WPI

Analyzing the Anti-Cancer Properties of *Artemisia annua*

Submitted by:

Thien-Kim Nguyen

Jillian Spera

Advisors:

Professor Michael Buckholt, PhD

Professor Jill Rulfs, PhD

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Abstract

As cancer treatments become more advanced, the need for new drugs to counter resistance is ever-growing. *Artemisia annua* is a novel subject in oncology for its cytotoxic abilities primarily from the compound artemisinin. Using T47D breast cancer cells, we studied the plant's effects on cell proliferation, and we examined markers of cell cycle regulation and oxidative stress to understand the potential mechanisms of action. Initial data showed notable reduction in cell numbers for samples treated with whole plant extracts, alongside weaker results using the compound alone. Our preliminary results suggest a killing mechanism via free radicals and apoptosis, but further study is needed to clarify the effects of the extract and the pure compound.

Acknowledgements

Our team would like to thank both of our advisors, Professor Michael Buckholt and Professor Jill Rulfs, for guiding us on the development of this project as part of the Biology & Biotechnology department's ProjectLab. The ProjectLab provided the lab resources, and our advisors provided the protocols for the blotting solutions and procedures. For our dosage test experiments, Professor Pamela Weathers provided the *Artemisia afra* tea extract. Samuel Furtado and Carter Lewis of our sister project in the ProjectLab provided the *Artemisia annua* tea extract, and they also shared their protocol for its extraction. Lastly, we shared T47D cell cultures with fellow WPI student Sarah Huber.

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Introduction/Background

Cell Lines: Breast Cancer Cells (T47D) and Ovarian Cancer Cells (OVCAR-3)

Breast cancer is abnormal, uncontrollable cell growth originating in breast tissue. Breast cancer is one of the most common types of cancer in women, but it can also affect men as well. In general, there are three main subclasses of breast cancer itself, most of these cancer cells contain either all, or at least one of the following: estrogen receptors, progesterone receptors and HER2 protein. Thus the types of breast cancer are split into three main groups: HER2 breast cancer, progesterone and estrogen fueled breast cancer, or triple negative breast cancer, which lacks all three receptor types (Breastcancer.org, 2020). In this day and age, breast cancer normally has a fairly good prognosis, but triple negative breast cancer, which accounts for about 10-20% of all cases, tends to be more aggressive and harder to treat, resulting in a worse prognosis than the other types. The cancer cells used in this project are T47Ds, which are estrogen-responsive epithelial cells from a 54-year-old female (ATCC, 2020). This hypotriploid cell line expresses the WNT7B oncogene, and it was derived from an infiltrating ductal carcinoma in the patient's breast.

Ovarian cancer is another type of abnormal and uncontrollable cell growth, but instead, it originates in the ovaries. Roughly 1 in 78 women will have ovarian cancer, and it is the fifth deadliest cancer in women ages 35-74 (NOCC, 2020). The OVCAR-3 cancer cells used in this project are epithelial cells from a 60-year-old Caucasian female, and the cells are androgen, estrogen, and progesterone receptor positive (ATCC, 2020). This aneuploid cell line has a largely irregular karyotype, with many normal chromosomes underrepresented and chromosome counts near the triploid range.

Anti-Cancer Compounds: Pure Artemisinin, *Artemisia annua*, and *Artemisia afra*

Artemisinin ($C_{15}H_{22}O_5$) is a sesquiterpene lactone containing a peroxide bridge, and its endoperoxide ring is the center of its mechanism of action. It works through the cleavage of the endoperoxide bridges by iron when in contact with red blood cells (heme), which produces free radicals that damage macromolecules and cause lethal oxidative stress in the cell (Greenshields, 2017). Artemisinin has poor solubility in oil and water, so it is typically applied orally or rectally to the digestive tract in humans. There have also been experiments to chemically modify the compound for intravenous/intramuscular injection, with some success for intravenous treatment on 19 cancer patients in a Phase 1 study at Georgetown University (Slezáková, 2017). In regards to the immune system, artemisinin has been found to regulate certain T-cells, block, and inhibit the activation of B-cells (Tin, 2012). Studies have also shown that artemisinin can regulate immune function by blocking the production of inflammatory cytokines along with cyclin-dependent kinase activity to arrest the cell cycle (Zhu, 2013). Unfortunately, it is difficult to work with in drug form because of its low bioavailability, poor pharmacokinetic properties, and high cost. In addition, usage of artemisinin alone has led to relapses requiring other drugs to

clear and prevent such damage. Though usually mild, its side effects include nausea, vomiting, loss of appetite, dizziness, and mild blood abnormalities.

The genus *Artemisia* (including sagebrush, mugwort, tarragon, and more) , consists of hundreds of daisy-like species found all over the globe that are used for their general chemical properties. The leaves of some species have been used to create beverages and medicines, such as artemisia tea. The subject of this research, the species *Artemisia annua* (commonly known as sweet/annual wormwood, sagewort, or mugwort) is an annual short-day plant native to temperate Asia and naturalized across the globe (Efferth, 2017). It is characterized by its brown/violet stem, green/yellow flowers, and brown seeds, and it is known for its strong antioxidant properties due to its high production of phenolic compounds (such as coumarins, flavones, and phenolic acids) (Carbonara, 2012). Artemisia originates as a Chinese medicinal herb used in tea and supplements for its antimicrobial and anti-inflammatory properties. Today, artemisia and its derivatives have had successful experimentation in treatments against malaria (Elfawal, 2012), auto-immune diseases (Langroudi, 2010), and viruses (Seo, 2017). Specifically, this project focuses on the plant's anti-cancer properties, which have been studied more thoroughly in recent years because of its cell-killing abilities through the production of free radicals and oxidative stress (Efferth, 2004; Ho, 2013; Kiani, 2020). It is believed that the artemisia family's vast pleiotropic effects can attack multi-drug resistant tumors that do not respond well to existing therapies. The plants also exhibit little bystander effect, possibly making them safer for normal cells in heterogeneous tumors compared to other treatments. Studies like Efferth's show that the plant's artemisinin is the core of its cytotoxic effects, but there has also been promising data on using raw extract because other plant compounds (like flavonoids) have been observed to give an additive effect on the cytotoxicity as well as aid in compound extraction from the plant (Carbonara, 2012). Their effects include inhibiting the proliferation of tumor cells and mediating the tumor-related signaling pathways (Zhang, 2014). Other notable effects are promoting apoptosis and disrupting cancer invasion and metastasis (Kadioglu, 2017). Lastly, studies on artemisia showed angiogenesis prevention as well as regulation of the tumor microenvironment (Langroudi, 2010). The artemisia family has been shown to use these methods in inhibiting at least 20 different cancers, including osteosarcoma (Isani, 2019), liver cancer (Liu, 2018), gastric cancer (Sun, 1992), and ovarian cancer (Wu, 2011). With fast absorption, wide distribution, quick excretion, little cross resistance, and some multi-drug resistance reversal, artemisia treatments continue to garner attention in oncology research. To compare their effectiveness, this project tested the effects of *A. annua* tea extract and pure artemisinin on cancer cells.

Artemisia afra (commonly known as African wormwood) is indigenous to Africa, and it is characterized by green, fern-like leaves along with white bristles and yellow flowers (Liu, 2009). All parts of the plant have various medicinal properties, including treatment for colds, malaria, cough, fever, asthma, and diabetes. While artemisinin is most abundantly found in *A. annua* (a close relative of *A. afra*), *A. afra* strains can be cultivated with trace amounts of the compound. In this project, an *A. afra* strain with no artemisinin was tested as well to see if the plant's other compounds alone still had strong cytotoxicity against cancer cells.

