Drug-Herb Interactions between *Artemisia annua* Tea, Caffeine, and Acetaminophen

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Abstract

Artemisia annua is a medicinal herb that is used around the world as a treatment for malaria and other diseases. It is an inhibitor of the Cytochrome-P450 enzyme 3A4. This CYP is responsible for metabolizing about 50% of currently used clinical drugs. Because of the wide variety of substrates of CYP3A4, it is important to explore any interactions between substrates when consumed simultaneously to prevent liver strain and other adverse effects. Here, the interactions between acetaminophen and caffeine (two drugs that are very commonly consumed worldwide) and *A. annua* tea infusion are investigated using the P450-Glo Assay from Promega with human liver microsomes (HLMs). Two checkerboard assays using acetaminophen vs. *A. annua* tea and caffeine vs. *A. annua* tea, respectively, were completed to determine the interaction between the two drugs. Based on fractional inhibitory concentration (FIC) and kinetics analysis of these assays, both acetaminophen and caffeine were found to be antagonistic and competitive inhibitors with *A. annua* tea. This means that both drugs affect *A. annua*'s ability to inhibit CYP3A4. Based on this, it is safe to co-consume acetaminophen/caffeine at the recommended dosage with *A. annua* tea.

1.0 Background

1.1 Artemisia use as medication

Artemisia annua L., of the class Magnoliopsida and family Asteraceae, is an annual herb that grows in temperate climates. It is native to China and Vietnam but is also cultivated globally, including in East Africa, the United States, Russia, India, and Brazil (de Ridder et al., 2008). *A. annua* contains the sesquiterpene lactone, artemisinin, a highly effective antimalarial drug used especially in sub-Saharan Africa to treat malaria. The total amount of artemisinin in different varieties of *A. annua* ranges from 0.01 to 1.4% by weight depending on the extraction method, collection period, sample preparation, and environmental influences (de Ridder et al., 2008).

The first description of *A. annua* as a medicinal herb dates back to China in 168 BC. In the fourth century, the Chinese scholar Ge Hong first described a method of preparing *A. annua* in which the plant was soaked in water, wrung out, and the resultant juice ingested (de Ridder et al., 2008). According to the Chinese medical handbook *Classified Materia Medica* in 1596, tea-brewed leaves were used to treat fever, chills, lice, wounds, and "lingering heat in the joints and bones" (de Ridder et al., 2008). Later, the tea was also used to treat acute convulsions. In 1972, as a result of antimalarial research by Project 523 sponsored by the Chinese government to support the Vietnamese army during the Vietnam War, artemisinin was identified as the active antimalarial constituent of *A. annua* (de Ridder et al., 2008). Artemisinin is now globally used as artemisinin combination therapy (ACT) to treat malaria. Today, *A. annua* is used to treat a variety of diseases including drug-resistant *Plasmodium* strains, cerebral malaria, malaria in children, *Schistosoma* spp., *Pneumocystis carinii*, *Toxoplasma gondii*, and human cytomegaloviruses (de Ridder et al., 2008).

1.2 Cytochrome P450s

1.2.1 CYP3A4 & Cytochrome P450s

Cytochrome P450s (CYPs) are a family of 57 hemeprotein enzymes that are essential for drug and xenobiotic metabolism (McDonnell and Dang, 2013; Sevrioukova and Poulos, 2013). While all CYPs share a common protein structure, they have unique substrate selectivity (Sevrioukova and Poulos, 2013). CYPs function by catalyzing a variety of monooxygenation and detoxifying reactions through the oxidation of drug substances and the addition of molecular oxygen. This added oxygen not only makes the molecule more water-soluble and easier to flush out of the body but also creates a handle for additional detoxifying enzymes to bind to the toxin for further modification (Sevrioukova and Poulos, 2013).

CYP3A4 is a specific cytochrome P450 enzyme that is a part of the CYP3A subfamily (which also includes CYP3A4, 3A5, and 3A7). This family is the most common enzyme in the liver, of which 3A4 is the most abundant and it is responsible for the metabolism of about 50% of clinically used drugs (de Wildt et al., 1999; Agrawal et al., 2010). One of the notable features of this enzyme is that its chemical structure allows it to be highly flexible in terms of substrate specificity and it is this promiscuity that allows CYP3A4 to have the vast number of substrates that it does (Agrawal et al., 2010; Sevrioukova and Poulos, 2013).

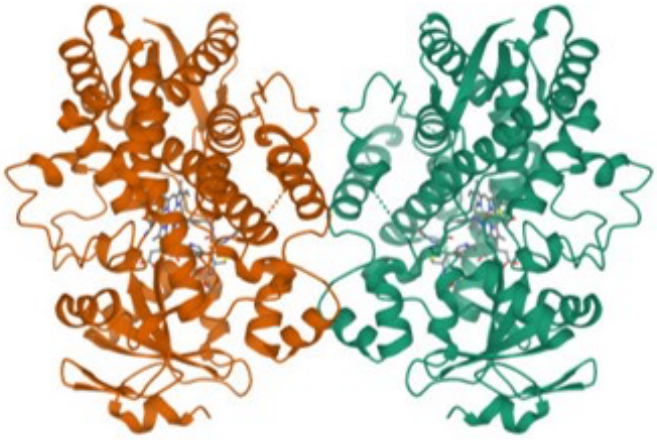

Figure 1: Protein 3-D structure of CYP3A4.
Image from the RCSB PDB (RCSB.org) of PDB ID 6UNE (Samuels and Sevrioukova, 2020).

Additionally, CYP3A4 is prone to cooperative substrate binding, which displays non-Michaelis-Menten kinetics (Sevrioukova and Poulos, 2013). This allosteric behavior is caused by the simultaneous bonding of more than one substrate molecule in or near the active site. These substrate and effector binding sites are usually adjacent to each other with a large cavity that is affected by the various interacting amino acid residues (Sevrioukova and Poulos, 2013). This

indicates that there are additional interactions, other than those at the active site, between CYP3A4 and substrates that impact function, which is vital for drug metabolism.

1.3 Enzyme Inhibition

1.3.1 Enzyme Inhibition

When an enzyme has more than one substrate, as is the case with CYP3A4, substrates can display several different inhibitory behaviors. These interactions impact the biochemical function and properties of the enzyme and the chemical reaction that is catalyzed by the enzyme.

1.3.2 Biochemical Kinetics & Constants

There are multiple biochemical constants used to describe the kinetics of a chemical reaction that is important to understand enzyme function. The first set of constants is those describing chemical reactions as a whole: K_m and V_{max}. V_{max} represents the maximum velocity of the reaction and K_m, known as the Michaelis constant, describes the concentration of substrate needed for the reaction to reach half maximum reaction velocity, V_{max} (Cheng and Prusoff, 1973).

The second set of biochemical constants is those that are specific to inhibitory reactions; including K_i and IC_{50}. K_i, known as the inhibition constant, is the dissociation constant of the enzyme-inhibitor complex and the IC_{50} value quantifies the concentration of inhibitor necessary to cause 50% inhibition of an enzyme (Cheng and Prusoff, 1973). The smaller the IC_{50} value, the less drug is necessary to inhibit the reaction by 50%, therefore indicating a more potent enzyme inhibitor.

1.3.3 Competitive Inhibition

In competitive inhibition, the binding of a competitive inhibitor and the binding of a substrate are mutually exclusive events as the two share the same active site (Strelow et al., 2012). Kinetically, competitive inhibition is characterized by an increase in K_m, but no change in V_{max}, which can result in an increase in the K_i value at higher concentrations of substrate. Physiologically, this behavior affects the efficacy of drugs by causing an increase in the local concentration of the substrate and the competitive inhibitor will lose potency (Strelow et al., 2012).

1.3.4 Noncompetitive Inhibition

Unlike competitive inhibition, noncompetitive inhibition occurs when the inhibitor binds to an allosteric site different from the active site. Kinetically, noncompetitive inhibition is characterized by a decrease in V_{max}, and no change in the K_m or K_i values (Strelow et al., 2012). Physiologically, this behavior is important in feedback inhibition and affects the efficacy of drugs because even though the substrate can still bind to the enzyme, the catalyzed metabolic

reaction will not occur, which increases the bioavailability of the drug (Delaune and Alsayouri, 2021).

1.3.5 Uncompetitive Inhibition

Uncompetitive inhibition occurs when the inhibitor binds to the enzyme-substrate complex and yields it inactive (Strelow et al., 2012). For this to occur, both the inhibitor and substrate must be bound to the enzyme. Kinetically, both the V_{max} and the K_m of the reaction decrease (Strelow et al., 2012). Physiologically, the substrate concentration is increased due to a lack of catalyzation which results in increased local drug concentration and potency (Heise et al., 2012).

1.3.6 Allosteric Inhibition

Allosteric inhibition occurs when an inhibitor binds to another site, an allosteric site, that is separate from the active site. This causes a conformational change of the active site that triggers inhibitory behavior by either affecting the shape of the active site, stabilizing the transition state, or reducing the ability to decrease catalysis activation energy (Strelow et al., 2012). This mechanism functions under the umbrella of noncompetitive and uncompetitive inhibition, which indicates that physiologically it will have the same effect of an increase in the local drug substrate concentration and a loss of therapeutic potency. (Wenthur et al., 2013).

1.3.7 Partial Inhibition

Partial inhibition is the result of an inhibitor binding to an enzyme-substrate complex that decreases the ease of function but does not completely inhibit the reaction. This means that the enzyme-substrate-inhibitor complex is unable to generate the product with less efficiency than the enzyme-substrate inhibitor alone. Unlike full inhibition which prevents all function, the active site retains some of its ability to bind to a substrate and catalyze a reaction (Strelow et al., 2012). Physiologically, any decrease in enzymatic activity will decrease metabolism and therefore require an increase in the target drug concentration. (Deodhar et al., 2020).

