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The Effects of Phytoestrogens Found in Promensil on Human Ductal Carcinoma

A Major Qualifying Project

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Degree of Bachelor of Science

In Biology and Biotechnology

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Abstract

During menopause, many women are prescribed hormone replacement therapy (HRT) to alleviate disruptive symptoms. Many assume that herbal HRTs are safer than prescription HRTs because they are "natural", even though they are not regulated in either composition or dose by the FDA. Promensil is one of these supplements. The proliferative effects of its specific components, biochanin A and formononetin were investigated using human ductal carcinoma cells. Results indicate that cell proliferation is inhibited by high concentrations of biochanin A (>10μM) and promoted at low concentrations (<10μM), while formononetin had no effect on cell proliferation. These results indicate that herbal HRTs may not be safe for women with breast cancer.

Acknowledgements

We would like to thank our advisors Professor Jill Rulfs and Professor Mike Buckholt. Their guidance and experience helped us throughout our project. We would also like to thank Professor JoAnn Whitefleet-Smith and Abby White for providing further guidance and materials to make our project successful. Professor Destin Heilman was a helpful presence in the project lab and for that we thank him. We would also like to thank our fellow Phytoestrogen Project groups for working with us and sharing technical knowledge. Our project could not have been what it was without the help of all of these people.

Introduction

Menopause is a normal bodily process that women go through in which menstruation and fertility come to an end. In the United States, the average age for women to go into menopause is about 51 years. As a woman ages there is a natural decline in the level of reproductive hormones in her body. More specifically, the levels of estrogen and progesterone decline during menopause. Menopause is associated with uncomfortable symptoms in addition to the decrease in menstruation and fertility. These symptoms include hot flashes, mood swings, sleep disturbances, and increased abdominal fat among others. To lessen and sometimes alleviate the symptoms of menopause there are several treatments, Hormone Replacement Therapy (HRT) being the most commonly known and effective of these (1). This therapy entails replacing the hormones the body no longer produces. Initially it was believed that there were long-term benefits to using HRT in addition to just reducing symptoms, including fewer incidences of osteoporosis and heart disease. However, longer term studies revealed that some of the more prevalent negative side effects of HRT were an increased risk of breast cancer, stroke, blood clots, and mammogram abnormalities (2).

Incidences of these negative side effects caused many doctors to not prescribe HRT as a treatment. In an effort to find relief, women began to turn to herbal supplements that were believed to be more natural. Herbal supplements contain phytoestrogens as a means of treating the symptoms of menopause, though there have been few studies that have provided conclusive evidence that these alternatives are any safer (1). Because phytoestrogens may mimic estrogens they could potentially cause hormone imbalances and lead to cancer.

Obtaining more of an understanding as to how phytoestrogens affect the human body is instrumental in providing women with the information needed to make healthy decisions. Some research has been done, but not enough to create a clear understanding of the effects of phytoestrogens. Some sources claim that phytoestrogens are able to induce the development of breast cancer; one of the major concerns associated with classic HRT (3). Other studies have suggested that there are anticancer effects of a diet rich in phytoestrogens (4). This divide in the understanding of how phytoestrogens actually affect the body is very evident. In order for women to make an educated and appropriate decision as to how to treat their menopause symptoms, there first need to be reliable data on which to base these decisions.

In an effort to close the gap of understanding about phytoestrogens, the primary hormones found in the popular supplement Promensil, biochanin A and formononetin, were tested on human breast cancer cells. These particular hormones are common to red clover, which is used in several herbal hormone replacement therapies (5). A major concern about these hormones is that they will have the same negative effects that are associated with classic HRT. By testing the phytoestrogens on human breast cancer cells,

their potential to cause increased proliferation of breast cancer can be assessed. This will provide a baseline and direction in which further research can be conducted.

Background

Menopause

Menopause is currently defined as the cessation of the menstrual cycle for a twelve month period (6). During this time, many chemical and physiological changes occur such as a decrease in endogenous estrogens. It has been found that women in menopause over the age of 60 have much less endogenous androstenedione, testosterone, estrone, and estradiol than women under the age of 40 still having their periods. Though these declines occur, stability of clearance and metabolism rates remain relatively stable (6). Also, the main form of estrogen in the body switches from estradiol to estrone (7). Physiologically, follicles within the ovary also decrease with age. At menopause, the ovary can become fibrotic, hyperplastic, or hypertrophic. Women with hypertrophic ovaries may still produce significant amounts of androstenedione and testosterone for years, therefore every woman experiences menopause differently (6).

Many women experience physiological changes including mood swings, drying of the mucous membranes, behavioral changes, sleep disturbances, bone loss, and a myriad of other issues (7). The most common side effects that women seek treatment for are hot flashes (8). This symptom is important to this study because the most commonly prescribed and effective form of drug treatment for this symptom is hormone replacement therapy. Hot flashes are characterized by heat, flushing, sweating, anxiety, and chills. This occurs most frequently in the first two years of menopause, but can last for much longer (7).

Few conclusions have been drawn by the scientific community as to the cause of hot flashes. Low levels of estrogen have been implicated, but prepubescent girls with low levels of estrogen do not have hot flashes. Luteinizing hormone and gonadatrophin releasing hormones also have shown little correlation. Some postulate, however, that it is estrogen withdrawal that causes these uncomfortable symptoms. Circulating levels of estrone and estradiol are lower in postmenopausal women with hot flashes than in women with normal hormone levels. Also, women with gonadal dysgenesis who have never had normal levels of estrogen do not experience hot flashes unless they are first exposed to, and then withdrawn from estrogen. Hormone replacement therapy, therefore, would be effective in abating the symptoms of estrogen withdrawal (8).

Hormone Replacement Therapy

Hormone replacement therapy was first popularized in 1966 by Robert A. Wilson, M.D. in a book called *Feminine Forever,* which advocated "estrogen replacement treatment". This school of thought believed that by replacing endogenous estrogen lost during menopause, women could avoid all or at least some of the symptoms. Women undergoing hormone replacement have reported a reduction in hot flashes, mood swings, osteoporosis, urogenital atrophy, atherosclerotic cardiovascular disease, lower blood pressure, lower cholesterol, and lower angina and myocardial infarction (7).

Prescription vs. Nonprescription

There are two main types of hormone replacement therapy: prescription and nonprescription. Even within these groups, hormone replacement therapies vary in types, formulations, doses, and routes of administration. A company seeking FDA approval for a prescription drug must conduct testing and provide information on its drug's efficacy and safety. The FDA does not conduct testing itself, but provides certified physicians, statisticians, chemists, pharmacologists, and other professionals who review the company's data. If this group determines that the drug's health benefits are greater than its known risks, the drug receives approval (9). In this way, the FDA does not ensure that no adverse effects can occur, but it does serve as a form of control.

In contrast, nonprescription, or over the counter drugs, are not as heavily regulated. These drugs only require a drug monograph, described by the FDA as a "recipe book covering acceptable ingredients, doses, formulations, labeling, and testing parameters". Products that conform to a monograph can be marketed without FDA pre-approval, taking out an important normalizing step (9).

Links to Breast Cancer

Though hormone replacement therapies have been shown to effectively mitigate the symptoms of menopause, there have been concerns over their long term safety. As early as 1975, reports were published in the New England Journal of Medicine linking exogenous estrogens to endometrial cancer (7).

Much of the concern over hormone replacement therapy surrounds the ability of estrogens to bind to estrogen receptors. According to Dip et. al., in the normal mammary gland, estrogen receptors are expressed in a small number of epithelial cells that are most non-dividing (10). In a majority of breast cancers (~70%), estrogen receptors are up regulated, and the tumor becomes estrogen responsive (11) (12) (13).

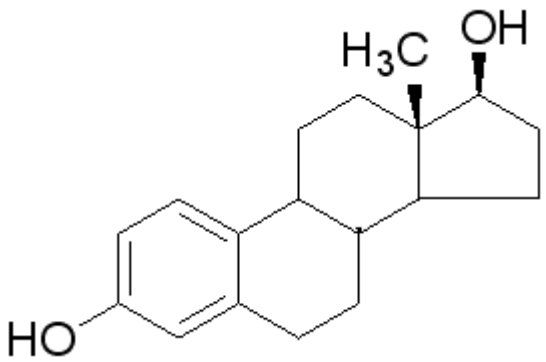
There are two major subtypes of the estrogen receptor: alpha (α) and beta (β). The estrogen receptor is part of the steroid/retinoid receptor gene super family, and is a soluble DNA-binding protein that acts as ligand-activated enhancer factor. When the receptor binds to an estrogenic compound, it activates transcription by then binding to palindromic sequences called estrogen-responsive elements in the promoters of target genes. In this way the receptor up regulates gene expression (14) (15). There can be dissimilar effects when different conformations of receptor ligand binding occur, making investigation of each hormone very important. Though the estrogen receptor is an important factor in cell responses to hormones, estrogens can act upon the cell in independent pathways. Estrogens have been shown to regulate cell-signaling pathways, reduce proliferation, and induce apoptosis in cells lines without estrogen receptors (16).

