Phytoestrogens and their Effects on Breast Cancer Cell Proliferation

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Advisors: Professor Mike Buckholt, PhD and Professor Jill Rulfs, PhD
Authors: Nathan Ferron, Sarah Gardinier, and Kathleen Tran

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Abstract

Over the counter (OTC) phytoestrogen supplements are marketed as an alternative to hormone replacement therapies but may increase the risk of hormone-sensitive breast cancers. To begin assessing this risk, phytoestrogens were extracted from OTC supplements, separated using HPLC, and tested using an estrogen responsive breast epithelial cell line (MCF7). Results suggest lipid components of the OTC supplement Promensil have an antiproliferative effect on cultured breast cancer cells, though future research is recommended to outline the biochemical cause of this effect.

Acknowledgments

The project authors would like to take this opportunity to thank some individuals without whom this project would not have been possible. First and foremost, to our advisors Professor Mike Buckholt and Professor Jill Rulfs, we thank you for your invaluable guidance and patience throughout the course of this project. To Jake Rogers, we thank you for your continued support as a peer and for your problem solving skills.

Background

Introduction

Research has shown that there is a link between estrogen receptor activity and breast cancer, a disease which affects one in eight adult, American women in their lifetime (DeSantis et. al, 2014). Though much research has been done to illuminate the relationship between human-produced estrogens and the onset of breast cancer, there is still much research to be done to understand the effects of phytoestrogens on the body, especially in relation to cancer growth.

In a number of previous projects at this institution, the researchers examined the effect that store-bought phytoestrogen supplements had on the proliferation of breast cancer cells. They found that the supplements largely do have a positive proliferative effect on the cells, although one product was repeatedly shown to have anti-proliferative effects on growth. (Bitzas et. al, 2015) The goal of this project is to study whether or not individual phytoestrogens, lipids, or other compounds soluble in methanol in these supplements are responsible for the observed effects on breast cancer cell culture.

To determine this, the supplements used in the previous project were extracted again. Using a high pressure liquid chromatography, the major components of the extracts

were separated and pooled fractions added to cell cultures. The effects of these substances on the proliferation of the breast cancer cells were measured. As many of the plants in the human diet contain a large variety of phytoestrogens within them, their effect in this regard was worth investigation.

Previous MQPs

In previous projects performed at this institution, the researchers examined the effect that store-bought phytoestrogen supplements had on the proliferation of breast cancer cells. They found that when the estrogen supplements were tested on breast epithelial cells, most of the supplements increased the proliferation of the cell cultures. The exception was that of the Promensil supplement, which did not increase the proliferation of the cells and, in fact, seemed to inhibit proliferation. The last group also analyzed the individual components of their supplements and found that one peak of note found in all other supplements was absent in the Promensil supplement (Bitzas et al, 2015) (Lesage et al, 2014).

Breast Cancer

In the United States, breast cancer is the second most common cancer that can occur in people of any gender, though it rarely occurs in men (Veronesi et. al, 2005). There are many types of breast cancer and the causes for all of them are currently not clearly understood. The hormone, estrogen, seems to have a connection with the development

of certain types of breast cancer. Estrogen mediates its functions through two specific intracellular receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Osborne et. al, 1996). Both ERα and ERβ have nearly identical DNA-binding domains but their N-terminal activation function 1 regions differ greatly, meaning that there can be selective stimulation of the two different receptors (Katzenellenbogen & Katzenellenbogen, 2000).

Estrogen

Estrogen is a steroid hormone produced primarily in the ovaries of female vertebrates, but is also produced in small amounts in fat cells, the liver, the adrenal glands, and breast tissues (Nelson et al, 2001). It is responsible for stimulating most of the growth and development of the female reproductive system. There are four main types of estrogen produced in humans: estradiol, estrone, estriol, and estetrol (Baird et al, 1969). Estradiol is produced the most in females between puberty and menopause. Estradiol generally has the highest affinity for the receptors compared to other types of estrogen, meaning it has the greatest effect on estrogen related protein production (Pfaff et al, 1973). Estrone is produced mainly by females after the onset of menopause and has a decreased function compared to estradiol. Menopause is essentially caused by the replacement of the estradiol production with estrone production (Cauley et al, 1989). Estriol and estetrol are only produced during pregnancy (Kaiser et al, 2000). In estrogen responsive cells of the female reproductive system, cell proliferation will accelerate in the presence of all types of estrogen in varying degrees and the steady flux of estradiol present in the female body between the times of puberty and menopause allow the

reproductive system to maintain a steady level of maintenance and function (Delemarre-van de Waal et al, 2000).

Action of estrogen on estrogen responsive cells is controlled by estrogen receptors (ERs) located in the cytoplasm of the cell. When ERs bind to estrogen, the receptor can then diffuse into the nucleus of the cell and begin to mediate protein production. There are two types of ERs: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). (Deroo et al, 2006)

ERα (Estrogen Receptor Alpha)

The ERα is an estrogen receptor that acts as a hormone-dependent transcriptional regulator for estrogen (Osborne et. al, 1996). This receptor is responsible for several functions, including mediating the mitogenic cancer promoting effects of estrogen (Rizza et. al, 2014). Assays of breast tissue cells show normal presence of alpha-receptors, but precursor cancer cells are shown to have a higher number of alpha-receptors in them (Allred et. al, 2004). Initial screening of known ER ligands shows that certain steroidal compounds exhibited moderate affinity and potency preference for ERα (Katzenellenbogen & Katzenellenbogen, 2000).

ERβ (Estrogen Receptor Beta)

The ERβ, like the ERα, is a cytoplasmic estrogen receptor. Unlike ERα, ERβ mediates the tumor suppression effects of estrogen, acting as an antagonist to the ERα effects (Rizza et. al, 2014). The expression of ERβ is lower in cancerous breast cells than in normal breast tissues, meaning that expressions levels of both ERα and ERβ have a

role in breast cancer carcinogenesis (Lazennec et. al, 2001). As is the case with ERα, there are certain estrogen compounds that have a higher affinity for ERβ than. Different phytoestrogen compounds show different levels of affinity to both ERα and ERβ (Morito et. al, 2001) but certain phytoestrogens and androgen-derived diols have a higher preference for ERβ than estradiols (Katzenellenbogen & Katzenellenbogen, 2000).

Hormone Replacement Therapy

Hormone replacement therapy (HRT) is a medical treatment in which the patient receives natural doses of synthetic hormones to make up for the lack of a hormone in the body (Laucella et. al, 1999). HRT can be used for different reasons, such as treating women dealing with the symptoms of menopause and people transitioning genders. At the beginning of the 21st century, there were about 6 million American people taking artificial estrogen and progestin as HRT for estrogen but the demographic has since declined in numbers due to studies showing a link between HRT and an increased risk of cardiovascular disease and breast cancer (Lerner et. al, 2011).

Male-to-Female Transition

As of now, there are not many studies regarding transgender individuals and their transition. HRT can be used to help transgender individuals to physically transition, but that brings to question whether transgender women undergoing this treatment will be at risk of breast cancer. HRT is known to have some adverse effects on menopausal women, but it is unknown if the same applies to transitioning women. There is one case of a transitioning woman with a complicated mental health history developing breast cancer. The patient had been taking HRT since 2002 and underwent a vaginoplasty in

2010 in Thailand, but did not have a breast augmentation. Though she died in 2011, the autopsy showed extensive metastatic tumors in her breasts and her liver. It is unknown whether HRT had a role in the patient's development of breast cancer and the development of estrogen receptors. (Pattison & McLaren, 2013).

