The Effects of Phytoestrogens on LNCaP Prostate Cancer Cells

A Major Qualifying Project Report submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science submitted by:

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Abstract:

Phytoestrogens found in plants have been reported to be therapeutically effective in treating prostate cancer, with reduced side effects compared to conventional hormone treatment. The structural similarities phytoestrogens share with estradiol 17-β suggest that they will bind to the ER-β region of LNCaP cells and induce downstream suppression much like estrogen does. This paper seeks to examine the effects of different types of phytoestrogen extracts on prostate cancer cells compared to conventional hormones used to treat prostate cancer.
Introduction:
Prostate cancer is a condition in which cancer cells form in the tissue of one of the male reproductive glands, the prostate. If the growth of this cancer is not suppressed, metastasis occurs and potentially spreads to the central nervous system, bones, lymph nodes and other organs and glands of the body (Adjakly et al., 2014). Prostate cancer is less frequent in men under age forty and in men on vegetarian diets, meaning age and diet are factors contributing to the risk of developing prostate cancer (National Cancer Institute, 2015). Male hormones are required for the proper functioning of the prostate gland. However, excess amounts of androgens can lead to mutations in the proteins expressed in the prostate, leading to the development of cancer. Reports have shown that in the U.S. alone, 2,707,821 men have prostate cancer, making it the most common type of cancer in the United States, followed closely by breast cancer (National Cancer Institute, 2013).

In order to effectively treat patients with prostate cancer, several conventional cancer therapies are used. These include surgery, complete removal of the tumorous growth, radiation therapy and chemotherapy to suppress tumor activities (American Cancer Society, 2014). Hormone therapies such as ADT (androgen deprivation therapy) lower androgen levels in the prostate, thereby suppressing the growth and metastasis of tumor cells. Various drugs can be used which either lower androgen levels or have anti-androgenic properties to inhibit cancerous activities (American Cancer Society, 2014). While anti-androgenic drugs are effective, there are certain risks that accompany them. Once a tumor has progressed to a certain extent, it may become androgen-independent and no longer respond to such treatments (Morrissey and Watson, 2003).

Estrogen treatment is one method of depriving the body of androgens. Estrogen hormones are attracted to two different isoforms of estrogen receptor, α and β. While ER-α is found predominantly in uterine cells, ER-β can be found in the prostate, ovaries, and salivary glands (Hewitt and Korach, 2002). Several prostate cancer cell lines, including LNCaP, PC-3, and DU-145, express ER-β (Adjakly, 2014). Estrogen, namely 17-β estradiol, is an agonist which binds to ER-β and induces tumor suppressor activity downstream (Singh et al. 2014).

Reported side effects from estrogen therapy in prostate cancer patients include cardiac dysfunctions, hot flashes, tenderness or enlargement of the breasts, and erectile dysfunction. As a
result, phytoestrogens, which suppress tumorous growth by binding to ER-β, have been used as an alternative method of treatment (Weng et al., 2013).

Previous Major Qualifying Project (MQP hereafter) research on phytoestrogens conducted by Bao suggested anti-proliferative activities from the commercial phytoestrogen supplement, Promensil, on LNCaP cells (Bao, 2013). Results from Bao’s paper showed that Promensil-treated cells and estrogen-treated ones both displayed lower cell proliferation by MTT assay and immunoblot against Proliferating Cell Nuclear Agent (PCNA). Bao explained in her paper that the p21 pathway, when up-regulated, promotes cell cycle arrest, and suggested phytoestrogen components in promensil as responsible agents for inducing actions downstream this pathway. Furthermore, research by Young et al. indicated that phytoestrogens genistein and biochanin A are active agents in promoting the up-regulation of the p21 pathway. The suggestions made in Bao’s paper served as crucial groundwork for this study.

**Phytoestrogens:**

Phytoestrogens, as their name suggests, are plant-derived estrogens. It has been reported that phytoestrogens are found in abundance in foods such as flax seed, grains, fruits, and broccoli. While various vegetables and fruits contain phytoestrogens, soy and red clover have the highest concentrations of phytoestrogens with reported therapeutic properties (Goetzl et al., 2007). Phytoestrogens derived from soy have been tested to inhibit the growth of breast cancer, and their therapeutic role has been extended to include prostate cancer as well (Weng et al., 2012).

Phytoestrogens can be largely divided into three categories: isoflavones, coumestans, and lignans (Patisaul & Jefferson, 2013). Experiments involving phytoestrogens in vitro and in vivo have shown that phytoestrogens are capable of both estrogenic and antiestrogenic activities. The estrogen-like properties of phytoestrogens are most likely the result of the structural similarities it shares with estrogen. Notably, isoflavones are structurally similar to 17-β-estradiol, and they also act as an agonist for ER-β (Adjakly et al., 2014).

The binding affinity of the isoflavone genistein to ER-β is comparable to the binding affinity of 17-β-estradiol (Hirose et al., 2002). Some isoflavones, therefore, have a high affinity towards ER-β, and will consequently induce suppression downstream by binding to it in the
same manner as 17-β-estradiol. (Goetzl et al., 2007). Figure 1 below displays the structural similarities between different types of isoflavones and 17-β-estradiol.

