

May 2014

# The Effects of Over-the-Counter Phytoestrogens on Breast Cancer Cells

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# The Effects of Over-the-Counter Phytoestrogens on Breast Cancer Cells

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## Abstract

Phytoestrogens, plant produced hormones, are marketed to alleviate menopausal symptoms because studies indicate increased incidence of breast cancer in women undergoing estrogen Hormone Replacement Therapy. We studied the potential proliferative effects of phytoestrogens on the breast cancer cell line T47D-KBluc. HPLC isolated components of black cohosh, red clover, and grape seed extracts were assessed for proliferative effects using the MTT assay. Our results suggest that grape seed demonstrated the greatest proliferative effects.

## Acknowledgements

Our team would like to recognize the people who were involved in making this project a possibility and who supported us throughout the duration of this project. We would like to thank our advisers Mike Buckholt and Jill Rulfs for their guidance and encouragement throughout the year. They gave our project direction and provided support (academic and emotional) that we cannot thank them enough for. We would also like to thank Abbie White for ordering and stocking supplies for us throughout the year as well as JoAnn Whitefleet-Smith, for sharing her lab equipment and expertise.

## Authorship

Primarily, Theresa Renna and Tracy Sinkewicz were responsible for the work related to black cohosh, Stephanie Lesage and Elena Musteata were responsible for the work related to red clover, and Taylor Manning and Nirali Parekh were responsible for the work related to grape seed. Each member equally participated in all aspects of lab work and writing from the project's inception to conclusion.

# Background

## Introduction

Menopause is defined by the permanent cessation of menstruation due to a loss of function in the ovaries, which produce eggs as well as the female hormone estrogen. Women going through menopause are typically in their forties or fifties and experience symptoms such as hot flashes, night sweats, sleep disturbances, and changes in vaginal and urinary tracts. In the past, a standard treatment to regulate these uncomfortable symptoms was hormone replacement therapy (HRT) which uses synthetic hormones such as estrogen and progesterone to normalize fluctuating hormone levels. Studies now show that HRT may have many negative and life-threatening side effects such as breast and ovarian cancer (“Menopausal hormone therapy and cancer”, 2011). This resulted in many women turning to phytoestrogens as alternatives to combat their menopausal symptoms. Phytoestrogens are phytochemicals naturally occurring in plant compounds that can mimic the effects of estrogen and thus have the potential to alleviate the symptoms of menopause. While phytoestrogens are part of a daily diet available in foods such as soy, red grapes, and legumes, they are marketed to menopausal women as dietary supplements that are a safe and natural alternative to estrogen. However, these supplements are not regulated as strictly by the Food and Drug Administration (FDA) as substances that are classified as drugs; therefore, their side effects are not well understood and may include an increased risk for breast cancer.

## Part 1: Hormones and Hormone Therapy

### Hormones

Hormones are the chemical messengers in the human body that control processes such as growth and development, metabolism, sexual function, reproduction, and mood. These chemical messengers are stored and released as needed by glands in the endocrine system. The major endocrine glands include the pituitary, pineal, thymus, thyroid, and adrenal glands, along with the pancreas, testes in males, and ovaries in females. Once the body signals the glands to release the hormones, they travel through the blood to reach tissues or organs that contain specific target cells. The hormones bind to the receptor molecule of the target cell, which can lead to a change in shape of the receptor and ultimately a cellular response. Many organs in the body, specifically reproductive organs and tissues, have estrogen and progesterone receptors. Estrogen is particularly important for sexual and reproductive development in women. It is mostly produced in the ovaries, but also in fat cells and in the adrenal gland. Estrogen fluctuations are linked with menstruation, pregnancy, and menopause, which is why they are found in birth control pills and are used in hormone replacement therapies during menopause.

### Estrogen

Estrogen is the primary sex hormone for women and is classified as a steroid. This allows it to directly diffuse through the cell membrane. Once in the cytoplasm, the hormone binds to the Estrogen Receptor (ER) forming an estrogen-ER complex. ERs, once activated by estrogen, are responsible for transporting the whole estrogen-ER complex to the nucleus where it binds to

estrogen response element (ERE) sequences (“Estrogen”, 2013). This binding controls protein synthesis by affecting the rate of transcription of estrogen responsive genes and thus the protein production and physiological response (Deroo, 2006). There are two known isoforms of cytoplasmic ERs, Estrogen Receptor  $\alpha$  (ER $\alpha$ ) and Estrogen Receptor  $\beta$  (ER $\beta$ ), which vary in distribution throughout tissues in a given organism and play different roles dependent on the affinity for a specific estrogen to bind to it (Deroo, 2006).

## Menopause

Estrogen levels fluctuate in women during menopause. Menopause is characterized by a time when a woman has been menstruation free for one year without being ill, pregnant, breast feeding or using medications. Approaching menopause, women go through a phase called perimenopause which lasts for a few years, causing irregular periods and unpredictable heavy bleeding. During perimenopause, the ovaries of a woman start to shrink leading to the fluctuation of estrogen and progesterone levels during hormone production. These hormones are responsible for menstrual cycle regulation and pregnancy and can cause a variety of symptoms for menopausal women (“Menopausal hormone therapy and cancer”, 2011). For example, estrogen deficiency causes vulvar-vaginal atrophy (VVA), and hormonal fluctuation causes vasomotor changes that lead to hot flashes experienced by 60% - 90% of menopausal women (Levine, 2011). Other common symptoms of menopause include night sweats, sleep disturbances, and changes in vaginal and urinary tracts (“Menopausal hormone therapy and cancer”, 2011).

## Hormone Replacement Therapy

HRT is often prescribed to women in order to help control or moderate their symptoms during menopause. HRT entails the intake of hormones such as estrogen, progesterone, or both in order to regulate fluctuating hormone levels. These hormones can be delivered in different forms such as pills, skin patches, creams, gels, or by intrauterine devices. Although helpful in treating some menopausal side effects, HRT can lead to vaginal bleeding, bloating, breast tenderness and swelling, headaches, mood changes, and nausea. Dangerous long term effects such as increased chance of blood clots, bone loss, heart attack, stroke, breast cancer, and gallbladder disease have also been observed (“Menopausal hormone therapy and cancer”, 2011).

### ET and EPT

If a woman has previously undergone a hysterectomy, a procedure to remove the uterus in order to end menstrual cycles, estrogen alone is prescribed as hormone therapy. Although estrogen alone reduces hot flashes, bone loss, vasomotor and VVA symptoms, it is deleterious to the endometrium in the uterus by stimulating hyperplasia (Levine, 2011). To prevent these harmful effects, a combination of estrogen and progesterone is prescribed to women who have not undergone uterus removal. The addition of progesterone in this estrogen-progesterone therapy (EPT) prevents overgrowth of cells in the lining of the uterus thereby preventing growths that can lead to uterine cancer. Progesterone alone, however, can cause breast discomfort, bloating, fatigue, and depression (Levine, 2011). Therefore, the optimal menopause



therapy should relieve vasomotor and vulvar-vaginal symptoms, prevent fractures, have beneficial or neutral cardiovascular effects, and should not stimulate breast or endometrium cancer cell growth (Levine, 2011).

To test the effects of HRT (estrogen therapy and EPT), randomized controlled Women's Health Initiative (WHI) trials were conducted with 27,000 healthy women ages 50-79 from 40 US clinical centers. The two studies conducted included therapy with Estrogen Alone Therapy (ET), and Estrogen Progestin Therapy (EPT). In both groups, some of the women received hormone therapy while the others were treated with a placebo. The study was completely randomized and double blind. There were 10,739 postmenopausal women involved in the ET study. These women had hysterectomies and took a daily estrogen dose in the form of conjugated equine estrogen (CEE) for about six years. The effects of estrogen were then compared to the effects of the placebo. It was observed that estrogen therapy had neutral effects on breast cancer and even showed lower risks of breast cancer in those without a family history of breast cancer. The study showed that the risk of breast cancer was reduced by 23% (26/10,000 people per year versus 33/10,000 people per year), and even if breast cancer did develop, the mortality rate was decreased (Anderson & Limacher, 2004).

When testing the effects of EPT, 16,608 postmenopausal women were involved where 8,506 women were given EPT (0.625 mg/dl conjugated equine estrogens plus 2.5 mg/dl medoxyprogesterone acetate) and 8,102 women were treated with a placebo. Out of those treated by EPT, there was a 26% increase in developing breast cancer (38/10,000 people per year versus 30/10,000 people per year) (Gebbie, 2002). This statistic is consistent with estimates from pooled epidemiological data from the Heart and Estrogen/Progestin Replacement Study Follow-Up (HERS II). This data reported a 15% breast cancer increase after using EPT for less than 5 years, and a 53% breast cancer increase after using EPT for more than 5 years, specifically showing a 27% breast cancer increase after 6.8 years of EPT use (Hulley *et al.*, 2002). EPT also increased breast density, which made it more difficult to find breast cancer using a mammogram (Gebbie, 2002).

Overall, when comparing the ET to the EPT, a cohort study of follow-up data from the Breast Cancer Detection Demonstration Project (1980-1995) shows a 1% increase in breast cancer with each year of ET, and an 8% increase in breast cancer with each year of EPT (Schairer, 2000). Although the WHI trial results for ET show a decreased risk in breast cancer, the question is raised as to how that is possible since many observational studies, such as the Breast Cancer Detection Demonstration Project follow-up, show that ET either increases the risk of breast cancer or has a neutral effect. A theory to explain this phenomenon is that breast cancer tumor cells can adapt to grow best at prevailing estrogen concentrations. For example, if estrogen concentrations are reduced in Michigan Cancer Foundation-7 (MCF-7) breast cancer cells, tumor cells will stop growing at first, but will eventually adapt to the reduced concentrations and begin growing again. When estrogen concentrations are suddenly increased again, tumor growth is inhibited (Howell & Cuzick, 2012). Therefore, it is possible that after menopause, estrogen concentrations decline, causing tumors to adapt and grow at the new level. However,

they are inhibited when high estrogen concentrations are introduced via hormone replacement therapy. Similarly, if a woman already has breast cancer and uses high-dose estrogen treatment, stopping the treatment will cause a withdrawal response, which would suggest that the tumor adapted to grow at the higher dose (Howell & Cuzick, 2012).

### Tissue Selective Estrogen Complexes

An ideal menopause treatment shows relief of hot flashes and VVA, maintain bone mass, and protect the endometrium and breast from estrogenic stimulation (Levine, 2011). An alternative method to ET or EPT is tissue selective estrogen complexes (TSEC). This is a new class of compounds that pairs a selective estrogen receptor modulator (SERM) with one or more estrogens in order to get tissue specific activity. This specificity should allow estrogenic action where it is beneficial (Mirkin & Komm, 2012).

A type of TSEC treatment uses a combination of SERM basedoxifene (BZA) and conjugated estrogens (CE). When tested on MCF-7 breast cancer cells, it was observed that BZA blocked the effects induced by CE, such as antiapoptotic effects, cell proliferation and growth, and protein and gene expression. In addition, the tissue selective agonist and antagonist activities of BZA in combination with CE help balance estrogenic activities in breast and endometrial tissues and have an overall neutral effect on these tissues. This was also supported when BZA/CE was tested in mouse and non-human primate models, where BZA inhibited stimulatory activity of CE in breast tissues (Mirkin & Komm, 2012).

Overall, BZA/CE treatment leads to improved post-menopausal symptoms such as reduction in hot flashes and VVA, increased bone mineral density, and improved quality of life, health and sleep. Preclinical and clinical studies show that the beneficial qualities of this treatment are maintained without endometrial or breast cell growth stimulation. To further test the safety of BZA/CE treatment, Selective Estrogen Menopause and Response to Therapy (SMART) trials were conducted. In these trials, the mean percent deviations from the baseline breast density at 24 months were compared between women treated with BZA/CE and a placebo. No increase in abnormal breast mammograms were observed at 24 months. No differences were observed in breast cancer over 2 years for the BZA 20 mg/CE 0.45 mg treatment or for the BZA 20 mg/CE 0.625 mg treatment compared with placebo. These trials, along with other studies, show that BZA/CE treatments are safe and effective (Mirkin & Komm, 2012).

HRT is successful in lessening the strength of some menopausal symptoms. However, research has shown that HRT can increase the risks of many detrimental side effects such as coronary heart disease, osteoporosis, and breast cancer. Being aware and afraid of the negative side-effects, less than 25% of women receive HRT during menopause. The majority of women undergoing menopause search for alternative ways to self-manage their treatment. The most popular alternative is replacing HRT with phytoestrogens. Although research on the side effects of phytoestrogens is limited, studies are now starting to address the gynecologic effects of these agents in order to better define them as treatment (Umland, 2000).

## Part 2: Phytochemicals

Phytochemicals are compounds produced by plants that can affect human health if consumed (Kurzer, 1997). They are found in many common fruits, vegetables, beans, and grains that are commonly included in most diets. There are various types of phytochemicals such as carotenoids, allyl sulfides, and polyphenols (“e.hormone”, 2012). Carotenoids are found in many vegetables and fruits such as carrots, yams, and cantaloupe. They are viewed as anti-cancer agents due to Vitamin A, retinoids, and antioxidants. Allyl sulfides, found in garlic and onions, are thought to strengthen the body’s immune system. The last type of phytochemical, polyphenols, includes a large subgroup known as flavonoids. Flavonoids are chemicals derived from fruits, vegetables, and grains that may prevent cancer and heart disease. Flavonoids can act as antioxidants which rid the body of harmful molecules such as ‘free radicals’ through the consumption of plant products like grapes and eggplant (Cornwell, 2004). Additionally, isoflavonoids are a specific type of flavonoid found in red clover, garbanzo beans, and other plant products and are suspected to imitate the actions of estrogen (Cornwell, 2004). There is no substantial evidence supporting the benefits of phytochemicals, but they are being researched further.