Cell Cycle Regulators, Apoptotic Factors, and Reactive Oxygen Species

In studying the possible mechanisms of action behind artemisia's cytotoxicity, we looked at three methods and their corresponding target proteins. First, we observed the effects of our extract and artemisinin on cyclin D1 levels in cancer cells. This protein was chosen because artemisinin has been shown to reduce cytokine production and CDK activity in order to arrest the cell cycle (Zhu, 2013). CD1 works with other cyclins to regulate CDKs, and binding to CDK4/CDK6 allows the cell cycle G1/S transition (Mohammadizadeh, 2013). CD1 is positively regulated by the tumor suppressor protein Rb since it phosphorylates and inhibits Rb. Mutations of the CD1 gene often lead to its overexpression and amplification. This promotes excessive cell cycle progression and even shortens the G1 stage, which can lead to cancer. CD1 overexpression is reported to occur in roughly 50% of human breast cancers, and CD1 gene amplification is reported in 20% of breast cancers (Roy, 2006). In our experiments, we predicted that there would be a decrease in CD1 levels for the cancer cells following treatment since the artemisinin should prevent the cell cycle from continuing as rapidly.

We also analyzed if artemisia affected superoxide dismutase 1 (SOD1) activity since it is suspected to kill cells via the free radicals and oxidative stress caused by the breakdown of artemisinin (Efferth, 2004). SOD1 destroys free superoxide radicals by binding copper and zinc ions and converting the superoxides into oxygen and hydrogen peroxide (Papa, 2014). These lethal reactive oxygen species are formed naturally in many metabolic processes, and SOD1 reduces this damage to allow proper cell function. This protein is typically found in the cytoplasm, nucleus, and intermembrane space of mitochondria, where reactive oxygen species (ROS) byproducts are often formed (Gomez, 2019). Recently, SOD1 has been found to have a crucial role in some cancers since the abnormal activity of tumor cells often leads to unbearably high ROS levels. Cancer cells have been observed to increase SOD1 levels to counteract ROS for their survival. For example, SIRT3 expression (a regulator of the SOD family) has been shown to decrease in 87% of breast cancers (Papa, 2014). This may correlate to an upregulation of SOD1 to maintain cell survival. We predicted that SOD1 presence would increase in the experimental cancer cells since artemisia would be causing more free radicals to form and kill the cells.

The last mechanism of action that we looked at involved the plant extract inducing apoptosis in the cancer cells (Efferth, 2004). We used the apoptosis regulator Bax protein (also known as bcl-2-like protein 4) as the main indicator of the process since it activates apoptosis by forming a heterodimer with a related protein called BCL2 (Carpenter, 2020). Bax functions by opening the mitochondrial voltage-dependent anion channel (VDAC), losing the membrane potential there and releasing cytochrome c and more pro-apoptotic factors from the mitochondria (Oltval, 1993). This mitochondrial outer membrane permeabilization activates caspases to terminate the cell. The Bax gene is upregulated by the tumor suppressor p53, which is why Bax deletions or loss-of-function mutations often lead to oncogenic gene activation and cancer progression (Carpenter, 2020). We predicted that Bax levels would increase in cancer cells after the treatment since artemisia should be promoting apoptosis for these cells.

Methods

Experimental Compound Preparation

The pure artemisinin solution was made following the artemisinin MSDS page by Cayman Chem. This solution contained 16mg of artemisinin and 1mL of pure ethanol (EtOH). Once the artemisinin was dissolved, the solution was diluted to 1% EtOH by adding 99mL of distilled water. The end solution was made to have an artemisinin concentration of about 470uM in order to match the tea extract.

The *A. annua* tea extract was made from the plant's leaves, and the artemisinin content of this tea extract was 470 uM. 2g of dried *A. annua* leaves was added to 200mL boiling water, and it was left for 10min. with a stirring rod. The mixture was strained and filtered, and the samples were stored as aliquots in a -20°C freezer (Bilia, 2006).

The *A. afra* tea extract was made from the plant's leaves, and the artemisinin content of this tea extract was negligible. Dried *A. afra* leaves were resuspended in dichloromethane (1g dried leaves per 20 mL DCM) and extracted for 30min. in a sonicating water bath at room temperature. Leaves were extracted twice more with fresh dichloromethane, then the extracts were pooled and dried under N_2. The final extract was resuspended in distilled water and stored as aliquots in a -20°C freezer (Martini, 2020).

Cell Culture and Maintenance

T47D cells were cultured in DMEM with 10% Fetal Bovine Serum and 0.2 units/mL bovine insulin, and they were maintained in a vented T75 flask. 1% PenStrep was also added to the flask. The flasks were stored in an incubator at 37°C, and 5% Carbon Dioxide. Cells were obtained from ATCC (cell line number HTB-133), and they were checked, split, and replated as needed.

OVCAR-3 cells were cultured in DMEM media with 20% Fetal Bovine Serum and 0.2 units/mL bovine insulin, and they were maintained in a vented T75 flask. 1% PenStrep was also added to the flask. The flasks were stored in an incubator at 37°C, and 5% Carbon Dioxide. Cells were obtained from ATCC (cell line number HTB-161), and they were constantly checked, split, and replated as needed.

Cellometer Cell Counting

Following trypsinization and resuspension, 50uL samples of the T47D or OVCAR-3 cell cultures were taken and mixed thoroughly with 50uL of Trypan blue. 20uL of this mixture was then put in a slide and inserted into a Cellometer® (Sigma-Aldrich) for observation and counting. The program calculated the number of live cells, and the counts used were the averages for each

sample. Cell counting was done before plating for the experiments, and it was also done after each dose response test to compare the starting and end live cell counts.

Dose Response Tests

Three dose response tests were conducted in total. The first used T47Ds and observed the effects of pure artemisinin and *A. annua* tea extract on cell counts (conducted twice). The second tested the effect of *A. afra* tea extract on T47Ds. The third used OVCAR-3s and observed the effects of the extract and artemisinin on the cells. Distilled water was the control for the *A. annua* and *A. afra* tea extracts, and 1% EtOH was the control for the pure artemisinin solution. The tea extract was used in more diluted doses compared to the artemisinin solution because an initial dosage test showed almost no surviving cells when the extract was not diluted to at least a 1:10 ratio. The well labels and artemisia dilutions for the tests are in Appendices A and B.

T47D or OVCAR-3 cells were plated at 1 x 10^5 cells per well in a 24-well plate. Each well had a total volume of 500uL, with 400uL of media/cells and the remaining 100uL split between the extract/artemisinin and their solvents depending on the dilution for that well. The plates were incubated at 30°C for 24 hours, removed from the plate with 1-2mL of trypsin, and then the cells were counted for each well. The cells were observed under a 100x lens.

Cell Harvesting and Protein Spectrometry

The media was aspirated off from the T47D dose response tests, and the cells were frozen at –80°C for 48hr. PBS was added to the wells, and then the cells were scraped off for use in protein spectrometry and analysis. The protein content was determined using the BioRad Quick Start Bradford protein assay using bovine serum albumin (BSA) as the protein standard.

Protein Dot Blotting and Densitometry

Immobilon membrane was wetted with methanol then sandwiched between the two layers of the dot blotter. 20ug of protein from each dosage test sample was deposited in the slots. The blotter was connected to an aspirator and left to sit for a few minutes as the excess liquid was removed. Once the protein was drawn into the membrane, it was removed.

The membrane was blocked using a blocking solution of 5% non-fat milk and left on a shaker for one hour. Next, the primary antibody solution was made with TBS-T (10mM Tris, 0.1% tween, 0.9% NaCl, pH~7.4) at their designated ratios, and the membrane was submerged in the solution on a shaker for one hour. The primary antibodies used were Bax (starting dilution of 1:200), CD1 (starting dilution of 1:200) and SOD (starting dilution of 1:5000), all of which were obtained from Santa Cruz Biotechnology. After washing the membrane 3-5 times with wash buffer (TBS-T) for 5 minutes each, it was placed in 10mL of secondary antibody solution (alkaline phosphatase conjugated Rabbit anti-mouse IgG from Sigma-Aldrich, and mixed in a

1:5000 dilution in TBS-T) for 1hr. The membrane was once again washed 3-5 times with wash buffer for 5 minutes each, and it was submerged in substrate solution (one Sigma-Aldrich Fast Tab dissolved in approximately 10 mL of TBS) for a few minutes until dark spots appeared where the samples were placed.