Menopause

Menopause is a condition that women undergo when they stop having a regular menstruation cycle due to a decrease in production of estradiol and progesterone. The average age range where menopause occurs is 40 to 50 years old and it is a natural biological condition that women experience. (Nelson, 2008)

The symptoms menopausal women experience vary by individual. Some common symptoms include hot flashes, vaginal dryness, and night sweats, among others (Nelson, 2008). Complications can arise from menopause due to the declining production of reproductive hormones, specifically estradiol. These complications include cardiovascular disease, osteoporosis, and pelvic organ atrophy (Laucella et. al, 1999). These symptoms and complications can be reduced and alleviated with the use of HRT by replacing the declining estradiol and progesterone in the body.

However, there are risks associated with using HRT to alleviate menopause symptoms. While HRT can alleviate the symptoms of menopause, there is also an increased risk of breast cancer. Cells in the body, particularly breast tissue cells, are stimulated by the presence of estrogen (Laucella et. al, 1999). HRT does not create *de novo* breast cancerous cells, rather, HRT stimulates pre-cancer cells to grow (Dietel, 2010). These

pre-cancerous cells may have active estrogen receptors, specifically ERα, that are stimulated by HRT to proliferate (Allred et. al, 2004). In the last decade, there has been a decline of breast cancer rates and studies have shown that this decline has a positive correlation with the declining use of HRT (Neutel & Morrison, 2010).

Phytoestrogens

Phytoestrogens are a class of compounds which plants naturally produce. These compounds are either structurally or functionally similar to the estrogen molecule, allowing them to potentially act as if they were estrogen within animal cells. There are many differing types of phytoestrogens grouped together based on their general structure. Varieties of phytoestrogens include flavonoids, isoflavonoids, and lignans. (Patisaul & Jefferson, 2010)

There is a wide variety of potential uses for phytoestrogens in a medical sense. They have been claimed to aid in relieving menopausal symptoms as a hormone replacement. They may prevent the development of osteoporosis due to the effects estrogen has on bone density. They have also been thought to improve cardiovascular health and prevent heart disease. However, many of these claims have yet to produce definitive results due to widely varying dosage, composition, compounds, and duration of use across the various studies into their effects. In addition, phytoestrogens may have many negative effects on the human body. There has been much debate in this topic over the years as to whether or not phytoestrogens affect breast cancer proliferation, and if they do, whether that effect is positive or negative. There is evidence

to suggest that phytoestrogens can disrupt natural hormone levels and ovulation in both animals and humans.(Patisaul & Jefferson, 2010)

Isoflavonoids

Isoflavonoids are mostly found in the subfamily *Papilionoideae*, which is within the family *Lefuminosae*. (Dixon, 2004) This form of phytoestrogen was the first to be discovered, being found in a study devoted to the negative effects that clover seemed to be having on cattle and sheep. These compounds are derived from flavanones. Most phytoestrogens that are used in dietary supplements tend to be simple isoflavones, with the exception of coumestrol. (Dixon, 2004) There are relatively few plants in a typical human diet that produce isoflavonoids. The main sources in the human diet of these phytoestrogens are soybeans, chickpeas, and alfalfa sprouts. Some specific isoflavonoids have decreased levels of estrogen-like effects, such as those that have undergone *O*-methylation. (Dixon, 2004)

Methylated isoflavones which have undergone isoprenylation may have greater effects similar estrogen, but are generally too uncommon in the human diet to be considered statistically important. (Dixon, 2004) The isoflavonoids formononetin and biochanin A have been shown to have lowered estrogenic effects when compared to daidzein and genistein, phytoestrogens shown to have a proliferative effect on estrogen responsive breast cancers in concentrations lower than 10µL/mL (de Lemos, 2001). Of special interest within the isoflavonoids is coumestrol. Coumestrol shows strong estrogenic activity, showing effects similar to that of estradiol. Coumestrol also shows a greater affinity for binding to the ER than genistein does. (Dixon, 2004)

Flavonoids

When compared to their isoflavonoid cousins, flavonoids tend to show negligible or low estrogenic activity. (Dixon, 2004) This is especially common among those which are typically a part of the human diet. Despite this, some flavonoids, especially flavones, are better than isoflavonoids at inhibiting the aromatase cytochrome P450 pathway which helps to convert androgens to estrogens. (Dixon, 2004) Within the flavonoid family, one compound of special interest is 8-prenylnaringenin. This compound, produced by hops, is one of the more potent flavonoids in a phytoestrogen sense. It can show ER-mediated activity at a magnitude lower concentration than its parent flavonoid. (Dixon, 2004)

Lignans

Defined as dimeric phenylpropanoid compounds, lignans function in a wide distribution within the plant kingdom as a defense mechanism. (Dixon, 2004) In the human diet, they are mostly present in cereals and grains, as well as berries and garlic. While not typically phytoestrogens themselves, these compounds can be converted to phytoestrogens by symbiotic microflora in the proximal colon. (Dixon, 2004)

Other Phytoestrogens

There are several other assorted compounds found in many plants used for medicinal purposes and found naturally in the human diet that may have significant estrogenic activity. (Dixon, 2004) For example, fresh corn was discovered to have a mitogenic compound after corn cob bedding was found to interfere with the estrus cycle and sexual behavior of rats. (Dixon, 2004) This compound stimulates proliferation of breast

cancer cells of both the ERα and β varieties. While no specific compound was identified, some preliminary studies suggest that safflower seeds can protect against ovariectomy-induced bone loss in rats. (Dixon, 2004) Other interesting phytoestrogens include the chalcone and isoflavonoids in licorice root, the phenylbutanone glucoside lindleyin found in rhubarb, and several bioactive triterpenoid compounds in ginseng. (Dixon, 2004)

Cell Line

In order to gather the data for the experiments performed in this study, it was necessary to maintain a cell line to perform these tests on. In this study, the cell line used was an MCF7 ATCC line. Obtained from a 69 year old Caucasian female with adenocarcinoma, these cells were gathered from a metastatic site from pleural effusion in the lungs. (MCF7 (ATCC HTB-22)) These cells are an epithelial line that is adherent. When the cells are examined with a karyotype, the modal number of chromosomes is 82, with individual specimens ranging from 66 to 87 chromosomes. (MCF7 (ATCC HTB-22)) Crucially, these cells are estrogen-responsive and process estradiol through standard receptor pathways, meaning that substances that are estrogen-like should upregulate the growth of the cells. (MCF7 (ATCC HTB-22))

Methods

Media

MCF-7 cells were incubated at 37°C and 5% CO_2 in a humidified incubator in DMEM with10% volume Fetal Bovine Serum, 300 µg/ml PenStrep, 300 µg/ml glutamine + 2 ng/ml insulin as outlined in Hamelers et al, 2003. Cells used for proliferation assays were harvested at 40-60% confluence with trypsin. Prior to use in the MTT assay, cells were maintained as described above. When they reached 60-70% confluence, the cells were plated in 36 wells of a 96 well culture plate with 10^4 cells/well. The cells were incubated in Phenol red-free medium with 5% dextran-coated charcoal-treated (DCC) serum for 24 hours to allow the cells to adhere to the plate. After 24 hours passed, the serum was replaced with phenol red-free, DCC serum-stripped media containing 0.2% bovine serum albumin, 10ug/mL of transferrin, and 30 nM sodium selenite and cells were incubated for another 24 hours. The serum-stripped media allowed the cells to synchronize their cell cycles in order to remove confounding factors from cells in different stages of the cell cycle.