1.3.8 Michaelis-Menten and Lineweaver-Burk Plots

These kinetics can be examined using a variety of analysis methods. Michaelis-Menten and Lineweaver-Burk plots are two such methods to calculate biochemical constants and compare the kinetics of enzyme reactions with different concentrations of inhibitors. In a Michaelis-Menten plot, the concentration of substrate is plotted on the x-axis and the reaction velocity is plotted on the y-axis. V_{max} and K_m can be determined from this plot (Cheng and Prusoff, 1973). However, Lineweaver-Burk plots show them more clearly. The Lineweaver-Burk plot is a reciprocal plot: 1/velocity is plotted on the y-axis and 1/substrate concentration is plotted on the x-axis. In a Lineweaver-Burk plot, the y-intercept of the line is equal to $1/V_{max}$ and the x-intercept of the line is equal to $-1/K_m$.

Both Michaelis-Menten and Lineweaver-Burk plots are used to investigate enzyme inhibition. Comparing these plots using different concentrations of inhibitors shows the type of inhibition occurring. An example of the Michaelis-Menten plots of different types of inhibitors is shown in Figure 1.

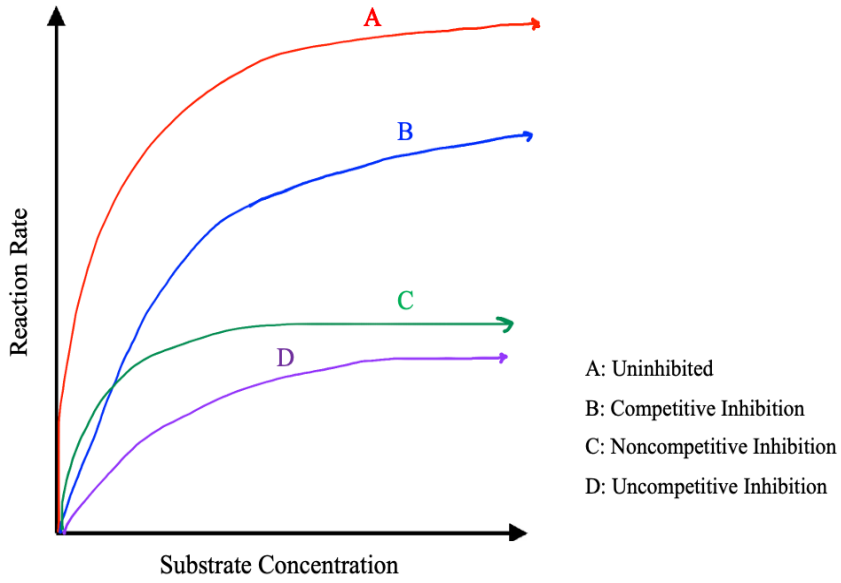

Figure 2: Graphical summary of effects of different types of enzyme inhibition.

In a Lineweaver-Burk plot, the type of inhibition is shown by the intersection of the lines and shifts of the x- and y- intercepts. The intersection of the lines at the y-axis (meaning a shift in the x-intercept, and therefore K_m) means the inhibitor participates in competitive inhibition. The intersection of the lines at the x-axis (meaning a shift in the y-intercept, and therefore V_{max}) means the inhibitor participates in noncompetitive inhibition. Parallel lines (meaning a shift in both the x- and y-intercepts and therefore both V_{max} and K_m) mean the inhibitor participates in uncompetitive inhibition.

1.4 Herb-Drug Interactions

Herb-drug interactions are an important safety issue that needs to be addressed as the co-consumption of clinical drugs with non-prescription herbal remedies. The World Health Organization reported that 80% of the world's population uses traditional medicine in some fashion (WHO, 2023). Looking specifically at the United States, a 2017 study reported that 35% of the US population used herbal medicine (Rashrash et al., 2017). The same study found that 38% of prescription medication users and 42% of OTC medication users reported herbal medicine use (Rashrash et al., 2017). Traditional remedy treatment continues to rise and therefore this investigation is important to examine the physiological impact of co-medication.

They are complicated interactions because there are multiple chemical components (phytochemicals) in herbal preparations and each phytochemical has unique pharmacological

activity (Singh & Zhao, 2017). There are two main classifications of interactions: pharmacokinetics and pharmacodynamics. Pharmacokinetic interactions occur when drugs interact at the macro-biological level of metabolism: absorption, metabolism, and excretion. On the other hand, pharmacodynamic interactions occur at the cellular level of receptor sites, where they can have additive or inhibitory effects (Richens, 1995). Aspects of each of these interaction classes were analyzed in this study. Combining herbal remedies like *A. annua* can increase or decrease the activity of conventional over-the-counter (OTC) drugs through antagonism or synergism or by impacting the metabolism of drugs, which can cause changes in functional drug concentrations within the body.

1.4.1 Fractional Inhibitory Concentration

The fractional inhibitory concentration (FIC) index range is used to analyze the combinatorial effect of two compounds. This index measures whether the interaction is synergistic, indifferent, or antagonistic (Scorzoni, et al., 2016). Antagonism means that the combination of the compounds lowers the overall activity of the compounds (Lorian, 2005). Synergy means that the combination of the compounds increases the overall inhibitory activity of the compounds alone and usually beyond an additive effect (Lorian, 2005). Indifference means that there is no increase or only a slight increase in inhibitory activity as compared to the compounds alone (Lorian, 2005). This number provides valuable insight into the relationship between the two compounds that might not necessarily be visible through just analyzing and graphing raw data.

1.4.2 *Artemisia*-Drug Interactions

While interactions with *A. annua* tea have not been widely investigated, artemisinin has been well-documented for its wide usage in ACTs for malaria treatment. In these ACTs, *Artemisia*-based substances like artemisinin, artesunate, artemether, and artemotil which are short-acting malarial agents are combined with long-acting agents like amodiaquine, mefloquine, or lumefantrine to eliminate the remaining infection (Maldonado & Grundmann, 2022).

When co-consumed with protease inhibitors and non-nucleoside reverse transcriptase inhibitors, two types of HIV antiviral drugs, artemisinin had negative interactions. Protease inhibitors inhibit CYP enzymes, which prevents the metabolism of ACT products. Oppositely, nonnucleoside inhibitors were found to induce CYP enzymes and drug transporters that metabolize ACTs, which increased artemisinin metabolism (Maldonado & Grundmann, 2022). These interactions are especially important in the clinical use of *A. annua* as the pharmacokinetic properties of the drugs that are administered can affect not only hepatic toxicity, but it can also impact drug efficacy. According to the University of Liverpool, although not distinctly studied, there are not expected to be any adverse effects of consuming ACTs and PrEP, which is a very important preventative HIV medication, because they are metabolized via two separate metabolism pathways. On the other hand, longer-term medications like maraviroc are metabolized via the CYP pathway and caution should be taken when consuming both because

while coadministration has not been studied here either, they share similar metabolic CYP pathways are shared and could minimize the efficacy of the HIV medication (University of Liverpool, 2023).

1.5 *Artemisia annua*

The primary active ingredient in *A. annua* L. is artemisinin. Artemisinin is a sesquiterpene lactone with an internal peroxide bridge, which can be seen in Figure 2 (Svensson and Ashton, 1999). With artemisinin, there are more than 600 identified secondary metabolites including additional sesquiterpenes, monoterpenes, and phenolic compounds (Abate et al., 2021). In malaria-endemic developing countries, *A. annua* is typically ingested through tea made from the dried leaves of *A. annua* (DLA, dried leaf *Artemisia*), which means that both artemisinin and all additional secondary metabolites are ingested *in toto* thereby aiding in improving artemisinin bioavailability and its downstream efficacy (Desrosiers et al., 2020).

Part of this enhanced bioavailability of artemisinin is due to its increased solubility and intestinal transport afforded by the essential oils in the plant. Some of these phytochemicals are hepatic inhibitors that inhibit liver P450s resulting in increased bioavailability of artemisinin. Those phytochemicals inhibit the ability of the P450s from metabolizing artemisinin to its therapeutically inactive metabolites, allowing it to pass through the liver and distribute to tissues and organs thereby increasing biological responses as compared to pure artemisinin (Desrosiers et al., 2020).

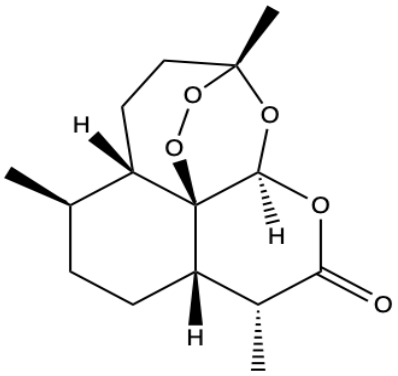

Figure 3: Chemical structure of artemisinin, the antimalarial compound in *Artemisia annua.*

1.5.1 *Artemisia annua* and Artemisinin Metabolism

The following described metabolic pathway is based on the metabolism of artemisinin by CYP3A4. Artemisinin is primarily metabolized by hepatic CYP2B6 with a secondary contribution of CYP3A4 in the liver (Gordi et al., 2005). As seen in Figure 3, when artemisinin is metabolized by CYP3A4 it results in a variety of products including deoxyartemisinin, deoxydihydroartemisinin, 9,10-dihydrodeoxyartemisinin, and crystal-7 (Lee and Hufford, 1990).

These metabolites are inactive as they lack the endoperoxide moiety that is mainly responsible for the antimalarial activity of the molecule (Lee and Hufford, 1990).