### Phytoestrogens

Phytohormones are phytochemicals that can mimic hormone signals in animals. There are twenty known types of phytohormones, including phytoestrogens, which mimic the female hormone estrogen. Phytoestrogens are studied for their potential protection against hormone dependent cancers, such as breast, prostate, and uterine cancer and are found in over 300 plants. Phytoestrogens are categorized into different chemical classes including isoflavonoids, lignans, coumestans, and stilbenes.

#### Isoflavonoids

Isoflavonoids are a subclass of flavonoids that are the most studied of the phytoestrogens. Isoflavonoids can exist as glucosides or as aglycones. As aglycones, they can be transported across intestinal epithelial cells, and as glucosides, they can be hydrolyzed to aglycones in the digestive system. Genistein, a commonly studied isoflavonoid, can interact with both ER $\alpha$  and ER $\beta$  to induce similar responses as estradiol in breast, ovarian, endometrial, prostate, vascular, and bone tissues. Genistein has also shown the ability to block cell proliferation of normal mammary cells, possibly decreasing the risk of breast cancer. However, when bound to ER $\alpha$  it has one third the potency of estradiol, and one thousandth the potency when bound to ER $\beta$ . The main source of isoflavonoids is in soy-based foods such as soy based infant formula, tofu, soy milk, kidney, navy and pinto beans, and chick peas (Cornwell, 2004).

#### Lignans

Lignans are phenylpropanoid dimers and oligomers, which are often not estrogenic by themselves, but can be converted to estrogenic mammalian lignans. This conversion of plant lignans (secoisolariciresinol and matairesinol) to mammalian lignans (enterodiols and enterolactone) occurs in the gastrointestinal tract as a result of bacterial action (Kurzer, 1997). Both enterodiols and enterolactone bind to ER $\alpha$  and ER $\beta$  with a higher affinity for ER $\beta$ . Lignans

are found in high concentrations in flaxseed, whole grain breads, vegetables, and tea. They can also be found in low concentrations in fruits; however, they are not found in strawberries or cranberries (Cornwell, 2004).

### Coumestans

Coumestans can be found in large numbers; however, very few (coumestrol and 4'-methoxycoumestrol) have been known to show estrogenic activity (Cornwell, 2004). Studies have shown that coumestrol has a higher affinity than estradiol for binding to ER $\beta$ . The highest concentration of coumestrol can be found in clover and soybean sprouts and low levels can be found in legumes, Brussels sprouts, and spinach (Cornwell, 2004).

### Stilbenes

The primary dietary source of stilbenes is resveratrol, a phytoalexin, which accumulates at areas of pathogen infection. Only the trans isomer of resveratrol has been found to be estrogenic, and it has a much higher affinity for interacting with ER $\beta$  than ER $\alpha$ . Resveratrol has also shown agonistic and antagonistic activity in MCF-7 cells in the hamster ovarian cell line (CHO-K1) that was transfected with human ERs. Resveratrol is primarily found in peanuts as well as the skin of red grapes and products such as juice and wine. However, the processing of these foods has an effect on the amount of resveratrol found in the final product. For example, grapes have a higher content of resveratrol than grape juices, and boiled peanuts have a higher concentration than peanut butter or roasted peanuts (Cornwell, 2004).

## Phytoestrogens in Daily Diets

Since phytoestrogens are found in various plants and plant products, humans ingest the chemicals naturally through diet. Daily intake from vegetables, beans, grains, and seeds is not detrimental to the body and may even be seen as beneficial; however, consuming excess phytoestrogens through dietary supplements may have varying effects. Infants who drink soy formula have the highest level of phytoestrogens while adults on vegetable diets intake the next highest amount (Kurzer, 1997). When comparing the health of Westerners and Asians, it appears that a diet including phytoestrogens can aid in cancer prevention and treatment of menopausal symptoms (Hilakivi-Clarke *et al.*, 2010). This conclusion was made when combining the work of Trock *et al.* and Wu *et al.* (Trock *et al.*, 2006; Wu *et al.*, 2008).

In the Trock *et al.* study, Westerners and Asians were given a high soy diet in order to observe the effects on breast cancer. The study indicated that the intake of soy by Westerners was associated with a reduced breast cancer risk and was most prevalent among premenopausal women. However, the Asian women did not see any significant reduction. Wu *et al.* performed a similar study using the categories Westerners and Asian women, but found the opposite results (Wu *et al.*, 2008). Western women did not have any risk reduction, but the Asian women did. The source of confusion stems from the classification of the women in the study; Trock *et al.* included Asian Americans in his Western category whereas Wu *et al.* included them in the Asian category. Therefore, when combining these results, it was concluded that Asian American women see a protective effect by consuming moderate levels of soy on a daily basis

whereas Asians who adopt the Western dietary habits later in life do not see protection (Hilakivi-Clarke *et al.*, 2010). This is because Asians would be going from a high soy intake to a low soy intake which would put them at a higher risk for breast cancer, whereas Asian Americans who already had a Western diet are eating more soy resulting in breast cancer risk reduction (Hilakivi-Clarke *et al.*, 2010).

In addition to the amount of phytoestrogens consumed, the time period in which someone is exposed may also be important. When exposed early in life through diet, phytoestrogens may be cancer preventative, but the effects of ingestion during adulthood are less clear (Warren, 2001). This is inconclusive due to studies that propose phytoestrogens as a health benefit and contradictory results showing them as a health risk. Results from some of these studies are presented below.

### Health Benefits

In a study done on postmenopausal Japanese women, participants have reportedly experienced reduced vasomotor symptoms of menopause such as hot flash frequency and severity. There was a 30% - 50% reduction in hot flashes when soy isoflavonoids were taken in several doses throughout the day. However, this total percentage includes the 10% - 20% of participants given a placebo who also reported a reduction. This limits the actual benefits to a 20% - 30% reduction of hot flashes which is still a significant percentage, but not as substantial as the study had postulated (Hilakivi-Clarke *et al.*, 2010).

Another possible benefit of phytoestrogens includes cardiovascular health. The effects of a diet low in saturated fat and cholesterol with 25g of soy protein per day on the risk of heart disease were tested (Lissin *et al.*, 2000). It was found that an average intake of 47g per day of soy protein decreased total cholesterol by 9%, decreased Low Density Lipoprotein (LDL) by 13%, and showed a trend toward increased High Density Lipoprotein (HDL). To determine the role of isoflavonoids in the control of cholesterol, a study compared participants receiving soy protein containing its natural isoflavonoids and participants receiving soy that was stripped of its isoflavonoids (Lissin *et al.*, 2000). These studies suggested that isoflavonoids were required for the desired results in cholesterol levels.

Although there have been studies to support both reduction in postmenopausal symptoms and increased cardiovascular health, the results may be the cause of other factors. Since the plant products consumed in these studies were not just phytoestrogens but also included protease inhibitors and antioxidants, the results could be due to these other ingredients (Kurzer, 2003). Additionally, since the study on reduced hot flashes was done on Asian women, there may be some adaptation that they have acquired through years of high soy intake providing them with a better response to phytoestrogen supplements than Westerners (Kurzer, 2003).

### Health Risks

Conversely, phytoestrogens can also pose a health risk. High exposure to estrogen over a lifetime is linked to increased breast cancer risk, so if phytoestrogens bind through the same pathway, they may also pose a risk. A study was conducted using estrogen responsive breast cancer cells treated with low doses of phytoestrogens extracted from soymilk (Dip *et al.*, 2013).

mRNA analysis revealed that the phytoestrogen induced tumor-promoting transcriptional signaling was indistinguishable from that of estrogen (Dip *et al.*, 2013). This increase in signaling led to proliferation of tumor cells showing that phytoestrogens can pose a health risk at certain doses (Dip *et al.*, 2013). Additionally, the estrogen-like properties may cause reproductive problems such as infertility and developmental issues (Kurzer, 2003). Many historical plants documented to prevent pregnancies or cause miscarriages were tested and found to contain phytoestrogens or other phytohormones. It is thought that plants began producing compounds that when ingested by herbivores would limit their reproduction, resulting in a reduced predation of that plant species (Kurzer, 2003).

The health benefits and risks mentioned above provide strong evidence that phytoestrogens need to be studied further. Although it is clear that phytoestrogens in an everyday diet are not detrimental to the body, there is little conclusive data regarding their use as dietary supplements marketed as a safe alternative to HRT.

### Part 3: Food and Drug Administration

In order for any drug to be legally sold in the United States, it must be approved by the Food and Drug Administration (FDA). Since its inception in 1906 under the Department of Health and Human Services, the FDA has regulated over \$1 trillion worth of products. The FDA is responsible for the safety of foods, tobacco, electronic device radiation, vaccines and drugs. The drug category encompasses over the counter and prescription medications for both human and veterinary use (“What does FDA do?”, 2013).

#### Over the Counter Drugs

Over the counter drugs (OTC) are medications that may be purchased without a prescription. The list of what constitutes an OTC is vast. Some examples include vitamins, dietary supplements, allergy medication, and painkillers. They are available at pharmacies as well as health supplement stores such as GNC and Vitamin World. The majority of phytoestrogens are sold this way.

Over the counter drugs are monitored by the Office of Drug Evaluation and the Nonprescription Drug Advisory Committee. These groups monitor the safety and efficacy of the drugs and the appropriateness of their labels as well as determine the contents of drug monographs. A drug monograph is like a recipe that describes the acceptable parameters and ingredients for medications by class. For instance, there may be a monograph simply for antacids, not a specific brand or type of antacid. If an OTC falls completely within the constraints of the correct monograph for its category, then that medication can go to market directly (“OTC, Nonprescription”, 2013).

The drug monograph is created through a three-phase process in which the findings are continually tested and reviewed. In the first phase, the active ingredients, appropriate dosages, side effects, and repercussions of misuse are examined and approved by advisory review (Over-the-Counter (OTC) Drug Monograph Process, 2013). The findings are published in the *Federal Register* in the format of an Advanced Notice Proposed Rulemaking (ANPR). Once the findings are published, they are once again

open for review and any qualified and interested person or group can formally submit comments. Following this review, the active ingredients may be categorized into one of three groups:

- Category I: generally recognized as safe and effective for the claimed therapeutic indication;
- Category II: not generally recognized as safe and effective or unacceptable indications;
- Category III: insufficient data available to permit final classification (“Over-the-Counter (OTC) Drug Monograph Process”, 2013).

Once the category is selected, phase two begins. Similar to the first phase, the review process begins. This time, reviewers must take the previous panel’s review, public comment, and any new relevant data into account. The OTC Drug Review panel then publishes its findings as a tentative final monograph (TFM) in the *Federal Register*. Once there, it is made public again and is up for review and comment from interested groups (“Over-the-Counter (OTC) Drug Monograph Process”, 2013).

The monograph is finalized in the third phase. Like in the previous phases, the comments, additional data, and anything else relevant brought to light must be considered. The final monograph contains the appropriate active ingredients, labeling guidelines, and dosage suggestions that were verified to safely treat the ailment the OTC drug is targeting. A final monograph is not set in stone. As new data and more safe and effective active ingredients emerge, it may become desirable to amend a monograph. The public can do this by submitting a petition, or it can occur more directly by the Drug Review Commissioner (“Over-the-Counter (OTC) Drug Monograph Process”, 2013).

If an OTC does not fall within the proper monograph, the manufacturer must go through the New Drug Evaluation process. Prescription drugs that are applying to become OTC may require going this route. This process is extremely long and requires FDA observation throughout development. Based on this, it is easier for OTC drugs to be made via the monograph process, which does not require FDA pre-approval before going to market (“OTC, Nonprescription”, 2013).

## FDA Regulations for Dietary Supplements

Phytoestrogens are typically sold as “dietary supplements,” which are not governed as strictly as substances classified as “drugs” by the FDA. Although the guidelines governing the production and composition of dietary supplements are much more lenient, evidence has shown that these supplements can have severe and lasting effects on the human body. The popularity of dietary supplements has skyrocketed over the last few decades, leading to an abundance of controversies regarding their potentially harmful effects. Due to the potential hazards of dietary supplement use, it is vital to test these medications and hold them to higher standards than those required by the FDA.

Today, all dietary supplements distributed in the United States must follow the guidelines described in the FDAs Current Good Manufacturing Practices (CGMPs). This set of rules prevents many potential hazards by requiring both foreign and domestic manufacturers to ensure supplements are free of contaminants and are accurately labeled. As a general rule, any substance marketed as a dietary supplement prior to October 15, 1994 that has been deemed safe by the FDA is allowed in modern supplements. Manufacturers must have a reasonable expectation that new dietary ingredients (anything

marketed as a supplement after October 15, 1994) are safe for consumption, and these ingredients must be reported to the FDA (“New Dietary Ingredients”, 2013). Additionally, any serious adverse reactions to dietary supplements must be reported to the FDA. The final ruling on these CGMPs was enacted on June 22, 2007, making all of these guidelines mandatory by law (“Final Rule”, 2013). A statement from the FDA summarizing CGMPs was then released to the public:

“The final rule aims to ensure that dietary supplements do NOT have wrong ingredients; too much or too little of a dietary ingredient; improper packaging; improper labeling; contamination problems due to natural toxins, bacteria, pesticides, glass, lead, or other substances” (“Final Rule”, 2013).

