Anti-Cancer Properties of Pure Artemisinin and *A. Annua*

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

Advisors: Mike Buckholt, PhD
Jill Rulfs, PhD

Author: Megan Cyr

Abstract

Artemisinin is the most common treatment for the rapid clearance of *Plasmodium falciparum*, the parasite known to cause malaria. Prior studies have supported that artemisinin may have anti-cancer properties. The goal of this study was to investigate 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. It was determined that the absolute cell number in T47D breast cancer cells was reduced in the presence of *A. annua* tea extract while there was minimal to no effect of the pure artemisinin and *A. annua* tea extract on LNCaP prostate cancer cells, L6 myoblast cells, and NIH3T3 fibroblast cells. Additionally, there was no effect seen on the T47D breast cancer cells when incubated with *A. afra* and *A. absinthium* tea extracts. Future studies should investigate the amount of artemisinin present in each of the tea extracts created from the artemisia plants and explore the mechanism of action that artemisinin may use to have an effect on the absolute cell number of cancer cells.

Acknowledgments

I would like to thank my advisors, Professors Jill Rulfs and Mike Buckholt for providing guidance throughout this project and helping develop this paper. I would like to specifically thank Jill Rulfs for splitting my cells on days when I could not come into the lab and for maintaining backup stocks of cell lines throughout the project. Additionally, I would like to thank Professor Pamela Weathers for providing dried Artemisia annua for this project. Finally, I would like to acknowledge my friends and family for all their support during the time spent on this project.

Table of Contents

Background

Introduction

Each year, approximately 1.8 million new cases of cancer are diagnosed in the United States, and approximately 600,000 people die from the disease (National Cancer Institute, 2020). Cancer is one of the leading causes of death worldwide. In 2018, there were 18.1 million new cases and 9.5 million cancer-related deaths. Cancer treatments range from chemotherapies, to surgery, to radiation treatment, to target therapies that all depend on the type of cancer that one has and how advanced it may be (National Cancer Institute, 2020). Recent literature has suggested that the active compound in antimalarial drugs, artemisinin, has anti-cancer properties and can be utilized as a cancer treatment (Kumari, Keshari, Sengupta, Sabat, & Mishra, 2017; (Willoughby et al., 2008). Previous undergraduate studies at WPI have attempted to replicate the relationship found between artemisinin and breast and ovarian cancer cell lines, but there have been conflict data found in student reports compared to that of literature. It is also largely unknown the exact mechanisms in which artemisinin can target cancer cells. This report looks at the effects of pure artemisinin solution, *A. annua* tea extract, *A. afra* tea extract, and *A. absinthium* tea extract and their effects on cancer cell lines as well as non cancer cell lines.

Breast and Prostate Cancer

Breast cancer is the out of control growth of cells originating in the breast tissue. In the United States, about 264,000 cases of breast cancer are diagnosed in women and about 2,400 in men each year. Of these cases approximately 42,000 women and 500 men in the U.S. die each year from breast cancer (CDC, 2020). Prostate cancer is also an out of control growth of cells but rather than originating in the breast, the cancer originates in the prostate glands. Prostate cancer is the second most common form of cancer in men, second to skin cancer. One out of every eight men will be diagnosed with prostate cancer each year, and approximately 34,000 men die from this cancer (CDC, 2019). Both breast and prostate cancer treatments are incredibly important to explore due to the number of people who are affected each year. Breakthroughs in potential treatments could provide relief to millions of people each year.

Mechanisms of Cancer

Cancer is a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body. In the human body, cells grow and multiply through cell division to form new cells as needed. When cells become damaged or grow old, they die, and new cells take their place. (National Cancer Institute, 2007). In normal cells, there are elaborate cell cycle checkpoints that prevent the progression of division to repair damage or, in case of irreparable damage, stimulate cell death. When cells with irreparable DNA or chromosome damage are allowed to progress through the cell cycle by the evasion of checkpoints, cancerous cells can multiply exponentially leading the build up of cells resulting in cancerous growths, known as tumors (Visconti, Della Monica, & Grieco, 2016). The tumors are able to invade and spread to

nearby tissues and cause new tumors. Cancer cells rely on unique properties to allow them to survive. Some of these include growing in the absence of growth signals or relying on different nutrients than a normal cell for growth. Researchers have taken advantage of this fact, developing therapies that target the abnormal features of cancer cells (National Cancer Institute, 2007).

Artemisia annua

Artemisia annua, as seen in Figure 1, is a plant that has been used for thousands of years for the prevention and treatment of infectious diseases, particularly in developing countries. The plant is grown in Asia, India, Central and Eastern Europe, and in the more temperate regions of America, Africa, and Australia. *A. annua* has been most traditionally and widely used as a dietary spice, herbal tea, and medicinal plant in Asia, specifically in areas of China and Korea. *A. annua* can be broken down into many different compounds with biological activity, including monoterpenes, sesquiterpenes, phenolics, and coumarins (Dib & El Alaoui-Faris, 2019). While these compounds have been most notably used in the treatment of malaria, they also have been described to have anti-hyperlipidemic (Dib & El Alaoui-Faris, 2019), anti-plasmodial (Suberu et al., 2013), anti-convulsant (Dib & El Alaoui-Faris, 2019), anti-inflammatory (Kim et al., 2015), anti-microbial (Kim et al., 2015), and antiviral properties (Lubbe, Seibert, Klimkait, & van der Kooy, 2012).

Figure 1. *A. annua* growing in nature (NCCIH Clearinghouse, 2021).

Artemisia afra

More common to South Africa, *A. afra* is also a plant that has been used for thousands of years with claims for use in treatment of bronchitis, diabetes, the common cold, headaches, and inflammation (Liu, Van der Kooy, & Verpoorte, 2009). In most cases, *A. afra* contains trace

levels of artemisinin, the active compound thought to be most effective against malaria and cause anticancer properties. Because *A. afra* contains little to no artemisinin, it serves as an important comparison to the *A. annua*. The goal of using *A. afra* is to determine if other active compounds present in both *A. afra and A. annua* cause the anti-cancer effects other than artemisinin.

Artemisia absinthium

The plant, *A. absinthium*, has a natural habitat in Europe, West Asia, and North Africa. As with both *A. annua* and *A. afra*, there are century old claims that say that the species can be effective against various ailments. Additionally, there is minimal research that supports the mechanism of action in which this plant functions to aid the immune system in fighting off different alignments (Szopa et al., 2020).

Artemisinin as a Malaria Drug

Malaria is caused by *Plasmodium falciparum*, a parasite that is found and transmitted through the bites of female Anopheles mosquitoes. This life threatening disease has been present for thousands of years, but the disease saw a major resurgence in the 1950s due to the emergence of parasites resistant to existing antimalarial drugs like chloroquine. In 1967, Chinese scientists began Project 523 which explored the extraction and isolation of constituents with possible antimalarial activities with a focus on Chinese herbal materials. Initially, more than 2,000 herbal preparations were investigated with little progress or significant results. The turning point came when the extract artemisinin from *A. annua* had promising inhibition effects against parasitic growth. The research team shared their findings with the rest of the world in the 1980s, but it took until 1993 for a western company to complete their own research and manufacture artemisinin-derived compounds (Tu, 2011).

