

Content and Effects of Over-The-Counter Phytoestrogen Supplements on Breast Cancer Proliferation

Worcester Polytechnic Institute
Department of Biology and Biotechnology



Advisors: Mike Buckholt, PhD and Jill Rulfs, PhD

Authors: Gabrielle Bitzas, Tyler Mathews, Sherman Peoples, Sarah Roth

Table of Contents

Abstract	3
Acknowledgements	4
Authorship	5
Background	6
Hormones and Estrogen Activity	6
Introduction to Hormones	6
Normal Estrogen Receptor Activity	6
Estrogen-Deficient Receptor Activity.....	7
Menopause and Hormone Replacement Therapy	7
Menopause Definition	7
Symptoms and Treatment.....	7
History of Hormone Replacement Therapy.....	8
Estrogen and Estrogen Receptors.....	8
Phytoestrogens	9
Coumestan.....	10
Lignans.....	10
Stilbenoids.....	10
Flavonoids	10
The Food and Drug Administration	11
History	11
Over the Counter Drugs vs. Dietary Supplements	11
What this means for Phytoestrogens.....	12
T47D-KBluc Cell Line	12
Origin.....	12
Effects of Soy Supplements on Cancer Cell Proliferation.....	13
Effects of Red Clover Supplements on Cancer Cell Proliferation	13
Methodology	14
T47D-KBluc Cell Line	14
Extraction	14
Supplement Extraction	14
High Performance Liquid Chromatography	15
Serial Dilution	15
MTT Assay	15
Cell Synchronization	16
Trypsinization	16
Results	17
Effects on Proliferation	17
HPLC	24
Discussion	29
Limitations of the study	31
References	32

Abstract

Phytoestrogens are plant compounds, which can be taken as an alternative to Hormone Replacement Therapy to treat symptoms of menopause. The proliferative effects of over the counter supplements containing two common phytoestrogens: red clover and soy, were tested on T47D-Kbluc cells using MTT assays. We observed differences in proliferation between the supplements tested. We have identified unique common peaks between supplement chromatograms and have discussed their potential effects on cell proliferation.

Acknowledgements

We would like to take this opportunity to thank some important people without whom, this project would not have been possible. To our advisors Mike Buckholt and Jill Rulfs we thank you for your continued guidance and support throughout the year, within the project and outside of it. We would also like to thank Abbie White for her help in acquiring supplies for us throughout the project.

Authorship

Gabrielle Bitzas and Sarah Roth were responsible for all work related to cell culture. Tyler Mathews and Sherman Peoples were responsible for the work related to HPLC. All members were involved in multiple aspects of the project from beginning to end.

Background

Breast cancer is one of the most common and deadly forms of cancer in the United States. The disease is expected to develop in 1 out of every 8 women in the U.S. (Breastcancer.org, 2014), and kills more women than all other cancers excluding lung cancer. Hormone Replacement Therapy (HRT), a process that introduces estrogen into a patient is often use to treat symptoms of menopause (Holli et. al., 1998). Recent studies about the effects of HRT on breast cancer have raised concerns that HRT may increase the patients' risk for breast cancer (Katalinic et. al., 2008). This study hopes to find other methods of successfully treating breast cancer by using phytoestrogens, or estrogen derived from plants.

Hormones and Estrogen Activity

Introduction to Hormones

Hormones are a class of circulatory signaling molecules that play an important role in many physiological functions. They are comprised of three classes: amine hormones, peptide hormones, or steroid hormones. While amine hormones are created via the typical formation of amino acids, peptide hormones are synthesized by the formation of short chains created by peptide bonds between many amino acids (Taylor et. al., 2000). Steroid hormones, otherwise known as steroids, are not amino-acid based and don't have to bind to a receptor on the cellular surface to enter the cell (Klinge et. al., 2008, Figure 5A). This makes the regulation of steroid hormones all that more important, as the lipid-based family of molecules can freely diffuse across the cell membrane.

Normal Estrogen Receptor Activity

Estrogen Receptors are responsible for regulating transcriptional activity (Simpson et. al., 2001). After an estrogen receptor binds with an estrogen molecule that elicits an agonistic response, coactivators dimerize with two ligand-bound complexes and bind to Estrogen Response Elements (EREs), which are types of DNA sequences activated in the presence of estrogen (Kimball, 2011). These hormone response elements are located near the promoter that activates transcription when bound (Hyder et. al., 2000). These regions can only be activated in the presence of an estrogen molecule that elicits an agonistic response, as transcription is naturally "turned off" (Hyder et. al., 2000). Estrogen molecules can elicit a full range of responses, from fully agonistic (completely activates the transcriptional region) to fully antagonistic (completely de-activates the transcriptional region) and everything in between (activates or de-activates the transcriptional region depending on cellular conditions) (Stovall et. al., 2008). A decrease in steroid

concentrations brought on by biological processes such as menopause (Simpson et. al., 2001) can make it difficult for proper metabolic activity.

Estrogen-Deficient Receptor Activity

Individuals that suffer from estrogen-deficient conditions like menopause force their estrogen receptors to adjust to a lower estrogen concentration. This presents two problems. First, an inability of ERs to adjust to low levels of estrogen can lead to a decrease in transcriptional activity that often manifests itself through symptoms such as bone loss (Riggs et. al., 1998). Second, individuals that have adjusted to lower levels of estrogen show increasing sensitivity to hormones (Riggs et. al., 1998). It's been noted that cancer cells express estrogen-sensitive markers, and the formation of estrogen-sensitive cells are theorized to lead to cancer cells (Diel et. al., 2002) Some treatments for those with low steroid hormone concentrations have already been tested, but with mixed results. Hormone Replacement Therapy can treat some menopausal symptoms by increasing the amount of circulating estrogen in an individual, but has also been shown to put recipients at risk for cancer (Cui et. al., 2014).

Menopause and Hormone Replacement Therapy

Menopause Definition

Menopause is defined as the end of menstruation due to reduced secretion of estrogen and progesterone (Nelson, 2008). There is a transitional period of roughly 4-5 years that is characterized by more irregular menstrual cycles, but women's ages at the time of their final cycle vary (Nelson, 2008). Menopause can be accompanied by unpleasant symptoms, the most common being "hot flashes", and "night sweats".

Symptoms and Treatment

There are many symptoms commonly associated with menopause. Symptoms can include, but are not limited to, vasomotor episodes ("Hot Flashes"), vaginal dryness, incontinence, mood changes, and reduced libido (Nelson, 2008).

Vasomotor episodes are one of the most commonly reported symptoms of menopause. They are characterized by a warming feeling in the face and chest, and often associated with perspiration, palpitations, and a feeling of anxiety (Nelson 2008). Patients report that vasomotor episodes affect sleep, which can lead to a decrease in overall health in the long-term. These episodes can occur for up to 5 years post-menopause, and are often more severe in patients who have surgically induced menopause (Bachmann, 1999). Patients who have severe symptoms often seek treatment, to preserve quality of life. In addition to the commonly reported symptoms, menopause can also cause more serious problems. These more severe conditions include heart disease, osteoporosis, and cancer (Watkins, 2007).

Osteoporosis is common in menopausal and post-menopausal women because of the reduction of hormone production in the ovaries (Lindsay, 1996). The reduced amount of estrogen causes an increase in reabsorption of skeletal mass, and leads to an increase in fractures from minor accidents or falls (Lindsay, 1996). These effects can be offset by using a replacement for estrogen, or a “bone specific agent” to reduce the reabsorption of the skeletal mass (Lindsay, 1996).

Physicians and researchers have developed several scales to gage the severity of symptoms in a specific patient relative to a larger group, and advise treatment plans. The menopause-specific quality of life questionnaire, Green climacteric scale, and Blat-Kupperman index are some of the most commonly used and referenced . Most of these scales and scores rely heavily on self-reporting of symptoms (Nelson, 2008). Self-reporting is not a reliable source for physicians to use to make a diagnosis, or to prescribe treatment (Nelson, 2008).

In the 2008 US Census the average life expectancy for females in the United States was 80.5 years (US Census Bureau, 2012). This would give a woman a minimum of 30 years of postmenopausal life (assuming that their last menstrual period occurred at the age of 60). The most common treatment for menopause is Hormone Replacement Therapy (HRT). Some “non-prescribed therapies” including red clover, soy, and black cohosh herbal supplements are commonly used, but there is not much data to support or refute their success (Nelson, 2008). There are more varied treatment methods that are shown to be no different than placebo when used to treat menopausal symptoms including magnets, aerobics, Chinese herbs, and “reflexology” (Nelson, 2008).

History of Hormone Replacement Therapy

Hormone Replacement Therapy (HRT) is a medical procedure where synthetic hormones are used to prevent or treat symptoms of a specific medical condition. Historically, HRT was prescribed as a preventative measure against conditions including osteoporosis, heart disease and other signs of aging especially in women, however in many cases the risks outweigh the benefits (Watkins, 2007). Long term HRT is most commonly prescribed for women who have entered menopause. HRT is also commonly used in male-to-female transitioning of transgendered individuals, and for alleviating symptoms of genetic conditions such as Turner Syndrome (Bolar, 2008).

Estrogen and Estrogen Receptors

Estrogen is the main female hormone used in menopausal HRT. It gained immense popularity in the 1960s as a way to alleviate the symptoms of menopause, however in 1975 it was discovered that the risk of endometrial cancer was increased (Watkins, 2007). Estrogen regained some popularity in the 1980s after the hormone

progestin was added in combination, however estrogen-progestin therapy leaves users with increased risk of breast cancer, blood clots, and stroke (Watkins, 2007).

There are two subtypes of human estrogen receptors, Estrogen Receptor α (ER α) and Estrogen Receptor β (ER β) (Morito, 2001). Both estrogen receptors alpha and beta are co-expressed in about half of breast cancer cases (Lattrich, 2014). The estrogen receptors are similar but differ in their C-terminal ligand-binding domain and in the N-terminal transactivation domain (Morito, 2001). The roles of ER alpha and ER beta in breast cancer pathogenesis are becoming increasingly clearer due to several clinical and *in vitro* studies (Rizza, 2014). The two estrogen receptors are responsible for opposite roles in regulating cell proliferation (Powell, 2012).

ER α

ER α is a nuclear hormone receptor and transcription factor that helps regulate gene expression and is involved in cell proliferation (Rizza, 2014). It has also been found that without the alpha-receptor the mammary ducts become truncated and do not fully develop. ER α is involved in cancer-promoting effects in response to estrogen and has been shown to be an effective therapeutic target for years (Powell, 2012). The alpha-receptor has been linked to some of the carcinogenic effects of estrogen in breast (Powell, 2012).

