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Measuring changes in bioavailability of artemisinin

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Measuring changes in bioavailability of artemisinin

in combination with flavonoids, phenolic acids, and monoterpene found in *Artemisia annua*

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Abstract

Malaria, the fifth leading cause of disease by infectious agents, has claimed the lives of approximately 584,000 people while posing a risk to 3.2 billion more worldwide, according to latest global estimates (World Health Organization, 2014). Artemisinin is a potent antimalarial found in *Artemisia annua* and is currently used in combination with other antimalarial drugs (artemisinin combination therapy; ACT), mainly against the infectious *Plasmodium* parasites. The use of artemisinin and its derivatives in ACT is currently the WHO recommended treatment for malaria, but is relatively expensive to many endemic regions. Artemisinin resistance in the *Plasmodium* parasite has recently developed, decreasing the potency of ACT, and initiated the search for alternative treatments. *A. annua* dried leaves taken orally has been found to be more efficacious than ACT, perhaps due to increased bioavailability of artemisinin.

In this current project, a Caco-2 intestinal model was used to test changes in the bioavailability of artemisinin. The experiments were performed to simulate the effects of specific phytochemicals (at various concentrations) on artemisinin transport from the intestinal lumen into the serum. Sixty minute transport studies were analyzed across the monolayer at fifteen minute intervals, comparing artemisinin transport in combination with two flavonoids (quercetin and rutin), two phenolic acids (chlorogenic and rosmarinic acid), and one monoterpene (camphor). Of all the performed transports, only camphor (at a 1:10 molar ratio to artemisinin) showed a significant increase in artemisinin transport compared to the transport of artemisinin alone. Additionally, artemisinin transport across Caco-2 monolayers was found to vary with artemisinin concentration.

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1.0 Introduction and Background

1.1 Malaria

Malaria is a life-threatening illness, which strains economic growth and reproduction in endemic regions. Globally, it is the fifth leading cause of disease by infectious agents (Centers for Disease Control and Prevention, 2010). The disease is caused by *Plasmodium* parasites, the most deadly strain being *P. falciparum,* which is transmitted to humans through the bite of an infected mosquito. Other strains that cause malaria in humans include *P. vivax, P. malariae,* and *P. ovale*. Latest global estimates from December 2014 confirmed 198 million cases (uncertainty range from 124 to 283 million) and 584,000 deaths (uncertainty range from 367,000 to 755,000) in 2013 were attributed to this disease. Of the many deaths associated with malaria, approximately 78%, were African children under the age of 5. Currently, 3.2 billion people world-wide are at risk of becoming infected with malaria. Risk extends into all 6 of WHO's territories, where 1.2 billion are at high risk, which is described as greater than a 1 in 1000 chance of contracting the disease (World Health Organization, 2014).

One of the most influential risk factors in contracting malaria is human behavior, specifically social and economic factors, surrounding the disease. In many poverty-stricken regions where malaria is endemic, there is often a lack of access to adequate housing or preventative equipment against mosquitos, lack of knowledge against disease, and cultural objection against treatments that leave the citizens vulnerable to infection (Centers for Disease Control and Prevention, 2012). Agricultural methods frequently foster mosquito habitats by generating pools of standing water, and supplying secondary blood meal sources including farm animals. Together these conditions entice greater populations of mosquitoes to those areas. Lastly, human activities such as war, migration, and travel expose otherwise healthy, non-immune individuals to areas where malarial transmission is high (Centers for Disease Control and Prevention, 2012). Interestingly, individuals with the sickle cell trait are relatively protected against *P. falciparum* and those with a negative Duffy blood group are resistant to *P. vivax* infection (Friedman, 1978; Langhi & Bordin, 2006).