Artemisinin is now the most common treatment for the rapid clearance of *Plasmodium falciparum* and the quick resolution of the symptoms caused. It has been difficult for scientists to determine the actual mechanism of action that allows artemisinin and its derivatives to have effective actions against this parasite (O'Neill, Barton, & Ward, 2010). Artemisinin's structure is a sesquiterpene trioxane lactone whose endoperoxide bridge, as seen in Figure 2, has been determined essential for antimalarial activity (Nti-Gyabaah, Gbewonyo, & Chiew, 2010). The mechanism of action is thought to be related to artimisinin's chemical ability to generate free radicals. Artemisinin is able to react with Fe^{2+} to be converted into oxygen centered free radicals from the peroxide bridge present in its structure. Then, the oxygen centered free radicals are converted into carbon centered free radicals which are suggested to be the principal intermediate in the parasiticidal process (Suberu et al., 2013).

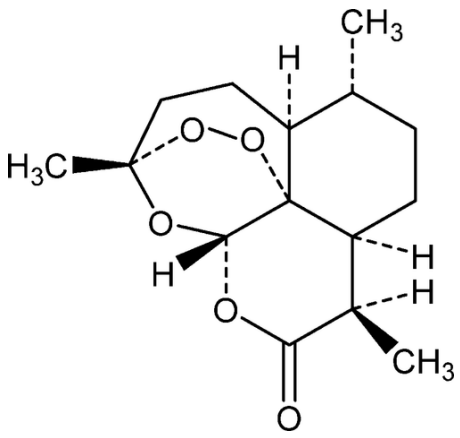

Figure 2. The chemical structure of artemisinin (Nti-Gyabaah, Gbewonyo, & Chiew, 2010).

Anti-Cancer Potential of Artemisia annua

In addition to being a strong anti-malarial drug, artemisinin has shown anticancer activity through in-vivo and in-vitro experiments (Kumari, Keshari, Sengupta, Sabat, & Mishra, 2017). An ideal anticancer drug should have high potency and specificity in killing cancer cells without significant or fatal toxicity on normal cells. Current cancer therapies have shown improving response to tumors and better patient survival, but side effects and poor quality of life, often leads to discontinuation, dose reduction and emergence of drug resistance. Similar to its antimalarial effects, artemisinin's anticancer mechanism of action is thought to be related to the reaction of its endoperoxide bond to create Fe^{2+} to be converted into oxygen centered free radicals. It is hypothesized that the addition of iron to cancer cells triggers the cytotoxic effects of artemisinin. Artemisinin also promotes cancer cells to undergo apoptosis rather than necrosis in most cases. Induction of apoptosis is a major advantage to the anti-cancer properties because it does not cause inflammation or cell damage which is seen with necrosis (Das, 2015). It is important to note that the solvent in which compounds found in Artemisia plants are extracted could result in different compounds being more successfully extracted in certain solvents. The active compounds of *A. annua* have been extracted in oils, alcohols, and aqueous solutions. Each extraction solvent could result in slightly different compounds due to their chemical properties. The artemisinin used in this report was dissolved in ethanol and the tea extracts were prepared using deionized water.

Researchers from the Cancer Biology Laboratory, Institute of Life Sciences in Odisha, India, as well as researchers from the National Central University in Chungli, Taiwan collaborated to explore the cell viability, apoptosis, and gene analysis of MCF-7, T47D, and MDA-MB-231 cells treated with varying concentrations of artemisinin. It is important to note that MCF-7 and T47D express estrogen receptors, while MDA-MB-231 cells do not. The different cell types were incubated with different concentrations of artemisinin (500 nM, 1, 10, 50 and 100 μM) and incubated for two different time periods (12 and 24 hours). Although there was no significant reduction in viability of cells after 12 hours of treatment, there was a dose-dependent reduction in viability and cell density after 24 hours of artemisinin treatment for

all three cell lines. The researchers were able to support the anti-cancer claims of artemisinin treatment, but concluded that further exploration is required to establish the contribution artemisinin had in cancer cell reduction (Kumari, Keshari, Sengupta, Sabat, & Mishra, 2017).

Researchers from the Department of Molecular and Cell Biology and Cancer Research Laboratory at the University of California at Berkeley partnered with researchers from the College of Veterinary Medicine at the University of Minnesota to explore the effects of artemisinin on prostate cancer cell lines. LNCaP, PC3, and DU145 human prostate cell lines were plated with varying concentrations of artemisinin for 48 hours. Additionally, LNCaP cells were injected into mice and artemisinin treatments were given through their drinking water. The *in vitro* and *in vivo* effects of the artemisinin treatment showed that artemisinin inhibits the proliferation of LNCaP cells. Both of these results suggest that artemisinin has a potent anti-proliferative effect on human prostate cancer cells, similarly seen in the breast cancer cell lines (Willoughby et al., 2008).

Previous MQP Research

The exploration of artemisinin as a cancer therapeutic is not a novel idea. Previous WPI undergraduate studies (known as Major Qualifying Projects, or "MQPs") have explored the cytotoxic effects of *A. annua* on cancer cell lines as well as explored the effects of artemisinin on Safe ESKAPE Pathogen Relatives (Furtado & Lewis, 2021; Nguyen & Spera, 2021). Specifically the research exploring the cytotoxic effects of *A. annua* on cancer cell lines compared the effects of *A. annua* tea extract and pure artemisinin on T47D cells. The students that completed this research found that the *A. annua* tea extract had a 65% reduction in cancer cell number and the pure artemisinin had a 25% reduction in cell number compared to the control indicating that both solutions were cytotoxic to the breast cancer cells (Nguyen & Spera, 2021). It was thought that the *A. annua* tea extract was more cytotoxic compared to the pure artemisinin due to the presence of other compounds in the plant. Nguyen and Spera also explored the effects of *A. annua* tea extract and pure artemisinin on OVCAR3 cells, an ovarian cancer cell line. The results of the experiment on the ovarian cancer cell supported that the two treatment groups were successful in reducing cell counts, but there were not enough replicates performed. Due to the minimal number of replicates performed on both the ovarian and breast cancer cell lines, it was difficult to truly understand the full effect the treatment groups had. Nguyen and Spera also only explored cancer cell lines. It is also important to understand the effects that artemisinin has on non-cancer cell lines. These cell lines can serve as a control to understand if artemisinin is able to have anti-proliferation effects on all cell lines indiscriminately or if the plant's underlying mechanisms target the specific attributes of cancer cells.

Cell Lines

There are 4 different cell lines being used in this study. The T47D cell line has shown promise in previous research with the focus on artemisinin on breast cancer (Kumari, Keshari, Sengupta, Sabat, & Mishra, 2017. Additionally, T47D was used in previous MQPs to explore

these claims (Nguyen & Spera, 2021). T47D cells are epithelial cells obtained from a 54 year old female with an infiltrating ductal carcinoma of the breast. In addition to T47D cells, the other cancer cell line being explored was LNCaP cells. Primary literature has also supported that artemisinin has anti-cancer effects on prostate cancer (Willoughby et al., 2008). LNCaP cells are prostate cancer cells obtained from a 50 year old male with a prostate carcinoma. NIH3T3 and L6 were chosen for research with artemisinin as an anti-cancer therapy to help determine if artemisinin is able to attack cancer cells specifically, or if it attacks all cells indiscriminately. L6 cells are myoblasts isolated from the skeletal muscle from a rat. Myoblasts are the undifferentiated cells that are capable of differentiating into muscle cells. NIH3T3 cells are fibroblasts that were isolated from a mouse embryo. Fibroblasts are a type of cell that contributes to the formation of connective tissue that connects tissues and organs in the body.