![Figure 1: Chemical composition of sample isoflavones and estradiol-17β (Structures from cyberlipid.org and tuscany-diet.net).](image)

Promensil:

Promensil is an over-the-counter dietary supplement made for the purpose of relieving women of the symptoms of menopause. Promensil Menopause Double Strength, which was used to test the effects of phytoestrogens on LNCaP cells, is particularly abundant in isoflavones, especially genistein, which is derived from red clover and soy (Setchell et al., 2010). One serving of NATROL’s Promensil Menopause Double Strength contains 80mg of isoflavones including genistein, daidzein, formononetin, biochanin A, and glycitein (Setchell et al., 2010). In order to observe significant and specific effects of the various components of Promensil, we used cultured cells and tested the tumor-suppressing abilities of the product and several of its constituent isoflavones.

LNCaP:

The Lymph Node Carcinoma of the Prostate (LNCaP) is a prostate cancer cell line. LNCaP cells contain androgen receptors and their growth is highly dependent on androgen binding. This cell line is used extensively for the in vitro study of the effect of androgens on human prostate cancer (van Steenbrugge et al., 1989). LNCaP is used as a model in research
because it grows relatively quickly, making it easy to culture. Furthermore, the cell line is highly sensitive to hormone activity, expressing both androgen and estrogen receptors (Horoszewicz et al., 1983). In our experiments, we tested the effects of phytoestrogens and Promensil on the growth and development of LNCaP cells compared to those of pure estrogen and testosterone.

**PCNA:**

Proliferating Cell Nuclear Antigen (PCNA hereafter) is a member of the DNA β-clamp family and a cofactor involved in DNA synthesis. These clamps form hexagonal rings that encircle and slide along DNA, facilitating the binding of polymerases and other replication proteins involved in DNA synthesis and repair (Moldovan et al., 2007). Proteins that associate with PCNA include topoisomerases, helicases, ATPases, DNA ligase, and protein kinases such as the p21 tumor suppressor (Moldovan et al., 2007). Since the presence of PCNA indicates cell proliferation, the amount of PCNA observed in a cell sample treated with phytoestrogens can be used to measure that cell line’s proliferation response to said phytoestrogens. A decrease in PCNA, and by extension in proliferation, with the addition of a raw hormone or phytoestrogen extract could signify a beneficial effect on mitigating the growth of cancer cells.

**MTT Assay:**

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a salt whose tetrazolium ring is cleaved by dehydrogenase enzymes in mitochondria (Mosmann, 1983). Normally the salt in solution has a faint yellow color, but once cleaved it yields a dark blue precipitate. Because the cleaving can only occur in live cells, MTT can be used for colorimetric assay purposes to assess cell numbers. Adding an alcohol such as isopropanol makes the resulting precipitate soluble, so by measuring the absorbance of the resulting solution, it is possible to determine how many living cells are present (Mosmann, 1983). This assay technique was used to quantify the number of live cells present before and after phytoestrogen treatment. Similarly to the PCNA tests we performed, the aim of the MTT assay was to observe a marked change in the number of live or active cancer cells following treatment with various hormones and phytoestrogen extracts.
Ongoing Research of the Effects of Phytoestrogens on Prostate Cancer:

Phytoestrogen therapies have been in the research spotlight for years as a result of studies on dietary factors that can reduce an individual’s chance of developing prostate cancer. In the United States, one in six men are at risk of developing prostate cancer at some point in their lives, whereas in parts of Asia, especially Japan, the risk is notably lower (Hörmann, 2012). This disparity is attributed to the significantly higher consumption of soy products in Asia. Soy inherently contains isoflavones such as genistein: a phytoestrogen that has been linked to decreased levels of growth in cancerous cells due to its ability to induce apoptosis (Hörmann, 2012). Another phytoestrogen under investigation is daidzein, which has displayed positive effects on human hepatic cancer SK-HEP-1 cells by causing apoptosis (Park et al., 2013).

In both studies, MTT analysis and cell proliferation assays were used alongside other techniques for measuring cell viability and apoptosis. Experimenting with phytoestrogen components individually and in various combinations and concentrations could prove useful in determining the most effective means of therapy while minimizing harmful side effects. A study performed by Dong et al. tested the effects of genistein, daidzein, and combinations of the two on three types of prostate cancer cell lines: BPH-1, a benign prostate hyperplasia, PC3, a malignant androgen-independent prostate cancer epithelial cell line, and LNCaP, the early stage androgen-dependent prostate cancer cell line used in our study. The study found that high doses of daidzein and genistein individually induced apoptosis and a halt in the cell cycles of all three strains (Dong, 2013).

Focusing on the effects of different combinations of phytoestrogens and phytochemicals in various cell lines could also yield further insight into how they target different types of cancers. According to a 2014 study, different flavonoids target different parts of the body and therefore could be used strategically to target specific locations where tumors and cancerous cells are an issue. For example, flavonol aglycones such as myricetin have a strong effect on lung cancer cells, whereas apigenin is more successful with cervical cancer cells due to how the body processes the two (Sak, 2014).

Based on previous reports and research on isoflavones’ abilities to inhibit tumor growth in prostate cancer (Adjakly et al., 2014), our research explored the effects of the over-the-counter phytoestrogen product Promensil as well as how individual phytoestrogen components induced changes in the prostate cancer cell line LNCaP. We chose to test the effects of genistein and
biochanin A specifically because a significant portion of our research examined those isoflavones.

