Assessing Artemisinin's Treatment Viability on MCF7 Breast Cancer Cells

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Abstract:

Artemisinin, an antimalarial treatment derived from *Artemisia annua*, reacts with ferrous compounds, generating free radicals unless hindered by antioxidants. High iron levels and low antioxidant levels of cancer cells make artemisinin a potentially effective treatment. We utilized MTT assays to assess artemisinin's effect on MCF7 breast cancer cells. Artemisinin upregulated cell proliferation and appeared to affect the cells' morphology, which could impact tumor development.

Introduction:

There have been several studies and compelling research conducted regarding the effect artemisinin has on the proliferation of breast cancer cells. It is estimated that around 1 in 8 women in the United States will have breast cancer at least once in their lifetime (U.S., 2016). Breast cancer is the second most diagnosed cancer in women in the United States, behind skin cancer (U.S., 2016). Breast cancers that are sensitive to estrogen can be treated with antiestrogens that do not contain steroids (Sundar et al, 2008), however these can have severe side effects. Breast cancers that are not responsive to estrogen are currently treated through surgery to remove the tumors, or chemotherapy (Sundar et al, 2008). Researchers are now looking at plant compounds utilized for other medicinal purposes as potential natural treatments for certain types of breast cancer. Specifically, researchers have been focusing on the malaria treatment that utilizes Artemisia annua, whole plant and extract, due to its selectivity for cancer cells and its low toxicity toward normal cells (Su et al., 2015). Thus, we examined the effects of these treatments, and the purified active ingredient artemisinin, on the breast cancer cell MCF7 as a model.

Artemisia annua contains a multitude of chemical compounds that can influence metabolic pathways. A study performed in 2008 studied the relative antioxidant and nutrient levels in *Artemisia annua* to assess the nutritional balance and chemical composition of the plant and quantify its viability as a potential herbal tonic and treatment (Brisibe et al, 2008). Experimenters grew *A. annua* plants in specified conditions and studied their composition among different tissues within the plant to identify chemical concentrations and variations. Researchers determined that *A. annua* leaves, the most common part of the plant utilized to brew teas used in disease treatments, have a relative protein content of 27.1%, compared to a mere 8.23% value found in roots (Brisibe et al, 2008). When analyzing mineral and amino acid values in different tissues, researchers concluded that leaves and inflorescence had the highest values of nearly all minerals, and leaves having significantly higher levels of amino acids (Brisibe et al, 2008). Finally, researchers studied antioxidant capacities, with antioxidants being vital to the mitigation of free radical production in organisms. The results illustrate that leaves being utilized to brew artemisinin teas have a wide range of additional compounds that may influence metabolic pathways in the organism or vector being exposed

(Brisibe et al, 2008). The focus of this particular study will then take into consideration the medicinal value of the *Artemisia annua* plant as well as the purified artemisinin compound.

Artemisinin and its derivatives cause the proliferation of free radicals when they react with iron compounds. This free radical proliferation allows for the destruction of infectious agents like malaria, which utilize host iron supplies (Torti et al, 2013). Despite being incredibly toxic to malaria producing protozoa, artemisinin seems to have little to no detrimental effect on the human body due to the presence of antioxidant enzymes. These enzymes may work to prevent the activity of free radicals, rendering them less detrimental to human somatic cells. While vital to the immune system, antioxidant presence in cancer cells could hinder the effectiveness of artemisinin as a treatment method, as the enzymes would prevent the free radicals from causing apoptosis (Cui et al, 2009). However, the direct mechanism of action of artemisinin in regard to this potency compared to traditional cancer therapeutic agents is still being determined (Lai et al., 2013).

Thus, studies were performed in order to understand the presence of antioxidants in cancer cell lines. One study utilized a wide variety of biochemical analyses to determine variance in antioxidant levels of different strains of cancer. Researchers performed *in vivo* experiments by stimulating estrogen induced hamster kidney tumors and then measuring endogenous antioxidant levels. Researchers then compared these antioxidant levels to those of control models to draw conclusions on how cancer affects antioxidant presence. Compared to the control tissues, nearly all cancers were found to have lower levels of several antioxidants, namely manganese superoxide dismutase, zinc superoxide dismutase and catalase, among several other superoxide dismutases. The data suggest that the utilization of antioxidants is decreased in cancer cells (Oberley and Oberley, 1997).

Researchers also focused on the effect iron levels had on cancer growth and development, as well as how regulation of these levels affect cellular processes within the body (Torti et al, 2013). It was observed that an abundance of transferrin (TF) receptors on the outside of cells mediate the uptake of iron compounds, and that increasing levels of TF receptors correlated directly with an increase in tumor development among patients. Further evaluations into the effects of dietary iron intake and accumulation in cells among the patients studied supported these initial conclusions. As well, it was observed that a reduction of iron stores within cancer

patients over time correlated directly with a decrease in cancer (Torti et al, 2013). This was shown through a long term experiment where over the course of 4.5 years, elderly men with peripheral artery diseases repeatedly donated blood (Torti et al, 2013). The results indicated that overall cancer risk and cancer-specific mortality was reduced. When comparing cancer lines to benign tissues, the study also demonstrated that the cancerous cell lines had higher levels of transferrin with respect to their normal counterparts (Torti et al, 2013).

To study the effects of artemisinin on cancer cells with respect to iron levels, researchers cultured Molt-4 cells with holotransferrin to bolster the cellular iron levels (Lai et al, 2004). According to their results, the presence of artemisinin directly correlated with a decrease in cell count. Experimenters utilized dihydroartemisinin (DHA), an artemisinin derivative, to study the effects of the compound on cells. Experimenters set up groups exposed to no compounds, transferrin, DHA, and both test compounds to study their effects and synergies. There was little variation between the control group and transferrin groups, while the DHA exposed groups showed a significant drop in cells present. Moreover, the DHA and transferrin group showed a much greater drop in cell counts, down to near 0% cell counts after 8 hours of incubation. In addition, cellular apoptosis rates also showed dramatic variation depending on treatment methods utilized, with nearly 30% of the DHA and transferrin cells undergoing apoptosis by 8 hours with a steadily increasing rate until that point in contrast to the only 5% from the control groups that was consistently maintained throughout the trials. The high rate of apoptosis strongly supports the researchers' claims that artemisinin is able to interfere with cancer growth, and its presence is particularly effective when bolstered by higher cellular iron levels (Lai et al, 2004).

In another study, polyphenols from Korean *Artemisia annua L* (pKAL) were isolated and their effects on MDA-MB-231 breast cancer cells were examined. Specifically this study focused on the adhesion of cancer cells to the endothelial cell and epithelial-mesenchymal transition (EMT), which is a process considered to facilitate metastasis. Through various assays (including adhesion, Matrigel invasion, and cell viability, along with gelatin zymography and western blotting) the researchers were able to see that pKAL inhibited the breast cancer cells from adhering to the endothelial cells of human blood vessels through suppressing the expression of a cell adhesion protein (VCAM-1), thus interfering with the EMT process (Ko et al., 2016). It is also important to note that, this study demonstrated that polyphenols derived from pKAL had

very little toxic effect on cells that were not the MDA-MB-231 breast cancer cells (Ko et al., 2016). In order for this to be an effective treatment, though, further investigations into the effects of artemisinin on cancer cells need to be performed.

