growth of an estrogen receptor positive tumor, however, at concentrations greater than 10 μmol/L, genistein was inhibitory (47). In the present study, the significant inhibitory effect on cell viability was obtained with the highest concentration of AA SPE of 1 μg/ml E equivalents.

Coumestrol has long been reported as the most abundant phytoestrogen in Alfalfa (68,76). However, it is apigenin that has been the proven anti-cancer agent on MCF-7 cells (68,69). Recently, two other phytoestrogens were characterized in Alfalfa sprouts, liquiritigenin and isoliquiritigenin (77). The Alfalfa SPE fraction used in this study is not a pure compound but rather a mixture of phenolics extracted from the plant tissues. It is possible that the inhibitory effect on MCF-7 cell viability is due to the combined effect of the phenolic compounds in the SPE fraction.

It is through the ERβ that phytoestrogen may act to inhibit breast cancer cell growth and the stimulatory effects of ERα on growth of tumors (35,78). Boue *et al.* were able to show preferential binding of the alfalfa extract to ERβ over ERα at 1 μg (76). Coumestrol, one of the phytoestrogens in alfalfa, has been shown to increase slightly cyclin D1 expression within 24 h at a 10 μM concentration in MCF-7 cells; the addition of 10 nM E_2 did not inhibit the effect (76). Coumestrol also induced a reduction in the Bcl2/Bax ratio in MCF-7 cells (76). However, coumestrol slightly increased colony

formation in a soft agar assay of MCF-7 cells and induced an increase in the expression levels of the pro-survival transcription factor NF-κB (nuclear factor kappa light chain enhancer of activated B-cells) (76). For the above stated reasons, it is unlikely that coumestrol contributed significantly to the anti-cancer effects seen with Alfalfa in this study.

Since phytoestrogens bind to ERβ with higher affinity than to ERα, ERβ protein expression level was examined by Western blot after 24 and 72 h treatment with AA SPE fraction. ERβ and ERβ isoform expression levels increased in all treatments with AA SPE fraction (100 ng E equivalents). The ERβ/ERβ isoform expression levels induced by 17β-estradiol and 100 ng E equivalents of AA SPE fraction were similar at roughly 1.4 indicating that 100 ng of SPE can elicit a similar response as 10 ng of 17-β estradiol in MCF-7 cells. After 72 h of treatment the ERβ/ERβ isoform ratio increased to a least 2.0 in all treatments and the control. However, 17β-estradiol treated cells showed a lower expression level ratio than the control which could be a result of receptor desensitization due to the prolonged stimulation of the ERβ receptor in the MCF-7 cells by the agonist 17β estradiol. Since only ERβ antibodies were used for Western blot analyses in this study, a strong preference for the transcriptional activation of ERβ versus ERα by the phytoestrogens in AA SPE cannot be confirmed. Future research should employ Western blot analysis with both ERβ and ERα antibodies.

In 2005, Zheng *et al.* showed induction of apoptosis as a result of a 48 h incubation of

HeLa (cervical cancer cell line), with apigenin giving an IC_{50} value of 35.89 μM (68). This current study demonstrated the up-regulation of protein expression of certain tumor suppressors and pro-apoptotic proteins in correlation with the AA SPE treatment of MCF-7 cells (Figures 9-10). These effects of the phytoestrogens in Alfalfa may be through the ERβ. Previously, ERβ has been shown to inhibit the ability of ERα to translocate the tumor suppressor p53 out of the nucleus and into the cytoplasm (78,79). As a result, the protein expression levels of the p53 downstream targets, p21 and bax, increased (78). The activation of ERβ with the phytoestrogen apigenin and subsequent induction of apoptosis has been previously reported in HeLa and HL-60 cancer cell lines (68,69). However, phytoestrogens act through both ER-dependent and ER-independent mechanisms in cancer cells (68). It is possible that different phytoestrogens in the AA SPE fraction acted through both ER-dependent and –independent mechanisms in inducing cell cycle arrest and apoptosis in MCF-7 cells. A proposed model of action of phytoestrogens in AA SPE fraction is diagramed in Figure 10.

