Effects of Genistein in Over-the-Counter Phytoestrogen Supplements on Ovarian Cancer Cells

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Abstract

β-Estradiol hormone replacement therapy (HRT) is used by perimenopausal and postmenopausal women to alleviate menopausal symptoms. HRT can upregulate cell proliferation in estrogen responsive tissues. We focused on using over-the-counter (OTC) phytoestrogen supplements that are high in genistein as an alternative to HRT. Using ovarian cancer cells OVCAR-3, we exposed the cells to various OTC phytoestrogen supplements, and performed cell proliferation assays to measure the response. We found OTC phytoestrogens had an antiproliferative effect on the OVCAR-3 cells, but there is no correlation between the amount of genistein and the strength of the antiproliferative effect of the phytoestrogen. In conclusion, we propose taking phytoestrogen supplements as an alternative to β-Estradiol HRT.

Acknowledgements

We would like to thank our project advisors, Professors Jill Rulfs and Mike Buckholt, who were instrumental in getting our project set up. Despite all of the experimental hiccups we faced, they always there to patiently support us. We would also like to thank Mike Boca for keeping us stocked with all of the cell culture supplies we needed throughout the year.

Introduction

Ovarian cancer is one of the most commonly occurring cancers in women, affecting approximately one in every sixty women, most of whom are over the age of 60 (Khon 2015). This cancer can manifest with a number of uncomfortable symptoms, mainly gastrointestinal or abdominal in nature (Furuya 2012). Often by the time the patient is symptomatic, the cancer is already in the late stages, which are more challenging to treat. Ovarian cancer originates from mutations in various ovarian cell types, and can vary in both the rate of proliferation and rate at which it metastasizes (Goff et al. 2007). For the purpose of this MQP, we focused on ovarian cancer of the epithelial cells in the ovary.

The most common type of ovarian cancer, epithelial ovarian cancer accounts for nearly 90% of all diagnosed cases. It is a particularly aggressive form of ovarian cancer, causing the highest number of gynecologic cancer related deaths in the United States at approximately 14,000 deaths per year. While this form of cancer can be caused by a mutation in the epithelial cells, researchers also noted that estrogen has proliferative effects on the cells (Kang et al. 2001).

Women most commonly diagnosed with epithelial ovarian cancer tend to be either perimenopausal or postmenopausal, with more than 80% of cases occurring in women over the age of 40. Many of these women would be experiencing symptoms of menopause, which may be relieved by taking estrogen in a treatment known as hormone replacement therapy. When women go through menopause, their bodies experience a significant drop in estrogens levels, which causes many uncomfortable symptoms that may be alleviated through the addition of supplemental estrogen treatments. However, studies have shown an increased risk of ovarian cancer in postmenopausal women undergoing estrogen replacement therapy (Rodriguez et al. 2001). A potential solution to relieving menopausal symptoms without increasing the likelihood of developing ovarian cancer could be found in over-the-counter (OTC) phytoestrogen products.

Estrogen

Estrogen is a naturally occurring steroidal hormone that is synthesized from androgen, a hormone responsible for sexual development. Estrogen plays a key role in the development and regulation of many structural aspects of the female anatomy, including primary and secondary sex characteristics, skin, fat deposits, and bone formation (Li et al. 2014). In order to for estrogen to act on a cell, it binds with an estrogen receptor, which is located either intracellularly or on the membrane (Li et al. 2014). Because it is a steroid, estrogen is able to diffuse across the cell membrane to reach intracellular estrogen receptors as well as the membrane receptors. Once estrogen binds to the receptor, the receptor undergoes a conformational change allowing the complex to enter the nuclear membrane and bind to a specific DNA locus, the estrogen response element, activating gene transcription (Kang et al. 2001). For our purposes, we are studying the response of estrogen receptor β (ERβ).

Many varieties of estrogen exists naturally in the body, however 17β-estradiol is the most prominent estrogen in terms of usage and binding affinity to receptors. This hormone is responsible for the regulation of female sex organs, namely the ovaries. In relation to ovarian cancer, it's been shown that 17β-estradiol exerts antiapoptotic effects on ovarian cancer cells (Ho 2003).

Menopause

In menopausal women, the circulating estradiol level in the bloodstream decreases significantly due to the depletion of oocytes in the ovaries (Ho 2003). However, the decrease of estradiol is not linear; systemic estradiol levels fluctuate dramatically throughout menopause, especially in premenopausal women when levels tend to spike. Thus, hormone replacement therapy (HRT) is not typically started until later in the menopausal process, most notably in postmenopausal women. While HRT is used to alleviate symptoms of menopause, such as hot flashes, and prevent long term effects like osteoporosis and vaginal atrophy, it also increases the risk of developing breast or ovarian cancer (Ho 2003). Phytoestrogens are a potentially feasible

alternative to HRT as they can also help alleviate symptoms of menopause and are found naturally in various plant types and are available OTC in tablets or capsules.

Phytoestrogen

Phytoestrogens are plant-based compounds that have estrogenic properties. These compounds have most notably been extracted from soy, but also from other plants such as red clover and black cohosh root. Phytoestrogens are able to interact directly with human estrogen receptors, which has led to the creation of an industry based on dietary phytoestrogen (Hwang et al. 2013a). One type of phytoestrogen, genistein, is widely found in OTC phytoestrogen supplements as it is the primary type naturally produced in soybeans.

Genistein has been shown to have antiproliferative effects on estrogen receptor-positive cancers, such as certain ovarian cancers (Hwang et al. 2013a). In a study by Kim et. al. in 2014, the chemotherapeutics effects of genistein on various cancers in animal models was studied (Kim et al. 2014). The researchers concluded that genistein acted as an estrogen antagonist at certain dosages against prostate cancer, and that this phytoestrogen may have agonistic effects depending on the type of cancer being affected. Another study performed by Hwang et. al. specifically tested the effects of genistein on BG-1 ovarian carcinoma cells, specifically because the cells were know to proliferate in the presence of 17β-estradiol (Hwang et al. 2013b). In this study, the researchers determined that by triggering apoptotic signaling cascades, genistein had effective antiproliferative effects on the cancer cells. Many of the phytoestrogen supplements used in this study contain genistein, suggesting that there is the potential for suppression of cell growth in the ovarian cancer cell line.