Using a different approach, a study in 2014 compared the effectiveness of *Artemisia annua* tea versus purified artemisinin as a cancer treatment for the MCF7 breast cancer cell line. It has been shown *in vitro* that metabolites of the tea are able to work with artemisinin increasing antiplasmodial activity (Suberu et al., 2014). A majority of the co-metabolites present in *Artemisia annua* tea are chlorogenic acids, also known as caffeoylquinic acids (CQAs). Similar to the polyphenols in the previous study, CQAs can act as antioxidants, and they also possess the ability to act against bacteria and histamines (Suberu et al., 2014). Furthermore, CQAs have been found to disrupt the RAB oncogene in human breast cancer cells through blocking of the methylation of the promoter region (Suberu et al., 2014). The researchers in this study chose to look at how chlorogenic acid (3-caffeoylguinic acid, 3CA) and *Artemisia* hot water infusion (tea) work as a treatment against MCF7 cancer cells compared to cisplatin, an anticancer compound. The results illustrated, using the percent of cell survival and the molarity of anticancer agent used, that cisplatin had much greater cytotoxic effects than artemisinin (Suberu et al., 2014). Combining artemisinin with 3CA at a 1:1 molar ratio caused a large decrease in cytotoxicity, while combining cisplatin with 3CA at a 1:1 molar ratio caused 2.5-fold increase in cytotoxicity over cisplatin by itself (Suberu et al., 2014). These results suggest that *Artemisia annua* tea may not be an effective anticancer therapy.

Artemisinin causes the level of free radicals to increase, however free radicals can be inhibited by antioxidants. Therefore, the high iron levels and low antioxidant levels of cancer cells makes artemisinin an interesting compound to pursue in the hopes to provide a natural cancer therapy for patients. Based on the literature, we developed the following hypotheses to research artemisinin's anti-proliferative effects. First, we predicted that artemisinin will inhibit the proliferation of MCF7 cells due to the high iron levels and low antioxidant levels present in these cancer cells. Our second hypothesis was that: pure artemisinin will be a more effective tumor therapy than whole plant extract due to additional antioxidants present in *Artemisia annua* that may interfere with the anti-proliferative effect of artemisinin on MCF7 cells.

Methodology:

Pure Artemisinin Solution

A pure artemisinin solution was made from Sigma- Aldrich artemisinin (Product #361593) to a final concentration of 12.5 mM stock in 100% EtOH.

Artemisia annua Tea Preparations

Following the protocol outlined in Subaru (2014) with slight modifications, water brewed tea was prepared by boiling water 1 liter and pouring it over 5 grams of dried, crushed *Artemisia annua* leaves to steep for 20 minutes. The *Artemisia annua* water brewed tea was then sterile filtered, and stored at 4℃. Ethanol brewed tea was prepared by macerating 1 gram of the dried *Artemisia annua* leaves, and adding it to 200 mL of 70% EtOH in water, then incubated on a shaker at room temperature overnight. The *Artemisia annua* ethanol brewed tea was then sterile filtered, and the ethanol was allowed to evaporate overnight, and the dry extract was resuspended in 200 mL of water (Suberu, J.O., et al., 2014).

Cell Culture: MCF7 Cell Line

MCF7 cells were maintained at 37℃ until they reached about 75% confluence at which time they were passaged 1:5 by trypsinization (Tchafa, A. M, et al., 2013). Cell counts were obtained using a Nexcelom Bioscience Cellometer® Auto T4 and 1% Trypan blue viability stain. MCF7 cells were grown in two types of media depending on the experiment. The first media, "Insulin" was made from Dulbecco's Modification of Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), 1% Penicillin/ Streptomycin (P/S), and 0.01 mg/mL bovine pancreas insulin (MCF7 (ATCC® HTB-22™)). The second media, "White," was made by combining phenol red free DMEM, 10% dextran charcoal stripped FBS, and 1% P/S (Vybrant® MTT Cell Proliferation Assay Kit).

MTT Assays

MCF7 cells were plated at 1 x 10^4 cells per 200 uL media /well in a 96 well plate and cultured for 24 hours in insulin medium as described previously (Vybrant® MTT Cell Proliferation Assay

Kit). In order to determine estrogen responsiveness, triplicate samples were treated with estrogen for 24 hours at either 2mM, 10mM, or 100 mM (1% total volume of ethanol per well) in phenol red free medium with untreated and EtOH controls. For artemisinin dose response testing, triplicate samples were treated with artemisinin for 24 hours at 5uM, 25uM, 50uM, 75uM, and 100 uM (1% total volume of ethanol per well) with an untreated sample as a control. A second dose response experiment was subsequently performed the same method previously described, with a different range of artemisinin concentrations: 25 uM artemisinin, 15 uM artemisinin, 5 uM artemisinin, and 2.5 uM artemisinin. Time course experiments were performed with triplicate samples treated with 15 uM artemisinin with incubation times of 24, 18, and 12 hours. Finally, to compare the effects of artemisinin and *Artemisia annua* tea, triplicate samples were treated with 15 uM artemisinin, 5 %, 7.5%, 10%, and 15% *Artemisia annua* tea (v/v) brewed in ethanol, and 5%, 7.5%, 10%, and 15% *Artemisia annua* tea (v/v) brewed in water with untreated and EtOH controls. It is important to note that the final concentration of ethanol in these samples were above the previously designated 1% per well since the tea was diluted in 70% ethanol originally. After another 24 hour incubation period, Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTT) reagent was added to each of the wells and incubated for four hours in 37°C (CellTiter 96® AQueous One Solution Cell Proliferation Assay System Protocol). Then the plate was read at 570 nm utilizing a BioTech Instruments® EL800 Microplate reader. Each MTT assay experiment (estrogen responsiveness, dose response, time course, and artemisinin vs. tea) was repeated twice as described above. Samples run in triplicate were averaged, and expressed as percent control. Due to small sample size (N=2), statistical analysis was not performed.

HPLC

High Pressure Liquid Chromatography (HPLC) was performed utilizing an Agilent 1100 Series HPLC system. Using the method of Ferreira et al, ultraviolet detection with a wavelength of 192 nm, bandwidth 3, and a reference wavelength of 592 nm, reference bandwidth 100, were used while running a C-18 column at a flow rate of 1 ml/min under isocratic conditions of 60% Acetonitrile/ 40% aqueous (0.1%) acetic acid. Between sample injections, the mobile phase (which was run for five minutes) and the data collection window (which was run for 15 minutes)

had a flow rate of 1mL/min (Ferreira, J.F., et al., 2009). Samples of 10 uL were analyzed, and elution times were used to identify and quantify peaks and confirm the eluted compound. The tested samples were 12.5 mM stock pure artemisinin, 5 uM artemisinin, 50 uM artemisinin, 100 uM artemisinin, *Artemisia annua* tea brewed in water, *Artemisia annua* tea brewed in ethanol. The water brewed and ethanol brewed *Artemisia annua* teas were then both spiked with pure 12.5 mM stock pure artemisinin and ethanol to identify the solvent peak. A standard curve was created using the pure artemisinin concentration and the area under the peak. The determined linear line of best fit was then used to approximate the levels of artemisinin in *Artemisia annua* tea samples.