ER β

Like the alpha-receptor the beta-receptor is a nuclear hormone receptor and a transcription factor that helps regulate gene expression (Rizza, 2014). Unlike the alpha-receptor that is believed to play a role in cell proliferation, the beta-receptor is believed to be responsible for the inhibition of cell proliferation (Morito, 2001). This was determined after many studies and experiments, produced results supporting the hypothesis of inhibiting cell proliferation (Powell, 2012).

Phytoestrogens

Available knowledge suggests phytoestrogens affect numerous processes related to reproduction, bone remodeling, skin, cardiovascular, nervous, immune systems and metabolism. This potentially leads to useful prevention of diseases (Alexander, 2014). Phytoestrogens are non-steroidal estrogens that are located found in many species of the plant kingdom. Phytoestrogens are similar in chemical structure to mammalian estrogen, estradiol. This results in their ability to bind to estrogen receptors alpha and beta with a preference for the beta receptor (Younes and Honma, 2011). After binding to the receptor in the cytoplasm, they are able to move from the cytoplasm to the nucleus of the cell where they bind estrogen response elements and affect transcription, resulting in control of expression of target genes (Sirotkin, 2014). Estrogen receptor alphas are considered as promoters of cell proliferation and estrogen receptor beta promote cellular apoptosis (Rietjens et al., 2013).

According to their chemical structure and biosynthesis pathways, phytoestrogens are divided into flavonoids, coumestan, lignans, stilbenoids, and miscellaneous

classes. This study will focus on flavonoids because of their widespread presence in over the counter supplements that advertise relief of menopause symptoms. Flavanoids are further divided into flavones, flavonols, flavanones, and isoflavonoids (Alexander, 2014).

Coumestan

Coumestan is a derivative of simple isoflavonoids and is generally found in alfalfa and clover. Because of its availability in mostly alfalfa, it is more linked to veterinary science than human nutrition (Dixon, 2004). It has strong estrogen-like activity and higher binding affinity for the estrogen receptor than genistein (Tinwell et al., 2000).

Lignans

Lignans are a diverse class of phenylpropanoid dimers and oligomers, which are a family of organic compounds that are synthesized by plants from phenylalanine. They are generally found in high amounts in flaxseed, whole grain bread, vegetables, and tea (Cornwell et al., 2004). Because of their availability in cereals and grains, they account for the majority of total phytoestrogen exposure (Fletcher, 2003). Evidence of health benefits come from epidemiological and intervention studies. In a study done by Peterson et al. (2010), intervention studies using flaxseed lignan supplements and sesamin supplements showed beneficial associations with C-reactive protein and meta-analysis which suggested a lowering effect on plasma total and low-density lipoprotein cholesterol, as well as, possible lipid and blood pressure lowering. Observational epidemiological studies showed some decreased risk of cardiovascular disease showing a promising association but not well established (Peterson et al., 2010). Benefits are equal with those of isoflavones that are thought to be wide, ranging from prevention of cardiovascular disease and cancer, to cognitive ability (Salter et al., 2012).

Stilbenoids

Stilbenoids, like isoflavonoids, are produced through the phenylpropanoid-acetate pathway. The main sources of stilbenoids are from resveratrol from red wine and peanuts. However, only the trans form of resveratrol has been shown to be estrogenic. Resveratrol has shown agonistic and antagonistic activity in MCF-7 cells and CHO-K1 cells transfected with human estrogen receptors (Cornwell et al., 2004). Like other phytoestrogens, principle health benefits of resveratrol ingestion are prevention of cardiovascular disease and cancer (Salter et al., 2012).

Flavonoids

Isoflavonoids are formed by a branch of the flavonoid biosynthetic pathway. They originate from naringenin in genistein biosynthesis and liquirtigenin in diadzein

biosynthesis both of which are flavonoid intermediates (Dixon, 2004). Genestein, one of the common isoflavonoids, has a wide variety of pharmacological effects in animal cells, including tyrosine kinase inhibition, chemoprevention of breast and prostate cancers, cardiovascular disease and post-menopausal ailment relief (Dixon et al., 2002). For example, a study done in perimenopausal women showed that soy isoflavones attenuated bone loss from the lumbar spine in patients. This was further speculated that the isoflavones rather than the soy protein attributed to this effect (Alekel et al., 2000). Genestein shares structural features with estradiol-17 β , specifically, in the area that confers the ability to bind estrogen receptors and secondary hormone binding proteins. This allows genestein to have both estrogenic and anti-estrogenic activity (Dixon et al., 2002). This is due to the activation and inhibition of the estrogen receptor types, which can induce or inhibit estrogen signaling (Mueller et al., 2004). The anti-estrogenic activity is due to competition binding between the isoflavonoid and estradiol. This is shown in an example by tamoxifen, which is a chemo preventive agent in women with high risk of breast cancer. Tamoxifen is structurally similar to genestein and thus competes with it (Dixon et al., 2002).

The Food and Drug Administration

History

The Food and Drug Administration (FDA) began to function as it is known today in 1906 after the passage of the Pure Food and Drugs Act. The FDA is responsible for the analysis and regulation through safety standards of human and veterinary drugs, the United States food supply, cosmetics, etc. The FDA is also charged with pressing for innovation in providing safe, affordable, and effective medicine to the public.

Over the Counter Drugs vs. Dietary Supplements

The FDA's classifications and regulatory procedures for Over the Counter (OTC) Drugs and Dietary supplements are different. These differences are part of what makes the classification of a product important to companies, more lax regulations can allow subpar products to pass under the radar.

An OTC Drug is one that has been proven to be safe to use without professional supervision. OTC drugs can be approved by the FDA by the submission of an application. More commonly they are marketed by following an "OTC drug monograph". An OTC drug monograph states appropriate usage and dosing for the specific ingredients used in a product. OTC drug monographs allow a product to bypass FDA review and go straight to the market (FDA, 2014).

According to the Center for Drug Evaluation and Research (CDER) the required information on OTC Drug Labels is as follows:

- ❖ Title (Drug Facts or Drug Facts (continued))
- ❖ Active Ingredient(s)
- ❖ Purpose(s)
- ❖ Use(s)
- ❖ Warning(s)
- ❖ Directions
- ❖ Other Information
- ❖ Inactive Ingredients
- ❖ Questions? or Questions or comments? (optional)

This information must be present in the specified order on the outside of the drug container (CDER, 2008).

Dietary supplements are classified as products containing one or more “dietary ingredients”, taken by mouth that enhances one’s diet. The ingredients may include:

- ❖ Vitamins
- ❖ Minerals
- ❖ Botanicals
- ❖ Amino acids
- ❖ Other substances found in the human diet

Dietary supplements may not be advertised as conventional foods, and in place of “Nutritional Facts” labels must have a “Supplement Facts” label. Additionally, because supplements are a type of “food” they are not required to be approved by the FDA before being brought to market. Instead it is the responsibility of the producer to ensure that the product is safe, contains no misleading or false claims, and otherwise follows all FDA standards for a food product. (FDA, 2014).

What this means for Phytoestrogens

Most common phytoestrogen supplements like Promensil are sold as dietary supplements rather than OTC drugs regardless of how they are being used. Due to the more lax regulatory standards set for dietary supplements these products can be brought to market and sold until such a time that the product is shown to be unsafe or it is reclassified as a drug, and held to those regulatory standards. Without the strict regulatory standards, there is a huge variety in the types of phytoestrogen supplements available on the market, and the effect or lack of effect of the products on consumers could be wildly variable depending on the brand, concentration of “active ingredient”, delivery system and many other variables.

T47D-KBluc Cell Line

Origin

T47D-KBluc cells are a line of human breast cancer cells that luminesce under exposure to estrogen (Wilson et. al., 2004). In order to form the T47D-KBluc line, T47D cells are infected with estrogen-responsive luciferase reporter gene constructs. After estrogen molecules bind to estrogen receptors, two of the ligand-

bound receptor complexes dimerize and bind to EREs. These EREs are DNA sequences upstream from a TATA box that regulates expression of the luciferase reporter gene (Wilson et. al., 2004). A luciferase enzyme assay can then be used to quantify the luminescence of the reporter gene brought on by estrogen exposure. After the cells have been plated and exposed to the proper assay conditions, they luminesce almost instantly (Biotium, 2014). The lack of background luminescence means that the measured cell luminescence can be fully attributed to luciferase expression (Biotium, 2014). Estrogen has a strong effect on the activity of the luciferase reporter gene, as increasing concentrations of 17β -Estradiol from 1×10^{-4} nM to 0.1 nM have been shown to increase the fold induction of the reporter gene by almost 10-fold (Wilson et. al., 2004, Figure 2A).

Effects of Soy Supplements on Cancer Cell Proliferation

Many dietary supplements use soy as an ingredient. Supplements containing soy have been shown to reduce proliferation of estrogen-dependent cancer in some studies (Kang et. al. 2009), but have resulted in no change in cancer proliferation for other studies (Shike et. al., 2014) (Moss et. al., 2014). The most common phytoestrogens found in soy supplements are genistein and daidzein. Genistein use has shown an increase in cancer proliferation when using large doses (Kuiper et. al., 1997), but may still cause a decrease in proliferation at lower concentrations (Setchell et. al., 2001). Daidzein has been shown to reduce cancer proliferation (Lepri et. al., 2014). The lack of definitive results on the effects phytoestrogens have on estrogen-dependent cancer are concerning due to the possibility that some components of the supplements could actually be harmful.

Effects of Red Clover Supplements on Cancer Cell Proliferation

Red clover is another plant commonly found in dietary supplements. Supplements containing red clover have caused decreases in cancer cell proliferation (Caron, 2007), as well as increased proliferation (Chen et. al., 2015). Daidzein has been shown to reduce cancer proliferation at the right concentration. Biochanin A has been shown to reduce proliferation in a number of studies (Johnson, 2010), but has also been shown to promote proliferation as well. Although treatment of estrogen-dependent cancer with phytoestrogens has led to mixed results, more research should be done on the subject as these supplements may provide a safer alternative to HRT if they have inhibitory properties such as with diadzen and biochannin A.

Methodology

T47D-KBluc Cell Line

Cells were maintained at 37 degrees Celsius in DMEM + 10% Fetal Bovine Serum + 1% PenStrep + Glutamine media. T25 flasks were maintained in 5mL, and T75 flasks were maintained in 10mL of media. Cells were fed new media as needed, and split as they approached approximately 85-90% confluence.