Previous Major Qualifying Projects (MQPs)

Previous MQP projects focused on the effects of OTC phytoestrogen products on breast cancer cell proliferation, but we refocused the phytoestrogen project to examine the effects genistein in OTC phytoestrogen products on the proliferation of ovarian cancer cells. Of the OTC phytoestrogen products previously chosen to be studied, only Promensil has consistently shown

evidence of anti-proliferative effects on breast cancer cells (Gergel et al. 2010). The primary chemical compounds found in Promensil are biochanin A and formononetin, which may be metabolized to genistein and daidzein respectively. These compounds have been the focus of a number of previous MQP studies, but only biochanin A has been shown to have significant effects on the breast cancer cells (Gergel et al. 2010). One such study showed that with higher concentrations of biochanin A (>10μM), cell proliferation is inhibited, but may be promoted at lower concentrations (<10μM) (Gergel et al. 2010). Given the particularly promising data acquired from previous MQPs, genistein was chosen for further investigation.

Ovarian Cancer Cell Line

Based on our research on estrogen and estrogen receptors in relation to phytoestrogens, we studied the effects of phytoestrogen on the ovarian cancer cell line OVCAR-3. This cell line derives from an adenocarcinoma on the epithelial tissue on the ovary of a 60-year-old female. OVCAR-3 is an adherent cell line that has estrogen and androgen receptors, making it ideal for evaluating hormone treatments (Hamilton et al. 1983). Containing high amounts of both ERα and ERβ, OVCAR-3 is especially sensitive to fluctuations in estrogen levels, specifically 17β-estradiol, which has been shown to stimulate growth relative to the dose of 17β-estradiol administered (Kang et al. 2001).

Methodology

Cell Culturing

OVCAR-3 cells were obtained from Sigma-Aldrich Corp. The cells were cultured in DMEM (with 4.5 g/L glucose and sodium pyruvate without L-glutamine, obtained from Corning Cellgro) supplemented with 20% Fetal Bovine Serum (FBS) obtained from Equinox, 10 µg/mL bovine pancreatic insulin, and 1% Penicillin Streptomycin (Pen/Strep) and incubated at 37°C and 5% CO_2. These cells were split every 2 to 4 days at no more than 1:2 or 1:3 following a 5 minute to 8 minute trypsinization.

Reflux

Extractions were performed on each of the OTC phytoestrogen products. Supplements were ground with a mortar and pestle, and each supplement was added to a 250 mL round bottom flask with 80 mL of methanol as the solvent. The sample was then refluxed using a water bath at 68°C for one hour. The refluxed samples were then filtered using Grade 3 Whatman filter paper with a 6µm pore size and stored at -20°C.

Phytoestrogens

The main active ingredient is listed on each of the phytoestrogen products, which would either be the plant name or the specific isoflavone that was sampled. As these products are not regulated by the Food and Drug Administration, most of the products did not clearly list the isoflavones utilized and in what amounts. As such, we decided to reflux the phytoestrogens based on the dosage on the packaging, adjusted to use the recommended daily dosage if it was not clearly stated.

Promensil

A one month supply of double strength Promensil was obtained from the company Real Health Laboratories. Two tablets, obtained from from lot #16156B, containing 160 mg of isoflavones combined were crushed using a mortar and pestle prior to being added to the round bottom flask. This was double the recommended daily dose of one tablet per day, due to the size of the tablet and the expectation.

Black Cohosh (Estroven)

One caplet, obtained from Novasoy Product # 16218EKM15, containing 56 mg of isoflavones was emptied directly into the round bottom flask and allowed to dissolve. This was the recommended daily dose.

Black Cohosh (Nature's Way)

Three capsules, obtained from Batch # 20006136, containing 540 mg of Black Cohosh root each were emptied directly into the round bottom flask. The recommended dosage was one capsule three times daily.

Soy Isoflavones (PipingRock)

Three capsules, obtained from Product # 08277, containing 13 mg of isoflavones each were emptied directly into the flat bottom flask. The serving size was one capsule, and it was recommended to take one capsule three times daily.

Red Clover Liquid Extract (Herb Pharm)

One hundred and sixty drops of extract containing 2,660 mg of Red Clover leaf and flower was added directly to the round bottom flask. The recommended daily dosage was 40 drops at four separate times.

Red Clover Capsules (Wild Harvest)

Three capsules, obtained from Lot # 104214RCL, containing a total 1050 mg of organic Red Clover was emptied directly into the round bottom flask. This was the recommended daily dosage.

Yellow Soybeans / Black Soybeans

Approximately ¼ cup of dry soybeans for both types was first crushed using a mortar and pestle. Then the crushed soybeans were added to the round bottom flask with the methanol. This total amount was used as it was the serving size listed on the packages the soybeans were sold in.

High Performance Liquid Chromatography (HPLC)

Following the initial filtering, 2 mL of each sample was further filtered using a 0.2 μm filter. Each HPLC run sampled either 20 μL or 100 μL of a given extract, depending on the purity of the sample, using an Agilent 1100 series HPLC system on column two (serial code SB-C18) at a flow rate of 1mL/min. The column was washed prior to running the extract using a 100% acetonitrile solution. The mobile phase, used to separate and quantify various components of the extracts, was a 10 mM ammonium acetate solution with 0.1% trifluoroacetic acid (TFA). The ammonium acetate (0.1% TFA) solution was eluted at 100% for 2 minutes, then decreased to 50% at a gradient from 2 to 24 minutes (Setchell et al. 2001). It was then run with 50% ammonium acetate (0.1% TFA) solution and 50% acetonitrile for 5 more minutes before returning to 100% ammonium acetate (0.1% TFA) solution (Setchell et al. 2001).

MTT Assay

The cells were plated in a CellTiter 96 well flat bottom plate at a density of 10,000 cells per well using 100 μL DMEM with 20% FBS, 10μg/mL insulin, and 1% Pen/Strep. After incubating for 24 hours to allow the cells to adhere to the plate, the media was removed and replaced with 100μL Phenol Red Free DMEM (PHRED, with 4.5g/L glucose and sodium pyruvate without L-glutamine, obtained from HyClone) with 20% FBS, 10μg/mL insulin, and 1% Pen/Strep. The

cells were given an additional 24 hours to equilibrate in PHRED.

