Artemisinin's Effect on Iron Metabolism in Breast Cancer Cells

A Major Qualifying Project Report
Worcester Polytechnic Institute
Department of Biology and Biotechnology

Advisors: Mike Buckholt and Jill Rulfs

Authors: Daisy Connors and Clare Nargi

Abstract

Artemisia annua is a wormwood plant with active compound artemisinin that has been used in medicine as a treatment of malaria and fever. Previous research has found that artemisinin has demonstrated antitumor activity by affecting immunosuppression and cancer metabolism. The goal of this study was to determine if there are specific concentrations and time periods of *A. annua* and pure artemisinin solution exposure that lead to changes in T47D cell growth and investigate if artemisinin promotes degradation of ferritin in the cell, leading to ferroptosis. When T47D breast cancer cells were treated with pure artemisinin and *A. annua* tea extracts, the live cell count after 24 hours was approximately half of the control. Additionally, there were similar effects seen with MDA-MB-231 triple negative, or late stage, breast cancer cells. There were no significant effects seen on treated NIH/3T3 fibroblast cells. The concentration of intracellular iron was found to be more than two-times higher in the artemisinin and *A.annua* tea-treated T47D cells compared to the controls, alluding to the possibility of ferritin degradation by artemisinin. Future studies should continue to investigate artemisinin's mechanism of action in cancer cells to validate claims related to ferroptosis.

Acknowledgments

We would like to thank our amazing advisors, Professors Jill Rulfs and Mike Buckholt for all the encouragement and guidance throughout this MQP and helping us develop this project from start to finish. Thank you to Jill Rulfs for splitting our cells when we were not on campus throughout the year. Thank you to Mike Bocka for keeping the Project Lab stocked with supplies and ordering items to help us be successful throughout our project. Lastly, we would like to thank our friends and family members for all of their support during the time spent on this project and at WPI overall.

Table of Contents

1. Background

1.1 Introduction

Breast cancer is one of the most common forms of cancer diagnosed in women in the United States every year. In 2023, it was estimated that in the United States 297,790 new cases of invasive breast cancer would be diagnosed in women across the country (American Cancer Society, 2023). Many different forms of cancer treatment can be pursued based on the needs of the patient. The health status of the patient and the stage of the cancer are two major factors that need to be considered. Recent literature has looked at the mechanism of action of the compound artemisinin in cancer cells, and its potential to be a therapeutic approach in suppressing tumors (Zhang et al., 2014). In the context of cancer, excess free iron in cells may react with artemisinin's organic peroxide to create many free radicals, or reactive oxygen species (ROS), which are lethal to cells and can lead to cell death. Following the work of previous undergraduate studies at WPI this report looks at the effects of pure artemisinin solution and an *A. annua* tea extract on cancer cell and non-cancer cell lines.

1.2 Mechanisms of Cancer

Cancer is a disease of cell proliferation. Each cell division is an opportunity for a new mutation to be acquired and passed on, cancer causing mutations can promote proliferation. Cancer is also predominantly a genetic disease that can occur throughout all areas of the human body. Cancer cells develop qualities that allow them to grow in the absence of growth signals, and avoid the immune system response (National Cancer Institute, 2007). Every type of cancer has its genetic differences and each individual person's cancer has its own unique qualities.

1.3 Breast Cancer Prevalence and Treatment

For women in the United States, breast cancer is the most common cancer besides skin cancer. In 2023, The American Cancer Society estimated that 297,790 new cases of invasive breast cancer would be diagnosed in women across the country (American Cancer Society, 2023). The average age of a woman diagnosed with breast cancer in the United States is 62 years old. Breast cancer death rates have been found to be decreasing since 1989, which is likely due to new developments in the screening and treatment of breast cancer. But incidence rates of breast cancer have also been observed to be increasing by 0.5% each year (American Cancer Society, 2023).

As you get older the risk for developing most types of cancer increases. This is because cancer arises from the accumulation of genetic changes over time and this combination of mutations leads to cell transformation. As cells continue to acquire mutations as the cancer evolves this contributes to the genetic heterogeneity in the tumor. There are other risk factors for breast cancer as well including but not limited to family health history, inheritance susceptibility,

alcohol intake, and breast tissue density (National Cancer Institute, 2023). Primary prevention measures against cancer have the goal of keeping the cancerous process from ever developing. Secondary prevention strategies use screening and early detection to identify cancer early in its development. There are tests and procedures such as mammography, ultrasonography, breast magnetic resonance imaging (MRI), and biopsy that can be done to diagnose breast cancer. Following a diagnosis, breast cancer is most commonly treated with a variety of strategies which can range from surgery, radiation, chemotherapy and hormone therapy (National Cancer Institute, 2023). What type of therapy is used depends significantly on the patient that is being treated. The health status of the patient and the stage of the cancer are two major factors that need to be considered.

1.4 Artemisia annua and Artemisinin

Artemisia annua is an annual herbaceous herb native to six continents; it belongs to the plant family of *Asteraceae*, commonly known as the daisy family (Septembre-Malaterre et al., 2020). The genus *Artemisia* is also referred to as “sweet wormwood” because the plants’ extracts have been anciently used as an anthelmintic medicine to treat worm diseases (Mravčáková et al., 2020).

Figure 1: *Artemisia annua* (Septembre-Malaterre et al., 2020).

The plant has been used in traditional Chinese medicine for thousands of years as an herbal remedy for the treatment of malaria and fever. *Artemisia annua* possesses antibacterial, antioxidant, anti-inflammatory, and antifungal activities which makes it a promising cytotoxic agent against cancer cells (*Do Artemisia Annua Benefits Outweigh the Potential Risks?*, n.d.).

Artemisinin is an active compound derived from the sweet wormwood, *Artemisia annua*; the structure of artemisinin makes it a sesquiterpene lactone and organic peroxide (PubChem, n.d.).

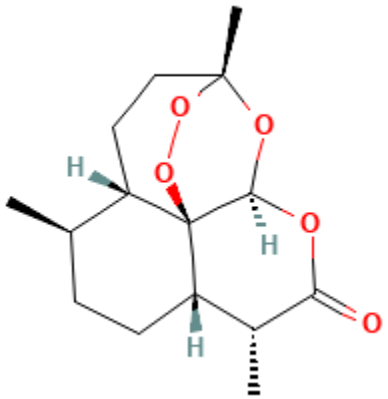

Figure 2: Organic chemical structure of Artemisinin (PubChem, n.d.).

A sesquiterpene lactone refers to a compound made up of three isoprene units bound to cyclic organic esters (Wen & Yu, 2011). Organic peroxides are characterized by a weak -O-O- structure and are considered to be structural derivatives of hydrogen peroxide. The weak peroxide bond makes organic peroxides predisposed to spontaneous decomposition, and these compounds are especially sensitive to catalysts that accelerate this decomposition process (Clark, 2001). Iron, for example, is a catalyst that can cleave artemisinin's endoperoxide bridge to decompose the peroxide into highly reactive hydroxyl radicals. The hydroxyl radicals that come from this reaction, known as the Fenton reaction, can cause detrimental biological damage in cells (Winterbourn, 1995).

1.5 Anti-Cancer Potential of *Artemisia annua*

1.5.1 Artemisinin and Immunosuppression

When thinking about the development of anticancer therapies, it is well-known that these therapies should have high potency and specificity in killing cancer cells without significant or fatal toxicity on normal, non-cancerous cells. Normal, non-cancerous cells are critical for the progression and spreading of a tumor throughout the body as they can reside in the tumor microenvironment. The tumor microenvironment denotes these non-cancerous cells and components that are presented in the tumor (i.e. blood vessels, extracellular matrix, etc.) which work together to inhibit immune function and reprogram the surrounding cells, ultimately enabling them to play a determinative role in tumor survival and progression (Hinshaw et al.).

In the tumor microenvironment, there are four types of non-cancerous cells that inhibit immune function including regulatory T cells, myeloid-derived suppressor cells, tumor-associated macrophages and cancer-associated fibroblasts. As a result, tumor-specific T cells are unable to enter tumor tissues or their functions are impaired after entering the tissues. This indicates that inhibiting immunosuppression will be beneficial for cancer therapy (Hinshaw & Shevde, 2019).

In a 2014 paper authored by Zhang et al, forty female mice were injected with cervical tumor ascites and randomly assigned artemisinin or distilled water as their treatments; After ten days, researchers found that artemisinin demonstrated antitumor activity by affecting immunosuppression and cancer metabolism in the experimental mice groups. Researchers involved in this study explored artemisinin's mechanism of inhibition of immune inhibitory molecules, including tumor growth factors. The 2014 paper illustrates artemisinin's capability of decreasing levels of growth factor PGE2 as well as decreasing the number of regulatory T-cells in the tumor microenvironment (Zhang et al., 2014). These results demonstrate a promising mechanism of action of the artemisinin compound in cancer cells, and pave a path for a potential therapeutic approach in suppressing tumors (Zhang et al., 2014).

