

Artemisia annua and its Effects on Cancer Proliferation

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Abstract

Tea brewed from *Artemisia annua* is commonly used as an antimalarial, but its effects on cancer are almost entirely unknown. To test these effects, breast (MCF7) and ovarian (OVCAR-3) cancer cells were cultured in the presence of tea extract and their growth measured. Results suggest that the tea promotes proliferation in both cell lines, raising concerns that use of the tea for malaria treatment may increase cancer risk.

Introduction

Artemisia annua is a type of wormwood plant used in the treatment of malaria. The plant has been used in Chinese traditional medicine, where it is known as *qinghao*, since at least the second century BC. In this tradition, it was believed to have a cooling and detoxifying effect, allowing it to effectively counteract the fevers brought on by malaria. Western medicine was introduced to the plant in the 1970's after the increasing prevalence of drug-resistant *Plasmodium* parasites drove the Chinese government to research traditional remedies for the disease (Hsu, 2006).

Since it was determined to have anti-malarial properties, the plant has been used primarily as a source of artemisinin, the major anti-malarial compound in the plant that has also been shown to have anti-proliferative effects on cancer. However, tea made from the plant is still used as a common malaria treatment and prevention measure in countries where it grows natively, and studies have therefore been conducted to test the effectiveness of the tea compared to artemisinin. Similarly, some tests have been conducted to determine its comparative effects on cancer based on the beneficial effects seen in the use of artemisinin and other compounds present in the plant in preventing cancer proliferation (Ko et. al., 2016). In order to expand upon this fairly limited body of research, we chose to analyze the effects of *A. annua* extract on the growth of breast and ovarian cancers.

Artemisinin

The major component in *Artemisia annua* used in malaria treatments is artemisinin. The artemisinin molecule has an endoperoxide bridge which allows it to interact with iron and form free radicals, shown in Figure 1 below (Tonmumphean 2001).

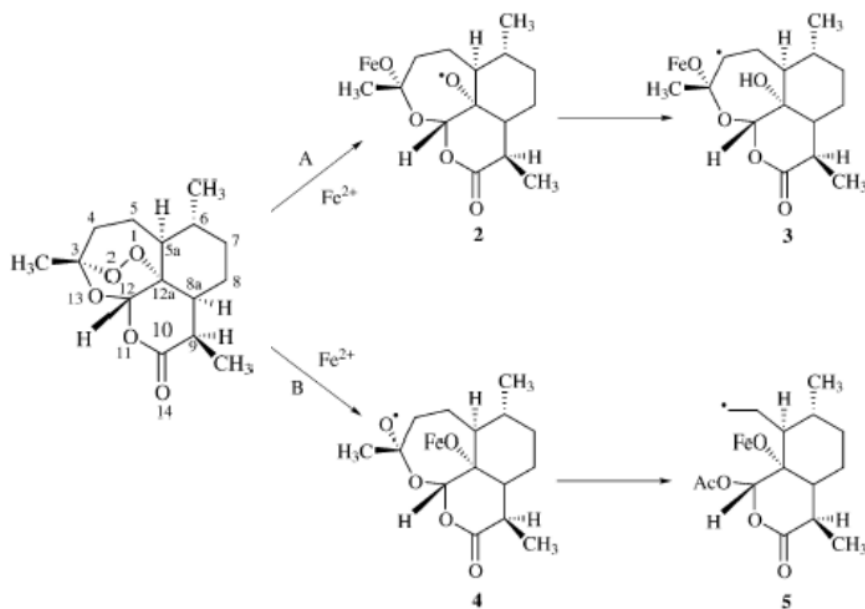


Figure 1: Diagram showing the cleavage of the endoperoxide bridge in the artemisinin molecule (Tonmumphean, 2001).

Free radicals are highly reactive, capable of causing large amounts of damage to cells when present in high concentrations (National Institute of Health, 2014). This makes artemisinin an effective antimalarial, as those parasites are abnormally high in iron due to their presence in their hosts' blood. It also has potential as a cancer therapy for the same reason; relative to healthy cells, cancer cells tend to have high concentrations of iron as discussed below. This potential has been studied extensively both in human cancer *in vitro* (Singh 2004, Sun 1995) and in rat models *in vivo* (Singh 2001), repeatedly demonstrating anti-proliferative effects on many types of cancers. Human trials have not been conducted due to decreased efficacy compared to current chemotherapeutic agents, but artemisinin derivatives are being explored for possible increased potency.

Iron and Cancer

There have been a number of studies that have identified various ways in which cancer is linked to iron in humans. One review of 33 studies on colorectal cancer concluded that higher levels of dietary or supplementary iron correlated with increased rates of cancer (Nelson 2001), while in a separate study clinically reduced levels of iron due to repeated phlebotomy were shown to

decrease the risk of cancer (Edgren 2008). Most relevant here, some researchers have discussed the apparent modification of the tumor environment to be particularly iron-rich, providing cancers growth benefits from the increased iron compared to more regulated iron levels in healthy tissues (Torti, 2013). These indicators show that iron is likely to be more highly concentrated in cancer cells, providing a target for artemisinin to generate free radicals and damage those cells.

Artemisia annua

Tea produced from *Artemisia annua* has been a part of Chinese traditional medicine for thousands of years for the treatment of intermittent fevers, a common symptom of malaria (Hsu 2006). This continues to be a common course of treatment for malaria, particularly in rural areas of countries where the plant grows naturally and access to purified artemisinin can be difficult (de Ridder 2008). Use of the tea may provide additional benefits compared to artemisinin alone; it is possible that other compounds present in the tea also have anti-malarial properties, increasing efficacy while also reducing the likelihood of resistance developing against artemisinin (Ferreira 2010). An *in vivo* study of malaria patients was conducted to analyze the efficacy of *A. annua* tea, showing that the tea preparation had equivalent effects to artemisinin in rapidly eradicating parasites (Mueller 2000).

Based on the observed efficacy of the tea on malaria and the research showing the effectiveness of artemisinin against cancer, some researchers have shown interest in *A. annua* tea as a form of cancer treatment. One study analyzed the effect of both artemisinin and *A. annua* tea on HeLa cancer cells and *Trypanosoma* parasites to determine lethal doses (Efferth 2010). Their results, generated using two different preparation techniques on seven different *A. annua* samples, consistently demonstrated lower IC₅₀ values by weight for the tea than artemisinin alone in both the cancer and the parasites.

Another study, however, found that the tea had higher IC₅₀ values than artemisinin in MCF7 breast cancer cells (Subaru 2014). To further test this, the researchers tested the cytotoxicity of artemisinin with another major compound in artemisia, 3-caffeoylquinic acid (3CA), and found that the antagonistic interactions almost completely nullified the effects of artemisinin. From this

they concluded that, while some compounds present in artemisia extract may act synergistically, the presence of 3CA decreases the overall cytotoxicity to cancer cells.