Three ten-fold serial dilutions were made for a total of four concentrations (stock, 1:10 dilution (D1), 1:100 dilution (D2), and 1:1000 dilution (D3)) of each of the phytoestrogen extracts; Promensil, Estroven, Nature's Way, Women's Moon Cycle Tea, PipingRock, Herb Pharm, Wild Harvest, and both yellow and black soybeans. Dilutions were made in the same manner for 1mM β-estradiol dissolved in methanol and 15mM genistein dissolved in methanol. Following the secondary 24 hour incubation period, the PHRED was aspirated and replaced with new PHRED, at which point 1 µL of each sample (stock extracts and dilutions) were applied to the wells as depicted in Table 1. As it is the solvent for the samples, methanol was chosen as a control, as well as a media only control chosen to show baseline cell growth. The 1mM β-estradiol and the three ten-fold dilutions were used as a positive control. The cells were given 24 hours to incubate with the samples in the media.

		1	2	3	4	5	6	7	8	9	10	11	12
(-)	A	PHRED only	PHRED only	PHRED only	PHRED only	PHRED only							
Methanol	B	1µL Methanol	1µL Methanol	1µL Methanol	1µL Methanol	1µL Methanol							
(+)	C	1µL stock	1µL stock	1µL stock	1µL D1	1µL D1	1µL D1	1µL D2	1µL D2	1µL D2	1µL D3	1µL D3	1µL D3
Sample 1	D	1µL stock	1µL stock	1µL stock	1µL D1	1µL D1	1µL D1	1µL D2	1µL D2	1µL D2	1µL D3	1µL D3	1µL D3
Sample 2	E	1µL stock	1µL stock	1µL stock	1µL D1	1µL D1	1µL D1	1µL D2	1µL D2	1µL D2	1µL D3	1µL D3	1µL D3
Sample 3	F	1µL stock	1µL stock	1µL stock	1µL D1	1µL D1	1µL D1	1µL D2	1µL D2	1µL D2	1µL D3	1µL D3	1µL D3
Sample 4	G	1µL stock	1µL stock	1µL stock	1µL D1	1µL D1	1µL D1	1µL D2	1µL D2	1µL D2	1µL D3	1µL D3	1µL D3

Table 1. Standard Plating Layout*. 96 well plate set-up for the MTT Assay with OVCAR-3 cells. Cell-only and methanol were used as a negative control. The 1mM β-estradiol was used as the positive control. Each well contained 100 µL of PHRED and approximately 10,000 cells.*

Time trials were performed in order to get more accurate results in terms of the doubling time of the OVCAR-3 cells. Several 24 hours trials were performed, as were several 48-hour trials and two 72-hour trials. In each case, the media was changed every 24 hours and the phytoestrogen

treatments were reapplied as well. To perform the MTT assay, the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Lot #: 0000220603) was used. 20 µL of the reagent was added to each well, and the plate was incubated for an additional 4 hours. Then, a spectrophotometric plate reader was used to read the absorbance for each well at 570 nm. The absorbances from wells containing no cells were averaged and subtracted from the averages from each sample set well reading to normalize the data. The results are expressed as percent proliferation in relation to the negative control, or the normalizing number.

Results

High Performance Liquid Chromatography (HPLC)

The procedure was performed as described in the methodology section. We performed HPLC on the extract samples of Black Soybeans, Estroven, Promensil, Wild Harvest, and genistein. The chromatograms were compared to determine genistein concentrations within each.

Figure 1 shows the chromatogram of genistein, showing a peak at 22.68 minutes.

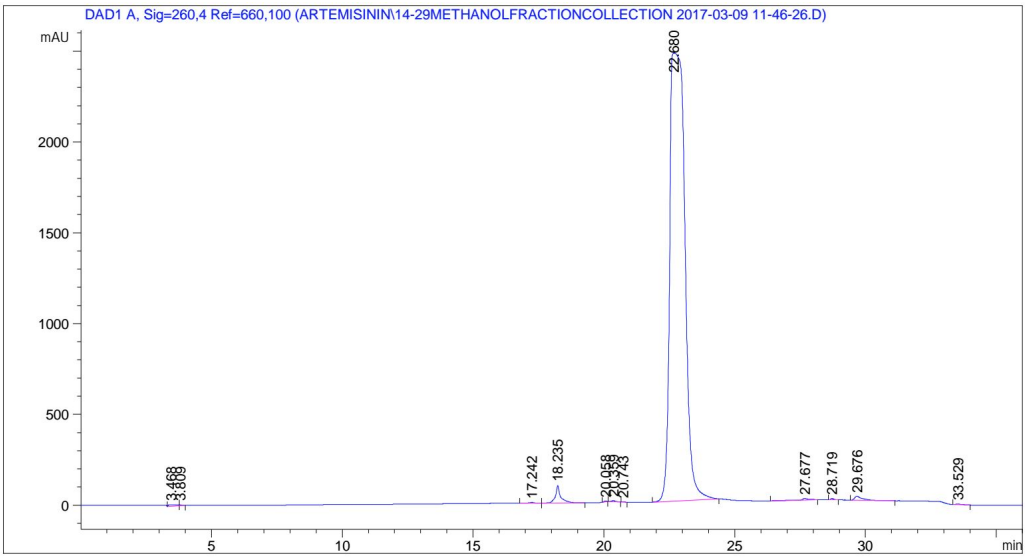

Figure 1. Chromatogram of genistein. *HPLC chromatogram of 2.7 ng of genistein in methanol. Time (min.) is shown on the x-axis and absorbance (mAU) is shown on the y-axis.*

Figure 2 shows the chromatogram of Wild Harvest, showing a peak at 22.55 minutes. This peak has an area of 6428.26 mAU*s and a percent area of 9.73. This means that nearly 10% of detectable peak area is from this peak, suggesting that the product may contain nearly 10% genistein. It is not an exact measurement, as the peak area of each detected compound depends on both the amount present and how well it absorbs at the detecting wavelength. This product showed the largest peak of all phytoestrogen products tested (refer to Table 2.)

