WORCESTER POLYTECHNIC INSTITUTE

Artemisinin and Breast Cancer

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Abstract

Antiproliferative effects of the compound, artemisinin, have been observed in several breast cancer lines. Whole plants extracts appear to be more efficacious than the pure compound in the treatment of diseases such as malaria. In our studies, MCF-7 breast cancer cells were treated with purified artemisinin and extracts of the whole plant *Artemisia annua* to compare the effects. These results suggest that extracted artemisinin extract contains compounds with antagonistic effects which may inhibit its effects in cancer therapy.

Acknowledgements

We would like to thank Pam Weathers for donating the plant material used in our experiment. We would like to further thank Jill Rulfs and Michael Buckholt for their advisory role in this project.

Background

Breast cancer can present itself in a variety of forms which means that treatment must be as versatile as the disease itself. This project will focus specifically on treating estrogen responsive breast cancer by antagonistically blocking estrogen receptor α. This antagonistic blocking of estrogen receptor α will be accomplished through treatment of a model cell system (MCF-7) with pure artemisinin and artemisinin extracts taken from the plant. Artemisinin is a chemical compound, naturally synthesized by the plant *Artemisia annua*, found to have antimalarial and anti-cancer properties. We will be further examining the effectiveness of artemisinin in preventing the proliferative activities of estrogen in our model cell system.

Breast Cancer and Current Forms of Treatment

Cells are considered cancerous or malignant when they undergo rapid and uncontrolled cellular division. This process is known as proliferation. When cancerous cells develop in the tissues of the breast, the patient has developed the disease breast cancer. Breast cancer can be classified several different ways depending on location and manner of development, as well as the traits possessed by the cancerous cells. These cancerous cells are then tested and classified so an accurate diagnosis and effective treatment plan can be created.

Types of Breast Cancer

Breast cancer can be classified into two broad categories: invasive and noninvasive. Invasive (infiltrating) breast cancer is not confined to a particular location of the breast or body. Instead, it has broken through the natural tissue barriers to spread throughout the body via the bloodstream and lymph nodes. Noninvasive (in situ) breast cancer remains in a particular location of the breast without spreading to other tissues (Henderson, 2003). These two categories can be used to classify how the other subtypes of breast cancer behave. For example, Ductal Carcinoma begins

in the milk ducts and is the most common breast cancer type. Though it originates in the milk ducts, as the disease progresses in some individuals, it begins to spread to other parts of the body, or metastasize. This means that this particular form of breast cancer can be classified as invasive or noninvasive. Lobular carcinoma begins in the glands that produce breast milk (lobules) and is then transported by the milk ducts. Inflammatory breast cancer is unique in that it starts in the soft tissues of the breast and blocks the lymph vessels of the skin. This causes the breasts to become firm, tender, warm, and itchy from the build-up of white blood cells. Papillary carcinoma is a rare form of breast cancer whose cells are arranged in finger-like projections known as papules. The final form of breast cancer is called triple negative breast cancer. Breast cancer cells typically contain receptors for estrogen and progesterone. In addition, "healthy" breast cancer cells contain receptors for a protein called human epidermal growth factor receptor 2 (HER2). HER2 stimulates normal cell growth in healthy cells. Triple negative breast cancer occurs when the cancer cells do not contain receptors for progesterone, estrogen, or HER2. Approximately 10-20% of all breast cancers are triple negative (Henderson, 2003). When breast cancer cells test negative for all three receptors traditional hormone therapies and medications are ineffective. Instead, triple negative breast cancer is typically treated through chemotherapy, radiation therapy, and non-HER2 targeted therapy (Henderson, 2003).

Hormone Therapy

Breast cancer cells are typically sensitive to hormones, particularly estrogen. This means that the cells are activated when hormones bind to receptors on the cell surface. Breast cancer cells that test positive for estrogen responsiveness are labeled estrogen receptor-positive (ER-positive). Many ER-positive cell lines are also progesterone receptor-positive (PR-positive). Typically, progesterone is responsible for the monthly thickening of uterine walls in preparation for

pregnancy in women. Hormone therapy works by preventing the body from producing estrogen and progesterone, the hormones which the tumor cells are most sensitive to. Several treatment strategies have been developed to treat hormone-sensitive breast cancer including suppressing estrogen production in the ovaries, preventing estrogen synthesis, and blocking estrogen's effects. Understandably, tumors that are not sensitive to hormones are unresponsive to this kind of treatment.

In premenopausal women, the ovaries are the main source of estrogen. As such, the ovaries are primarily targeted for reducing or suppressing their function in order to lower estrogen levels. There are several drugs that suppress the ovaries temporarily using gonadotropin-releasing hormone agonists, also known as luteinizing hormone-releasing hormone agonists (Ovarian Ablation, 2011). Both interfere with pituitary gland signals which target the ovaries and stimulate estrogen production. Additionally, one of the most extreme processes, called ovarian ablation, involves surgically removing the ovaries (oophorectomy) or using radiation. Unlike temporary suppression with drugs, both of these procedures are permanent.

Other hormone therapies focus on directly blocking estrogen production rather than suppressing the overall function of the ovaries. Aromatase inhibitors block the activity of aromatase, an enzyme which the body uses to synthesize estrogen in the ovaries and other tissues. Typically, this treatment is used in postmenopausal women as premenopausal women produce too much aromatase for the inhibitors to effectively block (Hormone Therapy for Breast Cancer, 2012). The final strategy of hormone therapy is to block estrogen's effects. Selective estrogen modulators (SERMs) bind to estrogen receptors and prevent estrogen from binding. Unfortunately, many SERMs act as agonists for estrogen receptors in other tissues. One example

of this, tamoxifen, acts antagonistically in breast tissue but agonistically in uterine and bone tissue (Hormone Therapy for Breast Cancer, 2012).

Treatment

Typical treatment of early stage breast cancer involves an initial surgery to remove the tumor cells, followed by an adjuvant therapy. Adjuvant therapy is typically in the form of radiation therapy, hormone therapy, targeted therapy, or chemotherapy. These adjuvant therapies typically occur daily for approximately 5 years. Treatment is typically a mix of hormone therapies and traditionally starts with several years of tamoxifen, a drug which acts antagonistically for estrogen receptors in breast cancer cells (Hormone Therapy for Breast Cancer, 2012). Treatment with tamoxifen is followed by treatments with other aromatase inhibitors, or treatment with tamoxifen is skipped entirely and other aromatase inhibitors are used for the full five years.

Side Effects

Due to tamoxifen's estrogen-like behavior, premenopausal women treated with it are at a higher risk for uterine cancer and weaker bones. Tamoxifen also puts patients at risk for stroke, blood clots, and cataracts (Riggs, 2000). Other SERMs like raloxifene are also known to increase the risk of blood clots and strokes. Ovarian suppression in patients increases their risk of bone loss and depression. Ovarian suppression leads to bone loss due to the effects estrogen deficiency has on osteoclasts. In estrogen deficient environments, osteoclast activity increases and more bone is reabsorbed (Riggs, 2000). In addition, ovarian suppression can also cause mood swings and depression. Other aromatase inhibitors are known to cause significant bone loss leading to possible osteoporosis, as well as increase the risk of heart attacks, heart failures, and depression (Ovarian Ablation, 2000).