Phytoestrogens can cross the plasma membrane and form complexes with ERs. Since phytoestrogens show preference for ERβ, the diagram in Figure 10 shows a phytoestrogen-ERβ complex formed in the cytoplasm and in the nucleus at the ERE of a target gene inducing transcription. This is the ER-dependent signaling pathway for phytoestrogens. It is also possible that phytoestrogens use ERα in triggering transcription of target genes, as shown by Sakamoto *et al.* (2010) when treating MCF-7 cells with pure dietary phytoestrogen compounds (80).

Up-regulation of p53, p21, Bax, and Cytochrome c in the cytoplasm is indicative of cell cycle arrest at the G_1 phase of the cycle followed by apoptosis (81). Disruption of the cell cycle progression is often a cause of intrinsic apoptosis. The protein p21 is a general Cyclin Dependent Kinase (CDK) inhibitor and a downstream target of the tumor suppressor protein p53 (81,82). In the face of DNA damage, the p53 protein acts as a transcription factor for p21, which in turn arrests the cell cycle by inhibiting the interaction between CDK and the appropriate cyclin (81). Cell cycle arrest is a possible result of p53 up-regulation. Tumor suppressor p53 can act as a transcription factor for the CDK inhibitor p21 (81,82). If p21 is upregulated, it can inhibit the cell cycle at any of the cell cycle phases (80-82). In this study, the proteins p53, p21, bax, and cytochrome c were all up-regulated compared to control when MCF-7 cells were treated with 1 μg E equivalents of AA SPE fraction. The tumor suppressor p53 has the ability to inhibit bcl-2, an anti-apoptotic protein, which helps to keep the levels of bax low (81). If p53 inhibits bcl-2 activity, it allows for the up-regulation of bax, the pro-apoptotic protein, part of the same protein family as bcl-2.

Bax and bcl-2 sit on the outer membrane of the mitochondria (81). The down-regulation of bcl-2 by p53 allows bax to oligomerize on the surface of the mitochondria thereby creating a pore in the outer membrane (81). This pore allows the escape of many apoptotic factors including cytochrome c. In 2010, Sakamoto *et al.* while working the leukemia cell line HL-60 showned apigenin-induced apoptosis through release of cytochrome c and subsequent caspase activation using a 30 μM concentration (80).