Extraction of supplements

Supplement extractions were done on the phytoestrogen supplements following the procedure outlined in Setchell et. al (2001). Briefly, supplement samples were added to a 250 mL round bottom flask and mixed with 80 mL of 80% methanol. The mixture in

the flask was refluxed with round bottom flask and a fractional column for one hour in boiling methanol. All extracts were filtered and stored in a -20 °C.

For this experiment, extracts of Oregon's Wild Harvest Red Clover (manufactured by Oregon's Wild Harvest, Lot # 104214RCL) and Promensil (manufactured by Natrol, Lot # 16156B) were used. One 80 mg tablet of Promensil was crushed in a sterile mortar and pestle while three 350 mg capsules of Wild Harvest were used. These quantities for both brands of supplements represent one recommended daily dose.

High Performance Liquid Chromatography (HPLC)

HPLC was performed on the extracts following the same procedure that was used for the previous MQP (Caron, 2007). Briefly, sample extracts were separated using a C18 250 X 4.6 mm column with a linear gradient of 10mM ammonium acetate-0.1% trifluoroacetic acid and 100% acetonitrile. The column was started with 100% acetonitrile and the solvent's percent composition gradually changed to 100% ammonium acetate-0.1% trifluoroacetic acid by the end of the run. For the first 2 minutes, the column was washed with with a solution of 10 mM ammonium acetate and 0.1% of trifluoroacetic acid at 100% concentration. Over the next 22 minutes, the column was washed with a linear gradient of 10 mM ammonium acetate-0.1% TFA and 100% acetonitrile. Over the this time period, the percentage of 10 mM ammonium acetate-0.1% TFA in the solution changed from 100% to 50% while the percentage of acetonitrile changed from 0% to 50%. Following this, for 5 minutes, the column was

washed with a 50% ammonium acetate-TFA and 50% acetonitrile solution. The remainder of the time in the procedure comprised of the column being washed with 100% ammonium acetate-TFA. The HPLC instrument operated for a total of 36 minutes with the flow rate being 1 mL/minute of solution eluded. A chromatogram of the samples was generated by measuring the absorbance (in mAU, or micro absorbance units) of the samples at 260 nm over the course of the HPLC. To test if the solvent had any effect on the cells, the HPLC instrument was run without any samples loaded in and the elution was collected in the same fractions as the elution for the supplements. The samples from this run were used in the cell culture experiment as outlined in the following section, Methods, MTT Assay.

MTT Assay

As described above, cells were counted, trypsinized and plated in 96 well plates at a concentration of $1x10^3$ cells per well in 100µl of media with serum. Cells were given 24 hours to adhere before media was aspirated and replaced with 100 µL of serum-starved media. This allowed the cells to all come to phase G_0 in their growth cycle. After 24 hours, this media was then replaced with 100µl of charcoal stripped serum media (DMEM + 10% volume Charcoal Stripped FBS + 300 µg/ml PenStrep + 300 µg/ml

Glutamine) along with 10µl of sample as outlined in Figure 1.

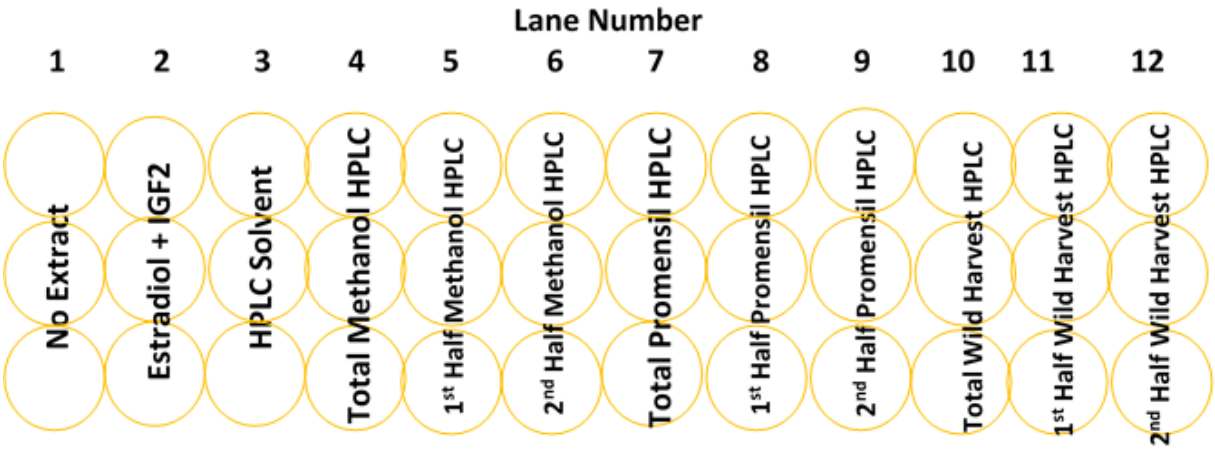

Figure 1: <u>Diagram for the Layout of 96-Well Plates Used in Assay</u>

Charcoal stripped serum was used for this step as this serum does not contain any lipids and therefore no hormones such as estradiol. After 24 hours incubation, 10µL of CellTiter 96 Aqueous One solution (manufactured by Promega) was added to each well, incubated for 1 hour and absorbance was read at both 450 and 570 nm as the optimum wavelength to measure the assay is 490nm and the project team did not have access to this wavelength filter.

Data Analysis

Once the data was read, an average was obtained for the absorbance of each triplicate. For each non-control triplicate, its average was expressed as a percentage of the positive (estradiol added) control triplicate average. The percent control for each sample condition was averaged across all test sets. A standard deviation was taken for each of these averages, and from the standard deviation a standard error was taken. This

process was then repeated using the negative (no extract added) controls. The data was separated between what wavelength was used to take the data.

Results

High Performance Liquid Chromatography (HPLC)

The HPLC procedure outlined in the methods section was used on extract samples of Promensil and Wild Harvest twice, each using the same sample. For each supplement during their HPLC runs, the eluate was collected in test tubes with each tube collecting a five minute interval throughout the entire HPLC run.

Figure 2 and Figure 3 show the two chromatograms for Promensil. The x-axis shows the time and the y-axis is the absorbance in micro absorbance units (mAU). The nearly identical traces show a number of large peaks between 15 and 36 minutes.

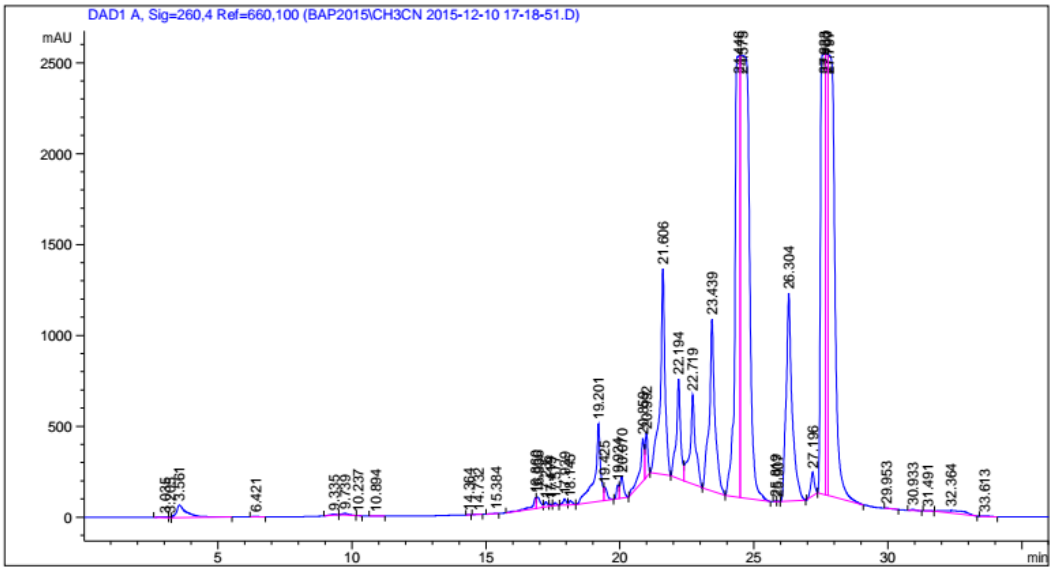

Figure 2: <u>Chromatogram for Promensil (1st run)</u>

Chromatogram of first HPLC run for Promensil. Eluate was collected in test tubes each collecting five minute interval throughout the entire run. X- axis shows time (min.) and y-axis shows absorbance (mAU)