1.5.2 Artemisinin and Regulated Cell Death

The mechanism of antitumor action of artemisinin that has been most confirmed by literature is the compound's role in regulated cell death. Artemisinin-induced cell death was first addressed as apoptosis, and it was discovered when artemisinin was being researched for its antimalarial effects. In malarial patients, artemisinin is activated by endoparasitic iron which catalyzes the cleavage of the compound's endoperoxide ring. When iron reacts with an organic peroxide, such as artemisinin's endoperoxide ring, a free radical intermediate is created which is thought to kill the parasite by alkylating and poisoning essential malarial proteins (Meshnick, 1994).

In the context of cancer, excess free iron in cells may react with artemisinin's organic peroxide to create many free radicals, or reactive oxygen species (ROS), which are lethal to cells. A surplus of ROS in cells activates regulated cell death due to the high oxidative stress the cell undergoes (Zhou et al., 2022).

Regulated cell death is a lethal process resulting in the elimination of unwanted cells through tightly structured signaling cascades and molecularly defined effector mechanisms due to intracellular or extracellular stress. Among all classified regulated cell death types, artemisinin-induced cell death has been linked to apoptosis, autophagy, and ferroptosis (Zhou et al., 2022).

1.6 Previous MQP Research

The Major Qualifying Project (MQP) is completed by every undergraduate at WPI prior to graduation. The MQP is a team-based, professional-level research experience. The ProjectLab is used by students to work on projects directly affiliated with department research. Previous WPI undergraduate students have explored the effects of *A. annua* and artemisinin on cancer cell lines. In the prior academic year a student investigated the relationship between pure artemisinin and *A. Annua* tea extract when cultured with various cancer cell lines and non cancer cell lines. Additionally, this study investigated the effects of *A. afra* and *A.absinthium* on a cancer cell line. These strains were used because they are plants from the same genus as A. annua, but have

different chemical properties. Notably the compound artemisinin is not found in A.afra. The results of this MQP determined that the absolute cell number in T47D breast cancer cells was reduced in the presence of *A. annua* tea extract (Cyr, 2022). Prior to this in the 2020-2021 academic year, students used T47D breast cancer cells and studied the plant's effects on cell proliferation, and examined markers of cell cycle regulation and oxidative stress to understand the potential mechanisms of action (Nguyen & Spera, 2021). Future directions from this MQP were to look into testing for the presence of free radicals following exposure to artemisinin.

1.7 Cell Lines (T47D, MDA MB 231, NIH/3T3)

There are three different cell lines being used in this MQP. T-47D cells are cells that have been isolated from a 54 year old female patient with an infiltrating ductal carcinoma of the breast (ATCC, n.d.). The cells are epithelial cells that can be used in breast cancer research like this MQP. T47D cells have shown promising results in previous MQP research. In 2022, it was found that pure artemisinin and A. annua tea extracts show statistically significant effects when plated with T47D cells. The compound and the tea extract had an effect on the absolute cell number of T47D cells (Cyr, 2022). As described above, beyond work at WPI, there has been other research completed elsewhere that also is investigating the anti-cancer claims about A. annua and artemisinins. Artemisinins have shown broad anticancer properties by being tested in a variety of different cell lines, including breast cancer cell lines (Yang, 2014). MDA MB 231 cells are cells that have been isolated from a 51 year old female patient with chromosome counts in the near-triploid range (ATCC, n.d.). Similar to T-47D cells, the cells are epithelial cells that can be used in breast cancer research. MDA MB 231 cells were chosen to be included in research to observe the effects of artemisinin and *A. annua* tea extracts on a more differentiated phenotype compared to T47D cells. NIH/3T3 cells are cells that have been isolated from a mouse NIH/Swiss embryo (ATCC, n.d.). NIH/3T3 are a non-cancerous fibroblast cell line. NIH/3T3 cells were chosen to be included in research with artemisinin and *A. annua* tea extracts to determine if these extracts had the same effect on all cell types or if they were able to attack cancer cells specifically.

1.8 Iron Metabolism Mechanism

Cytoplasmic iron is mainly stored in the protein ferritin, which is critical for the maintenance of iron metabolism and protection of cells from oxidative damage. Ferritin functions to release iron in a controlled manner or when the body needs it (Yang et al., 2014).

Excessive iron ions that are not coupled with ferritin, or another iron-storage protein, result in increased levels of reactive oxygen species (ROS). This is because concentrations of free iron in the cells will allow these ions to react with peroxides which facilitates the Fenton reaction. The Fenton reaction induces the oxidation of ferrous iron (Fe^{2+}) to form ferric iron (Fe^{3+}), and this happens alongside the conversion of hydrogen peroxide to form hydroxyl

radicals. Free iron can also react with organic peroxides to generate hydroxyl radicals (Yan et al., 2021).

Excess levels of these ROS hydroxyl radicals can cause significant damage to essential building blocks of the cell, including proteins, DNA, and lipids (Hu et al., 2022). A specific type of damage these hydroxyl radicals can cause is known as lipid peroxidation, which is the process in which free radicals attack and oxidize lipids containing a carbon-carbon double bond and results in the formation of lipid peroxyl radicals and hydroperoxides (Ayala et al., 2014). Excessive oxidation of lipids alters the physical properties of cellular membranes; this disruption of the cellular membrane stimulates a specific form of regulated cell death known as ferroptosis (Ayala et al., 2014). Ferroptosis is cell death from iron-dependent lipid peroxide accumulation, and it has been found to play a pivotal role in tumor suppression, thus providing new opportunities for cancer therapy (Zhang et al., 2014).

A specific cytotoxic compound that has potential of becoming a new cancer therapy is artemisinin. Artemisinin is known to induce lysosomal degradation of ferritin in an autophagy-dependent manner which releases free iron in the cells (Chen et al., 2020). As previously mentioned, artemisinin is an organic peroxide due to its -O-O- cyclic structure, and can be cleaved by this free iron at its endoperoxide bridge to form hydroxyl radicals. The metabolism and proliferation rate of cancer cells are generally higher than that of normal cells, so their demand for iron is also significantly higher than that of normal cells, leading to an excess of free iron in the cells to ultimately cause exceeding oxidative stress via these hydroxyl radicals. Notably, these hydroxyl radicals can partake in lipid peroxidation to ultimately induce ferroptosis in cells (Guo et al., 2021).

1.9 Hypothesis & Objectives

Looking into how potential cancer therapies can balance patient survival and resulting side effects that cause poor quality of life is an important endeavor. Many cancer treatments have been found to have negative side effects as they attack rapidly growing cancer cells. It is hypothesized that when T47D breast cancer cells are treated with a pure artemisinin solution and an *A. annua* tea extract, these cancer cells will undergo cell death. The mechanism of action that causes this cell death is a specific form of apoptosis, known as ferroptosis. Artemisinin promotes degradation of ferritin which leads to free iron in cells and promotes the formation of hydroxyl radicals; this mechanism results in regulated cell death by ferroptosis resulting in an observed change in cell numbers following treatment.

2. Methods

2.1 Experimental Compound Preparation

To develop the pure artemisinin solution, similar methods from previous MQP projects were followed. On the Cayman Chemical website the product information sheet for artemisinin was downloaded. From this sheet, the solubility of artemisinin in ethanol was found to be 16 mg/ml. Knowing the solubility of artemisinin in ethanol and the molar mass of artemisinin the maximum stock concentration of artemisinin that could be produced when dissolving artemisinin in pure ethanol was calculated. To produce a stock concentration of 50 mM artemisinin, 50 mg of powdered artemisinin was dissolved in 3.53 mL of pure ethanol.

To prepare the *Artemisia annua* tea, the hot water extraction method that was used in previous MQP projects was followed. To begin, 200 ml of water was brought to a boil in a beaker on a hot plate. Once boiling, ~2 grams of dried *A. annua* was measured out on a balance and added to the water, and thoroughly mixed with a stirring rod continuously. After steeping for 15 minutes, the beaker was taken off the hot plate, and large pieces of plant were sieved out using filter paper. Following the removal of larger pieces, the tea was then filtered through a 0.2μm filter in order to sterilize the tea. Following filtration, the tea was allotted into 50mL centrifuge tubes, and frozen at -20°C for future use.

2.2 Cell Culture and Maintenance

A frozen stock of T47D cells that were kept in liquid nitrogen vapor were thawed. The vial of cells were thawed in a 37 ℃ water bath, added to a vial containing culture medium, spun, resuspended, and then dispensed into the T75 flask. After being plated the culture was incubated at 37 ℃ in the lab humidified incubator with a 5% CO_2 atmosphere. Cells were split approximately every three days using trypsin to resuspend the cells once they reached 70-80% confluency which is when there is approximately 6.5 x 10^5 cells/mL in the T-75 flask. The T47D cells were cultured in DMEM with 10% Fetal Bovine Serum and 1% PenStrep. In order to determine the appropriate subcultivation ratio, a Cellometer ((Nexcelom (Cellometer K2 Fluorescent Cell Counter)) was used to complete cell counts. Cells were then seeded in T-75 flasks at a density of approximately 2 x 10^6 cells/mL.