The blotted membranes were scanned with a Biorad Imager for densitometry analysis. Using the computer program's "Volume" tool, the sample areas on the membrane were selected for analysis. After automatically adjusting the values with any background detected, the program provides an absolute dataset of their densitometry values. Graphs were made with this data to see any trends between the samples.

Results

First Dosage Test: Pure Artemisinin and *A. annua* vs. T47Ds

The main purpose of this experiment was to see if different dilutions of the pure artemisinin and *A. annua* tea extract (listed in Appendix A) affected the live cell counts of the breast cancer cells. We expected the cell counts to increase as the treatments became more diluted, and this trend helped show that the results were due to the extract/artemisinin's presence.

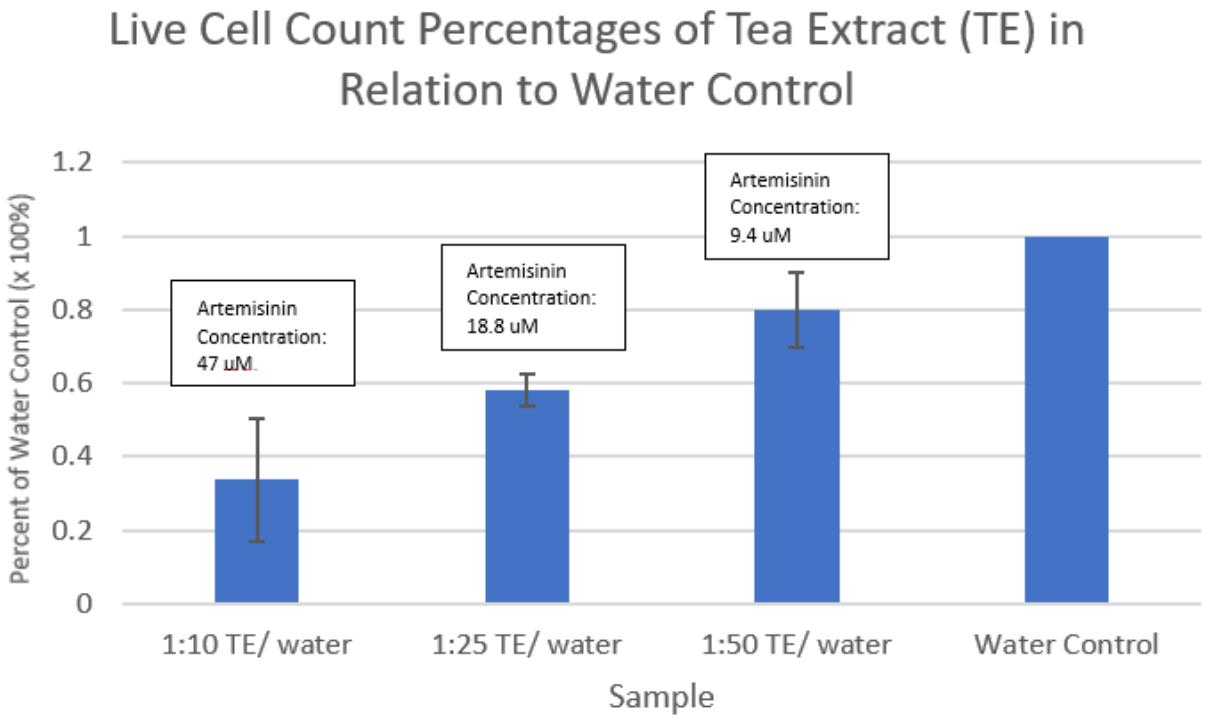


Figure 1: TE / Water Control for T47Ds. These are the average live cell counts for each extract dilution over the average cell count of the water control. Error bars show the standard deviation for the tea extract solutions. TE = *A. annua* tea extract. The ratios refer to Extract : Water. N = 2.

Figure 1 shows the end cell counts following 24hr treatments of different *A. annua* tea extract dilutions in comparison to the water control. The total number of cells in the wells increased as the extract decreased, with the cell percentages going from 35% for the 1:10 samples to 80% in the 1:50 samples. There was some variability in the triplicates for each sample, as shown by the error bars.

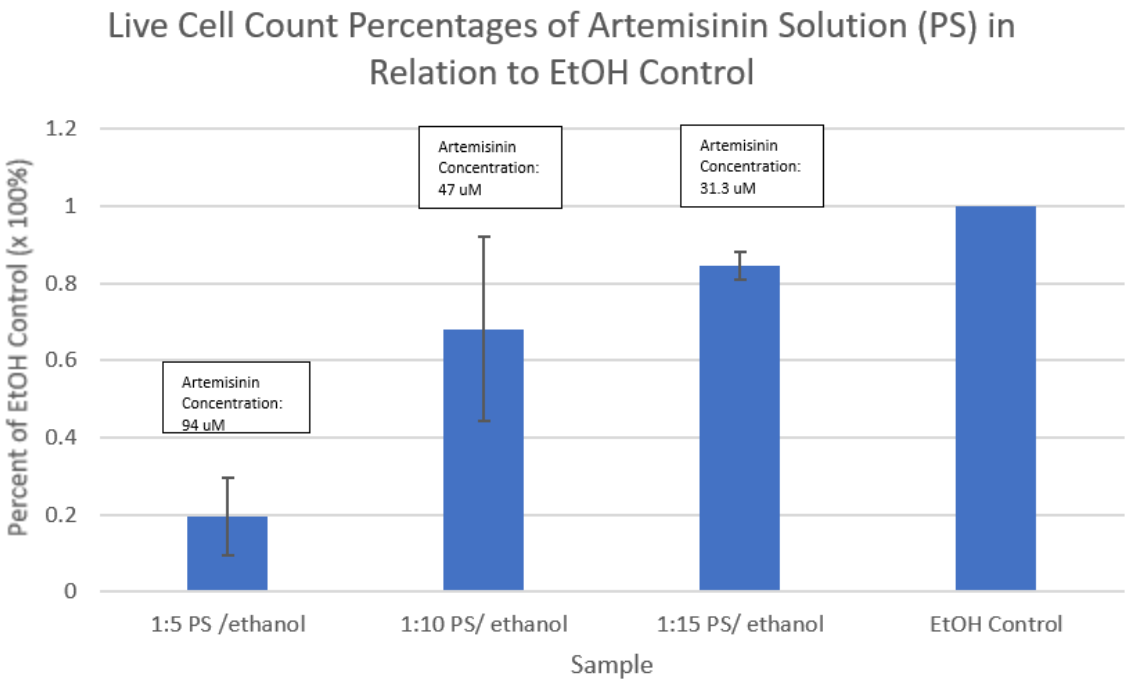


Figure 2: PS / EtOH Control for T47Ds. These are the average live cell counts for each artemisinin dilution over the average cell count of the EtOH control. Error bars show the standard deviation for the artemisinin solution dilutions. PS = pure artemisinin solution. The ratios refer to Artemisinin : EtOH. N = 2.

Figure 2 shows similar data to Figure 1, but for the artemisinin samples compared to their EtOH control. This data showed a similar trend of the live cell counts decreasing as the artemisinin increased, with a 20% total cell count compared to the control for the 1:5 samples. This percentage increased to 85% in the 1:15 samples. However, the triplicates varied more for this data, as depicted by the wide error bars.

From both experimental tests, the controls had generally low effects on cell count by themselves, which showed that the solvents of the treatments had little effect on the cells compared to the extract/artemisinin. *A. annua* seemed to have a larger effect on cell count compared to artemisinin at the same ratio (1:10). The tea extract ended with a cell count that was around one third of its control (~65% reduction in cell number) while artemisinin ended with almost three-quarters of the cell amount in its control (~25% reduction). Since the artemisinin solution was made to have the same amount of artemisinin as the tea extract (470uM), we suspected that the other compounds in the plant could have caused this data difference.

Second Dosage Test: *A. afra* vs. T47Ds

Since *A. afra* tea extract only has trace amounts of artemisinin, it was used to determine if the other compounds in artemisia plants also had strong effects on cells upon exposure.