Extraction

Solvent extractions were performed on the varying supplements, and then refluxed according to the procedures outlined in Setchell et. al. (2001). Due to the variation of phytoestrogen concentration across supplements different amounts of the supplements were used in the extractions, in order to get similar concentrations of phytoestrogens across all supplements. A given supplement was added to a 250-mL round-bottom flask. 80 mL of 80% methanol were added to the round-bottom flask. After the solution was mixed, the flask was attached to a reflux condenser. The solution was refluxed for 1 hour in a 65°C water bath. The solution was then purified using a syringe filter and stored at -20°C

Supplement Extraction

Spring Valley Soy Isoflavone

Three 80mg tablets underwent solvent extraction after being crushed in a sterile mortar and pestle. This represents 1.5 serving sizes as indicated on the product packaging. As the first supplement extracted, using one serving size was decided after this extraction had been completed.

Promensil

One 80mg tablet underwent solvent extraction after being crushed in a sterile mortar and pestle. This represents one serving size as indicated on the product packaging.

Wild Harvest Red Clover

Extraction was performed using three 350mg capsules. This represents one serving size as indicated on the product packaging.

Piping Rock Soy Isoflavone

Extraction was performed using three 650mg capsules. This represents one serving size as indicated on the product packaging.

Red Clover Plant

1 gram of dried red clover flowers were weighed and crushed in a sterile mortar and pestle. The crushed flowers were then extracted.

High Performance Liquid Chromatography

Samples were injected onto a reverse phase C18 250 X 4.6 mm column in 20 μ L and 100 μ L volumes. Following the HPLC procedure outlined in Caron (2007), the column was washed with a solution of 10 mM ammonium acetate and 0.1% of trifluoroacetic acid for two minutes. The column was then washed with a linear gradient of 10 mM ammonium acetate-0.1% TFA and 100% acetonitrile for the next 22 minutes. The percentage of the wash comprised of 10 mM ammonium acetate-0.1% TFA for this time period varied between 100% - 50%, while the percentage of the wash comprised of 100% acetonitrile varied between 0% - 50 % during this time period. The column was then washed with a solution containing 50% ammonium acetate-TFA and 50% acetonitrile for 5 minutes. Finally, the column was washed with 100% ammonium acetate-TFA for 6 minutes. The absorbance of the samples was measured at 260 nm, and used to generate a chromatogram. The peaks generated by the chromatogram show the absorbance (in mAu's, or micro absorbance units) of the sample at a given time during its run. The chromatograms of the Spring Valley, Promensil, Wild Harvest, and Piping Rock samples were compared to the chromatograms of genistein, daidzein, and biochanin-A to match the

Elution times of the known phytoestrogens with peaks found in the phytoestrogen supplements.

Serial Dilution

Five ten-fold dilutions were created from the red clover plant extraction and each supplement extraction. The dilutions were made by mixing 100 μ L of the stock extraction with 900 μ L of 90% methanol in a 1.5 mL microfuge tube. The dilution was vortexed thus becoming the first 10 fold dilution (10^{-1}) in the five dilution series. 100 μ L of the 10^{-1} dilution was mixed with 900 μ L of 90% methanol, vortexed and was labeled as the 100-fold dilution (10^{-2}). This process was repeated until the 10^{-5} dilution was created for each extraction.

MTT Assay

Cells were counted, trypsinised and plated in 96 well plates at a concentration of 1×10^4 cells per well in 100 μ L of media (DMEM+10% FBS+1% PenStrep+1% Glutamine). Cells were given 24 hours to adhere before media was aspirated and replaced with 80 μ L of Phenol-Red free DMEM + 5% Charcoal Stripped FBS + 1%

PenStrep + 1% Glutamine (Phred-Free). After 24 hours an additional 20ul of Phred-free media mixed with 1uL of desired supplement. After 48 hours 20uL of CellTiter 96 Aqueous One solution (manufactured by Promega) was added to each well, incubated for 2-4 hours, and absorbance was read using a plate reader at 570nm.

Cell Synchronization

For some experiments as noted in results, cells were synchronized to the same point of the cell cycle. After 24 hours of adhesion in the 96 well plates, the original media was replaced with 100uL of media without serum (DMEM+ 1% PenStrep+ 1% glutamine). After 24 hours this media was aspirated, and replaced immediately with 100uL of Phred-free media containing 1uL of the desired supplement. The rest of the protocol followed the normal MTT protocol listed above.

Trypsinization

For some experiments, as indicated in Results, to avoid inconsistencies in plating due to cell-to-cell adhesion, three T75 flasks were trypsinised daily for a 5-day period before cell counts began for MTT plating.

Results

In order to determine the effects of the supplements and B-estradiol on proliferation a series of different MTT assays were performed. These included a series of serial dilution, time-based, B-estradiol concentration, and stock treatments on the T47D-Kbluc line. To determine the makeup of each supplement, HPLC was used to analyze what known and unknown components were present. These results were compared with the determined effect on proliferation to base conclusions on how different components effect growth within the cell line.

Effects on Proliferation

An initial concentration curve for each extract was run using the dilution series as described in Methods to determine the optimal concentration to use in future assays. This assay consisted of all supplements and controls on one plate. The supplements and controls underwent serial dilutions to see the effects of differing concentrations on the cells. Concentrations that gave the best results would be used in the future assays. As seen in Figure 1, however, no conclusive results were derived from the assay. Figure one shows an example of a representative assay using the original MTT assay method.

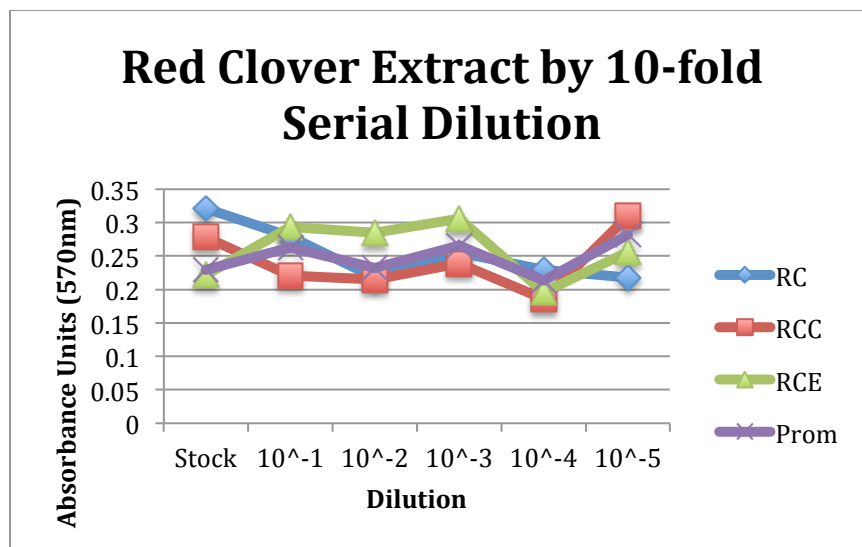


Figure 1: MTT Assay, Serial Dilutions of Red Clover Extracts: RC is the team-made red clover extract, RCC is the store-bought red clover capsule, RCE is the store-bought red clover extract, and Prom is the red clover based Promensil. The plate showed too much variability and no conclusions could be drawn from the dose response.

There did not appear to be an effect on proliferation from any tested dilutions of treatments. There was a high amount of variability between the assays performed. There did not appear to be dose dependent reactions in any of the assays.

Next, a time-based assay was performed to determine the ideal amount of time to run future assays. This was done by running the stock solutions of both the supplements and controls on one plate and leaving the additions on the cells for 24, 48, and 72 hours to see which period of time yielded the cleanest results. These assay too, however, had a high amount of variability on the plates and had no conclusive results.

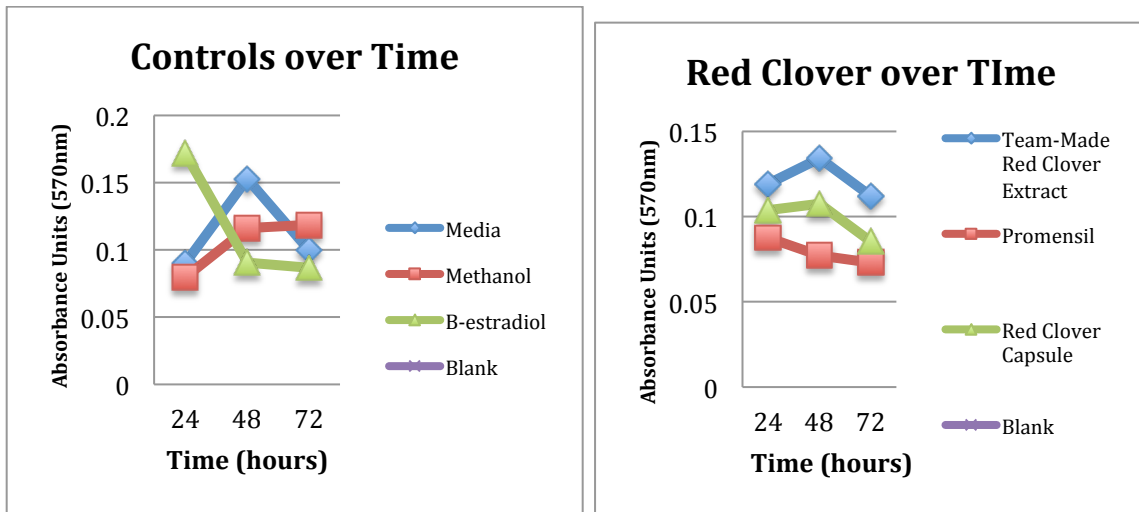


Figure 2: MTT Assay, Red Clover Extracts and Controls over time. The Red clover supplements and B-estradiol had increasing trends while the media and methanol controls had decreasing trends. However, the variability was too high on the plate to have a conclusive result

These results also did not appear to have a noticeable effect on proliferation due to the intra-assay variability within the plates in values taken from both the controls and supplements. Table 1 below shows the raw absorbance data for the time-based assay. This plate was, as stated previously read at 570nm after being exposed to the titer for 2-4 hours.

Table 1: Raw data for time-based MTT Assay. ST is the soy tablet, RC is the team-made red clover extract, SC is the soy capsule ,Prom is Promensil, and RCC is the red clover capsule. ** indicate an error in filling the wells, these values were not used in the averages to produce the graphs in Fig. 2.