MDA MB 231 and NIH/3T3 cells were also cultured in DMEM with 10% Fetal Bovine Serum and 1% PenStrep. They were maintained in T-75 flasks and stored at 37 ℃ in the lab incubator with a 5% CO_2 atmosphere. Cells were split approximately every three days using trypsin to resuspend the cells once they reached 70-80% confluency which is when there is approximately 6.5 x 10^5 cells/mL in the T-75 flask. Cells would be replated at the seeding density of the T-75 flask which is approximately 2 x 10^6 cells/mL or lower depending on the needs of experiments.

2.3 Cellometer Cell Counting

When the amount of cells present per milliliter of culture media was needed to be noted following resuspension of cells in media, equal volumes of the cell culture and Trypan blue were mixed to determine viability. Then 20 uL of that cell suspension was then pipetted into a Cellometer slide. This slide was then inserted into the Cellometer. The Cellometer then reports back the live cell count, dead cell count, mean diameter, viability (%), and live cell concentration (cells/mL). Cell counting was performed before plating in flasks or well plates to ensure the appropriate number of cells were plated.

2.4 Dose Response Tests

In order to evaluate the dose of pure artemisinin and *A. annua* tea required to produce an effect on T47D, MDA MB 231 and NIH/3T3 live cell count, a dose response assessment on all three cell lines was performed. For the pure artemisinin dose response assessment, cells were plated at a density of $1x10^5$ cells/ml into a multiwell plate. Triplicate wells were treated with varying concentrations of artemisinin. One set of triplicates was treated with 1% ethanol as a control. Cells were left to plate down overnight in a 37^{o}C incubator with a 5% CO2 atmosphere.

After cells plated down, all nine wells were treated with the appropriate solutions prepared earlier. The final concentrations in the wells were 1% ethanol as the control and either 10 or 50uM artemisinin. After treatment, the well plate was placed back in the incubator for 24 hours and all wells were harvested using the method described in section 2.2 and a cell count was performed using the Cellometer method described in section 2.3. Total cell count, live cell count, and percent viability for all cell wells were recorded and the averages of each of the three replicates was determined. Using the averages, percent control was calculated by dividing the average live cell count of cells treated with 10uM and 50uM artemisinin by the average live cell count of cells treated with the 1% ethanol control then multiplied by one hundred. Graphs were constructed based on the percent control.

For the *A. annua* tea dose response assessment, cells were plated at a density of $5x10^4$ cells/ml into a multiwell plate. Triplicate wells were treated with varying concentrations of *A.annua* treatments or distilled water. Cells were left to plate down overnight in a 37^{o}C incubator with a 5% CO2 atmosphere. The stock of *A. annua* tea prepared in section 2.1 was thawed completely in a 37^o C water bath. The *A. annua* tea stock was then filtered into a new 50mL conical tube using a disposable Steriflip with a pore size of 0.2 µm. Samples of distilled water control, 1:2 tea extract to distilled water, 1:5 tea extract to distilled water, and 1:10 tea extract to distilled water were prepared.

After cells plated down, all twelve wells were treated with the appropriate solutions prepared earlier. After treatment, the microplate was placed back in the incubator for 24 hours and all wells were harvested using the method described in section 2.2 and a cell count was performed using the Cellometer method described in section 2.3. Total cell count, live cell count,

and percent viability for all cell wells were recorded and the averages of each of the three replicates was determined. Using the averages, percent control was calculated by dividing the average live cell count of cells treated with 1:2 tea extract, 1:5 tea extract, and 1:10 tea extract by the average live cell count of cells treated with the distilled water control. Graphs were constructed based on the percent control.

2.5 Time Course Testing

The time course tests were conducted with the T47D cell line and different concentrations of pure artemisinin solution and *A. annua* tea extract to observe the effect on live cell concentration over time. As in section 2.4 cells were plated at a density of $1x10^5$ cells/ml or $5x10^4$ cells/ml into a multiwell plate and treated with chosen concentrations of artemisinin or *A. annua* tea. Based on results from dose response testing, the concentration of pure artemisinin solution tested over time was 50 uM, along with a control of 1% ethanol. The *A. annua* tea extract concentrations that were tested were 1:2 TE and 1:5 TE, along with a control of distilled water.

Once the T47D cells were subcultured and resuspended in fresh media, the appropriate number of cells for each well plate were plated in the necessary number of wells to satisfy the time points which were 24, 48, and 72 hours. All experimental time points were plated in duplicates. Cells were then left to plate down overnight in the 37 ℃ incubator with a 5% CO_2 atmosphere. After cells plated down, cells were treated with the appropriate solutions prepared earlier.

After being treated, the plates were incubated in the 37 ℃ incubator with a 5% CO_2 atmosphere in the lab for 24 hours. After 24 hours the necessary wells were harvested and a cell count was performed using the Cellometer method in section 2.3. After completion, the well plates were put back into the incubator. This same method was repeated at 48 and 72 hours. The following information was recorded for each time point: cell count pre-treatment, live cell concentration, total cell concentration, and viability. From these time points the averages were calculated from the two replicates. Using the averages, percent control was calculated by dividing the average live cell count of the treated cells by the average live cell count of the control cells. Graphs were then constructed based on the percent control.

2.6 Iron Assay

In order to determine if artemisinin promotes degradation of ferritin in the cell, an iron assay kit was purchased to evaluate the levels of iron in treated and non-treated T47D cells. The iron assay kit used was purchased from Abcam – Iron Assay Kit (Colorimetric) (ab83366). This kit can be used to measure ferrous and/or ferric ions in various types of samples including cell cultures (*Iron Assay Kit (Colorimetric) (Ab83366)*, n.d.). Cells were plated at a density of $2x10^6$ cells/ml into T-47D flasks and left to incubate overnight. Flasks were then treated with chosen concentrations of artemisinin and *A. annua* tea. Final concentrations in the flasks were 50 uM

artemisinin, 1:2 *A. annua* tea extract, 1:5 *A. annua* tea extract, and 1% ethanol and distilled water controls. These treated flasks were incubated for 24 hours and then cells were subcultured. Using a Cellometer, approximately 1 x 10^6 cells were isolated and resuspended in 100 uL of Iron Assay Buffer given with the kit. The Iron Assay Buffer was used to lyse the cells. Once the cells were resuspended in the buffer, the sample was passed through a 26 gauge needle and blue microfuge tube grinders were applied to the samples for additional lysing. Following this step, the protocol that came with the kit was followed exactly. Due to setting on our colorimetric microplate reader output was measured at OD 595 nm instead of OD 593 nm as recommended. An iron assay test using the same method described above was also run on all three cell types untreated to determine T47D, NIH3T3 and MDA-MB 231 iron stores. Readings from the colorimetric microplate reader were recorded and calculations were done as described in the protocol using the standard curve generated at the start of each assay. Graphs were constructed comparing the total iron concentrations with different treatments.

3. Results

3.1 Dose Response Assessments

The section below highlights results from the dose response testing which was conducted to determine if there were any observable effects of pure artemisinin and *A. annua* tea on T47D, MDA MB 231 and NIH/3T3 live cell counts pre- and post-treatment.

Table 1: Results from *A. annua* tea treated T47D cells after 24hrs.

Treatment Type	**Average Live Cell Concentration Pre-Treatment (cells/mL)**	**Average Live Cell Concentration Post-Treatment (cells/mL)**	**Average Total Cell Concentration Post-Treatment (cells/mL)**	**Average Viability Post-Treatment**	**Average Live Cell % Control**
Distilled water control	$2.42x10^4$	$9.11x10^4$	$9.70x10^4$	94.03%	100%
1:2 Tea Extract	$2.42x10^4$	$3.78x10^4$	$4.33x10^4$	86.97%	41.49%
1:5 Tea Extract	$2.42x10^4$	$5.78x10^4$	$6.89x10^4$	84.50%	63.45%
1:10 Tea Extract	$2.42x10^4$	$9.21x10^4$	$9.94x10^4$	92.87%	101.01%

As seen in table 1, the live T47D cell concentration pretreatment was $2.42x10^4$ for all possible treatments to ensure accurate comparisons amongst the various treatment types. After 24 hours of treatment, the average live cell concentration in the distilled water treated cells and the 1:10 tea extract treated cells went up to $>9x10^4$, meaning that the 1:10 tea extract cells were approximately 100% of the control. The 1:5 tea extract treated cells had a live cell concentration of $5.78x10^4$ after 24 hours which calculated a 63.45% control. Similarly, the 1:2 tea extract treated cells had a live cell concentration of $3.78x10^4$ after 24 hours of treatment and therefore had the lowest live-cell-percent-control of 41.49%. All of these experiments were done in triplicates, and the final percent controls calculated are the averages of all three experiments performed.