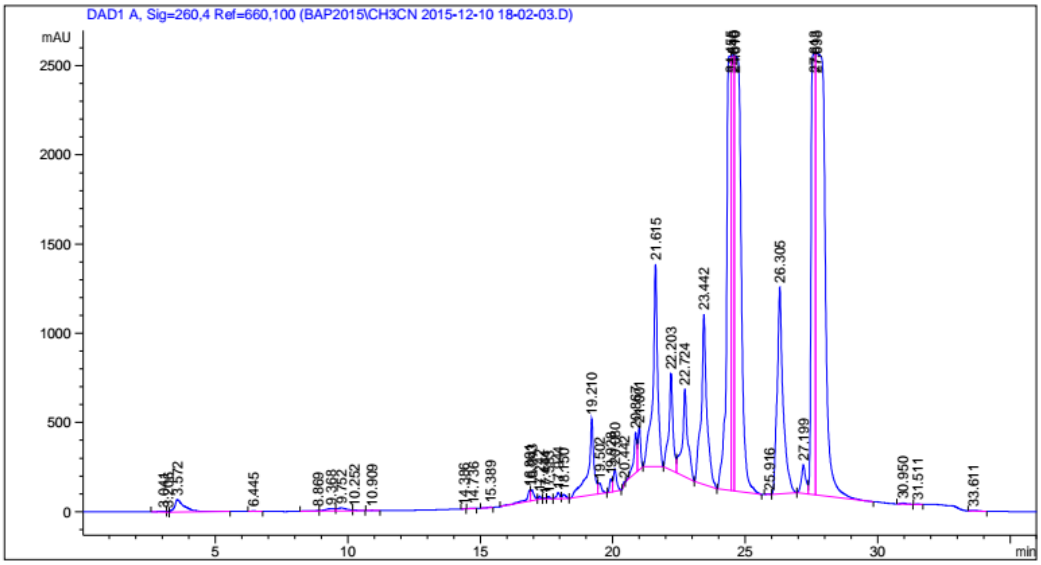

Figure 3: <u>Chromatogram for Promensil (2nd run)</u>
Chromatogram of second HPLC run for Promensil. Eluate was collected in test tubes each collecting five minute interval throughout the entire run. X- axis shows time (min.) and y-axis shows absorbance (mAU)

Figures 4 and 5 show the chromatograms for Wild Harvest.

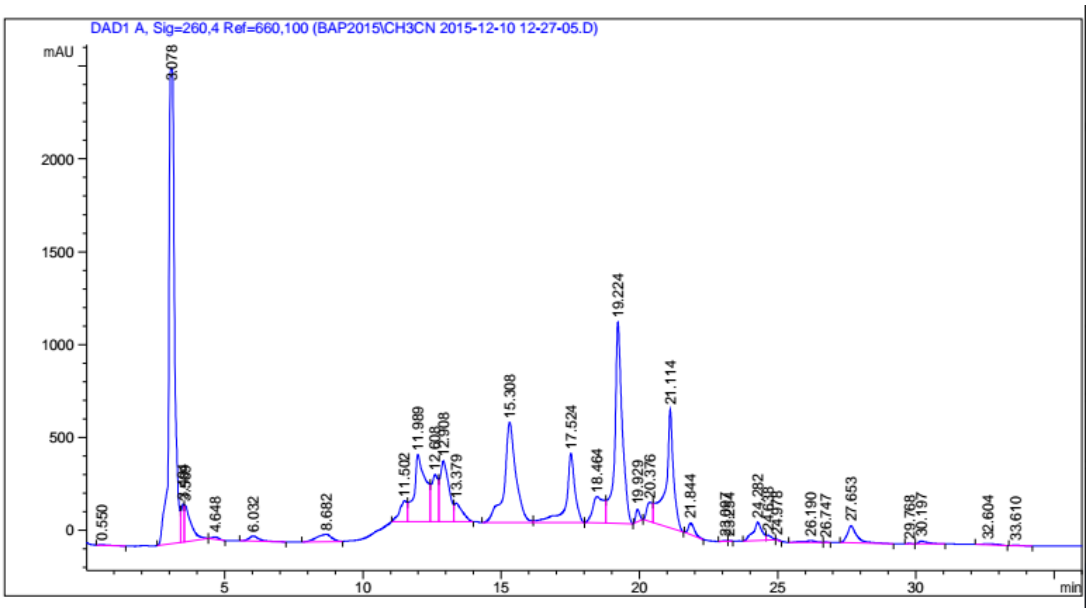

Figure 4: <u>Chromatogram for Wild Harvest (1st run)</u>
Chromatogram of first HPLC run for Wild Harvest. Eluate was collected in test tubes each collecting five minute interval throughout the entire run. X- axis shows time (min.) and y-axis shows absorbance (mAU)

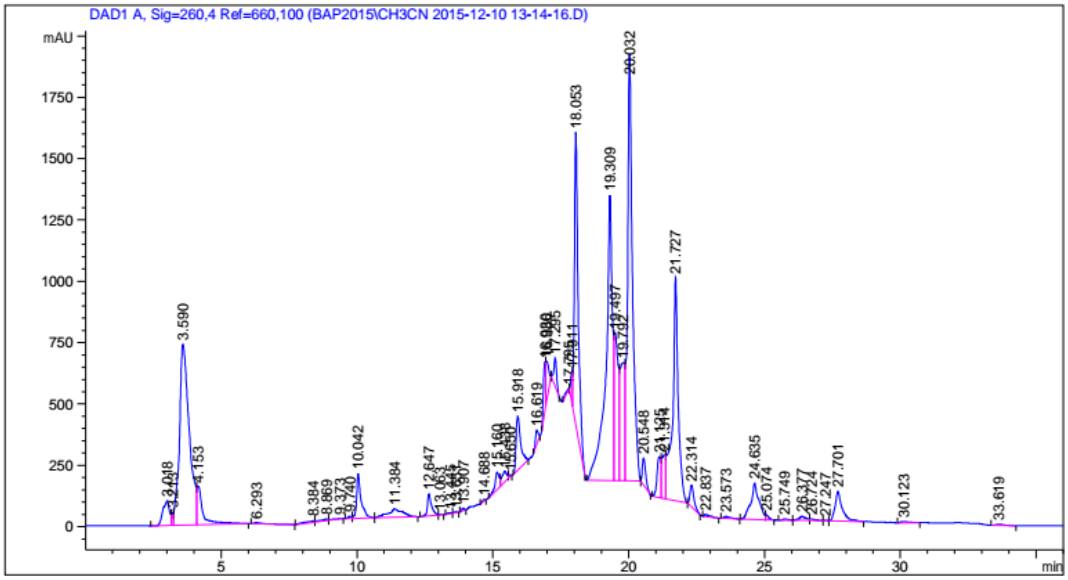

Figure 5: <u>Chromatogram for Wild Harvest (2nd run)</u>
Chromatogram of second HPLC run for Wild Harvest. Eluate was collected in test tubes each collecting five minute interval throughout the entire run. X- axis shows time (min.) and y-axis shows absorbance (mAU)

Due to the significant differences seen between the two runs, of the two Wild Harvest, a third run was performed. This chromatogram (not shown) was nearly identical to that shown in Figure 5. Due to the discrepancies seen in the first run (Fig.4), the extracts from it were discarded and those from the second set of Wild Harvest runs used for all subsequent experiments.