Research on *Artemisia princeps*

Research on the effects of *Artemisia annua* on cancer growth are rare, but some studies have been performed with a close relative, *Artemisia princeps*. In one study (Sarath, 2007), researchers tested the growth of MCF7 cells with two forms of treatment media: an *Artemisia* smoke media and an extraction of *A. princeps* in water. The smoke media was prepared by burning the leaves of the plant and collecting the smoke in 50ml complete culture medium. The water extract was prepared by mixing the ashes of the burned leaves with 1.5 liters of boiling water. MCF7 cells were cultured with either the smoke extract or water extract, and the effects were measured using MTT and TUNEL assays. The researchers concluded that *A. princeps* smoke and water extracts induced apoptosis by disrupting the mitochondrial membrane potentials. They suggested that these extracts reduces BCL-2, a molecule that protects the cell from various apoptotic molecules that disrupt mitochondrial membrane potential. When the smoke was used with doxorubicin, a chemotherapeutic drug, the number of living MCF7 cells decreased by 50%, suggesting that the smoke can be used with other drugs for synergistic effects.

Another study (Ju, 2012) examined the effects of the plant on HeLa (human cervical cancer), A5-49 (human lung cancer), T-47D (human breast cancer), and AsPC-1 (human pancreatic cancer) cells. *A. princeps* was dried and extracted in 95% ethanol at room temperature for 24 hours and then the ethanol solutions were filtered, evaporated, and freeze-dried into a powder. This powder was then solubilized in H₂O, extracted with hexane/ethyl acetate, and then evaporated to acquire a flavonoid-rich fraction (FRAP). The cell lines were then grown with FRAP or with cisplatin, a chemotherapeutic agent used as a positive control, using the MTT assay to assess the cell viability and determine IC₅₀ values. The researchers found lower IC₅₀ values in the cells treated with FRAP than those treated with cisplatin, suggesting that the flavonoids extracted from *Artemisia princeps* had strong apoptotic effects on cells. Their data also suggests that FRAP disrupts the mitochondrial membrane potential by reducing the presence of BCL-2.

Cancer Cell Lines

Breast cancer is both the second most common cancer in women and the second leading cause of cancer death in women. Roughly 12% of all women in the US will be diagnosed at some point in their lives, causing over 40,000 deaths each year. While the mortality rate has been decreasing every year, the incidence rates have been increasing (DeSantis et. al., 2016). In order to analyze the effects of *A. annua* on breast cancer, we used the MCF7 cell line, a well-studied estrogen receptor-positive breast cancer cell line. The cell line was chosen both because breast cancer is a common cancer likely to afflict any population, and also because it is a well-characterized model system.

Ovarian cancer, while not as common as breast cancer, is still contracted by over 21,000 women in the US each year. Because it is hard to diagnose early, it has a high mortality rate and is the most lethal gynecological malignancy (Huang, 2010). To determine the effects of *A. annua* on ovarian cancer, we used the estrogen-responsive OVCAR-3 cell line. This cell line was chosen mainly for comparison with MCF7 cells, to observe if the effects of tea extracts were consistent between multiple cancer models.

Our Project

Both artemisinin and *Artemisia annua* tea are currently in use as antimalarial treatments, and both have been studied and shown to be effective in killing the parasites (Mueller 2000). Artemisinin has also been shown to be effective in treating cancer, leading some researchers to hypothesize that *A. annua* tea may have similar effects. This has not been studied as extensively, however, and the current research is unclear as to how effective the treatment may be. Better understanding of these effects could potentially lead to the tea being used in new chemotherapy approaches, or it could reveal the adverse side effects of tea use against malaria and push for change in cultural practice. We therefore decided to study the effects of both *A. annua* tea and artemisinin on cancer growth, using two common preparations of the tea for comparison and two different cancer cell lines. We hypothesized that *A. annua* tea would inhibit cancer proliferation at greater levels than equivalent amounts of artemisinin alone. Our results suggest the opposite, showing that artemisinin decreases cell proliferation but the tea increases cell proliferation relative to the untreated controls.

Methods

Cell Culture

Both MCF7 and OVCAR-3 cell lines were obtained from the ATCC and cultured according to the procedures outlined in Hamelers, 2003. Briefly, the cells were cultured in Dulbecco's modified Eagle's medium (Mediatech, Inc), which contained 10% fetal bovine serum (Equitech) for MCF7 cells and 20% fetal bovine serum for OVCAR-3 cells, 100 µg/mL of bovine insulin (Sigma Aldrich), 100 IU/mL penicillin, 100 µg/mL streptomycin. The cells were incubated at 37°C with 5% CO₂ until they reached approximately 60% confluence, at which point they would be plated for the MTT assay.

Tea Extraction

Two forms of *Artemisia annua* tea were prepared, using dried leaves acquired from Dr. Pamela Weathers, following the protocol outlined in Subaru (2014), with slight modifications. For the water tea, 5 g of *Artemisia* leaves were crushed in a pestle and mortar and added to 1 L of water and boiled for twenty minutes, strained using filter paper, and stored at either 4°C or 20°C. For the ethanol tea, 1 g of *Artemisia annua* leaves were crushed in a pestle and mortar and added to 200 mL of 70% ethanol in water, then incubated overnight at room temperature with shaking. Plant matter was removed using filter paper, and ethanol was allowed to evaporate overnight. The dry extract was then resuspended in 200 mL of water.

High Performance Liquid Chromatography (HPLC)

HPLC was performed on the extracts following the protocol outlined in Lapkin (2009), with some modifications. Briefly, using an Agilent 1100 Series system HPLC, a C-18 250 x 4.6 mm column was run under isocratic conditions with a flow rate of 1 mL/min for 20 minutes, using 24% acetonitrile, 0.06% formic acid, 0.016% acetic acid and 75.92% water. This mobile phase was initially used due to experimental error, rather than the more common 60% acetonitrile/40% water, but was kept in use because it generated clearer data. Samples of both the water and ethanol teas were separated, and the artemisinin peak in each was confirmed by spiking the sample with additional pure artemisinin. Additional runs were performed with either 2.12 µg, 2.82 µg, 3.53 µg, or 4.23 µg of artemisinin to establish a correlation between peak area on the

HPLC chromatogram and the mass of artemisinin added to the column, which was then used to determine the artemisinin content of both the water and ethanol teas.