Many researchers have attempted to verify if hormone replacement therapy can cause or affect cancer growth, however, human studies are often plagued by conflicting results, and questions arise regarding the appropriateness of control groups, validity of exposure information, and role of selection biases. Also the ovarian status, duration of hormone use, intervals of use, subject subgroups and interactions with other breast cancer risk factors confound trials. Some risk factors are long term use (greater than 10 years), higher doses, and late-age menopause (17). Yet, over and over, people are unable to conduct medical reviews that show conclusively if hormone replacement therapy can cause breast cancer, or protect against it (18) (19) (20) (21).

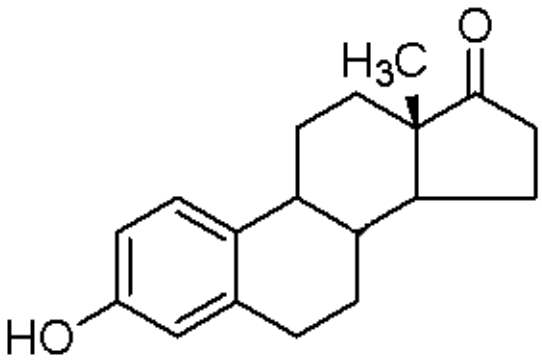
In the past decade, much data has surfaced pointing to the potentially harmful effects of hormone replacement therapy. A serious backlash against HRTs was created in 2003 by a study by the Women's Health Initiative on the effects of HRT containing estrogen and progestin on healthy women. This study was abruptly stopped because the risk of the trial was greater than the proposed health benefits (22). In an attempt to avoid the risks associated with traditional hormone replacement, many women began to search for alternative therapies.

Phytoestrogens

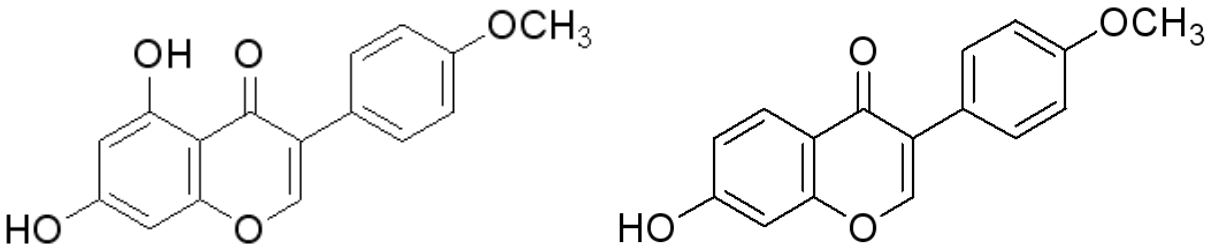
Phytoestrogens are plant hormones that have similar structure and function as mammalian estrogens. Shown below in Figure 1 are the two main forms of estrogen in the human body, estradiol and estrone, and the two phytoestrogens investigated in this project.



Estradiol



Estrone



Biochanin A Formononetin

Figure 1: Structure of mammalian estrogens and plant phytoestrogens

As can be seen in Figure 2, these hormones share very similar structures. Red lines denote common molecular bonds. The two phenol groups denoted by "P" are the proposed reason for the ability of phytoestrogens to bind to estrogen receptors in the human body (16). Other sources propose that only one aromatic ring and a hydroxyl group are necessary for effective binding of the estrogen receptor because the rest of the receptor will allow hydrophobic groups (22). Other important factors are the spacing between the hydrophobic and hydrogen bond interactions, the degree and size of the alkyl group, and its location on the phenolic ring.

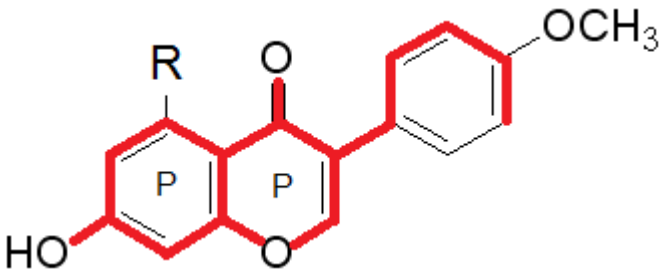


Figure 2: Structural similarities between mammalian estrogens and plant phytoestrogens

As they are so similar, phytoestrogens have been discussed as agonists and antagonists of the estrogen receptor depending on the tissue, concentration, and how much endogenous estrogen is already present. Because of this, they are sometimes called selective estrogen receptor modulators, or SERMS (22). The structural similarities to endogenous estrogens and the ability to act as SERMS have led people to believe that phytoestrogens may provide relief from menopause symptoms without the potential dangerous side effects.

A major drawback to phytoestrogens, however, is their variability. Most phytoestrogens are marketed as extracts contained in nonprescription medications. This nonprescription status allows companies great leeway in determining the components of their product. Formulations, dosages, and delivery methods vary between brands, and even between lots of the same product. Though many companies may attest to following their monograph, scientists have found high variability in even some of their most basic components. In one study, red clover products such as Promensil were analyzed for

hormone content, and how the carrier matrix of the product affected its intestinal deposition. The authors found that isoflavone content varied significantly between products, in addition to the hormone absorption rates. Two of the hormones most greatly affected by the matrix were biochanin A and formononetin. The rates of absorption, metabolism, permeability, and excretion were highly variable and greatly affected by the carrier matrix (23). This is alarming as women who decide to use hormonal therapies may be receiving more or less of their "prescribed" dosage with little knowledge of the actual safety and efficacy of the product which she has chosen.

The two phytoestrogens studied in this project were biochanin A and formononetin. Both of these are considered isoflavones, and are commonly found in hormone replacement therapies derived from red clover (Trifolium pretense). When ingested, biochanin A can be metabolized into genistein, and formononetin into daidzein. Figure 3 illustrates the metabolic changes that these hormones undergo. It has been noted that the metabolites of these first two hormones may not have the same effect as their precursors (22).

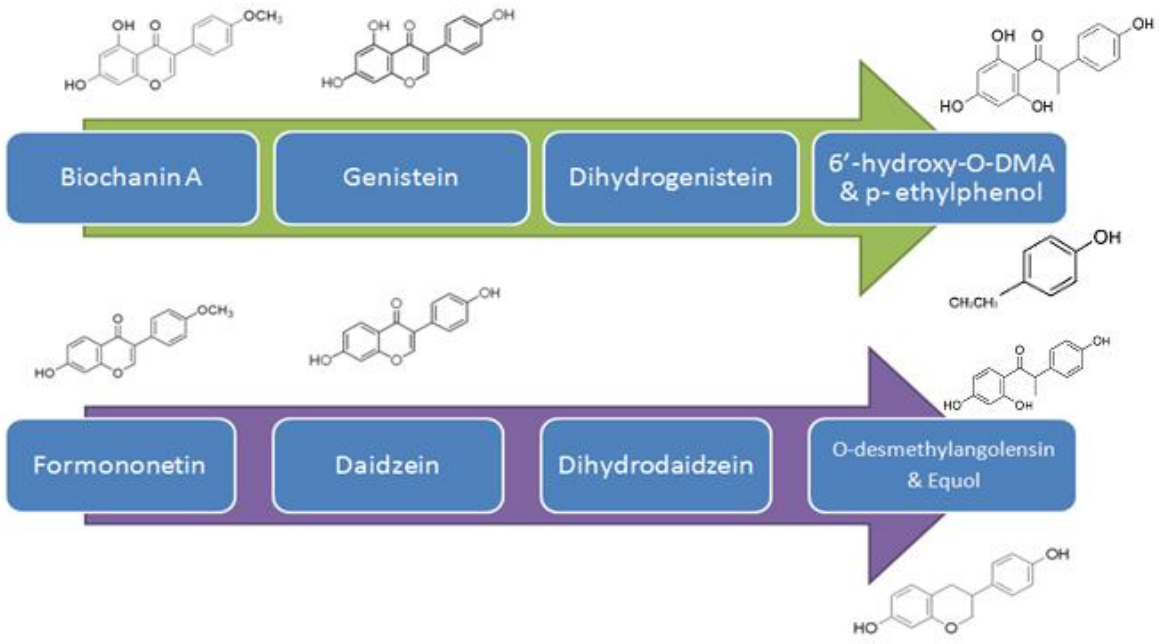


Figure 3: Metabolism of biochanin A and formononetin with structural images of each molecule

Biochanin A

Overall, biochanin A has been found to induce breast cancer cell proliferation at concentrations lower than 10µM and cause inhibition at concentrations greater than 20µM. It has also been found that an estrogen responsive MCF7 cell line proliferated in response to biochanin at concentrations less than

0.1uM, mediated through the estrogen receptor (24). Another study using an estrogen responsive MCF7 line showed that cell proliferation was inhibited at an IC50 value of 20µM (25). Taken together with other studies (26) (3) (27), these results suggest that concentrations lower than 0.1µM will cause proliferation, possibly including up to 10µM, but concentrations higher than 20µM may have inhibitory effects through estrogen receptor independent means.