Results:

Please refer to Appendices A-F for the raw data compiled throughout all experiments.

Estrogenic Response

A MTT assay was performed as previously described in order to determine if the MCF7 cell culture used in this study was estrogen responsive. The absorbance values for the pure media wells were subtracted from the values of the experimental wells. These new values were then averaged, compared to the positive control cells, MCF7 cells grown with no added estrogen. The data is expressed as percent control (refer to *Figure 1* below).

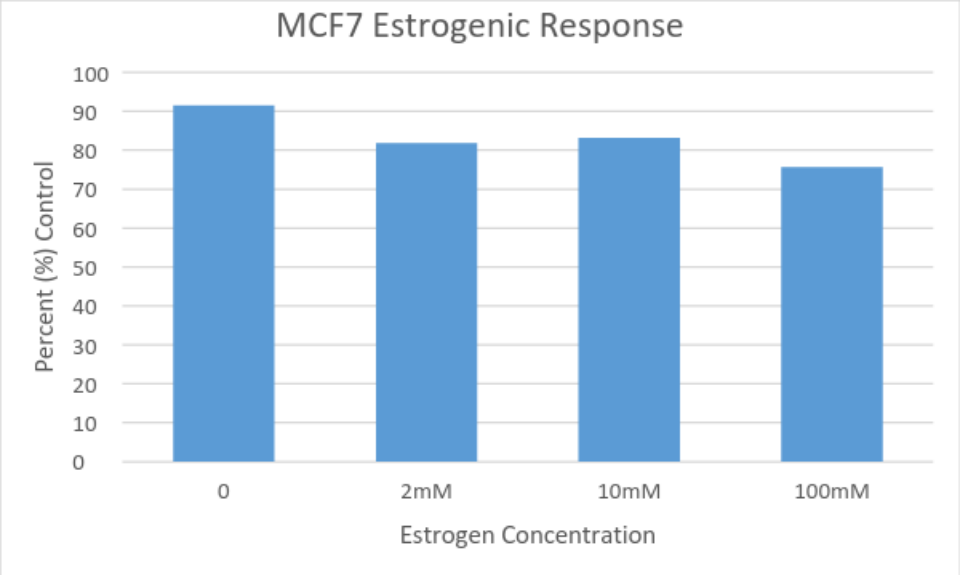

Figure 1: MTT assay (N=2) estrogenic response results for cells treated with ethanol, and 2mM, 10mM, and 100mM concentrations of estrogen.

As seen in *Figure 1*, there is a general trend decreasing the amount of cell proliferation compared to the control cells across the experimental treatments. Ethanol demonstrated about a 10% difference compared to control MCF7 cells, while the various dilutions of estrogen showed about a 20% decreased compared to the positive control. However, due to small sample size (N =2) and large variability in triplicate measures, no conclusion can be reached about the effect of estrogen on cell proliferation. Overall, the data consistently showed that the MCF7 cells appeared to have no proliferative response when exposed to various concentrations of estrogen, especially in comparison to the positive control.

Dose Responsiveness Artemisinin

An MTT assay was performed, as previously described, in order to determine the effective dose of pure artemisinin on MCF7 Breast Cancer cells. *Figure 2* below then represents the initial trials of the dose response testing.

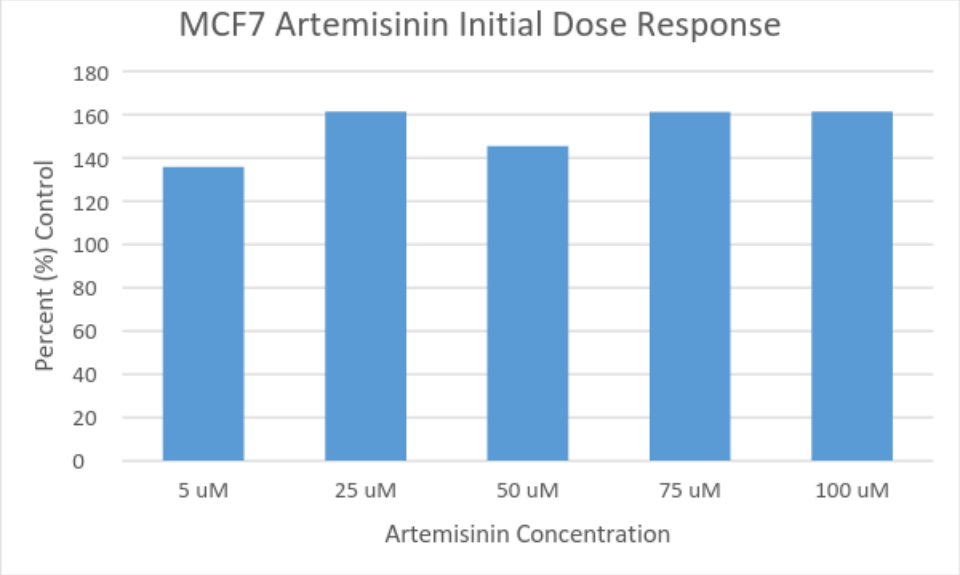

Figure 2: MTT assay (N=2) initial artemisinin dose response results for cells treated with 5 uM, 25 uM, 50 uM, 75 uM, and 100 uM concentrations of artemisinin.

The results in *Figure 2* do not conclusively demonstrate a difference in the response to doses of artemisinin from 5 uM to 100 uM; however, we observed an apparent plateau when cells were exposed to artemisinin concentrations higher than 25 uM. Thus, this procedure was repeated with decreased artemisinin concentrations: 25 uM, 15 uM, 5 uM, and 2.5 uM artemisinin (see *Figure 3*).

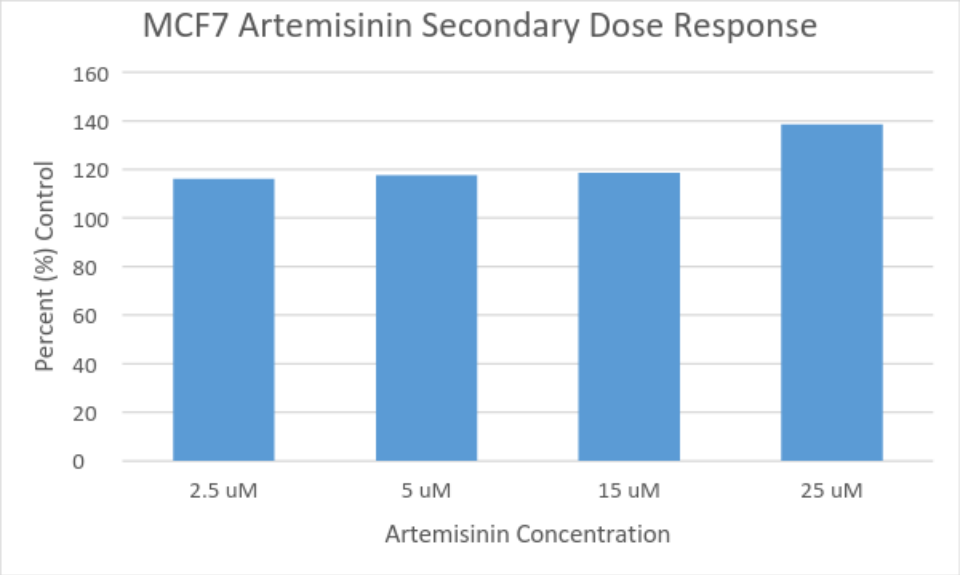

Figure 3: MTT assay (N=2) second artemisinin dose response results for cells treated with 2.5 uM, 5 uM, 15 uM, and 25 uM concentrations of artemisinin.