These guidelines have lead Americans to feel secure in their decisions to buy and consume dietary supplements, but leniencies in the laws have allowed dangerous substances to be marketed as supplements numerous times.

One potentially dangerous discrepancy in CGMPs is that manufacturers are not required to register new products with the FDA prior to production and distribution. Although the manufacturers are expected to follow the FDA’s guidelines, the Dietary Supplement Health and Education Act of 1994 (DSHEA) ruled that dietary supplements are regulated similarly to food rather than drugs, so there is no strict quality control by the government before new products are released. Instead, it is the manufacturers’ responsibility to provide the FDA with some evidence that their products are safe (“Final Rule”, 2013). This increases the chance of hazards resulting from companies that are negligent, dishonest, or unaware of dangers in their products. Dietary supplements are created and sold by many foreign or inexperienced companies that cannot be trusted to ensure the quality, potency, or purity of their products.

Poorly regulated dietary supplements have been the cause of many serious health problems for Americans. The most notable of these situations is the use of ephedra and ephedrine in weight loss supplements and athletic performance enhancers, which gained extreme popularity in the 90s (Beckner, n.d.). Ephedrine, the alkaloid extract from the plant *Ephedra sinica*, is still sought after today by weight loss fad followers, even though there has been no sufficient evidence showing that it is safe or even effective for long-term use. Controversies arose when studies showed that ephedrine use is associated with 2 to 3 fold increases in psychiatric symptoms, autonomic symptoms, upper gastrointestinal symptoms, and heart palpitations. A paper released by the Journal of the American Medical Association in 2003 pointed out 5 deaths, 5 myocardial infarctions, 11 cerebrovascular accidents, 4 seizures, and 8 psychiatric cases that were linked to the use of ephedrine in dietary supplements (“The Need for Regulation of Dietary Supplements”, 2003). Despite these telling studies, ephedrine and similar products are still popularized and desired by many Americans.

Even when dietary ingredients themselves are deemed “safe” by the FDA, other complications can pose health hazards to dietary supplement users. For example, the lack of regulation often leads to incorrect dosage. Additionally, the amount of each supplement listed on the label does not always accurately reflect the amounts in the supplements themselves. Interactions between botanical ingredients and prescription medications can also cause complications for users. Furthermore, irresponsible



manufacturers sometimes neglect to report adverse effects of their products. It is estimated that only about 10% of serious side effects are properly reported to the FDA (Marcus, 2002). Possibly the most concerning aspect of dietary supplement regulation is that pharmaceuticals and heavy metals can evade quality control measures, putting supplement users' health in serious danger. A comprehensive study of patented dietary supplements from Asia sold in California in 1998 produced alarming results. About 32% of these products were found to contain undeclared pharmaceuticals, such as clorpheniramine and phenacetin, or heavy metals like lead, mercury, and arsenic (Marcus, 2002). Although time has passed since the biggest controversies regarding dietary supplements occurred, there are still many loopholes in the regulation processes that endanger the American public.

By executing extensive safety tests on a series of phytoestrogen-based dietary supplements, this study aims to reveal unknown potential health benefits and hazards for those using these substances in their daily lives. The results of these tests, in addition to the information provided about current FDA regulations, could be used to suggest improvements in the government regulation processes for dietary supplements as a whole. A primary focus of this study was observing the effects of phytoestrogens on the T47D-KBluc breast cancer cell line.

## Part 4: Phytoestrogens and T47D-KBluc Cell Line

In this study, three commercial OTCs, black cohosh, red clover, and grape seed extract, were tested for their proliferative and anti-proliferative effects on T47D-KBluc breast cancer cells.

### Black Cohosh

Black cohosh, commonly known as *Cimicifuga racemosa* Nutt, is generally used as an herbal remedy of symptom relief during and after menopause-like flashes and sweats, joint aches, and headaches. Black cohosh is a part of the isoflavonone family and contains the ingredient *isoflavonone formononetin*.

*In vitro* studies have reported black cohosh to possess inhibitory effects on proliferation of estrogen responsive breast cancer cells. Gaube *et al.* performed experiments using black cohosh extract in dichloromethane (Gaube *et al.*, 2007). The extract was applied to MCF-7 breast cancer cells for 72 hours at which time the media was changed and the cells were allowed to grow for another 120 hours. The MTT assay conducted revealed that black cohosh treated cells responded conversely to treatment with  $\beta$ -estradiol, showing no cell proliferation. Additionally, the extract caused downregulation of the ER $\alpha$  mRNA which can result in antitumor activity (Gaube *et al.*, 2007).

A second study performed by Al-Akoum *et al.* demonstrated the anti-proliferative effects of black cohosh extract on MCF-7 cells (Al-Akoum *et al.*, 2007). Al-Akoum *et al.* also observed the effects of the ER antagonist tamoxifen which binds to the receptor and blocks the active site. Adding various concentrations of tamoxifen to  $\beta$ -estradiol treated cells inhibited the proliferative effects seen without the antagonist. Additionally, when added to black cohosh, tamoxifen contributed further to the inhibition of breast cancer cell growth. Black cohosh was also added to  $\beta$ -estradiol treated cells resulting in a dose-dependent inhibition of the  $\beta$ -estradiol's proliferative effects (Al-Akoum *et al.*, 2007).

There are contradicting findings by Ji *et al.* and Halabalaki *et al.* These studies used formononetin

extracted from black cohosh and purified. Both found that formononetin is estrogenic in a concentration dependent manner from 0.5 to 500  $\mu\text{M}$  on an estrogen responsive MCF-7 line (Ji *et al.*, 2005; Halabalaki *et al.*, 2006).

Although there were conflicting studies, the results of Al-Akoum *et al.* and Gaube *et al.* led to the decision to use black cohosh as a negative control.

### Grape Seed

Grape seed is a nutraceutical that belongs to the bioflavonoid family. It is composed of chains of procyanidins that are found in a wide variety of beverages and foods such as fruits and cereal grains.

A study conducted using grape seed focused on the effect of bioflavonoid on the highly metastatic MDA-MB231 breast cancer cell line, concentrating on cell invasion and migration. It was observed that high concentrations of flavonoid inhibited cell proliferation and apoptosis, while low concentrations of flavonoid decreased migration and invasion (Dinicola *et al.*, 2013). Another study observed the effect of grape seed on MCF-7 breast cancer cell proliferation and expression of the gene *survivin*. It also investigated the molecular, biological mechanism of inhibition by grape seed. The results revealed that grape seed inhibits the proliferation of the MCF-7 cells through arresting the cell cycle in S phase (Chen *et al.*, 2009).

From these studies, the effects on breast cancer activity caused by grape seed extract are concentration dependent. The effects of this extract on proliferation were varied; therefore, black cohosh was a more logical choice for a negative control.

### Red Clover

Red clover is a member of leguminosae family and is also known as *Trifolium pretense*. Red clover contains high levels of all four major forms of isoflavonoids – formononetin, biochanin, daidzein, and genistein. Of these, the most active components in red clover are biochanin and genistein (Albulescu, 2006).

Historically, red clover has been used by post-menopausal women for various reasons including hot flashes, night sweats, reduced libido, vaginal dryness, cyclic breast pain or tenderness, cancer prevention, indigestion, whooping cough, cough, asthma, bronchitis, and maintaining bone density.

A study conducted by Reiter *et al.* isolated isoflavonoids from red clover and observed their effects on eleven types of cancer cells, including breast cancer. Their findings showed that these isoflavonoids, primarily genestein, promoted cell cycle arrest and apoptosis (Reiter *et al.*, 2011). One study on red clover showed that women aged 49 - 65 taking 26 mg biochanin A, 16 mg formononetin, 1 mg genistein, and 0.5 mg daidzein daily for one year did not increase their mammographic breast density (Atkinson *et al.*, 2004). Another study on MCF-7 cells revealed increased proliferation in response to the biochanin A component of red clover at concentrations less than 0.1  $\mu\text{M}$ . However, the same cell line proliferation was inhibited when the concentration was increased to 20  $\mu\text{M}$  (Wang, 1995). Finally, a study showed that genistein activated an estrogen response element in Ishikawa cells and biochanin A induced luciferase in MCF-7 cells (Overk *et al.*, 2005).

These studies taken together show that red clover is also dose dependent at inducing breast cancer cell proliferation. The dose dependent response to red clover contributed to the choice of black cohosh as the negative control.

### T47D-KBluc Cell line

The T47D-KBluc cell line from American Type Culture Collection is used in these experiments (T47D-KBluc, 2013). T47D cells are from the ductal carcinoma of mammary gland, breast/duct derived from pleural effusion of a 54 year old female. This cell line is used to screen chemicals for estrogenic or anti-estrogenic activities as they are reported to have an affinity for ER $\alpha$  and ER $\beta$ . Various studies established that the cell line is highly responsive to estrogen, which is important because it allows research of phytoestrogenic responses as well (Wilson *et al.*, 2004).

T47D-KBluc is the fluorescent variation of the T47D cell line. It was developed by transfecting T47D cells that contain both ER $\alpha$  and ER $\beta$  with estrogen response element, the luciferase reporter gene construct (Wilson *et al.*, 2004).

### Conclusion

The effects of menopause on a woman's body due to hormone fluctuations are detrimental, and efforts to minimize these side effects are progressing. Replacing HRT with phytoestrogen treatment remains in the preliminary steps. Further research and development of phytoestrogens needs to be completed in order to classify them as a safe and effective treatment for menopause symptoms. One of the most detrimental side effects of HRT is the increased risk of breast cancer, which needs to be taken into account when dealing with phytochemicals that respond through the same receptors. There are very few conclusive studies on phytoestrogens' proliferative effects on breast cancer cells and many such studies contradict each other. The experiments described in this paper will shed light on the effects of three different over the counter phytoestrogen pills sold currently: black cohosh, red clover, and grape seed. Extracts of these supplements containing phytoestrogens will be tested for proliferative effects on breast cancer cells, then certain chemicals within the pill will be analyzed.

## Methods

### Extraction of Supplements

Based on the processes outlined by Setchell *et al.* (2001), the supplements were extracted from the capsules they were commercially available in. The purpose of this process was to obtain liquid samples of each phytoestrogen that could be used in the High Performance Liquid Chromatography (HPLC) process and administered onto cells.

#### Black Cohosh

One 540 mg capsule of Nature's Way Black Cohosh was opened and the contents were added to 80 mL of 80% volume per volume methanol in an Erlenmeyer flask and mixed. The solution was then transferred to a round bottom flask and attached to a water-jacketed reflux condenser. The solution was refluxed for approximately 60 minutes at a boiling point of 65°C, which is the boiling point of methanol. After the reflux, the sample was filtered using a Drummond 0.8 µL filter and stored at -20°C in two 50 mL tubes in the dark.

#### Red Clover

The same procedure as above was followed, however using one 400 mg capsule of Nature's Way Red Clover.

#### Grape seed Extract

The same procedure as above was followed, however using one 300 mg capsule of Nature's Way Grape seed Extract.

### High Performance Liquid Chromatography

HPLC was performed according to the procedures outlined in Setchell *et al.* (2001) and Jessica Caron's Thesis (2007) in order to determine the contents of each phytoestrogen supplement. 10 µL of grape seed extract, as well as the phytoestrogen standards genistein, diadzein, and resveratrol, were injected at a rate of 1 mL/min onto a reverse phase C18 250 X 4.6 mm column, while black cohosh and red clover were increased to 50 µL and 100 µL respectively due to initial lack of sufficient signal. The standards were at a concentration of 100 µM. Following this injection; the column was washed with a solution containing 10 mM ammonium acetate and 0.1% trifluoroacetic acid (TFA) for two minutes. For the subsequent 22 minutes, the column was eluted with a linear gradient of the ammonium acetate TFA solution and acetonitrile, during which time the ammonium acetate and TFA ran between 100% - 50% and acetonitrile ran between 0% - 50%. The column was then held isocratic for the remaining five minutes of the run, followed by 100% 10 mM ammonium acetate, 0.1% TFA for a six minute re-equilibration. The absorbances of the samples at 260 nm were recorded and generated in a chromatogram.

The chromatograms show peaks for the times at which different compounds were eluted, and the absorbances (mAU) of the compounds. The chromatograms of red clover, black cohosh and grape seed extracts were observed and compared to the chromatograms of phytoestrogen standards. In order to

identify whether the extracts contained phytoestrogen standards, the extracts were spiked with each standard. Since the extracts did not contain any of the phytoestrogen standards, then the most prominent peaks were collected. Prominent peaks were identified as those with the largest percentage of the total area. Peak collection started at the start of the peak of interest, and stopped 15 seconds after the end of the peak.