Formononetin

The other hormone investigated in this project was found to be a proliferation inducing agent at all concentrations in a dose dependent manner. One study found that formononetin has estrogenic activity in a concentration dependent manner from 0.5 to 500uM on an estrogen responsive MCF7 line. Inhibition by a known estrogen antagonist at 100nM pointed to great similarity to endogenous estrogen (28). Another study also found that formononetin binds both the alpha and beta forms of the estrogen receptor and induces cell proliferation and gene expression in breast cancer and endometrial cells in an estrogen receptor dependent manner. A known estrogen antagonist was also found to inhibit formononetin induced proliferation of estrogen receptor positive breast cancers (29).

The same types of results were found in a live model investigating rat mammary gland proliferation. Estrogenic effects were found in rats given formononetin at 40mg/kg subcutaneously. This study found mammary gland proliferation was enhanced 3.3 fold over saline treated controls when animals were treated with formononetin. Estrogen receptor expression in animals given formononetin was twofold higher than control, even though formononetin was found to have a binding affinity 15,000 times less than that of 17β-estradiol. The authors concluded that formononetin supports mammary gland proliferation, but estrogenic potency is extremely weak compared with 17β-estradiol (25). These results suggest that formononetin will increase estrogen responsive cancer cell proliferation in a dose dependent manner through the estrogen receptor.

Methods

Media

Different media compositions were used for different stages of the experiment. For cell maintenance of the T47D cell line the base media was Roswell Park Memorial Institute Media (RPMI), 10% Fetal Bovine Serum (FBS) and 2mg/ml insulin.

The media used during the Promega Cell Titer 96 AQ Non-radioactive Proliferation Assay One Step assay contained altered RPMI and FBS. Due to estrogenic effects associated with phenol red, experiments were conducted in phenol red free RPMI. Charcoal stripped serum was used in these experiments in an effort to reduce the steroid levels from the serum that could cause false positive and other incorrect findings.

Cells

The cell line used in the experiments was T47D. It was derived from a human epithelial breast duct carcinoma that was isolated and cataloged with the ATCC. These cells were initially plated from a frozen stock in a T25 flask to prepare for expansion and splitting. The cells were maintained about three times a week with fresh media and passaging, as needed. The cells were incubated at 37°C and 5% CO_2. Some of the cells were frozen down as a stock for future use.

Reagents

50 mg insulin derived from bovine pancreas was dissolved in 200ml of 18N glacial acetic acid and 10ml of distilled water. This provided a 2mg/ml concentration to work with. 17β – Estradiol (>98% pure), formononetin ($\geq$ 99% pure), and biochanin A were dissolved in dimethyl sulfoxide (DMSO) to create stock solutions of 0.10M. With this stock solution the needed concentrations for testing the hormones was achieved via serial dilution.

Assays

The Promega Cell Titer 96 AQ Non-radioactive Proliferation Assay One Step, also known as a MTS assay, was used to test the proliferation of the T47D cells after being treated with the respective hormones during each test. The cells were plated in Falcon brand 96 well plates and allowed to grow to approximately 80% confluence. The media was replaced with 100 µl of phenol red free assay media and the different concentrations of hormones were achieved via serial dilution. The total amount of DMSO (1%) was maintained across all wells as to insure more accurate hormone responses. The plate was left for 24 hours with the hormones. 20 µl of the Promega assay was added to each well and allowed to develop for 3 hours. After the three hours the plates were read at 450 nanometers with a BioTek EL800 plate reader. The plate design can be seen in Figure 4.

	100 µM	**N** 100 µM	**O** 10 µM	10 µM	**C** 1 µM	**E** 1 µM	**L** 0.1 µM	**L** 0.1 µM	**S** 0.01 µM	0.01 µM	
	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	
	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	
	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	
	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	
	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	
	100 µM	100 µM	10 µM	10 µM	1 µM	1 µM	0.1 µM	0.1 µM	0.01 µM	0.01 µM	
N	**O**		**H**	**O**	**R**	**M**	**O**	**N**	**E**	**S**	

Figure 4: Proposed set up of the hormone test plates

In an effort to confirm the results obtained with the MTS assay, immunoblotting for Proliferating Cell Nuclear Antigen (PCNA) was also conducted. To prepare the cells for blotting, they had all media removed and were frozen at -80°C. All wells for each hormone concentration were then scraped into Lawmmli sample buffer and added to a 1.5ml Eppendorf tube. The tubes were then placed in a heating block to denature the proteins and then loaded into prepared gels. The gels were made of 3.9% and 12% acrylamide in the stacking and separating sections, respectively. With the voltage set at 120 mV, the gels were run for approximately 60 minutes in running buffer until the dye was near the bottom of the gel. The proteins were then transferred to a Millipore Immobilon P™ membrane via semi-dry blotting technique. The blotting was run at 60 milliamps for 90 minutes. 5% non-fat dry milk in phosphate buffered saline (PBS) was used to block the membrane for about 30 minutes. The membrane was then incubated for 60 minutes in a 1:750 dilution of PCNA primary antibody (from Santa Cruz Biotechnology) in blocking buffer, after which the membrane was washed three times with PBS. Goat anti-mouse antibody (from Santa Cruz Biotechnology) was then applied to the membrane in a 1:750 dilution in blocking buffer. Extra antibody was removed with three rinses of PBS. Developing the band was done via dissolving 1 Sigma*FAST* BCIP®/NBT tablet in 10 ml of rdH_2O and applying it to the membrane until the bands developed.

Data Analysis

All wells from a given concentration were converted to percent difference from the control and then averaged with other wells of the same hormone and concentration. Error was calculated as standard deviation of the mean.

Results

Assay Validation

In order to assure that the MTS was functioning correctly, increasing cell concentrations were plated across a 96 well plate, as seen in Figure 5. The cells were allowed to establish themselves in the wells for 24 hours in the standard growth media. The media was replaced with phenol red free and cells were allowed to grow for another 24 hours, after which 20µl of the MTS assay was added to each well and allowed to develop for 3 hours. The plate was then read in the plate reader to provide the values seen in Figure 6. Figure 7 shows the graphed values from the plate demonstrating a trend-based increase in the values thus showing that the assay was functioning as intended.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		5,000	5,000	10,000	10,000	15,000	15,000	20,000	20,000	25,000	25,000	
C		5,000	5,000	10,000	10,000	15,000	15,000	20,000	20,000	25,000	25,000	
D	0,000											
E	0,000											
F		30,000	30,000	35,000	35,000	40,000	40,000	45,000	45,000	50,000	50,000	
G		30,000	30,000	35,000	35,000	40,000	40,000	45,000	45,000	50,000	50,000	
H												

Figure 5: MTS Assay Validation Experimental Layout for a 96 Well Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.039	0.039	0.039	0.04	0.039	0.039	0.04	0.043	0.039	0.041	0.04	0.04
B	0.04	0.34	0.634	0.785	0.741	1.164	1.233	1.273	1.478	1.472	1.386	0.04
C	0.039	0.489	0.511	0.615	0.838	1.182	1.098	1.357	1.41	1.388	1.417	0.041
D	0.324	0.041	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.039	0.04	0.042
E	0.312	0.041	0.04	0.042	0.042	0.041	0.042	0.041	0.038	0.041	0.04	0.041
F	0.04	1.654	1.703	1.698	1.604	1.853	1.782	1.792	2.253	1.696	1.355	0.039
G	0.043	1.695	1.673	1.721	1.801	1.727	1.795	1.974	1.671	1.417	1.29	0.041
H	0.04	0.041	0.043	0.039	0.04	0.04	0.04	0.041	0.04	0.042	0.04	0.039