MTT Assay

To measure cell proliferation, Promega CellTiter 96 Cell Proliferation Assays (MTT) were performed following the normal protocol. Briefly, MCF7 and OVCAR-3 cells were plated on a 96-well plate at a concentration of 10^4 cells/well in 200 μ L of normal growth media and incubated for 24 hours, again at 37°C with 5% CO₂. The media was then removed and replaced with 200 μ L of experimental media, which was growth media containing 5%, 7.5%, 10%, or 12.5% tea, or growth media containing 100 μ g/mL artemisinin, with normal growth media without tea as a control. Each experimental condition was run in triplicate for each experiment. After an additional 24 hour incubation, 40 μ L of the MTT labelling reagent was then added to each well and incubated for four hours, after which the absorbance of each well was read at 570 nm using a microplate reader. The data were expressed as percent control relative to cells in normal growth media and averaged across three trials.

Results

High Performance Liquid Chromatography

Both the water tea and ethanol tea were tested for artemisinin content using the HPLC procedure given in the methods section. In order to interpret the HPLC data, pure artemisinin was first run at several different concentrations to establish a curve comparing the concentration of artemisinin to the HPLC peak area in milli-absorbance units (mAU). An example of the chromatogram obtained for these HPLC runs is shown below in Figure 2.

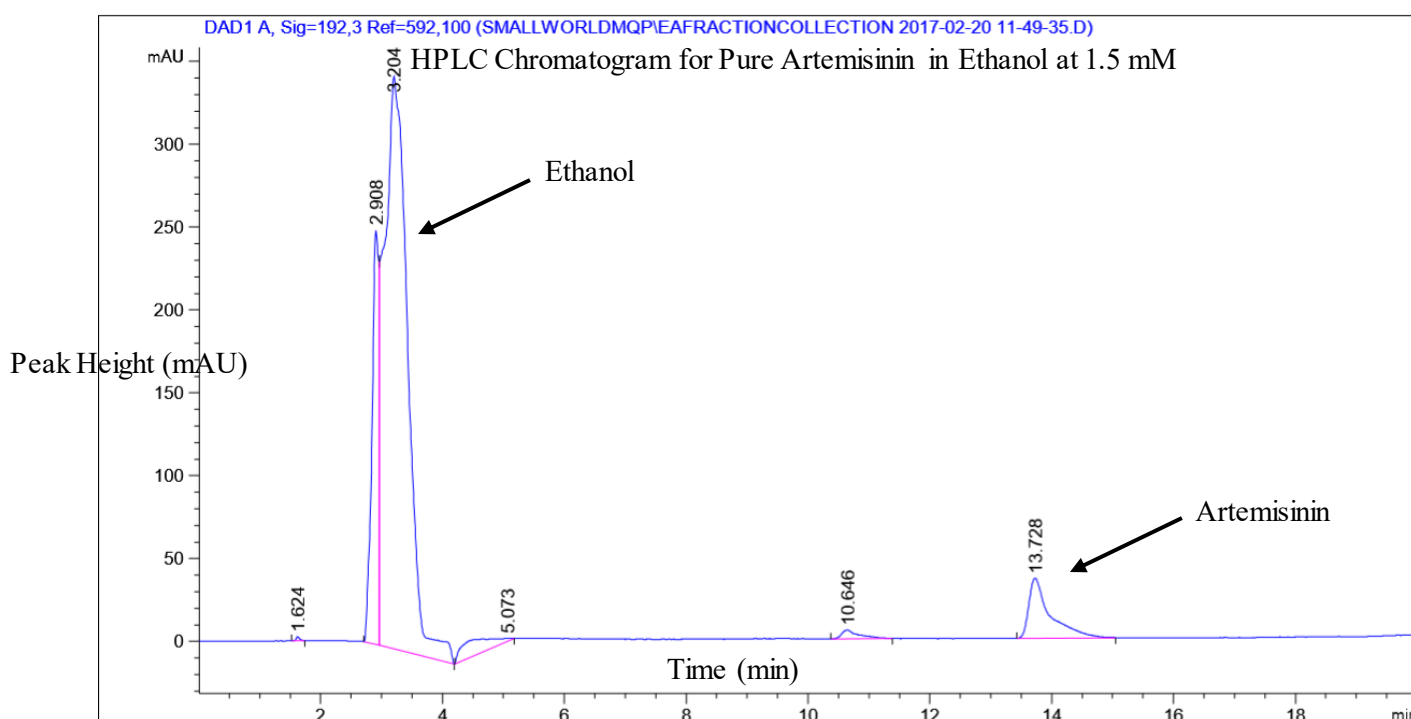


Figure 2: Chromatogram for pure artemisinin in ethanol at 1.5 mM. 10 μ L of solution was added to a C-18 250 x 4.6 mm column run under isocratic conditions with a flow rate of 1 mL/min for 20 minutes, using 24% acetonitrile, 0.06% formic acid, 0.016% acetic acid and 75.92% water, as described in methods.

The first labeled peak in Figure 2 was determined to be the solvent, ethanol, by running another HPLC test using only pure ethanol. The second labeled peak was determined to be artemisinin by varying the concentration of artemisinin relative to ethanol in subsequent trials.

After several different concentrations of pure artemisinin were tested, the chromatogram peak areas were plotted against mass of artemisinin added to the column to generate a model that would predict mass of artemisinin given any chromatogram area. This graph is shown in Figure 3 below.