Model Cell System

The cell line MCF-7 has been used as a model system for estrogen responsive breast cancer for decades. As an estrogen responsive cell line, they test positive for the presence of the two types of estrogen receptors, α and β. When estrogen binds to these receptors proliferative activity is induced.

Discovery of the MCF-7 Cell Line

The MCF-7 cancer line was originally isolated from an effusion of a patient with metastatic breast cancer. The cells were grown maintained for three years using radio and hormone therapy before a pleural effusion was sampled and tested. Further cytogenic studies showed that their cell line contained 88 chromosomes. Lippman found that when the MCF-7 cells were treated with tamoxifen, an antiestrogenic drug, their growth was inhibited (Levenson, 1997). Exposure to the hormone, estrogen, however, reversed the inhibition and the cells were once again able to proliferate. Until this point, the drug had been classified as a competitive estrogen inhibitor, which at high concentrations, was fatal to the cells. Dr. Lippman knew that his discovery aligned with this classification and he announced his belief that, "The potential value of a hormone-dependent human breast cancer in long term tissue culture for the study of mechanism(s) by which steroid hormones exert their trophic effects is significant, particularly in view of the likelihood of obtaining regulatory variants or mutants which are hormone independent" (Levenson, 1997).

Over the next several years, many other discoveries regarding the MCF-7 cell line were made including the identification of several different hormone receptors within the cells by Horwitz (Levenson, 1977). Making MCF-7 cells a model system for tumor response mechanisms and biological actions that occur during hormone therapy.

The MCF-7 cell line was a pivotal part in developing an understanding many aspects of breast cancer cells. Study of this cell line has revealed the organization of the ER and steroid hormone systems with breast cancer cells as well the effect of estrogen and antiestrogen on receptors. Information gathered from the studies of these cells has also contributed towards the progress of researcher's overall understanding of antiestrogen action.

Estrogen

Estrogen is often referred to as the female sex hormone and is responsible for the development and regulation of sex characteristics. In addition, it regulates the female reproductive system and promotes growth and differentiation of the mammary glands. Estrogen is classified as a steroid hormone and is able to readily diffuse across cell membranes. The most common type of estrogen produced within the cell is 17β-estradiol. Once the estrogen molecules have crossed the cell membrane, they can bind to estrogen receptors, which control a variety of gene expressions. Estrogen levels in women are constantly fluctuating, even after puberty, due to their menstrual cycle, pregnancy, and eventual menopause.

Estrogen Receptor-α

Estrogen Receptor (ER) α is one of the two forms of estrogen receptors encoded by the ESR1 gene. Estrogen has been recognized as a vital factor for growth in a large number of breast cancer cases. The presence of ER α has shown a direct correlation with positive prognosis (Ali, 2000). There is an overexpression of ER α in over 50% of all breast cancer cases. Of these cases, 70% respond to tamoxifen which is known to block ER α. In addition, a higher level of estrogen receptor α indicates an increased risk for breast cancer. A large portion of breast cancer cases, in which ER α is overexpressed, show a link between cell proliferation and estrogen binding to ER α (Ali, 2000). Treatment of the breast cancer tumor cells through hormone therapy causes a

resistance to develop. To combat this resistance, diverse hormonal therapies are constantly being developed and modified to prevent tumor resistance.

Estrogen Receptor-β

Estrogen Receptor (ER) β is the second form of estrogen receptor, identified several years after ER α (Hayashi, 2003). ER β presence has been detected in breast cancer tumor cells, especially when they tested positive for ER α. ER β has been found to support growth and proliferation in over 50% of primary breast cancers (Palmieri, 2002). When ER β was initially discovered in 1996, there were doubts of its importance in breast cancer. Further research has led to our current understanding that both estrogen receptors have distinct cellular distribution. Both receptors regulate separate genes and sometimes work against one another. ER β, when expressed in breast cancer cells, can cause proliferation independent of ER α activation (Palmieri, 2002). When treating breast cancer it is vital that treatment accounts for both receptors if they are expressed in the cells.

History and Effects of *Artemisia annua*/ Artemisinin

Artemisia annua is a plant, found throughout the world, with known medicinal properties. In recent years its function as an antimalarial agent has been further investigated, as well as any other medicinal properties it may possess. Special attention has been paid to determining the most potent form in which the plant can be administered in order to treat malaria. Additionally, further research of the plant has shown the plant may potentially kill cancer cells and behave as an antagonistic agent for estrogen receptors in breast cancer.

Artemisia Annua and Artemisinin

Artemisia annua is a species of wormwood found in Asia and several other temperate climates throughout the world. Medicinal use of the plant, known as “quighao” in traditional Chinese

medicine, dates back to 200 BC. It was believed that the cool (yin) of the herb would be beneficial in treating internal sources of heat (yang) such as fevers. In 1596, it was first suggested that tea made of *Artemisia annua* could be used to treat malaria. The modern study of the plant began in the 1960's when the Chinese government was searching for a new form of antimalarial. The researchers attempted to follow the guidelines of the original treatment protocol using hot water extracts of *A. annua*. They found that in this form, the plant had no antimalarial effect on mice infected with the rodent malarial parasite, *Plasmodium berghei*. They then treated the infected mice with cold ethereal extracts of *A. annua*. This method of extraction produced antimalarial activity within the mice (Brown, 2006).

In 1971, scientists began clinical trials on the use of extracted *A. annua* as an antimalarial for primates infected with the Plasmodium parasite (Brown, 2006). Chinese military personnel infected with the parasite were the first humans to be successfully treated with the extract. The trial produced extremely positive results. The majority of patients in the trial received therapeutic results and there did not appear to be any dangerous side effects to the treatment. Additionally, the extract was found to effectively treat both chloroquine-resistant falciparum malaria and cerebral malaria. Following the promising results of the cold ethereal extractions, the compound artemisinin, the active ingredient in the *A. annua* plant, was isolated and identified in 1972 (Brown, 2006).

The compound, artemisinin, was found to contain a 1,2,4-trioxane ring. It is the only naturally occurring substance with this structural format (Brown, 2006).

Current Uses for Artemisinin

Artemisia annua and its derivatives have quickly become gained notoriety as a cheap treatment of many types of malaria. They have been found to rapidly eliminate malaria parasites with a

reduction ratio of roughly 10,000 per erythrocytic cycle. The source of artemisinin's antimalarial properties is the endoperoxide bridge in the compound's trioxane structure. Though it has still not been confirmed, the most likely mechanism of action for artemisinin's treatment of malaria involves the decomposition of the endoperoxide bridge upon interaction with heme, a non-protein constituent of hemoglobin found within the parasite. The breakdown of the endoperoxide bridge releases carbon-centered free radicals which then likely kill the parasite by destroying essential malarial proteins. Artemisinin's unique mechanism of action has even allowed researchers to combat several drug-resistant strains of the disease (Singh, 2011).

One reason that artemisinin's mechanism of action is debated stems from the discovery that the compound is also effective in cases of other parasitic infections, such as tick-borne Babesia parasites. These parasites do not digest hemoglobin and therefore do not produce the heme responsible for the decomposition of artemisinin's endoperoxide bridge. This finding suggests that this mechanism of action is not responsible for the anti-parasitic properties of artemisinin. Instead, the efficacy of artemisinin treatment in these cases suggests the hypothesis that the sarco/endoplasmic reticulum um ATPases, SERCAs, found in these parasites are the main targets of artemisinin.