Figure 6: MTS Assay Validation Results

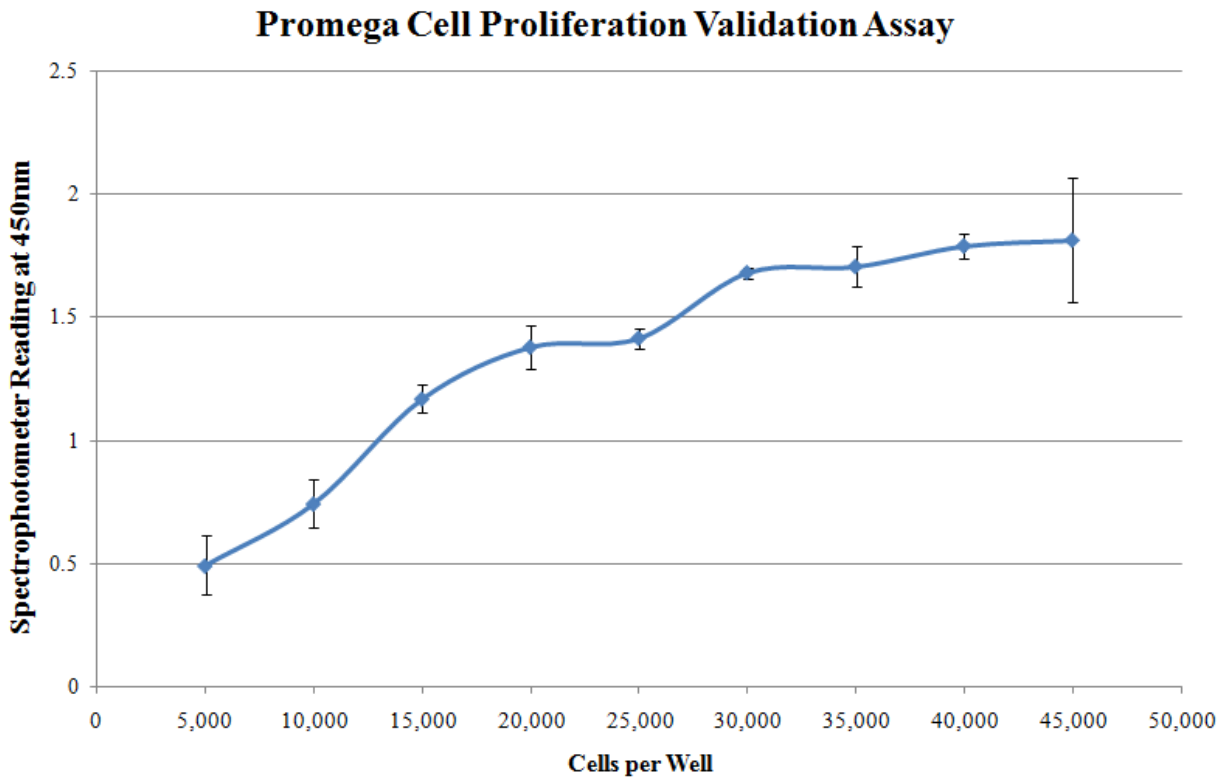


Figure 7: Graph of MTS One-Step Cell Proliferation Assay Validation

Estrogen Responsiveness

The T47D cell line was tested for estrogen responsiveness. This test was done with 17β-Estradiol dissolved in either ethanol or DMSO as a means of concluding which solvent was most appropriate for the cell line.

DMSO

As seen in Figure 8, 10µl and 1µl of DMSO were tested on the cells. 17β-estradiol concentrations from 0µM to 100µM were tested in both volumes of DMSO.

	10µL of DMSO Stock Solution per Well						1µL of DMSO Stock Solution per Well					
	1	2	3	4	5	6	7	8	9	10	11	12
A		No Cells	→					No Cells	→			
B		100µM	10µM	1µM	0.1µM	0.01µM		100µM	10µM	1µM	0.1µM	0.01µM
C		100µM	10µM	1µM	0.1µM	0.01µM		100µM	10µM	1µM	0.1µM	0.01µM
D	0µM	100µM	10µM	1µM	0.1µM	0.01µM	0 µM	100µM	10µM	1µM	0.1µM	0.01µM
E	0µM	100µM	10µM	1µM	0.1µM	0.01µM	0 µM	100µM	10µM	1µM	0.1µM	0.01µM
F	0µM	100µM	10µM	1µM	0.1µM	0.01µM	0 µM	100µM	10µM	1µM	0.1µM	0.01µM
G	0µM	100µM	10µM	1µM	0.1µM	0.01µM	0 µM	100µM	10µM	1µM	0.1µM	0.01µM
H	0µM	100µM	10µM	1µM	0.1µM	0.01µM	0 µM	100µM	10µM	1µM	0.1µM	0.01µM

Figure 8: Experimental Design for Estrogen Responsiveness using DMSO

Figure 9 shows the spectrophotometer results for each well in the testing plate outlined in Figure 8. It can be seen that the empty wells gave very similar readings, meaning the spectrophotometer performed correctly.

	10µL of DMSO Stock Solution per Well						1µL of DMSO Stock Solution per Well					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.04	0.323	0.326	0.327	0.329	0.33	0.047	0.326	0.318	0.323	0.309	0.291
B	0.04	0.405	0.455	0.492	0.569	0.508	0.043	0.555	0.69	0.619	0.488	0.561
C	0.04	0.38	0.486	0.514	0.531	0.501	0.041	0.54	1.296	0.636	0.722	0.74
D	0.372	0.38	0.523	0.683	0.633	0.537	0.682	0.605	0.745	0.838	0.993	0.658
E	0.524	0.473	0.591	0.694	0.547	0.589	1.078	0.594	0.824	0.958	0.814	1.343
F	0.554	0.457	0.505	0.591	0.594	0.55	0.735	0.5	0.751	1.038	0.842	0.876
G	0.453	0.46	0.586	0.474	0.501	0.676	0.732	0.609	0.747	0.829	0.879	0.957
H	0.359	0.446	0.582	0.617	0.599	0.535	0.799	0.6	0.805	0.753	0.842	0.643

Figure 9: Experimental Results for Estrogen Responsiveness Using DMSO

The spectrophotometer readings in Figure 9 were graphed to create Figure 10 showing the difference between using 10µL of DMSO and 1µL of DMSO in testing the phytoestrogen concentrations. Higher spectrophotometer results were produced using 1uL of DMSO, indicating it as a more viable option as the testing volume. However, estrogen responsiveness was seen at the lowest concentrations of estrogen.

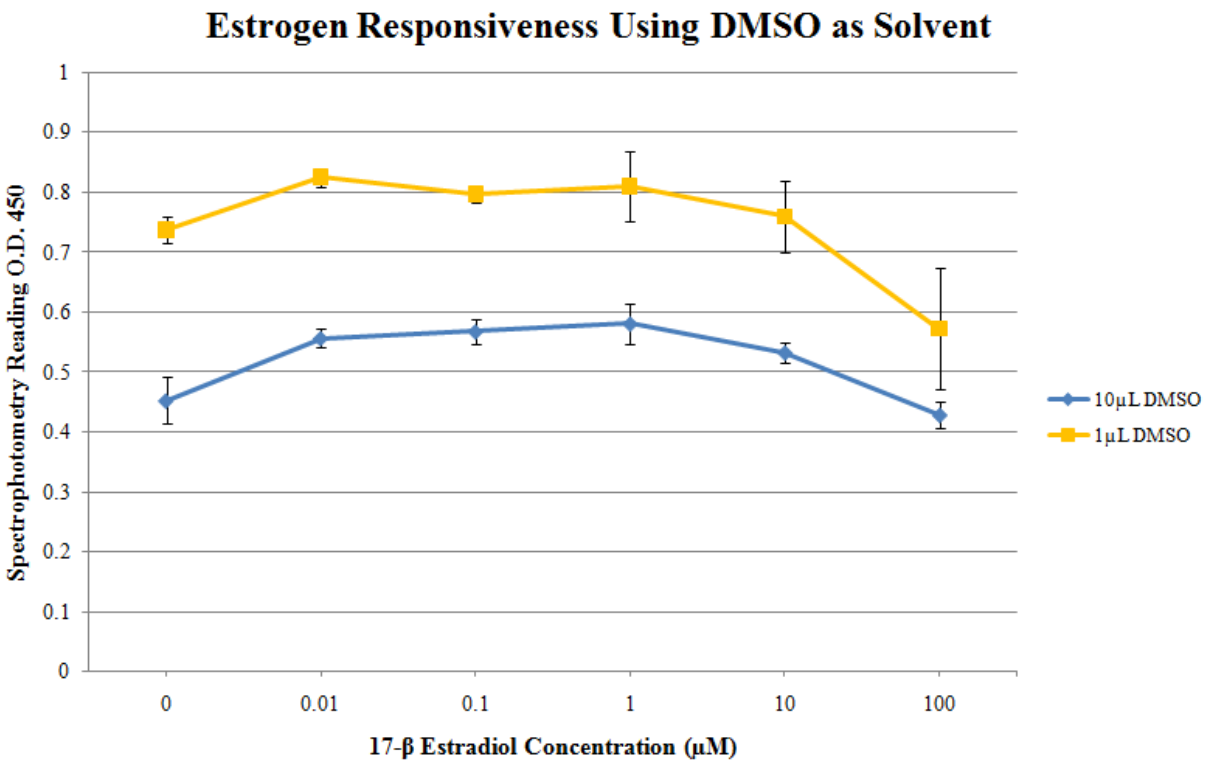


Figure 10: Graph of Results for Estrogen Responsiveness Test Using DMSO

Ethanol

As seen in Figure 11, 10µl and 1µl of ethanol were tested on the cells. 17β- Estradiol levels from only 0µM to 10µM were tested in both volumes of ethanol due to stock concentration.