Hypothesis

A successful cancer treatment would not only eliminate the cancer cells, but also keep adverse effects from the treatment to a minimum. The negative effects from cancer treatments result in pain and fatigue, anemia, nausea, hair loss, and many more symptoms. Cancer treatments move through the body quickly to attack the fast growing cancer cells. Because these drugs travel so fast, they can have an impact on normal, healthy cells that grow quickly too. It was hypothesized that when both cancer cells (T47Ds and LNCaPs) and non-cancer cells (L6s and NIH3T3s) are treated with a pure artemisinin solution and an *A. annua* tea extract, the cancer cells would decrease in density while the non-cancer cells would not be impacted by the treatment. Separately, the T47D cell line would be treated with tea extracts made from *A. afra* and *A. absinthium*. These additional tea extracts are made from plants that are in the same genus as *A. annua*, but have different chemical properties. By exploring the effects of the additional tea extracts, it can be determined which artemisia plant might have the most effect on the cell lines, or have no effect at all.

Methods

Experimental Compound Preparation

To develop the pure artemisinin solution and make the *A. annua* tea extract, the same protocol was followed as the previous WPI undergraduate studies completed by Jillian Spera and Thien-Kim Nguyen, as well as, Samual Furtado and Carter Lewis. This was done because the experiments performed were a continuation of Spera and Nguyen's work and there was collaboration between the group in regards to the pure artemisinin solution and *A. annua* tea extract development.

The pure artemisinin solution was made following the MSDS page outlined from Cayman Chem. 10mg of powdered artemisinin was added to 0.625mL of pure ethanol (EtOH). Once dissolved in the ethanol, 61.9mL of deionized water was added to dilute the solution to 1% EtOH (Nguyen & Spera, 2021).

The *A. annua* tea infusion was prepared using the hot water extraction method. First, 100mL of water was brought to a boil in a beaker on a hot plate. Then, 1g of dried *A. annua* was added to the boiling water and stirred thoroughly. After steeping for 15 minutes, the beaker was removed from the hot plate, and large pieces were sieved out using vacuum filtration and filter paper. It was also filtered through a 0.22µm filter, allotted into 15mL centrifuge tubes, and frozen at -20°C (Furtado & Lewis, 2021).

The *A. afra* tea infusion was prepared by removing leaves from the *A. afra* plant purchased. The leaves were placed in an aluminum disk in an incubator set to 100℃ for 24 hours. The tea was then prepared using the hot water extraction method. 7.5mL of water was brought to a boil in a beaker on a hot plate. Then, 0.0749g of dried *A. afta* was added to the boiling water and stirred thoroughly. After steeping for 15 minutes, the beaker was removed from the hot plate, and large pieces were sieved out using vacuum filtration and filter paper. It was also filtered through a 0.22µm filter, allotted into a 15mL centrifuge tube, and frozen at -20°C.

The *A. absinthium* tea infusion was also prepared using the hot water extraction method. The *A. absinthium* was ordered offline from Starwest Botanical Store. 14.8mL of water was brought to a boil in a beaker on a hot plate. Because the *A. absinthium* was already dried, 0.1480g of *A. absinthium* was added to the boiling water and stirred thoroughly. After steeping for 15 minutes, the beaker was removed from the hot plate, and large pieces were sieved out using vacuum filtration and filter paper. It was also filtered through a 0.22µm filter, allotted into a 15mL centrifuge tube, and frozen at -20°C.

Cell Culture and Maintenance

T47D breast epithelial cells were cultured in DMEM with 10% Fetal Bovine Serum and 1% PenStrep. They were maintained in a vented T75 flask with approximately 10mL of media. The flasks were stored in an incubator at 37°C and 5% Carbon Dioxide. Cells were split as

needed when they reached 70-90% confluency, approximately three times a week. This maintenance also kept the cells in log phase growth conditions.

LNCaP prostate epithelial cells were cultured in DMEM with 10% Fetal Bovine Serum and 1% PenStrep. They were maintained in a vented T75 flask with approximately 10mL of media. The flasks were stored in an incubator at 37°C and 5% Carbon Dioxide. Cells were split as needed when they reached 70-90% confluency, approximately three times a week. This maintenance also kept the cells in log phase growth conditions.

NIH3T3 fibroblast cells were cultured in DMEM with 10% Fetal Bovine Serum and 1% PenStrep. They were maintained in a vented T75 flask with approximately 10mL of media. The flasks were stored in an incubator at 37°C and 5% Carbon Dioxide. Cells were split as needed when they reached 70-90% confluency, approximately three times a week. This maintenance also kept the cells in log phase growth conditions.

L6 myoblast cells were cultured in DMEM with 10% Fetal Bovine Serum and 1% PenStrep. They were maintained in a vented T75 flask with approximately 10mL of media. The flasks were stored in an incubator at 37°C and 5% Carbon Dioxide. Cells were split as needed when they reached 70-90% confluency, approximately three times a week. This maintenance also kept the cells in log phase growth conditions.

Cell Counting

Following trypsinization and resuspension of cells in media, 20 uL samples of the desired cell culture was taken and mixed thoroughly with 20 uL of Trypan blue. A sample of 20 uL of the mixture was then placed in a slide and inserted into a Cellometer® for observation, cell counting, and viability calculations. The program counts four separate views of the cells and averages each sample. Cell counting was performed before plating for experiments to ensure equal numbers of cells. Additional cell counting was performed after each dose response test to compare initial live cell counts and final live cell counts

Dose Response Tests

There were 5 repeated dose response tests using pure artemisinin solution and *A. annua* tea extract conducted for each cell line. The dose tests were conducted with L6, NIH3T3, T47D, and LNCaP cell lines and observed the effects of pure artemisinin solution and *A. annua* tea extract on the density of live cells. Cells were plated at 1.0×10^5 cells per well on a 24-well plate. Each well had a total volume of 500uL, with 400uL dedicated to media and cells and the remaining 100uL dedicated to the *A. annua* tea extract or pure artemisinin solution and the indicated solvent. The experimental variables were plated in triplicates. The control for the pure artemisinin solution was 1% EtOH. The control for the *A. annua* tea extract was deionized water. The plates were incubated at 37.0℃ for 24 hours washed with 100uL of PBS, and then lifted from the plate with 100uL of trypsin. The trypsinizing agent was diluted with 200uL of fresh DMEM with 10% Fetal Bovine Serum and 1% PenStrep. The absolute cell count and viability of each experimental and control group were found using the Cellometer®. The dilution doses of

pure artemisinin solution can be found in Supplemental Table 1. The dilution of the *A. annua* tea extract can be found in Supplemental Table 2. The plate map can be found in Supplemental Table 3.

Additional dose response tests were completed with T47D cells and both *A. afra* tea extract and *A. absinthium* tea extract. Cells were plated at $1.0x10^5$ cells per well on a 24-well plate. Each well had a total volume of 500 uL, with 400 uL dedicated to media and cells and the remaining 100 uL dedicated to the *A. afra* tea extract or *A. absinthium* tea extract with deionized water. The plate map for *A. afra* tea extract and *A. absinthium* tea extract can be found in Supplemental Table 4. The plates were incubated at 37.0℃ for 24 hours washed with 100uL of PBS, and then lifted from the plate with 100uL of trypsin. The trypsinizing agent was diluted with 200uL of fresh DMEM with 10% Fetal Bovine Serum and 1% PenStrep. The absolute cell count and viability of each experimental and control group were found using the Cellometer®.

Results

Pure Artemisinin and *A. annua* Tea Extract versus Cancer Cell Lines

The main purpose of the dose response testing performed was to understand how varying concentrations of pure artemisinin and *A. annua* tea extract could affect the absolute cell number of both cancer and non-cancer cell lines. It was hypothesized that when both cancer cells (T47Ds and LNCaPs) and non-cancer cells (L6s and NIH3T3s) were treated with a pure artemisinin solution and an *A. annua* tea extract, the cancer cells would decrease in absolute cell number while the non-cancer cells would not be impacted by the treatment.