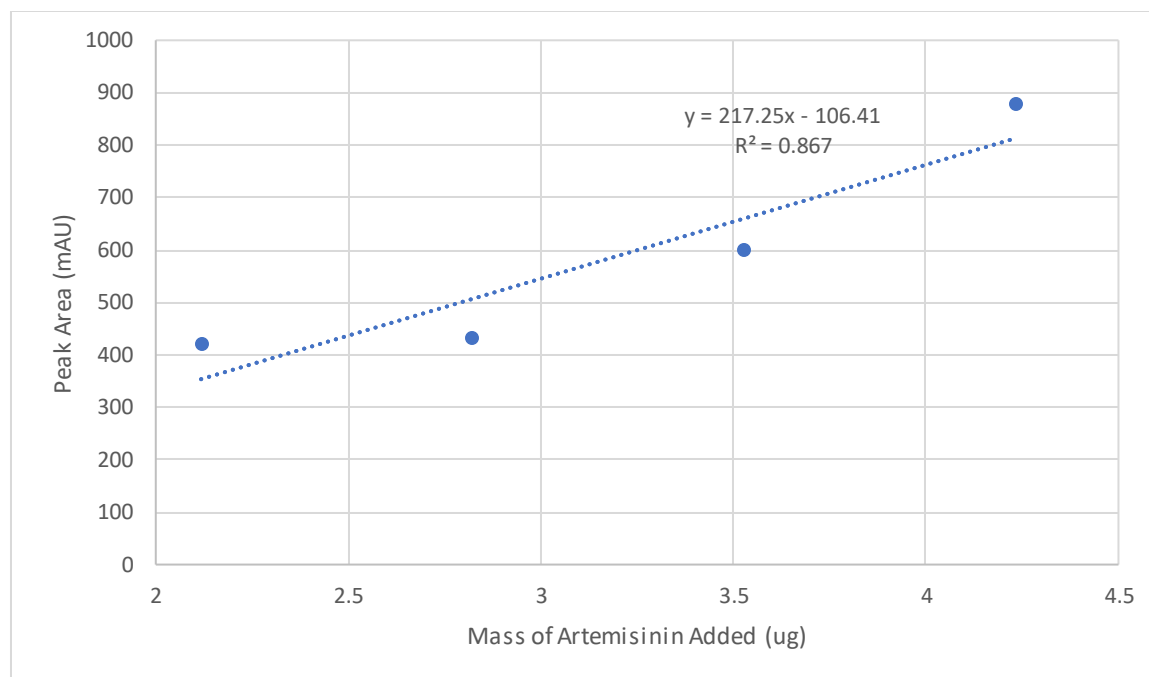


Figure 3: Graph displaying four HPLC results for artemisinin samples at different concentrations, with a line of best fit.

The four masses of artemisinin tested (2.12 μg , 2.82 μg , 3.53 μg , 4.23 μg) and their respective peak areas determined by HPLC could be used to determine the mass of artemisinin present in an unknown solution using the equation shown in Figure 3. The two *Artemisia* tea extracts were then tested to determine artemisinin content. A representative chromatogram is shown in Figure 4 below, along with a chromatogram showing the tea extract spiked with artemisinin to determine the correct peak.

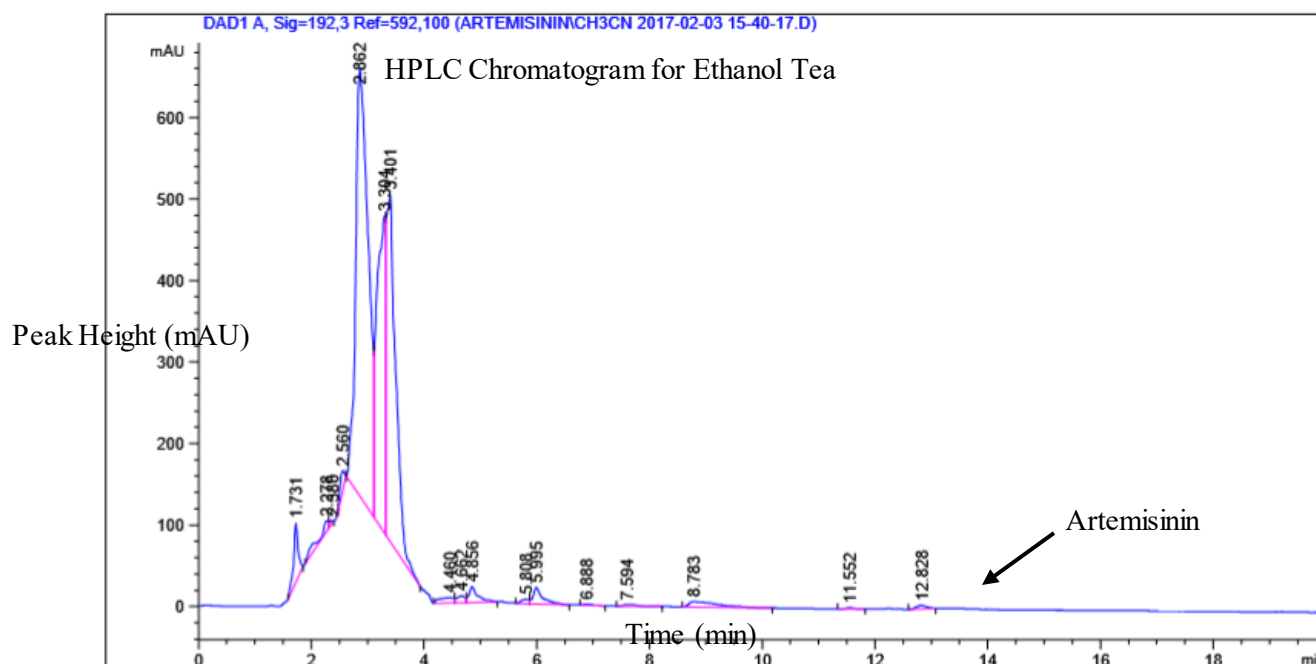


Figure 4: Chromatogram for *Artemisia* tea extracted in ethanol. 10 μ L of tea was added to a C-18 250 x 4.6 mm column run under isocratic conditions with a flow rate of 1 mL/min for 20 minutes, using 24% acetonitrile, 0.06% formic acid, 0.016% acetic acid and 75.92% water, as described in methods.