Once again, the results in *Figure 3* do not demonstrate a dose dependent response, although there appears to be a possible increase in cell number as compared to untreated controls, albeit somewhat less than the increase shown in *Figure 2*. We chose to pursue artemisinin at a concentration of 15 uM for future experiments in this study because 25 uM artemisinin caused a relatively higher proliferative effect than the lower doses.

Artemisinin Time Course

Results of a time course, performed as described in Methods, are shown in *Figure 4* below.

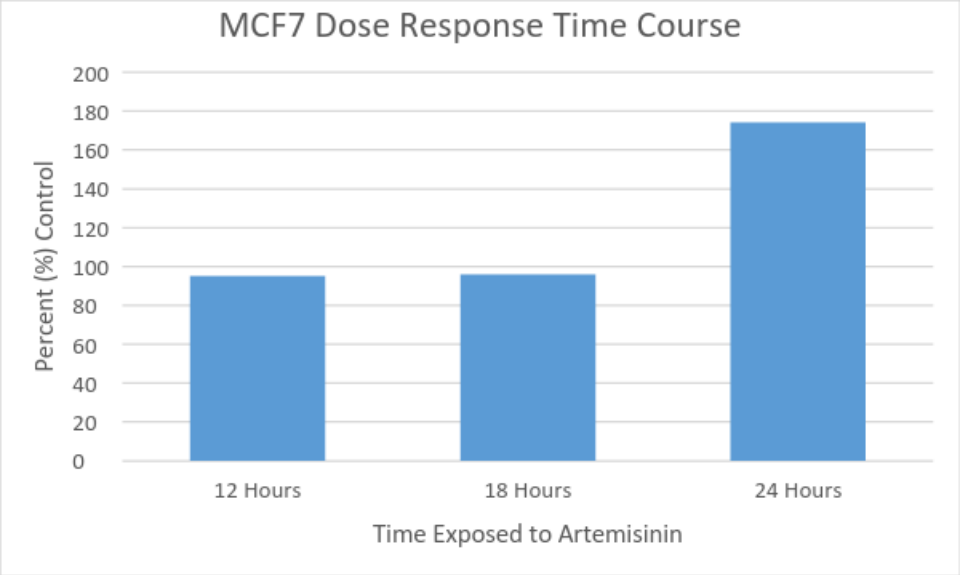

Figure 4: Time course experiment (N=2) over intervals of 12 hours, 18 hours, and 24 hours treated with 15 uM artemisinin.

The results presented in *Figure 4* demonstrate an apparent difference in growth after 24 hours in 15 uM artemisinin. Thus, 24 hours and 15 uM artemisinin were used in subsequent experiments.

HPLC

High pressure liquid chromatography (HPLC) was performed to identify artemisinin in both pure substance and plant extract. Both ethanol and water brewed *A. annua* tea were tested to determine artemisinin content in the two preparations. A representative chromatogram is shown in *Figure 5*.

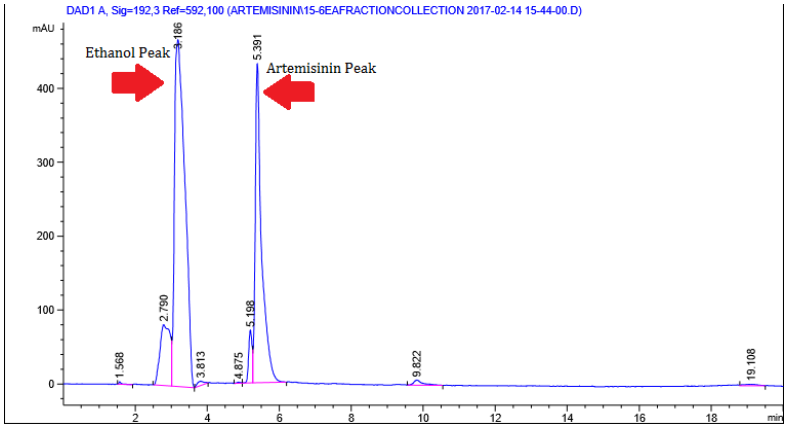

Figure 5: Sample of HPLC results for 12.5 uM stock pure artemisinin demonstrating the peak for ethanol at 3 minutes and the peak for artemisinin at 5.4-5.5 minutes (Ferreira, J.F., et al., 2009).

Table 1 below summarizes the areas under the artemisinin peak, as determined above, for pure artemisinin and *A. annua* teas tested by HPLC.

Table 1: HPLC results for artemisinin and *A. annua* teas (each various concentration of artemisinin was sampled at the same volume of 200uL)

Test group	Area (mAU*s)	Time of elution (minutes)
Artemisinin 5 uM	136.6	5.5
Artemisinin 25 uM	575.4	5.5
Artemisinin 50 uM	2391.5	5.4
Artemisinin 75 uM	1631.1	5.5
Stock artemisinin 12.5 mM	5282.5	4.4
Tea brewed in water (1)	43.9	5.5
Tea brewed in water (2)	32.0	5.4
Tea brewed in EtOH (1)	30.4	5.4
Tea brewed in EtOH (2)	50.1	5.5
Tea brewed in EtOH with 2.5mM artemisinin	512.1	5.5
Tea brewed in water with 2.5mM artemisinin	332.4	5.5

Using several concentrations of artemisinin, as well as teas spiked with artemisinin, it was concluded that artemisinin elutes at approximately 5.4-5.5 minutes (Ferreira, J.F., et al., 2009). This time of elution was used to identify peaks due to artemisinin in tea samples, although there was a poor correlation between artemisinin concentration and peak, a best fit plot was constructed (see *Figure 6*) and used to calculate approximate artemisinin concentrations in the two teas.