Table 2: Results from pure artemisinin treated T47D cells after 24hrs.

Treatment Type	**Average Live Cell Concentration Pre-Treatment (cells/mL)**	**Average Live Cell Concentration Post-Treatment (cells/mL)**	**Average Total Cell Concentration Post-Treatment (cells/mL)**	**Average Viability Post-Treatment**	**Average Live Cell % Control**
1% EtOH control	$5.15x10^4$	$1.27x10^5$	$1.36x10^5$	83.17%	100%
10 uM artemisinin	$5.15x10^4$	$1.40x10^5$	$1.54x10^5$	91.50%	110.24%
50 uM artemisinin	$5.15x10^4$	$6.80x10^4$	$8.36x10^4$	82.00%	53.54%

In table 2, for all possible treatment types, the live T47D cell contraction was $5.15x10^4$ cells pretreatment in order to accurately compare all treatment types after the 24 hour incubation period. The post-treatment live cell concentration in both the 1% EtOH treated cells and the 10uM artemisinin treated cells were similar, measuring over $1x10^5$ cells. The 10uM artemisinin cells interestingly have a greater live cell concentration than the control post-treatment, meaning that the live-cell-percent control was over 100% for these cells. After 24 hours of 50uM artemisinin treatments, however, the live cell concentration was measured at only $6.8x10^4$, ultimately giving a low live-cell-percent control at 53.54%. All of these experiments were done in triplicates, and the final percent controls calculated are the averages of all three experiments performed.

Table 3: Results from *A. annua* tea treated NIH 3T3 cells after 24hrs.

Treatment Type	**Average Live Cell Concentration Pre-Treatment (cells/mL)**	**Average Live Cell Concentration Post-Treatment (cells/mL)**	**Average Total Cell Concentration Post-Treatment (cells/mL)**	**Average Viability Post-Treatment**	**Average Live Cell % Control**
Distilled water control	$5.0x10^4$	$8.73x10^4$	$9.41x10^4$	93.40%	100%
1:2 Tea Extract	$5.0x10^4$	$8.42x10^4$	$9.25x10^4$	91.00%	98.30%
1:5 Tea Extract	$5.0x10^4$	$8.22x10^4$	$8.91x10^4$	92.20%	94.16%

Table 3 illustrates the results after 24 hours of distilled water control and *A. annua* tea treatments in NIH 3T3 cells when each well pretreatment started at the same live concentration of $5.0x10^4$. After 24 hours of treatment, all treatment types had similar live cell concentrations of ~$8x10^4$ cells. Due to this, the live-cell-percent-control for the 1:2 tea extract and 1:5 tea extract treated cells were both very high, calculating at 98% and 94%. All of these experiments were done in

triplicates, and the final percent controls calculated are the averages of all three experiments performed.

Table 4: Results from pure artemisinin tea treated NIH 3T3 cells after 24hrs.

Treatment Type	**Average Live Cell Concentration Pre-Treatment (cells/mL)**	**Average Live Cell Concentration Post-Treatment (cells/mL)**	**Average Total Cell Concentration Post-Treatment (cells/mL)**	**Average Viability Post-Treatment**	**Average Live Cell %Control**
1% EtOH control	$1.0x10^5$	$1.71x10^5$	$1.86x10^5$	91.70%	100%
50 uM artemisinin	$1.0x10^5$	$1.58x10^5$	$1.70x10^5$	92.70%	91.40%

As seen in table 4, the live NIH 3T3 cell concentration pretreatment was $1.0x10^5$ for all possible treatments in order to accurately compare the posttreatment cell counts. Similarly to the results seen in table 3, the 1% EtOH and 50uM artemisinin treated cells had very similar live cell concentrations after 24 hours: the 1% EtOH control cells had a live cell count of $1.71x10^5$, while the 50uM artemisinin cells were at $1.58x10^5$. Due to this, the live-cell-percent-control in the 50uM artemisinin treated NIH 3T3 cells was high, calculating at 91.4%. All of these experiments were done in triplicates, and the final percent controls calculated are the averages of all three experiments performed.

Table 5: Results from *A. annua* tea treated MDA MB 231 cells after 24hrs.

Treatment Type	**Average Live Cell Count Pre-Treatment (cells/mL)**	**Average Live Cell Concentration Post-Treatment (cells/mL)**	**Average Total Cell Concentration Post-Treatment (cells/mL)**	**Average Viability Post-Treatment**	**Average Live Cell % Control**
Distilled water control	$3x10^4$	$3.49x10^5$	$3.83x10^5$	91.30%	100%
1:2 Tea Extract	$3x10^4$	$1.24x10^5$	$1.52x10^5$	81.80%	35.50%
1:5 Tea Extract	$3x10^4$	$1.72x10^5$	$2.02x10^5$	84.90%	49.30%

As seen in table 5, the live MDA MB 231 cell concentration pretreatment was $5.10x10^4$ for all treatment types. After 24 hours of treatment, the average live cell concentration in the distilled water treated cells went up to $3.49x10^4$. Both of the experimental treatments, being the 1:5 and 1:2 tea extracts, the live cell concentration dwindled significantly after 24 hours of treatment. The 1:5 tea extract treated cells had a live cell contraction of $1.72x10^5$ posttreatment which calculated a live-cell-percent-control at only 49.3%. Similarly, the 1:2 tea extract treated cells had a live cell concentration of $1.24x10^5$ posttreatment, which measures a live-cell-percent

control at only 35.50%. All of these experiments were done in triplicates, and the final percent controls calculated are the averages of all three experiments performed.

Table 6: Results from pure artemisinin treated MDA MB 231 cells after 24 hrs.

Treatment Type	**Average Live Cell Count Pre-Treatment (cells/mL)**	**Average Live Cell Concentration (cells/mL)**	**Average Total Cell Concentration (cells/mL)**	**Average Viability Post-Treatment**	**Live Cell % Control**
1% EtOH Control	$1x10^5$	$3.71x10^5$	$4.46x10^5$	83.20%	100%
50 uM Artemisinin	$1x10^5$	$2.45x10^5$	$2.61x10^5$	93.60%	66%

In table 6, the pretreatments started at the same live cell concentrations of $1x10^5$ MDA MB 231 cells in order to accurately compare the post-treatment results. The 1% EtOH treated cells measured a live cell concentration of $3.71x10^5$ cells after 24 hours of treatment. The 50uM artemisinin treated cells, however, measured roughly ⅓ of this number at $2.45x10^5$ cells after the 24 hour treatment period. Due to this, the live-cell-percent-control for the 50uM artemisinin treated MDA MB 231 cells was approximately 66%. All of these experiments were done in triplicates, and the final percent controls calculated are the averages of all three experiments performed.

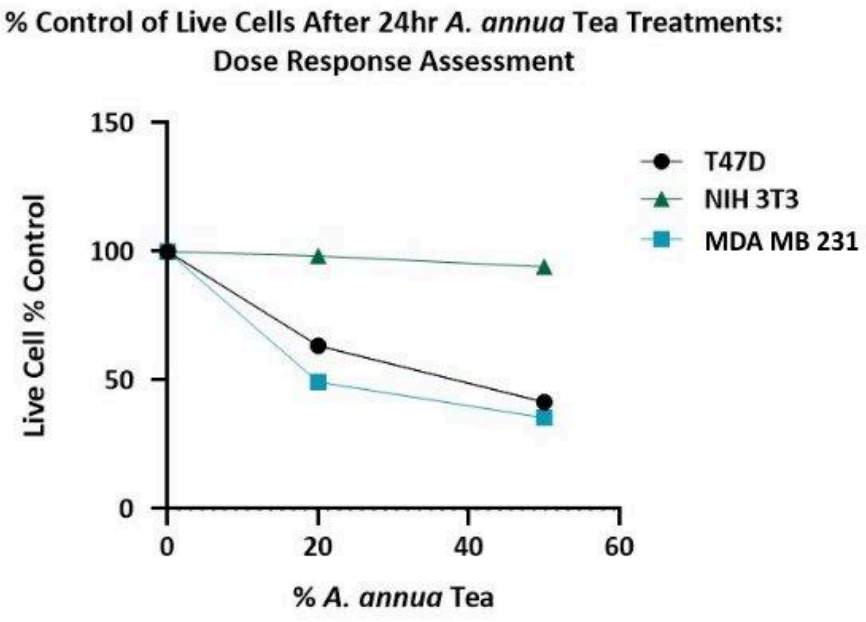

Figure 1: Line graph illustrating how 24 hour *A. annua* tea treatments compare in three different cell types: T47D, NIH3T3, MDA MB 231

Figure 1 shows the results from the *A. annua* tea dose response experimental results in the T47D, NIH 3T3, and MDA MB 231 cells which were all compared in order to visualize the treatment's effects on different stages of cancer cells vs. non-cancer cells. As seen in figure 1, the 1:5 tea extract (20% *A. annua* tea) treatments after 24 hours had similar effects on live-cell-percent-control in both the T47D and MDA MB 231 cancer cells, with the percent control being ~60% in T47D cells and ~50% in MDA MB 231 cells. Similarly, the 1:2 tea extract (50% A. annua tea) treatments after 24 hours in both the T47D and MDA MB 231 cells decreased the live-cell-percent-control significantly, with the percent control being ~40% in T47D and ~35% in MDA MB 231 cells. Conversely, figure 1 shows that the NIH 3T3 non-cancer cells stay relatively consistent after both 24 hour *A. annua* tea treatments; the live-cell-percent control was above 90% in both 1:5 tea extract and 1:2 tea extract treated NIH 3T3 cells.