## Serial Dilutions

After extraction, the stock solutions of black cohosh, grape seed, and red cohosh were diluted for concentration dependent testing later. Five 10-fold dilutions were completed using the stock solution and 95% methanol. Five 1.5 mL eppendorf tubes were labeled with the extract name and dilution number and 900  $\mu$ L of 95% methanol were added to each. 100  $\mu$ L of the stock solution is added to the first dilution tube raising the volume up to 1 mL with 1 part stock solution and 9 parts methanol. After mixing thoroughly, 100  $\mu$ L of the first dilution was added to the next tube containing 900  $\mu$ L of methanol making the second 10-fold dilution. This is done three more times until the fifth dilution was made and completed for the three extracts,  $\beta$ -estradiol, black cohosh peak, grape seed peak, and red cohosh peaks 1, 2, 3, and 4. The dilution is depicted in Table 1 below.

<b>Black Cohosh D1</b>	<b>Black Cohosh D2</b>	<b>Black Cohosh D3</b>	<b>Black Cohosh D4</b>	<b>Black Cohosh D5</b>
100 $\mu$ L BC Stock + 900 $\mu$ L Methanol	100 $\mu$ L BC D1 + 900 $\mu$ L Methanol	100 $\mu$ L BC D2 + 900 $\mu$ L Methanol	100 $\mu$ L BC D3 + 900 $\mu$ L Methanol	100 $\mu$ L BC D4 + 900 $\mu$ L Methanol

*Table 1: Black Cohosh 10-fold Dilution with 95% Methanol*

## MTT Assay

A CellTiter 96 well flat bottom plate was used for the MTT assay. Each well received  $10^4$  cells plated in 100  $\mu$ L DMEM with 10% FBS, 1% glutamine, and 1% Penicillin Streptomycin (Pen/Strep). The cells were allowed to attach to the plate for 24 hours at which time the media was aspirated and replaced with 100 $\mu$ L Phenol Red Free DMEM with 5% dextran-charcoal treated FBS (PHRED). The cells were allowed to equilibrate in PHRED for 24 hours before chemicals were applied.

Five 10-fold serial dilutions of  $\beta$ -estradiol, grape seed extract, black cohosh, and red clover stock solutions were made in 95% methanol. The layout of the 96 well plate is depicted in Table 2. Methanol was used as a control since it was the solvent used to make the dilutions of  $\beta$ -estradiol and the phytoestrogen samples. A media alone control (blank) was also run to observe the baseline cell growth. After the 24 hour equilibration, the PHRED was aspirated and replaced with fresh PHRED along with 1  $\mu$ L of each sample, according to Table 2, and incubated for another 24 hours.

<b>Control Blank</b>	<b>Control Methanol</b>	<b>b-estradiol</b>	<b>Grape Seed</b>	<b>Black Cohosh</b>	<b>Red Clover</b>
<b>100µL media +</b>	<b>100µL media +</b>	<b>100µL media +</b>	<b>100µL media +</b>	<b>100µL media +</b>	<b>100µL media +</b>
0µL Stock	0µL Stock	1µL Stock	1µL Stock	1µL Stock	1µL Stock
1µL Stock	1µL Stock	1µL D1	1µL D1	1µL D1	1µL D1
		1µL D2	1µL D2	1µL D2	1µL D2
		1µL D3	1µL D3	1µL D3	1µL D3
		1µL D4	1µL D4	1µL D4	1µL D4
		1µL D5	1µL D5	1µL D5	1µL D5

*Table 2: The layout for the 96 well plate used for the MTT assay*

Once the cells were exposed to each supplement for 24 hours, 20 µL of CellTiter 96 Aqueous One Solution Reagent (Promega) was added to each well followed by incubation for another 2 - 4 hours. At this time, the 96 well plate was placed in a spectrophotometric plate reader and the absorbance for each well was read at 570 nm. The absorbances from wells containing no cells were averaged and subtracted from each well reading to normalize the numbers.

Additionally, the MTT assay was run with the same controls and extracts used in Table 2, but with the addition of the peaks for each extract. Five 10-fold dilutions were made for the black cohosh peak, grape seed peak, and red clover peaks 1, 2, 3, and 4. Alternatively, the assay including the peaks was completed with a second brand of MTT reagent, TACS MTT Cell Proliferation Assays (Trevigen). After the 24 hours with each supplement, 10 µL of TACS reagent was added to each well and incubated for 2 - 4 hours. At this time, 100 µL of Detergent Reagent was added to each well and the plate was again incubated for 2 - 4 hours. The absorbances were read using the same plate reader at 570 nm.

## Results and Discussion

### HPLC Results

The purpose of this project was to analyze the components of the three over the counter phytoestrogen supplements: red clover, grape seed, and black cohosh. In order to gain a better understanding about their composition, the three extracts were separated by HPLC according to the HPLC procedure in the Methods Section. After the HPLC was completed, a chromatogram was generated, and used to analyze the data. The x-axis on the chromatogram depicts the time in minutes that it took to separate the components of the sample. The y-axis shows the mass absorbance unit (mAU), which represents the height of the peaks. In addition, the chromatogram analyzes the width and height of the peak, the area beneath the peak, and the percent of the total area that each peak takes up. These measurements, found in Table 3, were used in order to determine the major peaks, which represent the main components of the extracts.

Sample	Peaks	Time (min)	Height (mAU)	Width (min)	Total Area (mAU*s)	% Total Area
Black Cohosh	11	16.8	45.37	0.46	1711.52	35.00
Grape Seed	1	3.6	2454.15	0.40	72231.50	89.56
Red Clover	1-2	3.26-3.71	181.17	0.40	5138.70	21.03
Red Clover	13-19	15.56-18.31	305.10	0.84	2752.94	11.27
Red Clover	28-29	21.56-22.14	241.47	0.55	5150.69	21.09
Red Clover	35	27.6	226.74	0.32	5567.18	22.79

Table 3: Chromatogram Peak Measurements

Figure 1 below depicts a chromatogram showing the HPLC of black cohosh extract. Based on the measurements shown in Table 3, the most prominent peak is Peak 11 at 16.80 minutes. This peak is the largest and comprises 35% of the total area.

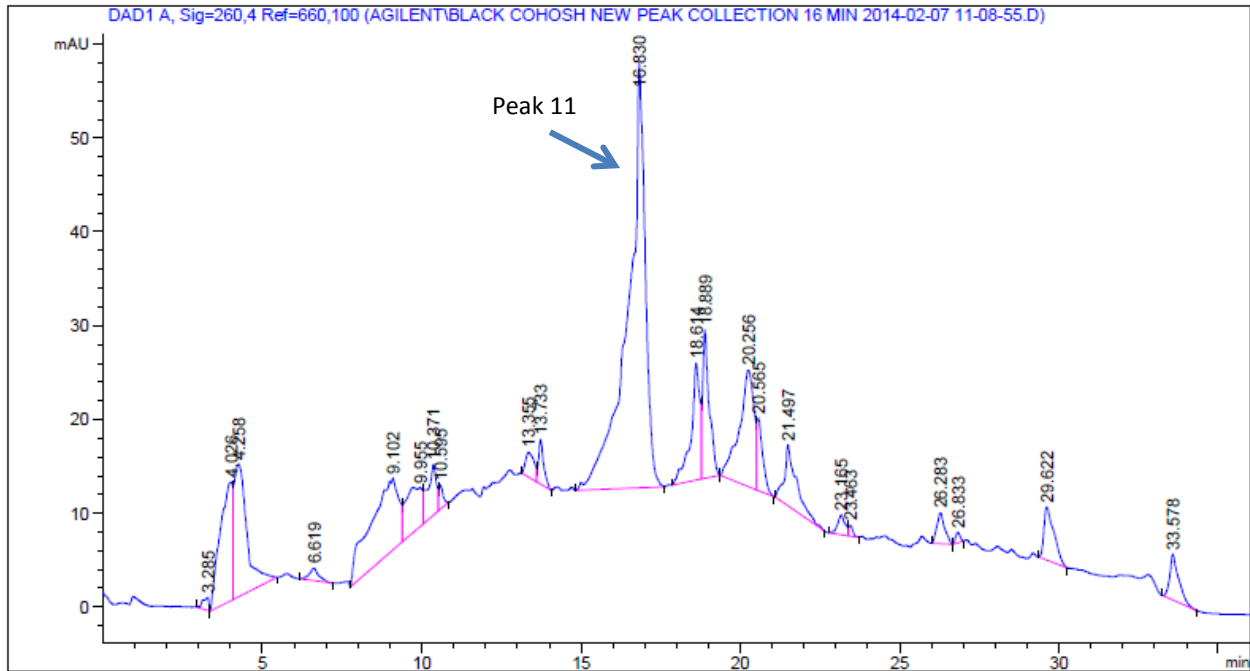


Figure 1: Chromatogram of Black Cohosh Extract

Figure 2 below depicts a chromatogram showing the HPLC of grape seed extract. Based on the measurements shown in Table 3, the most prominent peak is Peak 1 at 3.60 minutes. This peak is the largest and comprises 89.56% of the total area.

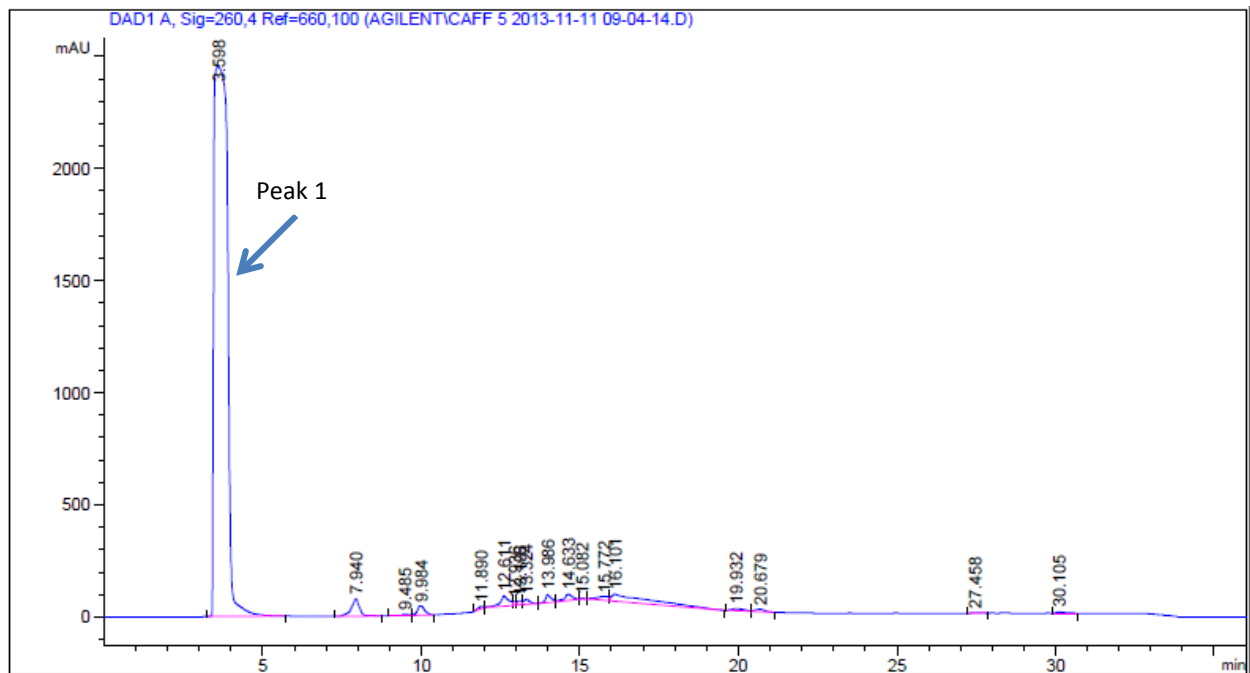


Figure 2: Chromatogram of Grape Seed Extract



Figure 3 below depicts a chromatogram showing the HPLC of red clover extract. According to the measurements shown in Table 3, the most prominent peaks are Peaks 1 and 2 at 3.26 to 3.71 minutes, Peaks 13 through 19 at 15.56 to 18.31 minutes, Peaks 28 and 29 at 21.56 through 22.14 minutes and Peak 35 at 27.60 minutes. The combination of Peak 1 and 2 takes up 21.03% of the total area. The combination of Peaks 13 through 19 comprises 11.27% of the total area. The combination of Peaks 28 and 29 comprise 21.09% of the total area. Finally peak 35 comprises 22.79% of the total area, making it the largest peak.