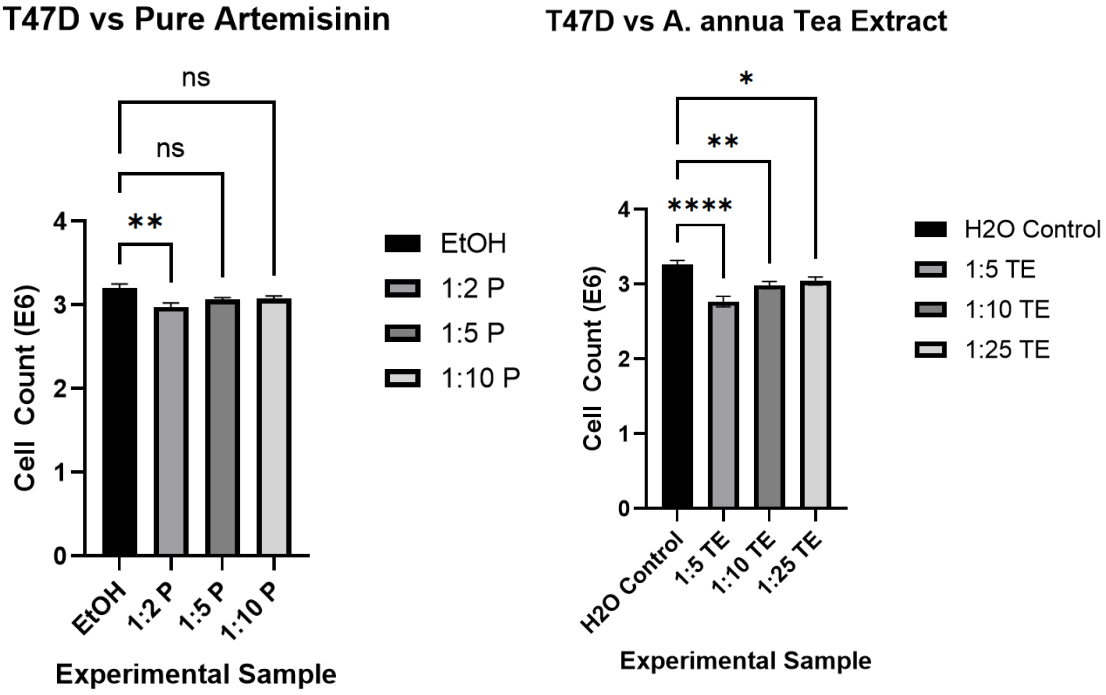

Figure 3. T47D cells vs Pure Artemisinin and *A. annua* Tea Extract. Average cell counts of five dose response tests each with triplicate controls and experimental variables for T47Ds when plated with pure artemisinin and *A. annua* tea extract. Error bars show the standard error of the mean. P = pure artemisinin. TE = *A. annua* Tea Extract. The ratios refer to the experimental variables to water or ethanol respective to the experiment. Pairwise comparison represented by brackets and asterisks of statistically significant differences between control groups and experimental groups.

Figure 3 shows the averaged cell counts of T47D cells following 24 hour treatments of pure artemisinin dilutions and *A. annua* tea extract dilutions with comparison to an ethanol and water control, respectively. The pure artemisinin was dissolved in 100% ethanol and diluted to a 10% concentration with deionized water, as noted in the methods. The *A. annua* tea extract was made through steeping in deionized water. When the T47D cells were plated with pure artemisinin dilutions, there was a statistical difference between the ethanol control and the 1:2 pure artemisinin to water dilution. The mean amount of cells present in the ethanol control wells

were approximately $3.20x10^6$ cells while the mean cells present in the 1:2 pure artemisinin to water wells had approximately $2.98x10^6$ cells. Through an unpaired t test, there was a P value of 0.0038, which is less than 0.05, indicating a statistically significant difference in the amount of cells between these two wells. Comparatively, the 1:5 pure artemisinin to water dilution had approximately $3.06x10^6$ cells per well which resulted in a P value of 0.0265. The 1:10 pure artemisinin to water dilution had approximately $3.07x10^6$ cells per well, which resulted in a P value of 0.0504. Based on the dilution well comparisons to the ethanol control well, only the 1:2 pure artemisinin dilution had a small but significant effect on the T47D breast cancer cells.

The *A. annua* tea extract dilutions had the most notable effect on the absolute cell number of the T47D cells. The water control had an average cell count of $3.27x10^6$ cells, while the 1:5 tea extract to water dilution had an average cell count of $2.77x10^6$. Using an unpaired t test, the P value comparison for these wells was less than 0.0001. Furthermore, the 1:10 tea extract to water dilution had an average cell count of $2.99x10^6$ and a P value of 0.0004. The 1:25 tea extract to water dilution had an average cell count of $3.05x10^6$ and a P value of 0.0051. The P value comparisons to the water control were all less than 0.05, indicating that the *A. annua* tea extract has a significant effect on the T47D breast cancer cells.

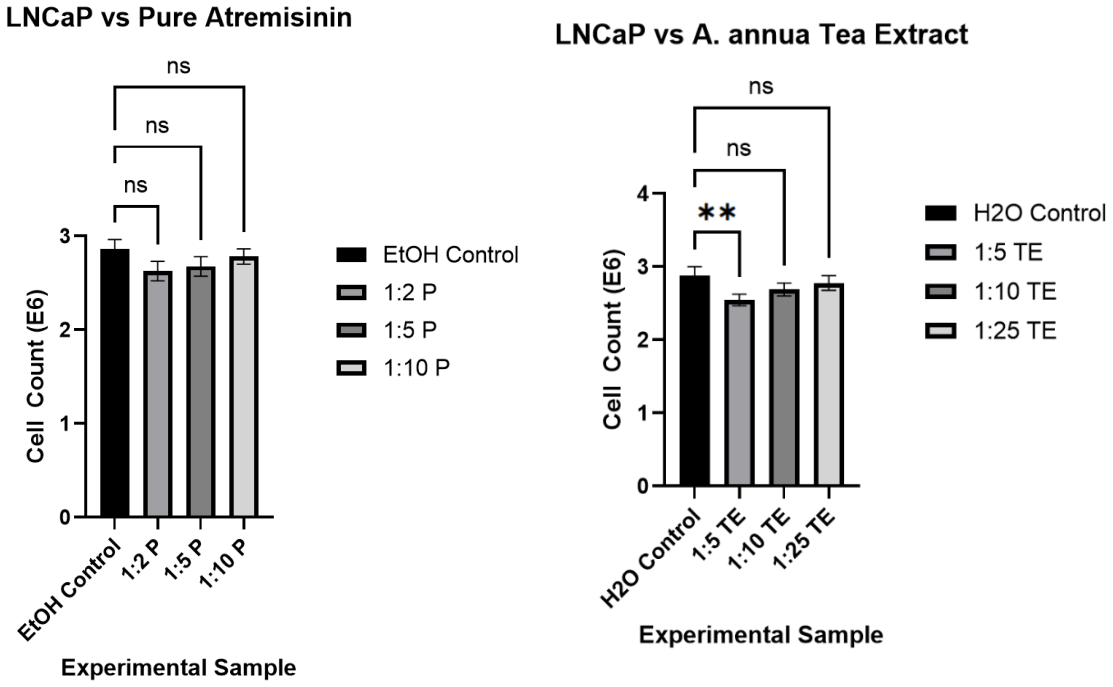

Figure 4. LNCaP cells vs Pure Artemisinin and *A. annua* Tea Extract. Average cell counts of five dose response tests each with triplicate controls and experimental variables for LNCaPs when plated with pure artemisinin and *A. annua* tea extract. Error bars show the standard error of the mean. P = pure artemisinin. TE = *A. annua* Tea Extract. The ratios refer to the experimental variables to water or ethanol respective to the experiment. Pairwise comparison represented by brackets and asterisks of statistically significant differences between control groups and experimental groups.