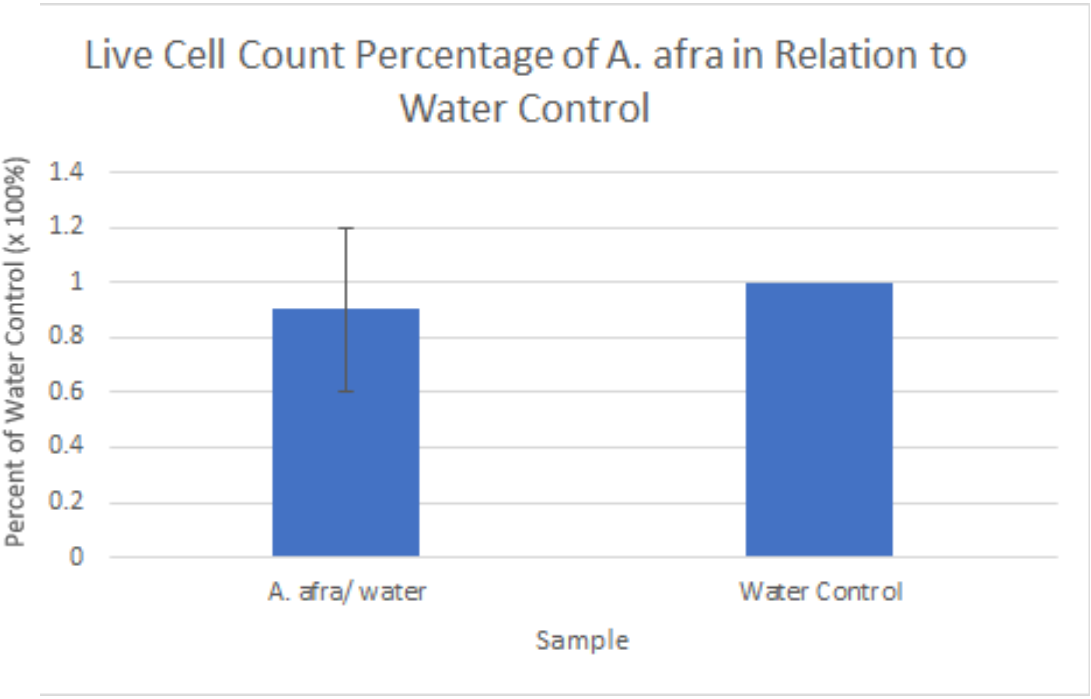


Figure 3: *A. Afra* / Water Control for T47Ds. These are the average live cell counts for *A. afra* over the average cell count of the water control. Error bars show the standard deviation for *A. afra.* N = 1.

Since *A. afra* had similar cell counts to the water control according to Figure 3 (the remaining cell counts of *A. afra* were roughly 90% of the cell numbers seen in the water control), this data seems to show that the other plant compounds had little to no effect on the cells. Compared to the ~40% difference in cell counts between the extract and artemisinin 1:10 samples in the First Dosage Test, this 10% cell reduction does not fully explain the data. We speculate that artemisinin could have an additive effect on cancer cells when combined with the other plant compounds, which would make the compounds more effective together than on their own.

Third Dosage Test: Pure Artemisinin and *A. annua* vs. OVCAR-3s

This experiment was performed not only to see if pure artemisinin and *A. annua* tea extract affect the cell viability of ovarian cancer cells, but also to support the data gathered from the First Dosage Test. Although there were no abnormal color changes to the media, it was suspected that the data differences amongst those triplicates were due to either unequal cell distribution at the start of the experiment, or improper cell counting for the results. In order to obtain more standardized data to draw stronger conclusions, the dosage test was repeated once more with OVCAR-3s.

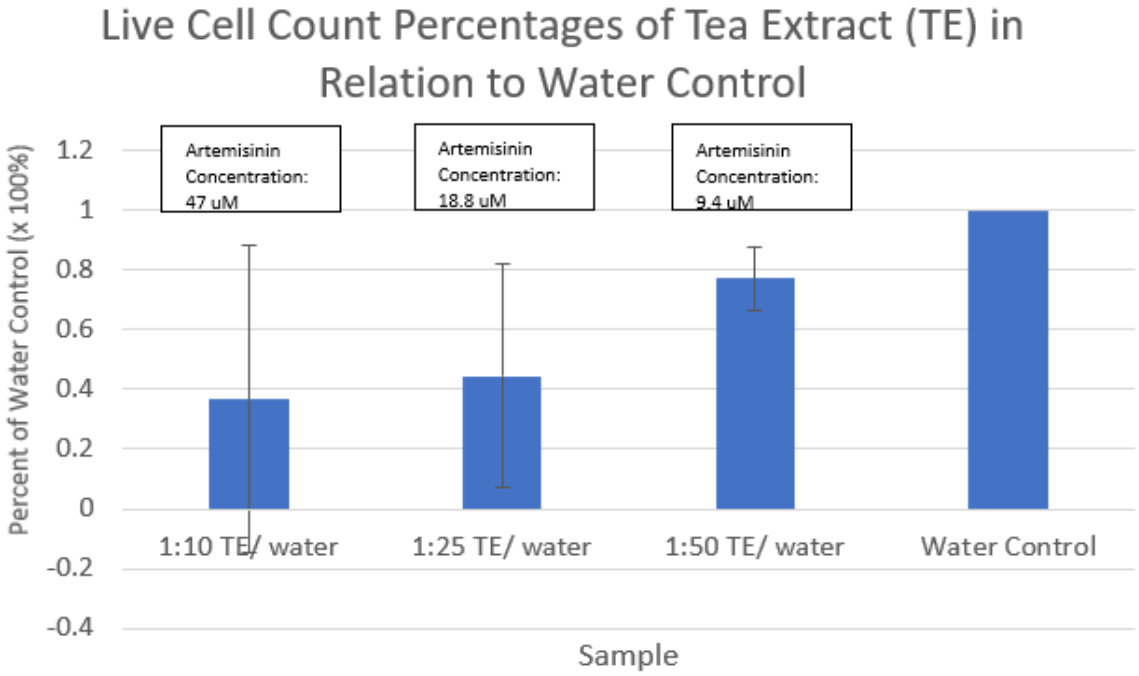


Figure 4: TE / Water Control for OVCAR-3s. These are the average live cell counts for each extract dilution over the average cell count of the water control. Error bars show the standard deviation for the tea extract dilutions. TE = *A. annua* tea extract. The ratios refer to Extract : Water. N = 1.

The results in this test were similar to the first experiment, but with a generally larger variety in the data. In Figure 4, the difference in total cell count between the 1:10 and 1:25 ratios (5% difference) was much smaller than what was seen for the T47Ds in Figure 1 (25%). The similar data in these two ratios also made the lower cell count trend less prominent. The ratio of live cells in the dosed wells compared to the control did not decrease in the expected intervals, but instead had a sudden drop between the 1:25 and 1:50 ratios (~35% difference).