MTT Assays

The MTT assays were conducted as described in the MTT assay methods section. HPLC fractions were collected at 5 minute interval and pooled together with half 1 comprised of the pooled samples collected from 0-15 minutes and half 2 from 15-36 minutes. An additional sample was comprised of all of the fractions from each run and

labeled as “pooled”. The absorbance reading is measured in micro absorbance units (mAU). Appendix B shows all of the raw data of MTT assay results for the plates.

HPLC fractions were collected at 5 minute interval and pooled together. Half 1 = pooled samples collected from 0-15 min; half 2 = pooled samples collected from 15-36 min; total = all of the fractions from total HPLC run. Average values from five MTT assays of Promensil and Wild Harvest were expressed as a percent of the negative control where no samples were added.

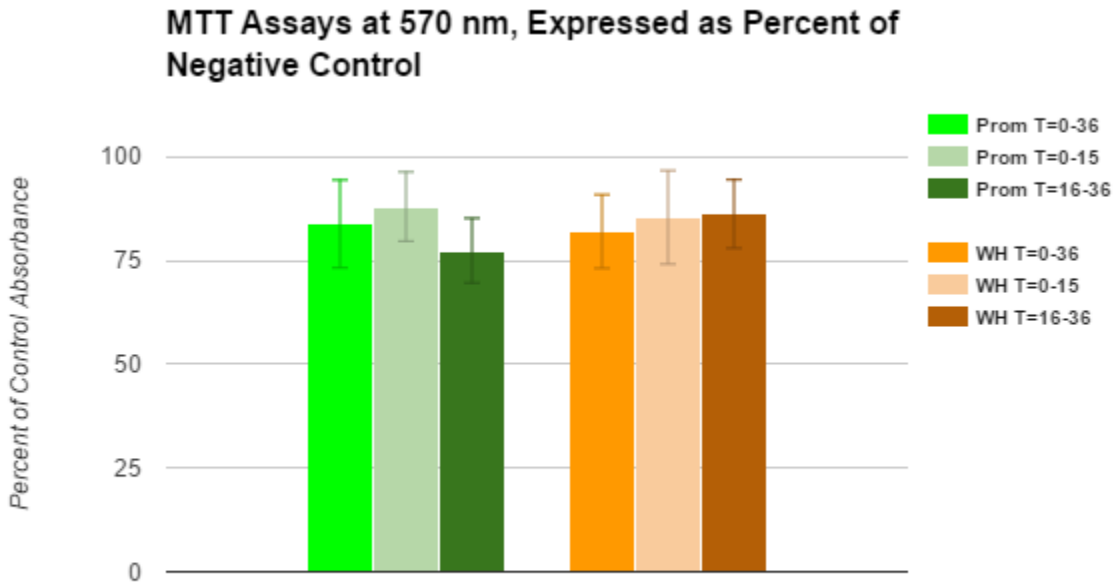

Figure 5: <u>MTT Assays Expressed as Percent of Negative Control (No Extract Added)</u>

Figure 5 shows the average values from five MTT assays of Promensil and Wild Harvest expressed as a percent of the negative control, which had nothing added to the growth media. If the absorbance of a sample is shown as 100%, then the sample would

have the same absorbance readings as the control meaning that the supplement had no effect on cell proliferation relative to the untreated control. For Promensil, the percent control for the total fraction was 83.8%, for the 0 to 15 minute fraction 88.0%, and for the 16 to 36 minute 77.4%. For Wild Harvest, the total fraction value was 82.0% of the control value, the 0 to 15 minute fraction 85.4%, and the 16 to 36 minute fraction 86.2%. These data are graphically represented in Figure 5. As seen in Figure 5, both Promensil and Wild Harvest samples show a lower absorbance reading than the negative control. The second half sample from the Promensil extract resulted in the lowest value, indicating the most inhibition of proliferation average, about 23%.

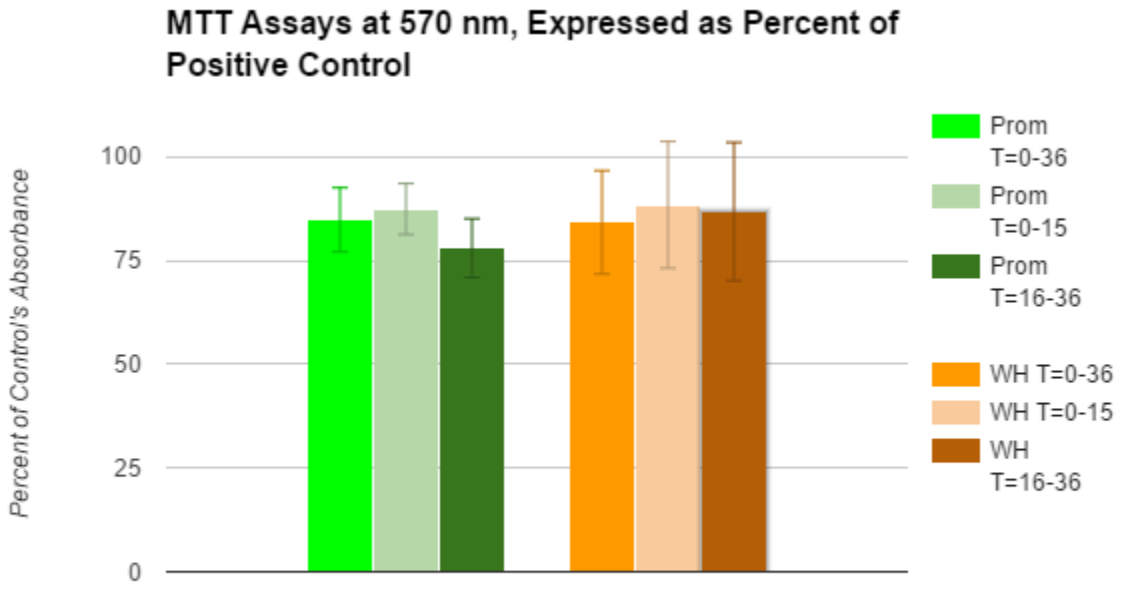

Figure 6: <u>MTT Assays Expressed as Percent of Positive Control (Estradiol added)</u>
HPLC fractions were collected at 5 minute interval and pooled together. Half 1 = pooled samples collected from 0-15 min; half 2 = pooled samples collected from 15-36 min; total = all of the fractions from total HPLC run. Average values from five MTT assays of Promensil and Wild Harvest were expressed as a percent of the positive control, estradiol.

Figure 6 shows the MTT assays of Promensil and Wild Harvest samples of the plates expressed as a percent of the positive control (samples treated with estradiol). As with the case in Figure 5, if the sample value is 100% then it would have the same absorbance as the positive control, meaning that the sample and the positive control had similar effects on growth of the MCF7 cells. For Promensil, the total fraction value was 84.8% of positive control, the 0 to 15 minute 87.4%, and the 16 to 36 minute fraction 78.0%. For Wild Harvest, the total fraction value was 84.2% of the positive control, the 0 to 15 minute fraction 88.4%, and the 16 to 36 minute fraction 86.8%. Again the second half sample from Promensil has the largest impact, resulting in a 22% inhibition of proliferation relative to the estradiol treated sample.