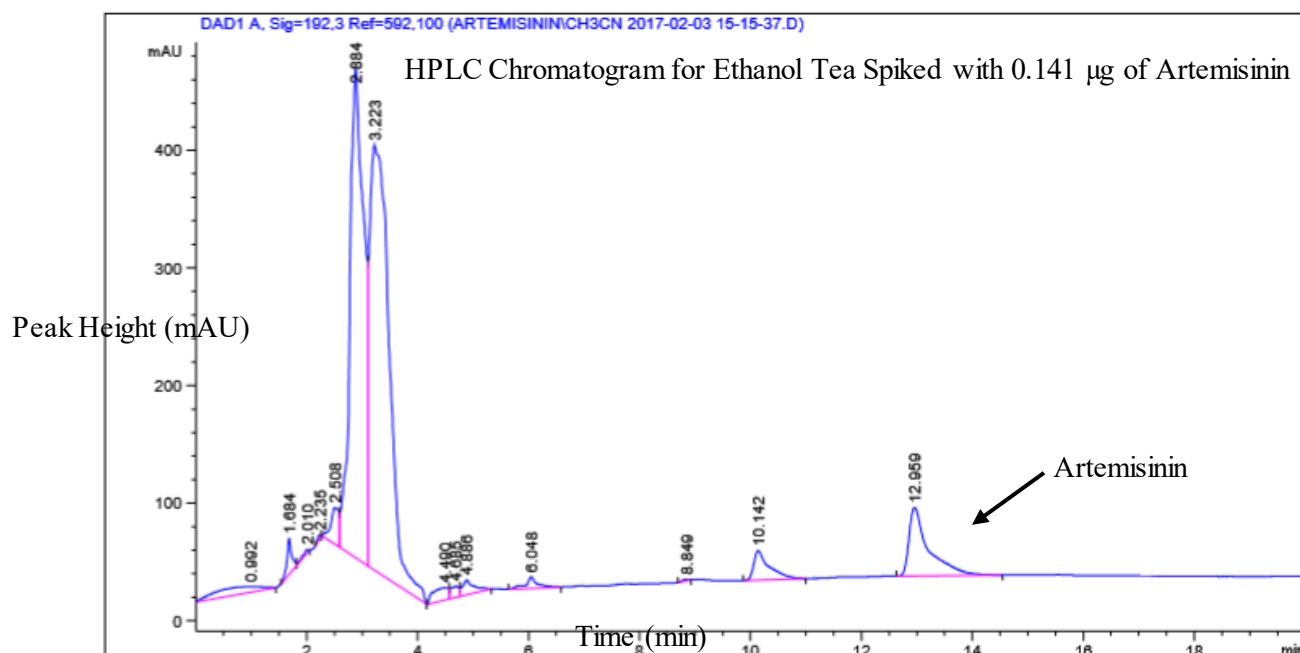


Figure 5: Chromatogram for ethanol tea spiked with 0.141 μ g of artemisinin. 10 μ L of the spiked tea was added to a C-18 250 x 4.6 mm column run under isocratic conditions with a flow rate of 1 mL/min for 20 minutes, using 24% acetonitrile, 0.06% formic acid, 0.016% acetic acid and 75.92% water, as described in methods.

Both the ethanol and water teas were tested, and confirmation tests in which the teas were spiked with additional pure artemisinin were performed to correctly identify the artemisinin peak. The peak area for the ethanol tea, shown in Figure 4, was found to be 52.42 mAU, which corresponds to 0.731 μg or 259 μM artemisinin in the tea. The peak area for the water tea was found to be 50.11 mAU, corresponding to 0.720 μg or 255 μM artemisinin in the tea.

MTT Assay

Both water and ethanol extracted teas were mixed in media at different concentrations as described in methods. The concentrations of teas present in media were 5%, 7.5%, 10%, and 12.5%. A negative control of 0% tea in media was run as well. Figure 5 below shows the normalized data in percent control with standard error bars. An increase in cell proliferation was shown at all concentrations for both cell lines in both teas, as high as a 70% increase, with no apparent dose response. However, there is significant error for many of the test conditions.

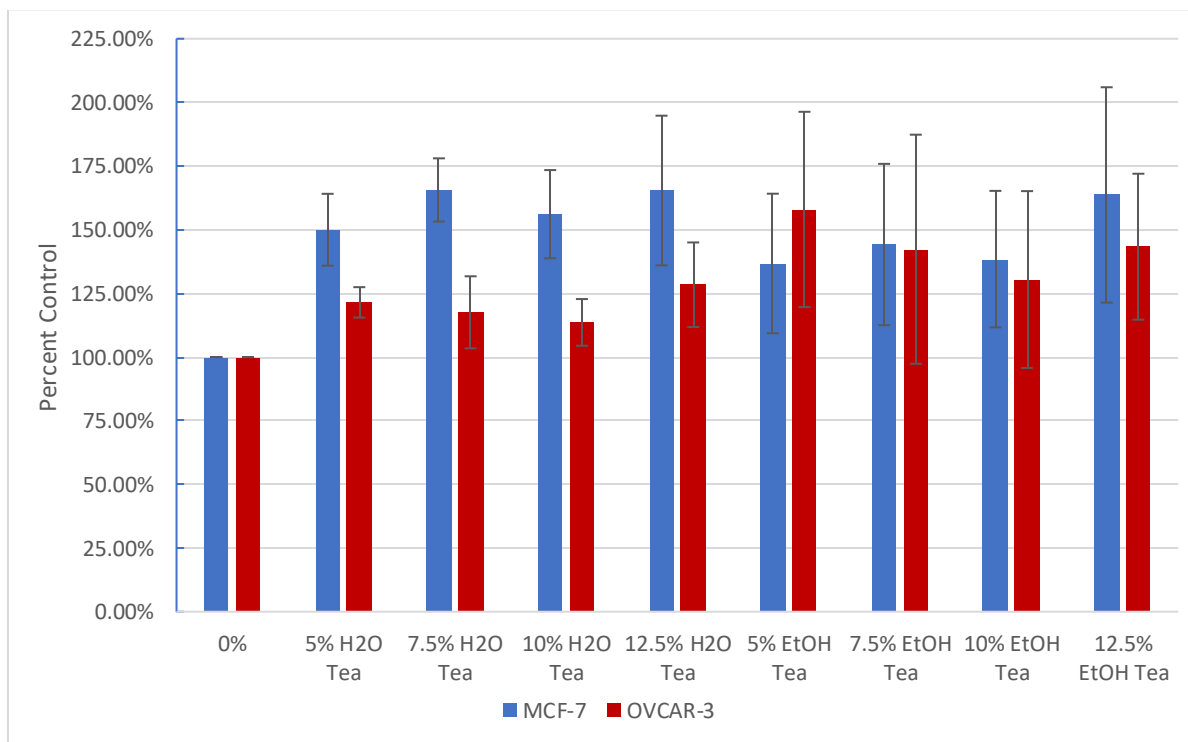


Figure 5: Cellular response to various amounts of water tea and ethanol tea in media after growth for 24 hours. Relative numbers of cells were measured as absorbance 4 hours after addition of MTT reagent. Samples were run in triplicate for each experiment, with $n = 3$ individual trials for each condition normalized to the control (normal growth media).

This experiment was also run with pure artemisinin. The pure artemisinin concentration that was present in the media for this experiment was $100\mu\text{M}$ of artemisinin in culture media. Figure 6 below shows the normalized data in terms of percent control. The reduction in cell proliferation was less than 10% for both cell lines, although with only two trials significance cannot be determined.