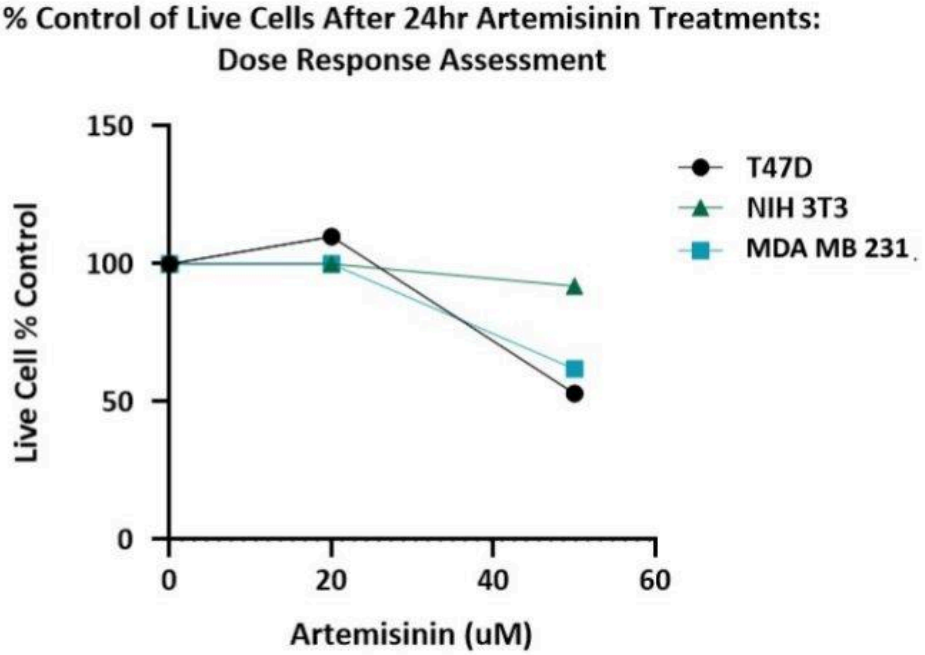

Figure 2: Line graph illustrating how 24 hour pure artemisinin treatments compare in three different cell types: T47D, NIH3T3, MDA MB 231

In Figure 2, the pure artemisinin dose response experimental results in the T47D, NIH 3T3, and MDA MB 231 cells were compared in order to visualize the compound's effect on different stages of cancer vs. noncancer cells. As seen in figure 2, the 10uM Artemisinin treated cells had little to no effect on all cell types as the live-cell-percent-control was measured to be at or over 100% in both the cancer and noncancer cell lines. The 50uM artemisinin treated cells in the T47D and MDA MB 231 cells had a significant effect on live-cell-percent control after 24 hours.

The live-cell-percent-control went down to ~50% in the T47D cells and ~60% in the MDA MB 231 cells. The NIH 3T3 cells, however, had little to no effect on the 50uM artemisinin treatments as the live-cell-percent-control stayed above 90% after the 24 hour treatment period.

3.2 T47D Time Course Tests

The below section highlights results from the time course testing which was conducted with the T47D cell line and different concentrations of pure artemisinin solution and *A. annua* tea extract to determine if there was an observable effect on live cell concentration over time.

Table 7: Results from *A. annua* tea treated T47D cells after 24, 48, and 72 hours

Treatment Type	**Average Live Cell Concentration Pre-Treatment (cells/mL)**	**Average Live Cell Concentration Post-Treatment (cells/mL)**	**Average Total Cell Concentration Post-Treatment (cells/mL)**	**Average Viability Post-Treatment**	**Average Live Cell % Control**
24 Hours Distilled Water Control	$4.64x10^4$	$5.135x10^4$	$5.275x10^4$	97.75%	100%
24 Hours 1:2 Tea Extract	$4.64x10^4$	$1.64x10^4$	$2.19x10^4$	75%	31.94%
24 Hours 1:5 Tea Extract	$4.64x10^4$	$1.665x10^4$	$1.945x10^4$	89%	32.42%
48 Hours Distilled Water Control	$4.64x10^4$	$8.58x10^4$	$8.72x10^4$	98.60%	100%
48 Hours 1:2 Tea Extract	$4.64x10^4$	$4.44x10^4$	$4.71x10^4$	94.50%	51.70%
48 Hours 1:5 Tea Extract	$4.64x10^4$	$5.66x10^4$	$6.48x10^4$	87.10%	66.00%
72 Hours Distilled Water Control	$4.64x10^4$	$8.48x10^4$	$9.03x10^4$	93.90%	100%
72 Hours 1:2 Tea Extract	$4.64x10^4$	$2.93x10^4$	$3.48x10^4$	83.75%	34.60%
72 Hours 1:5 Tea Extract	$4.64x10^4$	$5.54x10^4$	$5.815x10^4$	95.25%	65.33%

As seen in table 7, the live T47D cell concentration pretreatment was $4.64x10^4$ cells/mL for all possible treatments and times to ensure accurate comparisons amongst the various treatments and time points. After 24 hours of treatment, the 1:5 tea extract treated cells had a live cell concentration of $1.665x10^4$ which calculates to 32.42% of control. Very similarly, the 1:2 tea extract treated cells had a live cell concentration of $1.64x10^4$ which calculates a 31.94% control. After 48 hours of treatment, the 1:5 tea extract treated cells had a live cell concentration of $5.66x10^4$ which calculates a 66.0% control. Similarly, the 1:2 tea extract treated cells had a live cell concentration of $4.44x10^4$ which calculates a 51.70% control. After 72 hours of treatment, the 1:5 tea extract treated cells had a similar live cell concentration to 48 hours of $5.54x10^4$ which calculates a 65.33% control. And the 1:2 tea extract treated cells had an live cell concentration of $2.93x10^4$ cells which calculates a 34.60% control. All of these experiments were done in triplicates, and the final percent controls calculated are the averages of all three experiments performed.

Table 8: Results from pure artemisinin treated T47D cells after 24, 48, and 72 hours

Treatment Type	**Average Live Cell Concentration Pre-Treatment (cells/mL)**	**Average Live Cell Concentration Post-Treatment (cells/mL)**	**Average Total Cell Concentration Post-Treatment (cells/mL)**	**Average Viability Post-Treatment**	**Average Live Cell % Control**
24 Hours 1% Ethanol Control	$9.99x10^4$	$2.44x10^5$	$2.62x10^5$	93.40%	100%
24 Hours 50 uM artemisinin	$9.99x10^4$	$1.06x10^5$	$1.18x10^5$	90.80%	43.70%
48 Hours 1% Ethanol Control	$9.99x10^4$	$2.09x10^5$	$2.23x10^5$	93.30%	100%
48 Hours 50 uM artemisinin	$9.99x10^4$	$7.32x10^4$	$8.01x10^4$	91.40%	35.02%
72 Hours 1% Ethanol Control	$9.99x10^4$	$1.97x10^5$	$2.04x10^5$	96.70%	100.00%
72 Hours 50 uM artemisinin	$9.99x10^4$	$1.30x10^5$	$1.39x10^5$	92.95%	65.90%

In table 8, the live T47D cell concentration pretreatment was $9.99x10^4$ cells/mL in each of the multi wells for all possible treatments and times to ensure accurate comparisons amongst the various treatments and time points. After 24 hours of treatment, the 50 uM artemisinin treated cells had a live cell concentration of $1.06x10^5$ which calculates a 43.70% control. After 48 hours of treatment, the 50 uM artemisinin treated cells had a live cell concentration of $7.32x10^4$ which

calculates a 35.02% control. All of these experiments were done in triplicates, and the final percent controls calculated are the averages of all three experiments performed.