	Time (Hours)								
	72	72	72	48	48	48	24	24	24
media	0.111	0.094	0.095	0.127	0.143	0.188	0.08	0.093	0.097
meth	0.104	0.157	0.095	0.09	0.089	0.17	0.063	0.098	0.078
ST	0.084	0.105	0.102	0.086	0.079	0.172	0.098	0.08	0.078
RC	0.099	0.125	0.105	0.091	0.119	0.193	0.124	0.114	0.12
SC	0.063	0.078	0.079	0.083	0.113	0.105	0.076	0.073	0.079
Prom	0.07	0.073	0.076	0.077	0.074	0.081	0.072	0.093**	0.103
RCC	0.079	0.081	0.096	0.1	0.086	0.137	0.105	0.094	0.112
B-estradiol	0.102	0.076	0.082	0.083	0.094	0.094	0.08	0.076**	0.264

Inconsistencies with the above results led to us modifying the protocol to include a period of serum starving the cells to synchronize them in G0 before stimulating proliferation. . The first assay with this new protocol was a test of four different B-estradiol concentrations to determine if the T47D-Kbluc line to determine whether we could demonstrate estrogen responsiveness. . B-estradiol concentrations tested included $1 \times 10^{-8} \text{M}$, $1 \times 10^{-9} \text{M}$, $1 \times 10^{-10} \text{M}$, and $1 \times 10^{-11} \text{M}$. after 48 hours of exposure the plate was read at 570nm.

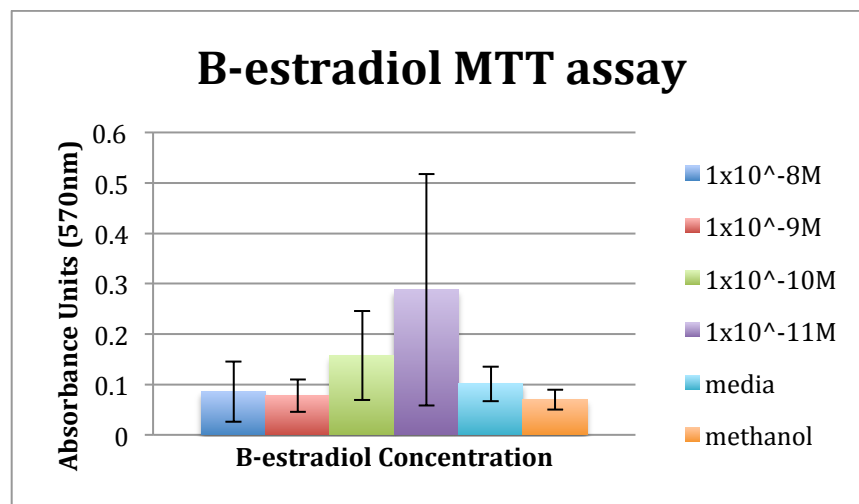


Figure 3: B-estradiol only MTT assay. While there was a increasing trend in absorbance as the concentration of estradiol decreased, the variability on the plate and the error present did not allow any conclusions to be drawn from the B-estradiol only MTT assay.

Error bars represent the standard deviation among the six wells at each concentration, a measure of intra assay variability. While this level of variability makes any analysis of these data impossible, there certainly is no clear effect on proliferation.

Because of continuous issues with variability and based on our observations that the cells tended to form tight cell-to-cell adhesion which resulted in difficulty in obtaining a single cell suspension for plating, we decided to trypsinize the cells daily for a week prior to plating in 96 well plates in an attempt to avoid cellular clumping.

Finally, two identical assays were run testing the four B-estradiol concentrations, undiluted stock solutions for store-bought red clover extract, red clover capsule, Promensil, the team-made red clover extract, media control, and methanol control.

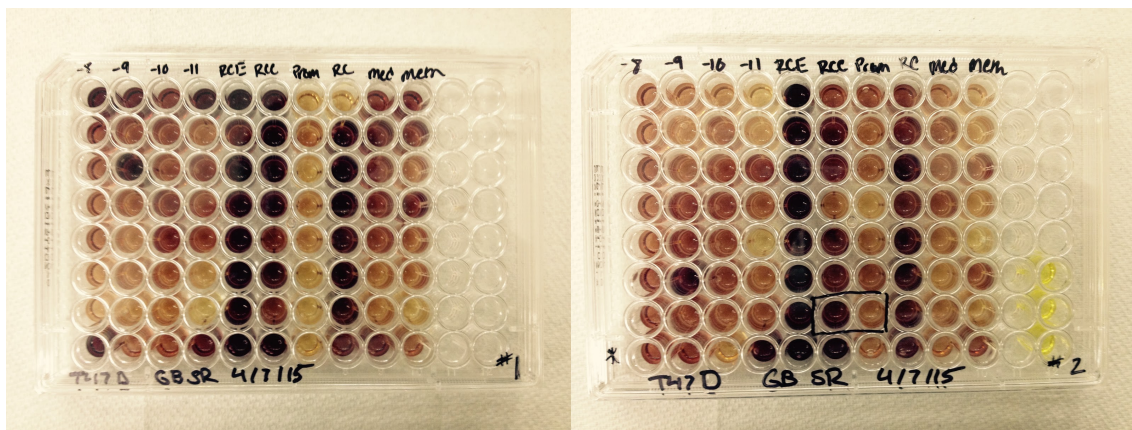


Figure 4: MTT Assay post-incubation. The first four columns labeled “-8-11” contain $1 \times 10^{-8} \text{M}$, $1 \times 10^{-9} \text{M}$, $1 \times 10^{-10} \text{M}$, and $1 \times 10^{-11} \text{M}$ B-estradiol. RCE is the store bought red clover extract, RCC is the red clover capsule, Prom is Promensil, RC is the team-made red clover extract, med is media control, and meth is the methanol control. The two boxed wells on plate 2 were to indicate potential plating error.

The color variation between columns indicates the difference in proliferation. Darker color indicates higher proliferation. Lighter color indicates little or no proliferation. The assay shown in Figure 4, unlike previously run assays, showed noticeable differences in proliferation by eye before quantifying the data with absorbance values. The columns containing B-estradiol as previously found experienced little proliferation, and high variability between wells.

The supplements appear to be less variable by eye. The store-bought red clover extract showed the highest proliferation, while Promensil does not appear to have experienced any when compared to the media or methanol controls. The red clover capsule and team-made red clover extract also increased proliferation.

Absorbance values were as a result more indicative of trends than in the previously run assays. Table 2 and 3 below show the raw absorbance data for plate 1 and plate 2. As before these were read at 570nm after incubation with titer for 2-4 hours.

Table 2: Raw data for final MTT assay, plate 1. RCE is the store-bought red clover extract, RCC is the red clover capsule, prom is Promensil, RC is the team-made red clover extract, med is media, and meth is methanol. ** indicate potential outliers that were not used in any averages or standard deviations to produce the graphs in Figures 5-7.

Plate 1	Treatment									
	B-estradiol				Supplements				Control	
	1×10^{-8} M	1×10^{-9} M	1×10^{-10} M	1×10^{-11} M	RCE	RCC	Prom	RC	Med	Meth
A	1.646**	1.195**	0.793	1.524**	2.136	1.531	0.197	0.177**	0.798	0.842
B	0.772	0.577	0.673	0.413	1.118	1.535	0.377	1.889	1.005**	0.876
C	0.635	2.111**	0.586	0.767	2.208	1.698	0.176	2.054	0.75	0.428
D	0.512	0.492	0.804	0.568	1.687	1.68	0.309	1.978	0.759	0.975
E	0.508	0.345	0.737	0.491	1.996	0.65	0.287	1.62	0.552	0.417
F	0.369	0.408	0.376	0.175	1.983	1.527	0.187	1.59	0.328	0.374
G	0.279	0.265	0.306	0.112	1.559	0.668	0.145	1.465	0.284	0.138

Table 2 showed more variation in data, there were six wells that had values well out of the range of other wells in the same treatment group. These varied values are potentially due to error and are indicated with ** in the table. As in the image of this plate shown in Figure 4, the store bought red clover extract showed the most effect on proliferation with the highest overall absorbance units on the plate.

Table 3: Raw data for final MTT assay, plate 2. RCE is the store-bought red clover extract, RCC is the red clover capsule, prom is Promensil, RC is the team-made red clover extract, med is media, and meth is methanol. ** indicate potential outliers that were not used in any averages or standard deviations to produce the graphs in Figures 5-7.

Plate 2	Treatment									
	B-estradiol				Supplements				Control	
	-8	-9	-10	-11	RCE	RCC	Prom	RC	Med	Meth
A	0.549	0.408	0.356	0.189	2.464	0.599	0.54	0.803	0.419	0.281
B	0.544	0.379	0.526	0.298	2.031	1.17	0.385	0.996	0.576	0.286
C	0.744	0.567	0.575	0.744**	2.335	1.038	0.371	2.14**	0.502	0.613
D	0.61	0.634	0.563	0.368	2.611	0.361**	0.205	1.188	0.567	0.275
E	0.504	0.617	0.514	0.102	2.378	1.397	0.374	1.522	0.342	0.118
F	0.658	1.849**	0.549	0.352	2.302	1.045	0.493	1.806	0.233	0.56
G	0.598	0.537	0.431	0.428	2.052	1.265	0.372	1.221	0.371	0.251

Table 3 shows less variation than table 2. There are 4 wells that had values well out of the range of other wells in the same treatment group. These varied values are potentially due to error and are indicated with ** in the table. As in the image of this plate shown in Figure 4, the store-bought red clover extract showed the most effect on proliferation. The store-bought red clover extract group on plate two also had higher absorbance values than the same group on plate one.

The absorbance values for each treatment group on a single plate were averaged together, and standard deviation was calculated to produce the graphical representations shown in Figures 5 and 6. Figure 5 shows the results for the B-estradiol treatments.