Figure 4 shows the averaged cell counts of LNCaP cells following 24 hour treatments of pure artemisinin dilutions and *A. annua* tea extract dilutions with comparison to an ethanol and

water control, respectively. When the LNCaP cells were plated with pure artemisinin, the experimental wells compared to the ethanol control well showed no statistically significant difference between the amount of cells. The average cell count for the control wells was $2.87x10^6$, while the average cell count for the 1:2, 1:5, and 1:10 pure artemisinin to ethanol dilutions were $2.63x10^6$, $2.68x10^6$, and $2.78x10^6$, respectively. Similarly, the unpaired t test showed P values that did not support statistically significant differences between the control wells and experimental wells.

The *A. annua* tea extract dilution had a differing effect on the LNCaP cells compared to the pure artemisinin solution. There was an effect on the absolute cell number at the highest concentration of *A. annua* tea extract. The 1:5 tea extract to water dilution resulted in a cell count of approximately $2.55x10^6$ cells per well compared to the water control well that had $2.89x10^6$ cells per well. The unpaired t test supported that these values were significantly different and the P value for this comparison was 0.0231. The 1:10 and 1:25 tea extract to water dilution did not result in a statistically significant difference between the amount of cells in each well compared to the water control. The amount of cells in the 1:10 tea extract to water dilution was approximately $2.69x10^6$ cells and the 1:25 tea extract to water dilution had approximately $2.78x10^6$ cells. Additionally, the 1:10 and 1:25 tea extract to water dilution had P values of 0.1895 and 0.4959, respectively, supporting that these wells compared to the control wells did not show significant effects of the tea extract on LNCaP cells.

Pure Artemisinin and *A. annua* Tea Extract versus Non-Cancer Cell Lines

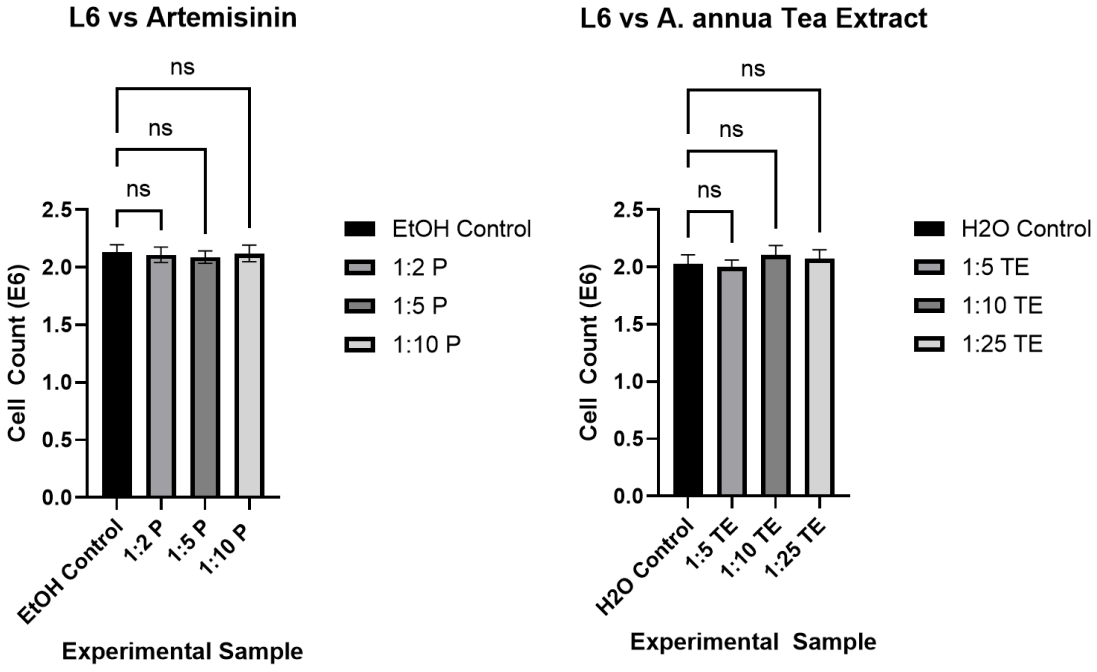

Figure 5. L6 cells vs Pure Artemisinin and *A. annua* Tea Extract. Average cell counts of five dose response tests each with triplicate controls and experimental variables for L6s when plated with pure artemisinin and *A. annua* tea extract. Error bars show the standard error of the mean. P = pure artemisinin. TE = *A. annua* Tea Extract. The ratios

refer to the experimental variables to water or ethanol respective to the experiment. Pairwise comparison represented by brackets of statistically significant differences between control groups and experimental groups.

Figure 5 shows the averaged cell counts of L6 cells following 24 hour treatments of pure artemisinin dilutions and *A. annua* tea extract dilutions with comparison to an ethanol and water control, respectively. When L6 cells were plated with pure artemisinin, the experimental wells compared to the control wells showed no statistically significant difference in terms of cell counts. The mean L6 cell count for the ethanol control wells was $2.13x10^6$ cells. The wells that contained L6 cells and a 1:2 pure artemisinin to ethanol dilution had a mean cell count of approximately $2.11x10^6$ cells. The wells that contained L6 cells and a 1:5 pure artemisinin to ethanol dilution had a mean cell count of approximately $2.09x10^6$ cells. The wells that contained L6 cells and a 1:10 pure artemisinin to ethanol dilution had a mean cell count of approximately $2.12x10^6$ cells. Because the means of the control wells and the experimental wells were incredibly close, an unpaired t test resulted in large P values that supported the claim that there was no statistical difference between the L6 cells plated with ethanol versus the L6 cells plated with varying dilutions of pure artemisinin.

Similarly to the L6 cells plated with pure artemisinin, the L6 cells plated with *A. annua* tea extract did not show a statistically significant difference when comparing the cell counts for experimental wells to control wells. The mean L6 cell count for the water control wells was $2.03x10^6$ cells. The wells that contained L6 cells and a 1:5 *A. annua* tea extract to water dilution had a mean cell count of approximately $2.00x10^6$ cells. The wells that contained L6 cells and a 1:10 *A. annua* tea extract to water dilution had a mean cell count of approximately $2.32x10^6$ cells. The wells that contained L6 cells and a 1:25 *A. annua* tea extract to water dilution had a mean cell count of approximately $2.12x10^6$ cells. It is valuable to note that the 1:10 *A. annua* tea extract to water dilution had a mean cell count that was higher than the amount of cells present on average in the control water control wells.