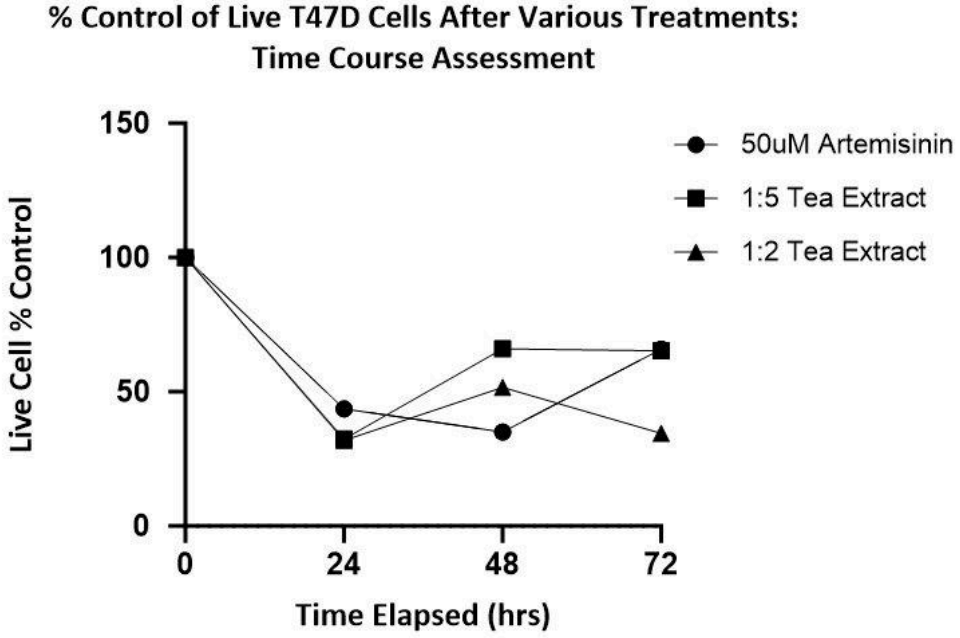

Figure 3: Line graph illustrating how pure artemisinin and *A. annua* tea treatments compare over time in T47D cells

The time course experiment with data from T47D cells was conducted to visualize the compound's effect on cancerous cells over time. By constructing this graph the time point where we see the greatest effect on the T47D cells can be visualized. As seen above, there was a significant effect on the live-cell-percent control with all three treatment types at the 24 hour mark. That significant effect was also seen at time points 48 and 72 hours. With all treatments and time points the percent control was 66.0% or lower.

3.3 Iron Assay Experiments

The final series of experiments that were performed were the iron assay tests in order to determine the total iron concentration in treated T47D cells as well as the total iron concentration in untreated T47D, NIH 3T3, and MDA MB 231 cells.

In order to ensure that the Abcam iron assay kit worked as intended, a standard curve was constructed using varying dilutions of the provided iron standard reagent.

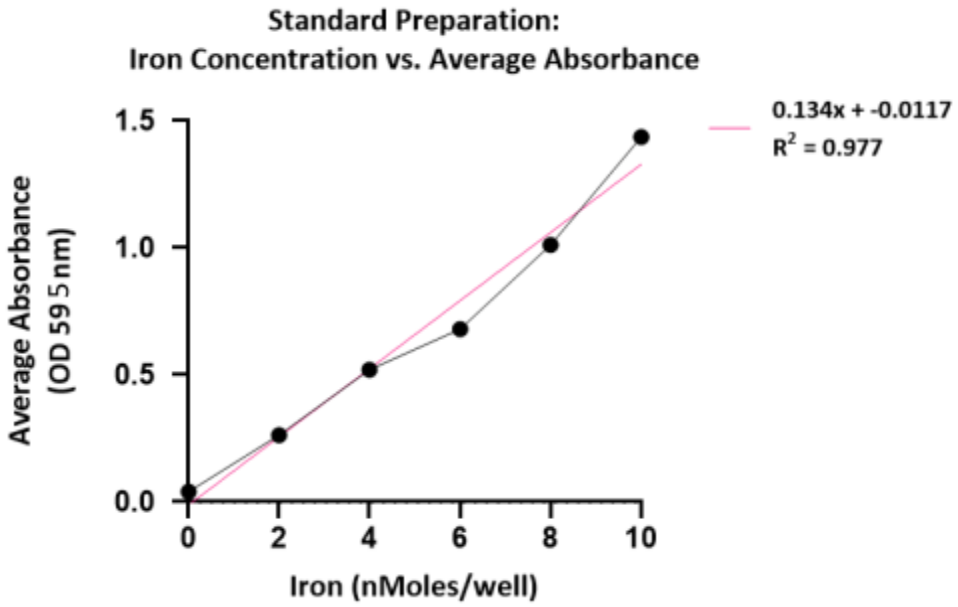

Figure 4: Standard curve of OD595 readings after measuring the iron concentration in various dilutions of the iron standard given in the Abcam iron assay kit.

As seen in figure 4, the average absorbance measured at 595 nm went up in a linear fashion as the amount of iron increased in the wells. The R^2 value was calculated at 0.977, demonstrating that this model can allow for accurate calculations of iron concentration in cells based on the OD 595 value. Notably, the average absorbance at OD 595 nm for 0 nMoles/well of iron was 0.0405, so this number was used to obtain the corrected absorbance of all samples to ensure accurate iron concentration calculations. The trendline equation was calculated to be 0.134x + (-0.117), where "x" will represent the corrected absorbance of each of the samples obtained after each iron assay, and the result will yield the iron concentration in nMoles per well.

The iron assay was run as described in section 2.6 with $2.0x10^6$ T47D cells in each well to ensure for accurate comparisons amongst treatment types.

Table 9: Total iron concentration in T47D cells after 24 hour *A. annua* tea treatments based on OD 595 readings

Treatment Type	**Average Absorbance (OD 595 nm)**	**Average Corrected Absorbance**	**Average Iron Concentration (nMol/well)**	**Average Iron Concentration (pMol/well)**
DW Control	0.053	0.0125	0.18059	180.60
1:2 Tea Extract	0.159	0.1185	0.97164	971.64
1:5 Tea Extract	0.083	0.0425	0.40447	404.45

Table 9 illustrates the obtained optical densities at 595 nm for each distilled water control and *A. annua* tea samples. The average optical density obtained in the distilled water control sample was 0.053, which correlates to a corrected absorbance of 0.0125. When the 0.0125 absorbance was used in the trendline equation seen in figure 4, the total average iron concentration for T47D cells treated with a distilled water control was calculated at 180.60 picoMolar per well. The same steps were performed to obtain the total iron concentration in the experimental samples, and it was calculated that cells treated with a 1:5 *A. annua* tea extract for 24 hours contained 404.45 pMol/well of iron. The highest amount of iron from the A. annua tea experiments was seen in cells treated with a 1:2 tea extract treatment, as these wells yielded an average iron concentration of 971.64 pMol/well.

Table 10: Total iron concentration in T47D cells after 24 hour pure artemisinin treatments based on OD 595 readings

Treatment Type	**Average Absorbance (OD 595 nm)**	**Average Corrected Absorbance**	**Average Iron Concentration (nMol/well)**	**Average Iron Concentration (pMol/well)**
1% EtOH Control	0.112	0.072	0.62089	620.90
50 uM Artemisinin	0.269	0.229	1.79253	1,792.5

Iron assays were also performed on T47D cells treated with a 1% EtOH control and the 50uM artemisinin solutions. As seen in table 10, the 1% EtOH cells had an average absorbance of 0.112 at OD 595 nm, or a corrected absorbance of 0.072. From this, the iron concentration of the 1% EtOH treated cells was found to be 620.90 pMol/well. The 50uM artemisinin treated cells had a very high absorbance in comparison to the control, with the corrected absorbance being 0.229. Using the trendline equation, it was determined that the average iron concentration in T47D cells treated with 50uM artemisinin was approximately 1,793 pMol/well.

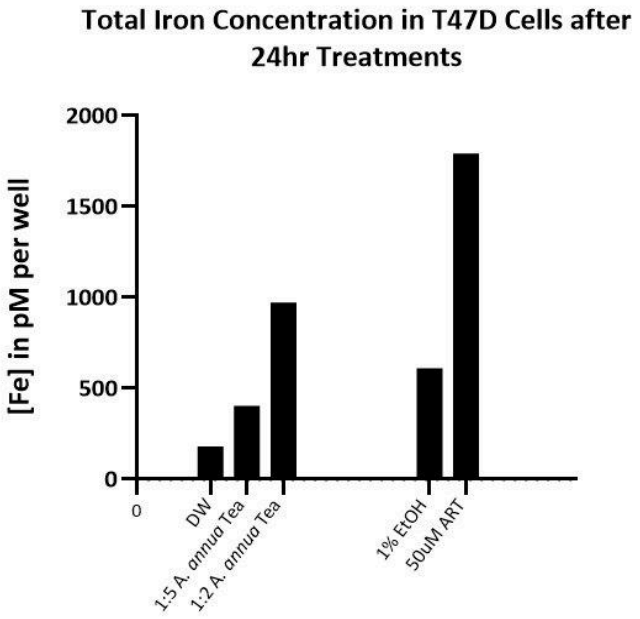

Figure 5: Bar graph illustrating the total iron concentration in picoMoles per well across various 24hr treatments.