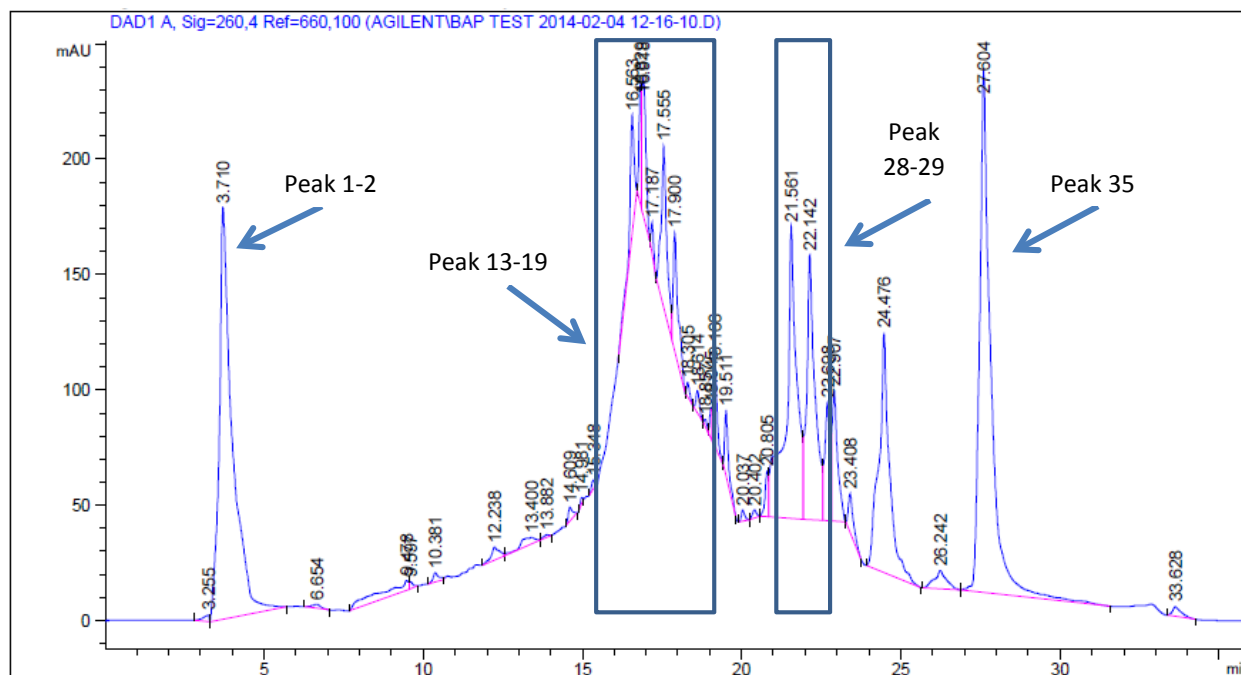


Figure 3: Chromatogram of Red Clover Extract

In order to compare the prominent peaks with known phytoestrogen samples, known standards were run. These standards were daidzein, resveratrol, and genistein and can be found in Figures 4, 5, and 6 respectively.

Figure 4 below shows the standard daidzein. The only peak in the daidzein chromatogram occurred at 19.903 minutes. This peak did not correspond to the prominent peaks determined for any of the extracts, meaning that daidzein is either not present in these extracts, or if it is present, it does not occur in large amounts. For example, in the grape seed extract the most prominent peak is at 3.598 minutes, but there is a small peak observed at 19.932 minutes which only amounts to 0.3247% of the total area. This peak could be daidzein, however it is present in very small amounts compared to the other compounds.

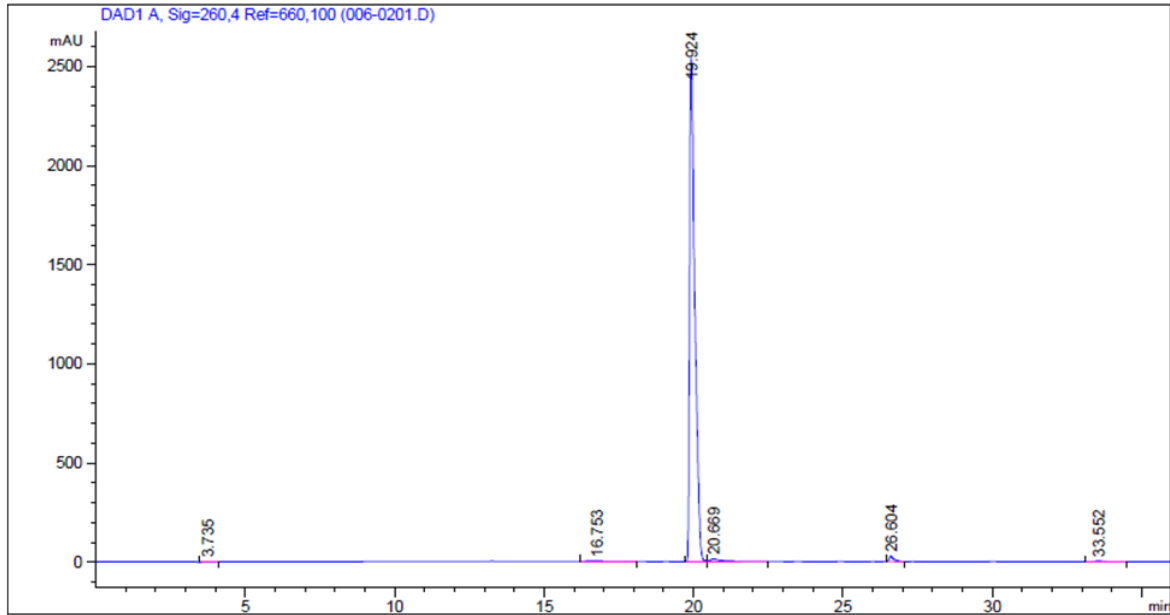


Figure 4: Chromatogram of Daidzein

Figure 5 below shows the HPLC data for the standard resveratrol. Based on this HPLC, resveratrol contains four overlapping peaks that occurred in the short span between 18.819 minutes and 19.111 minutes. This collection of peaks did not correspond to the prominent peaks determined for any of the extracts, meaning that resveratrol is either absent from these extracts, or if it is present, it does not occur in large amounts. For example, in the red clover extract, the most prominent peaks are from 15.563 to 18.305 minutes and 21.561 through 22.142 minutes, but there are small peaks observed at 18.857 and at 19.045 minutes which only amount to 0.0793% and 0.2766% of the total area respectively. These peaks overlap with part of the resveratrol peak and therefore could be resveratrol; however, it would be present in very small amounts.

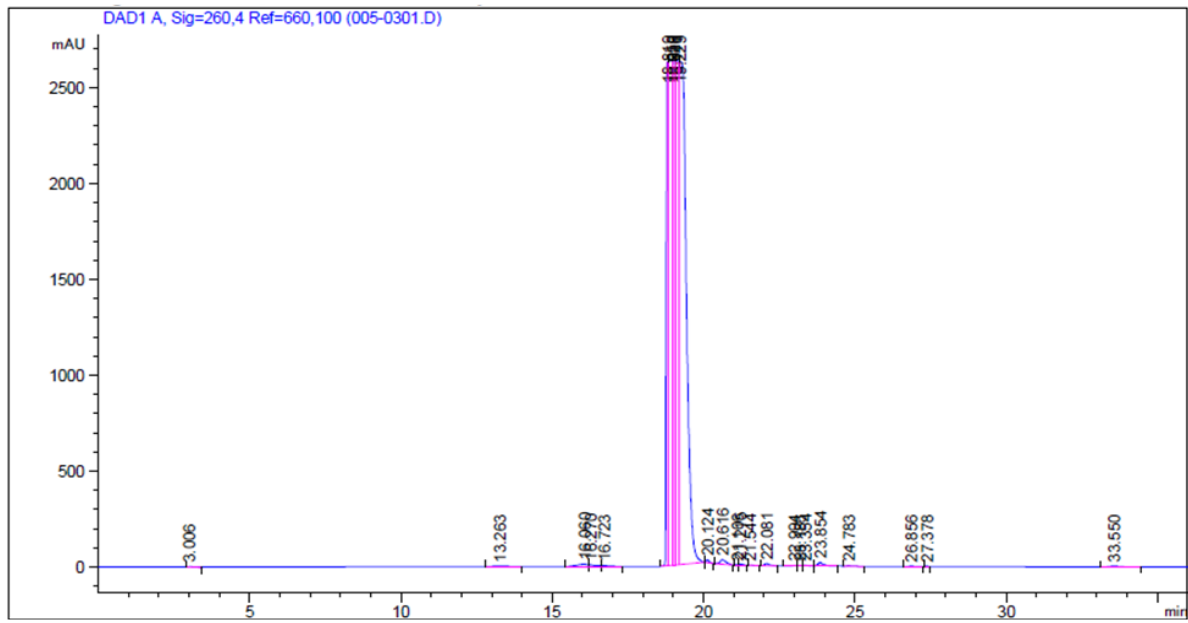


Figure 5: Chromatogram of Resveratrol

Figure 6 below shows the HPLC data for the standard genistein. The only peak in the genistein chromatogram occurred at 22.633 minutes. This peak did not correspond to the prominent peaks determined for any of the extracts, meaning that genistein is not visibly present in any of the extracts.

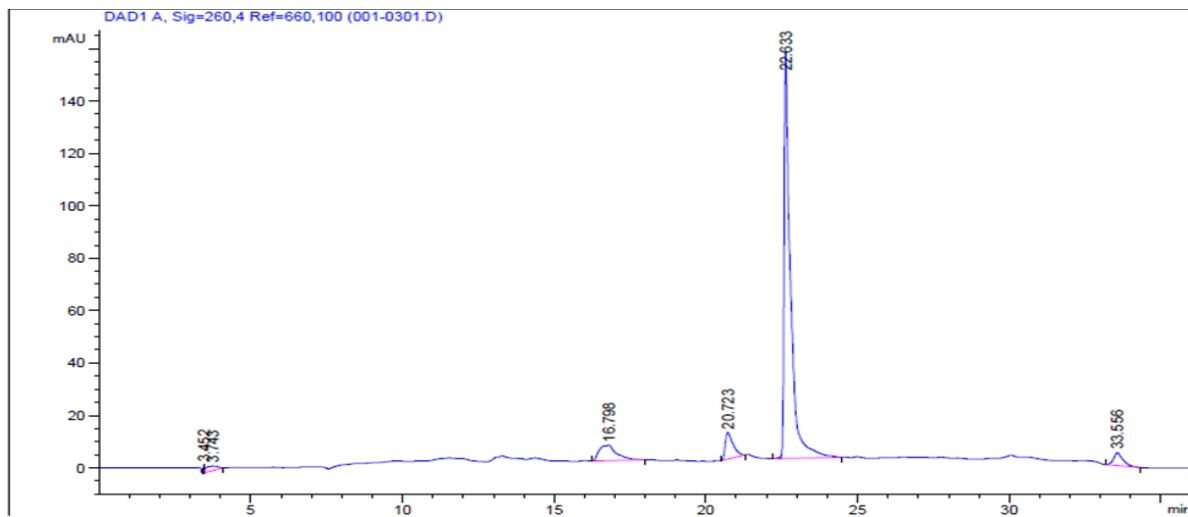


Figure 6: Chromatogram of Genistein

The absence of peaks in the red clover extract HPLC corresponding to genistein and daidzein is inconsistent with the findings in the background research (Albulescu *et al.*, 2006; Reiter *et al.*, 2011). Albulescu *et al.* reports isolating daidzein and genistein from purified red clover extract, but did not provide HPLC data for comparison purposes. Reiter *et al.* reported the isolation of isoflavonoids, primarily genistein, from red clover without including HPLC data either. The previous research conducted on black cohosh and grape seed components did not reveal the presence of the three

standards tested (Chen, 2009). Therefore, the absence of peaks during these experiments was as expected. Overall, the absence of daidzein, genistein, and resveratrol in the samples only rules out the lack of three known phytoestrogens, but others may be present.

Although the phytoestrogen supplements did not contain the known phytoestrogen standards in large amounts, the prominent peaks determined were collected for each supplement to test the effects of each compound present on the breast cancer line T47D-KBluc. Most of the smaller peaks in the chromatograms were not collected because the large peaks were focused on. It is possible that the compounds represented by the smaller peaks have greater effect on the cells. In the case of resveratrol, four overlapping peaks were observed in Figure 5 as one large peak. However, these peaks could be analyzed separately by using alternative solvents, slowing the flow rate, and using a different column. Similarly, red clover had many overlapping peaks making the collection process difficult. Further resolution of these peaks is necessary to see the individual effects of the overlapping peaks on cell proliferation.

Table 4 below shows the comparison between the peaks found in at least two extracts, which eluted less than 0.05 minutes apart. Black cohosh and red clover had the most compounds in common. The size and shapes of the compound peaks were also similar. These peaks should be analyzed in future experiments.

<b>Black Cohosh</b>	3.29	6.62		9.96	10.37	13.36		16.83	18.61	18.89	26.28
<b>Red Clover</b>	3.26	6.65	9.48		10.38		14.61	16.8	18.61	18.85	26.28
<b>Grape Seed</b>			9.49	9.98		13.32	14.63				

Table 4: Compound comparison among the extracts

## MTT Assays

An MTT assay is used to determine cell proliferation in the presence of phytoestrogen extracts at various concentrations. The peaks collected from HPLC were also tested on the cells to observe effects on proliferation. In this section, the extract and peak effects on proliferation will be compared to the proliferative effects of  $\beta$ -estradiol, the positive control. After the assay was completed, the plates were read using a spectrophotometer at 570 nm and these results were graphed versus the proliferation of untreated cells (blank). Each data point was graphed based on the stock concentration then three or four subsequent tenfold dilutions.

## CellTiter 96

The first MTT assay performed used the CellTiter 96 method (see MTT Assay Methods) for  $\beta$ -estradiol, black cohosh extract, grape seed extract, and red clover extract.

$\beta$ -estradiol was used as a positive control for this experiment because it is known to cause proliferation of breast cancer cells. The maximum concentration of  $\beta$ -estradiol used in this experiment was 0.1 nM,

which is directly comparable to the estrogen concentration of Wilson *et al.* The stock solution of  $\beta$ -estradiol in this assay had an absorbance reading of 0.715, which was the highest proliferation detected during this assay and can be seen below in Figure 7. When comparing the results shown here to Wilson *et al.*, the same proliferative effects were observed. For both assays, the proliferation stays at baseline from concentrations  $1 \times 10^{-5}$  nM to  $1 \times 10^{-3}$  nM and began its ascent at  $1 \times 10^{-3}$  nM (Wilson *et al.*, 2004). This similarity validates the CellTiter 96 assay results presented here.