For both Figure 5 and 6, the averages of the solvent control were not shown due to the averages being similar to the averages of the positive and the negative control, thus clarifying that the solvent does not have an effect on the growth of the cells. Appendix A shows the graphs with the averages of solvent control included.

Discussion and Recommendations

This study was performed to test the proliferative effect of OTC phytoestrogen supplements on breast cancer cell culture. The data showed that Promensil samples consistently showed lowered cell proliferation relative to both untreated and estradiol treated cells. This is consistent with previous data suggesting that the OTC product has anti-proliferative effects on breast cancer cells. The results using the Wild Harvest samples were less consistent and generally showed little or no overall effect on cell growth, as none of the data were statistically significant.

Of note amongst the Promensil samples was the 15-36 minute time fraction. Compared to the rest of the samples, this portion of the Promensil sample seemed to have the greatest antiproliferative effect of all tested fractions, including the Wild Harvest fractions. Whereas all other phytoestrogen supplement fractions tested resulted in effective cell counts that were 85-90% of control samples, the second fraction of Promensil values were 77.4% of the negative control and 78.0% of the positive control. While no analysis of the significance of this was done due to the small sample size, this is noticeably lower than any other sample tested and bears future attention.

While the Wild Harvest samples had an average absorbance under 90% of that of either control, the standard error for the Wild harvest samples was very high. This can be

attributed to one Wild Harvest sample out of the five having an absorbance much lower than that of the rest of the plate in any other samples. The absorbance of this particular plate can be found in Appendix B. If these data were omitted from the analysis, it seems likely that Wild Harvest would have had no demonstrable effect at best, or potentially even a somewhat proliferative effect. Again, these data bear repeating.

The highly irregular first HPLC run of the Wild Harvest sample was not explored further by this team, but could have been caused by any number of issues. It could have been that the sample had settled to the bottom of the vial used to inject the sample into the HPLC instrument, resulting in not all of the compounds being injected into the column. Alternatively, the results of that specific run could have been due to contamination in the line from an experiment performed by another group using extracts of different substances. Regardless, the result was assumed to be a form of error rather than a valid result and was summarily ignored.

It is the recommendation of this team that future experiments and MQPs should focus on the proliferative effects of the Promensil samples, specifically on extracts collected in the 15-36 minute eluted HPLC fraction. The samples containing fractions from the second half of the Promensil chromatography consistently show lower absorbance against the absorbance of estradiol samples. This would seem to indicate that there is some compound in the Promensil supplement which may suppress the proliferation of estrogen responsive cancer cells. Future experimenters may wish to plate and perform similar experiments as outlined in the Methods, MTT Assay section, but instead, plate wells with Promensil HPLC samples from shorter fraction times - ie. have triplicates of

0-5 minutes, 5-10 minutes, 10-15 minutes, etc. for the Promensil samples.

The fact that the same fraction of Promensil resulted in similar effects on cells both untreated cells and cells treated with estradiol may suggest that the effect is not being mediated through the estrogen receptor. The use of T47D KBluc cells which have a luciferase gene under the control of the estrogen response element would allow further analysis in this regard.

Additionally, further identification of the peaks for these extracts would be of use in future projects. Previous groups have made efforts at identifying several of the peaks as phytoestrogens, but these only account for a select few of the peaks in each sample. Many of the peaks still remain unidentified, and a study specifically focused on identifying all of the compounds in the samples could be of benefit for future studies focused on only analyzing the effects of specific peaks.

In addition, there are limitations with the methodology that was used in this project for measuring cell proliferation in the wells of the plate. The assay used to measure cell proliferation measures absorbance based on the total activity of the mitochondria. Strictly speaking, this is not a direct measure of proliferation, but can be indicative of it. If possible, it might be useful to use a different assay in future iterations of the project that offer a more direct measure of the quantity of cells in a well. This would come with other issues, as previous iterations of the project have used this assay, making it harder to compare results between research teams. However, it would offer a more accurate way to tell proliferative effects of these substances.

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Appendix A

Additional Assay Analysis Figures

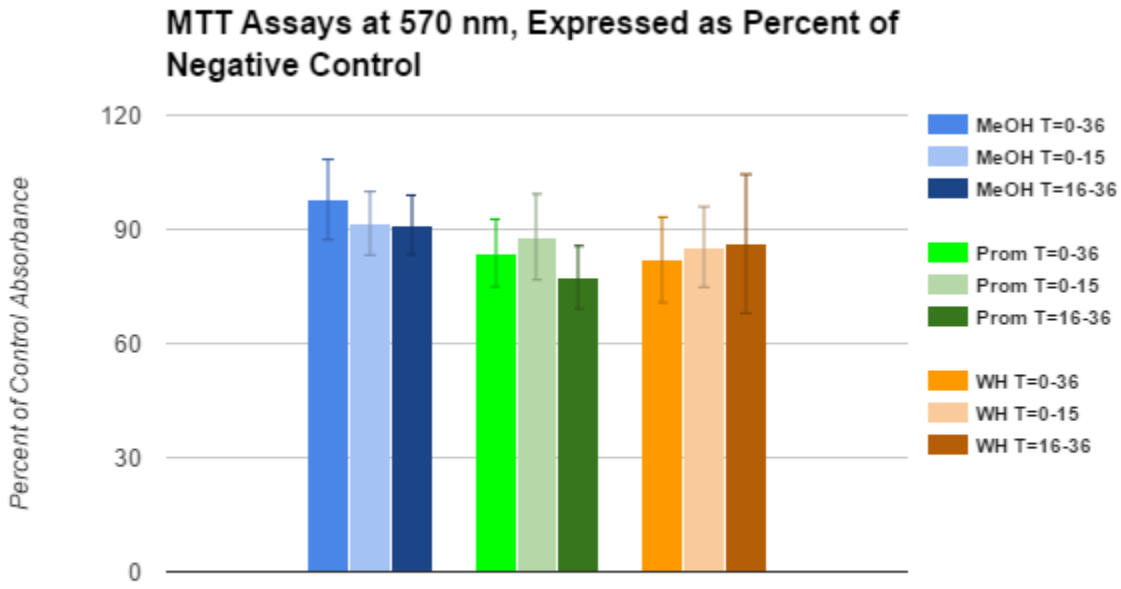

Figure 7: MTT Assays at 570 nm Expressed as Percent of Negative Control (No Extract Added), Including Solvent Control