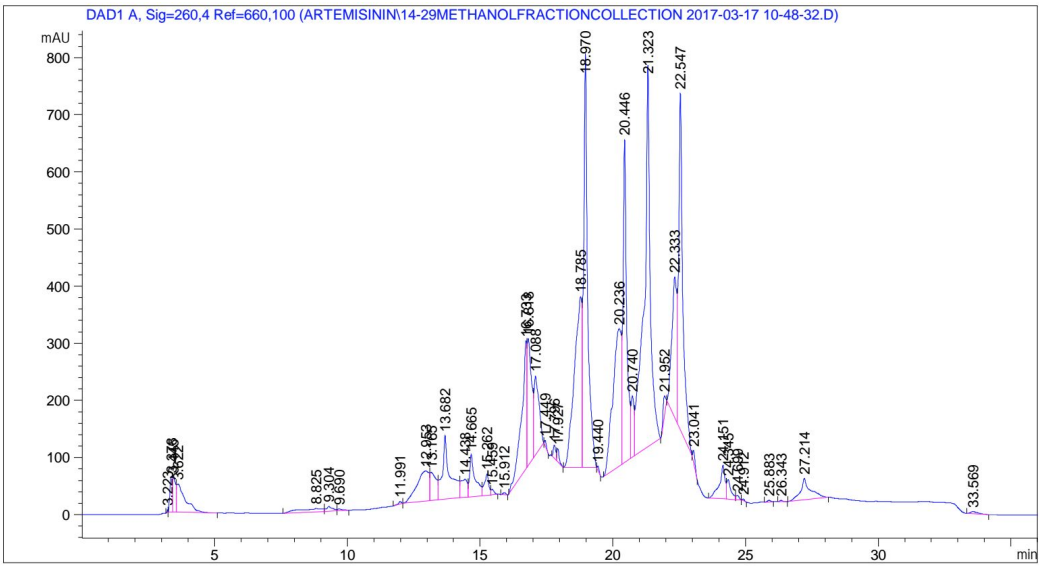

Figure 2. Chromatogram of Wild Harvest Extract. *HPLC chromatogram of 20µL of Wild Harvest extract in methanol. Time (min.) is shown on the x-axis and absorbance (mAU) is shown on the y-axis.*

Figure 3 shows the chromatogram of Black Soybean, showing a genistein peak at 22.68 minutes. This peak has an area of 747.29 mAU*s and a percent area of 0.55%.

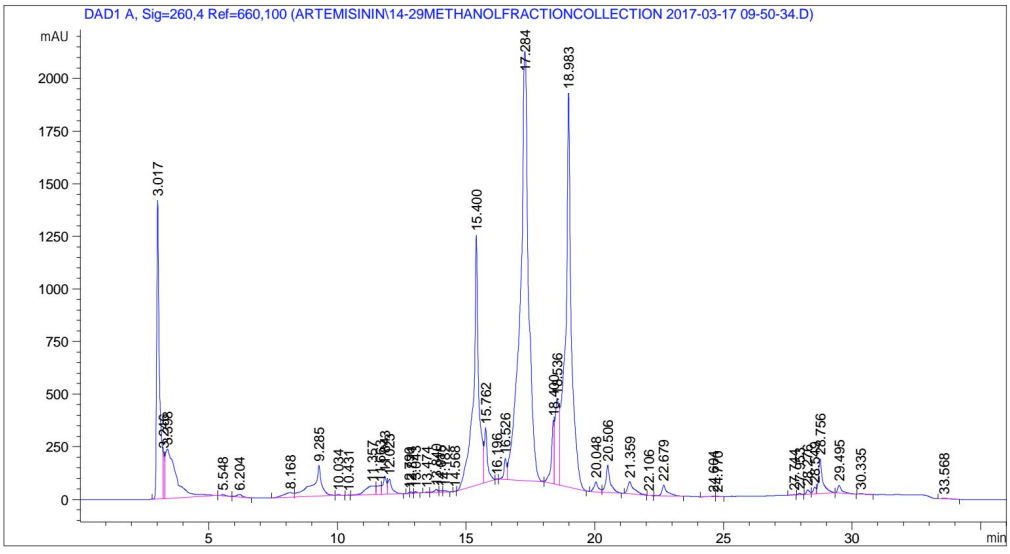

Figure 3. Chromatogram of Black Soybean Extract. *HPLC chromatogram of 50µL of black soybean extract in methanol. Time (min.) is shown on the x-axis and absorbance (mAU) is shown*

on the y-axis.

Table 2 shows genistein the relative mass in all of the phytoestrogen products. Genistein is shown at the top of the table for reference, and is shown to elute at 22.68 minutes. The genistein peaks in all other phytoestrogen products were determined by which peak appeared to be closest to that time point, so there is some variation, but all within approximately 30 seconds of one another. The mAU*s is the area per second, and the percent area is how much of a given peak makes up the total area of all detectable peaks. From this we see that Wild Harvest had the largest area under the genistein peak, and while black soybean did not have the smallest area under the peak, it was also closest to the elution time of genistein.

Product	Time (min.)	mAU*s	Percent Area
Genistein	22.68	98957	97.58 %
Black Soybean	22.68	747.29	0.55 %
Estroven	22.74	1018.3	0.62 %
PipingRock.com	22.61	408.57	0.42 %
Promensil	22.43	4219.22	2.25 %
Red Clover	22.66	244.42	0.60 %
Wild Harvest	22.55	6428.26	9.73 %
Yellow Soybean	22.75	2799.6	1.28 %

Table 2. Table of phytoestrogen products and amount of genistein. *The phytoestrogen products are listed alphabetically, and time genistein likely eluted, mAU*s, and percent area of total are shown.*

MTT Assay

The MTT assays were performed following the methods as described in the MTT assay methods section. The data were recorded every 24 hours over 72 hours. This time course experiment was run twice. Absorbance values are expressed as a percent of the negative control (cells in 1% methanol in PHRED). In Figures 4 - 6, the positive control, estrogen, is shown in blue. The three phytoestrogens shown in the figures are genistein, Wild Harvest, and black soybean, shown in red, green, and purple, respectively. The 4 dilutions tested are shown along the x-axis, the stock concentration is unknown and varies for each of the phytoestrogen products. The details of stock preparation can be found in Methods. Raw data from the MTT assays is shown in Appendix A.