Artemisinin Anti-Cancer Potential

Research has shown artemisinin demonstrates anti-cancer potential even for cell lines that are drug and radiation resistant (Amir, 2015). Cancer cells typically uptake larger amounts of iron than healthy cells in order to proliferate. Artemisinin reacts with iron to form free radicals which cause cell death. The increased iron uptake of cancer cells leaves them susceptible to the free radicals artemisinin creates (Lai, 2005). Many cancer cells have a larger percentage of transferrin receptors on the cell surface. These receptors increase uptake of the iron carrying protein

transferrin via endocytosis. When artemisinin covalently bonds to transferrin, both are taken into the cell together. Once endocytosed, the artemisinin reacts with the iron, killing the cancer cells. Artemisinin and transferrin bonded to artemisinin have been found to be extremely potent and selective in causing cancer cell death (Lai, 2005). It is hypothesized that artemisinin's anti proliferative properties in cancer cell lines originates with artemisinin's interactions with iron and heme intermediaries (Zhang, 2009). Artemisinin has also been found to suppress vascular endothelial growth factor C in lung cancer, increase calcium levels and activate p38 in lung cells, and block estrogen receptors in breast cancer.

Artemisinin Anti-Breast Cancer Properties

A standard model for estrogen responsive breast cancer is MCF-7 cells, which express both types of estrogen receptors (E_2 and ER-β). Early testing of MCF-7 cells with artemisinin, an antimalarial agent, demonstrates its ability to block estrogen induced cell progression and proliferation. Sundars et al demonstrated that artemisinin blocked the proliferation of cells teated with either E2 or PPT, a known ER alpha agonist.

Further research has shown that artemisinin dimers synthesized with liposomal nanoparticles demonstrate improved anti proliferative effects. These nanoparticles become more water soluble as the surrounding pH decreases, as tumors typically have a more acidic environment (Zhang, 2013). This improved delivery of artemisinin shows a down regulation of the HER2 protein in HER2+ cell lines. The dimer has also been shown to decrease the growth factor receptor activity in triple negative breast cancer, a notoriously hard types to treat through hormone therapy (Zhang, 2013). This research further demonstrates the potential potency of the treatment options further investigation of artemisinin presents.

Introduction

Breast cancer is the most common type of cancer present in women. There are over 203,000 new diagnoses and more than 45,000 deaths associated with breast cancer every year. Over 70% of breast cancer cell samples test positive for estrogen responsiveness. The cells' responsiveness to hormones determines the best treatment plan for the patient. Traditional methods of treatment for breast cancer involve surgery, tamoxifen, and chemotherapy (Henderson, 2003).

Artemisia annua is a form of wormwood plant found throughout the world. The species naturally produces a compound called artemisinin, which, for centuries, has been known to have antimalarial properties (Brown, 2015). Recently, these properties have been investigated much further (Brown, 2015). New research has suggested that the natural artemisinin compound may be effective in the treatment of some forms of cancer (Shyam, 2008).

Hundreds of years ago, the leaves of the artemisinin plant were consumed in the form of a tea as a primitive treatment for malaria. Recent studies have shown that the plant is an effective malaria treatment when made into pill form (Gogtay, 2013). Gogtay found that artemisinin is an effective alternative to treating chloroquine resistant strains of malaria. This lead researchers to believe that there are other compounds at work in the plant, whose potency is greatly reduced when made into a tea. Another study found that when brewed in a tea, artemisinin flavonoids casticin and artemetin were found at a 40% and 0% yield, respectively (Weathers et. al., 2012). These compounds are likely responsible for the compound's efficacy in the treatment of several forms of cancer (Weathers et. al., 2012). Artemisinin has been shown to possess strong antagonistic behavior towards estrogen receptor α (Sundar, 2008). Artemisinin not only blocks estrogen receptor α, it also possesses strong cytotoxic traits (Shahbazfar, 20104). Artemisinin's interaction

with iron, a substance taken in by cancer cells in large quantities, creates free radicals which kill cells (Zhang, 2009).

For patients with hormone responsive breast cancer, the typical treatment protocol is surgery followed by, usually, around 5 years of hormone and combined drug therapy. Tamoxifen is the most typical drug used to treat breast cancer. While tamoxifen is ineffective in its base form, onceit is digested by the liver into afimoxifene and endoxifenits, affinity for estrogen receptor α increases by more than 50 times (Henderson, 2003). This high affinity makes it a great antagonist for the receptor. Some of the other hormones and drug treatments include gonadotropin-releasing hormone agonists, selective estrogen receptor modulators, and aromatase inhibitors (Hormone Therapy for Breast Cancer, 2012). These therapies are designed to target the estrogen cycle in the body, specifically the production of the hormone or the estrogen receptors. While drug and hormone therapies are effective against estrogen responsive breast cancer they are not without side serious effects. Common side effects include heart problems, increased risk of uterine cancer, increased risk of osteoporosis, mood swings, depression, fertility issues, etc (Shahbazfar, 2014).

Hormone therapy also presents the risk of creating a treatment resistant strain of breast cancer. To prevent development of a hormone resistant strain of breast cancer the treatments are typically composed of several types of treatments that work in concert in method known as combination therapy. Combination therapy treats the patient with a combination of drugs to help prevent resistant strains from developing. Artemisinin is being studied as the base for a combination therapy to help better prevent drug resistance (Gogtay, 2013). The inclusion of artemisinin in the arsenal of treatments available to combat hormone responsive breast cancer cell lines would help to reduce the risk of treatment resistant strains of breast cancer developing.

Treatment with the compound has also been shown to lack the negative side effects that accompany the usual forms of therapy (Riggs, 2000).

The use of artemisinin as a treatment option is still being studied (Suberu, 2014). While the benefits have already become clear, there are many aspects of the compound that are still unknown such as the exact method of action that gives the plant its anti-proliferative properties. We hypothesize that the extract of artemisinin will contain compounds that will increase the plant's anti-proliferative abilities to be more effective than purified forms of the extract. This hypothesis is based on the research into its antimalarial abilities have shown that raw forms of the plant are more successful in treating malaria (Weathers, 2012).

Methodology

Cell Culture: MCF-7 Cell Line

MCF-7 ATCC cells were cultured according to the procedures outlined in (Hamelers, 2003). Briefly, the cells were cultured in Dulbecco's modified Eagle's medium which contained 10% fetal bovine serum (FBS) and 100 µg/ml of bovine insulin, 100 IU/ml of penicillin and 100 µg/ml of streptomycin. The cells were maintained at 37^0C. until they reached 60-70% confluence. After plating at 10^3 cells/well in 96 well culture plates, the cells were incubated in phenol red-free medium with 5% dextran-coated charcoal-treated (DCC) serum for 24 hours followed by another 24 hours in phenol red-free, DCC serum stripped media containing 0.2% bovine serum albumin, 10 µg/ml of transferrin and 30 nM sodium selenite.

Extraction

Extractions were performed following procedures outlined in Cambra (2014). 75 mg. of pre-dried and crushed *Artemesia annua* leaves were placed in a 15 mL conical tube and 4 mL of methanol was added to the tube. The tube was placed in an ultrasonic bath for 30 minutes on the highest setting. The organic matter was then removed from the solution via filtration with filter paper. The remaining methanol was left to evaporate under nitrogen. Following the nitrogen evaporation of the methanol, only the organic extract remained.