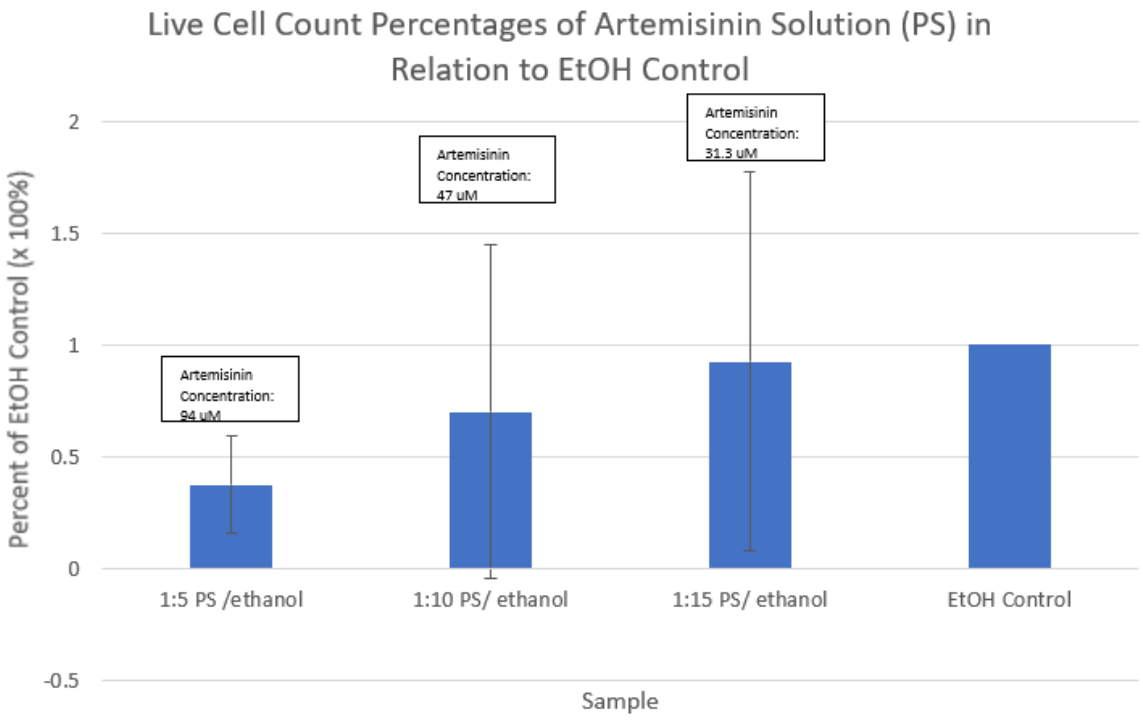


Figure 5: PS / EtOH Control for OVCAR-3s. These are the average live cell counts for each artemisinin dilution over the average cell count of the EtOH control. Error bars show the standard deviation for the artemisinin dilutions. PS = pure artemisinin solution. The ratios refer to Artemisinin : EtOH. N = 1.

Figure 5 shows a more standard cell count trend compared to Figure 4. Once again, *A. annua* dosed cells were found to have fewer cells compared to those dosed with artemisinin at equivalent concentrations, with a 32% difference in the cell count to control ratio at the 1:10 dilution. The error bars were substantially larger than those in the first experiment, which means that the data differences were even more likely to have been caused by inexact cell plating or cell count mistakes. However, it is still notable that *A. annua* and artemisinin were successful in reducing cell counts in a different cancer cell line.

Dot Blotting and Densitometry Analysis

The purpose of these protein analyses was to see if exposure to the extract/artemisinin caused changes in the amounts of these specific proteins. Each of the three chosen proteins is strongly correlated to a cytotoxic mechanism of action that artemisinin is believed to affect.

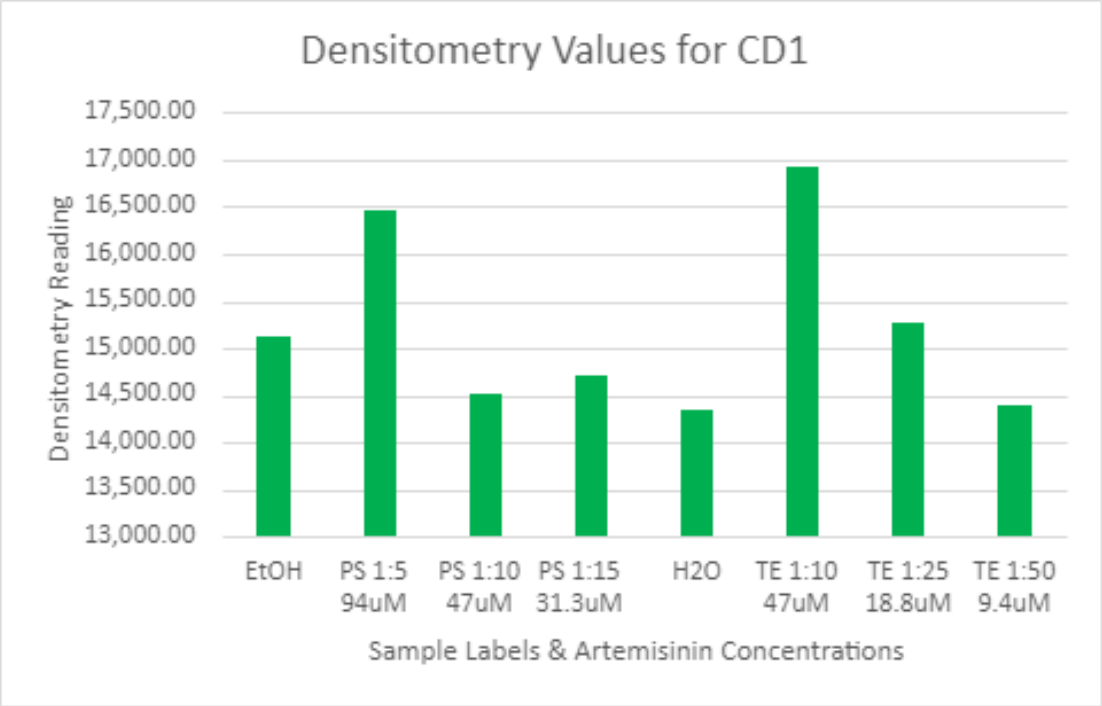


Figure 6: Densitometry Values for CD1. These are the Biorad densitometry readings of CD1 for the dosage test samples. The readings are absolute values. TE = *A. annua* tea extract. PS = pure artemisinin solution. The ratios refer to Extract/Solution : Solvent. N = 1.

Densitometry gives quantified measurements of the optical density of materials, so it was used to provide numerical data from the dot blot protein readings. Figure 6 shows that CD1 increased when cells were exposed to the lowest dilution of the treatments (1:5 for artemisinin and 1:10 for *A. annua*). The tea extract seemed to affect CD1 levels more since it showed higher protein presence in almost all of its ratios, and it had a larger difference between its lowest dilution and control. This increase was the opposite of what was expected to occur if the reagents prevented cell cycle progression, so we looked to our other protein analyses to determine our conclusions.

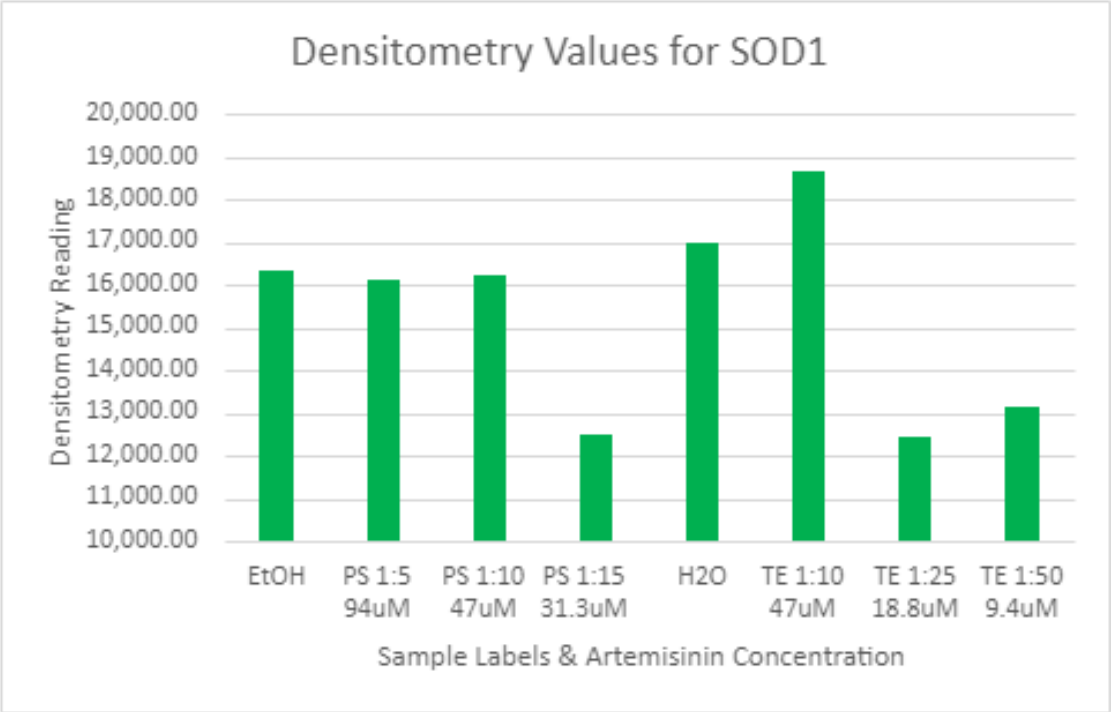


Figure 7: Densitometry Values for SOD1. These are the Biorad densitometry readings of SOD1 for the dosage test samples. TE = *A. annua* tea extract. PS = pure artemisinin solution. The ratios refer to Extract/Solution : Solvent. N = 1.