Figure 4 shows the absorbance values expressed as percentage of the negative control. The 24-hour assay was run three times for genistein, Wild Harvest, and black soybean. There is only one data point for estrogen as we needed to change the concentration of the estrogen (from 10mM to 1mM). The data show cell proliferation of less than 70% in cells treated with either Wild Harvest or black soybeans. It should also be noted that the dilution series does not appear to show dose responsive trends for any of the products the cells were exposed to.

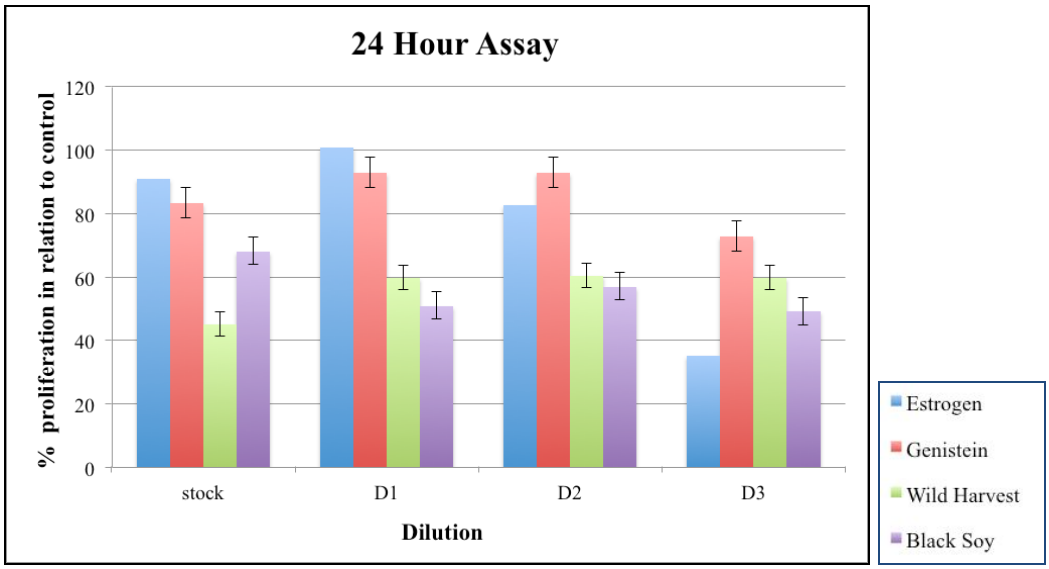

Figure 4. 24-Hour MTT Assay. *Percent of negative control shown on the y-axis. On the x-axis, estrogen stock was at a concentration of 1 mM and the stock genistein was at a concentration of*

5 mM. D1 is a 10-fold dilution, D2 is a 100-fold dilution, and D3 is a 1000-fold dilution. with 10-fold serial dilutions shown on the x-axis.

Figure 5 shows the absorbance values expressed as percentage of the negative control. The 48-hour assay was recorded three times for genistein and estrogen, once individually and twice as part of a 72-hour time course. Wild Harvest and black soybean were recorded only once as part of a 72-hour time course. The data show that nearly all of the cells treated with the phytoestrogen products and estrogen have a greater than 100% proliferation rate after 48 hours of incubation. We do not generally see any dilution effects for estrogen, genistein, or black soybeans, however, some dilution effects are observed for Wild Harvest. Here we see that exposure to stock Wild Harvest extract appears have a biphasic curve, showing a difference notable antiproliferative effect between the stock and 10-fold dilution, with a slight decrease with the 100-fold dilution. There is a notable increase in proliferation at the 1:1000 dilution.

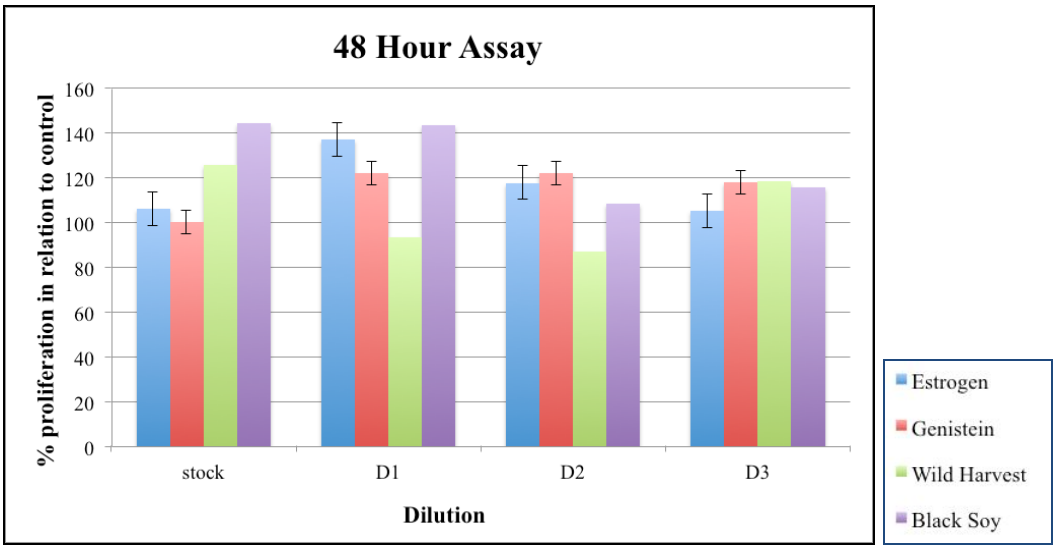

Figure 5. 48-Hour MTT Assay. *Percent of negative control shown on the y-axis. On the x-axis, estrogen stock was at a concentration of 1 mM and the stock genistein was at a concentration of 5 mM. D1 is a 10-fold dilution, D2 is a 100-fold dilution, and D3 is a 1000-fold dilution. with 10-fold serial dilutions shown on the x-axis.*

Figure 6 shows the absorbance values expressed as percentage of the negative control. The 72-hour assay was recorded twice with genistein and estrogen, and once with Wild Harvest and black soybean. The data show that the cells treated with the phytoestrogen products have a proliferation rate less than 90% after 72 hours, while estrogen has a proliferation rate greater than 90%. In this experiment, we see some dilution effects for estrogen, genistein, and Wild Harvest. The main dilution effect seen from estrogen exposure is seen in an increase at the 10-fold dilution compared to the stock or negative control. For genistein, the 5mM, 0.5mM, and 0.05 concentrations appear to have stronger antiproliferative effects; the 0.005mM exposure to genistein does not appear to have an effect on proliferation. Exposure to stock Wild Harvest extract appears to have a slight antiproliferative effect, but a notable decrease in proliferation at the 10-fold and 100-fold dilutions, with a slight increase at the 1:1000 dilution.