Figure 5 displays a visual representation of the total iron concentrations seen in T47D cells with the five different treatment types. Towards the left of figure 5 are the distilled water control and *A. annua* tea treated cells. The distilled water control was found to have the least amount of total iron in these experiments, totaling at ~180 pMoles/well. The 1:5 tea extract treated cells had over double this amount of iron, measuring at ~400 pMoles/well. Notably, the 1:2 tea extract treated cells, which is the least diluted *A. annua* tea and thus should contain the highest concentration of artemisinin, had almost five-times the iron concentration of the distilled water control, measuring at almost 1,000 pMoles/well of iron.

Towards the right of figure 5 shows the 1% EtOH treated cells as well as the 50uM artemisinin treated cells. The 1% EtOH treated cells contained ~600 pMoles/well of iron based on the trendline equation obtained from figure 4. The 50uM artemisinin treated cells, however, almost tripled that number as these cells contained nearly 1,800 pMoles/well of iron after the 24 hour treatments.

Table 11: Total iron concentration in untreated T47D, NIH3T3 and MDA-MB 231 cells

Treatment Type	**Average Absorbance (OD 595 nm)**	**Average Corrected Absorbance**	**Average Iron Concentration (nmol/well)**	**Average Iron Concentration (pM/well)**
NIH 3T3	0.086	0.0455	0.427	427
T47D	0.11	0.0695	0.606	606
MD MBA 231	0.45	0.4095	3.143	3143

Iron assays were performed on untreated T47D, NIH3T3 and MDA-MB 231 cells. As seen in table 11, the NIH 3T3 cells had a corrected absorbance of 0.0455. From this, the iron concentration was calculated using the trendline equation in figure 4 to be 427 pMol/well. The T47D cells had a similar corrected absorbance in comparison to the NIH 3T3 cells of 0.0695. The iron concentration is then calculated to be 606 pMol/well. The MD MBA 231 cells had a very high absorbance in comparison to the other two cell types, with a corrected absorbance of 0.409. This absorbance was used to calculate an iron concentration of 3143 pMol/well.

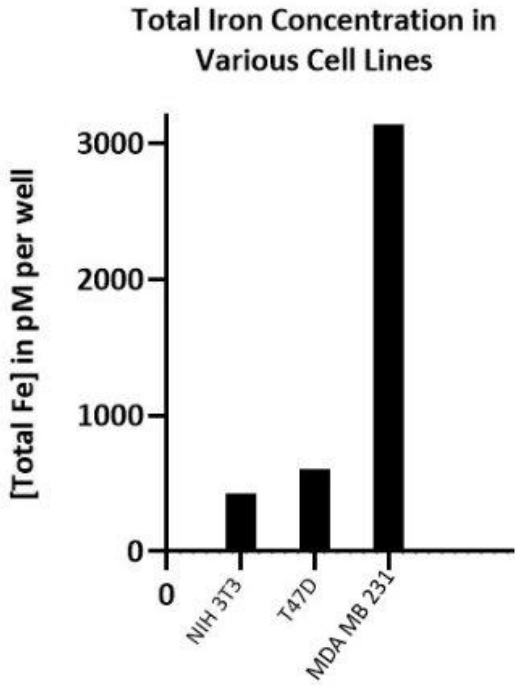

Figure 6: Bar graph illustrating how the total iron concentration varies in untreated T47D, NIH3T3 and MDA-MB 231 cells

Figure 6 displays a visual representation of the total iron concentrations seen across the three cell types all untreated. The first two cell types NIH 3T3 and T47D have relatively the same calculated concentration of total iron – which can be observed in table 11 as well. The last bar shows the calculated concentration of total iron in MDA MB 231 which can be observed to be significantly higher in comparison to the other two cell types.

4. Discussion

The purpose of the T-47D, NIH 3T3, and MDA MB 231 dose response experiments were to visualize the change in cell growth after 24-hour treatments. The live cell concentration before and after treatment was chosen for observation to look at the survival rate of cells. When pure artemisinin and *A. annua* tea extracts were plated with T-47D cells there was a significant difference in the average live cell concentration post-treatment between the control wells and experimental wells after 24 hours of treatment. There was no observable effect seen in the 1:10 *A. annua* tea extract to water dilution or 10 uM artemisinin treated cells; it is hypothesized that these weak dilutions do not contain enough artemisinin to have an effect on cell growth. The greatest effect was seen with the 1:2 *A. annua* tea extract to water dilution, 1:5 *A. annua* tea extract to water dilution, and 50 uM artemisinin. Similar results were also seen with the MDA MB 231 cells after 24 hours of treatment. The data collected from these experiments supported the literature that suggests that artemisinin has an effect on cancer cells (Zhang et al., 2014). Therefore, with these results from the effects of artemisinin and *A. annua* tea extracts, the hypothesis that pure artemisinin and *A. annua* tea extracts cause a growth defect in cancer cells can be supported. The pure artemisinin and *A. annua* tea did not have an effect on the live cell concentration post-treatment of NIH 3T3 cells. Therefore data collected from this experiment also supported the literature that suggests that artemisinin should not have an effect on non-cancerous cell lines (Zhang et al., 2014). The hypothesis that suggests that artemisinin and *A. annua* tea extracts should not have an effect on non-cancerous cell lines can be supported.

The results from the dose response testing in section 3.1 indicated that the greatest effect on T-47D cells was with the prepared 1:2 *A. annua* tea extract to water dilution, 1:5 *A. annua* tea extract to water dilution, and 50 uM artemisinin concentrations. Dose response testing was completed with different treatments for 24 hour periods. To test the effect of these concentrations over time, testing was completed with these chosen concentrations for 24, 48, and 72 hours. With the 1:2 and 1:5 *A. annua* tea extract the greatest effect was seen at the 24 hour mark. The average live cell count represented as % distilled water control increased and then decreased again at the 48 and 72 hour mark respectively. It is possible that the effect of *A. annua* tea either wore off or the tea's active ingredients started to degrade as time passed, therefore further testing with *A. annua* tea extracts was completed with 24 hour treatment periods. With the 50 uM concentration of artemisinin, the greatest effects were seen at 24 and 48 hours mark. As with the *A. annua* tea treatments the average live cell count represented as % ethanol control increased at 72 hours alluding to the possibility that the effects of pure artemisinin wore off as time passed. As with the *A. annua* tea extracts, further testing with pure artemisinin was completed with 24 hour treatment periods. Compared to the data collected from the dose response experiment completed above, these results supported the literature that suggests that artemisinin has an effect on cancer cells (Zhang et al., 2014).

Iron assay tests were performed using the Abcam iron assay kit in order to calculate total iron concentrations in treated T-47D cells as well as untreated T-47D, NIH 3T3, and MDA MB 231 cells. If artemisinin and *A. annua* tea have an effect on the iron metabolism of cancer, it is expected that the iron concentration increases in cells as the number of cells decreases after 24-hour treatments. As described in section 1.3, artemisinin is a compound derived from *A. annua* that may have tumor-suppressing potential due to the compound's endoperoxide bridge. In T-47D cells treated with a 1:5 and 1:2 *A. annua* tea extract to distilled water, the iron concentration went up 2-fold in 1:5 treatments and 5-fold in 1:2 treatments compared to the distilled water control treated cells. In T-47D cells treated with 50uM of a pure artemisinin solution, the iron concentration went up 3-fold compared to the 1% EtOH solution. The observable differences in total iron in the *A. annua* and artemisinin treated cells compared to the control cells positively correlates with the hypothesis that the excess iron in cells could be cleaving the artemisinin's endoperoxide bridge and causing ferroptosis in these cancer cells. As seen in section 3.1, the dose response experiments showed that T-47D cells treated with *A. annua* or pure artemisinin for 24 hours had over a 50% decrease in live cells compared to that of the controls. The hypothesis that pure artemisinin and *A. annua* tea extracts cause a growth defect in cancer cells due to increasing amounts in total intracellular iron concentration can be supported.

The total iron concentration found in untreated T-47D, NIH 3T3, and MDA MB 231 cells was measured in order to compare the iron concentration in cells at various stages of cancer. It is hypothesized that cancer cells, due to their higher intracellular iron concentrations, are more susceptible to the effects of artemisinin. Interestingly, there was no observable difference between the NIH 3T3 and T-47D total iron concentrations. The NIH 3T3 cells are a non-cancer, fibroblast cell line, so it was hypothesized that the T-47D breast cancer cells would have contained higher amounts of intracellular iron due to iron's important role in maintaining a tumor microenvironment for cancer progression. The MDA MB 231 cells, however, had over 10-fold more intracellular iron in comparison to the NIH-3T3 and T-47D cells. MDA MB 231 cells are a triple-negative breast cancer cell line which are more aggressive cancer cells than T-47D cells as they have a faster growth rate and higher risk of metastasis. It was anticipated that there would be higher iron concentrations in both cancer cell lines, and although this can be supported from the total iron results found in the MDA MB 231 cells, more research needs to be done to determine if T47D cancer cells have a higher intracellular iron concentration compared to non-cancer cells.