As mentioned previously, conventional therapies used to treat prostate cancer, especially androgen deprivation therapy, come with numerous side effects. Estrogens used in androgen deprivation therapy modulate androgen activity via estrogen receptors of prostate cancer cells (Weng et al., 2013). Despite the inhibition of prostate cancer proliferation, continual estrogen therapy carries the risk of inducing cardiovascular side-effects, namely thrombosis and cardiac events. A paper by Weng et al. discusses the mechanism by which estrogen and its receptor binding induce cardiovascular symptoms. In one of their experiments, HAEC (human aortic endothelial cells), LAPC-4 and LNCaP cells were stimulated by dihydrotestosterone (DHT) and treated with aE2, BE2, DES, ICI, genistein, and tamoxifen. Both HAECs and prostate cancer cell lines treated with DHT showed significant increases in viable cell numbers. ER ligands introduced to the cell lines had opposing effects and regulated the DHT-induced cell proliferation (Weng et al., 2013). Findings showed that when ER ligands were dispensed simultaneously with DHT, they resulted in a dose-dependent inhibition of DHT-induced cell proliferation. Knockdown of ER-β alone decreased estrogen inhibition of DHT-induced cell proliferation in LAPC-4 cells. The same effect was not observed when ER-α was knocked down. Moreover, binding affinities to ER differ between estrogen ligands, and this results in significant differences in their ability to suppress DHT-induced cell proliferation. Notably, the two ER receptors (α and β) were shown to have different modulating effects and function independently from one another (Weng et al., 2013). Taken together, this paper states that modulation of androgen action occurs in a receptor-ligand and receptor-isoform specific manner and thus the ability to inhibit proliferation depends on ER receptor ligands and receptor isoforms.

Epigenetic regulation by phytoestrogens which silence prostate cancer cell proliferation via demethylation of a gene promoter site have been suggested from a study conducted by Adjakly et al. This study reports an epigenetic modulating ability of soy phytoestrogens, specifically genistein and daidzein. Estradiol-β binding to ER reverses the methylation responsible for inhibiting oncosuppressors in prostate cancer cell lines. The authors of the paper experimented with three different cell lines of prostate cancer and observed whether a similar mechanism of estrogen binding to ER-β occurred with phytoestrogen binding to ER by reversing methylation at a DNA promoter site. Based on the known ability of genistein to induce apoptosis
and activation of the caspase pathway in prostate cancer cell lines, the writers hypothesized that this would be the case. Results from Adjakly’s experiment indicated that soy phytoestrogens, genistein and daidzein were significant actors in the demethylation of gene promoter sites. This was a prevalent pattern observed in all three prostate cancer cells lines, LNCaP, DU-145, and PC-3.

In a study conducted by Szliszka et al., LNCaP and DU-145 cells were treated with biochanin A and the effects were measured. The results from the experiment showed that biochanin A was able to induce cytotoxic and apoptotic effects in both cell lines. Tumor necrosis factor related apoptosis inducing ligand (TRAIL) used in combination with biochanin A was shown to have the strongest apoptotic effect, suggesting that biochanin A is capable of sensitizing TRAIL-resistant prostate cancer cells to TRAIL-induced apoptosis (Szliszka et al., 2013).

Several studies focusing on genistein found that it is related to many important processes in pathways for cell survival and apoptosis. Genistein affects a number of other proteins, including protein-tyrosine kinase, topoisomerase I and II, NF-κB, protein kinase B, protein histidine kinase and others (Brunet et al., 1999, Cardone et al., 1998, Van Antwerp et al., 1996 and Wu et al., 1996). Below shows a diagram of just a couple of the pathways genistein can affect.
Looking at the PKB or Akt pathway, genistein up-regulates XIAP (an apoptotic inducer) and inhibits Bax (an apoptotic suppressor). Genistein’s effect on these two proteins leads to apoptosis (Brunet et al., 1999). Genistein also inhibits NF-κB (Brunet et al., 1999); a class of proteins responsible for promoting cell survival and proliferation. Topoisomerases I and II, which are crucial enzymes for successful DNA replication, are inhibited by genistein as well, preventing viability in the cancer cells (Brunet et al., 1999). Seeing as a single phytoestrogen is capable of such activity, genistein in combination with other isoflavones such as daidzein or biochanin A could be substantially more effective when dealing with cancerous cells, prostate or otherwise.

A study in 2013 predicted that combinations of phytoestrogens with lower individual concentrations could be more effective, more efficient, and safer than using higher concentrations of single isoflavones when treating prostate cancer cells. The study also found that daidzein and genistein both had dose-dependent antiproliferative effects on LNCaP and C4-
2B cell lines. However, genistein was shown to be the more effective of the two (Dong, 2013). In another study, LNCaP and PC3 cells were treated with genistein, selenium, and a combination of genistein and selenium. All three treatments were able to induce apoptosis in both of the two cell lines. Results between the two cell lines were near-identical, whereas results between treatments showed that the combination of genistein and selenium was most effective at inhibiting cell growth compared to either individual treatment (Kumi-Diaka, 2010).

The studies explored above pertains to how phytoestrogens in different combinations and concentrations can influence cell proliferation in its early stages. It is evident from the researches that phytoestrogens and commercial phytoestrogen products inhibit tumor cell proliferation and could potentially replace conventional hormones in treating prostate cancer. However, understanding the most effective components and concentrations is important and figuring out whether or not they will be safe for use in cancer patients is paramount.
Materials and Methods:

Promensil Product and Preparation (Reflux):

One tablet (daily recommended dose by the manufacturer) of Promensil Double Strength ® (Novogen Ltd., Australia) was ground up and transferred to a round-bottom flask. A solution of 80% volume per volume methanol in water was prepared and used for the transfer of the ground-up product into the flask. The pestle and mortar used in grinding the tablet was rinsed with 80% methanol three times and the total volume in the flask was 100mL. The flask was then attached to a reflux apparatus and the water bath was brought to a temperature of 65 degrees Celsius. The Promensil solution was refluxed for one hour and then stored in the freezer. One tablet of Promensil contains 80mg of isoflavones (genistein, daidzein, formononetin, biochanin A), standardized red clover extract of 25:1, as well as calcium.