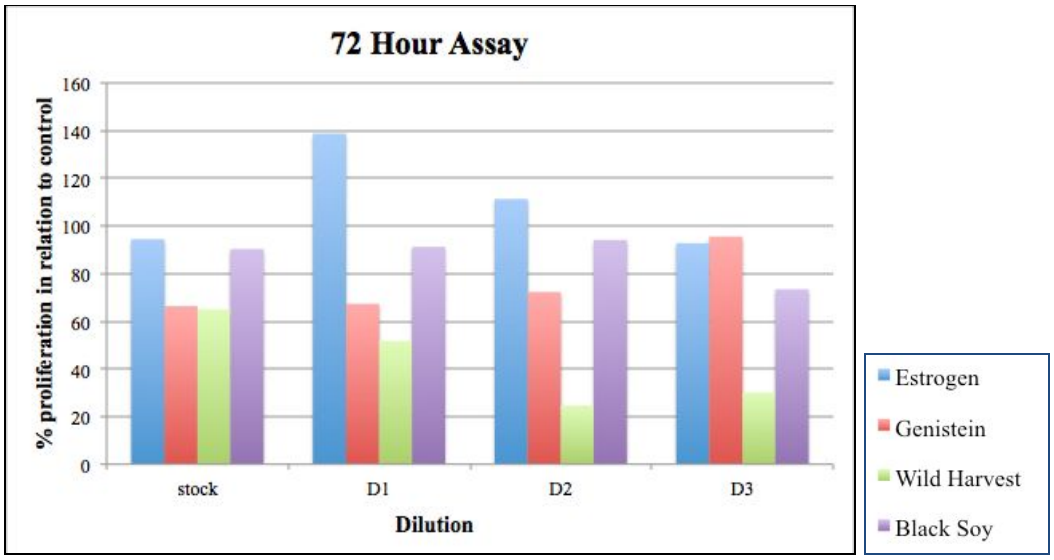

Figure 6. 48-Hour MTT Assay. *Percent of negative control shown on the y-axis. On the x-axis, estrogen stock was at a concentration of 1 mM and the stock genistein was at a concentration of 5 mM. D1 is a 10-fold dilution, D2 is a 100-fold dilution, and D3 is a 1000-fold dilution. with 10-fold serial dilutions shown on the x-axis.*

In Figure 7, the amount of genistein in a given product based on milli Absorbance Units (mAU) per second is compared to the percent proliferation normalized to the negative control. The amount of proliferation was measured by an MTT assay after 24 hours of exposure to a 1:100

dilution factor of phytoestrogen products. We see an R^2 value of 0.135, which indicates little correlation between percent genistein and percent proliferation. This is likely because we have one outlying data point, and used the 24-hour time point for reference.

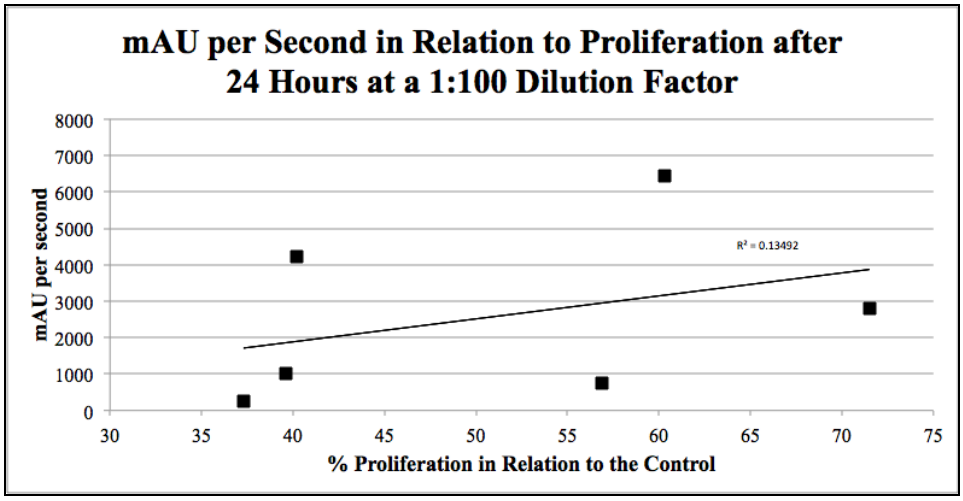

Figure 7. mAU per second compared to the percent proliferation. *Percent proliferation is shown of the 24 hour time point at dilution factor 2 (1:100). The x-axis shows the percent proliferation normalized to the negative control (cells in PHRED plus 1% methanol). The y-axis shows the percent of genistein in various phytoestrogen products, as determined by the HPLC chromatograms.*

To test our original hypothesis that higher levels of genistein in OTC phytoestrogen products would inhibit cellular growth, we compared the effects of Wild Harvest and black soybeans to pure genistein and estrogen. However, at the doses and time points we examined, we could not correlate increased genistein concentrations in OTC phytoestrogen products with stronger anti-proliferative effects, so we are unable to support our hypothesis. The data show that all phytoestrogen products tested decreased cell proliferation by >23%. Interestingly, pure genistein appeared to have a weaker anti-proliferative effect than phytoestrogen products that contain genistein.

Discussion

The data do not support our hypothesis, as there is no correlation between genistein concentration in phytoestrogen samples and percent cell proliferation. The phytoestrogen products we chose to show in our results, Wild Harvest and black soybeans, were chosen based on their relative genistein concentration. As shown in Table 2, Wild Harvest had the highest genistein concentration, while black soybean had one of the lowest genistein concentrations. Based on those concentrations, we expected to see a lower amount of proliferation in Wild Harvest treated cells than seen in black soybean treated cells. While this was true for the 72-hour assay, the reverse was true for the 24-hour assay, and generally cells treated with pure genistein showed higher proliferation than those treated with either Wild Harvest or black soybean. To support our hypothesis, we would have expected the lowest amount of proliferation from treating with a relatively high concentration of pure genistein, followed by Wild Harvest, and finally black soybean in relation to the negative control.