Pure Artemisinin Solution

A pure artemisinin solution was created using 100 mg of 98% pure artemisinin (Sigma). Artemisinin was dissolved in 20 mL of 70% aqueous ethanol to reach a final concentration of 5 mg/mL.

High Pressure Liquid Chromatography

HPLC was performed according to the procedure outlined in Ferreira (2008). An Agilent 1100 Series system HPLC with ultraviolet detection scanning from 190-400 nm was used. A C-18 column was run under isocratic conditions using a 40% 0.1% aqueous acetic acid/60% acetonitrile mobile phase at a flow rate of 1 ml/min. Ethanol , pure artemisinin in ethanol, sample plant extract in ethanol, and an extract sample spiked with pure artemisinin extract in ethanol were analyzed. Between sample injections, the mobile phase was run alone for 5 minutes. The data collection window was 15 minutes. The artemisinin peak in the extracted sample was identified by elution time (Ferreira, 2008) and confirmed by spiking the samples with purified compound.

Determining Artemisinin Content

To determine the artemisinin content of the sample plant extract a linear regression analysis was performed using purified artemisinin as the standard. Four dilutions of the pure artemisinin sample (1:10, 1:20, 1:30, and 1:40) were created and then run through the HPLC. The concentrations (0.5 mg/ml, 0.25 mg/ml, 0.166 mg/ml, and 0.125 mg/ml) were plotted versus the area under the artemisinin peak. A line of best fit was added to the graph and the line's equation was used to solve for the concentration of artemisinin in the plant extract sample, using the area under the artemisinin peak of the sample. Since the sample was originally suspended in 10 mL and the mass used in the extraction was 150 mg, it was possible to determine the artemisinin content extracted from the plant.

MTT Assay

A Promega CellTiter 96 Non-Radioactive cell Proliferation Assay (MTT) was run to analyze the effects of different concentrations of artemisinin, both pure and extract samples, as well as the effects of estrogen on cell proliferation. Each sample was run in triplicate. The different wells

contained $1x10^3$ cells and 10µL of the sample solution and were incubated for 48 hours in the experimental condition solutions before 10µL of assay buffer was added to each well. After adding the assay buffer, the plate was left to incubate for three hours and then read with a Biotek EL800 plate reader at 450 nm. The conditions tested were: ethanol, 300 µM artemisinin, 300 µM artemisinin combined with 1nM estrogen, 1nM estrogen alone, 30 µM pure artemisinin, 30µM pure artemisinin, 300 µM pure artemisinin and estrogen, 30µM pure artemisinin and estrogen, 300µM extract sample of artemisinin, 300µM extract sample of artemisinin and 1nM estrogen, ethanol and 1nM estrogen, 30 nM extract sample of artemisinin, 30µM extract sample of artemisinin, and 30 µM extract sample artemisinin and 1nM estrogen. There were also wells containing only media and a blank well containing only cells and media. Once the absorbances were determined, the value for wells plated without cells (media only), was subtracted from the absorbance of each well to ensure that the cell metabolism and overall cell amount were accurately measured. The results between the different plates were then averaged. To eliminate interplate variability, a percent control was calculated by dividing the average readings for each sample by the average readings for the untreated control and multiplying by 100.

Results

HPLC

High performance liquid chromatography (HPLC) was used to confirm and quantify artemisinin presence in the sample plant extract.

To identify peaks which result from absorbance by the solvent, ethanol, a solvent only control was run (see Figure 1)

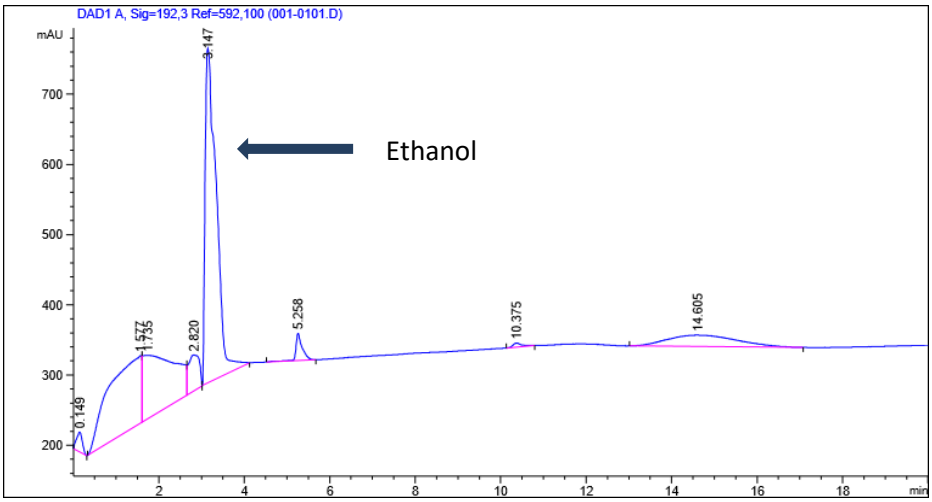

Figure 1: Chromatogram of Solvent Control

Figure 1 shows the peak elution time at 3.1 minutes for 70% aqueous ethanol.

The chromatogram for the pure artemisinin sample at a concentration of 5 mg/ml of artemisinin in 70% aqueous ethanol is shown in Figure 2.

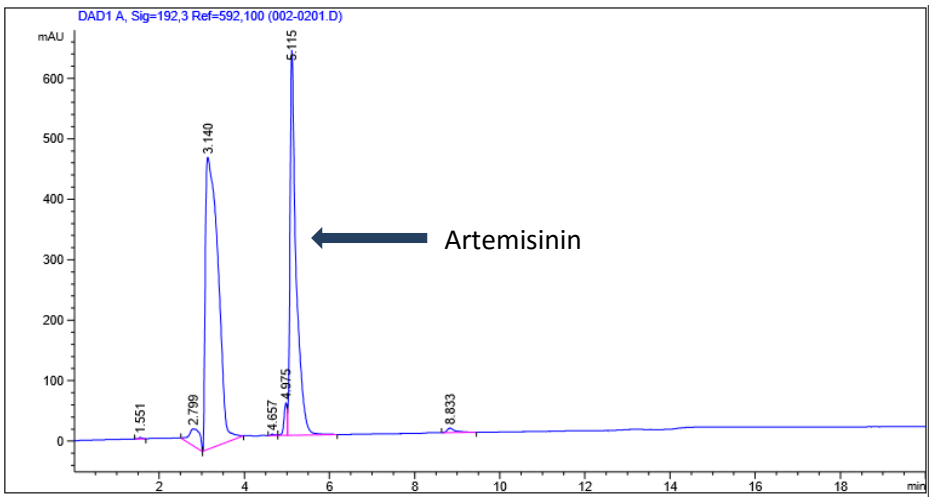

Figure 2: Chromatogram of Pure Artemisinin

Figure 2 shows two major peaks at 3.1 and 5.1 minutes. As the peak at 3.1 minutes was previously identified as ethanol, the peak at 5.1 minutes falls at the predicted elution time for artemisinin according to previous research (Ferreira, 2008). The area under the peak was determined to be $1.1*10^4$ mAu*s.

Next, an HPLC of sample extract from the dried plant matter was run and the results are shown below in Figure 3.