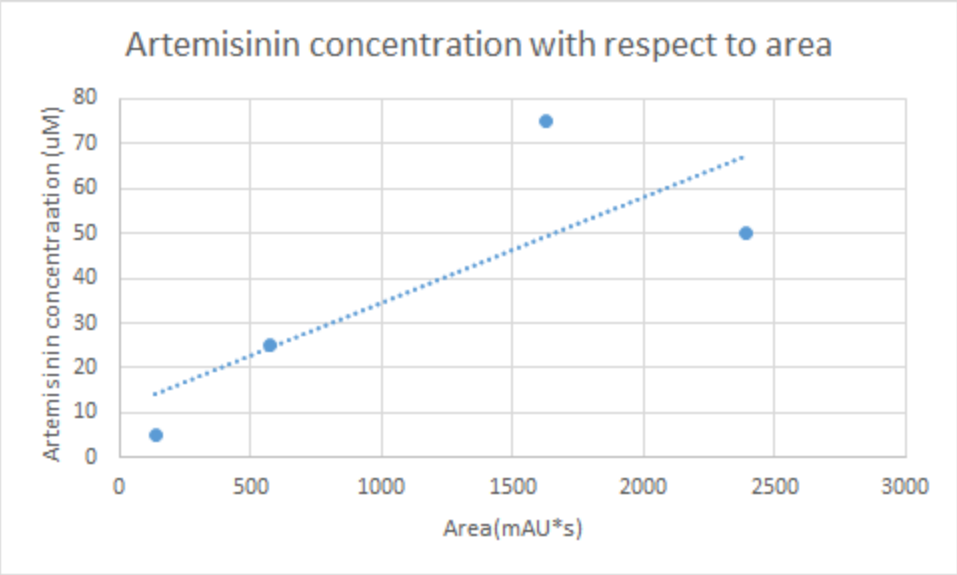

Figure 6: Artemisinin concentration in Artemisia annua tea with respect to HPLC recorded areas of trials tested in this study (note: the more trials performed at different concentrations, the more accurate the trend line and predictions will be).

When using the above denoted equation (y=0.0235x+10.912 where "x" represents the calculated area and "y" represents the calculated artemisinin molarity), the calculated tea values seemed disproportionately high (12 uM for the average EtOH tea and water brewed tea). It is also apparent that the values for the two teas fall well below the range of the standard curve. To re-approximate the molarity of the two tea values, a proportion of the peak area for 5 uM artemisinin was calculated using the peak areas for the two samples, resulting in approximately 1.5 uM artemisinin in EtOH brewed tea and 1.4 5 uM artemisinin in water brewed tea.

Artemisia annua Tea vs. Pure Artemisinin

An MTT assay was performed as outlined in the methodology in order to determine the effectiveness of *Artemisia annua* tea brewed in both ethanol and water on MCF7 breast cancer cells. Morphologically, there was a noticeable difference between cells grown in regular media and those treated with 15 uM artemisinin (see *Figure 7*). The MCF7 cells incubated in regular media (left) are adhered to the plate, as well as flat in appearance, as expected; while the cells incubated in the artemisinin are rounded up (right).

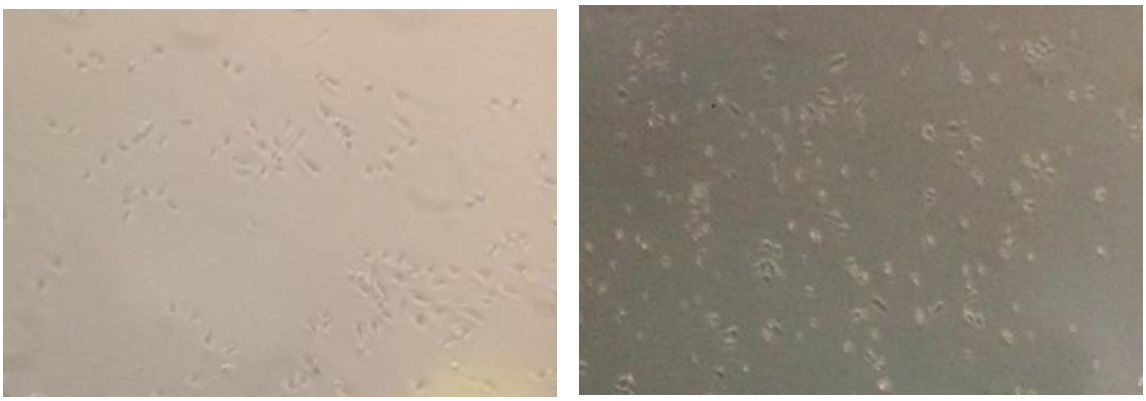

Figure 7: (Left) Image of MCF7 cells incubated in media after 24 hours. (Right) Image of MCF7 cells incubated in 15 uM artemisinin after 24 hours (400x magnification).

As seen in *Figure 8* below, demonstrating the MTT assay results for MCF7 cellular response when exposed to *Artemisia annua* tea, there is a noticeable difference from the control cells compared to cells incubated in the various percentages of *Artemisia annua* tea brewed in ethanol. However, ethanol alone appears to have an equivalent effect. There is an average decrease of about 50% in the cellular proliferation overall. By contrast, cells grown in *Artemisia annua* tea brewed in water, show average increase in cell number of about 18%.when compared to untreated controls the result of the cells treated with 15 uM pure artemisinin was also similar to the tea brewed in water results.

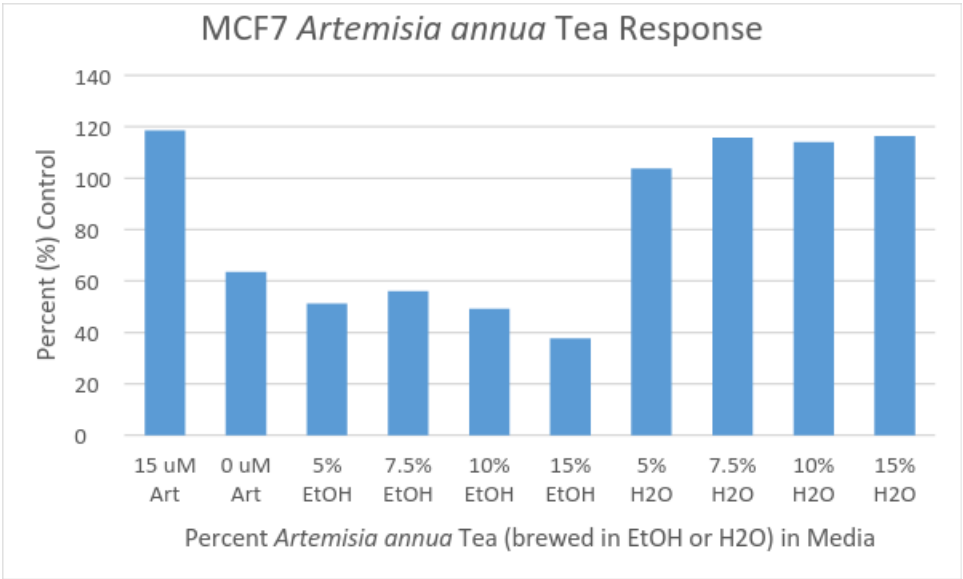

Figure 8: MTT assay (N=2) MCF7 cells treated with an array of EtOH and H2O teas containing 5%, 7.5%, 10%, and 15% artemisinin, as well as 15uM pure artemisinin and 1% EtOH as a negative control.

As previously mentioned in the methodology section, the final concentration of the ethanol in the Artemisia annua tea samples for both water and ethanol brewed were higher than the 1% designated per well. This was due to the tea originally being placed in 70% ethanol, and when added to the wells of the experiment, the 15% tea medium resulted in over 10% ethanol per well.