	10µL of Ethanol Stock Solution per Well						1µL of Ethanol Stock Solution per Well					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A		No	Cells	→				No	Cells	→		
B		10µM	1µM	0.1µM	0.01µM			1µM	0.1µM	0.01µM		
C		10µM	1µM	0.1µM	0.01µM			1µM	0.1µM	0.01µM		
D		10µM	1µM	0.1µM	0.01µM			1µM	0.1µM	0.01µM		
E	0µM	10µM	1µM	0.1µM	0.01µM			1µM	0.1µM	0.01µM		
F	0µM	10µM	1µM	0.1µM	0.01µM		0µM	1µM	0.1µM	0.01µM		
G	0µM	10µM	1µM	0.1µM	0.01µM		0µM	1µM	0.1µM	0.01µM		
H	0µM	10µM	1µM	0.1µM	0.01µM		0µM	1µM	0.1µM	0.01µM		

Figure 11: Experimental Design for Estrogen Responsiveness Using Ethanol

Figure 12 shows the spectrophotometer results for each well in the testing plate outlined in Figure 8. It can be seen that the empty wells gave very similar readings, indicating the spectrophotometry was performed correctly.

	10µL of Ethanol Stock Solution per Well						1µL of Ethanol Stock Solution per Well					
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.042	0.368	0.325	0.32	0.318	0.04	0.04	0.303	0.309	0.304	0.04	0.041
B	0.046	0.363	0.371	0.401	0.386	0.051	0.041	0.584	0.474	0.611	0.046	0.039
C	0.044	0.352	0.388	0.429	0.402	0.049	0.045	0.59	0.498	0.474	0.038	0.038
D	0.043	0.374	0.372	0.371	0.388	0.044	0.055	0.77	0.639	0.435	0.041	0.043
E	0.394	0.356	0.372	0.376	0.397	0.042	0.049	0.678	0.685	0.896	0.044	0.044
F	0.426	0.37	0.365	0.413	0.469	0.05	0.846	0.738	0.688	0.638	0.04	0.047
G	0.384	0.358	0.388	0.401	0.488	0.046	0.69	1.145	0.976	0.931	0.045	0.047
H	0.539	0.419	0.469	0.517	0.56	0.044	0.749	0.749	0.421	0.687	0.039	0.041

Figure 12: Experimental Results for Estrogen Responsiveness Using Ethanol

The spectrophotometer readings in Figure 12 were graphed to create Figure 13; showing the difference between using 10µL or 1µL of ethanol in testing the phytoestrogen concentrations. Higher spectrophotometer results were produced using 1uL of ethanol as compared to 10µL. The cells did not appear to be estrogen responsive at any concentration.

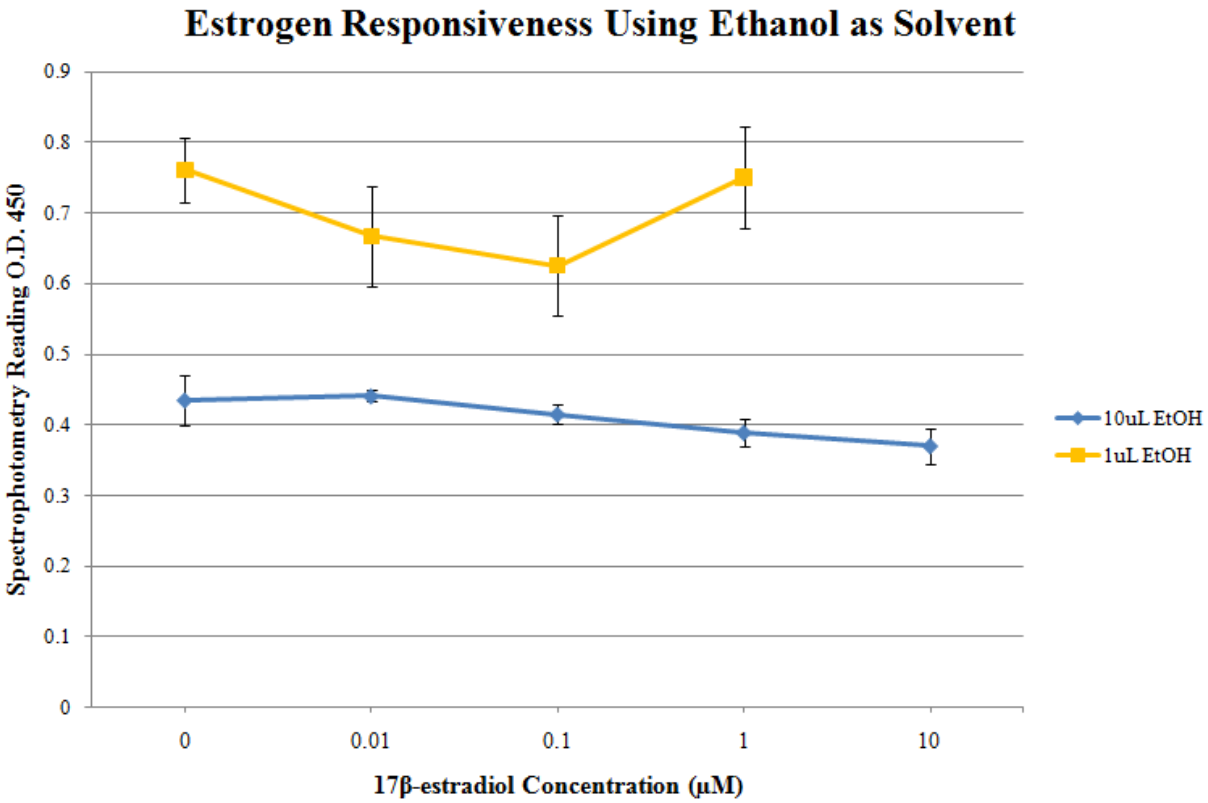


Figure 13: Graph of Results for Estrogen Responsiveness Test Using Ethanol

Phytoestrogen Testing

Biochanin A and formononetin were tested on T47D cells in 96 well plates in concentrations ranging from 0µM to 100µM. Biochanin was tested on two plates and formononetin was tested on three to provide a broad enough base to provide reliable conclusions from the data.

Biochanin A

Figure 14 shows the plate design for the biochanin A plates. Hormone levels of 0µM to 100µM were tested. The values achieved, seen in Figures 15 and 16, were complied to create the graph seen in Figure 17. Figure 15 shows the values for the first biochanin A hormone test. Some inconsistencies were observed in the blank wells seen in columns 1 and 12. Possible inconsistencies were also found in the test wells in the remaining columns. Figure 16 shows the values for the second biochanin A hormone test. Much more consistent values were achieved in the blank wells of columns 1 and 12 as compared to Figure 15. In Figure 17 proliferation was seen at the concentrations of 10µM and lower, as compared to the decrease in cell number of those treated with 100µM of biochanin A.