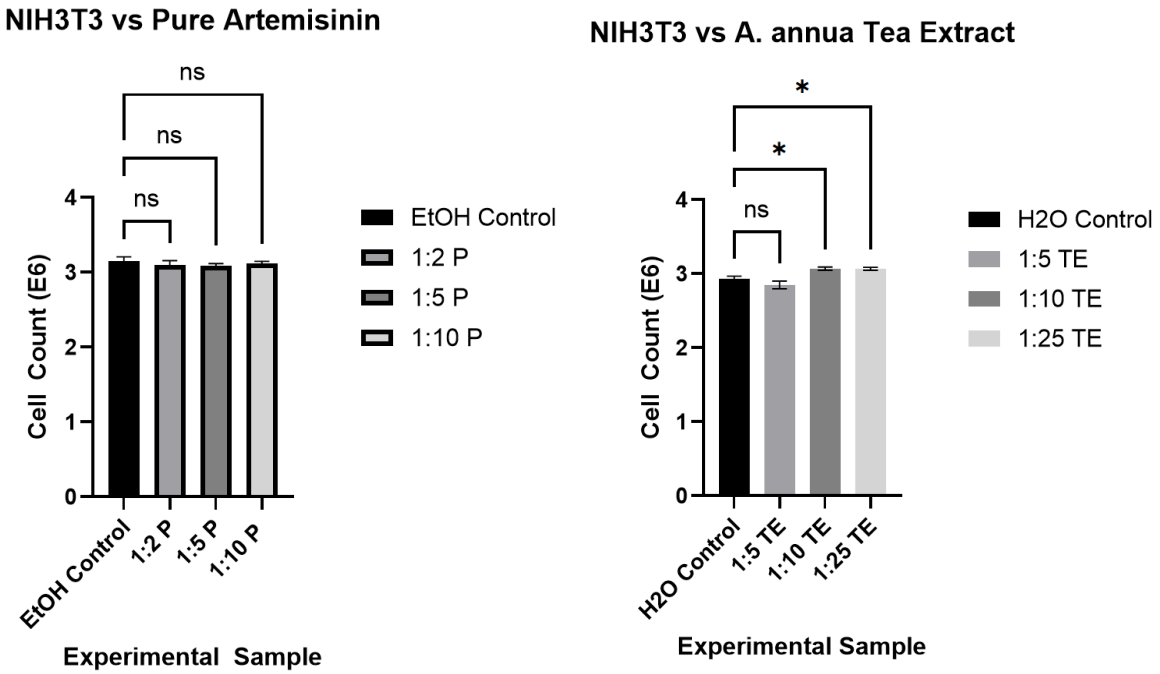

Figure 6. NIH3T3 cells vs Pure Artemisinin and *A. annua* Tea Extract. Average cell counts of five dose response tests each with triplicate controls and experimental variables for NIH3T3 when plated with pure artemisinin and *A. annua* tea extract. Error bars show the standard error of the mean. P = pure artemisinin. TE = *A. annua* Tea Extract. The ratios refer to the experimental variables to water or ethanol respective to the experiment. Pairwise comparison represented by brackets of significant differences between control groups and experimental groups.

Figure 6 shows the averaged cell counts of NIH3T3 cells following 24 hour treatments of pure artemisinin dilutions and *A. annua* tea extract dilutions with comparison to an ethanol and water control, respectively. When NIH3T3 cells were plated with pure artemisinin, the experimental wells compared to the control wells showed no statistically significant difference in terms of cell counts. The mean NIH3T3 cell count for the ethanol control wells was 3.15×10^6 cells. The wells that contained NIH3T3 cells and a 1:2 pure artemisinin to ethanol dilution had a mean cell count of approximately 3.10×10^6 cells. The wells that contained NIH3T3 cells and a 1:5 pure artemisinin to ethanol dilution had a mean cell count of approximately 3.08×10^6 cells. The wells that contained NIH3T3 cells and a 1:10 pure artemisinin to ethanol dilution had a mean cell count of approximately 3.12×10^6 cells. The control wells and experimental wells yielded close absolute cell counts, but an unpaired t test revealed P values greater than 0.05. This indicates there is no statistically significant difference between the NIH3T3 cells that were plated with an ethanol control and the NIH3T3 cells plated with varying dilutions of pure artemisinin.

The NIH3T3 cells plated with *A. annua* tea extract revealed that the absolute cell count of the water control wells was not statistically significant in the way that was hypothesized compared to the experimental wells. In the water control wells, the average cell count for the NIH3T3s was 2.93×10^6 cells. Comparatively, the 1:5 *A. annua* tea extract tea to water dilution had a mean cell count of approximately 2.85×10^6 cells. This was not a statistically significant decrease in absolute cell number compared to the mean. To support this, an unpaired t test

revealed a P value of 0.2454. Interestingly, the 1:10 *A. annua* tea extract tea to water dilution and 1:25 *A. annua* tea extract to water dilution had mean cell count of $3.07x10^6$ cells for both experimental wells. The absolute cell count for both of these experimental wells is greater than the absolute cell count of the control wells. The unpaired t test revealed P values that indicate that absolute cell counts of the 1:10 *A. annua* tea extract tea to water dilution and 1:25 *A. annua* tea extract to water dilution had a small, but statistically significant difference.

A. afra and *A. absinthium* Tea Extract versus T47D Cancer Cell Line

Two additional tea extracts were tested against the T47D cell line as a preliminary dose response test. The goal of these experiments were to determine if plants from the same genus as *A. annua* could also affect a cancer cell line. T47D cells were chosen in the preliminary test of *A. afra* tea extract and *A. absinthium* tea extract because the *A. annua* tea extract had the most statistically significant effect on the cancer cells. One dose response test performed in triplicates was completed on each additional artemisia tea extract.

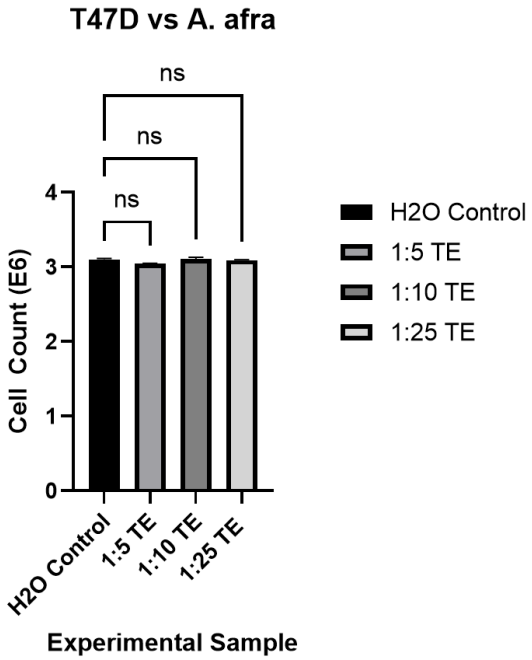

Figure 7. T47D cells vs *A. afra* Tea Extract. Average cell counts of one dose response tests with triplicate controls and experimental variables for T47D cells when plated with *A. afra* tea extract. Error bars show the standard error of the mean. P = pure artemisinin. TE = *A. afra* Tea Extract. The ratios refer to the experimental variables to water. Pairwise comparison represented by brackets of significant differences between control group and experimental group.

Figure 7 shows the averaged cell counts of T47D cells following 24 hour treatments of *A. afra* tea extract dilutions with comparison to a water control. The mean absolute cell count for the water control was approximately $3.10x10^6$ cells. The mean absolute cell count for the 1:5 *A. afra* tea extract versus the water dilution, the 1:10 *A. afra* tea extract versus the water dilution, and the 1:25 *A. afra* tea extract versus the water dilution was $3.04x10^6$, $3.11x10^6$, and $3.09x10^6$

cells, respectively. The unpaired t test revealed that the absolute cell number of the experimental wells compared to the control wells did not have a statically significant difference.