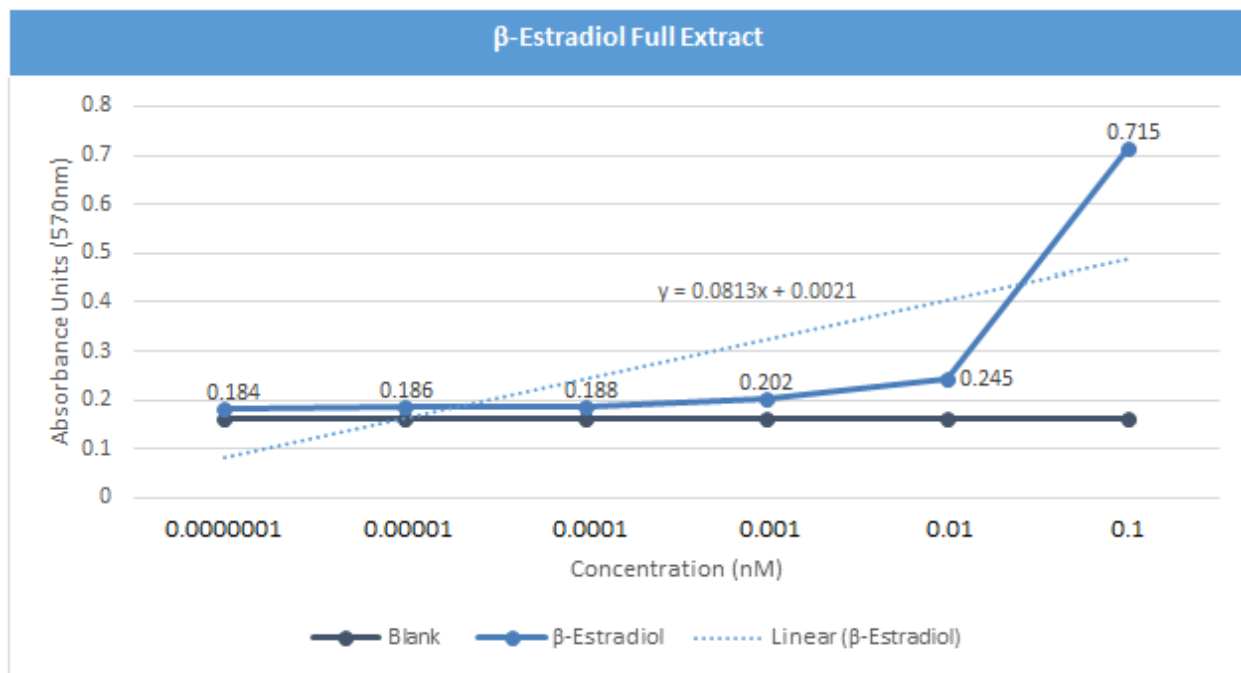


Figure 7: CellTiter 96 MTT Assay Reading of  $\beta$ -estradiol Extract at 570nm

The results for proliferation of breast cancer cells treated with black cohosh extract consistently stayed near baseline. The linear trendline of black cohosh, seen in Figure 8, extract indicates little difference in growth of treated cells compared to those not treated. Black cohosh was used as a negative control due to research performed by Al-Akroum *et al.* and Gaube *et al.* (Al-Akroum *et al.*, 2007; Gaube *et al.*, 2007). Through these studies, it was concluded that black cohosh has antiproliferative effects. The negligible deviation from baseline in comparison to the substantial growth of the positive control,  $\beta$ -estradiol, supports these findings. Although the results were similar, the extraction procedures and exposure time differed between experiments. Gaube *et al.* exposed the breast cancer cells to black cohosh extract in dichloromethane for 72 hours then allowed growth for an additional 120 hours, whereas this experiment exposed the cells to black cohosh extract in methanol for 24 hours. These discrepancies, in addition to the unknown concentrations of the components within the supplements, need to be taken into account.

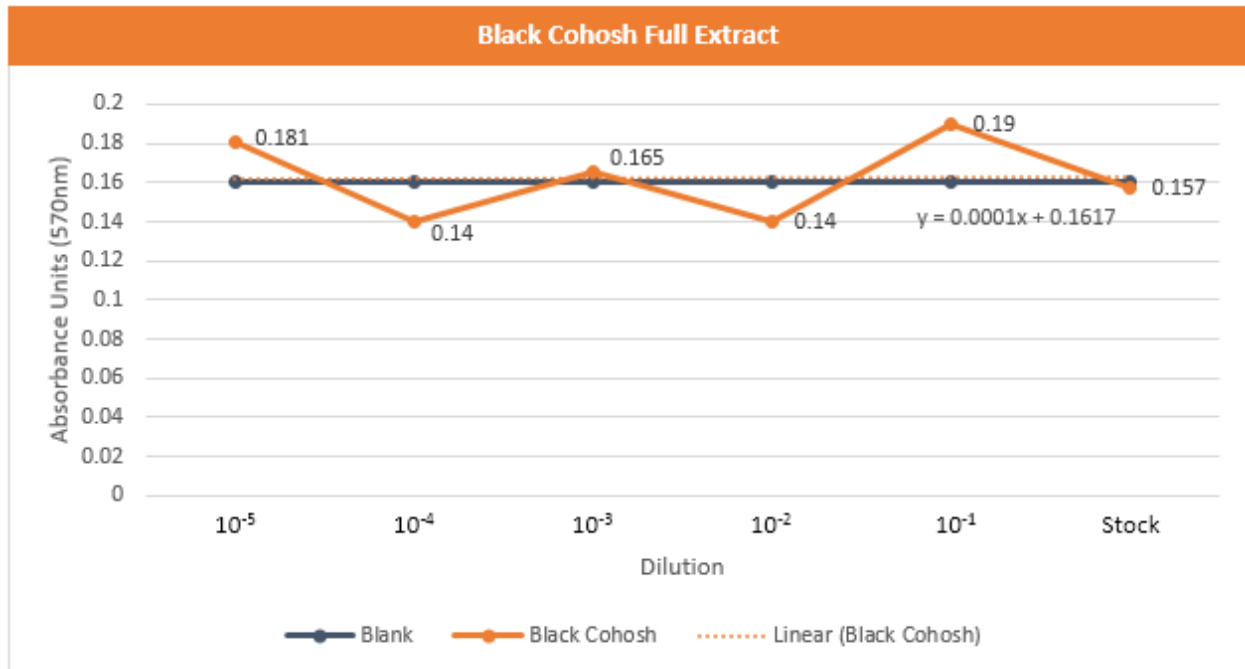


Figure 8: CellTiter 96 MTT Assay Reading of Black Cohosh Extract at 570nm

MTT assay results for the grape seed extract had a similar pattern to  $\beta$ -estradiol, depicted below in Figure 9. The stock solution eluted a high proliferation rate and was read at 570nm, revealing an absorbance of 0.608. The reading remained close to baseline until cell proliferation began at one hundredth of the stock solution concentration. The high proliferation rates seen in this experiment oppose the conclusions drawn from the study of Chen *et al.* (Chen *et al.*, 2009). The concentrations used in that study included increments from 40 to 200 mg/L and resulted in the inhibition of the proliferation of MCF-7 breast cancer cells. Their study suggested that grape seed extract had an overall antiproliferative effect. The results in this MTT assay are contradictory to those findings.

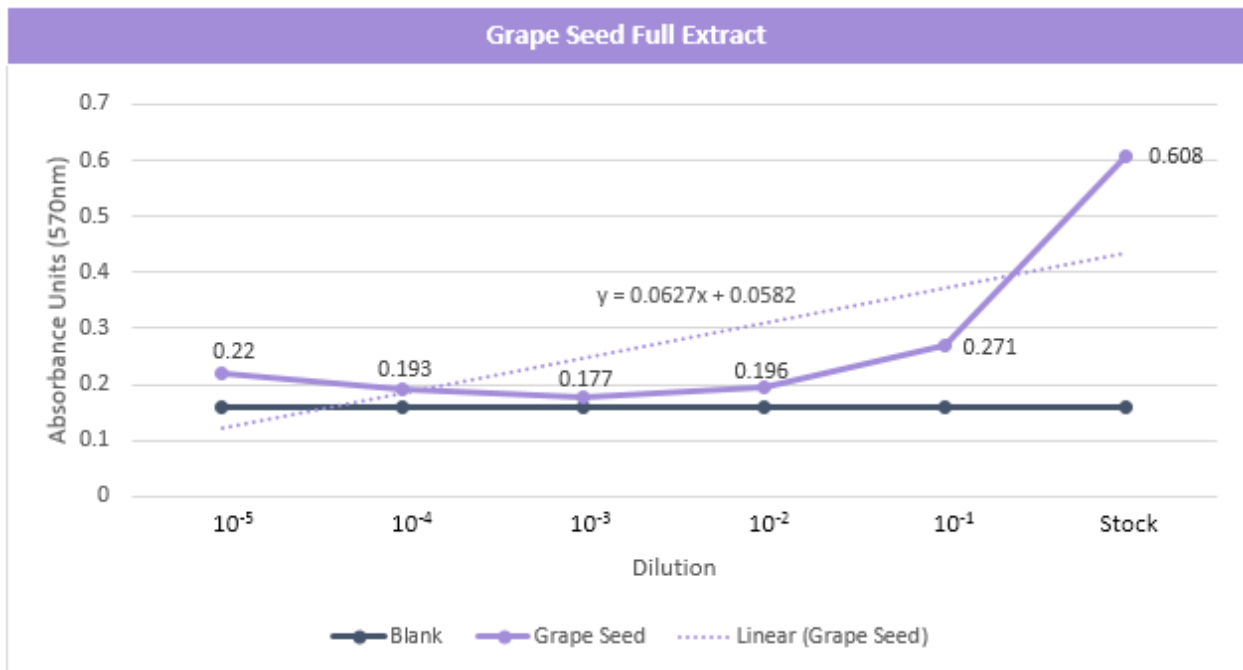


Figure 9: CellTiter 96 MTT Assay Reading of Grape Seed Extract at 570nm

Red clover-induced proliferation remained close to the baseline throughout the assay, showing that there are no proliferative effects, as shown in Figure 10. Reiter *et al.* studied the effects of isoflavonoids such as genestein on cancer cells. They tested growth promotion, cell cycle arrest and apoptosis. Their findings suggested that the isoflavonoids from red clover inhibited cell proliferation. Although they cannot directly be compared due to the use of full red clover extract in this experiment rather than the isoflavonoids isolated from the red clover, the results from this MTT assay are consistent with the findings of Reiter *et al.* (Reiter *et al.*, 2011). Similar to the other extracts, the concentration of the components of red clover were unknown causing difficulties when analyzing dose dependent effects.

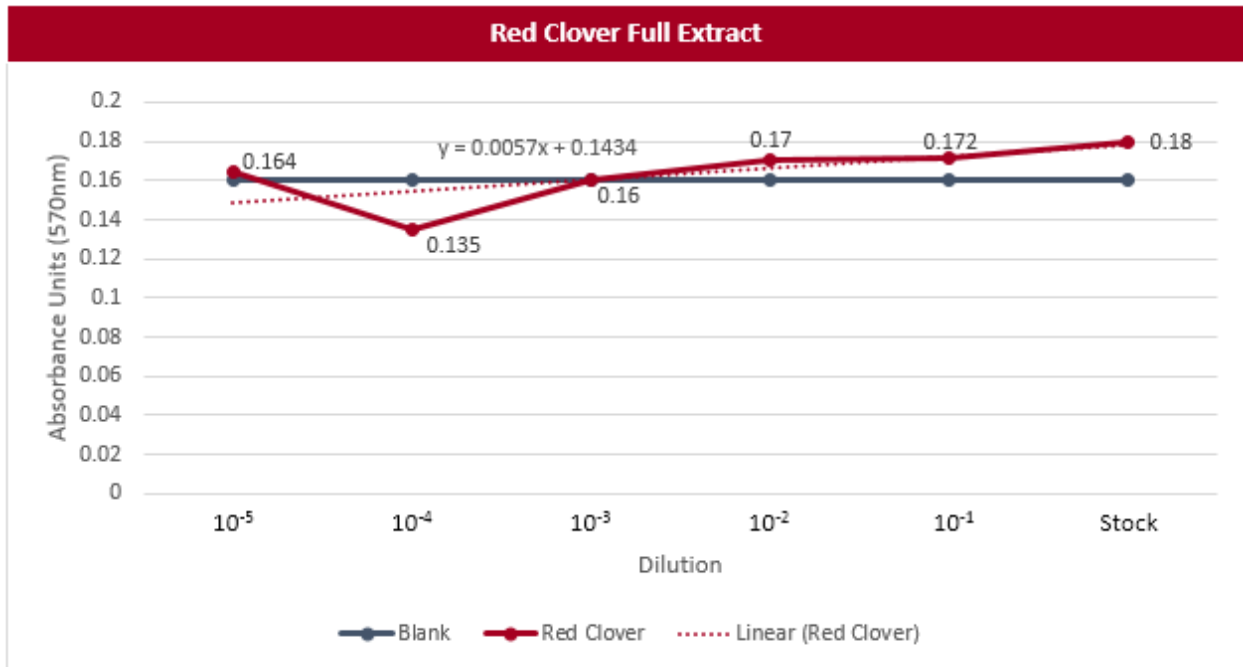


Figure 10: CellTiter 96 MTT Assay Reading of Red Clover Extract at 570nm

### TACS MTT

The TACS MTT assay was used to analyze the proliferative effects of each of the extracts and the peaks collected from them. These results are summarized below in Figures 11 through 16. The results using this assay were unreliable because neither the positive control of  $\beta$ -estradiol nor the negative control black cohosh were consistent with known results. For instance, the proliferation of cells treated with  $\beta$ -estradiol using the TACS MTT assay showed an opposite trend to the work done by Wilson (Wilson *et al.*, 2004).