	1	2	3	4	5	6	7	8	9	10	11	12
A		No	Cells		→	→	→	→	→	→	→	
B		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.01µM	0.01µM	
C		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.01µM	0.01µM	
D		100µM	100µM	10µM	10µM	1.0µM	1.0.µM	0.10µM	0.10µM	0.01µM	0.01µM	
E		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.01µM	0.01µM	
F		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.01µM	0.01µM	
G		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.01µM	0.01µM	
H		0µM	0µM	0µM	0µM	0µM	0µM	0µM	0µM	0µM	0µM	

Figure 14: Experimental design for testing of Biochanin A

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.04	0.069	0.09	0.32	0.337	0.334	0.323	0.345	0.331	0.365	0.315	0.039
B	0.057	0.929	0.592	1.501	0.652	0.926	0.724	1.084	0.434	0.378	0.403	0.057
C	0.075	1.067	1.223	1.597	1.323	1.518	1.454	0.643	1.022	0.571	1.332	0.07
D	0.06	1.035	0.685	1.688	1.283	1.569	1.192	1.671	1.755	1.01	1.388	0.089
E	0.049	0.808	0.904	1.259	1.395	1.187	1.285	1.469	1.527	1.663	1.255	0.074
F	0.071	1.324	1.211	1.348	1.491	1.127	1.613	1.377	1.59	1.36	1.721	0.05
G	0.053	0.992	0.985	1.402	1.591	1.52	1.716	1.346	1.429	1.74	1.434	0.076
H	0.075	1.299	0.54	1.6	1.346	1.434	1.518	1.257	1.582	0.345	1.36	0.068

Figure 15: Test plate 1 for the Biochanin A

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.04	0.333	0.344	0.336	0.34	0.332	0.339	0.333	0.338	0.339	0.333	0.04
B	0.039	0.609	0.624	1.217	1.366	1.102	1.139	1.347	1.16	0.992	1.033	0.04
C	0.044	0.614	0.634	1.392	1.263	1.253	1.053	1.471	1.089	1.092	1.011	0.042
D	0.045	0.606	0.651	1.292	1.368	1.204	1.238	1.291	1.218	1.073	1.122	0.06
E	0.042	0.632	0.655	1.056	0.951	1.228	1.665	1.416	1.072	1.292	1.097	0.049
F	0.043	0.641	0.659	1.304	1.167	1.176	1.151	1.157	1.025	1.125	1.034	0.065
G	0.046	0.662	0.722	1.143	1.247	1.162	1.121	1.013	1.248	1.468	1.304	0.045
H	0.085	0.842	0.785	0.785	0.795	0.797	0.963	1.096	1.111	1.477	1.233	0.082

Figure 16: Test plate 2 for the Biochanin A

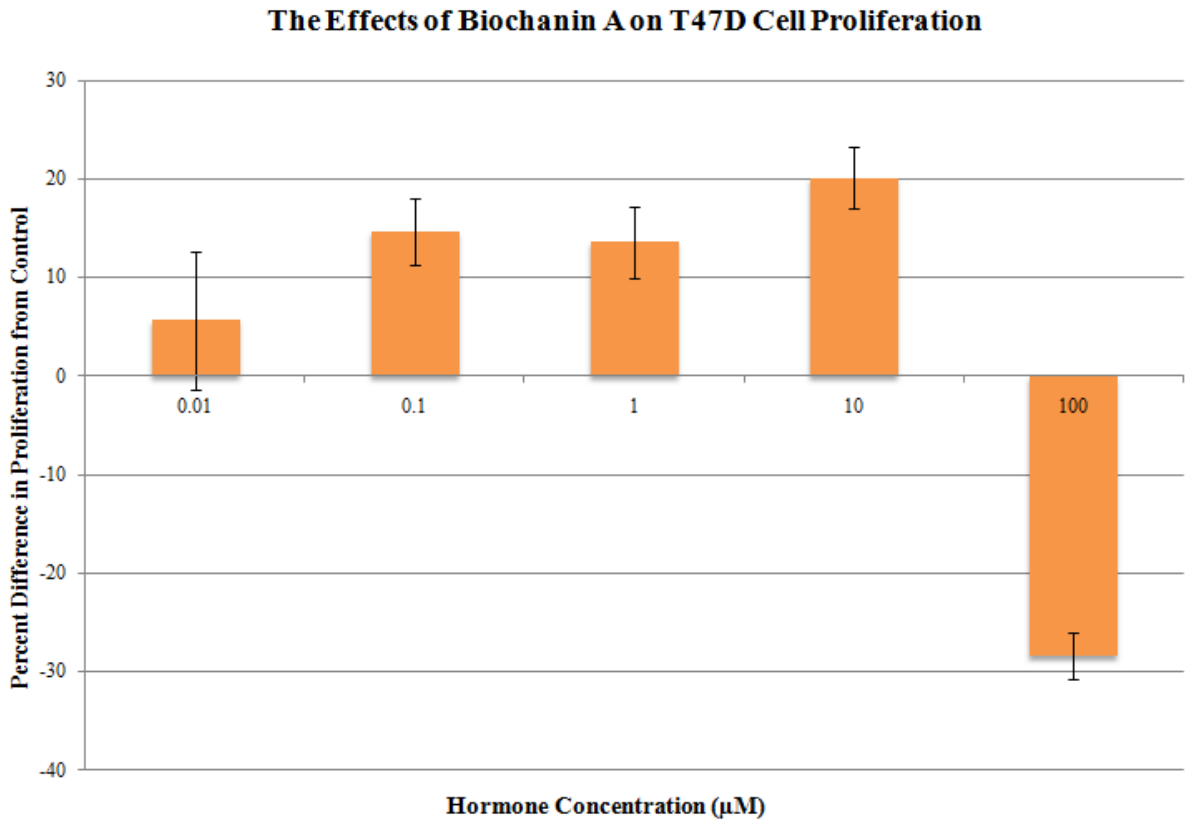


Figure 17: Graphed spectrophotometer readings for Biochanin A plates graphed as percent difference from the control

Formononetin

Figure 18 shows the plate design for the formononetin plates. Hormone levels of 0µM to 100µM were tested. The values achieved, seen in Figures 19 through 21, were used to create the graph seen in Figure 22.

	1	2	3	4	5	6	7	8	9	10	11	12
A		No	Cells	→								
B		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.0100µM	0.01µM	
C		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.0100µM	0.01µM	
D		100µM	100µM	10µM	10µM	1.0µM	1.0.µM	0.10µM	0.10µM	0.0100µM	0.01µM	
E		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.0100µM	0.01µM	
F		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.0100µM	0.01µM	
G		100µM	100µM	10µM	10µM	1.0µM	1.0µM	0.10µM	0.10µM	0.0100µM	0.01µM	
H		0µM	0µM	0µM	0µM	0µM	0µM	0µM	0µM	0µM	0µM	

Figure 18: Experimental design for testing of Formononetin

Figure 19 shows the first values for the formononetin hormone test. Consistent values achieved in the blank wells of columns 1 and 12 indicate a properly run assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.04	0.36	0.611	0.342	0.348	0.348	0.322	0.355	0.333	0.34	0.326	0.04
B	0.056	1.263	0.982	1.21	0.542	0.394	0.395	0.422	0.401	0.4	0.528	0.046
C	0.06	1.217	1.458	1.527	0.436	0.404	0.385	0.348	0.4	0.378	0.396	0.05
D	0.052	1.282	1.389	1.492	1.196	0.881	0.684	0.381	0.398	0.395	0.389	0.05
E	0.069	1.243	1.442	1.66	1.544	1.706	1.715	0.422	0.434	0.573	0.458	0.05
F	0.056	1.159	1.525	1.778	1.676	1.609	1.599	0.617	0.41	1.213	1.397	0.06
G	0.052	1.672	1.359	1.572	1.788	1.738	1.684	1.309	0.83	1.506	1.249	0.065
H	0.051	0.411	0.431	0.374	0.406	0.407	0.37	0.411	0.416	0.483	0.422	0.055

Figure 19: Test plate 1 for Formononetin

Figure 20 shows the second values for the formononetin hormone test. Consistent values achieved in the blank wells of columns 1 and 12 indicate properly run assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.04	0.364	0.359	0.358	0.351	0.356	0.354	0.364	0.357	0.356	0.355	0.04
B	0.048	0.939	0.909	0.978	1.12	1.244	1.318	0.821	1.272	1.797	0.872	0.041
C	0.053	0.941	1.018	1.115	1.022	0.974	0.907	0.923	1.245	0.87	1.052	0.042
D	0.045	0.989	1.118	1.199	1.101	1.033	1.364	1.304	1.018	1.393	0.958	0.038
E	0.056	1.073	1.076	1.219	1.102	1.157	0.998	1.156	0.88	1.012	1.119	0.04
F	0.041	1.025	1.172	1.176	1.125	1.083	1.145	1.357	0.958	1.007	1.137	0.047
G	0.043	0.998	1.127	1.125	1.118	1.136	1.082	1.293	0.86	0.922	1.163	0.044
H	1.048	1.053	1.042	1.049	1.098	1.218	1.117	1.207	1.77	0.932	0.947	1.116

Figure 20: Test plate 2 for Formononetin

Figure 21 shows the third values for the formononetin hormone test. Some inconsistencies were observed in the blank wells seen in columns 1 and 12. Possible inconsistencies also found in the test wells in the remaining columns.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.035	0.345	0.357	0.343	0.057	0.057	0.059	0.056	0.058	0.057	0.057	0.035
B	0.042	0.92	0.966	0.998	0.994	0.814	1.094	1.042	1.103	1.212	0.992	0.039
C	0.041	0.996	1.155	1.038	1.217	1.207	1.302	1.007	1.221	0.967	1.289	0.04
D	0.047	0.943	0.915	1.076	1.148	1.048	1.024	1.175	1.07	0.993	0.964	0.041
E	0.047	0.765	0.743	1.037	0.851	0.948	0.918	0.914	0.842	0.849	0.893	0.048
F	0.044	0.744	0.748	0.825	1.013	0.946	0.923	0.973	0.884	0.784	0.861	0.045
G	0.052	1.17	0.733	0.692	0.787	0.832	0.816	0.711	0.901	0.796	0.851	0.048
H	0.055	0.71	0.686	0.71	0.647	0.689	0.647	0.398	0.791	0.774	0.21	0.046

Figure 21: Test plate 3 for Formononetin

Figure 22 shows the compiled and graphed data of the formononetin plates. No discernable pattern is evident in the cells response to the hormone stimulus.