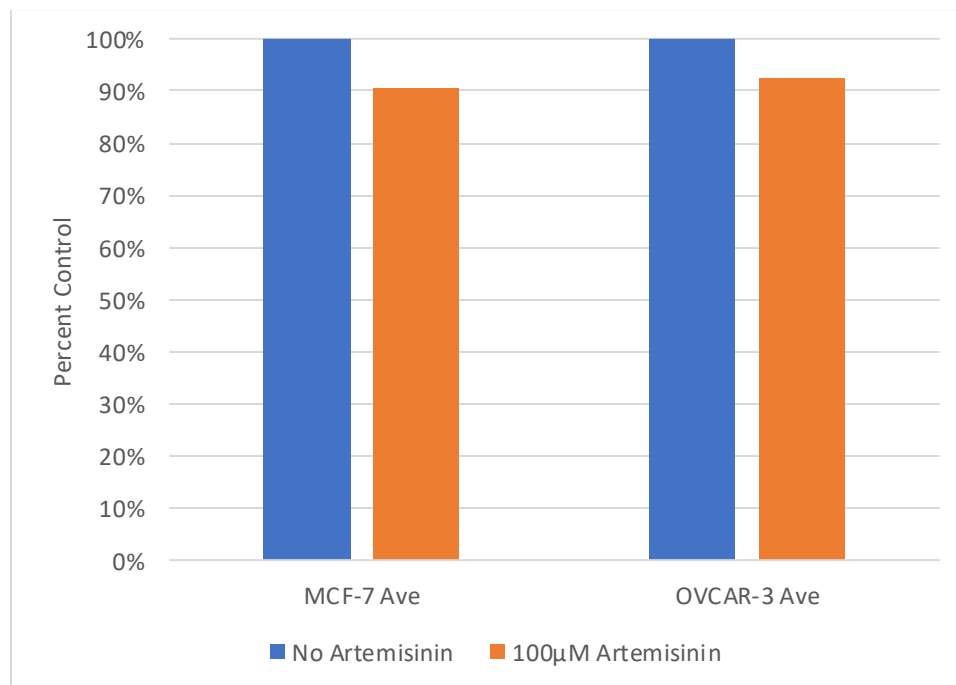


Figure 6: Cellular response to $100\mu\text{M}$ artemisinin in culture media after growth for 24 hours. Relative numbers of cells were measured as absorbance 4 hours after addition of MTT reagent. Samples were run in triplicate for each experiment, with $n = 2$ individual trials for each condition normalized to the control (normal growth media).

Discussion

The purpose of these experiments was to observe the effects of *Artemisia annua* extracts in the form of water tea and ethanol tea on MCF-7 and OVCAR-3 proliferation. Based on the data presented in Figure 5, both forms of tea promoted proliferation in both cell lines. This result is surprising, as previous research had indicated that the tea was lethal to cancer cells at lower concentrations than artemisinin. Our own test of pure artemisinin, shown in Figure 6, supported the literature and displayed decreased proliferation of both cell lines, suggesting that these results are not simply due to our particular cell lines, though with only two trials more research would be needed to confirm this. However, the response shown in both cell lines appeared to be lower than what would be expected from the literature, as other researchers have determined an IC_{50} value of artemisinin to be $9.13 \pm 0.07 \mu M$. The methods for our MTT assay are not entirely analogous to the methods those researchers used to determine the IC_{50} , particularly in the fact that our experiments involved a significantly decreased growth period after the addition of artemisinin, which may partially explain this discrepancy. Still, this decreased efficacy of artemisinin on our cell lines may also partially explain our surprising results for tea media.

While one study did report decreased efficacy of tea relative to artemisinin, likely due to competitive inhibition from other compounds present in the tea, the data still supported the suggestion of some researchers that *A. annua* had promise as a chemotherapeutic agent. Our project refutes this, potentially revealing a serious side effect of *A. annua* tea usage as an antimalarial. Given that purified artemisinin and its derivatives have been shown to effectively treat malaria and decrease cancer risk, use of the drug should certainly be advised over use of the tea, even in rural areas with much greater access to the plant.

Our HPLC results suggested that the artemisinin content of both the water tea and ethanol tea was roughly equivalent, at $255 \mu M$ and $259 \mu M$ respectively. These results may not be accurate, as the only standard curve that could be generated used masses of artemisinin an order of magnitude larger than what was found for the tea samples. Future research should replicate these tests with lower masses of artemisinin corresponding to the amounts in the tea samples to better determine their concentrations. Additionally, the elution time for artemisinin appeared to vary

between the pure compound and the tea samples. To verify that the peaks observed in the tea samples were artemisinin, HPLC tests were performed on tea samples spiked with artemisinin, confirming that the peak was artemisinin despite eluting more than a minute earlier. This discrepancy is likely due to some other compound or compounds in the tea interacting with the artemisinin and causing it to elute earlier, though this should not affect the expected correlation between peak area and mass of artemisinin. Assuming our calculated concentrations are accurate, our tea experiments were using significantly lower concentrations of artemisinin in the media than our pure artemisinin tests – only 32.3 μM even at the highest concentration tested, less than a third of the pure artemisinin tested. While this low concentration present in the tea media tests is greater than observed IC_{50} values in the literature, our cells seemed to display a decreased responsiveness to artemisinin even at the much higher value tested, meaning that the amount present may not have had any significant effect.

The potential difference in artemisinin concentration between the water tea and ethanol tea does not greatly affect our conclusions, however. Due to the large standard error for each experimental condition, neither water tea nor ethanol tea could be found to be more potent, nor could a specific tea concentration be determined to have had the greatest effect on proliferation. One possible source of the large variance in samples was that the cells were not nutrient starved before adding the experimental media to force them into the G_0 phase. If this was done for every experiment run, the cells may have grown at more similar rates from experiment to experiment. Going forward, this technique should be applied to produce more consistent results, and more trials should be conducted. Additionally, other forms of cancer should be studied to determine if the same effects can be demonstrated, and *in vivo* studies of cancer risk with tea consumption should also be conducted to verify that these results translate to disease outcomes.