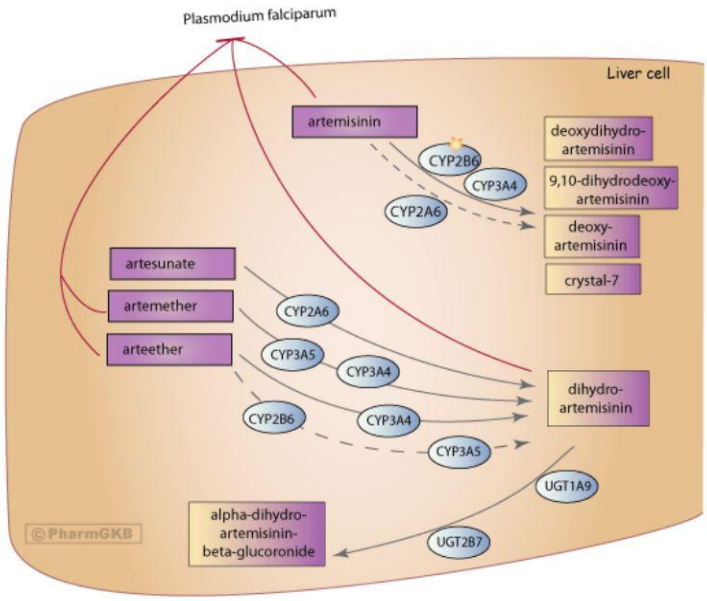

Figure 4: Metabolism of artemisinin in hepatocytes.
(Source: https://www.pharmgkb.org/pathway/PA165378192)

1.6 Drug Selection for Study

Drugs were selected for study based on popularity and the probability of interaction with compounds in *A. annua*. To be considered for use, the drug must be an inhibitor or substrate of CYP3A4. All commercially available CYP3A4 inhibitors were narrowed to only over-the-counter drugs, and then this list was further narrowed to drugs that were the most soluble in water. Over-the-counter drugs are both more popular and more accessible. Drugs that are soluble in water are preferred because they would dissolve in the *A. annua* tea infusion, which results in higher artemisinin content available for use in the assay. The two most popular global drugs that were chosen for study were caffeine and acetaminophen.

1.7 Caffeine

Caffeine is the most widely used psychoactive stimulant in the world. In the United States alone about 85% of the population consumes at least one caffeinated beverage (tea, coffee, soda, etc.) per day (Temple et al., 2022; Mitchell et al., 2014). The structure of caffeine, as seen in Figure 4 is a methylxanthine alkaloid that consists of a purine double-ring alkene structure with two amine groups, and two amide groups (https://ncithesaurus.nci.nih.gov/ncitbrowser/ConceptReport.jsp?dictionary=NCI_Thesaurus&ns=ncit&code=C328). In terms of dosage, the maximum recommended dose is 400 mg/day which is the equivalent of 4 to 5 cups of coffee in 8 hours, with the warning of not consuming more than 4,000 mg/day. The peak plasma concentration after a 500 mg dose is 17.3 µg/mL which is the equivalent of 89.1 µM caffeine (Willson, 2018).

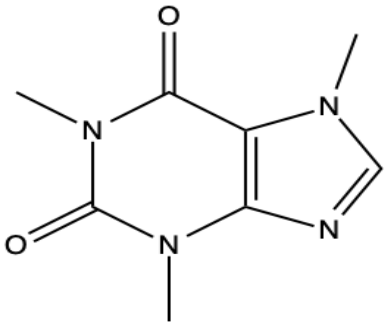

Figure 5: Chemical structure of caffeine.

1.7.1 Caffeine Metabolism

Caffeine is almost completely metabolized with only 3% or less being excreted unchanged (Thorn et al., 2012). The primary hepatic metabolic route of caffeine in humans, which accounts for 70-80% of caffeine metabolism, is through N-3-demethylation to paraxanthine (1,7-dimethylxanthine/17X) (Thorn et al., 2012). CYP1A2 carries out 95% of the metabolism of caffeine but is aided by CYP2C8, CYP2C9, and CYP3A4. As seen in Figure 5, CYP3A4 assists in the metabolism of caffeine in a variety of steps into secondary metabolic products including 1,3,7-trimethyl-uric acid and theophylline.

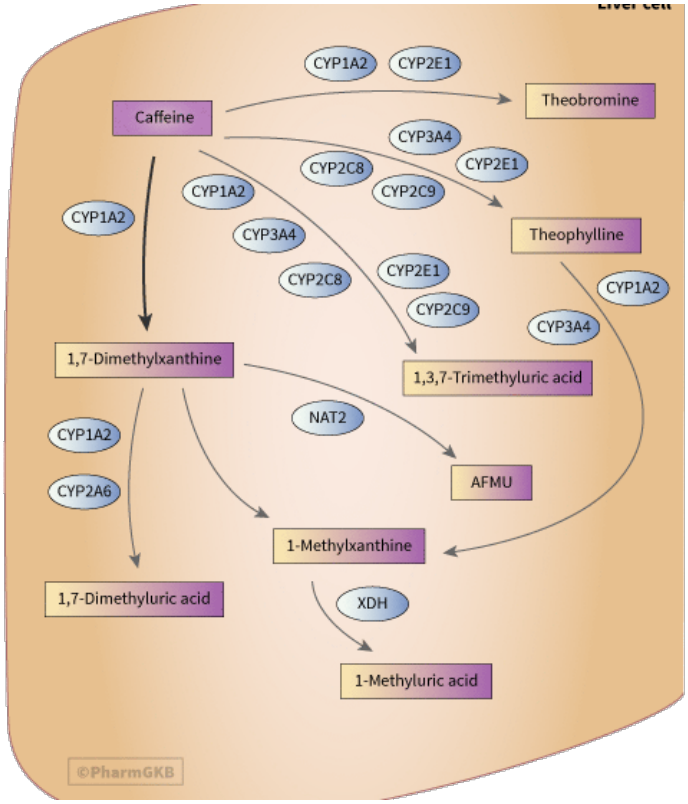

Figure 6:: Metabolism of caffeine within hepatocytes.
(Source: https://www.pharmgkb.org/pathway/PA165884757)

1.8 Acetaminophen

Acetaminophen (APAP) is one of the most widely used drugs in the world. In the United States alone 24.6 billion doses were sold in 2008 (McGill and Jaeschke, 2013). However, acetaminophen hepatotoxicity is the number one cause of acute liver failure in the United States, causing 50,000 - 80,000 emergency room visits annually (McGill and Jaeschke, 2013). This widespread use and the tendency towards hepatotoxicity are two of the reasons why acetaminophen and *A. annua* interactions were examined; co-consumption could have drastic impacts on liver enzyme function.

The structure of acetaminophen, as seen in Figure 6, contains a benzene ring with an amide group and a hydroxyl group positioned at opposite carbons on the ring. In terms of dosage, the maximum recommended dose is 1,000 mg every 8 hours, with the warning of not consuming more than 4,000 mg per day. The peak plasma concentration after a 1,000 mg dose (2 “extra strength” acetaminophen pills) is 12.3 μg/mL which is the equivalent of 81.37 μM APAP (Ohashi & Kohno, 2020).

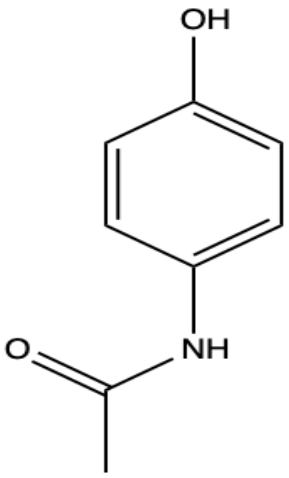

Figure 7: Chemical structure of acetaminophen.

1.8.1 Acetaminophen Metabolism

The metabolic pathway being described is for the hepatic metabolism of a therapeutic dosage of acetaminophen. Metabolism occurs primarily in the liver, with minor contributions by the kidney and intestine (Mazaleuskaya et al., 2015). Hepatically, APAP is primarily converted to pharmacologically inactive glucuronide (52-57%) and sulfate (30-44%), which are transported to the kidneys, and the remaining 5-10% is oxidized to a reactive NAPQI metabolite, which is the main compound of cytotoxic concern and involves CYP3A4 (Mazaleuskaya et al., 2015). The entire hepatic metabolic pathway can be seen in Figure 7.

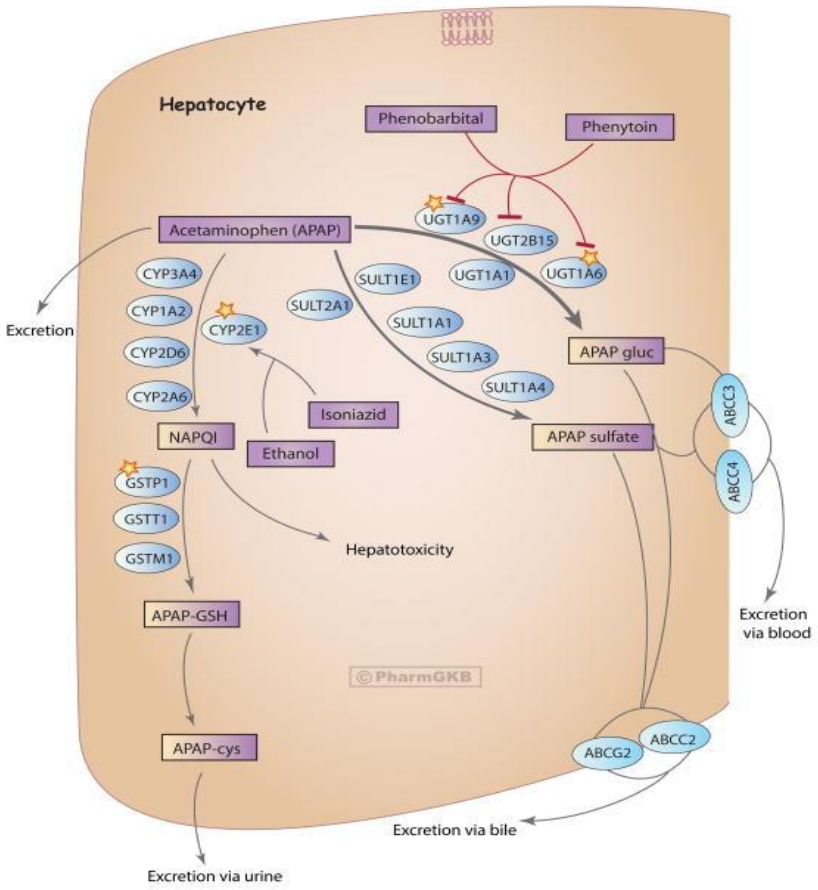

Figure 8: Metabolic pathway of acetaminophen within hepatocytes. (Source: https://www.pharmgkb.org/pathway/PA165986279)

1.9 Significance

According to the World Health Organization, 88% of all countries are reported to use traditional medicinal practices, with 80% of the world's population estimated to use herbal medicines (WHO, 2023). These are widely used among developing nations around the globe, but there is also an increased number of people using herbal medicines in other developed countries. Looking at the United States alone, 35% of the population reported using herbal medicine and specifically 64% of these users reported simultaneously using OTC medications (Rashrash et al., 2017). Specifically, *A. annua* has been used for thousands of years across Southeast Asia and China and more recently also across Africa as an antimalarial treatment. Besides being a malaria treatment, *A. annua* has also been found to be a promising therapeutic for SARS-CoV-2, tuberculosis, Lyme disease, and schistosomiasis (Weathers, 2022). With such a wide variety of potential uses, it raises the question asking can people using *A. annua* to treat these conditions also consume common OTC drugs (especially those with millions of users such as acetaminophen and caffeine) to alleviate symptoms.