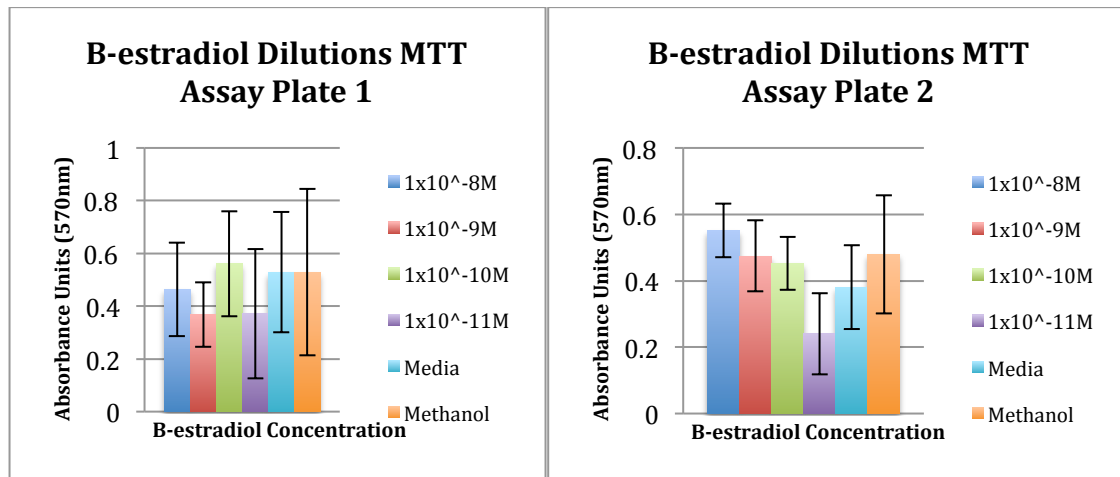


Figure 5: MTT assay, B-estradiol Concentrations and Controls for plates 1 and 2

Due to variance within the data, error bars for the B-estradiol concentrations were large. Plate one does not appear to show any trends across treatment groups. Plate 2 does show a slight trend that suggests that proliferation decreases as B-estradiol concentration decreases, however there was so much variance that this can only be speculation.

Although we do not have enough proliferation data to run accurate statistical analysis to prove significance, there is a trend present that could suggest the supplements having an effect on proliferation if supplemental data could be acquired. Figure 6 shows the graphical representation of the supplement treatment groups from tables 2 and 3

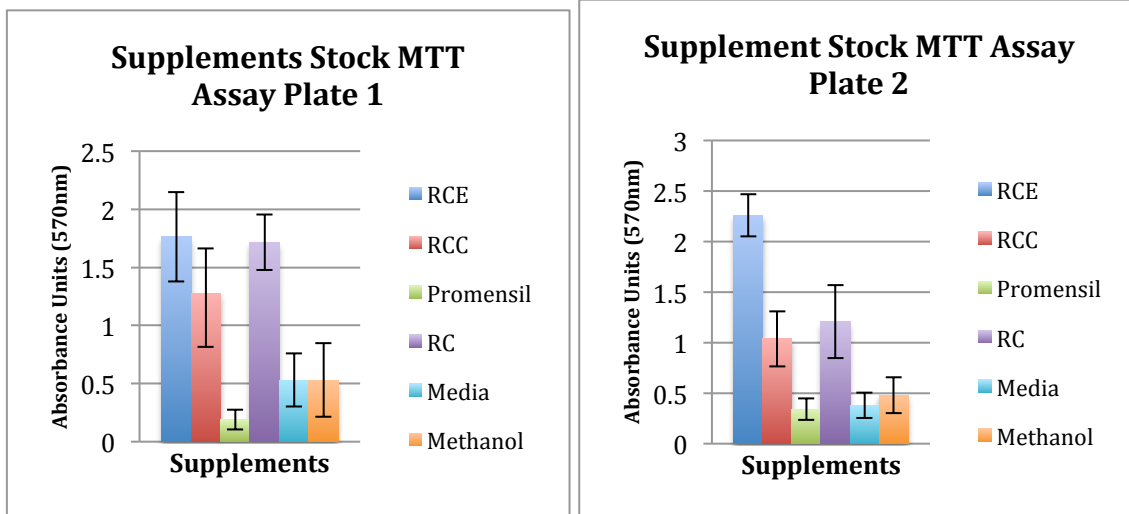


Figure 6: MTT Assay, Red Clover Supplements and Controls for plates 1 and 2, RCE is the store-bought red clover extract, RCC is the red clover capsule, RC is the team-made red clover extract.

The store-bought red clover extract showed the largest increase in proliferation. The red clover capsule, and the team-made red clover extract also showed an increase in proliferation. Promensil appears to have no effect on cell proliferation.

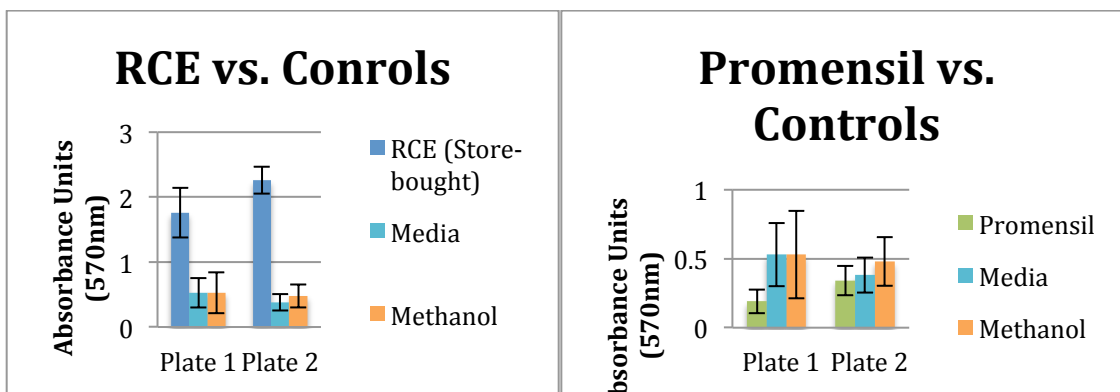


Figure 7: MTT Assay Comparison of Store Bought Red Clover Extract and Promensil with respect to media and methanol controls.

Figure 7 shows us a closer look at the effect of the store bought red clover extract, and Promensil compared to the controls from both assays shown in figure 4. These supplements exhibited the most extreme effects on proliferation out of all treatment groups in this assay. Following this result, we examined the HPLC analysis of Promensil and the red clover extracts to determine whether differences in components within the supplement could cause these discrepancies.

HPLC

High performance liquid chromatography (HPLC) was used to confirm the presence of known phytoestrogens (daidzein, genestein, and biochannin A) in the OTC phytoestrogen supplements using known phytoestrogen standards. The data collected from performing HPLC on the supplements was examined to see if any of the supplement components matched with the known standard components. Figure 8, shown below, is the HPLC run for the phytoestrogen standard daidzein.

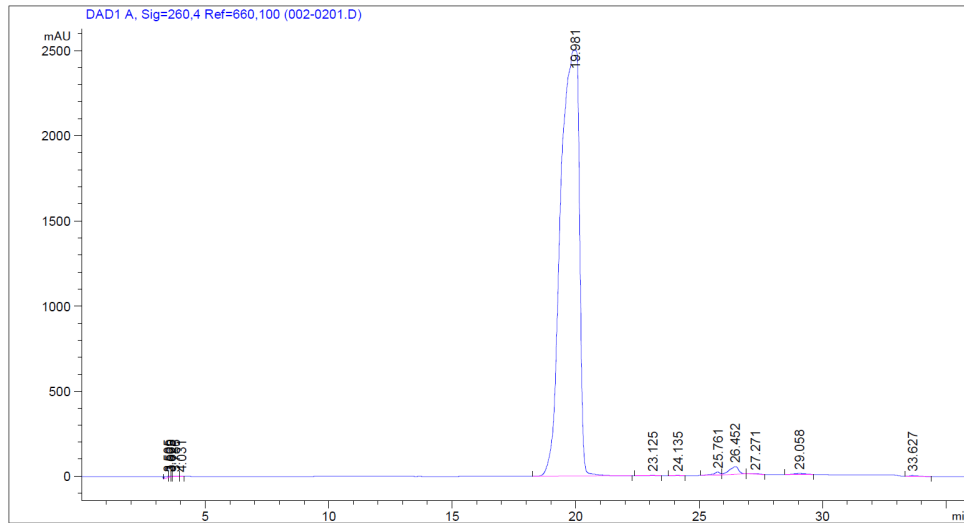


Figure 8: HPLC profile of Daidzein

Figure 8 shows that daidzein had a component with the peak elution time of 19.98 minutes and comprises 98.43% of the total area of the daidzein trace. Figure 9, shown below, is the HPLC run for the phytoestrogen standard genestein.

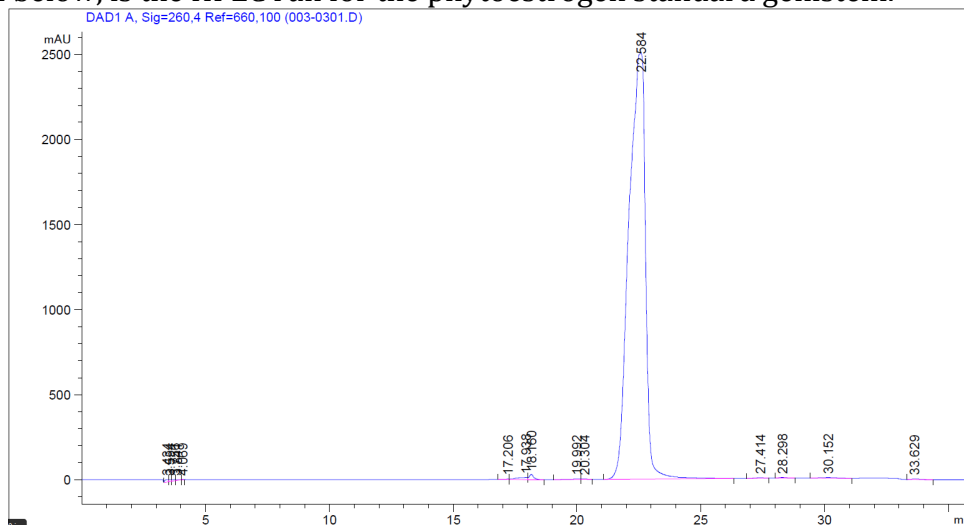


Figure 9: HPLC profile of Genistein

Figure 9 shows, the most prominent peak for genistein is found at 22.58 minutes and comprises 98.5% of the trace area. Figure 10, shown below, is the HPLC run for the final phytoestrogen standard biochanin A.