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Appendix A

HPLC Chromatograms

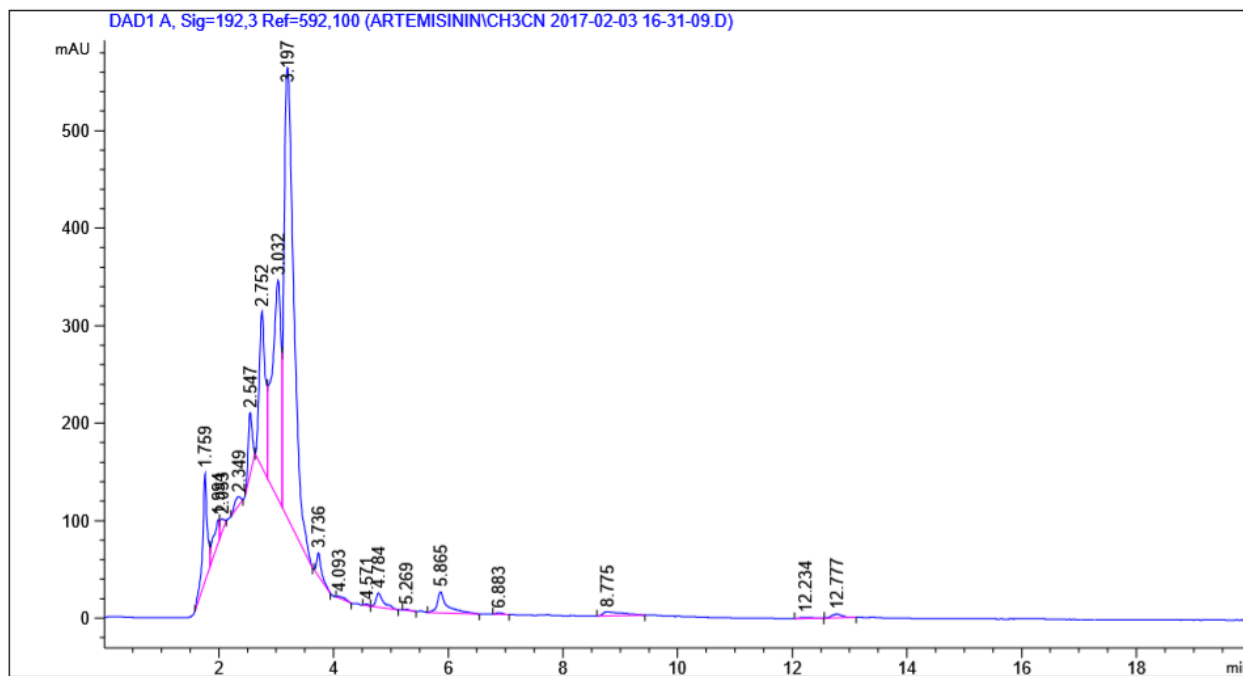


Figure 7: Chromatogram for the water tea, obtained as described in methods.

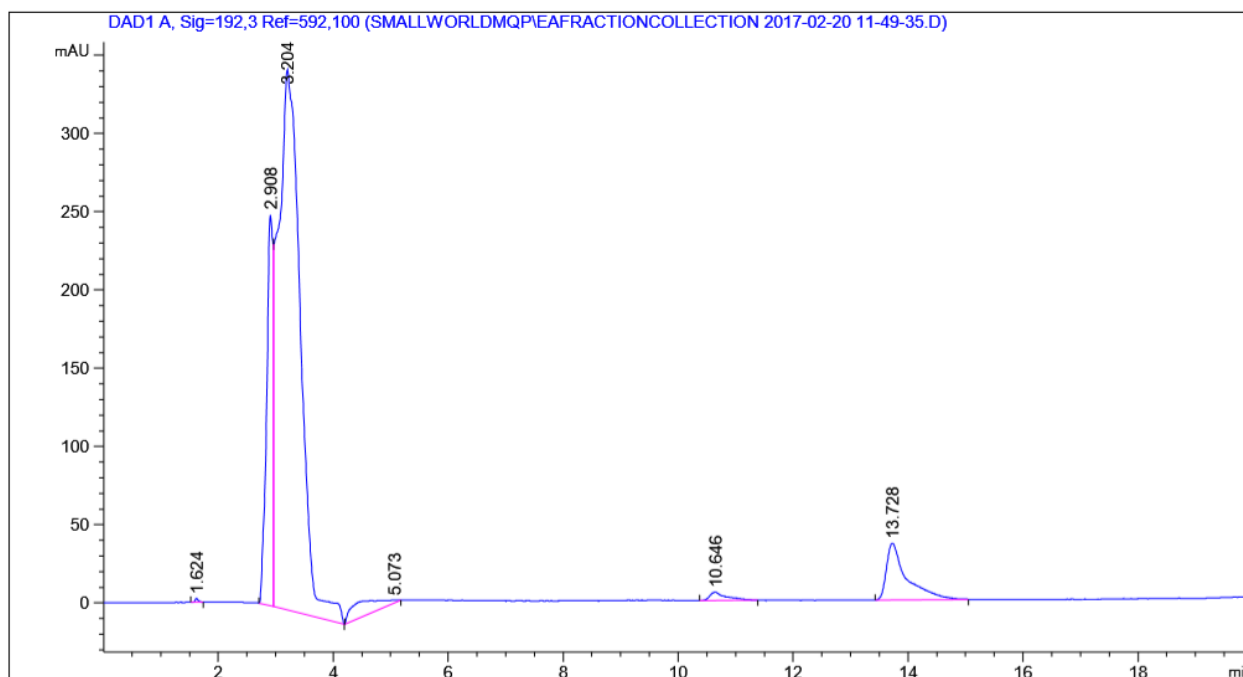


Figure 8: Chromatogram for pure artemisinin in ethanol at 1.5 mM, obtained as described in methods.

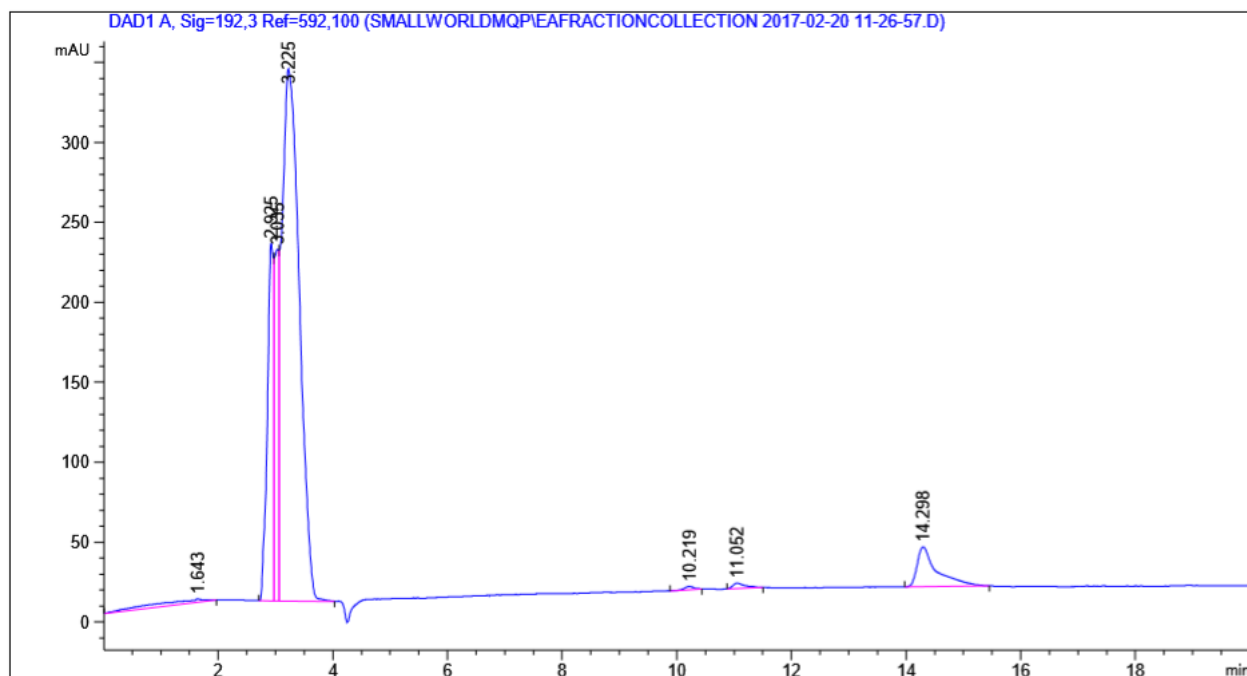


Figure 9: Chromatogram for pure artemisinin in ethanol at 1.25 mM, obtained as described in methods.