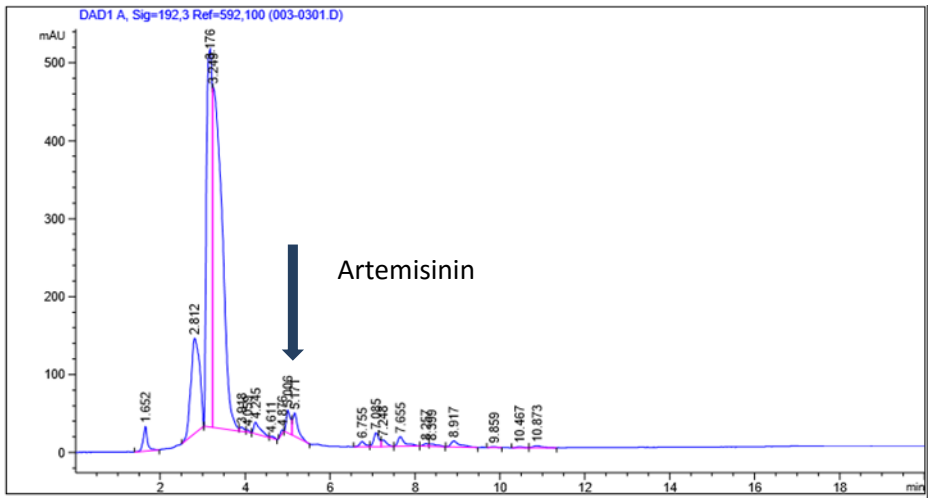

Figure 3: Chromatogram of Sample Extract

The chromatogram in Figure 3 showed the expected peaks for ethanol at 3.1 minutes and a peak eluted at 5.1 minutes, conditionally identified as artemisinin with an area of 257 mAu*s. To verify the identity of this peak as artemisinin, the sample solution was spiked with pure artemisinin at a 1:1 ratio. The results of the spiked extract can be seen in Figure 4, below.

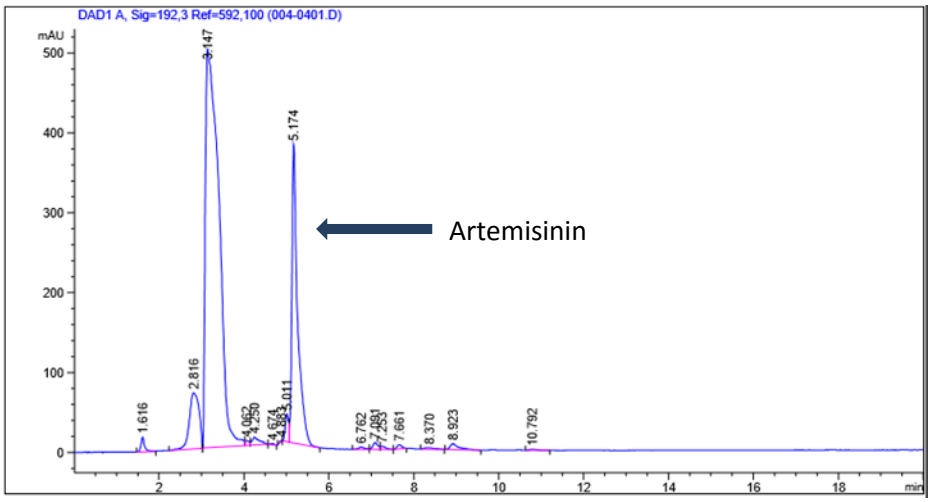

Figure 4: Chromatogram of Spiked Extract

Figure 4 shows the chromatogram of the spiked extract in which the peak located at 5.1 minutes went from an area of 257 mAu*s to 3,488 mAu*s. The spiked sample demonstrates that the compound eluted at 5.1 minutes was definitely artemisinin. Once the identification of artemisinin was confirmed, the amount of artemisinin in the sample extract needed to be compared to the pure sample to control the amount of artemisinin the cells were treated with.

Once the artemisinin peak in the sample was successfully identified, the total artemisinin content was determined as details in Methods. An HPLC was run on four different dilutions of pure artemisinin (1:20, 1:30, and 1:40). The chromatograms of the dilutions are shown in Figures 5, 6, and 7, below.

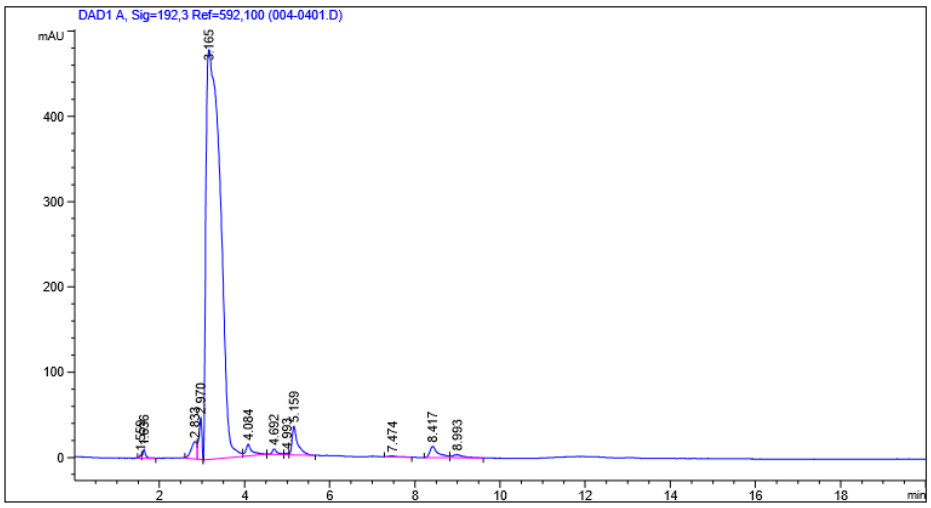

Figure 5: Chromatogram of 1:20 Dilution

The peak area of artemisinin at 0.25 mg/ml, shown above in Figure 5, was found to be 308 mAu*s.

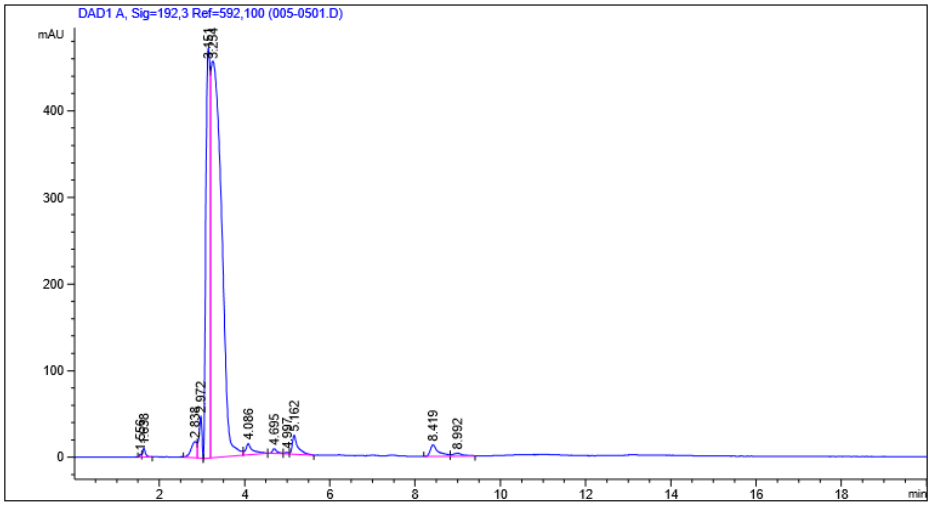

Figure 6: Chromatogram of 1:30 Dilution

The peak area of artemisinin at 0.166 mg/ml, shown above in Figure 6, was found to be 201 mAu*s.