2.0 Hypothesis and Objectives

2.1 Hypothesis

A. annua tea and caffeine are both metabolized via metabolic pathways that contain the enzyme CYP3A4. If a tea infusion of *A. annua* is combined with caffeine, then the overall CYP3A4 activity will be inhibited to a greater extent than either substance alone because of competitive inhibition at the active site.

If a tea infusion of *A. annua* is combined with acetaminophen, then the overall CYP3A4 activity will be inhibited the same amount as if only the tea was present. This is expected because acetaminophen is a substrate of CYP3A4 and it is only allosterically inhibitory at high concentrations.

2.2 Objectives

1. Determined how *A. annua* (DLA) combinations affect CYP3A4 metabolism of acetaminophen.
    a. Using liver microsomes and the Promega P450-Glo Assay specific to CYP3A4 compare luminescence of different ratios of *A. annua* to over-the-counter (OTC) medication levels to measure CYP3A4 activity.
    b. Determine what type of inhibition is occurring.
    c. Use statistical analysis to determine the overall CYP3A4 activity and observe the impacts of co-medication.
2. Determined how *A. annua* (DLA) combinations affect CYP3A4 metabolism of caffeine using the Promega P450-Glo Assay
    a. Using liver microsomes and the Promega P450-Glo Assay specific to CYP3A4 compare the luminescence of different ratios of *A. annua* to OTC medication to measure CYP3A4 activity.
    b. Determine what type of inhibition is occurring.
    c. Use statistical analysis to determine the overall CYP3A4 activity and observe the impacts of co-medication.
3. Analyzed the type of interactive inhibitory relationship that occurs between the combinations of acetaminophen and *A. annua* tea and caffeine and *A. annua* tea.
    a. Created Michaelis-Menten and Lineweaver-Burke plots to determine the type of inhibition caused by these combinations.
    b. Calculated an FIC index that describes the relationships of co-consumption of the tea and each of the two substrates.

3.0 Materials and Methods

The activity of CYP3A4 was measured via luminescence using the Promega P450-Glo Assay specific to CYP3A4 (PRV9910) with human liver microsomes (HLMs from a 200-donor pool of males and females (Sekisui XenoTech, Kansas City, KS, USA)) (Desrosiers et al., 2020).

3.1 Plant Material

A. annua L. cv SAM (voucher MASS 317314) tea infusion - 10 g DW/L boiled 10 min, sterile filtered, frozen (-20°C) - Batch #1.Sh6.01.15.20 - prepared 14Jan2021 - 581.69 µM artemisinin (artemisinin content determined via GC-MS (Weathers and Towler, 2012))

3.2 Chemicals & Reagents

Acetaminophen (as 4-Acetamidophenol, 98%; 102330050) was from Thermo Fisher Scientific (Waltham, MA, USA) and Caffeine (C-0750) was from Sigma Aldrich (St. Louis, MO, USA). Luciferin-IPA, NADPH, and Luciferin Detection Reagent were included in the P450-Glo Assay Kit (V9002), which was purchased from Promega (Madison, Wisconsin, USA).

3.3 Determination of IC_{50} for *A. annua* tea

To determine the CYP3A4 IC_{50} concentration for *A. annua* DLA tea infusion, the Promega P450-Glo assay was run using a concentration series shown in Table 1 and ranging from 0.57-581.69 µM ART. The test solutions were created by serial diluting *A. annua* tea with DI water. This experiment was completed once in technical triplicate according to the SOP detailed in Appendix A.

3.4 P450-Glo Assay Method Development - 100 µL vs. 50 µL Total Volume

The method of Desrosiers et al. (2020) was modified in this study for the P450-Glo assay to maximize the number of assays that could be performed per Promega P450-Glo Assay kit (V9002). The previous SOP (Appendix A) used a total well volume of 100 µL. The new SOP (Appendix B) used a total well volume of 50 µL.

Because CYP3A4 activity in the P450-Glo assay is quantified using luminescence, testing was necessary to determine a) whether the luminescence of a 50 µL well was high enough to be read accurately by the plate reader and b) whether the luminescence of materials decreases proportionally to decreases in luminescent material. The luminescence of wells containing 100 µL and 50 µL of luminol was measured using the protocol "Matt Luminescence" on the PerkinElmer Victor3 Multilabel Plate Reader and which was developed by Desrosiers et al. (previously 2020). Three 100 µL wells and three 50 µL wells were read, and the percent difference in luminescence was calculated between the 100 µL and 50 µL wells. This experiment was repeated three times.

Table 1: Concentrations of A. annua tea used in IC50 testing.

Dilution Factor	Concentration of ART in tea (μM)
N/A	0
1:1024	0.57
1:512	1.14
1:256	2.27
1:128	4.54
1:64	9.09
1:32	18.17
1:16	36.36
1:8	72.71
1:4	145.42
1:2	290.85
1	581.69

3.5 *A. annua* - Caffeine Drug-Herb Interactions Checkerboard Assay

To determine interactions between *A. annua* tea and caffeine, a checkerboard assay using Promega P450-Glo kits was performed. Seven concentrations of caffeine were tested against seven concentrations of *A. annua* tea. The concentration range for caffeine was 5.57-178 μM and was chosen to bracket the peak plasma concentration: 500 mg (or 89.1 μM), equivalent to 4-5 cups of coffee (Willson, 2018). The concentration range for *Artemisia* DLA tea was 3.47-111 μM of ART in the tea and was chosen based on the previously completed IC_{50} determination assay.

The test solutions were created using a highly concentrated caffeine solution and diluting with tea in different ratios. First, a caffeine/tea stock solution was made: 2 μL of 20.76 mg/mL solution of caffeine in DI water was added to 298 μL of *A. annua* tea. Then, 2 μL of this caffeine/tea stock solution was added to various ratios of tea and water to create the test solutions each with 178 μM caffeine and a total volume of 100 μL. These test solutions were serially diluted using the same ratios of tea to water to vary the concentration of caffeine. The test solutions with 0 μM ART (no *A. annua* tea) were made by adding 2 μL of 20.76 mg/mL solution of caffeine in DI water to 298 μL of DI water. A 2 μL aliquot of the resulting solution was added

to 98 µL of DI water, and this solution was then serially diluted to the desired concentration of caffeine. The test solutions with 0 µM caffeine were created by serially diluting the *A. annua* tea stock. The concentrations of these test solutions are summarized in Table 2.

Table 2: Test Solution Ratios of A. annua tea (as µM ART) and caffeine (µM). These solutions were serially diluted (keeping the ratio of tea and water constant) to vary the concentration of caffeine.

Concentration Caffeine (µM)	Concentration ART (µM)	Percent *A. annua* tea (%)	Percent water (%)	Volume *A. annua* tea (µL)	Volume DI Water (µL)	Volume CAF/tea stock
178	3.47	2.4	97.6	2.35	96	2
178	6.95	4.8	95.2	4.7	93	2
178	13.9	9.5	90.5	9.3	89	2
178	27.8	19	81	18.6	79	2
178	55.5	38	62	37	61	2
178	111	76	24	74	24	2

This experiment was repeated twice. The first assay was run according to the SOP in Appendix A, and the second assay was run according to the SOP in Appendix B. The plate was set up according to Table 3.

3.6 *A. annua* - Acetaminophen Drug-Herb Interactions

As with the *A. annua* and Caffeine study, the Promega P450-Glo kit specific to 3A4 was used to determine interactions between *A. annua* DLA and acetaminophen (APAP). Six concentrations of APAP were tested against six concentrations of *A. annua* tea. The concentration range for acetaminophen was 0 - 325 µM and chosen to bracket the peak plasma concentration after the maximum recommended single therapeutic dose (1,000 mg): 123 mg/mL or 81.4 µM (Ohashi and Kohno, 2020). The concentration range for tea was chosen based on 0 - 111 µM of ART in the tea from the previously completed IC_{50} determination assay.

The test solutions were prepared using a highly concentrated APAP solution and diluted with tea in different ratios. First, an APAP/tea stock solution was made: 2 µL of 12.3 mg/mL solution of APAP in DI water was added to 98 µL of *A. annua* DLA tea. Then, 2 µL of this APAP/tea stock solution was added to various ratios of tea and water to create the test solutions each with 325 µM APAP and a total volume of 140 µL. These test solutions were serially diluted using the same ratios of tea to water and added to vary the concentration of APAP. The test solutions with 0 µM ART (no *A. annua* tea) were made by adding 2 µL of 12.3 mg/mL solution of APAP in DI

water to 98 µL of DI water. A 2 µL aliquot of the resulting solution was added to 138 µL of DI water, and this solution was then serially diluted to the desired concentration of APAP. The test solutions with 0 µM APAP were created by serially diluting the *Artemisia* DLA tea. The concentrations of these test solutions are summarized in Table 4.

Table 3: *A. annua* & caffeine checkerboard set-up. *A. annua* tea (ART, top values): Caffeine (CAF, bottom values) Concentration Ratios (concentrations are in µM ART and µM caffeine). "NIC" = no inhibitor control, "NMC" = no microsome control, and "Keto" = ketoconazole, a positive inhibitor control.