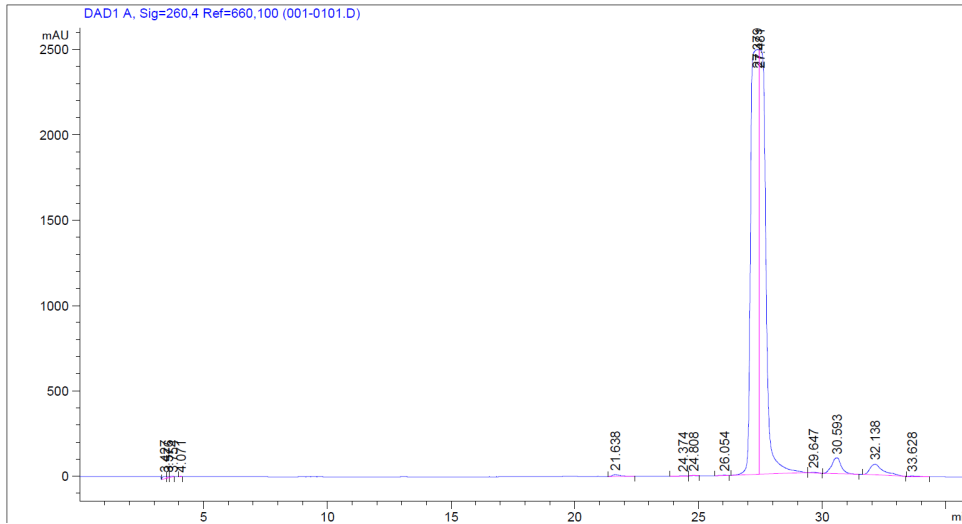


Figure 10: HPLC profile of Biochanin A

As seen in Figure 10, the trace showed two prominent elution peaks, one at 27.37 minutes that comprised 51.86% of the total trace area, and another at 27.48 that comprised 43.00% of the total trace area. As the elution peaks are so close and other runs showed the two elutions as a single elution, this peaks likely represent one component. Figure 11 shown below, is the HPLC run of the store-bought red clover extract.

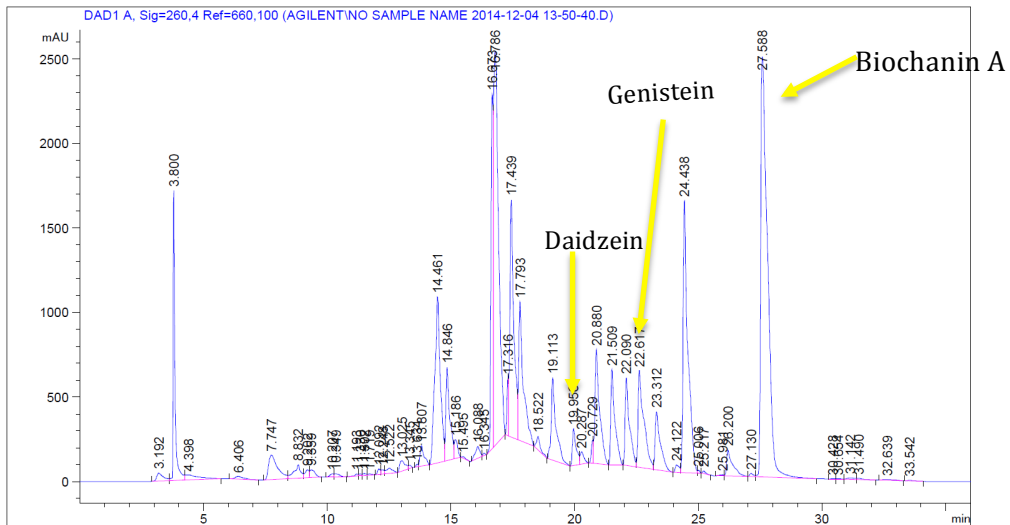


Figure 11: HPLC profile of the store-bought red clover extract

As shown in Figure 11, many components were identified in this supplement, including the phytoestrogen standards. This supplement contained peaks that aligned with peaks of the standards daidzein (0.94% of total trace area), genistein

(3.82% of total trace area), and biochanin A (19.81% of total trace area). Figure 12 shown below, is the HPLC run of the team-made red clover plant extract.

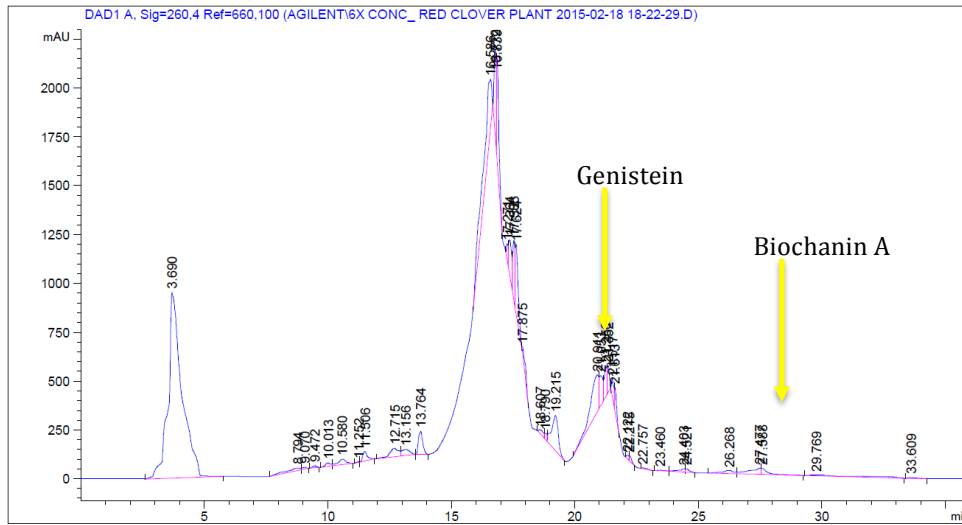


Figure 12: HPLC profile of the team-made red clover plant extract

As shown in Figure 12, the plant extract contained components with peak elution times of 22.75 minutes (0.064% of total trace area), and 27.58 minutes (0.63% of total trace area), showing that there are likely traces of the phytoestrogen standards in the plant extract. Figure 13, shown below, is the HPLC run for the Promensil supplement.

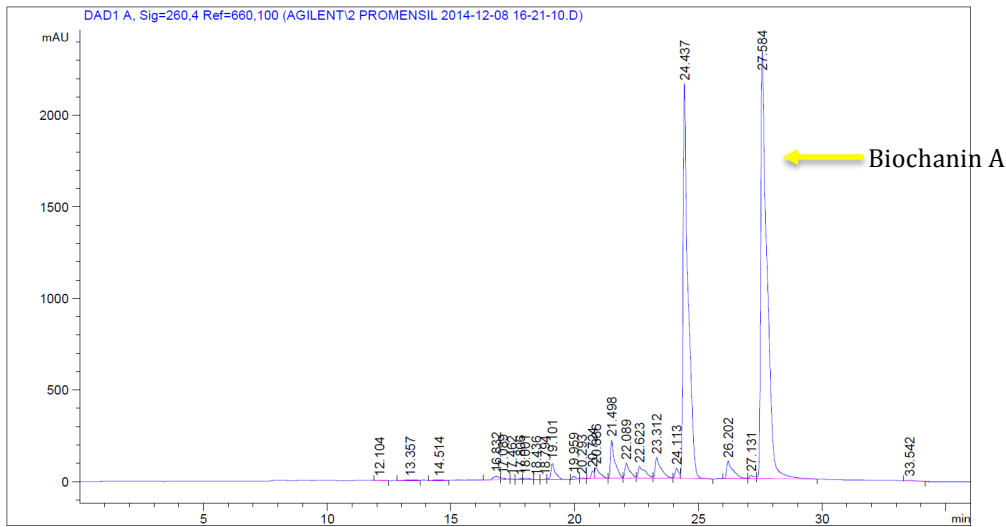


Figure 13: HPLC profile of Promensil

As shown in Figure 13, the Promensil contained components with peak elution times of 24.437 minutes (36.3757% of total trace area), and 27.584 minutes (48.8476% of total trace area). Figure 14, shown below is the HPLC run of the Red Clover Capsule.

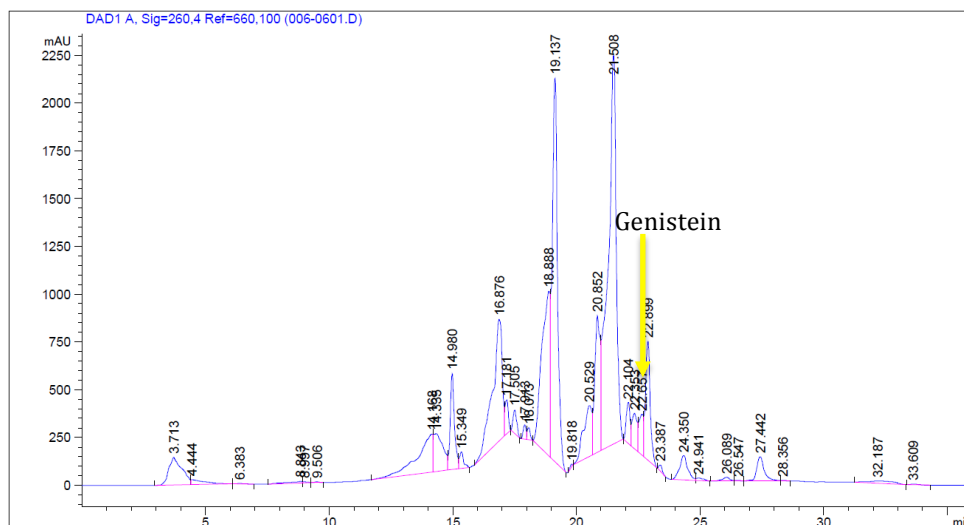


Figure 14: HPLC profile of the red clover capsules

As shown in Figure 14, the red clover capsule contained components with peak elution times of 18.888 minutes (10.4639% of total trace area), 19.137 minutes (16.1240% total trace area), and 21.508 minutes (25.2635% of total trace area). Figure 15, shown below is the HPLC run for the soy capsule supplement.