Treatment and Control Preparation:

Estrogen and testosterone were selected as negative and positive controls for our experiments, respectively. 17-β-Estradiol, a type of estrogen selected for our study, was diluted in methanol, as was methyltestosterone; a derivative of testosterone. The stocks of estrogen and testosterone were first dissolved in methanol followed by a serial dilution. The isoflavones genistein and biochanin A were also diluted from a stock of 200μM using methanol as the solvent.

Cell Culture:

The cells used for our experiments were LNCaP clone FCG, ATCC CRL-1740 from the American Type Culture Collection, and were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (Pen/Strep) and 1% glutamine at 37°C and 5% CO₂.

MTT Assay:

To observe the effects of different concentrations of genistein, biochanin A, and Promensil on proliferation, MTT Cell Proliferation Assays were conducted according to the protocol described by the manufacturers of Cell Titer 96 ® Aqueous One-Solution Reagent
(Promegna). The cells were plated in a 96-well plate at a density of $10^4$ cells/well. Normal media (DMEM, 10% FBS, 1% penicillin-streptomycin) was added to all the wells and was later replaced with phenol red-free medium described above after 24 hours of incubation. Following this incubation period, treatments were added to the wells as indicated for each of our respective experiments. 24-48 hours after treatment, 20μL of Cell Titer 96 ® Aqueous One-Solution Reagent (Promegna) was added to each well and the plates were left to incubate for 1-4 hours. At this point, optical density readings were taken at 595nm using a spectrophotometer. Table 1 below displays a sample assay plate with the volume of treatments and media added to each well.

**Table 1. 96 Well Plate Design**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein 0.1uM</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>100μL media (No cells)</td>
<td>No Treatment</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
</tr>
<tr>
<td>Genistein 1μM</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>100μL media (No cells)</td>
<td>No Treatment</td>
<td>2μL+100μL Media</td>
<td>2μL+100μL Media</td>
<td>2μL+100μL Media</td>
<td>2μL+100μL Media</td>
</tr>
<tr>
<td>Genistein 10μM</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>100μL media (No cells)</td>
<td>No Treatment</td>
<td>2μL+100μL Media</td>
<td>2μL+100μL Media</td>
<td>2μL+100μL Media</td>
<td>2μL+100μL Media</td>
</tr>
<tr>
<td>Genistein 50μM</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>100μL media (No cells)</td>
<td>No Treatment</td>
<td>Empty</td>
<td>Empty</td>
<td>Empty</td>
<td>Empty</td>
</tr>
<tr>
<td>Biochanin A 0.1μM</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>100μL media (No cells)</td>
<td>No Treatment</td>
<td>Empty</td>
<td>Empty</td>
<td>Empty</td>
<td>Empty</td>
</tr>
<tr>
<td>Biochanin A 1μM</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>100μL media (No cells)</td>
<td>No Treatment</td>
<td>Empty</td>
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<td>Empty</td>
</tr>
<tr>
<td>Biochanin A 10μM</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>100μL media (No cells)</td>
<td>No Treatment</td>
<td>Empty</td>
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<tr>
<td>Biochanin A 50μM</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>2μL+100μL media</td>
<td>100μL media (No cells)</td>
<td>No Treatment</td>
<td>Empty</td>
<td>Empty</td>
<td>Empty</td>
<td>Empty</td>
</tr>
</tbody>
</table>

*Table 1: Displays the sample design of a 96 well plate used for MTT assay. Each well shows 2μL of treatment and 100μL of the media. The final amount of treatment concentration and media concentration is indicated in the chart. Columns 10-12 (not shown) are empty.*

**Immunoblot:**

In order to confirm the results acquired from the MTT assay, an immunoblot for PCNA was conducted. A 6-well and a 12-well plate were filled with 100,000 cells/well and 50,000 cells/well respectively. After a 24-hour incubation period, the media was changed to phenol red free DMEM with 10% dextran-coated charcoal stripped FBS, 1% Pen/Strep, and 1% glutamine. After another 24 hours of incubation, treatments were added and the plates were incubated for 48
hours. The 6-well plate contained two treatments each of 50uM genistein, 0.1uM biochanin A, and 100% Promensil extract (concentration of 800ug/mL). The 12-well plate was treated with testosterone, estrogen, methanol, and no treatment (three wells of each). After the medium was aspirated, the wells were washed with PBS. After removing the PBS, the plates were frozen at -80 degrees Celsius overnight. The cells were then resuspended in 100uL of PBS by using a rubber policeman to scrape the bottoms of the wells. The proteins acquired at this point were measured through a Bradford Assay using Coomassie Plus Reagent (Pierce) to determine the total protein concentration of each sample. The sample absorbances were read at 595nm and were compared to a BSA standard curve of known concentrations. The results of the Bradford assay are found in Appendix section B, Figure A.