5. Recommendations

This MQP focused primarily on T-47D, NIH 3T3, and MDA MB 231 cell lines. Future experimentation may look at the effects of both pure artemisinin solution and *A. annua* tea extracts on other cancerous and non-cancerous cell lines to broaden the conclusions made.

To start, it would be helpful to determine how much of the artemisinin compound is present in the stock tea extract in order to see if there is a correlation with the amount of pure artemisinin tested. Methods of high performance liquid chromatography (HPLC) could help to separate, identify, and ultimately quantify the amount of artemisinin present in the *A. annua* tea.

This MQP started research into the mechanism of action of artemisinin in cancer cells by conducting the iron assay described in section 2.6 with treated and untreated T-47D, NIH 3T3, and MDA MB 231 cells. Current results show that treatment with *A. annua* tea extracts and artemisinin had a significant effect on the concentration of iron found in the T-47D cell extracts compared to the controls. This supports current research that suggests that free iron in cells may react with artemisinin's organic peroxide leading to a surplus of ROS in cells that activates regulated cell death due to the high oxidative stress the cell undergoes (Zhou et al., 2022).

The results from the iron assay with non-treated T-47D, NIH 3T3, and MDA MB 231 cells indicate that the relative concentration of iron in T-47D and NIH 3T3 is similar. More iron assays should be performed for accurate intracellular iron concentrations of T-47D, NIH 3T3, and MDA MB 231 cells. Future testing could look into how the average live cell concentration of treated T-47D cells and NIH 3T3 can be significantly different while the relative intracellular iron concentrations are close in value. Non-cancer and cancer cell types may have similar amounts of iron storage proteins which could be a reason for the similar intracellular iron concentrations seen in the T-47D cancer cells and NIH 3T3 non-cancer cells. Measuring the protein abundance of iron storage proteins, such as ferritin or transferrin may be helpful in determining possible differences between the cell lines.

6. References

American Cancer Society. (2023, September 14). *Key Statistics for Breast Cancer*. American Cancer Society. https://www.cancer.org/cancer/types/breast-cancer/about/how-common-is-breast-cancer.html

ATCC. (n.d.). *MDA-MB-231*. ATCC. Retrieved April 3, 2024, from https://www.atcc.org/products/htb-26

ATCC. (n.d.). *NIH/3T3*. ATCC. Retrieved April 3, 2024, from https://www.atcc.org/products/crl-1658

ATCC. (n.d.). *T-47D*. ATCC. Retrieved October 10, 2023, from https://www.atcc.org/products/htb-133

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxidative Medicine and Cellular Longevity*, *2014*, 360438. https://doi.org/10.1155/2014/360438

Chen, G.-Q., Benthani, F. A., Wu, J., Liang, D., Bian, Z.-X., & Jiang, X. (2020). Artemisinin compounds sensitize cancer cells to ferroptosis by regulating iron homeostasis. *Cell Death and Differentiation*, *27*(1), 242–254. https://doi.org/10.1038/s41418-019-0352-3

Clark, D. (2001). Peroxides and peroxide-forming compounds. *Elsevier Science Inc*, *1074–9098*(01).

Cyr, M. (2022). *Anti-Cancer Properties of Pure Artemisinin and A. Annua*.

Do Artemisia Annua Benefits Outweigh the Potential Risks? (n.d.). Dr. Axe. Retrieved October 10, 2023, from https://draxe.com/nutrition/artemisia-annua-benefits/

Hinshaw, D. C., & Shevde, L. A. (2019). The Tumor Microenvironment Innately Modulates Cancer Progression. *Cancer Research*, *79*(18), 4557–4566. https://doi.org/10.1158/0008-5472.CAN-18-3962

Hu, Y., Guo, N., Yang, T., Yan, J., Wang, W., & Li, X. (2022). The Potential Mechanisms by which Artemisinin and Its Derivatives Induce Ferroptosis in the Treatment of Cancer. *Oxidative Medicine and Cellular Longevity*, *2022*, 1458143. https://doi.org/10.1155/2022/1458143

Iron Assay Kit (Colorimetric) (ab83366). (n.d.). Abcam. Retrieved April 3, 2024, from https://www.abcam.com/products/assay-kits/iron-assay-kit-colorimetric-ab83366.html?pageNumber=2&productWallTab=ShowAll&PageSize=10&SortOrder=VoteDesc

Meshnick, S. (1994). The mode of action of antimalarial endoperoxides. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *88*(Supplement 1), 31–32.

Mravčáková, D., Komáromyová, M., Babják, M., Urda Dolinská, M., Königová, A., Petrič, D., Čobanová, K., Ślusarczyk, S., Cieslak, A., Várady, M., & Váradyová, Z. (2020). Anthelmintic Activity of Wormwood (Artemisia absinthium L.) and Mallow (Malva sylvestris L.) against Haemonchus contortus in Sheep. *Animals : An Open Access Journal from MDPI*, *10*(2), 219. https://doi.org/10.3390/ani10020219

National Cancer Institute. (2007, September 17). *What Is Cancer?* National Cancer Institute. https://www.cancer.gov/about-cancer/understanding/what-is-cancer

National Cancer Institute. (2023, September 29). *Breast Cancer Treatment (PDQ®) - Health Professional Version* [National Cancer Institute]. https://www.cancer.gov/types/breast/hp/breast-treatment-pdq

Nguyen, T.-K., & Spera, J. (2021). *Analyzing the Anti-Cancer Properties of Artemisia annua*.

PubChem. (n.d.). *Artemisinin*. Retrieved October 10, 2023, from https://pubchem.ncbi.nlm.nih.gov/compound/68827

Guo, Q., Li, L., Hou, S., Yuan, Z., Li, C., Zhang, W., Zheng, L., & Li, X. (2021). The Role of Iron in Cancer Progression. Frontiers in oncology, 11, 778492. https://doi.org/10.3389/fonc.2021.778492

Septembre-Malaterre, A., Lalarizo Rakoto, M., Marodon, C., Bedoui, Y., Nakab, J., Simon, E., Hoarau, L., Savriama, S., Strasberg, D., Guiraud, P., Selambarom, J., & Gasque, P. (2020). Artemisia annua, a Traditional Plant Brought to Light. *International Journal of Molecular Sciences*, *21*(14), 4986. https://doi.org/10.3390/ijms21144986

Wen, W., & Yu, R. (2011). Artemisinin biosynthesis and its regulatory enzymes: Progress and perspective. *Pharmacognosy Reviews*, *5*(10), 189–194. https://doi.org/10.4103/0973-7847.91118

Winterbourn, C. C. (1995). Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicology Letters*, *82–83*, 969–974. https://doi.org/10.1016/0378-4274(95)03532-x

Yan, H., Zou, T., Tuo, Q., Xu, S., Li, H., Belaidi, A. A., & Lei, P. (2021). Ferroptosis: mechanisms and links with diseases. *Signal Transduction and Targeted Therapy*, *6*(1), 1–16. https://doi.org/10.1038/s41392-020-00428-9

Yang, N.-D., Tan, S.-H., Ng, S., Shi, Y., Zhou, J., Tan, K. S. W., Wong, W.-S. F., & Shen, H.-M. (2014). Artesunate induces cell death in human cancer cells via enhancing lysosomal function and lysosomal degradation of ferritin. *The Journal of Biological Chemistry*, *289*(48), 33425–33441. https://doi.org/10.1074/jbc.M114.564567

Zhang, C., Liu, X., Jin, S., Chen, Y., & Guo, R. (2022). Ferroptosis in cancer therapy: a novel approach to reversing drug resistance. *Molecular Cancer*, *21*(1), 47. https://doi.org/10.1186/s12943-022-01530-y

Zhang, L., Liu, Z., Ye, J., Sha, M., Qian, H., Bu, X., Luan, Z., Xu, X., Huang, A., Yuan, D., Wu, Y., Wang, X., Wang, J., Huang, J., & Ye, L. (2014). Artesunate exerts an anti‐immunosuppressive effect on cervical cancer by inhibiting PGE_2 production and Foxp3 expression. *Cell Biology International*, *38*(5), 639–646. https://doi.org/10.1002/cbin.10244

Zhou, X., Suo, F., Haslinger, K., & Quax, W. J. (2022). Artemisinin-Type Drugs in Tumor Cell Death: Mechanisms, Combination Treatment with Biologics and Nanoparticle Delivery. *Pharmaceutics*, *14*(2), 395. https://doi.org/10.3390/pharmaceutics14020395