		Increasing TEA INFUSION concentration											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Increasing CAFFEINE Conc	**A**		3.47µM ART 0µM CAF	6.95µM ART 0µM CAF	13.9µM ART 0µM CAF	27.8µM ART 0µM CAF	55.5µM ART 0µM CAF	111µM ART 0µM CAF			NIC	NIC	NIC
	B	0µM ART 5.57µM CAF	3.47µM ART 5.57µM CAF	6.95µM ART 5.57µM CAF	13.9µM ART 5.57µM CAF	27.8µM ART 5.57µM CAF	55.5µM ART 5.57µM CAF	111µM ART 5.57µM CAF			NMC	NMC	NMC
	C	0µM ART 11.1 µM CAF	3.47µM ART 11.1 µM CAF	6.95µM ART 11.1 µM CAF	13.9µM ART 11.1 µM CAF	27.8µM ART 11.1 µM CAF	55.5µM ART 11.1 µM CAF	111µM ART 11.1 µM CAF			Keto	Keto	Keto
	D	0µM ART 22.3µM CAF	3.47µM ART 22.3µM CAF	6.95µM ART 22.3µM CAF	13.9µM ART 22.3µM CAF	27.8µM ART 22.3µM CAF	55.5µM ART 22.3µM CAF	111µM ART 22.3µM CAF					
	E	0µM ART 44.6µM CAF	3.47µM ART 44.6µM CAF	6.95µM ART 44.6µM CAF	13.9µM ART 44.6µM CAF	27.8µM ART 44.6µM CAF	55.5µM ART 44.6µM CAF	111µM ART 44.6µM CAF					
	F	0µM ART 89.1µM CAF	3.47µM ART 89.1µM CAF	6.95µM ART 89.1µM CAF	13.9µM ART 89.1µM CAF	27.8µM ART 89.1µM CAF	55.5µM ART 89.1µM CAF	111µM ART 89.1µM CAF					
	G	0µM ART 178µM CAF	3.47µM ART 178µM CAF	6.95µM ART 178µM CAF	13.9µM ART 178µM CAF	27.8µM ART 178µM CAF	55.5µM ART 178µM CAF	111µM ART 178µM CAF					
	H												

Table 4: Test Solution Ratios of A. annua tea (µM ART) and APAP (µM). These solutions were serially diluted (keeping the ratio of tea and water constant) to vary the concentration of APAP.

Concentration APAP (µM)	Concentration ART (µM)	Percent *A. annua* tea (%)	Percent water (%)	Volume *A. annua* tea (µL)	Volume DI Water (µL)	Volume APAP/tea stock
325	6.95	4.8	95.2	4.7	133.3	2
325	13.9	9.5	90.5	11.3	126.7	2
325	27.8	19	81	24.6	113.4	2
325	55.5	38	62	51.2	86.8	2
325	111	76	24	104.4	33.6	2

This experiment was repeated twice. The first assay was run according to the SOP in Appendix A, and the second assay was run according to the SOP in Appendix B. The plate was set up according to Table 5.

3.7 Data Analysis

3.7.1 IC_{50} Calculations

The average of the NMC (no microsome control) wells was subtracted from the raw values of the test wells. The difference between the average of the NIC (no inhibitor control) wells and the average of the NMC wells is the CYP inhibitor's comparator. The data were expressed as "fraction NIC" (experimental value/NIC value) and plotted as "fraction NIC" vs log concentration.

The resulting data were analyzed using a nonlinear regression analysis by GraphPad Prism 7 (San Diego, CA, USA) to provide an IC_{50} value that was used for further analysis.

3.7.2 Fractional Inhibitory Concentration (FIC) Index Calculations

The fractional inhibitory concentration (FIC) Index is a method of quantifying the interactions between two test drugs in a checkerboard assay (Lorian, 2005). It is a measure of how much a combination of drugs differs from each drug's minimum inhibitory concentration (MIC) (Lorian, 2005). The minimum inhibitory concentration (MIC) is the minimum concentration of each drug that results in a change in luminescence from the no inhibitor control (NIC) wells.

The FIC Index for each well of the plate was calculated using this equation:

$$\frac{A}{MIC_A} + \frac{B}{MIC_B} = FIC_A + FIC_B = FIC\ Index$$

Table 5: *A. annua* & acetaminophen checkerboard set-up. A. annua tea (ART, top values): Acetaminophen (APAP, bottom values) Concentration Ratios (concentrations are in µM ART and µM caffeine). "NIC" = no inhibitor control, "NMC" = no microsome control, and "Keto" = ketoconazole, a positive inhibitor control

		Increasing TEA INFUSION concentration											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Incr APAP Conc	**A**		6.95µM ART 0µM APAP	13.9µM ART 0µM APAP	27.8µM ART 0µM APAP	55.5µM ART 0µM APAP	111µM ART 0µM APAP				NIC	NIC	NIC
	B	0µM ART 20.3µM APAP	6.95µM ART 20.3µM APAP	13.9µM ART 20.3µM APAP	27.8µM ART 20.3µM APAP	55.5µM ART 20.3µM APAP	111µM ART 20.3µM APAP				NMC	NMC	NMC
	C	0µM ART 40.7 µM APAP	6.95µM ART 40.7 µM APAP	13.9µM ART 40.7 µM APAP	27.8µM ART 40.7 µM APAP	55.5µM ART 40.7 µM APAP	111µM ART 40.7 µM APAP				Keto	Keto	Keto
	D	0µM ART 81.4µM APAP	6.95µM ART 81.4µM APAP	13.9µM ART 81.4µM APAP	27.8µM ART 81.4µM APAP	55.5µM ART 81.4µM APAP	111µM ART 81.4µM APAP						
	E	0µM ART 163µM APAP	6.95µM ART 163µM APAP	13.9µM ART 163µM APAP	27.8µM ART 163µM APAP	55.5µM ART 163µM APAP	111µM ART 163µM APAP						
	F	0µM ART 325µM APAP	6.95µM ART 325µM APAP	13.9µM ART 325µM APAP	27.8µM ART 325µM APAP	55.5µM ART 325µM APAP	111µM ART 325µM APAP						
	G												
	H												

For this experiment, A is equal to the luminescence of the tea, and B is equal to the luminescence of the OTC drug (either acetaminophen or caffeine, respectively) in the well. MIC_A is the MIC of *A. annua* tea, and MIC_B is the MIC of acetaminophen or caffeine, respectively. The MIC of *A. annua* tea is 0.57 µM ART, the MIC of acetaminophen is 325 µM, and the MIC of caffeine is 5.57 µM. An FIC Index less than 0.5 means the drugs are synergistic; in other words, the drugs in combination are more inhibitory than either drug alone (Lorian, 2005). An FIC Index greater than 4 means the drugs are antagonistic; one drug impairs the ability of the other to inhibit, resulting in overall less inhibition from the combination of drugs than from either drug alone (Lorian, 2005). An FIC Index between 0.5 and 4 means the drugs are additive or there is no interaction between them: there is slight or no difference in the inhibitory effects of the drugs in combination in comparison to either drug alone (Lorian, 2005).

Data from the checkerboard assays were used to create Michaelis-Menten and Lineweaver-Burk analyses for each OTC drug vs. tea. In the Michaelis-Menten plots for this experiment, the y-axis is the velocity in luminescence units per minute and the x-axis is the concentration ART (in μM). The velocity of the reaction was calculated by dividing the luminescence of the well by the total reaction time (20 minutes). Each series represents different concentrations of inhibitor (acetaminophen or caffeine, respectively). Nonlinear regression analysis by GraphPad Prism 7 (San Diego, CA, USA) was used to create the Michaelis-Menten and Lineweaver-Burk plots, as well as to determine K_m and V_{max}.

4.0 Results

The goal of the two checkerboard assays was to analyze the interactions between acetaminophen/caffeine and *A. annua* DLA. The FIC index was used to determine the type of interaction between the two drug compounds. The kinetics of the assay was examined using Michaelis-Menten and Lineweaver-Burk plots to determine the inhibitory effects, if any, of the test drug on *A. annua* tea.

4.1 IC_{50} Determination for *A. annua* tea

The IC_{50} for *A. annua* tea was determined by plotting the "fraction NIC" calculated from the P450-Glo Assay versus the log concentration of ART (μM). The "fraction NIC" was calculated by dividing the luminescence value from each well by the average of the luminescence of the NIC (no inhibitor control) well. A nonlinear regression analysis of this plot by GraphPad Prism 7 (San Diego, CA, USA) was used to determine the IC_{50} of the *A. annua* tea. The plot is shown in Figure 8 below.

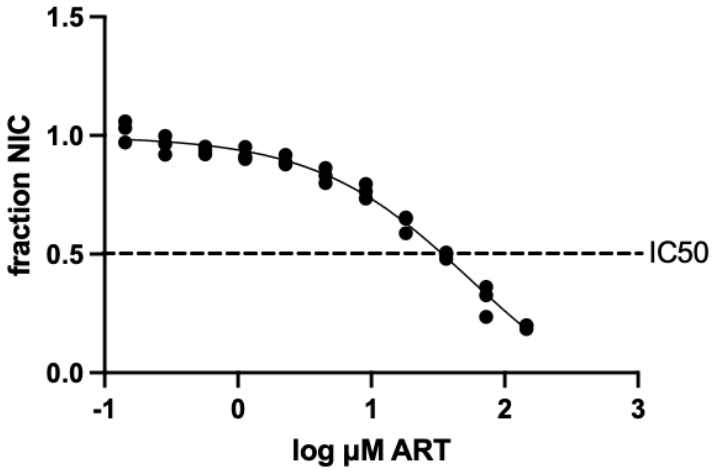

Figure 9: Fraction NIC vs. log concentration ART (μM) for A. annua tea. Fraction NIC was calculated to normalize the luminescence data to the no-inhibitor controls. It is calculated by subtracting the average of the no microsome control (NMC) wells from all luminescence data and then dividing by the average of the no-inhibitor control (NIC) wells. The result is plotted against log concentration to yield an IC50 value.

The maximum concentration of artemisinin in the tea limited analysis of activity at higher concentrations, nevertheless it was still possible to calculate an IC_{50} for the tea based on its artemisinin content. The IC_{50} of *A. annua* tea was determined to be 55.96 µM ART. This value was used to establish the concentration range of ART within *A. annua* tea to use in the checkerboard assays.