Figure 11 below depicts the MTT assay results of the positive control  $\beta$ -estradiol in comparison to the blank. The linear trendline of the data shows that with increased concentration of  $\beta$ -estradiol, there was a decrease in proliferation. This pattern of proliferation does not coincide with what was expected. The research conducted by Wilson *et al.* showed an increase in proliferation when cells were treated with 0.1 nM of  $\beta$ -estradiol. The results of this assay show that at 0.1 nM of  $\beta$ -estradiol, the cells had the lowest amount of proliferation, while the lowest concentration produced the highest proliferation. According to the findings of Wilson *et al.*, as well as the findings from the CellTiter 96 MTT assay, the highest concentration of  $\beta$ -estradiol should contribute to the highest proliferation of T47D cells (Wilson *et al.*, 2004).



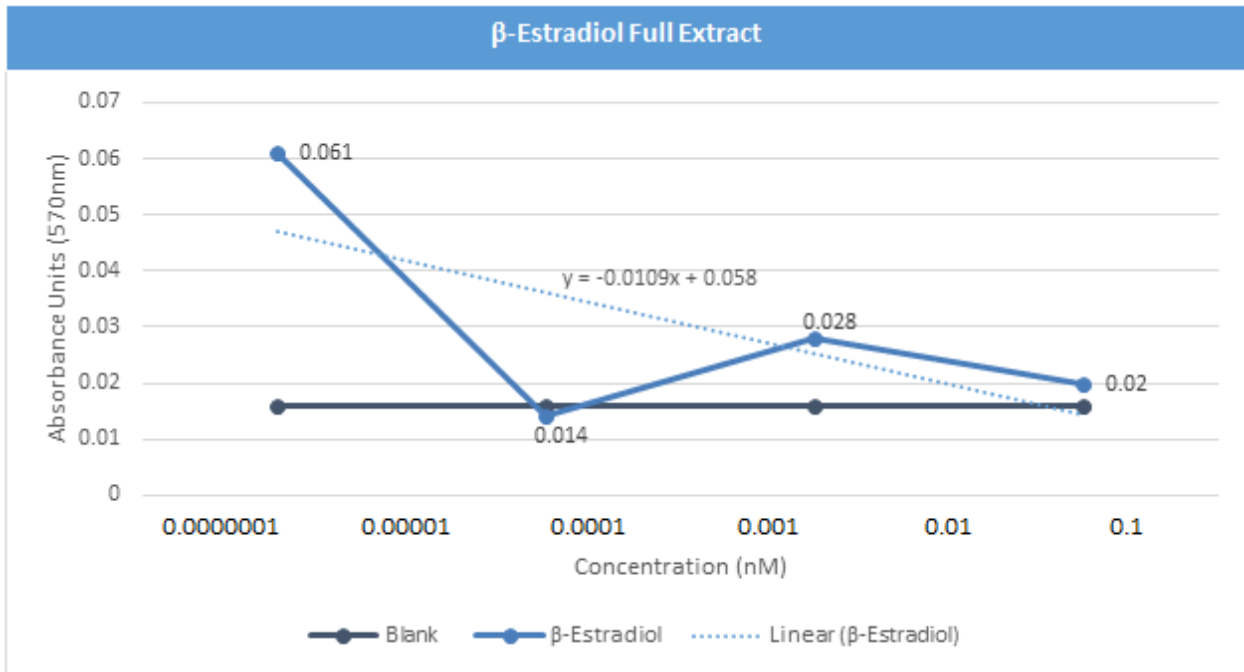


Figure 11: TACS MTT Assay Reading of  $\beta$ -Estradiol Extract at 570nm

As depicted in Figure 12, the black cohosh treated cells showed a slight increase in proliferation over the baseline growth. This increase in proliferation is not consistent with the findings of Al-Akoum *et al.* and Gaube *et al.* which both found black cohosh to cause antiproliferative effects (Al-Akoum *et al.*, 2007; Gaube *et al.*, 2007). However, in comparison to  $\beta$ -estradiol, the black cohosh proliferation rates were much lower shown by the slope of the linear trendline.

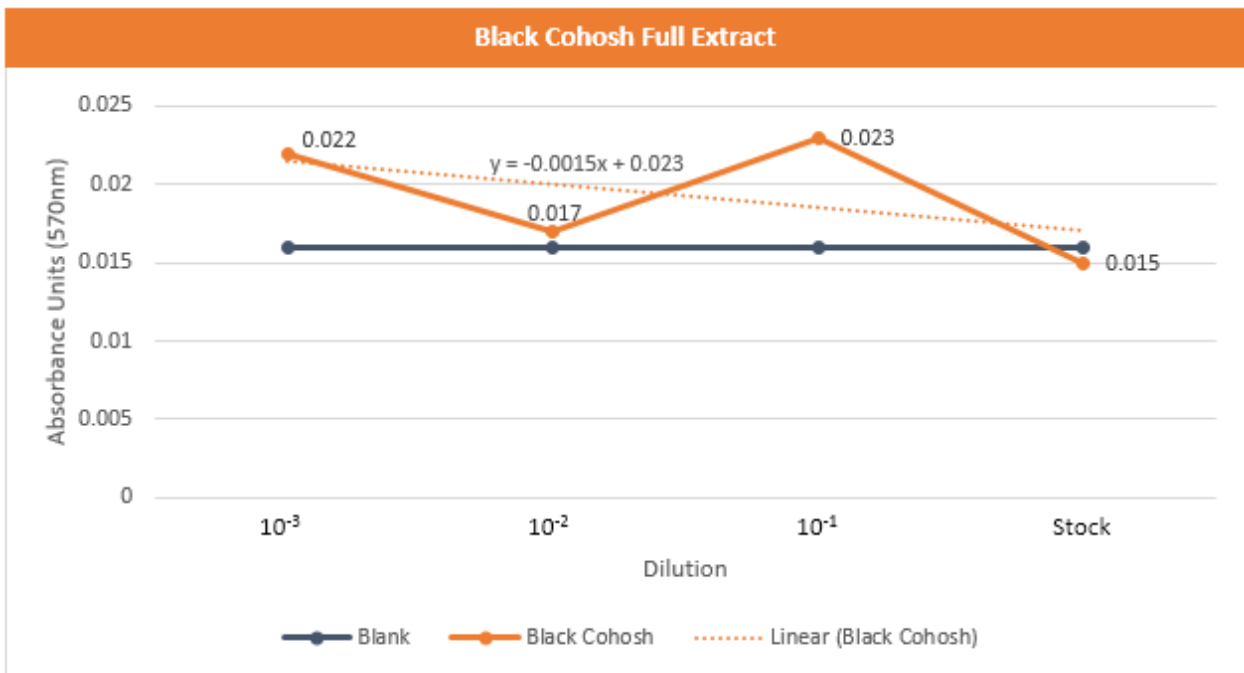


Figure 12: TACS MTT Assay Reading of Black Cohosh Extract at 570nm

Through the conflicting results of black cohosh and  $\beta$ -estradiol, the validity of the TACS MTT assay was called into question. For this reason, the results of grape seed extract and red clover extract are graphed together in Figure 13 below. At higher concentrations, the grape seed extract shows high cell proliferation (0.06) when compared to the positive control (0.09). As the grape seed extract was diluted, its impact on cell growth decreased. The stock solution of red clover extract matched baseline proliferation levels. The proliferation rate increased at one hundredth of the stock concentration. The red clover extract resulted in greater proliferation than the positive control.

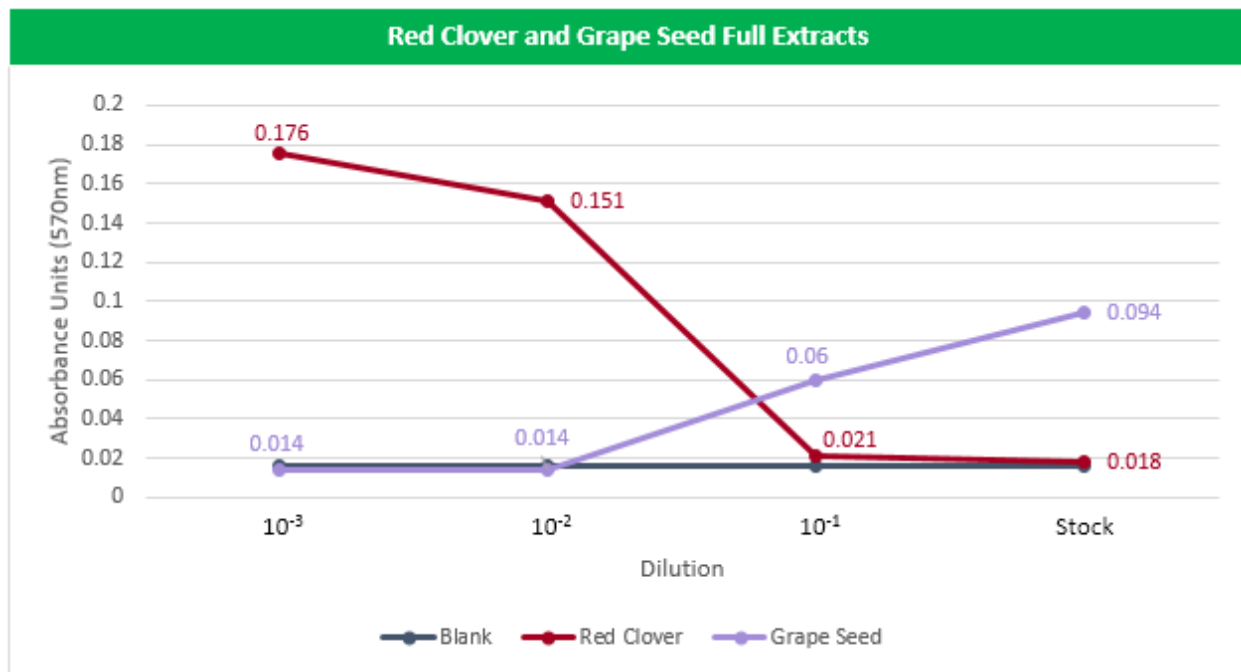


Figure 13: TACS MTT Assay Reading of Red Clover and Grape Seed Extracts at 570nm

The peak extracted from black cohosh stayed at baseline proliferation level until one tenth of the stock concentration, where proliferation increased. This is reflected in the positive slope of the trendline in Figure 14.

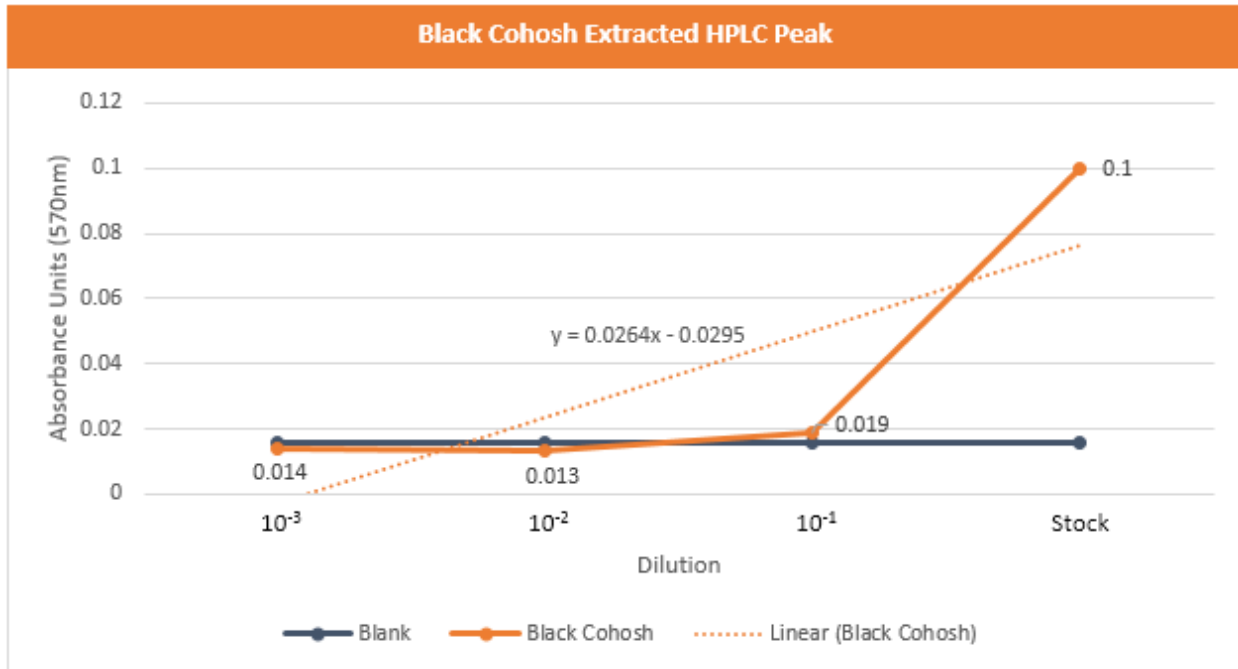


Figure 14: TACS MTT Assay Reading of Black Cohosh Peak at 570nm

When compared to the full grape seed extract, the peak collected had reverse effects on cell growth. The trendline is negative in Figure 15, showing a decrease in proliferation as concentration increased. The lowest concentration of the grape seed extract peak resulted in the highest reading (0.24) of any other tested in this experiment.