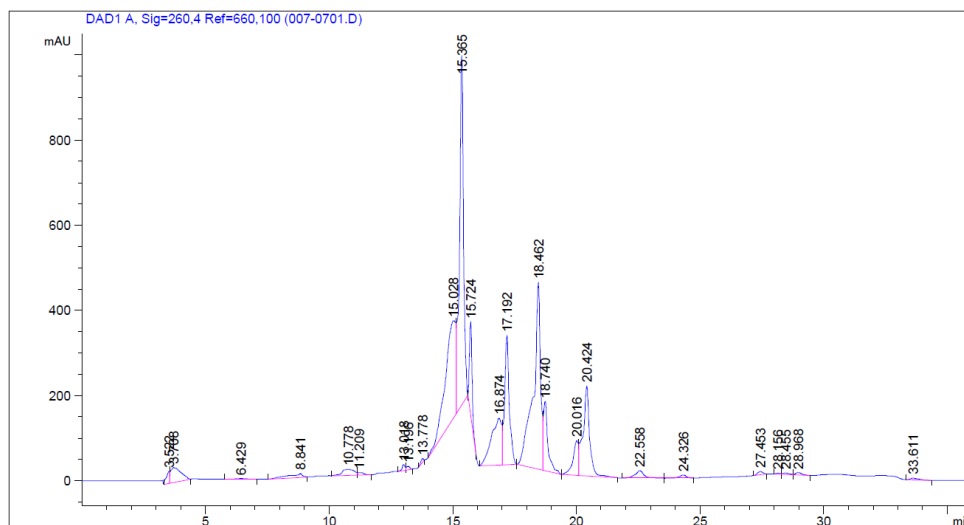


Figure 15: HPLC profile of Piping Rock Soy Isoflavone capsules

As shown in Figure 15, the Soy Capsule contained components with peak elution times of 15.365 minutes (21.4738% of total trace area), 15.028 minutes (14.7738% total trace area), and 18.462 minutes (19.9833% of total trace area). Figure 16, shown below, is the HPLC data for the Spring Valley tablet.

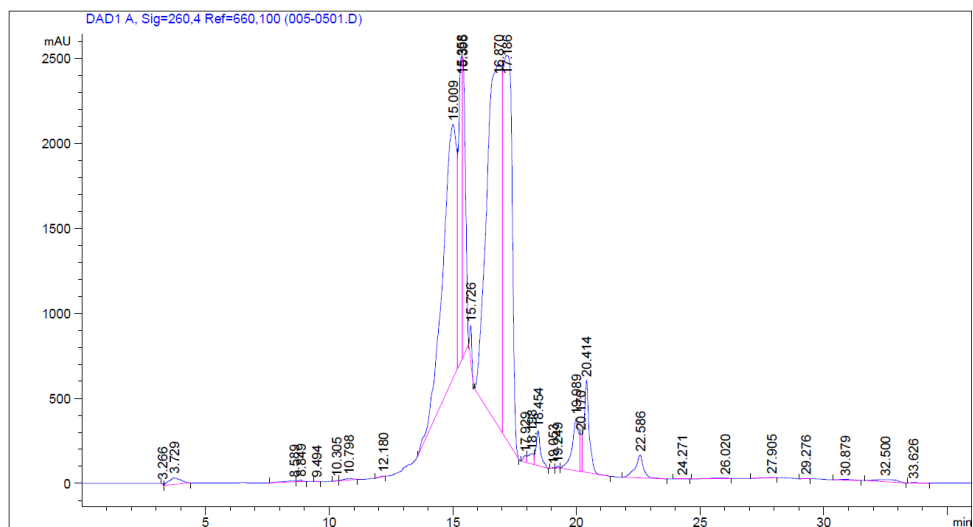


Figure 16: HPLC profile of Spring Valley Soy Isoflavone tablets

As shown in Figure 16, the Soy Valley Tablet contained components with peak elution times of 15.009 minutes (22.1919% of total trace area), 16.870 minutes (33.1452% of total trace area), and 17.186 minutes (22.0611% of total trace area). Other notable components were found at peak elution times of 15.358 minutes (6.6485% of total trace area) and 15.395 minutes (5.9622%).

Other notable peaks for each tested supplement are summarized in table 4. Each peak is described by elution time in minutes, and by % of total trace area.

Table 4: Additional Notable Peaks by Supplement

Other Notable Peaks									
Store-Bought Red Clover Extract		Team-Made Red Clover Extract		Red Clover Capsules		Soy Tablets		Soy Capsules	
Elution Time (min)	Trace area (%)	Elution Time (min)	Trace area (%)	Elution Time (min)	Trace area (%)	Elution Time (min)	Trace area (%)	Elution Time (min)	Trace area (%)
3.8	3.56	3.69	45.69	16.876	9.2918	16.874	7.1059	15.538	6.6485
14.46	6.39	16.84	4.49	20.852	5.8238	17.192	8.9611	15.395	5.9622
16.78	14	17.36	1.96			20.424	8.8716		
19.11	2.76	20.91	5.67						
20.88	3.74	21.51	0.81						
21.51	2.76	24.51	0.23						
22.09	2.67								
23.31	2.12								
24.44	8.95								
26.2	1.08								

Table 5, shown below, was created to show which supplements shared components with the standards and also the components that were shared amongst the supplements being tested.

Table 5: HPLC Summary for Tested Red Clover Supplements

	store-bought red clover extract	team-made red clover extract	red clover capsules	Promensil
Do cells proliferate in supplement's presence?	Yes	Yes	Yes	No
Known Phytoestrogens:				
Daidzein	X		X	
Genistein	X	X		
Biochanin A	X	X		X
Shared unidentified peaks (Retention time in minutes)				
3.66	X	X	X	
16.87	X	X	X	X
20.53	X	X	X	X
21.51	X	X	X	X
24.34	X	X	X	X
27.58	X	X	X	X
Other notable peaks (Retention time in minutes)	14.46, 19.11, 22.06, 23.31, and 26.20	17.35	14.46, and 14.98	23.31 and 26.20

Table 4 gives a summary of all chromatograms for the tested red clover supplements and compares the presence of both known and unknown components. Biochanin A is found in three of the four supplements, while daidzein and genestine are found in two each. Both the red clover capsules and Promensil only contain one known component. All components share unidentified peaks.

Discussion

Based on both the MTT, and HPLC results, we came to the following conclusions regarding the effects of these dietary supplements on breast cancer. Across all tested red clover supplements, there was variation in which known components were present. Three tested supplements, store-bought red clover

extract, team-made red clover extract, and red clover capsules shared six unidentified peaks, while Promensil only shared five peaks in common.

The supplement that caused the largest increase in proliferation according to MTT assay was the store-bought red clover extract. In addition to having the largest impact on proliferation, it had the largest number of peaks on its HPLC. The extract contained all three known phytoestrogen components tested for, diadzen, genestein, and biochannin A, six unknown peaks in common with other tested supplements, and six further notable unknown peaks that it does not share with other tested supplements. Of these peaks the peak of greatest interest is the peak found at 24.34 minutes, which is found in all tested red clover supplements, as well as some tested soy supplements.

Biochannin A, the only standard found in Promensil, is known to have anti-proliferative effects (Johnson, 2010). This is supportive of the idea that Promensil can be used to ease symptoms of menopause with less fear of the potentially harmful effects of traditional hormone replacement therapy (HRT).

Both extracts (store-bought and team-made) contained biochannin A, however the store-bought extract also contained genestein and daidzen, while the team-made extract contained genestine but no daidzen. This suggests that because both extracts saw an increase in proliferation, that diadzen and genestein both have proliferative effects. It is unclear whether the presence of both causes a sort of multiplied effect.

Future teams should test standards, alone and in combination to see what the effects isoflavones have on proliferation without the outside factors present in testing manufactured supplements, which do not contain pure phytoestrogens.

The most surprising trend observed in the results was the difference between the store-bought red clover extract, and the team-made red clover extract. At face-value these two extracts were the most similar treatments used in our experiments as they were (according to the label) both made from red clover flowers and leaves, and extracted in alcohol. The store-bought extract had minimal information on the label regarding ingredients, and is taken orally. We also have no information on what point within the plant's life cycle flowers and leaves were harvested during or where the plants were grown. For our own extract the red clover plants were a wild cultivar grown in Massachusetts.

In the future a way to explore the effects of the potentially very different ages of the plant, and cultivars, teams can extract red clover from a variety of points in the season in one area. Additionally testing wild cultivars versus greenhouse, or genetically modified cultivars could provide more answers as to why there may have been differences in the isoflavones present between the two extracts tested in this study.

Limitations of the study

Our study had some impactful limitations. Due to difficulties in acquiring meaningful data, the sample size of our final assay is very small (N=2). We were also unable to identify any proliferative effects in our cell line in response to B-estradiol. This was likely due to high variance in cell number between wells despite cell counting. Our cell line experienced issues with extreme cell-to-cell adhesion, which made consistency while plating difficult. The week-long period of trypsinization and subsequent synchronization by serum starvation presented us with promising results. Future groups should consider synchronization from the beginning of the experiment to reduce variability.

It is possible that other factors could have played a role in the difference of proliferation for the store-bought red clover extract and the Promensil. One of these factors is a possible difference in cell number between the wells because of the excessive clumping. While the trypsinization period did separate most of the cells, there still was a possibility of continued clumping.

Future groups should focus on identifying some of the unknown peaks that appear within more than one supplement, like the one at 24.37 minutes. These unknown peaks could be the reason similar supplements like the red clover capsule and Promensil have different effects on proliferation.

Known and unknown components can be collected and added to the cells to determine which, if any have proliferative or anti-proliferative effects. This can further solidify what makes these supplements different, while they are marketed as coming from the same plant.

After these components have been identified, the estrogen promoter regulated reporter can be used to determine which estrogen receptor the compound is interacting with, which can give more insight into that compounds' proliferative or anti-proliferative effects. Additionally, immunoblotting can determine if any of these compounds rather than being anti-proliferative are actually triggering the apoptotic cascade.

The small sample size of our results leaves us unable to say anything definitively, however, we can use the trends presented to us to form a basis for future studies. The conclusions and recommendations in this paper will help other groups to shoot past the point of tentative trial assays, and delve deeper into learning more about the effects of these components on breast cancer. This is a problem that must continue to be explored to allow the public to make informed decisions about the safety of these unregulated supplements, and take greater responsibility in their healthcare.

References

Albulescu, Mariana , and Marinela Popovici. "Isoflavonoids–Biochemistry, Pharmacology and Therapeutic Use." *Revue Roumaine de Chimie*. N.p., 15 May 2006. Web. 23 Nov. 2013

Alder, E. (1998). The Blatt-Kupperman menopausal index: a critique. *Maturitas: Journal of the Climacteric & Postmenopause*, 29(1), 19–24. doi: 10.1016/S0378-5122(98)00024-3

Alekel, D. L., Germain, A. S., Peterson, C. T., Hanson, K. B., Stewart, J. W., & Toda, T. (2000). Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. *The American Journal of Clinical Nutrition*, 72(3), 844.

Alexander V, S. (2014). Phytoestrogens and their effects. *European Journal of Pharmacology*, 741, 230-236. doi:10.1016/j.ejphar.2014.07.057

Bachmann, G. A. (1999). Vasomotor Flushes in Menopausal Women. *American Journal of Obstetrics and Gynecology*, 180(3).