Discussion:

The literature describes MCF7 cells as able to express high levels of the alpha estrogen receptor, therefore estrogen should stimulate the growth of this cell line (Lee et al., 2015). However, the MTT assays for the estrogenic response yielded no conclusive results since only two trials were performed and aggregated, allowing no statistical analysis to be done. As well, the variability among the replicates was so high as to negate any direct interpretation of the results. Overall though, there are consistent aspects of the data that may be addressed in regard to: artemisinin's effect on cellular proliferation, the minimal amount of artemisinin in *Artemisia annua* tea, and morphological effects of artemisinin on MCF7 cells.

Although artemisinin does not seem to have a clear impact on cellular proliferation as indicated by the contrasting results in literature, and our results from this study; there is indication that it may have a slight proliferative effect. Our results for MCF7 cells exposed to artemisinin in the dose response trials had higher cellular counts than the control group up to 25 uM artemisinin, where our data consistently demonstrated that the proliferation plateaus, suggesting this dose as a possible threshold. In hindsight though, we believe that using a higher concentration (such as 25uM) where cell proliferation increases noticeably, could be effective in further understanding what role artemisinin can play in proliferation.

In addition, our results indicated that while there were no noticeable changes at the 12 and 18 hour mark in the time course when exposed to artemisinin, at the 24 hour interval cells exposed to artemisinin had a much higher level of cellular proliferation. We believe that our cells did not have any observable differences during the first two time intervals because the cells had not completely undergone a mitotic cycle at those times. Thus, artemisinin may have an effect on cellular proliferation based on exposure and time available for mitotic divisions.

When the *Artemisia annua* tea was brewed in ethanol it consistently had the greatest effect on the decrease in cellular proliferation. When the tea was brewed in water, the cells seemed to increase in proliferation at a similar rate to 15uM pure artemisinin. Overall, the MCF7 cells that were exposed to 15% *Artemisia annua* tea brewed in ethanol exhibited the largest decrease in cell proliferation. One explanation for these results could be that the level of artemisinin that the cells were exposed to was too low to have a significant effect on cellular proliferation. Moreover, to reach an artemisinin concentration equivalent to 15 uM pure

artemisinin media, large amounts of ethanol brewed tea needed to be added to media. At the 15 uM equivalent, the test media was approximately 10% ethanol. This high level of alcohol could kill a large portion of the cells, resulting in an inaccurate reading of the tea's role in proliferation. Our hypothesis also predicted that artemisinin values would be higher in ethanol tea than water tea since artemisinin is soluble in alcohol, and our data does not support our hypothesis. We did not observe any noticeable differences between the two types of tea with respect to artemisinin concentration. We observed noticeable differences in tea composition aside from artemisinin content, with several compounds eluting in different concentrations. This finding could be due to several factors. One primary factor could be that, while ethanol acts as a much more effective solvent for pure artemisinin, compounds in the plant aside from artemisinin could affect how the solvent functions in dissolving compounds. Another key factor could be that the dried plant leaf that we used simply had low levels of artemisinin in it, so the variance between the effectiveness of the solvents could not be discerned through HPLC readings of the tea. This can explain why, in addition to having so many other eluting compounds within the tea, the artemisinin readings themselves were so low.

When utilizing pure artemisinin to create a trend line, we calculated a set of outlier values at the 50 and 75uM concentrations. Due to the limited HPLC calculations that we performed, we were not able to effectively assess the variance in these data points, which could influence the final values determined for approximate artemisinin concentrations within the two teas. We believe this outlier value was due to sampling errors that could have occurred when performing HPLC, leading to a relatively higher amount of artemisinin in the extracted 50uM sample or a relatively low extracted amount in the 75uM sample. By performing several replications of these readings, a more concise trend line can be calculated to eliminate potential outliers and produce a more accurate trend line for calculating artemisinin concentrations. Moreover, by measuring artemisinin elution values at concentrations lower than 5uM, we may more accurately determine the trend line and the amount of artemisinin in *Artemisia annua* tea.

Finally, we observed throughout the MTT assay experiments that cells exposed to artemisinin seemed to be rounded in shapes that resembled cells in trypsin, and were not growing in larger masses as readily as control cells. While the cells were not apparently suspended in the test media, their morphology and growth patterns suggest that they were not exhibiting behaviors

of cancer cells forming larger tissues. It is possible that the when the cells were viewed under the microscope, they were in a stage of mitosis that caused the shape change. It would be beneficial to view the cells during different growth stages and compare the morphologies to better understand whether the shape change was from artemisinin or due to the stage of the growth they were currently in. However, if cells are unable to plate down, in theory they may not be able to metastasize and form a tumor. This has been shown through cell adhesion studies, such as the 2016 study which concluded that Korean *Artemisia annua* L (pKAL) suppressed the expression of a cell adhesion protein, therefore breast cancer cells were unable to attach to the endothelial lining of blood vessels (Ko et al., 2016). The results of this paper suggested that pKAL could be a potential form of therapy against cancer metastasis (Ko et al., 2016).

Conclusions & Recommendations:

We concluded that we have not produced any evidence that supports the claim of artemisinin as a cancer therapy, as our results were not able to be statistically analyzed and cell counts did not seem to be affected. Due to this conclusion, we have developed the following recommendations for future studies regarding artemisinin's potential as a natural cancer therapeutic in areas of: materials used, procedures performed, and topics to explore.

Since the MCF7 cells did not seem to respond to estrogen, a compound known for increasing proliferation in MCF7 cells, we recommend that future experimenters utilize an array of different cell lines when studying artemisinin. As well, we recommend that future studies should compare cellular proliferation levels against those of previously tested compounds with known effects to comparatively assess the testability of their cells and the artemisinin treatment. In addition, a recommendation for the *Artemisia annua* tea versus artemisinin experiment is to use dimethyl sulfoxide (DMSO) as the solvent so that it will not kill the cells and can be ruled out as a confounding variable. Since DMSO does not affect cells as adversely as ethanol, it could be used as a potential solvent when attempting to brew teas that can be added at higher quantities to test mediums.

Along with this, we recommend utilizing a different method to quantify the concentration of cells in each well aside from or in addition to the MTT Assay. MTT Assays are able to detect viable cells, however they do not specifically detect cellular proliferation (Riss et al., 2016). Using an assay to assess the amount of damaged DNA within a well could be utilized to quantify apoptosis levels, rather than simply measuring the total amount of DNA within the wells.

We noticed that cellular morphology changed when cells were exposed to artemisinin, therefore we recommend that future experimenters assess the adhesion of cells exhibiting this behavior to understand how this phenomenon can be used in potential therapies, such as hindering the formation of more complex tissues or lowering the potential for metastatic cells to adhere and grow into additional tumors. If this morphological change affects cellular adhesion factors, it would prevent cells from successfully attaching to somatic tissues and developing into tumors. We suggest that future experimenters develop in vitro experiments to model cellular adhesion and metastatic properties to identify potential benefits and consequences of this as a treatment option. We also suggest that future researchers investigate specific cell adhesion factors and how they may change when exposed to artemisinin.