Once the protein concentration of the cells treated with each sample was found, SDS-PAGE electrophoresis was conducted by loading an equal amount (9.17ug) of protein into each lane of a 4-20% Mini-PROTEIN TGX precast gels (BIO-RAD, Hercules, CA, USA) and electrophoresis was carried out at 300-350 volts for approximately 20 minutes in 1X running buffer (Tris, glycine, SDS). Once the proteins were separated, semi-dry blotting technique was conducted to transfer proteins from gel to Immobilon-P membrane using CAPS transfer buffer (10 mM CAPS, 10% methanol, pH 11) at 400 amps/cm² for 20 minutes. Afterwards, the membrane was washed in Tris Buffer Saline (TBS: 10 mM Tris, 150 mM NaCl, pH 7.4). After washing, the membrane was incubated in anti-PCNA mouse antibody (Santa Cruz Biotechnology, Inc) at a 1:100 dilution in 5% nonfat dry milk overnight at 4 degrees Celsius. Once the incubation period was over, the membrane was washed with TBS twice and once with TBS/Tween 1%. The membrane was incubated with alkaline phosphatase conjugated goat anti-mouse IgG + IgM (Pierce) for two hours at room temperature and washed with TBS and TBS/Tween 1% as above. After washing was complete, the membrane was developed in SigmaFAST NBT/BCIP® reagent and reaction was stopped with water once the bands were manifested on the membrane. Lastly, densities of the protein bands displayed on the membrane were quantified by using ImageJ (National Institute of Health).
Statistical Analysis:

In order to validate the differences and patterns of the experimental samples, statistical analysis was conducted. When two sets of data were compared to one another, an unpaired t-test was used to determine the existence of statistically significant differences between groups. When more than two groups were compared against each other, analysis of variance (ANOVA) was performed in order to confirm the existence of statistical differences between multiple groups.

The formula for an unpaired t-test is shown below. A P-value was determined from these statistical tests, which represents the probability that the null hypothesis can be rejected. A P-value greater than 0.05 is not strong enough to reject a null hypothesis.

\[
 t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(N_1-1)S_1^2 + (N_2-1)S_2^2}{N_1+N_2-2} \left( \frac{1}{N_1} + \frac{1}{N_2} \right)}}
\]

[Formula 1]: T-test formula. Formula above displays t-value which indicates statistical significance between two data groups (Formula from chem.utoronto.ca).
Results:

LNCaP cells were exclusively used for these experiments and were treated with genistein and biochanin A; phytoestrogens that have had the greatest suppressing effects on cancer cell proliferation in previous studies (Weng et al., 2013). One of the objectives of our experiments was to observe the effects that genistein and biochanin A have on LNCaP cells. In order to conclusively state the results from the proliferation assay, an immunoblot assay was also performed using monoclonal anti-PCNA mouse antibodies. The presence of PCNA indicates cell proliferation, thus antibody binding of PCNA was predicted to be the greatest in testosterone treated cells (positive control), and lowest in cells treated with estrogen (negative control) and phytoestrogen components (Bao, 2013). Overall, our hypothesis that individual phytoestrogens would yield equal or similar anti-proliferative effects to estrogen and Promensil was not supported by the cell proliferation assay, but was partially supported by our immunoblot observations. We predicted that lower levels of PCNA would be present in cells treated with genistein, biochanin A and Promensil. Anti-proliferative effects of 50uM genistein were signified by a lower level of PCNA expression. However, cells treated with 0.1uM biochanin A, Promensil extract, and estrogen all lacked significant differences in their levels of expression compared to methanol- and testosterone-treated cells.

The cell morphology throughout our experiments was consistent with that published by the ATCC. Figure 3 below shows the standard morphology of our LNCaP cells in DMEM media compared with the ATCC image.
[Figure 3]: Cell morphology of LNCaP cells. Our cells are on the left and the ATCC image on the right for comparison (ATCC, 2014). Cell morphology at 50X microscope magnification, plated in normal media (DMEM, 10% FBS, 1% antibiotics).

LNCaP Proliferation Assay #1:

Our LNCaP cells were treated with varying concentrations of either genistein or biochanin A at concentrations of 0.1uM, 1uM, 10uM, and 50uM. 17-β estradiol and methyltestosterone (a testosterone analog) were also used at a concentration of 50uM.

1% Methanol controls were run to assess the effect of the solvent on cell proliferation. Proliferation was assessed using an MTT assay. The proliferative effects of each treatment, expressed as % methanol control, are shown in Figure 4 below.
[Figure 4]: Average % Methanol Control of Treatments on LNCaP cells. The error bars show the standard deviation of each treatment. The data shown is the average data of each treatment as compared to percent methanol used as control from each experiment, finally all the percent methanol values of each treatments were averaged, as shown in the figure. The N values are shown on the right side of the figure.

The figure above displays high variability of each treatment as indicated by the error bars. Due to the high variability, we cannot draw decisive conclusions about any inhibitory or proliferative patterns of the phytoestrogen treatments on LNCaP cells. An unpaired T-test was performed in order to determine the statistical significance of each treatment compared to the control. The results indicated that none of the phytoestrogen treatments were statistically different from estrogen, testosterone, or the methanol control, with the exception of biochanin A at 10uM. Biochanin A at 10uM had the smallest standard deviation when compared to that of the other treatments, and was shown to be statistically significant in promoting cell growth, with a P-value of 0.0048 as shown in table A of the appendix. Though no significant proliferative behaviors were observed from genistein-treated cells at any of our tested concentrations, there were indications of some inhibitory trends at concentrations of 0.1uM, 1uM and 10uM as shown
in appendix section A, table A. In addition, the undiluted Promensil extract also showed an inhibitory trend compared to methanol, but the significance of this trend cannot be determined due to the N value being only 1. Previous data collected by other groups from our lab, as well as other labs, indicates anti-proliferative effects of genistein and Promensil, but our results from the MTT assay could not fully support these findings.