4.2 Acetaminophen Fractional Inhibitory Concentration (FIC) Index

For this experiment, A is equal to the luminescence of the tea in the well, B is equal to the luminescence of acetaminophen in the well, MIC_A is the MIC of *A. annua* tea and MIC_B is the MIC of acetaminophen. The MIC of *A. annua* tea was 0.57 µM ART, and the MIC of acetaminophen was 325 µM. A representation of the FIC Indexes for each well of the acetaminophen checkerboard assay is shown in Table 6. These calculations use the luminescence data from the second checkerboard assay (conducted at half volume).

Table 6: FIC Index values for Acetaminophen Checkerboard Assay. Wells that showed synergy are shown in yellow, wells that showed additive/no interaction are shown in blue, and wells that showed antagonism are shown in orange.

		Increasing ART Conc (µM)				
		6.85	13.7	27.4	54.7	109
Increasing APAP Conc. (µM)	20.3	9.86	17.7	21.1	26.3	31.3
	40.7	10.5	13.4	19.7	25.8	29.9
	81.4	11.3	14.4	21.1	27.2	30.7
	163	10.8	21.5	22.3	27.5	31.1
	325	12.3	17.9	21.3	25.4	31.0

The FIC Index table shows that acetaminophen and *A. annua* tea have an antagonistic interaction regardless of the concentration of either drug. This means that acetaminophen decreases the ability of *A. annua* tea to bind CYP3A4.

4.2.1 Acetaminophen Kinetics Analysis

The kinetics of the acetaminophen checkerboard assays were investigated using Michaelis-Menten and Lineweaver-Burk plots. The Michaelis-Menten plot was created by plotting the velocity (in luminescence units/minute) of each concentration of acetaminophen (APAP) against varying concentrations of ART (µM) within *A. annua* tea. A nonlinear regression analysis by

GraphPad Prism 7 (San Diego, CA, USA) was used to determine the values for K_m and V_{max} for each concentration of APAP. A representative Michaelis-Menten plot for the acetaminophen checkerboard assay is shown in Figure 9, and representative K_m and V_{max} values are shown in Table 7. These figures and calculations use the luminescence data from the second checkerboard assay (conducted at half volume).

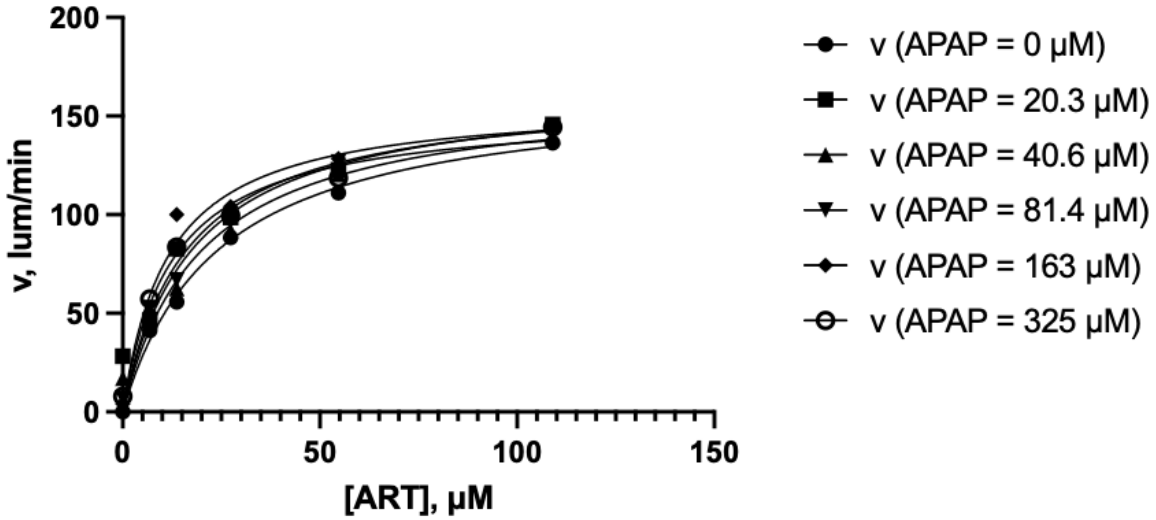

Figure 10: Michaelis-Menten Plot of Acetaminophen Checkerboard Assay. Velocity (lum/min) is plotted against the concentration of ART (µM) for each concentration of acetaminophen.

Table 7: Km and Vmax values for Acetaminophen Checkerboard Assay

APAP Conc. (µM)	V_{max}	K_m
0	164.5	24.27
20.3	163.4	16.07
40.6	163.6	20.00
81.4	166.4	17.97
163	158.0	11.57
325	153.3	12.50

The Michaelis-Menten plot matches the shape of the competitive inhibition curve from Figure 7, indicating that acetaminophen and *A. annua* tea appear to be competitive inhibitors. While the V_{max} values ranged from 164.5 luminescence units/minute at 0 µM APAP to 153.3 luminescence units/minute at 325 µM APAP, the measured K_m values decreased from 24.27 µM ART at 0 µM

APAP to 12.50 µM ART at 325 µM APAP. The consistent V_{max} values and range of K_m values also support that acetaminophen and *A. annua* tea participate in competitive inhibition.
A Lineweaver-Burk plot of the kinetics of APA vs. *A. annua* tea is shown in Fig. 10 and is a representative Lineweaver-Burk plot of data from the acetaminophen checkerboard assay conducted at half volume.

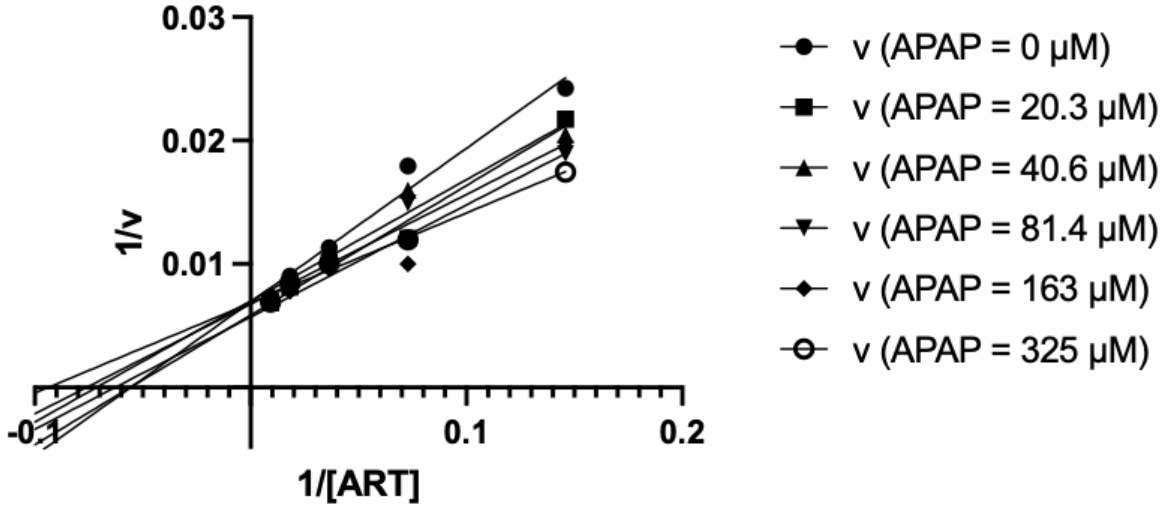

Figure 11: Lineweaver-Burk plot for Acetaminophen Checkerboard Assay

In the Lineweaver-Burk plot, the lines intersect, which eliminates uncompetitive inhibition as the inhibition method for acetaminophen and *A. annua* tea. The lines intersect at about the same y-value, which indicates competitive inhibition. This supports the idea that acetaminophen and *A. annua* tea participate in competitive inhibition as was suggested by the Michaelis-Menten plot.

4.3 Caffeine Fractional Inhibitory Concentration (FIC) Index

For this experiment, A is equal to the luminescence of the tea, B is equal to the luminescence of caffeine in the well, MIC_A is the MIC of *A. annua* tea and MIC_B is the MIC of caffeine. The MIC of *A. annua* tea was 0.57 µM ART, and the MIC of caffeine was 5.57 µM. A representation of the FIC Indexes for each well of the caffeine checkerboard assay is shown in Table 8. These calculations use the luminescence data from the second checkerboard assay (conducted at half volume).

4.3.1 Caffeine Kinetics Analysis

The kinetics of the caffeine checkerboard assays were investigated using Michaelis-Menten and Lineweaver-Burk plots. The Michaelis-Menten plot was created by plotting the velocity (in luminescence units/minute) of each concentration of acetaminophen (APAP) against varying concentrations of ART (µM) within *A. annua* tea. A nonlinear regression analysis by GraphPad Prism 7 (San Diego, CA, USA) was used to determine the values for K_m and V_{max} for each concentration of CAF. A representative Michaelis-Menten plot for the caffeine checkerboard

assay is shown in Figure 11, and representative K_m and V_{max} values are shown in Table 9. These figures and calculations use the luminescence data from the second checkerboard assay (conducted at half volume).

Table 8: FIC Index values for Caffeine Checkerboard Assay. Wells that showed synergy would be in yellow, wells that showed additive/no interaction would be in blue, and those that showed antagonism would be in orange.

		Increasing ART Conc. (µM)					
		3.45	6.90	13.8	27.6	55.1	110.
Increasing CAF Conc. (µM)	5.57	7.70	9.68	8.03	12.1	13.7	14.4
	11.1	9.23	10.2	11.6	12.5	14.2	15.7
	22.3	9.66	12.4	11.3	13.6	13.9	15.7
	44.6	9.87	10.1	11.3	13.0	14.9	15.9
	89.1	10.5	11.7	12.4	13.6	15.0	16.2
	178	11.4	11.3	12.7	13.9	15.5	16.3

The FIC Index table shows that caffeine and *A. annua* tea had an antagonistic interaction regardless of the concentration of either drug. This means that caffeine decreases the ability of *A. annua* tea to bind CYP3A4.