The results for SOD1 in Figure 7 were more difficult to draw conclusions from. As shown in the figure, the readings for the controls (EtOH and water) were as high as some of the samples. Even though slight trends can be found from the lowest treatment dilutions generally having the highest protein amounts, data discrepancies can be seen from both treatments. Most notably, the 1:5 and 1:10 artemisinin dilutions had SOD1 levels equal to the control, and the 1:50 ratio for *A. annua* was noticeably higher than the 1:25 dilution. Since we only performed this experiment once (N = 1), we believe that more trials are necessary to follow-up on this possible trend.

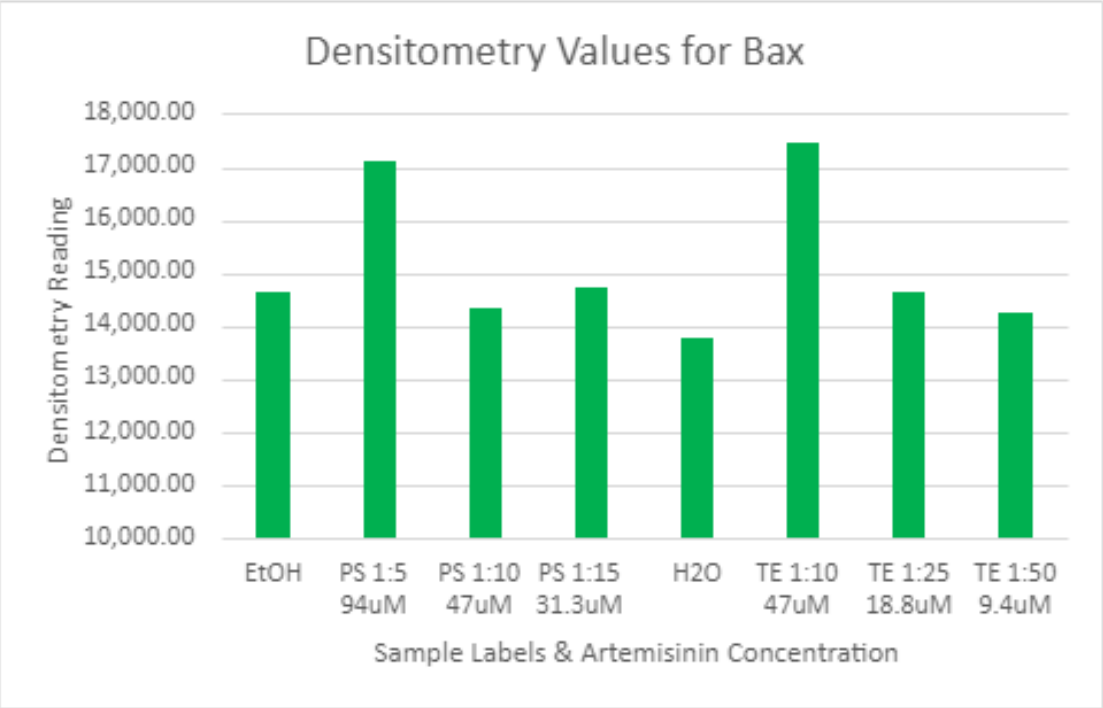


Figure 8: Densitometry Values for Bax. These are the Biorad densitometry readings of Bax for the dosage test samples. TE = *A. annua* tea extract. PS = pure artemisinin solution. The ratios refer to Extract/Solution : Solvent. N = 1.

The results for Bax in Figure 8 were the most expected out of the three protein analyses. *A. annua* and artemisinin showed nearly identical effects on Bax levels. Both treatments peaked in protein amount at their lowest dilution, and they had little effect on the amounts at their other ratios (which were around the same level as their respective controls). However, the tea extract may have affected Bax levels more since it had a 20% higher reading when the treatments are compared at the same ratio (1:10). Since this data showed the clearest trend, we believed that this was our best lead for determining artemisinin's mechanism of action.

Discussion

Comparison Between *A. annua* and Artemisinin

In general, the artemisinin solution affected the cell counts less than *A. annua*, but both showed some effects on the breast and ovarian cancer cells. Across all cancer cells tested at the 1:10 ratio, the tea extract had an average cell reduction rate of around 65% while artemisinin only had a rate of 25%. We thought that this could be explained by the other compounds in *A. annua*, such as flavonoids, giving the tea a slightly stronger effect on the cells. Our dosage test with *A. afra* (Figure 3) may support this hypothesis since the data showed a 10% reduction rate compared to the water control. We believe that the rest of the differences between the reduction numbers could be because the compounds have a synergistic effect when they are combined rather than when they are used alone. More tests analyzing the chemical makeup of *A. annua* are necessary to flesh out this concept. A dosage test using both *A. afra* and the artemisinin solution would confirm these findings if they show similar cell count reductions compared to *A. annua*.

The treatments also seemed to affect breast cancer cells more than ovarian cancer cells since the extract and artemisinin had lower cell counts for the breast cells at their lowest dilutions. However, we are unable to draw definitive conclusions based on our small data pool, especially since the experiments with ovarian cells produced extremely large error bars. Our data discrepancies were likely due to inaccurate cell plating at the start of each dosage test, along with poorly representative cell counting of the end results. More dosage testing with these and other cancers could help prove artemisinin's efficacy against them. If the treatments work better against certain cancers, determining the reasons behind this could help us further understand the mechanisms behind the compounds as well. In addition, it is still possible that the dilutions of the treatments may not have been optimal to show their effects on the cells. For future tests, it may be better to use our dilutions as guidelines for determining the ideal ratio for tumor treatment.

Mechanism of Action

In understanding the potential of artemisia and its derivatives in cancer treatment, it is necessary to know how these compounds affect cells and their processes. Our research into the mechanisms of action behind artemisinin may have narrowed down the primary activities of the compound, but our small data pool (N = 1) only allows us to draw preliminary conclusions. Starting with CD1 and cell cycle regulation, the results in Figure 6 showed the opposite of what was predicted. If artemisinin inhibited tumor growth by preventing cell cycle progression, then we would see decreases in CD1 since it is necessary to bypass the checkpoints. Instead, we saw peaks in protein amount at the lowest dilutions with little activity at the other ratios. One reason for this may be that the cancer cells were increasing the production of CD1 to counteract the damage caused by the extract/artemisinin. The experiment was only completed once so we cannot draw definitive conclusions, but our other protein analyses lead us to believe that artemisinin does not have much influence on CD1 or the cell cycle. We determined it would be best to look at our other proposed mechanisms involving cytotoxicity and apoptosis.

Changes in SOD1 activity is suspected to accompany the presence of artemisinin as the compound produces free radicals and oxidative stress when degraded by cells. Lethal reactive oxygen species are a normal byproduct from normal cell function, and they have been known to increase in cancer due to heightened proliferation. When the breast cancer cells are exposed to artemisinin, it is possible that oxidative stress increases beyond the threshold that cancer cells can control. Since SOD1 is responsible for keeping the cell alive by preventing these lethal oxygen species from destroying it, it is reported that artemisinin could increase SOD1 amounts. From the SOD1 results in Figure 7, we do not believe that there was enough conclusive evidence supporting free radical production as the primary method of cell killing in these experiments. Most of the lowest treatment dilutions had similar SOD1 presence to their controls, with low protein readings at the other ratios. A possible explanation for this data is that breast cancer cells naturally have high SOD1 amounts because they constantly need to keep their free radical levels in check from their abnormal activity. Unlike for CD1, SOD1 activity could tie in with our last studied mechanism of action, so we believe that these results could be further pursued.