**LNCaP Proliferation Assay Response to Testosterone Concentrations:**

In order to confirm the proliferative activity of testosterone on LNCaP cells, as well as the responsiveness of these androgen-dependent cells to testosterone, an MTT assay was performed which solely tested various concentrations of testosterone. Figure 5 below displays the effects of the various testosterone concentrations compared to a methanol control.

![Percent Methanol Control of Testosterone Concentrations](image)

**[Figure 5]:** Percent Methanol Control of Testosterone Concentrations. Displays relative proliferation of testosterone concentrations of 0.1uM, 1uM, 10uM and 50uM on LNCaP cells compared to that of methanol control. Percent methanol control was calculated by dividing average readings of each testosterone concentration by average of methanol control readings.

Testosterone at all concentrations showed no significant effect on the proliferation of LNCaP cells when compared to the control. Since this finding was inconsistent with our expectations, we were concerned that our cells had been inadvertently selected to be non-responsive to androgens. As a result, a new cell stock was ordered from ATCC. Subsequent experimental results continued to fail to show any significant increases in proliferation, as shown
in table C of the appendix. An analysis of variance test was carried out to observe any statistical differences between the various testosterone concentrations, but a resulting P-value of 0.325 indicates that no significant difference exists between the groups. Overall, no substantial conclusion can be made from the data above.

**PCNA Immunoblot:**
An immunoblot for PCNA was conducted in order to validate the findings from the multiple MTT assays. Concentrations of genistein and biochanin A with the strongest anti-proliferative effects were selected to treat the cells. 50uM genistein and 0.1uM biochanin A were used, as well as undiluted Promensil extract, 50uM estrogen, 50uM testosterone, and 1% Methanol. The results of this immunoblot are included in Figure 6 shown below. To confirm the band intensities displayed on the membrane, we quantified each band using ImageJ, and the area under the curve of each protein band are shown in figure 7 below.

![Figure 6: Immunoblot results. Probed with monoclonal anti-PCNA mouse antibody. Well #1 and #7: 50uM genistein; Well #2: 0.1uM biochanin A; Well #3: undiluted Promensil extract; Well #4: testosterone (positive control); Well #5: methanol (control); Well #6: estrogen (negative control); Well #8: marker. Well #7 showed the faintest band and Well #6 showed the darkest band. Molecular weight (kDa) shown to the right in yellow. MW (PCNA) = 36 kDa.](image-url)
[Figure 7]: Graph values from ImageJ indicate varying protein densities of immunoblotting for PCNA.
Discussion:

Previous research conducted on phytoestrogen compounds, especially genistein and biochanin A, reported their anti-proliferative effects on prostate cancer cell lines (Adjackly et al., 2014) with reduced side effects compared to conventional hormone therapy (Weng et al., 2013). Based on their findings as well as other literature reviews, we hypothesized that phytoestrogens could replace estrogen used to suppress LNCaP cell proliferation. In addition, suggestions from MQP research by Bao on Promensil suppression of LNCaP cells were also implemented to preliminary stages of our research and served as a directional guideline in defining our project’s objectives: testing individual Promensil components, using the same concentrations of samples she used for comparison, as well as the same controls (Bao, 2013). Taken together, we predicted that individual phytoestrogen components would yield anti-proliferative effects on LNCaP cells similar to those of pure estrogen and the commercial product, Promensil.

Due to the high variability of our data and the lack of statistical significance for the majority of treatments compared to the control, we cannot conclusively state the effects of the treatments on LNCaP cell proliferation. However, of all the treatments, the most promising results were shown by biochanin A at 10μM. At this concentration, biochanin A had the most consistent percent control and lowest standard deviation among all treatments, demonstrating statistical significance compared to methanol. Looking at Figure 4 though, biochanin A at all concentrations had a proliferative effect on the cells relative to the control, which refutes our hypothesis. Furthermore, other papers that reported a successful inhibition of cell proliferation used biochanin A at 100μM (Seo et al., 2011), a 10-fold higher concentration than what we used. It is possible that treatment with a more concentrated dose of biochanin A is responsible for our cells reacting in the manner observed.

There was some evidence of anti-proliferative activity of genistein and Promensil over the course of our experiments, although the composite results had high variability. MTT results of Promensil and 50μM of genistein observed in the immunoblot could support that phytoestrogens used in combinations could be more effective than using higher concentrations of individual phytoestrogens, as hypothesized in other research.

The proliferation measurements from the controls were highly variable, in addition, when measured against methanol for any statistical significance, none was found. Testosterone treatments (positive control) were hypothesized to increase cell proliferation due to LNCaP cells
being androgen-dependent. However, there were no significant differences between testosterone- and methanol-treated cells as indicated by a P-values of 0.1528 and 0.3399 at concentrations of 50uM and 200uM respectively; shown in table A of the appendix. This could have been due to the cell line becoming non-responsive to testosterone over time. To test this, an MTT assay was conducted with varying concentrations of testosterone. A new batch of LNCaP cells was also utilized to compare to the results from the first batch. The results showed that sensitivity to testosterone of both lines were similar, as indicated by ANOVA of testosterone concentrations in table C of the appendix. This reduced the possibility of the cells becoming androgen-independent or desensitized to testosterone. There also was the possibility of methyltestosterone, the testosterone derivative used, being inactive or unable to give the same effects as testosterone itself. Overall, our inability to observe testosterone-induced proliferation at any of our concentrations also substantiates the inconsistency behind our data.