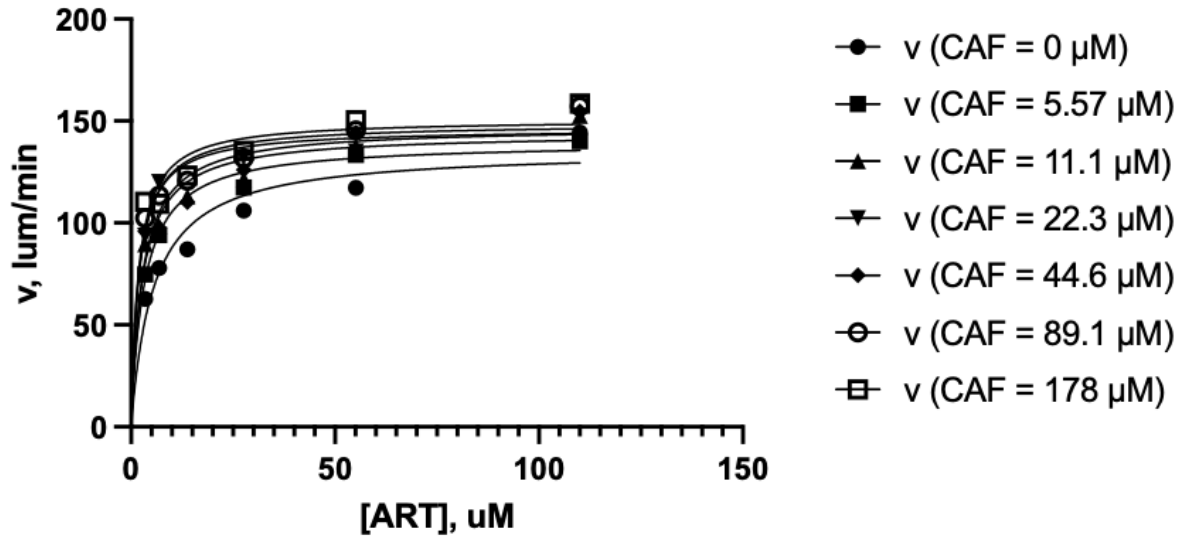

Figure 12: Michaelis-Menten Plot of Caffeine Checkerboard Assay. Velocity (lum/min) is plotted against the concentration of ART (µM) for each concentration of caffeine.

Table 9: Km and Vmax values for Caffeine Checkerboard Assay.

CAF Conc. (µM)	V_{max}	K_m
0	135.9	5.481
5.57	139.5	3.238
11.1	143.9	2.741
22.3	146.3	1.832
44.6	147	2.742
89.1	148.8	1.95
178	150.8	1.833

While the V_{max} values ranged from 135.9 luminescence units/minute at 0 µM CAF to 150.8 luminescence units/minute at 178 µM CAF, the measured K_m values decreased from 5.481 µM ART at 0 µM CAF to 1.833 µM ART at 178 µM CAF. The consistent V_{max} values and ranging K_m values support that caffeine and *A. annua* tea participate in competitive inhibition.
The Lineweaver-Burk plot was created by plotting the 1/velocity of each concentration of caffeine (CAF) against varying 1/concentrations of ART (µM) within *A. annua* tea. A linear regression analysis by GraphPad Prism 7 (San Diego, CA, USA) was used to fit the resulting plot. A representative Lineweaver-Burk plot for the caffeine checkerboard assay is shown in Figure 12. This figure uses the luminescence data from the second checkerboard assay (conducted at half-well volumes).

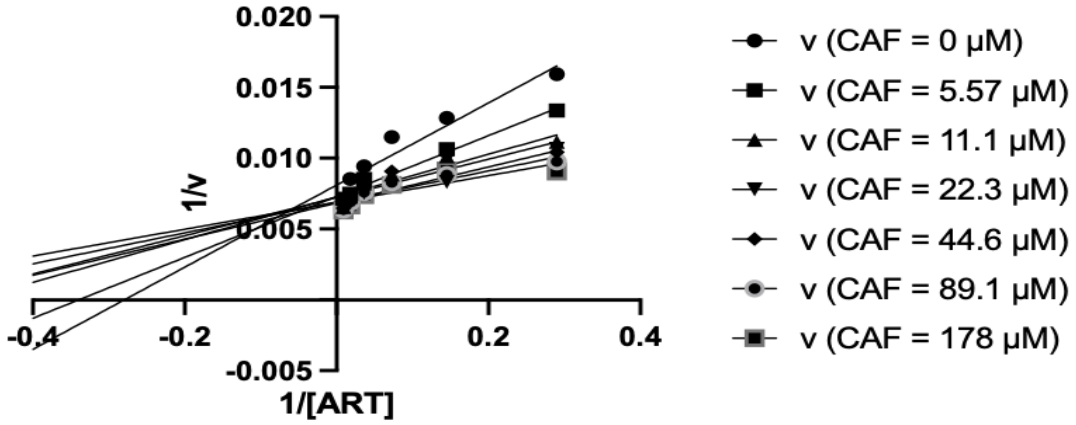

Figure 13: Lineweaver-Burk plot for Caffeine Checkerboard Assay.

In the Lineweaver-Burk plot, the lines intersect, which quickly eliminates uncompetitive inhibition as the inhibition method for caffeine and *A. annua* tea. The lines intersect at about the same y-value, which suggests competitive inhibition. This supports the idea that caffeine and *A. annua* tea participate in competitive inhibition as suggested by the decreasing K_m values from the Michaelis-Menten plot.

4.4 Comparison of full and half-volume P450-Glo Assays

From the luminol testing, all wells produced luminescence at high enough levels to be reliably read by the plate reader (Table 10). Overall, decreasing the total well volume to 50 µL instead of 100 µL resulted in an average of 52% decrease in the luminescence of the well. Luminescence decreased proportionally to the decrease in luminescent reagent; a 50% decrease in volume resulted in about a 50% decrease in luminescence.

Data from the checkerboard assays were normalized to the NIC wells before enzyme kinetics analysis, which made it possible to compare data from checkerboard assays run using both the 100 µL and 50 µL well volumes.

Table 10: Results from Luminol Testing for Method Development.

	Trial 1		Trial 2		Trial 3		Average
Volume (µL)	100	50	100	50	100	50	
Luminescence	14391	6694	16077	7979	15456	7494	
Percent Decrease (%)	54.7		51.0		52.2		**52.5**

5.0 Discussion

According to the World Health Organization, 80% of the world's population uses herbal medicines (WHO, 2023). While herbal medicine use is not as prevalent in the United States as it is in other parts of the world, according to Rashrash et al. 2017, 35% of the US adult population reported the use of at least 1 herbal medicine, with the average number of supplements used being 2.6 (Rashrash et al., 2017). In addition, 64% of herbal remedy users also reported concurrently using OTC medications (Rashrash et al., 2017). However, based on the FDA Guidelines for Clinical Drug Interaction Research (2017), any drug-use instructions must contain drug interaction data (Qiang et al., 2021). However, *A. annua* is not classified as a drug substance by the FDA because it is an herbal substance, which means that there is extremely limited if any instruction on the potential effects of drug interactions. This only further emphasizes the importance of examining the physiological impacts that OTC medications have on the metabolism of herbal medications for the safety and efficacy of these drugs.

Specifically with herb substances, it is important to study the pharmacokinetic and pharmacodynamic interactions between drugs and herbal medicine because medicinal herbs contain many different phytochemicals that may be present in trace amounts but still participate in the interaction (Singh & Zhao, 2017). For example, there is significantly greater bioavailability of artemisinin in the body when consuming *A. annua* DLA tea infusion versus pure artemisinin (Desrosiers, 2020). This may be due to inhibition of the hepatic CYPs (such as CYP3A4) that metabolize artemisinin by other anti-inflammatory phytochemicals in *A. annua* DLA, such as flavonoids, monoterpenes, rosmarinic and chlorogenic acids (Desrosiers, 2020). This can become a safety issue if the herb alters the metabolism of a secondary drug, e.g. acetaminophen, and the concentration of the drug in the plasma is different than would be expected per the dose taken. This could lead patients to have more of the drug in the system than expected, or possibly take more of the drug than is safe because the effects are not being experienced as strongly as expected. For example, *Echinacea purpurea* has been shown to induce CYP3A4 activity, which leads to decreased bioavailability of 3A4 substrates, such as acetaminophen (Awortwe et al., 2015). This may warrant the need to increase the dose of such drugs, which could lead to increased tolerance and system toxicity (Awortwe et al., 2015).

Recent research into *Artemisia annua* is mostly focused on the different effects that it has and has shown promising results as a therapeutic against SARS-CoV-2, tuberculosis, Lyme disease, and schistosomiasis (Weathers, 2022). However, to our knowledge, there is little research being done on the potential interactions between OTC medications and *A. annua* and this research aimed to begin filling that gap.

For acetaminophen, we hypothesized that acetaminophen and *A. annua* tea would exhibit competitive inhibition, which was supported by the collected data. On the other hand, for

caffeine, we hypothesized that caffeine would show no inhibition and only allosteric inhibition at high concentrations, and this was not supported by the data. Both the *A. annua* tea and acetaminophen and *A. annua* tea and caffeine combinations showed competitive inhibition.

A. annua has a complex chemical structure that contains over 600 secondary metabolites all of which could play a role in metabolism (Septembre-Malaterre et al., 2020). However, for this experiment, all data for both acetaminophen and caffeine were analyzed based on the assumption that *Artemisia* tea was the substrate and caffeine/acetaminophen was the inhibitor impacting the metabolism of the tea. When considering that, the data showed that physiologically, acetaminophen was acting as an antagonist against the metabolism of the two combined drugs. This was supported by the information from the FIC Index calculations. The FIC Index calculations showed that acetaminophen and *A. annua* tea were antagonistic, meaning that acetaminophen decreased the inhibitory effect of *A. annua* tea on CYP3A4. The kinetics analysis showed that acetaminophen and *A. annua* tea were competitive inhibitors of CYP3A4.

Acetaminophen binds to CYP3A4 (Strelow et al., 2012), and because *A. annua* is a competitive inhibitor that suggested they were competing for the same binding site on CYP3A4. The same concept applied to caffeine, as they both displayed competitive inhibition in the Lineweaver Burke plots. In addition, the data showed that physiologically, caffeine and acetaminophen acted as antagonists against the metabolism of the two-drug combination. These kinetics were corroborated by the FIC Index calculations.