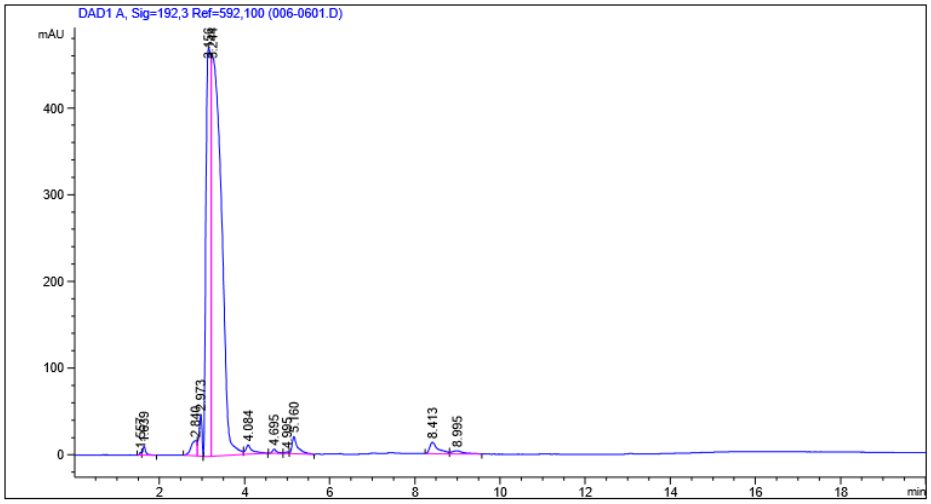

Figure 7: Chromatogram of 1:40 Dilution

The peak area of artemisinin at 0.166 mg/ml, shown above in Figure 7, was found to be 183 mAu*s. With this data, the area versus the concentration of the sample was plotted. The linear regression of this data is shown in Figure 8, below.

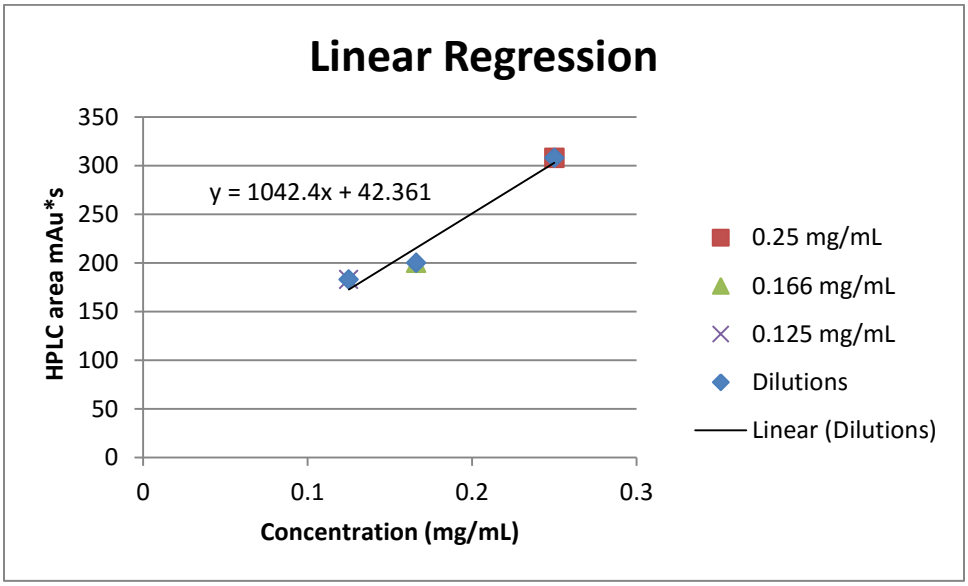

Figure 8: Linear Regression

In Figure 8, a line of best fit for area versus known concentration of artemisinin was determined. Using this line of best fit and the area under the artemisinin peak for the sample (257 mAu*s) as

the y value, it was possible to solve for the artemisinin concentration (x value) in the sample plant extract. This value was determined to be 0.2083 mg/ml in the sample, which was initially suspended in 10 ml of ethanol. From the 150 mg of Artemisia used to produce the sample extract, 1.4% artemisinin was extracted . Once this was determined, it was possible to control the amount of artemisinin the cell treated with the plant extract received.

MTT Assay

Each well was initially plated with 10^3 cells and after cell synchronization they were treated with several different experimental conditions. The control group (None) received no extra compounds. Ethanol, at a concentration of <1% (Ethanol or EtOH), was used to treat another group. The pure artemisinin (Pure Art or P) solution, dissolved in aqueous ethanol, was used to treat the cells at 300 μM. The sample extract solution (Sample or S) was also used to treat the cells at 300 μM. A 1:10 dilution of both artemisinin samples were also used as conditions (30 μM). All of the conditions were repeated with the addition of estradiol (E2) at 2 ng/ml. A set of control wells was also set up where media was added without the cells. The conditions were tested in triplicate for each 96 well plate. The organization of the conditions in the 96 well plates is shown below in Table 1.

Table 1: MTT Assay Results Key

	1	2	3	4	5	6	7	8	9	10	11	12
A	None	None	None	S+E2								Media Only
B	Ethanol	Ethanol	Ethanol	S+E2								Media Only
C	Pure Art	Pure Art	Pure Art	S+E2								
D	Pure Art	Pure Art	Pure Art	P+E2								
E	10% Pure	10% Pure	10% Pure	P+E2	EtOH+E2							
F	Sample	Sample	Sample	P+E2	EtOH+E2							
G	Sample	Sample	Sample	E2	EtOH+E2							
H	10% S	10% S	10% S	E2	E2							

All plates were set up according to the key shown in Table 1. Plates were read 3 hours after CellTiter administration. Original data for each experiment can be found in Appendix.

Table 2 shows the absorbance readings of the first plate, read at 450 nm. Once another plate was ready for assaying, the one step assay solution was applied and the plate was incubated for an additional 3 hours before being read. The results found in Table 3 were, as a whole; lower than the ones of the previous assay. Possible reasons for the lowered value include cell count not being as high in the previous assay, despite efforts to control cell number, or lower cell viability when added to the plate. The absorbance values in the third plate, shown in Table 4, were not as low as the ones observed in Table 3 and bore more similarity to the values initially observed in Table 2

Individual plate readings were adjusted by subtracting the value for the no cell blank for each plate. Then the average for each condition was determined (plate 1, 2, and 3) for each experiment and finally the average for each condition across three experiments was calculated (Ave). Each value was then expressed as 5 control with the no treatment control set at 100%. (See Table 2 below).