BIOTIUM. Firefly and Renilla Dual Luciferase Assay Kit. BIOTIUM. <http://biotium.com/wp-content/uploads/2013/07/PI-30005.pdf> 2014.

Berger, G. E. (1999). *Menopause and Culture*. London, GBR: Pluto Press.

Bolar K, Hoffman AR, Maneatis T, Lippe B. Long-term safety of recombinant human growth hormone in turner syndrome. *J Clin Endocrinol Metab*. Feb 2008;93(2):344-51.

CDER. "Guidance for Industry Labeling OTC Human Drug Products – Questions and Answers" December 2008

Chen, J., et. al. "Biochanin A Promotes Proliferation That Involves a Feedback Loop of MicroRNA-375 and Estrogen Receptor Alpha in Breast Cancer Cells." - *FullText*. *Cellular Physiology and Biochemistry*, Jan. 2015. Web. 28 Apr. 2015.

CHLEBOWSKI, R. T. et al. Breast cancer after use of estrogen plus progestin in postmenopausal women. *New England Journal of Medicine*, v. 360, n. 6, p. 573-587, 2009.

Cornwell, T., Cohick, W., & Raskin, I. (2004). *Dietary phytoestrogens and health*. United States: Elsevier Ltd. doi:10.1016/j.phytochem.2004.03.005

Cui, Y., et al. (2014). "Interactions of Hormone Replacement Therapy, Body Weight, and Bilateral Oophorectomy in Breast Cancer Risk." *Clinical Cancer Research* 20(1169).

DIEL, P. et al. Effects of the environmental estrogens bisphenol A, o,p'-DDT, p-tert-octylphenol and coumestrol on apoptosis induction, cell proliferation and the expression of estrogen sensitive molecular parameters in the human breast cancer cell line MCF-7. *The Journal of Steroid Biochemistry and Molecular Biology*, v. 80, n. 1, p. 61-70, 2002.

Dietary Supplements: A Framework for Evaluating Safety. (2005). Washington, DC, USA: National Academies Press. Retrieved from <http://www.ebrary.com>

- Dixon, R. A., & Ferreira, D. (2002). Genistein. *Phytochemistry*, 60(3), 205-211. doi:10.1016/S0031-9422(02)00116-4
- ER, S.; SR, D. Minireview: aromatase and the regulation of estrogen biosynthesis-some new perspectives. *Endocrinology*, v. 142, n. 11, p. 4589-4594, 2001.
- FDA. "Dietary Supplements" Web. 14 October 2014
<<http://www.fda.gov/AboutFDA/Transparency/Basics/ucm193949.htm>>
- FDA. "What are Over the Counter Drugs, and How are they Approved?" Web October 13 2014 <http://www.fda.gov/AboutFDA/Transparency/Basics/ucm194951.htm>
- FDA. "What do We Do?" Web. 10 October 2014.
<<http://www.fda.gov/AboutFDA/WhatWeDo/default.htm>>
- Holli, K., et al. (1998). "Low biologic aggressiveness in breast cancer in women using hormone replacement therapy." *Journal of Clinical Oncology* 16(9): 3115-3120.
- HYDER, S. M. et al. Identification of Functional Estrogen Response Elements in the Gene Coding for the Potent Angiogenic Factor Vascular Endothelial Growth Factor. *Cancer Research*, v. 60, n. 12, p. 3183-3190, 2000. Disponível em: < <http://cancerres.aacrjournals.org/content/60/12/3183.abstract> >.
- Johnson, Tara L. "Inhibition of Cell Proliferation and MAP Kinase and Akt Pathways in Oral Squamous Cell Carcinoma by Genistein and Biochanin A." Jan. 2010.
- Kang, Jong S., et. al. "Low Dose Estrogen Supplementation Reduces Mortality of Mice in Estrogen-Dependent Human Tumor Xenograft Model." Bioevaluation Center, Korea Research Institute of Bioscience and Biotechnology And College of Pharmacy, Chungbuk National University, 01 Jan. 2009
- Katalinic, A. and R. Rawal (2008). "Decline in breast cancer incidence after decrease in utilisation of hormone replacement therapy." *Breast Cancer Research and Treatment* 107(3): 427-430.
- KIMBALL, J. Steroid hormone receptors and Their Response Elements.
<http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/S/SteroidREs.html> 2011.
- KLINGE, C. M.; RAO, C. V. The Steroid Hormone Receptors.
http://www.glowm.com/section_view/heading/The%20Steroid%20Hormone%20Receptors/item/280, 2008.
- Kuiper, G. G., et. al. "Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta." *National Center for Biotechnology Information*. U.S. National Library of Medicine, Mar. 1997.
- Latrich, C., Schüler, S., Häring, J., Skrzypczak, M., Ortmann, O., & Treeck, O. (2014). Effects of a combined treatment with tamoxifen and estrogen receptor β agonists on human breast cancer cell lines. *Archives of gynecology and obstetrics*, 289(1), 163-171.
- Lesage, S., et al. (2014). The Effects of Over-The-Counter Phytoestrogens on Breast Cancer.
- Lindsay, R. (1996). The menopause and osteoporosis. 87(2), 16S–19S. doi: 10.1016/0029-7844(95)00430-0

- Lutter, S., et al. (2014). "The isoflavone irilone contributes to the estrogenic potential of dietary supplements containing red clover." *Archives of Toxicology* 88(2): 309-321.
- Morito, K., Hirose, T., Kinjo, J., Hirakawa, T., Okawa, M., Nohara, T., ... & Masamune, Y. (2001). Interaction of phytoestrogens with estrogen receptors alpha and beta. *Biological & pharmaceutical bulletin*, 24(4), 351-356.
- Mueller, S. O., Simon, S., Chae, K., Metzler, M., & Korach, K. S. (2004). Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor α (ER α) and ER β in human cells. *Toxicological Sciences*, 80(1), 14-25.
- Nelson, H. D. (2008). Menopause. *The Lancet*, 371(9614).
- Patisaul, H. B., & Jefferson, W. (2010). The pros and cons of phytoestrogens. *Frontiers in Neuroendocrinology*, 31(4), 400–419. doi:10.1016/j.yfrne.2010.03.003
- Peterson, J., Dwyer, J., Adlercreutz, H., Scalbert, A., Jacques, P., & McCullough, M. L. (2010). Dietary lignans: physiology and potential for cardiovascular disease risk reduction. *Nutrition reviews*, 68(10), 571-603.
- Powell, E., Shanle, E., Brinkman, A., Li, J., Keles, S., Wisinski, K. B., ... & Xu, W. (2012). Identification of estrogen receptor dimer selective ligands reveals growth-inhibitory effects on cells that co-express ER α and ER β . *PLoS one*, 7(2), e30993.
- Power, K. A., and Thompson, L. U. (2003). Ligand-induced regulation of ER α and ER β is indicative of human breast cancer cell proliferation. *Breast Cancer Res. Treat.* 81, 209–221.
- Rietjens, Ivonne M C M, Sotoca, A. M., Vervoort, J., & Louisse, J. (2013). Mechanisms underlying the dualistic mode of action of major soy isoflavones in relation to cell proliferation and cancer risks. *Molecular Nutrition & Food Research*, 57(1), 100.
- RIGGS, B. L.; KHOSLA, S.; MELTON, L. J. A Unitary Model for Involutional Osteoporosis: Estrogen Deficiency Causes Both Type I and Type II Osteoporosis in Postmenopausal Women and Contributes to Bone Loss in Aging Men. *Journal of Bone and Mineral Research*, 1998.
- Riley, T. H. (2010). *Nutrition and Diet Research Progress : Dietary Supplements*. New York, NY, USA: Nova Science Publishers, Inc.. Retrieved from <http://www.ebrary.com>
- Rizza, P., Barone, I., Zito, D., Giordano, F., Lanzino, M., De Amicis, F., ... & Andò, S. (2014). Estrogen receptor beta as a novel target of androgen receptor action in breast cancer cell lines. *Breast Cancer Res*, 16, R21.
- S, W. V.; K, B.; E, G. L. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol. Sci.* 2004.
- S., L.; AL., E. The isoflavone irilone contributes to the estrogenic potential of dietary supplements containing red clover. *Archives of Toxicology*, v. 88, n. 2, p. 309-321, 2014.
- Salter, A., Tucker, G., & Wiseman, H. (Eds.). (2012). *Phytonutrients*. Hoboken, NJ, USA: John Wiley & Sons. Retrieved from <http://www.ebrary.com>

Sirotkin, A. V., & Ebrary Academic Complete. (2011). *Regulators of ovarian functions*. New York: Nova Science Publishers.

Shike, M., et al. (2014). "The Effects of Soy Supplementation on Gene Expression in Breast Cancer: A Randomized Placebo-Controlled Study." *Journal of the National Cancer Institute* 106(9).

Shuman Moss, L. A., et al. (2014). "Dietary intake of a plant phospholipid/lipid conjugate reduces lung cancer growth and tumor angiogenesis." *Carcinogenesis*.

Setchell, K. D. R., et al. (2001). "Bioavailability of Pure Isoflavones in Healthy Humans and Analysis of Commercial Soy Isoflavone Supplements." *The Journal of Nutrition* 131(4): 1362S-1375S.

STOVALL, D. W.; PINKERTON, J. V. Estrogen Agonists/Antagonists in combination with Estrogen for Prevention and Treatment of Menopause-associated Signs and Symptoms. *Women's Health*, v. 4, n. 3, p. 257-268, 2008.

TAYLOR, J. M. et al. Growth hormone response to an acute bout of resistance exercise in weight-trained and non-weight-trained women. *Journal of Strength and Conditioning Research*, v. 14, n. 2, p. 220-227, 2000.

WATANABE, T. et al. In vitro sensitivity test of breast cancer cells to hormonal agents in a radionucleotide-incorporation assay. *Jpn. J. Cancer Res.*, v. 81, p. 536-543, 1990.

Wilson VS, Bobseine K, Gray LE. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol Sci* 2004;

Watkins, Elizabeth Siegel. *The Estrogen Elixir: A History of Hormone Replacement Therapy in America*. Baltimore: The Johns Hopkins University Press, 2007. *Project MUSE*. Web. 16 Oct. 2014. <<http://muse.jhu.edu/>>.

Watanabe, T., Wu, J. Z., Morikawa, K., Fuchigami, M., Kuranami, M., Adachi, I., Yamaguchi, K., and Abe, K. (1990). In vitro sensitivity test of breast cancer cells to hormonal agents in a radionucleotide-incorporation assay. *Jpn. J. Cancer Res.* 81, 536-543.