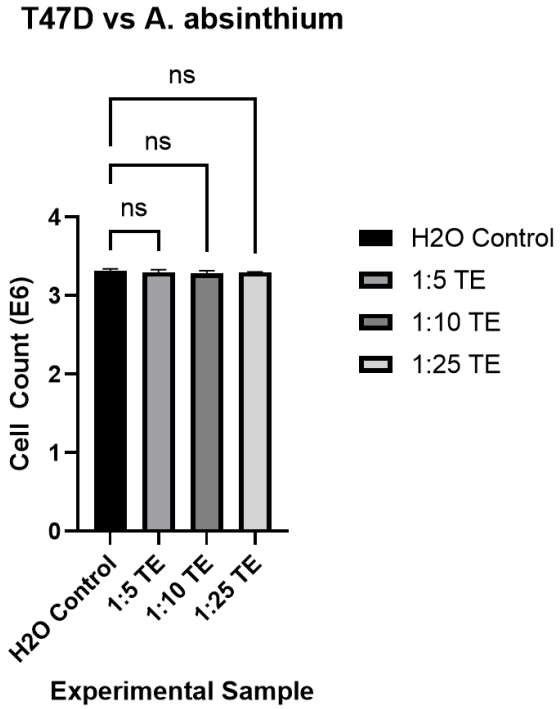

Figure 8. T47D cells vs *A. absinthium* Tea Extract. Average cell counts of one dose response tests with triplicate controls and experimental variables for T47D cells when plated with *A. absinthium* tea extract. Error bars show the standard error of the mean. P = pure artemisinin. TE = *A. absinthium* Tea Extract. The ratios refer to the experimental variables to water. Pairwise comparison represented by brackets of significant differences between control group and experimental group.

Figure 8 shows the averaged cell counts of T47D cells following 24 hour treatments of *A. absinthium* tea extract dilutions with comparison to a water control. The mean absolute cell count for the water control was approximately $3.32x10^6$ cells. The mean absolute cell count for the 1:5 *A. absinthium* and the 1:25 *A. absinthium* tea extract versus the water dilution was $3.30x10^6$, $3.29x10^6$, and $3.30x10^6$ cells, respectively. The unpaired t test revealed that the absolute cell number of the experimental wells compared to the control wells did not have a statically significant difference.

Discussion

The purpose of these experiments was to observe the effects of *A. annua* tea extract and pure artemisinin solution on the absolute cell number of cancer cells, T47Ds and LNCaPs, and non cancer cells, L6s and NIH3T3s. The absolute cell number was chosen to be observed because it is not understood how the active compounds present in the tea extracts or pure artemisinin solution are able to affect the cancer cells. There is not enough evidence to conclude that the active compounds are affecting the proliferation rate of the cancer cells or are cytotoxic to the cancer cells themselves. Because the mechanism of action is unknown, the absolute cell number was chosen to be observed and used to determine if the dose response tests are able to affect the cell lines chosen. It is also important to note that all the absolute cell counts determined from the experiment were incredibly close in value resulting in very low standard errors compared to the mean. This also indicates that mean cell counts of control wells compared to mean cell counts of the experimental wells would have very minimal amounts of variation but statistically significant differences can still be determined through unpaired t tests coupled with calculating P values. In fact, minimal amounts of variability among samples makes the statistical analysis performed more reliable because it is unlikely that the data gathered is due to chance alone.

When pure artemisinin and *A. annua* tea extracts were plated with T47D cells, the result was a small, but statistically significant difference between the control wells and experimental wells. The pure artemisinin only had a statistically significant effect when plated at its highest concentration while the *A. annua* tea extract had a statistically significant effect at all concentrations. The data collected from this experiment support the literature that suggests that the active compound artemisinin has an effect on T47D cancer cells (Kumari, Keshari, Sengupta, Sabat, & Mishra, 2017).In addition to having an effect on the absolute cell number of T47D cells, the *A. annua* tea extract also had a small, but statistically significant difference on LNCaP cells at the highest concentration only. The pure artemisinin did not have an effect on the absolute cell number of LNCaPs. This data does not support the evidence found in literature that suggests that artemisinin should have an effect on prostate cancer cell lines (Willoughby et al., 2008). Therefore, in combination with the results from the effects of pure artemisinin solution on the absolute cell count of T47D cells, the hypothesis can be rejected. The pure artemisinin only had an effect on the T47D cells at the highest concentration and no effect on the LNCaP cells. This indicates that there may be additional active compounds found in the *A. annua* tea extract that can have a greater effect on the absolute cell number of the cancer cell lines. Additionally, the *A. annua* tea extract had a greater effect on the absolute cell count of T47D cells compared to the LNCaP cells. This could indicate that there may be compounds in the *A. annua* tea extract that interact more successfully with the T47D cells rather than the LNCaP cells.

Equally important, when pure artemisinin was plated with both L6 cells and NIH3T3 cells, there was no statistically significant difference between the ethanol control wells and the experimental wells. When L6 cells were plated with *A. annua* tea extract, there was also no

statistically significant difference between the water control wells and the experimental wells. Interestingly, when NIH3T3 cells were plated with *A. annua* tea extract, there was a statistically significant difference between the absolute cell count of the water control wells and the absolute cell count of two of the experimental wells. The 1:10 *A. annua* tea extract to water dilution and 1:25 *A. annua* tea extract to water dilution had absolute cell counts that were greater than the water control wells' absolute cell count. This could indicate that the *A. annua* tea extract has active compounds that interact uniquely with the NIH3T3 cells and result in an increase in cell number. If this were true, then it would have been expected that the high concentration of tea extract in the 1:5 *A. annua* tea extract to water dilution would have also resulted in a higher absolute cell count compared to the mean. It is possible that the *A. annua* tea extract has active compounds that uniquely interact with the NIH3T3 cells and lead to an increase in absolute cell number compared to the mean. In conclusion, the hypothesis that suggested that the pure artemisinin and *A. annua* tea extract would not have an effect on non-cancer cell lines can be supported.

Finally, T47D cells were also plated with *A. afra* and *A. absinthium* tea extracts as a preliminary experiment to determine if other plants in the artemisia genus can affect the absolute cell count of a cancer cell line. Both *A. afra* and *A. absinthium* tea extracts did not result in a statistically significant difference between the absolute cell number of the water control wells compared to the absolute cell number of the experimental wells. These two additional plants most likely have different active compounds compared to the *A. annua* plant used in the previous experiments and have a different effect on the T47D cells. Although it is important to note that only one dose response experiment was completed with the *A. afra* and *A. absinthium* tea extracts and further experimentation is recommended.

Recommendations

Future studies should repeat the dose response experiments with *A. afra* and *A. absinthium* tea extracts on T47D cells since only one trial of this protocol was performed. Further exploration of these tea extracts on LNCaP cells, L6 cells, and NIH3T3 cells could reveal that another member of the artemisia genus could have an effect. Experimentation with both the pure artemisinin solution and all three tea extracts should be conducted on additional cancer cell lines available to broaden the results and help to determine if there is an effect on the absolute cell number of other cancer cell lines.

Additionally, if this study is repeated in the future, the total artemisinin concentration as well as the total concentration of other active compounds found in *A. annua*, *A. afra,* and *A. absinthium* plants should be determined through UV-Vis spectrophotometry coupled with gas chromatograph-mass spectrometry. In the interest of time, this was not completed. In doing so, the exact concentrations of the active compounds present in each of the plants could be determined and compared. Additionally, this could allow for the concentration of the pure artemisinin solution to match the concentration of artemisinin present in the tea extracts. This would serve as an additional variable that could be controlled throughout the experimentation. Moreover, the extraction methods used for the pure artemisinin solution was ethanol and water for all of the tea extracts. Literature indicates that active compounds of *A. annua* have been extracted in oils, alcohols, and aqueous solutions. Each extraction solvent could result in slightly different compounds due to their chemical properties. Using different extraction methods in combination with the previously mentioned techniques for determining concentrations, could reveal different active compounds present that may have an effect on the absolute cell number of the cell lines explored.

Furthermore, analysis could be performed to determine some aspect of the mechanism of action of artemisinin in cancer cells. Scientists are still attempting to determine the mechanism of action for artemisinin when it functions as an antimalarial drug and attacks the parasite *P. falciparum* (O'Neill, Barton, & Ward, 2010). Current evidence suggests that artemisinin is able to attack cancer cells through the reaction of its endoperoxide bond to create Fe^{2+} to be converted into oxygen centered free radicals. It is hypothesized that the addition of iron to cancer cells triggers the cytotoxic effects of artemisinin (Das, 2015). Experimentation could be performed to understand the underlying biochemical function of this active compound.