Table 2: MTT Assay Averages (Adjusted for Media)

Condition	Plate 1 (Au)	Plate 2 (Au)	Plate 3 (Au)	Avg (Au)	Percent control (%)	Standard Deviation	Standard Error
None	0.321	0.087	0.301	0.236	100	0.13	0.08
Ethanol	0.338	0.092	0.340	0.257	109	0.14	0.08
E2	0.393	0.110	0.321	0.275	116	0.15	0.09
Pure	0.441	0.087	0.350	0.293	124	0.18	0.11
Sample	0.328	0.090	0.420	0.279	118	0.17	0.10
10% S	0.329	0.093	0.340	0.254	107	0.14	0.08
10% Pure	0.301	0.103	0.315	0.240	101	0.12	0.07
EtOH+E2	0.570	0.099	0.463	0.377	160	0.25	0.14
P+E2	0.221	0.116	0.240	0.192	81	0.07	0.04
S+E2	0.329	0.111	0.350	0.263	111	0.13	0.08

These data are graphically represented in Figures 9 and 10.

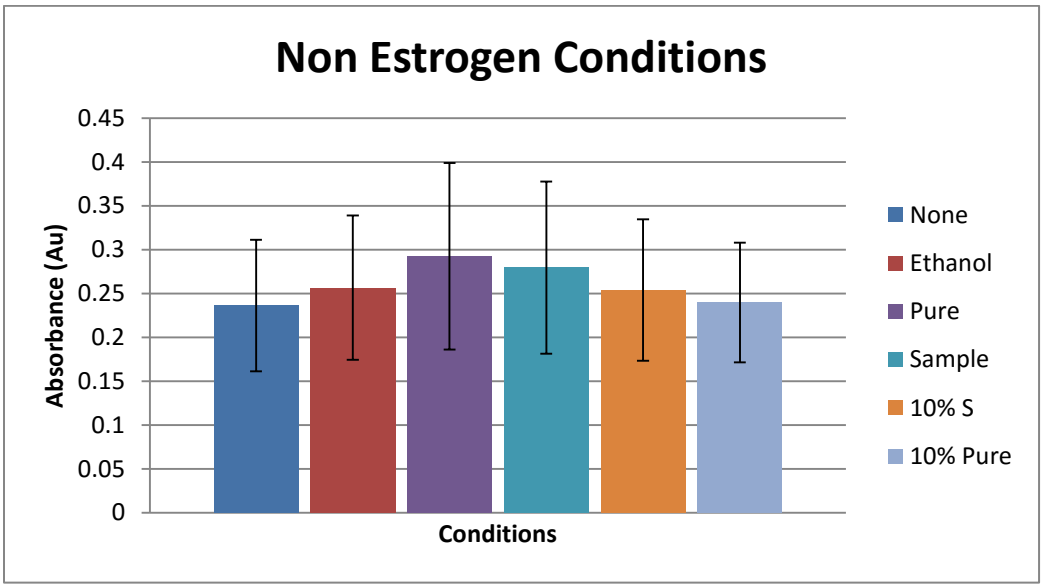

Figure 9: MTT Assay Average (Non Estrogen Conditions)

Figure 9 shows a graphic representation of the absorbance values shown in Table 2 for the samples in which no estrogen was present. The data for samples to which estrogen was added are

shown in Figure 10. The interplate variability among experiments resulted in large statistical errors which prevent any valid interpretation of the data.

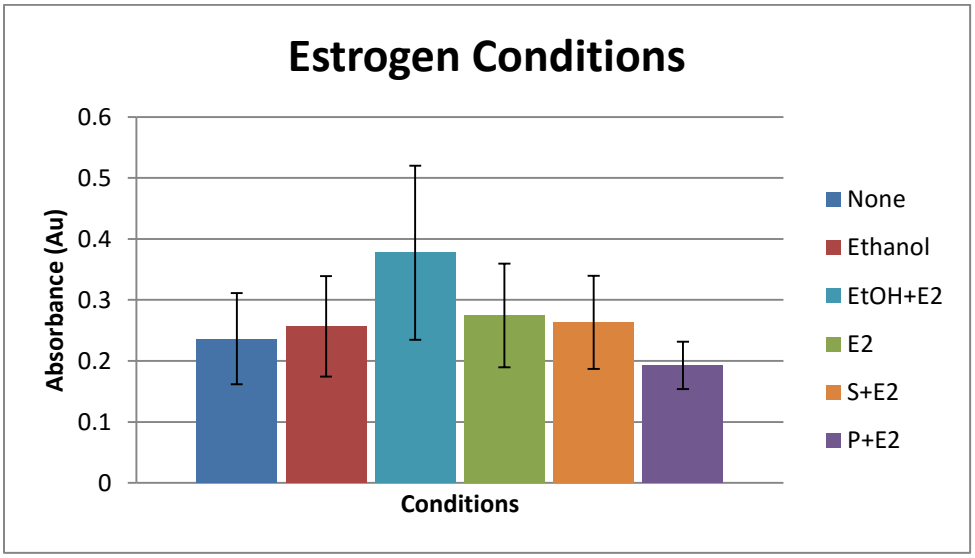

Figure 10: MTT Assay (Estrogen Conditions)

To help limit its effects on the results, a percent control of the results shown in Table 2 was calculated by comparing all the conditions to the control average. These data are shown in Table 2 above and graphically represented in Figure 11.

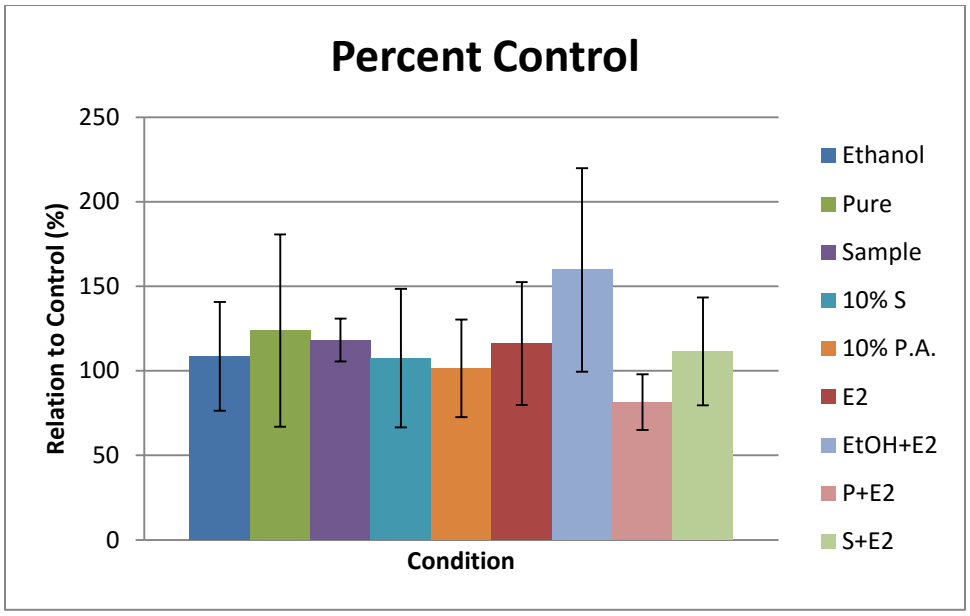

Figure 11: Percent Control of MTT Assay Averages

Examination of the percent controls shows two distinctly different results for the ethanol with estrogen condition and the pure artemisinin with estrogen condition. The ethanol and estrogen condition had a percent control value of 160% while the pure artemisinin with estrogen had a percent control value of 81%. The pure artemisinin value is the only one below 100% with all other conditions at percent controls above 100% as shown in Table 5 and Figure 11. A t-test of the values for estrogen plus ethanol and artemisinin plus ethanol shows a p-value of 0.28, however, which is not enough to prove statistical significance of these results. However, the other outcome worth noting is that the extract sample, which has the equivalent amount of artemisinin, does not appear to have an equivalent effect to the pure compound.