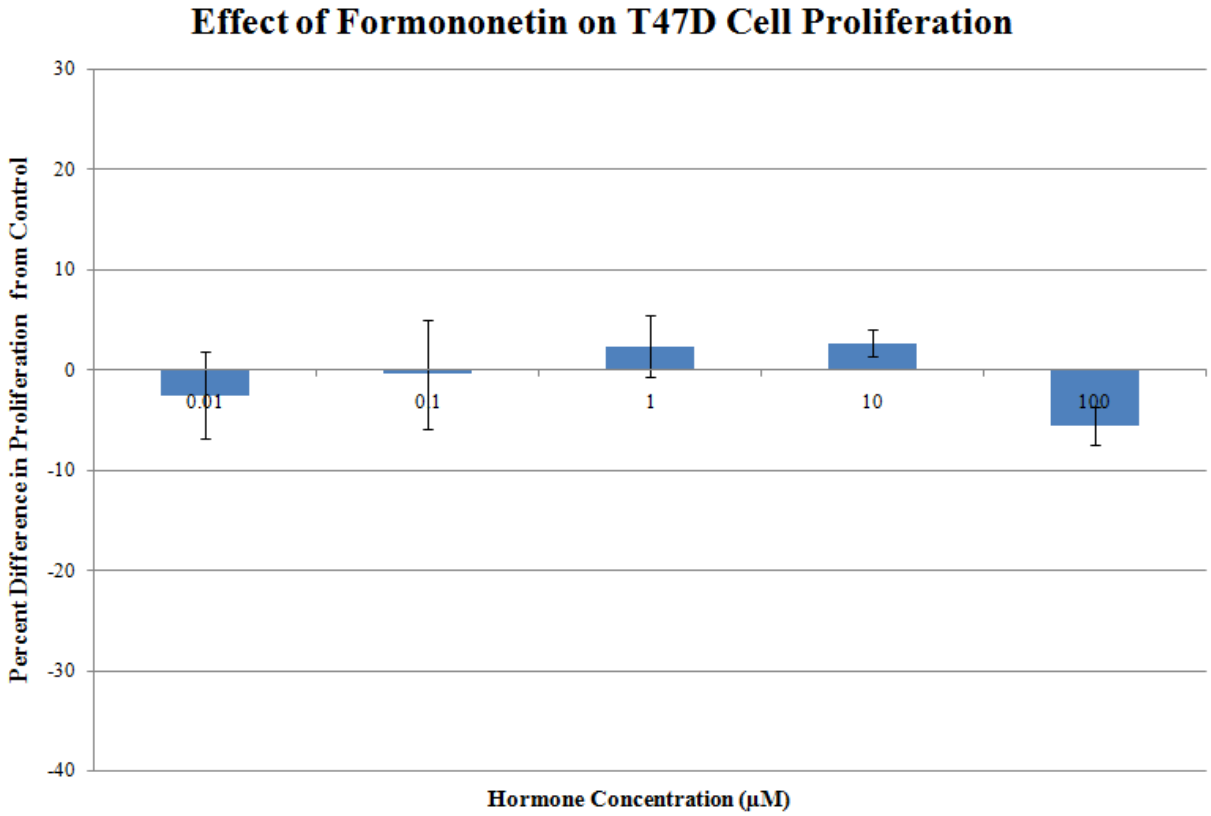


Figure 22: Graphed spectrophotometer readings for Formononetin plates graphed as percent difference from the control

Immunoblotting for PCNA

In an effort to validate the results collected via MTS assays, immunoblotting for PCNA was conducted. Dark bands were observed, as seen in Figure 23. Analysis of the bands was done based on color intensity with the ImageJ computer program, as seen in Figures 24 and 25. Further analysis of the formononetin bands could not be done due errors with the gel.

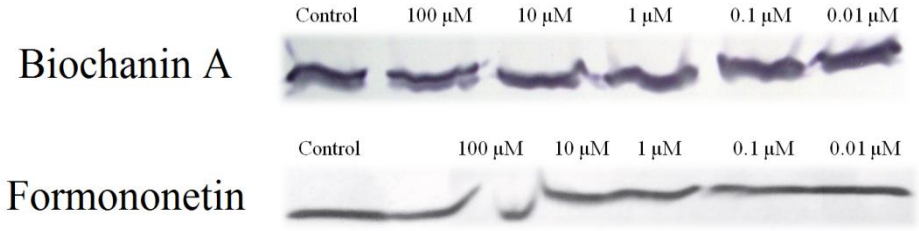


Figure 23: Immunoblot of PCNA of Biochanin A and Formononetin treated cells

Concentration (µM)	Area Sampled (Pixels)	Sample 1	Sample 2	Sample 3	Average	Standard Deviation of the Mean
0	16640	111.121	111.151	110.842	111.038	0.098382
0.01	16640	96.238	97.827	97.534	97.19967	0.488216
0.1	16640	93.199	93.437	93.674	93.43667	0.137121
1	16640	84.614	84.475	84.129	84.406	0.144195
10	16640	82.7	83.494	82.353	82.849	0.337699
100	16640	92.346	92.508	93.604	92.81933	0.395111

Figure 24: Values collected via ImageJ for Biochanin A PCNA bands

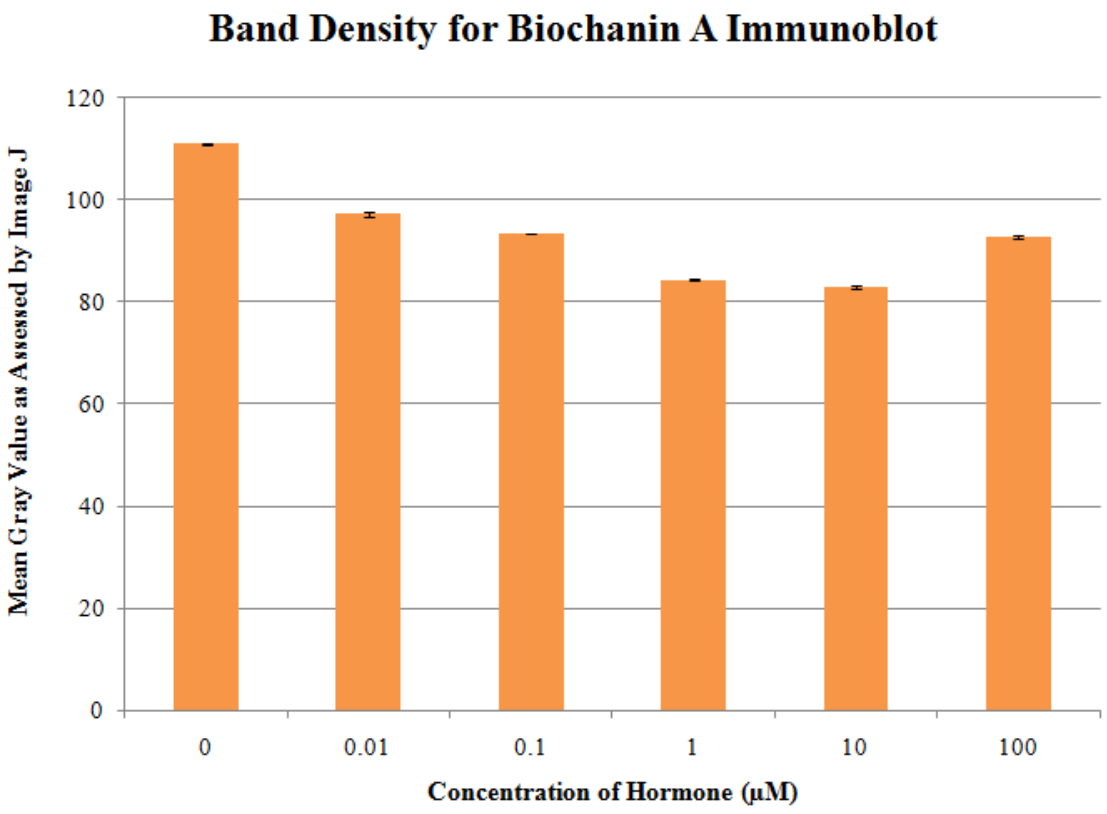


Figure 25: Graphed mean gray value assessed by ImageJ showing different intensities of bands achieved via immunoblotting for PCNA

Densitometry values suggest that there may be a slight inhibitory effect of biochanin A on PCNA expression at all concentrations tested. This is not consistent with the trends seen for cell proliferation; however the immunoblotting was only done once, thus limiting any significant interpretation of the results.