Proliferation in the cells treated with phytoestrogen samples at the 72-hour mark is lower than that of the positive control. We would expect to see the greatest difference in proliferation by the 72-hour mark as opposed to the 48-hour or 24-hour time points because we found that we needed to split the OVCAR-3 cells roughly every three days. This means that by the 72-hour time point, the OVCAR-3 cells would have doubled once. Additionally, the media was changed every 24 hours, so there may be some discrepancy in the data as the treatment dosage was very small (1μL per well) and was administered independently. Despite these possible confounding variables, the data show that overall, each phytoestrogen product decreased cell proliferation by at least 23%, indicating that they may prevent unregulated cell growth. Noting the results in Figure 6, we see that there is a decrease in proliferation at the 10-fold and 100-fold dilutions. In comparison, there is little difference between the 1:100 and 1:1000, indicating that by the 1:1000 dilution there was too little extract in the dilution to cause an antiproliferative effect.

We observed that pure genistein has a weaker anti-proliferative effect than the OTC products that contain genistein. This could be because the OTC products also contain other phytoestrogen

compounds such as daidzein or glycitein, which could be the true source of anti-proliferation seen in the experiments. It is also possible that another phytoestrogen compound is working in conjunction with the genistein to have a stronger anti-proliferative effect. However, the phytoestrogens were expected to have a stronger anti-proliferative effect than the positive and negative controls. The controls consistently had unexpected results, as the estrogen control was lower than the methanol control and the cell only control. Estrogen treated cells should have had a higher level of proliferation than both of the negative controls, but it seemed that the methanol and estrogen controls displayed the opposite results. Cells treated with methanol generally showed higher levels of proliferation than the cell only control, which could be due to some contamination of the methanol stock with organic compounds in the methanol that encouraged cell growth. Regardless of the problems with the controls, the phytoestrogen treated cells displayed overall antiproliferative effects.

In Figure 7 of our results section, we see that there is an R^2 value of 0.103 for correlation between percent genistein and percent proliferation. This tells us that there is little correlation between the two. We see that there is one specific outlier, which is from Wild Harvest. It is curious that this is the data point that skewed the data, as it showed the least proliferation by the 72-hour time point. If the outlier Wild Harvest at the 24-hour time point was removed from the figure showing genistein concentration compared to the percent proliferation, the result shows there is zero correlation between the two (see Appendix B). If we had run proliferation assays after 72-hours of exposure to each phytoestrogen product, we might have a clearer picture the 24-hour time point for reference.

Future projects should investigate other compounds in OTC phytoestrogens that could have an effect on proliferation. It would be worthwhile to determine if another compound in OTC products is augmenting the efficacy of genistein or if it is responsible for the decrease in proliferation itself. As mentioned earlier, it may be interesting to determine if daidzein is a stronger compound in terms of antiproliferative effects than genistein, both in its pure state and as a component of OTC phytoestrogen supplements. Regardless of what compound future project choose to focus on, it would be advantageous to spike the phytoestrogen supplements

with pure genistein to confirm that the peaks we used in our HPLC results were in fact genistein peaks. Also, isolating a sample from the time of the known genistein peak would give a much more specific sample to test during the MTT assays. Instead of applying 1µL of the original refluxed phytoestrogen supplement to each well, 1µL of the isolated sample would be added (as well as the subsequent dilutions). We believe that any or all of these suggestions would be incredibly beneficial to the future of this project.

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Appendix A

Experiment 4: 24 Hour Assay

		1	2	3	4	5	6	7	8	9	10	11	12
Cell Only	A	0.465	0.884	0.878	0.84	0.751	0.031	0.031	0.03	0.031	0.03	0.031	0.036
Methanol	B	0.612	0.851	0.962	0.872	0.815	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Genistein	C	0.461	0.561	0.677	0.628	0.536	0.545	0.7	0.524	0.617	0.645	0.577	0.676
Yellow Soy	D	0.607	0.409	0.445	0.523	0.425	0.468	0.557	0.361	0.398	0.467	0.438	0.619
Black Soy	E	0.693	0.509	0.521	0.629	0.459	0.561	0.63	0.49	0.414	0.474	0.433	0.426
Estrogen	F	0.442	0.443	0.47	0.463	0.523	0.701	0.454	0.467	0.331	0.334	0.464	0.179
	G	0.032	0.031	0.03	0.032	0.031	0.031	0.031	0.031	0.03	0.031	0.03	0.03
	H	0.031	0.03	0.03	0.03	0.03	0.03	0.03	0.031	0.03	0.029	0.03	0.03

Experiment 4 Repeat: 24 Hour Assay

		1	2	3	4	5	6	7	8	9	10	11	12
Cell Only	A	0.373	0.499	0.7	0.656	0.599	0.031	0.031	0.03	0.031	0.03	0.03	0.032
Methanol	B	0.419	0.583	0.674	0.647	0.573	0.029	0.03	0.03	0.031	0.03	0.03	0.03
Genistein	C	0.622	0.736	0.715	0.616	0.625	0.672	0.684	0.608	0.601	0.59	0.591	0.393
Yellow Soy	D	0.521	0.387	0.448	0.377	0.404	0.457	0.532	0.487	0.539	0.624	0.502	0.259
Black Soy	E	0.631	0.433	0.49	0.537	0.492	0.313	0.355	0.555	0.52	0.435	0.4	0.291
Estrogen	F	0.596	0.317	0.346	0.26	0.606	0.782	0.645	0.505	0.338	0.326	0.469	0.289
	G	0.031	0.033	0.031	0.031	0.031	0.031	0.031	0.031	0.03	0.028	0.031	0.03
	H	0.032	0.03	0.029	0.031	0.03	0.03	0.031	0.031	0.031	0.03	0.03	0.03