From our composite MTT assay results, estrogen treated cells showed one of the highest proliferation levels however, no conclusion of estrogen’s effect on LNCaP can be made due to the high variability between MTT assay trials. We can only conclude that estrogen had a proliferating trend on the LNCaP cells which refutes our predictions that estrogen modulates androgen activity by 17-β-estradiol binding to ER-β therefore suppressing androgen-dependent LNCaP cells. Again we see high variability in the data therefore the effect of estrogen on LNCaP cannot be substantiated.

Despite the comparable genistein bands observed in wells #1 and #7 of the immunoblot, when the genistein sample was loaded onto well #1, there was an experimental error with loading, which caused disruption of the well #1. Thus there exists a possibility of well #1 puncturing, which may have led to mixing of solutions at well #1 and #2. The intensities of the bands show that 50uM genistein contained less PCNA for antibody binding, which indicates that genistein-treated LNCaP cells had the most reduced growth out of the treatments tested. In order to validate our observation, protein density quantification was conducted using ImageJ. The area of each protein as shown in figure 7 correlates with the visible band densities in figure 6 above.

Methanol was selected as an appropriate solvent for the hormones from literature reviews and its use as a control solvent in previous MQP with LNCaP cells. Nonetheless, the control solvent, methanol, expressed unexpected results by having a tendency to proliferate the cells when compared to No-treatment cells. It appeared as though methanol was causing cell
proliferation however, statistically, a P-value of 0.2588 tells us no concrete conclusion can be made (Figure A in the appendix). Moreover, the high variability in the proliferation assay trials of methanol-diluted hormones, as well as proliferation from methanol-diluted biochanin A, suggests that a different solvent might yield more reliable data.
Conclusions and Recommendations:

Based on the collected data, we were unable to observe any conclusive trends from each treatment or the controls with the exception of 10 uM biochanin A. Despite the lack of statistically significant trends, comparable results from our MTT assays and immunoblotting suggest some consistency between the assays.

For further research on phytoestrogens’ effects on LNCaP cells, as well as other prostate cancer cell lines, some experimental modifications can be made in addition to performing assays that measure cell cycle arrest as suggested by multiple phytoestrogen research papers. Conducting these experiments with numerous control solvents such as methanol, ethanol, water, and/or methyl sulfate (MeSO₄), all of which are capable of dissolving the compounds, could increase data consistency and decrease the chance of one solvent’s contamination.

Conducting an assay in addition to MTT assay that measures lactate dehydrogenase (LDH) leaked from dead cells whose membranes had been degraded would be another method to promote consistent results as well as measure cell apoptosis. In addition, carrying out immunoblots in parallel to MTT assays could not only validate the results from both experiments, but allow one to compare and contrast between viable and nonviable cells. Lastly, to lower the amount of variability between different trials of the experiments, the cells being treated could first be serum-starved and forced into the G0 phase of the cell cycle before adding treatments. In doing so, the cells would all begin their entry to the cell cycle at the same time, and the effect of particular treatments would be more evident.

We suggest that future studies incorporate the suggestions made above as well as explore the epigenetic regulatory mechanisms of isoflavones on LNCaP and PC3 prostate cancer cell lines (Adjackly, 2012). In order to observe the demethylating properties of isoflavones that cause G2/M cycle arrest and prevent cell proliferation, future projects can use PCR to observe cell cycle arrest and perform methylation quantification using methyl-profiler-DNA-methylation analysis. As with any experiment, more data will validate and strengthen the findings. In conclusion, repeating these experiments multiple times, finding the most effective concentrations of treatments to add, and monitoring the cells over a longer period of time, we predict, will lead to promising and positive results that phytoestrogens have an anti-proliferating effect on LNCaP cells.
References:


Park, Hyun Jin, Jeon, Young Keul, You, Dong Hun and Nam, Myeong Jin (2013, October). Daidzein causes cytochrome c-mediated apoptosis via the Bcl-2 family in human hepatic cancer cells. *Food and Chemical Toxicology: 60*. 542-549.


Singh, Vishal, Sharma, Vikas, Verma, Vikas, Pandey, Deepti, Yadav, Santosh K., Maikhuri,


Appendix Section A: MTT Assay Results

Table A.