The kinetics analysis of the data gathered from the checkerboard assays supported the pharmacokinetic interactions between *A. annua* and both of the OTC drugs tested. Pharmacokinetic interactions occur at the macro-biological level of metabolism, which impacts the absorption of a drug (Richens, 1995). Both acetaminophen and caffeine competitively inhibited CYP3A4, which increases the bioavailability of artemisinin within the body. Data from the FIC analysis showed pharmacodynamic interactions between *A. annua* and both OTC drugs tested. Pharmacodynamic interactions occur at the cellular level of receptor sites, where they can have additive or inhibitory effects (Richens, 1995). Both acetaminophen and caffeine were antagonistic toward *A. annua* tea, meaning that the OTC drug inhibited the ability of *A. annua* to inhibit CYP3A4 and thereby increasing the bioavailability of artemisinin from the tea into the body.

The data suggested the prevalence of cooperative substrate binding. On their own, neither acetaminophen nor caffeine displayed Michaelis-Menten kinetics with CYP3A4, which is characteristic of CYP3A4 as it is prone to cooperative substrate binding (Sevrioukova and Poulos, 2013). This is a form of allosteric behavior that results from the simultaneous binding of more than one substrate in the active site and CYP3A4 is specially equipped for this function as it has large and flexible substrate pockets that are capable of accommodating multiple small

organic molecules, as is this case with *A. annua* tea and caffeine/acetaminophen (Denisov et al., 2009). P450s in general can simultaneously bind with several substrate molecules, and in this study CYP3A4 showed changes in kinetic behavior in the presence of plant phytochemicals. An example of this cooperative binding was seen in the Lineweaver Burke plots for both caffeine and acetaminophen. The kinetics suggested that there was a competitive interaction, however, the K_m values did not increase as anticipated but rather decreased indicating that while the binding was still occurring, the metabolism of the substance was not. We are hypothesizing that this was due to negative cooperative binding. The lower the value of Km, the more efficient the enzyme was at metabolizing its substrate. If caffeine prevented *A. annua* from binding to CYP3A4, the enzyme would not metabolize *A. annua* tea as efficiently thereby increasing its bioavailability, and is less inhibited, thereby increasing efficiency. While this conclusion was supported by the caffeine data at the lower concentrations, at higher concentrations, there seemed to be less drastic competitive inhibition at the higher caffeine concentrations where the Lineweaver-Burke plot began creating parallel lines suggesting there was a shift to a more non-competitive inhibition.

Noncompetitive inhibition is characterized by the inhibitor binding to an allosteric site different from the active site (Strelow et al., 2012). Ekroos and Sjögren (2006) reported that CYP3A4 bound to progesterone in an effector site adjacent to the active site. The small size of the progesterone molecule compared to a similarly sized molecule, erythromycin, another CYP3A4 substrate that does not induce a significant conformational change in the enzyme. Caffeine is also a small molecule in comparison to artemisinin. By itself, caffeine may be metabolized in the active site of 3A4, but in combination with artemisinin, it may be outcompeted and moved to an effector site. This would have the effect of noncompetitive inhibition where caffeine is in the role of the inhibitor bound to an allosteric site.

Physiologically, this means that acetaminophen and caffeine both impacted the metabolism of *Artemisia* tea, albeit only slightly. The overall function of CYP3A4 was somewhat decreased, which meant that drug metabolism was slowed for *A. annua*. This data suggests a greater bioavailability of *A. annua* tea due to of decreased CYP3A4 metabolism efficiency resulting in overall lessened liver function.

One of the most important considerations when co-consuming both these compounds is overall liver function. However, because the noticeably decreased function was only observed at concentrations above the recommended dosages of OTC drugs, co-consumption concerns are only important when caffeine and acetaminophen are consumed at levels 2-3 times the recommended dose.

6.0 Conclusion

Based on our results from the checkerboard assays, both acetaminophen, and caffeine competitively inhibited *A. annua*'s ability to inhibit CYP3A4. This data would suggest increased bioavailability of the *A. annua* tea. The inhibitory effects of acetaminophen and caffeine become significant at concentrations higher than the recommended dose. Therefore, it is safe to consume *A. annua* tea and acetaminophen or caffeine simultaneously, but it is important to note that it is only safe to do so at the recommended dose of acetaminophen or caffeine. Further consideration should be taken when consuming 2-3 times the recommended dose of these drugs, as the inhibitory effects may become more extreme and create strain on the liver.

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Appendix A: P450-Glo Assay SOP

Reagents:
Promega P450-Glo Kit

Reagents not included in Promega P450-Glo kit:
200 mM sucrose
671 mM KPO_4 buffer
Ketoconazole
Methanol
300 µM NADPH

Supplies:
2 µL, 10 µL, 20 µL, 100 µL, 1000 µL micropipettors and tips
96-well plate

1. Add 2.5 µL of 0.1 µg/µL ketoconazole in methanol to Keto control wells and dry down using an aquarium pump or allow to air dry
2. Add 12.5 µL sterile H_2O to NIC (no inhibitor control), NMC (no microsome control), and ketoconazole controls
3. Add 12.5 µL of test drug solution to corresponding test wells
4. Add 12.5 µL of *A. annua* tea sample to corresponding test wells
5. Make 4X CYP reaction mixture w/ microsomes using the ratio below per well for the number of drug test wells and NIC and ketoconazole controls
    a. 0.13325 µL 3 mM luciferin IPA stock (final concentration 32 uM)
    b. 7.3754 µL 671 mM KPO_4 buffer (final concentration 400 mM)
    c. 5 µL human liver microsomes in 250 mM sucrose (thaw and add last)
6. Make a 4X CYP reaction mixture without microsomes for the NMC wells using the ratio below per well
    a. 0.13325 µL 3 mM luciferin IPA stock (final concentration 32 uM)
    b. 7.3754 µL 671 mM KPO_4 buffer (final concentration 400 mM)
    c. 5 µL 250 mM sucrose
7. Add 12.5 µL appropriate 4X CYP reaction mixture to wells
8. Mix the plate for 10 seconds on a microplate mixer
9. Preincubate for 10 min in 37°C incubator
10. Add 25 µL 300 µM NADPH to each well with a multichannel pipette
11. Mix the plate for 10 seconds on a microplate mixer
12. Incubate for 10 min in 37°C incubator
13. Add 50 µL Luciferin Detection Reagent w/ multichannel pipette
14. Mix the plate for 10 seconds on a microplate mixer
15. Incubate for 20 min at room temperature

16. Record luminescence using PerkinElmer Victor3 Multilabel Plate Reader in Worcester Polytechnic Institute Gateway Park Room 3221
    a. Log in to the computer and open the program “PerkinElmer 2030 Manager”
    b. Select the protocol “Matt Luminescence”
    c. Edit the protocol to measure the wells used
    d. Close the protocol editor and click Start
    e. Export the luminescence data as an Excel file

Appendix B: Half-Volume P450-Glo Assay SOP

Reagents:
Promega P450-Glo Kit

Reagents not included in Promega P450-Glo kit:
200 mM sucrose
671 mM KPO_4 buffer
Ketoconazole
Methanol
300 µM NADPH

Supplies:
2 µL, 10 µL, 20 µL, 100 µL, 1000 µL micropipettors and tips
96-well plate

1. Add 2.5 µL of 0.1 µg/µL ketoconazole in methanol to Keto control wells and dry down using an aquarium pump or allow to air dry
2. Add 6.25 µL sterile H_2O to NIC (no inhibitor control), NMC (no microsome control), and ketoconazole controls
3. Add 6.25 µL of test drug solution to corresponding test wells
4. Add 6.25 µL of *A. annua* tea sample to corresponding test wells
5. Make 4X CYP reaction mixture w/ microsomes using the ratio below per well for the number of drug test wells and NIC and ketoconazole controls
    a. 0.13325 µL 3 mM luciferin IPA stock (final concentration 32 uM)
    b. 7.3754 µL 671 mM KPO_4 buffer (final concentration 400 mM)
    c. 5 µL human liver microsomes in 250 mM sucrose (thaw and add last)
6. Make a 4X CYP reaction mixture without microsomes for the NMC wells using the ratio below per well
    a. 0.13325 µL 3 mM luciferin IPA stock (final concentration 32 uM)
    b. 7.3754 µL 671 mM KPO_4 buffer (final concentration 400 mM)
    c. 5 µL 250 mM sucrose
7. Add 6.25 µL appropriate 4X CYP reaction mixture to wells
8. Mix the plate for 10 seconds on a microplate mixer
9. Preincubate for 10 min in 37°C incubator
10. Add 12.5 µL 300 µM NADPH to each well with a multichannel pipette
11. Mix the plate for 10 seconds on a microplate mixer
12. Incubate for 10 min in 37°C incubator
13. Add 25 µL Luciferin Detection Reagent w/ multichannel pipette
14. Mix the plate for 10 seconds on a microplate mixer
15. Incubate for 20 min at room temperature

16. Record luminescence using PerkinElmer Victor3 Multilabel Plate Reader in Worcester Polytechnic Institute Gateway Park Room 3221
    a. Log in to the computer and open the program “PerkinElmer 2030 Manager”
    b. Select the protocol “Matt Luminescence”
    c. Edit the protocol to measure the wells used
    d. Close the protocol editor and click Start
    e. Export the luminescence data as an Excel file

Appendix C: Materials & Catalog Numbers

Material	**Catalog Number**
Artemisia annua tea infusion	Sourced from Atelier Temenos B#1.Sh6.01.15.20; dried material added to stirred boiling water at a ratio of 10 g per L for 10 minutes; 0.22 um filter sterilized
Acetaminophen (4-acetamidophenol)	Thermo Fisher Scientific 102330050
Caffeine	Sigma Chemical Company C-0750
Luciferin-IPA	Promega V9002
Ketoconazole	Cayman Chemical 15212
KPO_4 buffer	KH_2PO_4 Sigma Chemical Company P-5379; KH_2PO_4 Fisher Scientific BP363-500
Sucrose	Phytotechnology Laboratories S391
Human liver microsomes	XenoTech H2620
NADPH tetrasodium salt	EMD Millipore Corp 481973-50MG
Luciferin Detection Agent	Promega V9002