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Appendices

Appendix A.

Estrogenic Response Raw Data (Absorbance Values at 570 nm)

Trial #1 & 2

	Cells (W)	Eth	Est 1/5x	Est 1x	Est 10x	
	8	9	10	11	12	
A	1.234	1.613	1.358	1.324	1.26	570
B	1.588	1.548	1.457	1.31	1.346	570
C	2.119	1.365	1.046	1.266	1.249	570
D	0.032	0.032	0.033	0.033	0.032	570
E	0.031	0.035	0.031	0.032	0.03	570
F	0.033	0.033	0.032	0.033	0.031	570
G	0.031	0.032	0.031	0.032	0.031	570
H	0.031	0.032	0.223	0.273	0.2	570
			W Media	W Media	W Media	

	Cells (W)	Eth	Est 1/5x	Est 1x	Est 10x	
	8	9	10	11	12	
A	0.519	0.54	0.524	0.547	0.78	570
B	1.077	0.976	1.119	1.13	0.897	570
C	0.995	0.975	0.927	0.935	0.526	570
D	0.031	0.03	0.031	0.032	0.029	570
E	0.031	0.029	0.03	0.033	0.031	570
F	0.03	0.031	0.031	0.03	0.03	570
G	0.029	0.031	0.03	0.03	0.029	570
H	0.03	0.031	0.165	0.411	0.211	570
			W Media	W Media	W Media	

Appendix B.

Artemisinin Dose Response Initial Raw Data (Absorbance Values at 570 nm)

Trial #1 & 2

	Cells R	5 Art	25 Art	50 Art	75 Art	100 Art
	1	2	3	4	5	6
A	1.721	2.041	2.225	2.294	2.575	2.79
B	2.04	1.736	2.017	2.454	2.174	2.419
C	1.829	2.247	2.459	2.341	2.351	1.952
D	0.04	0.031	0.031	0.032	0.034	0.032
E	0.032	0.031	0.032	0.033	0.033	0.03
F	0.033	0.033	0.032	0.031	0.032	0.031
G	0.031	0.033	0.031	0.032	0.029	0.032
H	0.788	0.64	0.689	0.031	0.032	0.032
	Red M	Red M	Red M			

	Cells R	5 Art	25 Art	50 Art	75 Art	100 Art
	1	2	3	4	5	6
A	1.072	1.498	1.573	1.184	1.415	2.004
B	1.109	1.791	2.399	1.856	2.117	1.786
C	1.152	1.577	1.618	1.287	1.649	1.344
D	0.029	0.03	0.03	0.032	0.031	0.03
E	0.03	0.029	0.03	0.031	0.03	0.032
F	0.031	0.03	0.03	0.03	0.03	0.031
G	0.03	0.03	0.03	0.031	0.03	0.032
H	0.437	0.445	0.436	0.029	0.03	0.031
	Red M	Red M	Red M			

Appendix C.

Artemisinin Dose Response Secondary Raw Data (Absorbance Values at 570 nm)

Trial #3 & 4

	Cells R	25 Art	15 Art	5 Art	2.5 Art	Red M
	1	2	3	4	5	6
A	0.99	1.292	1.508	1.487	1.717	0.523
B	1.628	2.347	1.93	2.017	1.884	0.509
C	1.015	1.718	1.732	1.712	1.65	0.543

	Cells R	25 Art	15 Art	5 Art	2.5 Art	Red M
	1	2	3	4	5	6
A	1.605	1.751	1.38	1.415	1.572	0.47
B	1.692	2.21	1.664	1.491	1.54	0.474
C	1.801	1.625	1.588	1.619	1.289	0.471

Appendix D.

Artemisinin Dose Response Time Course (Absorbance Values at 570 nm)

Trial #1

	24 C	24 A	24 M		18 C	18 A	18 M		12 C	12 Art	12 M
	1	2	3	4	5	6	7	8	9	10	11
A	1.23	1.947	0.434	0.031	2.17	2.36	0.482	0.032	1.957	2.054	0.662
B	1.301	1.761	0.704	0.03	1.399	1.854	0.787	0.031	1.999	1.625	0.593
C	1.322	2.057	0.7	0.031	2.514	2.189	0.719	0.031	1.822	2.246	0.502
D	0.031	0.031	0.031	0.032	0.032	0.033	0.032	0.031	0.031	0.031	0.031
E	0.032	0.03	0.03	0.03	0.03	0.03	0.03	0.032	0.03	0.031	0.031

Trial #2

	24 C	24 A	24 M		18 C	18 A	18 M		12 C	12 Art	12 M
	1	2	3	4	5	6	7	8	9	10	11
A	1.438	1.259	0.45	0.032	2.567	2.444	0.454	0.03	1.967	1.755	0.474
B	1.426	2.289	0.458	0.03	3.157	2.269	0.641	0.031	2.032	2.113	0.507
C	1.48	2.589	0.45	0.03	2.406	2.674	0.584	0.031	2.331	1.894	0.809
D	0.031	0.031	0.031	0.032	0.032	0.033	0.032	0.031	0.031	0.031	0.031
E	0.032	0.03	0.03	0.03	0.03	0.03	0.03	0.032	0.03	0.031	0.031

Appendix E.

Artemisia annua Tea vs Artemisinin (Absorbance Values at 570 nm)

Trial #1

	1	2	3	4	5	6	7	8	9	10
F	1.472	1.275	0.997	1.082	0.979	0.938	1.645	1.906	1.504	2.468
G	1.542	0.796	1.036	1.175	0.895	0.947	1.579	1.839	1.838	2.126
H	1.727	1.303	1.008	1.174	0.964	0.916	1.832	1.605	1.838	1.472
	Cells R	EtOH	5E	7.5E	10E	15E	5W	7.5W	10W	15W

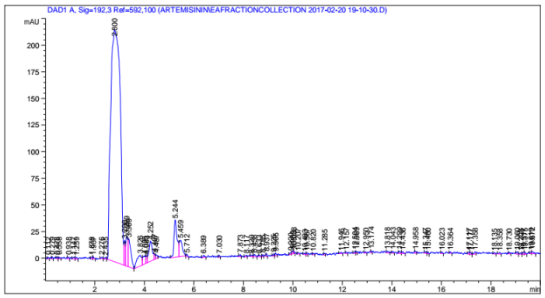
Trial #2

	1	2	3	4	5	6	7	8	9	10
F	1.706	1.301	1.127	1.164	1.181	0.913	1.651	1.828	1.824	1.661
G	1.726	1.331	1.145	1.07	1.147	0.874	1.639	1.853	2.027	1.734
H	1.647	1.323	1.182	1.154	1.183	0.972	1.729	1.862	1.75	1.474
	Cells R	EtOH	5E	7.5E	10E	15E	5W	7.5W	10W	15W

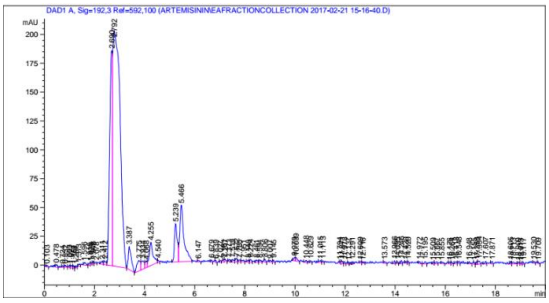
Appendix F.