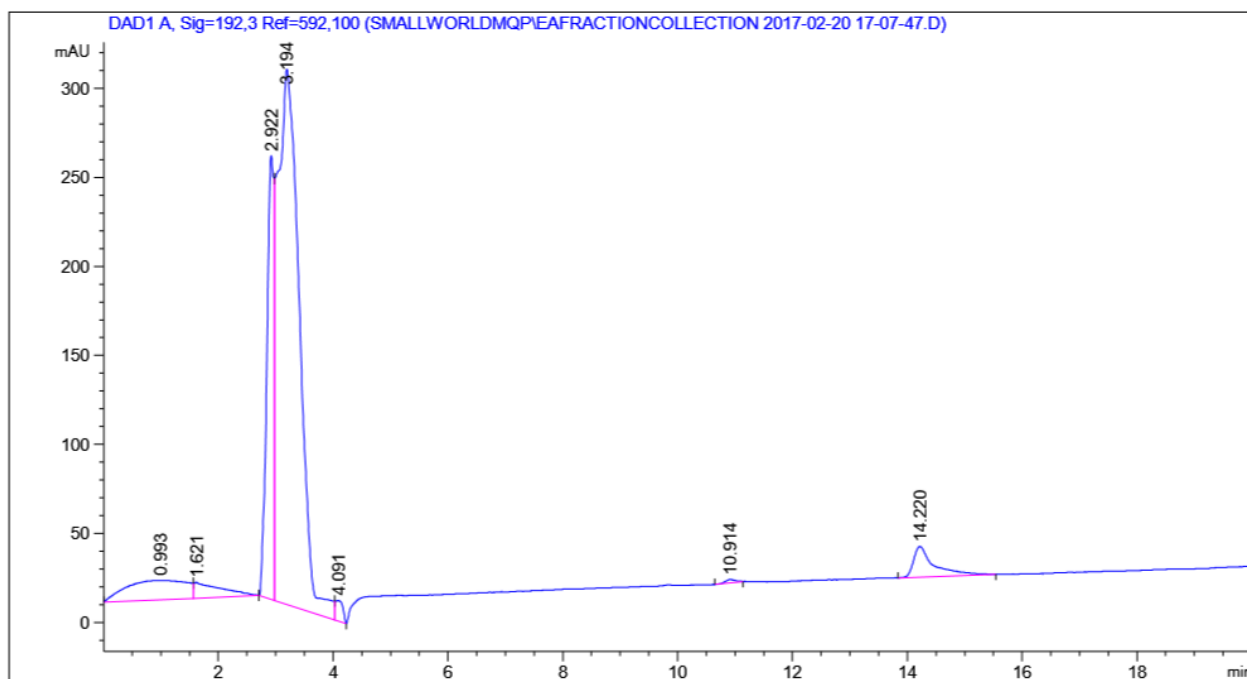


Figure 9: Chromatogram for pure artemisinin in ethanol at 1 mM, obtained as described in methods.

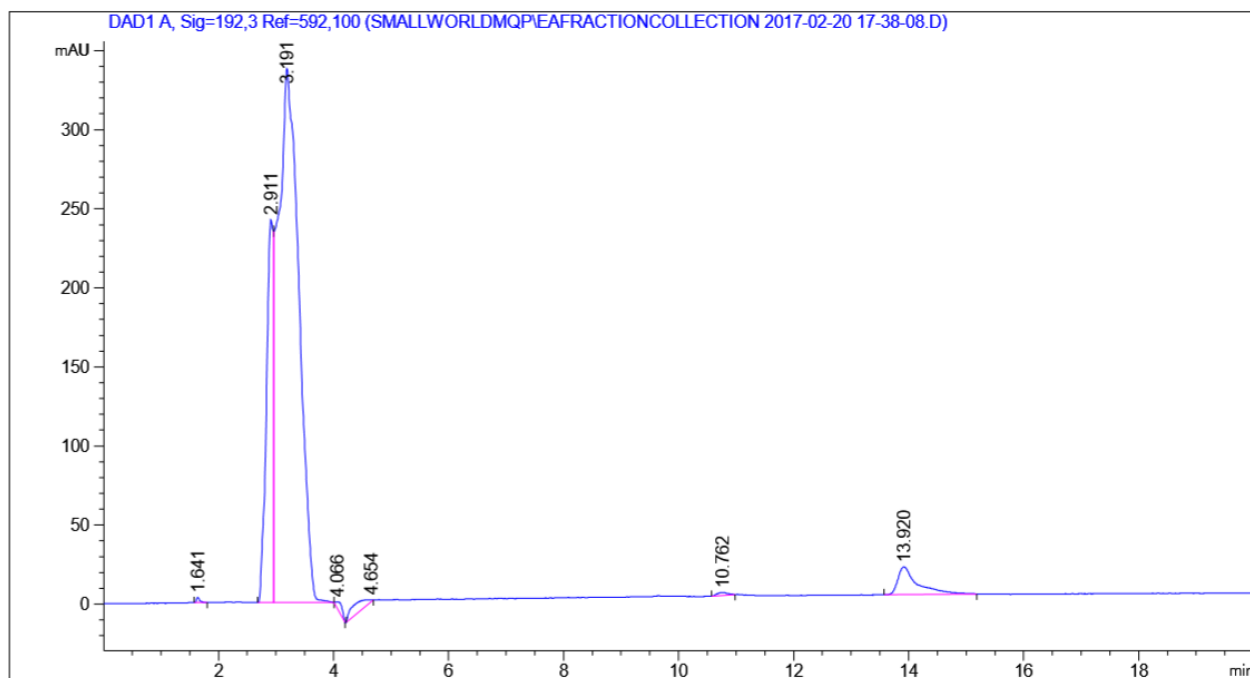


Figure 10: Chromatogram for pure artemisinin in ethanol at 0.75 mM, obtained as described in methods.