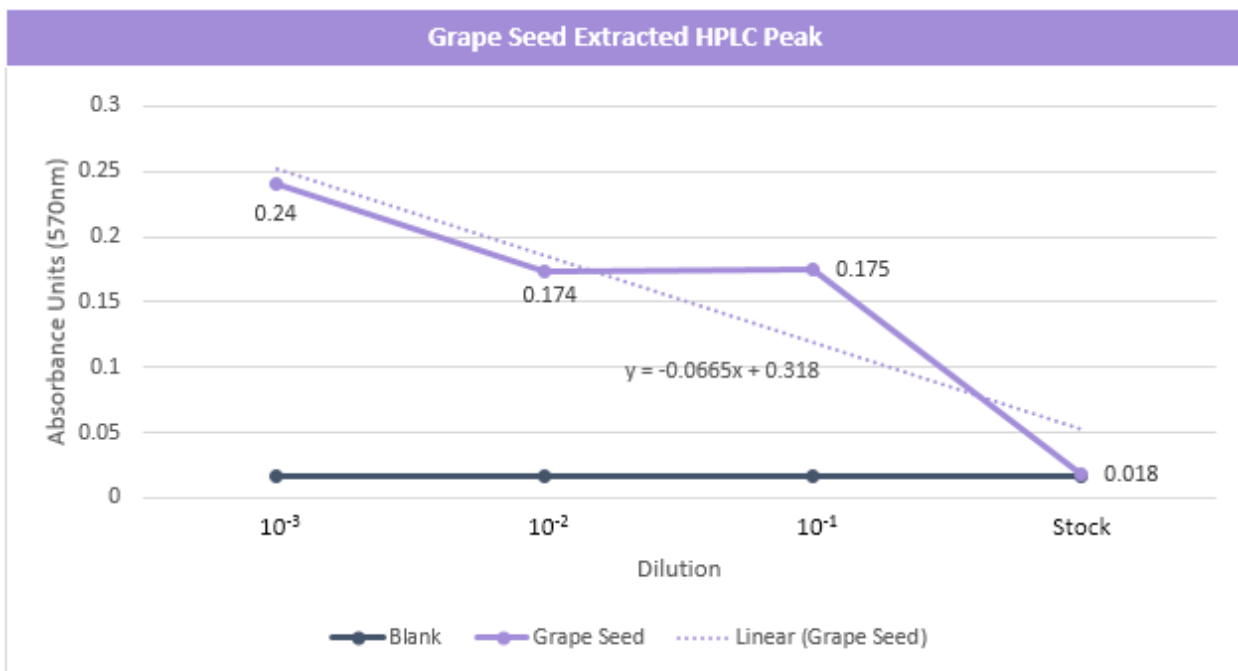


Figure 15: TACS MTT Assay Reading of Grape Seed Peak at 570nm

Figure 16 shows all four red clover extracted peaks for comparative analysis. Peak 1, peak 3, and peak 4 all show a decreasing proliferation with increasing concentration. Peak 2 does not have a prominent effect on cell growth when compared to the others in this experiment. Both peak 1 and peak 3 have similar trends of growth comparative to the complete red clover extract, having the highest proliferation at the lowest concentration.

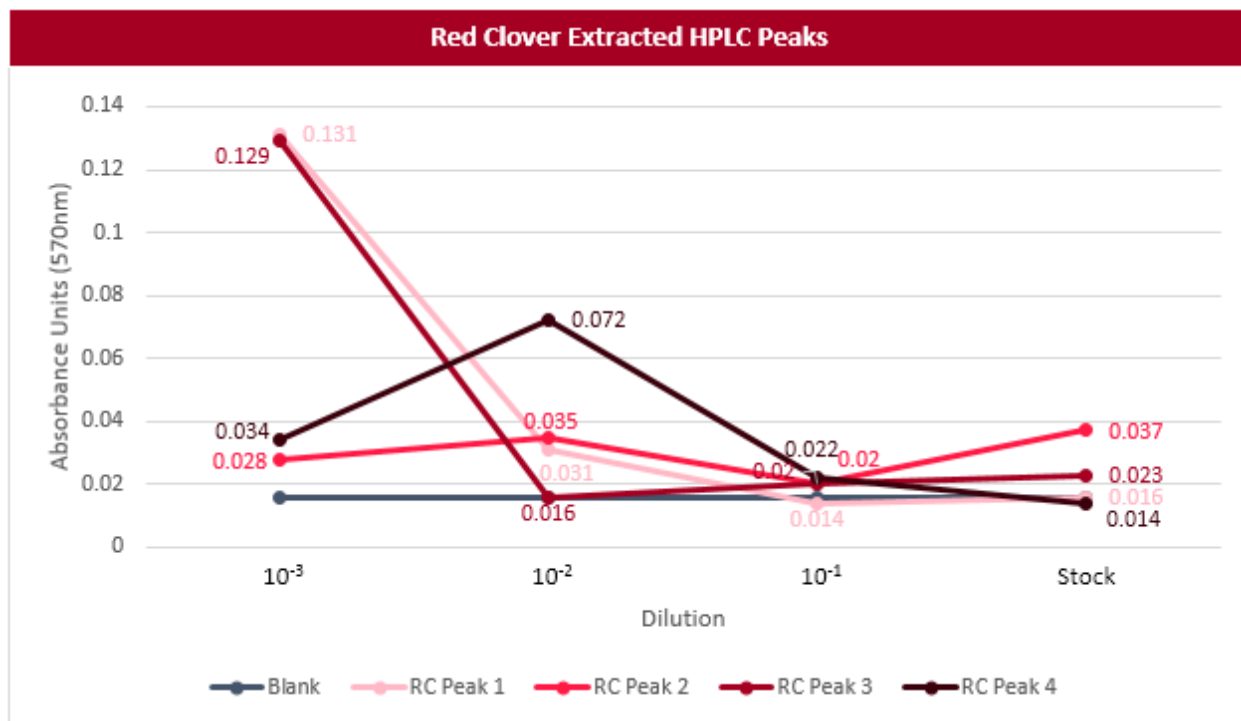


Figure 16: TACS MTT Assay Reading of Red Clover Peaks at 570nm

The slope of each linear trendline and the range for each extract for the TACS MTT assay are recorded below in Table 5. Black cohosh extract has the smallest range and lowest absolute value of the slopes recorded. As the positive control,  $\beta$ -estradiol has the second lowest range and slope. Grape seed extract is the only supplement that resulted in a positive slope. The largest range in proliferation readings was seen in red clover with a range of 0.158. Together, the data summarized in Table 5 are inconsistent and generally uninterpretable.

Sample	Slope	Range
$\beta$ -estradiol	-0.0109	0.0409
Black Cohosh extract	-0.0015	0.0070
Grape Seed extract	0.0286	0.0800
Red clover extract	-0.0604	0.1580

Table 5: T47D-KBluc Breast Cancer Cell Proliferation Trends in Various Phytoestrogens

## Conclusions

Based on analysis of the HPLC and MTT assay results, the following conclusions were drawn regarding the black cohosh, red clover, and grape seed supplements.

Through HPLC, the most prominent components of the extracts were observed and isolated.

The HPLC data showed only minor correlation between phytoestrogen standards tested and red clover and grape seed extracts. Thus, the identities of the compounds included in these dietary supplements remain largely unknown. Although the most prominent peaks were collected, they may not be phytoestrogens or have notable effects on cell proliferation. Additionally, the compounds depicted in the HPLC data may not independently impact breast cancer cell growth, but collectively could have agonistic or antagonistic effects. As previously mentioned, the study performed by Al-Akoum *et al.* provided evidence of antagonistic effects of tamoxifen, blocking the estrogen receptor (Al-Akoum *et al.*, 2007). When cells were treated with tamoxifen and  $\beta$ -estradiol together, tamoxifen prevented the proliferative effects previously seen with  $\beta$ -estradiol by binding the ER $\alpha$ . Similarly, the various components in the supplements could counteract each other. For example, Ji *et al.* isolated the phytoestrogen formononetin from black cohosh and applied only the purified sample to the cells. This experiment demonstrates that formononetin causes estrogenic effects within the cell (Ji *et al.*, 2005). Alternatively, Gaube *et al.* and Al-Akoum *et al.* show that black cohosh as a whole has no effect on proliferation of breast cancer cells (Gaube *et al.*, 2007; Al-Akoum *et al.*, 2007). This discrepancy may be explained by the various agonistic and antagonistic components within black cohosh along with any of the other supplements used in this study. Therefore, it is important to identify the variety and level of individual phytoestrogens in each supplement.

In addition to the effects of varying supplement components, the ratios of each component within the pill can differ. According to Albulescu *et al.*, daidzein and genistein could be isolated from red clover extract but in this HPLC, neither was present (Albulescu *et al.*, 2006). These differences can be due to plant growth conditions or varying harvest and preparation protocols. The metabolic production can be effected by these inconsistencies. This can pose a health threat for women ingesting the supplements if the levels of estrogenic components fluctuate among batches of the same supplement.

The results of the CellTiter 96 MTT assay were more consistent with recently published literature than the TACS MTT assay. The increase in proliferation of the breast cancer cells treated with the positive control  $\beta$ -estradiol confirmed this conclusion. In comparison, the cells treated with  $\beta$ -estradiol stock solution did not show any proliferation when using the TACS MTT assay; thus, this assay was considered unreliable. Therefore, the main conclusions were drawn from the CellTiter 96 MTT assay.

The growth of black cohosh treated cells resembled that of untreated cells. Additionally, compared to the growth of  $\beta$ -estradiol there was little proliferation. Therefore, this supplement properly served as the negative control chosen based on the work of Gaube *et al.* and Al-Akoum *et al.* (Gaube *et al.*, 2007; Al-Akoum *et al.*, 2007). Overall, black cohosh stock solution did not appear to have an effect on the breast cancer cells. According to Reiter *et al.*, red clover should have had antiproliferative effects on the

breast cancer cell growth. However, this was not possible to replicate due to the unknown concentrations of components in the extracted supplements. The cells treated with red clover in this study showed slight variance in cell growth from untreated cells. Similar to black cohosh, these treated cells resulted in very little proliferation compared to  $\beta$ -estradiol. Testing revealed that cells treated with grape seed caused cell proliferation similar to  $\beta$ -estradiol. The stock solution of grape seed caused a higher amount of cell growth than both red clover and black cohosh. This opposes the findings of Chen *et al.* which found grape seed to cause antiproliferative effects on the breast cancer cell line. However, further studies should be done in order to conclusively determine that grape seed extract had proliferative effects similar to that of  $\beta$ -estradiol.

In the future, the HPLC data should be looked at more closely. The extracts should be run through a different column at a slower elution rate to allow better extraction of peaks. Using mass spectrometry, the specific characteristics of each peak could be analyzed and compared to known compounds. This could help identify certain peaks. Additionally, HPLC can be used to compare the components of supplements from different manufacturers as well as the composition of supplements from the same bottle in order to observe variation from pill to pill.

To definitively observe the effects of treating breast cancer cells with the extracts, immunoblots can be used instead of MTT assays. Proliferating cell nuclear antigen (PCNA) is a protein that indicates the synthesis of DNA and therefore cell proliferation. Caspase-3 is an enzyme in the apoptotic cascade showing cell death. Through the use of these proteins, immunoblots would reveal cell production of proliferative or apoptotic cell signals.

In addition to viewing the effects of immunoblots, the extracts should be applied to breast cancer cells for various time periods. According to Howell and Cuzick, MCF-7 breast cancer cells experienced different effects depending on the duration of estrogen exposure. When estrogen levels declined, tumors adapted to the growth at the new level, but when estrogen was reintroduced, the cell growth was inhibited (Howell & Cuzick, 2012). These experiments could indicate that estrogen receptor binding may fluctuate with exposure time.

In order to accurately determine how these phytoestrogens will react in a biological organism, further experiments should be conducted to see which receptors are activated and how the supplements are absorbed into the cell. First, it should be confirmed that phytoestrogens enter the cells through the same receptor as  $\beta$ -estradiol. Additionally, it would be beneficial to have a thorough study of phytoestrogens' effects on DNA replication once it enters the cell.

Due to loose regulation of dietary supplements, products with questionable contents are made available and falsely advertised. Since the compounds in the supplements have not been identified, these products need regulation and should not be sold as dietary supplements until further studies are conducted. Proof of safety and efficacy should be provided prior to advertisement of these products as a safe alternative to hormone replacement therapy.

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