Discussion

The goal of this project was to determine if the phytoestrogens biochanin A and formononetin had proliferative effects on the T47D human ductal carcinoma cell line. Based on a thorough literature review, it was hypothesized that both of the tested phytoestrogens would cause proliferation at concentrations less than 10µM, and inhibit at higher concentrations such as 100µM.

This project was a continuation of previous phytoestrogen projects completed at WPI. A review of methodologies from these projects prompted the examination of each part of the experimental design: cell line estrogen responsiveness, proliferation assay, hormone solvent, and hormone concentrations were all verified. The T47D cell line was chosen because the MCF7 cell line owned by WPI had been shown unresponsive to estrogen (30).

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) was chosen because it was not radioactive like other assays, simple to use, and readily repeatable. A stock of assay was available from previous experiments, and needed to be validated prior to experimentation. On the principal that the MTS assay relates the relative amount of viable cells in a population, it can be validated by application to increasing cell concentrations. Figure 7 displays the relative linearity in absorbance readings, following increasing cell concentration. This plate also served as a test for optimal cell seeding density. Wells were inspected for confluence at the assay time point, and it was determined that a seeding density of 20,000 cells per well was sufficient as it would allow for a range of readings within the linear range of the assay as cell density changed with hormone treatment.

In creating the experimental design for this project, an appropriate hormone solvent was investigated. Both DMSO and ethanol were utilized equally in the literature, and both are cytotoxic. Also, the Promega protocol for the MTS assay states that strong reducing substances like DMSO can reduce tetrazolium salts nonenzymatically and lead to increased background absorbance values. Because of these concerns, an experiment was conducted to determine the optimal solvent, and concentration of that solvent that T47D cells could tolerate without affecting the MTS assay.

In an effort to maximize results from this experiment, estrogen responsiveness of the T47D cell line was also tested. A preparation of 100µM estrogen in ethanol was already available from a concurrent MQP project, and a stock solution of 10,000 µM of estrogen in DMSO was created. It was found that ethanol at 10µL per well or 10% of final volume, was cytotoxic and produced very low spectrophotometric readings. Also, 1µL of ethanol produced highly variable results, and no estrogen responsiveness was observed in either case. Figure 13 shows the negative results of the estrogen-ethanol test. In contrast, estrogen responsiveness was demonstrated with DMSO at both 1% and 10% total volume. Figure 10 illustrates how the T47D cells increased proliferation from 0.01µM to approximately 10µM. These results also confirmed that DMSO did not act as a significant reducing agent. If DMSO

was effectively reducing the tetrazolium compound in the MTS assay, the wells with 10% DMSO would be expected to show higher readings than those wells with 1% DMSO. This was not the case as 1% DMSO produced higher readings. DMSO was chosen as the hormone solvent for this project because it was the least toxic to cells at a concentration of 1%.

In order to determine the proliferative effects of biochanin A and formononetin, the MTS assay and a PCNA assay were utilized. Figure 17 shows the averaged effects of both MTS assays for the effects of biochanin A. Biochanin A increased T47D cell proliferation at concentrations between 0.01µM and 10µM. This proliferative effect was significantly greater in the 10µM concentration than the 0.01µM populations, and cannot be accounted for by standard deviation of the mean. At 100µM however, proliferation decreased greatly from the control.

The same test was conducted with formononetin, and repeated three times. The first plate, shown in Figure 19 had significant errors. Many wells in each concentration failed to develop, including wells in the control lane. A reasonable explanation for this error was not found, so the plate was excluded from analysis. The second plate developed as expected, and results are shown in Figure 20.

Error also occurred with the third plate. During the change to clear media, the control wells were left without media for approximately fifteen minutes in the incubator. This resulted in greatly decreased readings in the control from all other wells. Because of this laboratory error, it was impossible to have an accurate control for that plate, and it was excluded. However, the results for the hormone concentrations in this plate mirrored those results found in the second plate. Overall, both plates showed no increased or decreased proliferation between all formononetin concentrations and the control.

Though many studies support the idea that formononetin causes proliferation (28) (29) (25), others have found results similar to the ones presented here. In a large study of phytoestrogens, one of the only phytoestrogen that could not induce MCF7 cell proliferation was formononetin. The authors actually suggested that formononetin was toxic to the cells at the tested concentrations. They also found that biochanin A increased estrogen responsive MCF7 cell proliferation at concentrations less than 10µM, but inhibited at concentrations greater than 25µM (31). These results mirror those found in this study.

Another interesting study which used very similar assay methodology to this project also found comparative results. T47D and MCF7 cells were exposed to concentrations of biochanin A and formononetin ranging from 0.01µM to 10µM in 1% DMSO. They were assayed for proliferation using Alamar Blue, and estrogenic activity using a luciferase reporter construct. In the MCF7 cell line, the scientists found no concentration of formononetin under 100µM could stimulate proliferation. Biochanin A, however, caused the same proliferative effects as 10 or 100µM of estradiol at 0.2µM and 0.3µM respectively. This shows that in MCF7 cells, formononetin has no proliferative effect, but biochanin has very high proliferative capabilities (32). These results are also similar to the ones presented above.

Umehara et al repeated the same testing with the T47D cell line. Formononetin was found to have some proliferative effects: only 0.03 µM was needed to produce the same results as 10µM estradiol. However, no amount of formononetin could still produce effects similar to 100uM estradiol (32). This change in the effects of low levels of formononetin seemingly conflicts with their MCF7 results. However, these results can be explained by the differences in the two cell lines. MCF7 cells have become notorious recently as a line that has become highly variable due to laboratory selection. If we assume that the T47D cell line used by Umehara has retained estrogen responsiveness, and the MCF7 line has become unresponsive, it would point to biochanin A working through an estrogen receptor independent pathway, and formononetin working through the estrogen receptor.

Despite the consistent and repeatable results garnered from the MTS assay, PCNA data contradicted the original findings. The immunoblot showed, with little analytical error, that biochanin A inhibited PCNA expression in a dose dependent manner from 0.01 µM to 10 µM, and then showed slightly elevated but still inhibitory effects at 100µM. This shows an almost inverse relationship to the MTS data. PNCA has been used consistently to classify various types of breast cancer in *vivo* with great accuracy for poor prognostic cases (33) (34). It is a well known and trusted mode of assaying for cell proliferation. However, authors have found that that in certain breast cancers, the relationship between PCNA expression and cell proliferation is lost, but is increased in tissues adjacent to tumors (35). This could be a possible explanation for the discrepancy between the MTS assay and PCNA assay results. More testing would be needed to verify this.

The formononetin PCNA immunoblot had significant errors such that it could not be analyzed. The wells did not form correctly, and so samples ran together before voltage was applied. Also, air bubbles were trapped in the gel and distorted two of the possible bands. It is proposed that both of these gels should be interpreted with caution as protein normalization through laboratory means could not be undertaken as all samples were combined with sample buffer immediately post-aspiration of media.

For subsequent projects, it is suggested that concentrations of phytoestrogens between 10µM and 100uM be tested in addition to the ones used in this study. For biochanin A, this would help determine the exact concentration at which proliferation becomes inhibited. It is also suggested that a normal breast biopsy, possibly from a reduction surgery, should be used as a control for normal cells. These would not be identical to the cancer cells, but would serve as a control, allowing researchers to determine the effects of phytoestrogens not only on cancer cells, but also on healthy breast tissue. The final suggestion is a replication of the PCNA immunoblot as only one out of four gels developed for each hormone tested.

In conclusion, Biochanin A induces T47D cell proliferation from concentrations of 0.01µM to 10µM, and inhibits at 100µM. Formononetin has no affect on proliferation at any of the tested concentrations. If these results are statistically significant, they have important implications for over the

counter hormone replacement therapies. They indicate that the effects of certain phytoestrogens can be dose dependent, or devoid of estrogenic activity. Because there is little consistency between products and even lot numbers of the same product, women may be receiving different concentrations than the product labeling indicates. Also, if one hormone is more estrogenic than the other, the same variability will result. So, despite their "natural" label, women should be skeptical and discerning when it comes to over the counter phytoestrogen therapies.

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