References

CDC. (2020, September 14). Basic Information About Breast Cancer. Retrieved from Centers for Disease Control and Prevention website: https://www.cdc.gov/cancer/breast/basic_info/index.htm#:~:text=Each%20year%20in%20the%20United

CDC. (2019). What Is Prostate Cancer? Retrieved from Centers for Disease Control and Prevention website: https://www.cdc.gov/cancer/prostate/basic_info/what-is-prostate-cancer.htm

Dib, I., & El Alaoui-Faris, F. E. (2019). Artemisia campestris L.: review on taxonomical aspects, cytogeography, biological activities and bioactive compounds. *Biomedicine & Pharmacotherapy*, *109*, 1884–1906. https://doi.org/10.1016/j.biopha.2018.10.149

Furtado, S., & Lewis, C. (2021). *Artemisinin Project: Effect of Artemisia annua Extract on Safe ESKAPE Pathogen Relatives*.

Kim, W.-S., Choi, W. J., Lee, S., Kim, W. J., Lee, D. C., Sohn, U. D., … Kim, W. (2015). Anti-inflammatory, Antioxidant and Antimicrobial Effects of Artemisinin Extracts from Artemisia annua L. *The Korean Journal of Physiology & Pharmacology : Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology*, *19*(1), 21–27. https://doi.org/10.4196/kjpp.2015.19.1.21

Kumari, K., Keshari, S., Sengupta, D., Sabat, S. C., & Mishra, S. K. (2017). Transcriptome analysis of genes associated with breast cancer cell motility in response to Artemisinin treatment. *BMC Cancer*, *17*. https://doi.org/10.1186/s12885-017-3863-7

Lubbe, A., Seibert, I., Klimkait, T., & van der Kooy, F. (2012). Ethnopharmacology in overdrive: The remarkable anti-HIV activity of Artemisia annua. *Journal of Ethnopharmacology*, *141*(3), 854–859. https://doi.org/10.1016/j.jep.2012.03.024

Liu, N. Q., Van der Kooy, F., & Verpoorte, R. (2009). Artemisia afra: A potential flagship for African medicinal plants? *South African Journal of Botany*, *75*(2), 185–195. https://doi.org/10.1016/j.sajb.2008.11.001

National Cancer Institute. (2007, September 17). What Is Cancer? - National Cancer Institute. Retrieved from www.cancer.gov website: https://www.cancer.gov/about-cancer/understanding/what-is-cancer#:~:text=Cancer%20is%20a%20disease%20caused

National Cancer Institute. (2020, September 25). Cancer Statistics. Retrieved from National Cancer Institute website: https://www.cancer.gov/about-cancer/understanding/statistics

NCCIH Clearinghouse. (2021, January). Mugwort. Retrieved from NCCIH website: https://www.nccih.nih.gov/health/mugwort

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

Nti-Gyabaah, J., Gbewonyo, K., & Chiew, Y. C. (2010). Solubility of Artemisinin in Different Single and Binary Solvent Mixtures Between (284.15 and 323.15) K and NRTL Interaction Parameters. *Journal of Chemical & Engineering Data*, *55*(9), 3356–3363. https://doi.org/10.1021/je100125x

O'Neill, P. M., Barton, V. E., & Ward, S. A. (2010). The Molecular Mechanism of Action of Artemisinin—The Debate Continues. *Molecules*, *15*(3), 1705–1721. https://doi.org/10.3390/molecules15031705

Suberu, J. O., Gorka, A. P., Jacobs, L., Roepe, P. D., Sullivan, N., Barker, G. C., & Lapkin, A. A. (2013). Anti-Plasmodial Polyvalent Interactions in Artemisia annua L. Aqueous Extract – Possible Synergistic and Resistance Mechanisms. *PLoS ONE*, *8*(11), e80790. https://doi.org/10.1371/journal.pone.0080790

Szopa, A., Pajor, J., Klin, P., Rzepiela, A., Elansary, H. O., Al-Mana, F. A., … Ekiert, H. (2020). Artemisia absinthium L.—Importance in the History of Medicine, the Latest Advances in Phytochemistry and Therapeutical, Cosmetological and Culinary Uses. *Plants*, *9*(9), 1063. https://doi.org/10.3390/plants9091063

Tu, Y. (2011). The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. *Nature Medicine*, *17*(10), 1217–1220. https://doi.org/10.1038/nm.2471

Visconti, R., Della Monica, R., & Grieco, D. (2016). Cell cycle checkpoint in cancer: a therapeutically targetable double-edged sword. *Journal of Experimental & Clinical Cancer Research*, *35*(1). https://doi.org/10.1186/s13046-016-0433-9

Willoughby, J. A., Sundar, S. N., Cheung, M., Tin, A. S., Modiano, J., & Firestone, G. L. (2008). Artemisinin Blocks Prostate Cancer Growth and Cell Cycle Progression by Disrupting

Sp1 Interactions with the Cyclin-dependent Kinase-4 (CDK4) Promoter and Inhibiting CDK4 Gene Expression. *Journal of Biological Chemistry*, *284*(4), 2203–2213. https://doi.org/10.1074/jbc.m804491200

Supplemental Materials

Supplemental Table 1. Pure Artemisinin Solution Dilution for Dose Response Testing

Well Label	Added Volume of EtOH	Added volume of Artemisinin Solution
EtOH control	100 µL	0 µL
1:2 PS	50 uL	50 uL
1:5 PS	80 uL	20 uL
1:10 PS	90 uL	10 uL

*Total Well Volume = 500uL

Supplemental Table 2. *A. annua* tea extract Dilution for Dose Response Testing

Well Label	Added Volume of H_2O	Added Volume of *A.annua* Tea Extract
DI Water control	100 µL	0 µL
1:5 TE	80 uL	20 uL
1:10 TE	90 uL	10 uL
1:25 TE	96 uL	4 uL

*Total Well Volume = 500uL

Supplemental Table 3. Dose Test Well Labels for Pure Artemisinin Solution and *A. annua* Tea Extract

	1	2	4	3	5	6
A	0.2% EtOH Control	0.2% EtOH Control	DI Water Control	0.2% EtOH Control	DI Water Control	DI Water Control
B	1:2 Pure	1:2 Pure	1:5 Pure	1:5 TE	1:5 TE	1:5 TE
C	1:5 Pure	1:5 Pure	1:10 Pure	1:10 TE	1:10 TE	1:10 TE
D	1:10 Pure	1:10 Pure	1:25 Pure	1:25 TE	1:25 TE	1:25 TE

Pure = Pure Artemisinin Solution; TE = *A. annua* Tea Extract

Supplemental Table 3. Dose Test Well Labels for *A. afra* Tea Extract and *A. absinthium* Tea Extract

	1	2	3	4	5	6
A	DI Water Control	DI Water Control	DI Water Control	DI Water Control	DI Water Control	DI Water Control
B	1:5 AF TE	1:5 AF TE	1:5 AF TE	1:5 AB TE	1:5 AB TE	1:5 AB TE
C	1:10 AF TE	1:10 AF TE	1:10 AF TE	1:10 AB TE	1:10 AB TE	1:10 AB TE
D	1:25 AF TE	1:25 AF TE	1:25 AF TE	1:25 AB TE	1:25 AB TE	1:25 AB TE

AB TE = *A. absinthium* Tea Extract; AF TE = *A. afta* Tea Extract