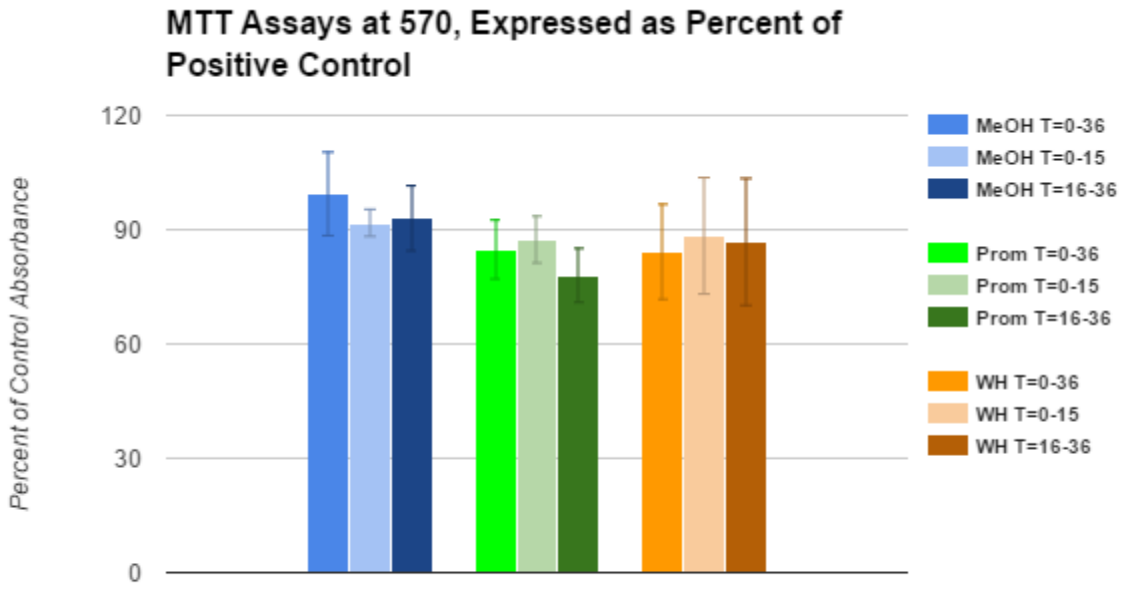

Figure 8: MTT Assays at 570 nm Expressed as Percent of Positive Control (Estradiol Added), Including Solvent Control

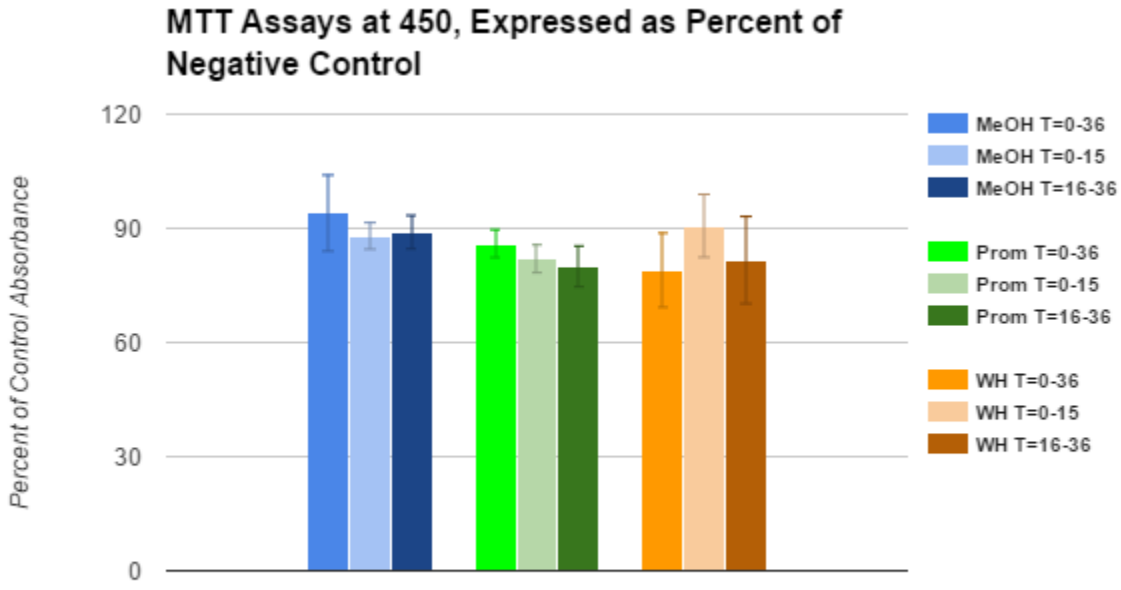

Figure 9: MTT Assays at 450 nm Expressed as Percent of Positive Control (Estradiol Added), Including Solvent Control

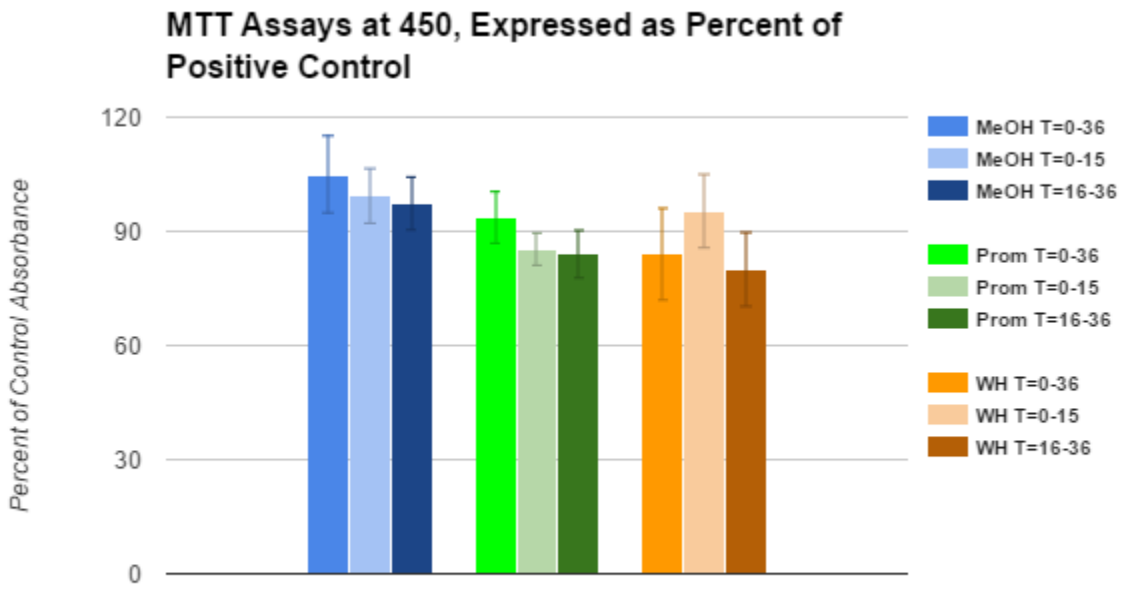

Figure 10: MTT assays compared to Positive Control (Estradiol) at 450 nm, Including Solvent Control

Appendix B

Calculated Data for MTT Assays

Negative Control 570									
	Blank Total	Blank First	Blank Second	Prom Total	Prom First	Prom Second	WH Total	WH First	WH Second
First Plate, % of Control	60	71	72	85	80	77	83	112	106
Second Plate, % of Control	90	91	89	90	87	84	44	69	43
Third Plate, % of Control	107	80	80	55	55	49	74	63	70
Fourth Plate, % of Control	115	96	117	79	93	77	105	73	65
Fifth Plate, % of Control	117	120	98	110	125	100	104	110	147
Average %	97.8	91.6	91.2	83.8	88	77.4	82	85.4	86.2
Standard Deviation	23.65797963	18.60913754	17.39827578	19.86705816	25.23885893	18.44722201	25.10975906	23.64952431	40.82523729
Standard Error	10.58017013	8.322259309	7.780745466	8.884818512	11.28716085	8.249848483	11.22942563	10.5763888	18.25760116

Table 1: <u>Raw Data for Plates Read at 570 nm, Compared to Negative Control</u>

Positive Control 570									
	Blank Total	Blank First	Blank Second	Prom Total	Prom First	Prom Second	WH Total	WH First	WH Second