Out of all of our protein analyses, we believe that Bax showed the most promising results. If artemisinin promoted apoptosis in some way, it was likely that Bax would increase since it is a primary apoptotic marker. Figure 8 showed strong peaks in protein amount at the reagents' lowest dilution, with Bax levels similar to the controls at the other ratios. This data could mean that high extract/artemisinin presence causes enough damage to the cancer cells that it induces apoptosis in many of them. The damage could be caused by increasing oxidative stress, which could possibly explain the high SOD1 levels seen as well. However, these concepts are hypothetical and would require confirmations of oxidative stress, free radicals, and ROS being present in these cells.

Future Direction

Our results were far from conclusive and require further experimentation to confirm and clarify our findings. We suggest more trials of our dosage tests and protein analyses to increase the data pool for these studies. This would help confirm or disprove our results along with ensuring that our methodology and results are replicable. We also propose tests measuring oxygen free radicals to follow-up on our protein analyses. Artemisinin's most well-understood effect on cells is producing free radicals and causing death via oxidative stress. By testing for the presence of free radicals following exposure to artemisinin, this data could shed more light on our findings on SOD1 and Bax. Our SOD1 data was unclear because our controls showed high readings for the protein, but we still saw slight increases at the lowest treatment dilution. Coupled with the positive Bax data, proving free radical production could solidify this as a primary mechanism of action for artemisinin. Lastly, a later experiment should also study the bystander effect of artemisinin because it may be dangerous to normal cells if it kills cancer cells via free radicals. Even if cytotoxicity against cancer cells is proven, artemisinin cannot be used as treatment if it kills cells indiscriminately.

References

ATCC. (2020). “NIH:OVCAR-3 [OVCAR3] (ATCC® HTB-161™).” https://www.atcc.org/products/all/HTB-161.aspx.

ATCC. (2020). “T-47D (ATCC® HTB-133™).” https://www.atcc.org/products/all/HTB-133.aspx.

Bilia, A. R., Gabriele, C., Bergonzi, M. C., de Malgalhaes, P. M., & Vincieri, F. F. (2006). Variation in artemisinin and flavonoid content in different extracts of Artemisia annua L. Natural Product Communications, 1(12), 1934578X0600101208.

Breast Cancer Information and Support. (2020, October 13). Retrieved October 16, 2020, from https://www.breastcancer.org/

Carbonara, T., Pascale, R., Argentieri, M. P., Papadia, P., Fanizzi, F. P., Villanova, L., & Avato, P. (2012). Phytochemical analysis of a herbal tea from Artemisia annua L. Journal of pharmaceutical and biomedical analysis, 62, 79-86.

Cao, Y., Feng, Y., Gao, L., Li, X., Jin, Q., Wang, Y., . . . Wei, M. (2019). Artemisinin enhances the anti-tumor immune response in 4T1 breast cancer cells in vitro and in vivo. International Immunopharmacology, 70, 110-116. doi:10.1016/j.intimp.2019.01.041.

Carpenter, R., & Brady, M. F. (2020). BAX Gene. StatPearls.

Cayman Chemical. (2018, March 14). Safety Data Sheet: Artemisinin. https://www.caymanchem.com/msdss/11816m.pdf.

Crespo-Ortiz, M. P., & Wei, M. Q. (2012). Antitumor activity of artemisinin and its derivatives: from a well-known antimalarial agent to a potential anticancer drug. Journal of Biomedicine and Biotechnology, 2012.

Efferth, T., Benakis, A., Romero, M. R., Tomicic, M., Rauh, R., Steinbach, D., ... & Marschall, M. (2004). Enhancement of cytotoxicity of artemisinins toward cancer cells by ferrous iron. Free Radical Biology and Medicine, 37(7), 998-1009.

Efferth, T. (2017, October). From ancient herb to modern drug: Artemisia annua and artemisinin for cancer therapy. In Seminars in cancer biology (Vol. 46, pp. 65-83). Academic Press.

Elfawal, M. A., Towler, M. J., Reich, N. G., Golenbock, D., Weathers, P. J., & Rich, S. M. (2012). Dried whole plant Artemisia annua as an antimalarial therapy. PloS one, 7(12), e52746.

Gomez, M. L., Shah, N., Kenny, T. C., Jenkins, E. C., & Germain, D. (2019). SOD1 is essential for oncogene-driven mammary tumor formation but dispensable for normal development and proliferation. Oncogene, 38(29), 5751-5765.

Greenshields, A. L., Shepherd, T. G., & Hoskin, D. W. (2017). Contribution of reactive oxygen species to ovarian cancer cell growth arrest and killing by the anti‐malarial drug artesunate. Molecular carcinogenesis, 56(1), 75-93.

Ho WE.., Peh H.Y., Chan T.K., Wong W.S. (2013). Artemisinins: pharmacological actions beyond anti-malarial. Pharmacol Ther. 2014 Apr;142(1):126-39. doi: 10.1016/j.pharmthera.2013.12.001. Epub 2013 Dec 6. PMID: 24316259.

Isani, G., Bertocchi, M., Andreani, G., Farruggia, G., Cappadone, C., Salaroli, R., ... & Bernardini, C. (2019). Cytotoxic effects of Artemisia Annua L. and pure artemisinin on the D-17 canine osteosarcoma cell line. Oxidative Medicine and Cellular Longevity, 2019.

Kadioglu, O., Chan, A., Cong Ling Qiu, A., Wong, V. K. W., Colligs, V., Wecklein, S., ... & Hsiao, W. L. W. (2017). Artemisinin derivatives target topoisomerase 1 and cause DNA damage in silico and in vitro. Frontiers in pharmacology, 8, 711.Lang, S. J., Schmiech, M., Hafner, S., Paetz, C., Steinborn, C., Huber, R., . . . Simmet, T. (2019). Antitumor activity of an Artemisia annua herbal preparation and identification of active ingredients. Phytomedicine, 62, 152962. doi:10.1016/j.phymed.2019.152962.

Kiani, B.H., Kayani, W.K., Khayam, A.U. *et al.* Artemisinin and its derivatives: a promising cancer therapy. *Mol Biol Rep* 47, 6321–6336 (2020). https://doi.org/10.1007/s11033-020-05669-z

Langroudi, L., Hassan, Z. M., Ebtekar, M., Mahdavi, M., Pakravan, N., & Noori, S. (2010). A comparison of low-dose cyclophosphamide treatment with artemisinin treatment in reducing the number of regulatory T cells in murine breast cancer model. International immunopharmacology, 10(9), 1055-1061.

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A., . . . Stadtman, E. R. (1990). [49] Determination of carbonyl content in oxidatively modified proteins. Oxygen Radicals in Biological Systems Part B: Oxygen Radicals and Antioxidants Methods in Enzymology, 464-478. doi:10.1016/0076-6879(90)86141-h.

Liu, Y., Gao, S., Zhu, J., Zheng, Y., Zhang, H., & Sun, H. (2018). Dihydroartemisinin induces apoptosis and inhibits proliferation, migration, and invasion in epithelial ovarian cancer via inhibition of the hedgehog signaling pathway. Cancer medicine, 7(11), 5704-5715.

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.

Martini, M. C., Zhang, T., Williams, J. T., Abramovitch, R. B., Weathers, P. J., & Shell, S. S. (2020). Artemisia annua and Artemisia afra extracts exhibit strong bactericidal activity against Mycobacterium tuberculosis. Journal of Ethnopharmacology, 262, 113191.

Meghri Katerji, Maria Filippova, Penelope Duerksen-Hughes, "Approaches and Methods to Measure Oxidative Stress in Clinical Samples: Research Applications in the Cancer Field", Oxidative Medicine and Cellular Longevity, vol. 2019, Article ID 1279250, 29 pages, 2019. https://doi.org/10.1155/2019/1279250.