HPLC Raw Results

5 uM Artemisinin



25 uM Artemisinin



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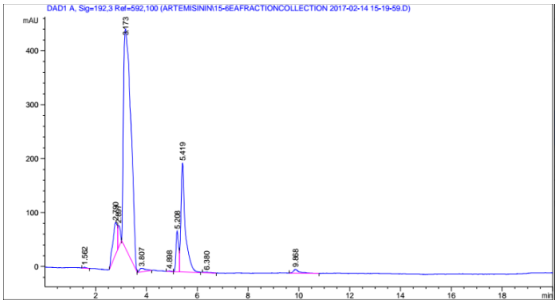
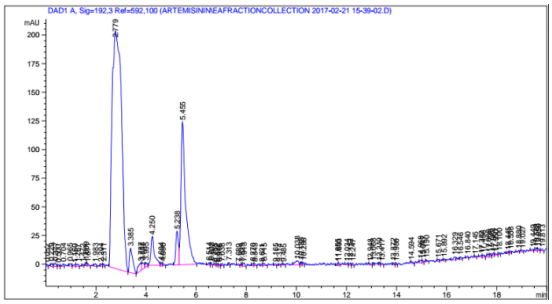
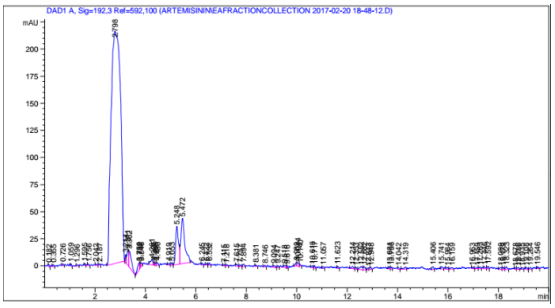


75 uM Artemisinin

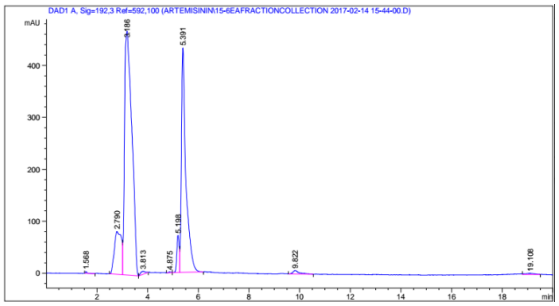


100 uM Artemisinin

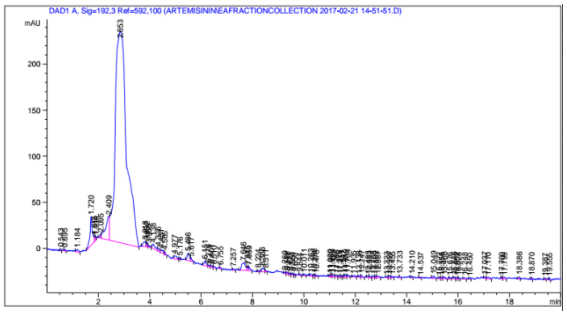
12.5 mM Artemisinin



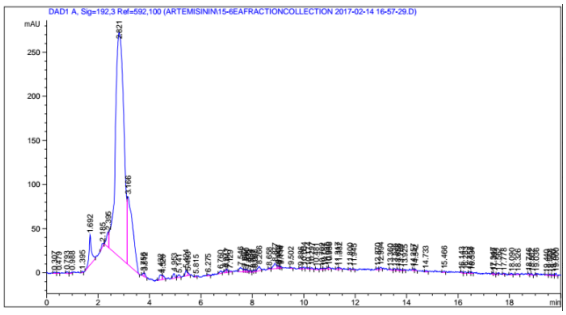
Artemisia annua Tea Brewed in Ethanol (1)



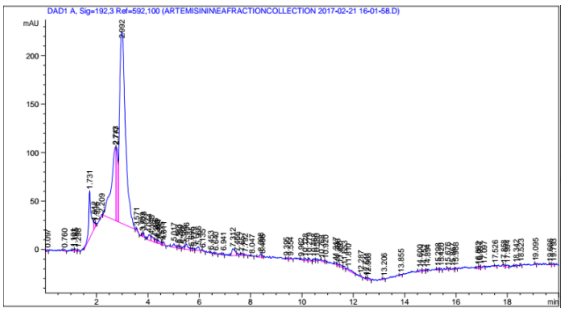
Artemisia annua Tea Brewed in Ethanol (2)

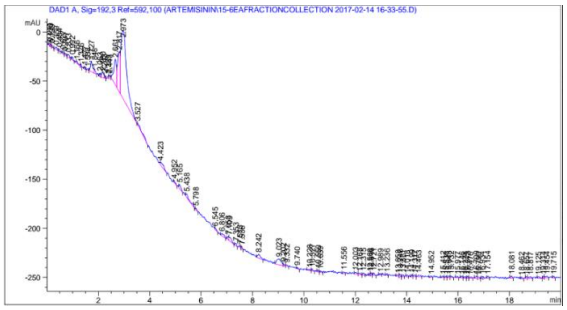


Artemisia annua Tea Brewed in Water (1)

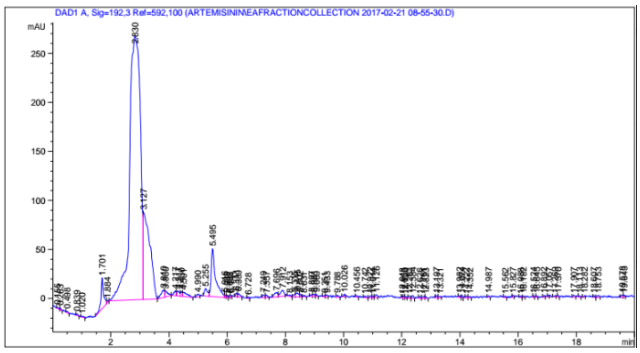


Artemisia annua Tea Brewed in Water (2)

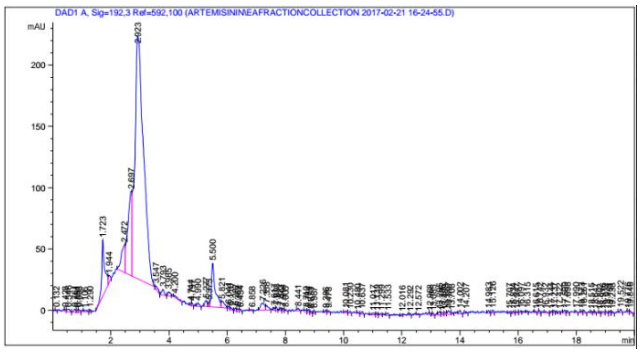




Artemisia annua Tea Brewed in Ethanol + 50 uL 12.5 mM Artemisinin



Artemisia annua Tea Brewed in Water + 50 uL 12.5 mM Artemisinin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1.944
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