First Plate, % of Control	80	94	95	113	106	102	110	149	141
Second Plate, % of Control	88	88	86	88	84	82	43	67	42
Third Plate, % of Control	134	100	100	69	69	61	92	79	88
Fourth Plate, % of Control	116	97	118	80	94	78	106	73	65
Fifth Plate, % of Control	79	80	66	74	84	67	70	74	98
Average %	99.4	91.8	93	84.8	87.4	78	84.2	88.4	86.8
Standard Deviation	24.47039027	7.949842766	19.07878403	17.28293956	13.70401401	15.82719179	27.84241369	34.1438135	37.23842102
Standard Error	10.94349122	3.555277767	8.532291603	7.729165544	6.128621378	7.078135348	12.45150593	15.2695776	16.65352815

Table 2: <u>Raw Data for Plates Read at 570 nm, Compared to Positive Control</u>

Negative Control 450									
	Blank Total	Blank First	Blank Second	Prom Total	Prom First	Prom Second	WH Total	WH First	WH Second
First Plate, % of Control	76	84	86	95	91	92	94	112	106
Second Plate, % of Control	87	83	81	84	80	80	54	81	55
Third Plate, % of Control	119	97	100	79	75	68	89	79	84
Fourth Plate, % of Control	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Fifth Plate, % of Control	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Average %	94	88	89	86	82	80	79	90.66666667	81.66666667
Standard Deviation	22.3383079	7.810249676	9.848857802	8.185352772	8.185352772	12	21.79449472	18.50225212	25.57994006

Standard Error	9.989994995	3.492849839	4.404543109	3.660601044	3.660601044	5.366563146	9.746794345	8.274458693	11.43969697

Table 3: Raw Data for Plates Read at 450 nm, Compared to Negative Control

Positive Control 450									
	Blank Total	Blank First	Blank Second	Prom Total	Prom First	Prom Second	WH Total	WH First	WH Second
First Plate, % of Control	99	111	102	111	96	100	102	120	94
Second Plate, % of Control	86	81	80	83	78	78	53	80	55
Third Plate, % of Control	130	106	110	87	82	74	97	86	91
Fourth Plate, % of Control	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Fifth Plate, % of Control	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Average %	105	99.33333333	97.33333333	93.66666667	85.33333333	84	84	95.33333333	80
Standard Deviation	22.60530911	16.07275127	15.53490693	15.14375559	9.451631253	14	26.96293753	21.57158625	21.70253441
Standard Error	10.10940156	7.187952884	6.947421584	6.772493386	4.226897996	6.260990337	12.05819224	9.647106647	9.705668447

Table 4: Raw Data for Plates Read at 450 nm, Compared to Positive Control

Appendix C

Raw Absorbance Data from MTT Assays

	-	+	Solvent	MeOH Total	MeOH 0-15	MeOH 16-36	Prom Total	Prom 0-15	Prom 16-36	WH Total	WH 0-15	WH 16-36
A	0.282	0.199	0.153	0.126	0.029	0.029	0.03	0.03	0.031	0.031	0.033	0.031
B	0.611	0.559	0.131	0.148	0.029	0.03	0.03	0.03	0.03	0.032	0.033	0.031
C	0.031	0.032	0.031	0.031	0.031	0.031	0.03	0.03	0.03	0.033	0.033	0.033

Table 5: First Assay Absorbance Raw Data at 570 nm

	-	+	Solvent	MeOH Total	MeOH 0-15	MeOH 16-36	Prom Total	Prom 0-15	Prom 16-36	WH Total	WH 0-15	WH 16-36
A	1.104	1.101	0.548	1.384	0.825	1.488	0.551	0.853	0.807	0.618	0.428	0.527
B	1.028	0.893	1.174	1.043	1.044	0.997	1.302	1.09	0.859	1.124	0.677	0.538
C	0.051	1.18	1.522	1.251	1.204	1.248	0.685	1.04	0.796	1.619	1.215	1.011

Table 6: Second Assay Absorbance Raw Data at 570 nm

	-	+	Solvent	MeOH Total	MeOH 0-15	MeOH 16-36	Prom Total	Prom 0-15	Prom 16-36	WH Total	WH 0-15	WH 16-36

A	0.87	0.954	0.9	1.013	0.984	0.982	0.936	0.915	0.749	0.917	0.911	0.872
B	1.187	0.687	1.053	1.082	0.914	1.048	0.669	0.722	0.729	0.742	0.616	0.596
C	0.811	0.986	1.037	1.323	0.884	0.848	0.67	0.51	0.469	0.893	0.735	0.934

Table 7: Third Assay Absorbance Raw Data at 450 nm

	-	+	Solvent	MeOH Total	MeOH 0-15	MeOH 16-36	Prom Total	Prom 0-15	Prom 16-36	WH Total	WH 0-15	WH 16-36
A	0.751	0.766	0.722	0.811	0.745	0.753	0.707	0.666	0.54	0.692	0.643	0.689
B	1.078	0.462	0.876	0.854	0.657	0.769	0.374	0.461	0.48	0.448	0.384	0.36
C	0.67	0.777	0.848	1.017	0.6	0.475	0.295	0.257	0.194	0.697	0.547	0.709

Table 8: Third Assay Absorbance Raw Data at 570 nm

	-	+	Solvent	MeOH Total	MeOH 0-15	MeOH 16-36	Prom Total	Prom 0-15	Prom 16-36	WH Total	WH 0-15	WH 16-36
A	1.233	1.358	1.358	1.105	1.241	1.241	1.415	1.153	1.229	0.818	1.564	0.629
B	1.513	1.471	1.349	1.235	1.151	1.067	1.011	1	0.996	0.76	1.039	0.865
C	1.469	1.46	1.361	1.338	1.087	1.11	1.122	1.2	1.126	0.692	0.829	0.844

Table 9: Fourth Assay Absorbance Raw Data at 450 nm

	-	+	Solvent	MeOH Total	MeOH 0-15	MeOH 16-36	Prom Total	Prom 0-15	Prom 16-36	WH Total	WH 0-15	WH 16-36

A	1.373	1.659	1.464	1.368	1.605	1.526	1.647	1.431	1.506	0.771	1.636	0.585
B	1.801	1.675	1.508	1.333	1.408	1.348	1.286	1.238	1.215	0.709	0.916	0.79
C	1.636	1.614	1.496	1.63	1.354	1.395	1.413	1.511	1.319	0.636	0.768	0.688

Table 10: Fourth Assay Absorbance Raw Data at 570 nm

	-	+	Solvent	MeOH Total	MeOH 0-15	MeOH 16-36	Prom Total	Prom 0-15	Prom 16-36	WH Total	WH 0-15	WH 16-36
A	0.324	0.307	0.265	0.268	0.291	0.308	0.381	0.318	0.332	0.298	0.469	0.373
B	0.368	0.304	0.294	0.269	0.303	0.294	0.324	0.33	0.351	0.328	0.364	0.396
C	0.391	0.315	0.273	0.289	0.319	0.328	0.329	0.341	0.309	0.388	0.383	0.379

Table 11: Fifth Assay Absorbance Raw Data at 450 nm

	-	+	Solvent	MeOH Total	MeOH 0-15	MeOH 16-36	Prom Total	Prom 0-15	Prom 16-36	WH Total	WH 0-15	WH 16-36
A	0.214	0.196	0.146	0.158	0.174	0.185	0.257	0.204	0.196	0.183	0.337	0.271
B	0.264	0.19	0.154	0.143	0.184	0.172	0.194	0.196	0.206	0.191	0.253	0.281
C	0.284	0.186	0.15	0.154	0.182	0.189	0.197	0.206	0.183	0.258	0.261	0.252

Table 12: Fifth Assay Absorbance Raw Data at 570 nm