Average % Methanol Acquired from Single Trials

<table>
<thead>
<tr>
<th>Average % Methanol</th>
<th></th>
<th></th>
<th></th>
<th>Average</th>
<th>Stdev</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Runs</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein 0.1uM</td>
<td>210.3664</td>
<td>103.9039</td>
<td>18.54447</td>
<td>110.9383</td>
<td>96.10424</td>
<td>0.89</td>
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<tr>
<td>Genistein 1uM</td>
<td>131.4738</td>
<td>82.58258</td>
<td>18.38275</td>
<td>77.47971</td>
<td>56.71795</td>
<td>0.6312</td>
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<tr>
<td>Genistein 10uM</td>
<td>103.3686</td>
<td>103.9134</td>
<td>121.0811</td>
<td>31.26685</td>
<td>89.90749</td>
<td>39.94949</td>
</tr>
<tr>
<td>Genistein 50uM</td>
<td>84.50433</td>
<td>120.8229</td>
<td>109.3093</td>
<td>38.0593</td>
<td>88.17396</td>
<td>36.68606</td>
</tr>
<tr>
<td>Biochanin A 0.1uM</td>
<td>189.1757</td>
<td>156.957</td>
<td>78.81402</td>
<td>141.6489</td>
<td>56.75102</td>
<td>0.3959</td>
</tr>
<tr>
<td>Biochanin A 1uM</td>
<td>189.7585</td>
<td>136.4865</td>
<td>80.8092</td>
<td>135.682</td>
<td>54.48324</td>
<td>0.4427</td>
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<tr>
<td>Biochanin A 10uM</td>
<td>157.54</td>
<td>145.4955</td>
<td>140.7316</td>
<td>147.9224</td>
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<td>0.0048</td>
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<td>Biochanin A 50uM</td>
<td>178.3097</td>
<td>122.2222</td>
<td>129.4571</td>
<td>143.3297</td>
<td>30.50882</td>
<td>0.1528</td>
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<tr>
<td>Testosterone 50uM</td>
<td>84.11935</td>
<td>140.8408</td>
<td>106.8693</td>
<td>132.345</td>
<td>116.0436</td>
<td>25.71618</td>
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<tr>
<td>Testosterone 200uM</td>
<td>102.9279</td>
<td></td>
<td></td>
<td>102.9279</td>
<td></td>
<td></td>
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<tr>
<td>Estrogen 50uM</td>
<td>85.37055</td>
<td>194.9865</td>
<td>18.0593</td>
<td>252.1772</td>
<td>137.6484</td>
<td>105.5762</td>
</tr>
<tr>
<td>No treatment</td>
<td>70.60783</td>
<td>92.49249</td>
<td>18.0593</td>
<td>60.38654</td>
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<td>100% Promensil</td>
<td>64.69609</td>
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<td>64.69609</td>
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<td></td>
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<td>Promensil 1:5</td>
<td>129.7252</td>
<td></td>
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<td>129.7252</td>
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<tr>
<td>Promensil 1:10</td>
<td>120.1499</td>
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<td>120.1499</td>
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<td></td>
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</tbody>
</table>

[Table A]: The table above displays average percent methanol acquired from each run as well as standard deviation and p-value of treatments compared to control. 10uM of biochanin A with p-value of 0.0048 was statistically significant when compared to the control.

Table B.

Testosterone Concentration Proliferation Assay O.D Readings

<table>
<thead>
<tr>
<th>Testosterone [uM]</th>
<th>O.D Readings</th>
<th>Avg</th>
<th>Stdev</th>
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</thead>
<tbody>
<tr>
<td>0.1uM</td>
<td>0.055 0.056 0.058</td>
<td>0.056333</td>
<td>0.001528</td>
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<tr>
<td>1uM</td>
<td>0.056 0.069 0.057</td>
<td>0.060667</td>
<td>0.007234</td>
</tr>
<tr>
<td>10uM</td>
<td>0.055 0.055 0.055</td>
<td>0.055 0</td>
<td>0</td>
</tr>
<tr>
<td>50uM</td>
<td>0.056 0.056 0.052</td>
<td>0.054667</td>
<td>0.002309</td>
</tr>
</tbody>
</table>

[Table B]: Displays the O.D readings of testosterone treated cells (10^3 per well) at concentrations of 0.1uM, 1uM, 10uM and 50uM. Average of the O.D readings and standard deviations are shown.

Table C.

ANOVA of Testosterone Concentrations
### Anova Analysis for Testosterone $10^3$

**Anova: Single Factor**

#### SUMMARY

<table>
<thead>
<tr>
<th>Groups</th>
<th>Count</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
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</thead>
<tbody>
<tr>
<td>0.055</td>
<td>2</td>
<td>0.114</td>
<td>0.057</td>
<td>0.000002</td>
</tr>
<tr>
<td>0.056</td>
<td>2</td>
<td>0.126</td>
<td>0.063</td>
<td>0.000072</td>
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<tr>
<td>0.055</td>
<td>2</td>
<td>0.11</td>
<td>0.055</td>
<td>0</td>
</tr>
<tr>
<td>0.056</td>
<td>2</td>
<td>0.108</td>
<td>0.054</td>
<td>8E-06</td>
</tr>
</tbody>
</table>

#### ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>9.75E-05</td>
<td>3</td>
<td>3.25E-05</td>
<td>1.585366</td>
<td>0.325361</td>
<td>6.591382</td>
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<tr>
<td>Within Groups</td>
<td>8.2E-05</td>
<td>4</td>
<td>2.05E-05</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Total               | 0.00018 | 7   |         |         |         |        |

**[Table C]:** Analysis of variance was conducted to observe statistical differences among the various testosterone concentration. The P-value of 0.325 indicates that there are no significant differences that exists between the different groups (different concentrations).
Appendix Section B: Bradford Assay and Immunoblotting Results

Figure A.

Bradford Assay Graph

[Figure A]: Displays graph of a diluted Albumin Standard (BSA) as well as treatment concentration that fall within the graph of the standard. The Y value indicates linearity of the standard curve.

Appendix Section C: ImageJ Reading

Table D.

Protein Density Quantification

<table>
<thead>
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<th>Protein Density Quantification</th>
<th>Area Under the Peak (pixels)</th>
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</thead>
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<tr>
<td>Genistein (Error)</td>
<td>91119.79</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>91415.05</td>
</tr>
<tr>
<td>Promensil</td>
<td>87043.1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>73797.07</td>
</tr>
<tr>
<td>Methanol</td>
<td>103476.5</td>
</tr>
<tr>
<td>Estrogen</td>
<td>86383.53</td>
</tr>
<tr>
<td>Genistein (No error)</td>
<td>65137.57</td>
</tr>
</tbody>
</table>

[Table D]: Displays protein density quantification of each bands shown in figure 6.