Experiment 5: 48 Hour Assay

		1	2	3	4	5	6	7	8	9	10	11	12
Cell Only	A	0.486	0.906	0.838	0.929	0.892	0.029	0.029	0.029	0.03	0.029	0.029	0.03
Methanol	B	0.564	1.028	1.408	0.891	1.123	0.03	0.03	0.029	0.029	0.029	0.029	0.029
Positive Control	C	0.772	1.17	1.133	1.098	1.155	1.051	0.925	0.974	1.005	0.971	0.992	0.623
Black Soy	D	1.301	1.391	1.631	1.121	1.301	0.883	1.089	0.878	0.929	0.785	0.901	0.69
Estroven	E	0.977	0.847	0.919	0.744	0.746	0.802	0.752	0.753	0.665	0.968	0.855	0.516
Genistein	F	0.907	0.435	0.46	0.571	0.471	0.413	0.369	0.385	0.425	0.961	0.908	0.627
	G	0.055	0.03	0.03	0.03	0.031	0.029	0.029	0.03	0.03	0.029	0.03	0.03
	H	0.031	0.03	0.031	0.031	0.031	0.03	0.029	0.029	0.03	0.032	0.031	0.03

Time Course 1: 24 Hour Data

		1	2	3	4	5	6	7	8	9	10	11	12
(-)	A	0.796	0.826	0.907	0.862	0.865	0.034	0.03	0.031	0.031	0.033	0.031	0.031
Methanol	B	0.893	1.668	1.151	1.039	1.003	0.03	0.029	0.029	0.032	0.032	0.03	0.031
Positive Control	C	0.69	1.158	0.961	1.27	0.888	0.964	0.516	0.948	1.087	0.524	0.165	0.396
Black Soy	D	1.183	0.964	1.079	0.198	0.835	0.841	1.022	0.826	0.625	0.733	0.908	0.589
Estroven	E	1.09	1.22	0.98	0.721	0.665	0.54	0.432	0.891	0.423	0.513	0.59	0.765
Genistein	F	0.939	1.135	1.25	1.411	1.716	1.184	1.36	1.408	1.41	1.177	0.925	0.641
Wild Harvest	G	0.824	1.11	0.975	1.18	1.205	1.049	1.182	1.09	1.184	1.128	1.329	0.939
	H	0.032	0.031	0.03	0.032	0.031	0.032	0.031	0.031	0.032	0.031	0.031	0.033

Time Course 1: 48 Hour Data

		1	2	3	4	5	6	7	8	9	10	11	12
(-)	A	1.051	0.742	0.772	0.742	0.66	0.031	0.031	0.031	0.031	0.031	0.03	0.033
Methanol	B	0.996	0.941	1.251	0.896	1.499	0.031	0.031	0.03	0.032	0.03	0.031	0.03
Positive Control	C	0.778	1.585	1.484	2.164	1.492	1.548	1.575	1.114	1.394	1.563	1.168	0.909
Black Soy	D	0.834	1.985	2.071	1.783	2.142	1.929	1.234	1.3	1.473	1.678	1.843	1.471
Estroven	E	1.24	1.391	1.127	1.022	1.17	0.613	1.101	0.497	0.588	0.767	1.046	0.883
Genistein	F	1.109	1.364	1.655	1.58	1.628	1.89	1.737	1.875	1.561	1.797	1.591	1.09
Wild Harvest	G	1.331	1.32	1.456	1.054	1.045	0.952	0.976	0.886	0.977	1.297	1.357	1.219
	H	0.032	0.03	0.032	0.031	0.03	0.031	0.031	0.03	0.03	0.031	0.03	0.031

Time Course 1: 72 Hour Data

		1	2	3	4	5	6	7	8	9	10	11	12
(-)	A	0.768	0.969	0.94	1.026	0.52	0.031	0.031	0.03	0.032	0.031	0.03	0.033
Methanol	B	1.605	1.729	1.662	1.407	1.534	0.031	0.031	0.031	0.031	0.03	0.03	0.033
Positive Control	C	1.02	1.094	1.35	1.788	1.708	1.211	1.501	0.845	0.594	0.981	0.995	0.173
Black Soy	D	1.186	0.897	0.793	1.212	0.797	0.896	1.086	0.777	1.135	0.753	0.917	0.668
Estroven	E	1.182	1.062	0.821	0.71	0.582	0.727	0.773	0.765	0.79	0.941	0.752	0.807
Genestein	F	0.615	0.66	0.411	0.364	0.332	0.293	0.436	0.502	0.358	0.419	0.509	0.47
Wild Harvest	G	0.809	0.274	1.035	0.626	0.578	0.483	0.236	0.223	0.347	0.365	0.369	0.245
	H	0.035	0.031	0.03	0.033	0.03	0.03	0.031	0.031	0.032	0.03	0.031	0.032

Time Course 2: 48 Hour Data

		1	2	3	4	5	6	7	8	9	10	11	12
	A						(-)	0.41	1.086	0.883	0.986	0.255	0.035
	B						(methanol)	0.319	0.204	0.26	0.697	0.227	0.03
	C												
	D												
Genistein	E	0.498	0.982	1.129	1.379	1.222	1.25	1.274	1.355	1.427	1.161	0.834	1.1
Estrogen	F	0.651	0.738	0.985	1.265	1.244	1.364	1.179	1.464	1.297	1.485	0.953	0.334
	G												
	H												

Time Course 2: 72 Hour Data

		1	2	3	4	5	6	7	8	9	10	11	12
(-)	A	0.381	0.718	0.541	0.918	0.786	0.032	0.037	0.039	0.037	0.04	0.035	0.036
Methanol	B	0.181	0.905	1.216	1.133	0.93	0.034	0.037	0.036	0.035	0.041	0.034	0.033
Genistein	C	0.491	0.736	0.862	1.464	0.75	0.628	0.86	0.915	1.04	1.391	1.507	1.139
Estrogen	D	0.544	1.067	1.318	1.31	1.491	1.407	1.277	1.552	1.105	0.985	1.242	1.31
	E	0.038	0.04	0.039	0.044	0.042	0.042	0.039	0.044	0.043	0.043	0.04	0.042
	F	0.047	0.044	0.045	0.044	0.047	0.047	0.049	0.047	0.046	0.048	0.046	0.039
	G	0.05	0.033	0.033	0.032	0.034	0.036	0.034	0.033	0.034	0.035	0.032	0.031
	H	0.032	0.031	0.03	0.031	0.034	0.032	0.031	0.035	0.033	0.032	0.03	0.032

Appendix B

Graph of Genistein Concentration vs Proliferation without Wild Harvest