Discussion

From the MTT assay results it has not been possible to show that the pure artemisinin has a significantly greater effect on MCF-7 cell growth than the sample extracts, directly from the *Artemesia annua* plant, with other compounds present. Unfortunately we have also not been able to demonstrate a significant anti-proliferative effect of pure artemisinin. However, while none of the data were statistically significant they do suggest that there could be an effect if more trials were conducted and the sample size was increased to more than the three plates performed in this study. While no strong trends were found in the average of the plate results shown in Figure 9 and Figure 10, there are some observable trends found in the percent control data shown in Figure 11, which limits the effect of interplate variability.

When viewed as percent control, it appeared that there was a lower cell number when the cells were treated with the pure artemisinin and estrogen as compared to the ethanol and estrogen condition with no artemisinin present. With ethanol concentration kept below 1%, its toxic effects on the cells was shown to be negligible, suggesting that the estrogen and ethanol condition's high percent control value (160%) is likely should be due solely to the estrogen presence. The pure artemisinin with estrogen had a percent control value of 81%, while the other conditions were all over 100% in the percent control calculations, shown in Table 6.

With any antiproliferative effect limited to the pure sample with estrogen, the prediction that artemisinin acts as an estrogen receptor antagonist is supported (Sundar, 2008). The same effect was not observed with the sample extract of the plant *Artemisia annua*. Since the artemisinin amount was controlled, this suggests that there may be other compounds in the sample extract that have either agonistic behaviors or prevent the artemisinin from acting as an antagonist. If ,

with further validation, this result persists it may suggest that any use of the *Artemisia annua* plant in a form that is not pure, for treatment of estrogen responsive breast cancer, would be useless, if not possibly detrimental, to the patient. While our initial expectations were that there might be other compounds in the plant which would act synergistically to support the anti-proliferative effects of artemisinin, unlike what has been shown for treatment of malaria, this does not appear to be the case. Furthermore, the use of *Artemisia annua* to treat non-estrogen responsive cancer would have either no effect or a positive effect on breast cancer cell proliferation. Clearly more work needs to be done before these conclusions can be validated.

Future Recommendations

Future teams will first want perform a dose responsiveness curve for artemisinin and its effects on the cells. This will help future teams confirm that they are treating the cells with the appropriate amount of artemisinin in order to produce an observable response. In addition, future groups will want to replicate any artemisinin responsiveness in the cells before proceeding and expanding the experiment. Future research will want to monitor responsiveness of the cells with an MTT assay at different time intervals, rather than just the 48 hours used in this experiment as an incubation time. This will help any future researchers determine the optimal time frame in which the artemisinin effects on the cells are observable. It is also possible an MTT assay may not be a sensitive enough assay to monitor cell proliferation if the artemisinin effect is not as strong initially expected.

In the future, research can be expanded to use different cultivars of artemisinin to determine if the effect is unique to each batch of artemisinin. Future research can also examine the effects of digested artemisinin on the cancer cells. Any further research will want to isolate the compounds present in the artemisinin extracted from the plant that were not found in the pure sample and

attempt to determine the individual effects each compound may have on the cells. Additionally, though this experiment plated 10^3 cells in each well, it may not have been the optimal number to use so any future research will first want to determine the appropriate amount of cells needed in order to produce the best results.

Limitations of the Study

The sample size of the research completed in this study was very small (N=3), and further repetitions of the experiment will be required in order to produce any meaningful and statistically significant data. Several plates of data were lost due to error in following experimental procedure. Maintaining consistency in cell plating between wells is also extremely difficult, despite careful cell counting and resuspension procedures. While multiple forms of research literature were used to determine the correct artemisinin concentration to treat the cells with, it was not necessarily the optimal amount to use on the cells. Determining the best concentrations to use may yield stronger results. While these results did not produce any statistically significant findings, they did suggest further research is warranted to determine and prove the difference in the effects of pure artemisinin and an extract taken directly from plant samples. Further research will help demonstrate which forms of artemisinin can be a viable treatment for breast cancer and if using raw extracts taken from the plant will actually harm a patient.

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Appendix

Table 3: MTT Assay Plate 1

Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	0.596	0.599	0.442	0.25	0.038	0.039	0.039	0.038	0.043	0.039	0.039	0.219
B	0.592	0.384	0.711	0.691	0.039	0.04	0.04	0.04	0.04	0.039	0.04	0.228
C	0.434	0.747	0.774	0.718	0.041	0.041	0.04	0.04	0.039	0.039	0.043	0.042
D	0.607	0.724	0.717	0.458	0.04	0.045	0.04	0.043	0.042	0.041	0.044	0.04
E	0.508	0.685	0.382	0.318	0.816	0.04	0.043	0.04	0.041	0.041	0.042	0.042
F	0.336	0.556	0.621	0.377	0.83	0.04	0.039	0.043	0.04	0.052	0.04	0.042
G	0.561	0.602	0.637	0.63	0.737	0.041	0.04	0.041	0.043	0.041	0.044	0.044
H	0.608	0.626	0.425	0.603	0.498	0.039	0.04	0.039	0.04	0.041	0.04	0.041

Table 4: MTT Assay Plate 2

Plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A	0.23	0.243	0.268	0.257	0.272	0.038	0.039	0.038	0.038	0.041	0.038	0.16
B	0.251	0.247	0.258	0.285	0.265	0.047	0.038	0.039	0.038	0.039	0.041	0.17
C	0.209	0.247	0.262	0.268	0.259	0.039	0.038	0.04	0.038	0.038	0.04	0.042
D	0.236	0.248	0.277	0.291	0.252	0.04	0.039	0.04	0.039	0.042	0.04	0.041
E	0.244	0.26	0.286	0.268	0.039	0.04	0.039	0.04	0.041	0.042	0.039	0.039
F	0.246	0.178	0.283	0.292	0.039	0.04	0.04	0.041	0.043	0.041	0.042	0.042
G	0.235	0.284	0.276	0.267	0.039	0.038	0.04	0.04	0.042	0.042	0.043	0.045
H	0.235	0.235	0.289	0.255	0.039	0.039	0.04	0.038	0.039	0.044	0.043	0.044

Table 5: MTT Assay Plate 3

Plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.494	0.475	0.473	0.557	0.039	0.039	0.039	0.038	0.044	0.039	0.044	0.188
B	0.532	0.527	0.536	0.562	0.041	0.04	0.039	0.04	0.04	0.039	0.04	0.196
C	0.543	0.555	0.563	0.531	0.041	0.041	0.04	0.04	0.039	0.041	0.043	0.042
D	0.607	0.511	0.531	0.435	0.04	0.045	0.044	0.044	0.042	0.041	0.044	0.04
E	0.508	0.685	0.382	0.424	0.649	0.04	0.041	0.044	0.041	0.041	0.04	0.039
F	0.656	0.621	0.588	0.431	0.653	0.04	0.039	0.043	0.044	0.039	0.04	0.042
G	0.64	0.597	0.601	0.525	0.657	0.041	0.04	0.041	0.043	0.041	0.044	0.044
H	0.701	0.554	0.34	0.503	0.512	0.039	0.04	0.039	0.04	0.041	0.04	0.041