Mesquita, C. S., Oliveira, R., Bento, F., Geraldo, D., Rodrigues, J. V., & Marcos, J. C. (2014). Simplified 2,4-dinitrophenylhydrazine spectrophotometric assay for quantification of carbonyls in oxidized proteins. Analytical Biochemistry, 458, 69-71. doi:10.1016/j.ab.2014.04.034.

Mohammadizadeh, F., Hani, M., Ranaee, M., & Bagheri, M. (2013). Role of cyclin D1 in breast carcinoma. Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences, 18(12), 1021–1025.

NOCC. (2020). “What is Ovarian Cancer?” http://ovarian.org/index.php.

Oltval, Z. N., Milliman, C. L., & Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programed cell death. cell, 74(4), 609-619.

Papa, L., Manfredi, G., & Germain, D. (2014). SOD1, an unexpected novel target for cancer therapy. Genes & cancer, 5(1-2), 15.

Riss TL, Moravec RA, Niles AL, et al. Cell Viability Assays. 2013 May 1 [Updated 2016 Jul 1]. In: Markossian S, Sittampalam GS, Grossman A, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK144065/

Roy, P. G., & Thompson, A. M. (2006). Cyclin D1 and breast cancer. The Breast, 15(6), 718-727.

Seo, D. J., Lee, M., Jeon, S. B., Park, H., Jeong, S., Lee, B. H., & Choi, C. (2017). Antiviral activity of herbal extracts against the hepatitis A virus. Food Control, 72, 9-13.

Slezáková, S., & Ruda-Kucerova, J. (2017). Anticancer activity of artemisinin and its derivatives. Anticancer Research, 37(11), 5995-6003.

Sun, W. C., Han, J. X., Yang, W. Y., Deng, D. A., & Yue, X. F. (1992). Antitumor activities of 4 derivatives of artemisic acid and artemisinin B in vitro. Zhongguo yao li xue bao= Acta pharmacologica Sinica, 13(6), 541-543.

Tin, A. S., Sundar, S. N., Tran, K. Q., Park, A. H., Poindexter, K. M., & Firestone, G. L. (2012). Antiproliferative effects of artemisinin on human breast cancer cells requires the downregulated expression of the E2F1 transcription factor and loss of E2F1-target cell cycle genes. Anti-cancer drugs, 23(4), 370-379

Wu, B., Hu, K., Li, S., Zhu, J., Gu, L., Shen, H., ... & Di, W. (2011). Dihydroartiminisin inhibits the growth and metastasis of epithelial ovarian cancer. Oncology reports, 27(1), 101-108.

Zhu, X. X., Yang, L., Li, Y. J., Zhang, D., Chen, Y., Kostecká, P., ... & Zídek, Z. (2013). Effects of sesquiterpene, flavonoid and coumarin types of compounds from Artemisia annua L. on production of mediators of angiogenesis. Pharmacological Reports, 65(2), 410-420.

Zhang, H. T., Wang, Y. L., Zhang, J., & Zhang, Q. X. (2014). Artemisinin inhibits gastric cancer cell proliferation through upregulation of p53. Tumor Biology, 35(2), 1403-1409.

Zhang, Y., Xu, G., Zhang, S., Wang, D., Prabha, P. S., & Zuo, Z. (2018). Antitumor research on artemisinin and its bioactive derivatives. Natural products and bioprospecting, 8(4), 303-319.

Zhang, Y. J., Zhan, X., Wang, L., Ho, R. J., & Sasaki, T. (2015). pH-responsive artemisinin dimer in lipid nanoparticles are effective against human breast cancer in a xenograft model. *Journal of pharmaceutical sciences*, *104*(5), 1815–1824. https://doi.org/10.1002/jps.24407

Appendices

Appendix A: Experimental Compound Dilutions for Dosage Testing

Well Label	Added Volume	Artemisinin Concentration
EtOH Control	100uL	N/A
Water Control	100uL	N/A
1:5 PS	100uL	94uM
1:10 PS	50uL	47uM
1:15 PS	33uL	31.3uM
1:10 TE	50uL	47uM
1:25 TE	33uL	18.8uM
1:50 TE	25uL	9.4uM

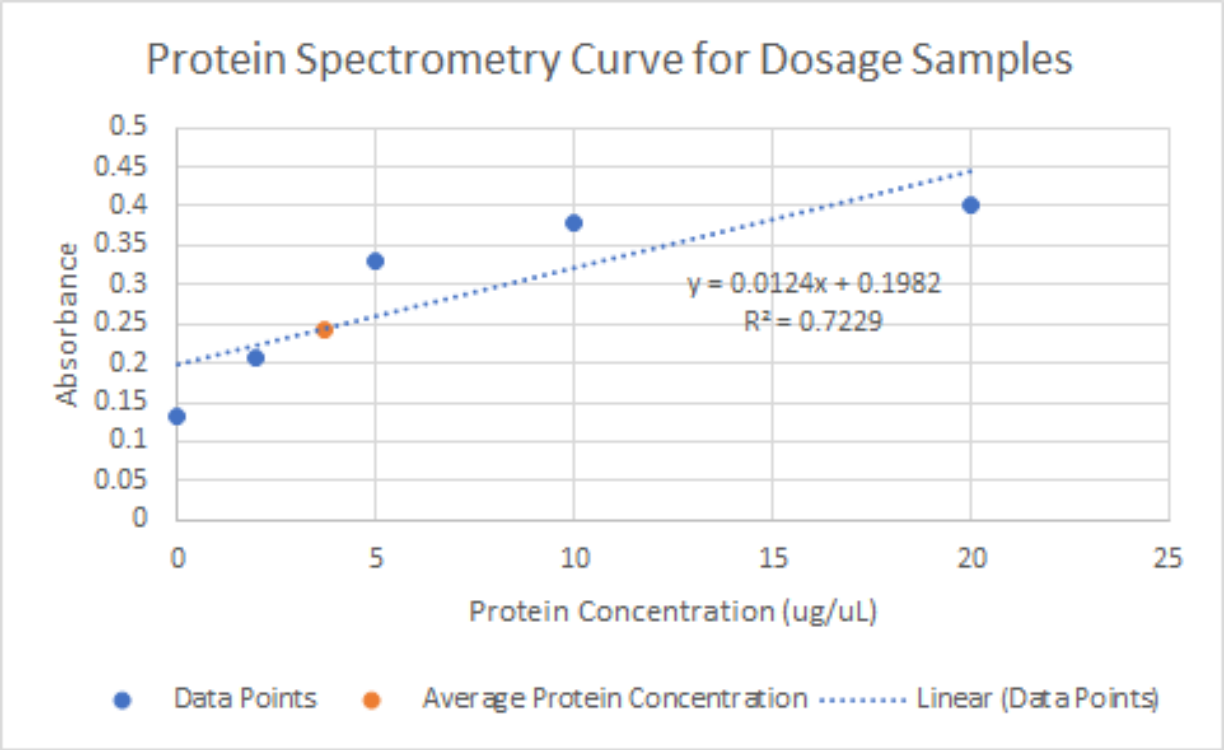
*Total Well Volume = 500uL

Appendix B: Dosage Test Well Labels

	1	2	3	4	5	6
A	0.2% EtOH Control	0.2% EtOH Control	0.2% EtOH Control	Water Control	Water Control	Water Control
B	1:5 PS	1:5 PS	1:5 PS	1:10 TE	1:10 TE	1:10 TE
C	1:10 PS	1:10 PS	1:10 PS	1:25 TE	1:25 TE	1:25 TE
D	1:15 PS	1:15 PS	1:15 PS	1:50 TE	1:50 TE	1:50 TE

*PS = Pure Artemisinin Solution ; TE = Tea Extract

Appendix C: Protein Spectrometry Curve for Dosage Samples



A standardized curve was made with different BSA concentrations (blue dots). Protein samples of T47Ds exposed to *A. annua* tea extract or artemisinin solution (as described in the dosage tests previously) were measured along this curve (the average protein amount is depicted as the orange dot).