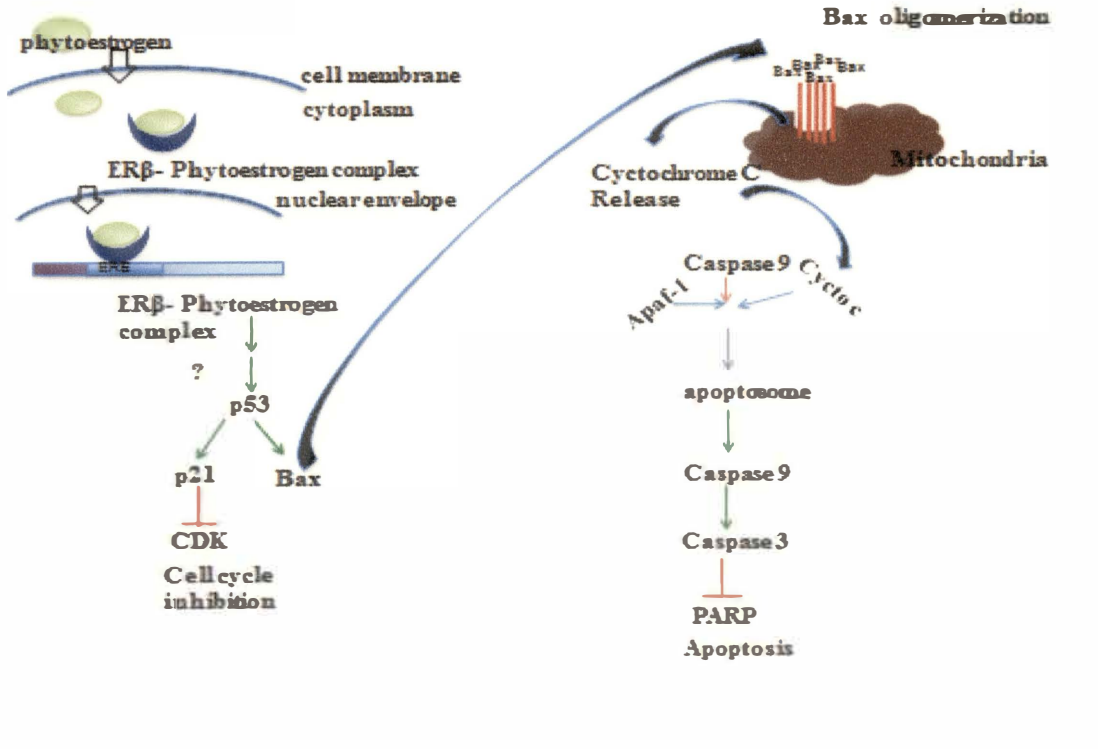

Figure 11. Proposed model of Alfalfa phytoestrogen action in inducing apoptosis in MCF-7 cells. The process is initiated by phytoestrogens-ERβ complex; p53-protein 53, p21-protein 21, CDK-2- cyclin dependent kinase 2, PARP- poly (ADP-ribose) polymerase, Apaf-1- apoptotic activating factor-1, ERE-estrogen response element, ERβ-estrogen receptor beta (68,72, 73, 82).

Cytochrome c joins with Apaf-1 in the cytoplasm causing Apaf-1 to unmask its CARD domain (81). The CARD domain of Apaf-1 can attract caspase 9, the intrinsic pathway initiator caspase, through its CARD domain. All three proteins together are called the apoptosome (69,81) (Figure 11). As a result, caspase 9 is activated and mature caspase 9 is then released from the apoptosome, subsequently activating caspase-3, an effector caspase in the apoptosis pathway (69) and therefore an indicator of an active

apoptosis cascade.

PARP is involved in DNA repair, as well as other functions in the cell, cleavage is an indicator of a cell undergoing apoptosis (72,73). In the current study, PARP cleavage was observed after 72 h treatment with 100 ng E equivalents of the AA SPE fraction. These results were consistent with the cell viability data. Additional Western blots results revealed further PARP cleavage after 24 h treatment with 500 ng and 1 μg for 24 h of AA SPE. PARP can be cleaved by caspase 3 (69). Cleavage of caspase 3 signals activation of the caspase. It is likely that PARP was cleaved after caspase 3 activation. Though PARP is a main target of caspase 3 it cannot be ruled out that other caspases may have cleaved PARP (73,82). Other proteins more upstream in the apoptosis cascade should be investigated to determine how apoptosis began. Future study is needed to determine the exact initiation point of apoptosis. These results are based on one or two Western blot analyses and therefore more replicates are needed to support the above model of action of the Alfalfa phytoestrogens in MCF-7 cells.

In summary, the results of this study indicate that Alfalfa SPE fraction was able to decrease cell viability of the MCF-7 breast cancer cells, most likely *via* both ER-dependent and ER-independent signaling pathways. Although this study found significantly enhanced transcriptional activity of cell cycle arrest and apoptosis marker proteins, further research and data analysis are needed to understand the detailed mechanism of apoptosis regulation by Alfalfa phytoestrogens in MCF-7 breast cancer cells. In this study, a complex mixture of Alfalfa phytoestrogens at a lower-concentration

treatment than previously reported may have potential anti-cancer activity. It has previously been reported that phytoestrogens can function as AIs by inhibiting the conversion of androstenedione and testosterone to estradiol and thus reducing the risk of breast cancer reoccurrence (44,45). It has also been previously reported that certain phytoestrogens contained within the Alfalfa fraction can reduce cell viability and induce macroautophagy in MCF-7 cells at much higher concentrations than dietary consumption concentrations of phytoestrogens (70). Alfalfa SPE fraction may be a good candidate for a dietary source of SERMs and for the development of novel treatments of breast cancer and HRT in postmenopausal women.

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