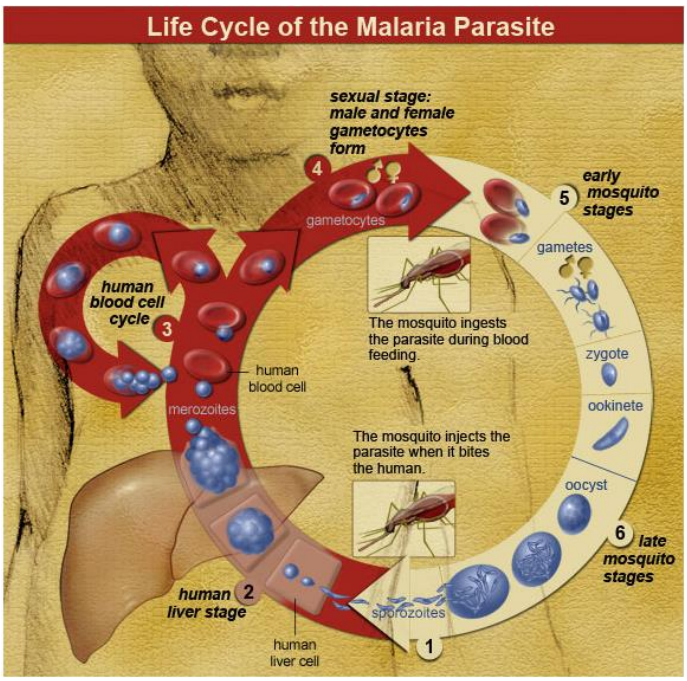


Figure 1: Life cycle of the malaria parasite in the human and mosquito hosts (National Institute of Allergy and Infectious Diseases, 2012)

The life cycle of malaria begins after an infected, female *Anopheles* mosquito bites and injects sporozoites into the blood stream (Figure 1; National Institute of Allergy and Infectious Diseases, 2012). The sporozoites then invade liver cells where they continue to mature and divide for a period of 5-16 days. Afterwards, the parasites invade red blood cells where they reproduce as merozoites and also form gametocytes. Symptoms are seen in humans approximately 10 to 15 days after the initial mosquito bite.

Malaria cases are typically categorized as uncomplicated or severe (Centers for Disease Control and Prevention, 2010). Uncomplicated malaria is usually characterized by an episode of 6-10 hours consisting of a cold stage (chills, shivering), a hot stage (fevers, headaches, vomiting), and a sweating stage (sweats, fatigue, tiredness). It is important to keep in mind that this disease is entirely treatable as long as timely and appropriate treatment is administered. In severe malaria, there is organ failure, abnormalities in blood, and/or afflictions with metabolism (Centers for Disease Control and Prevention, 2010). If the brain becomes infected, cerebral malaria results in symptoms of seizures, paralysis, epilepsy, and other neurological impairments. Of cerebral malaria survivors, 5-20 % may develop permanent disabilities due to brain damage (Breman et

al., 2001). Respiratory and renal systems also may be targeted, resulting in acute respiratory distress syndrome and kidney failure, respectively. Blood complications may include severe anemia, hemoglobinuria (blood in urine), and coagulation irregularities (Centers for Disease Control and Prevention, 2010). In metabolism, metabolic acidosis (high acidity in blood and organs) and hypoglycemia (low blood glucose) may occur in patients. To prevent fatality, patients experiencing severe malaria must seek immediate, dynamic medical care.

Besides the burden of illness and death, malaria can have secondary burdens including changes in household roles, deficits in education, migration, treatment expenditures, and forgone incomes. Malaria may be responsible for as much as 50% of medically-related school absences, including loss of 11% of school days in primary school, and 4.3% of secondary school days. Cognitive deficiencies from cerebral malaria brain damage, including inability to carry out executive functions, can also lead to educational deficits (Sachs & Malaney, 2002).

Increased preventative measures and the use of standard drug combination treatments have resulted in the decrease of worldwide malaria (Bhattarai et al., 2007). Safety measures include vector control, insecticide-treated mosquito nets (ITN) and sprays, chemoprevention (preventing blood stage infections in humans), and case management. Control and elimination efforts totaled 2.7 billion dollars in 2013, but most notably reached three times that in 2005 (World Health Organization, 2014). Since 2013, artemisinin-based combination therapies (ACTs) have been adopted in 79 of the 88 countries endemic to *P. falciparum* (World Health Organization, 2014). Although mortality rates from malaria have decreased globally by 47% since 2000, the disease still remains a devastating problem in many regions of the world including Africa, Asia, and South America (Figure 2; World Health Organization, 2014).

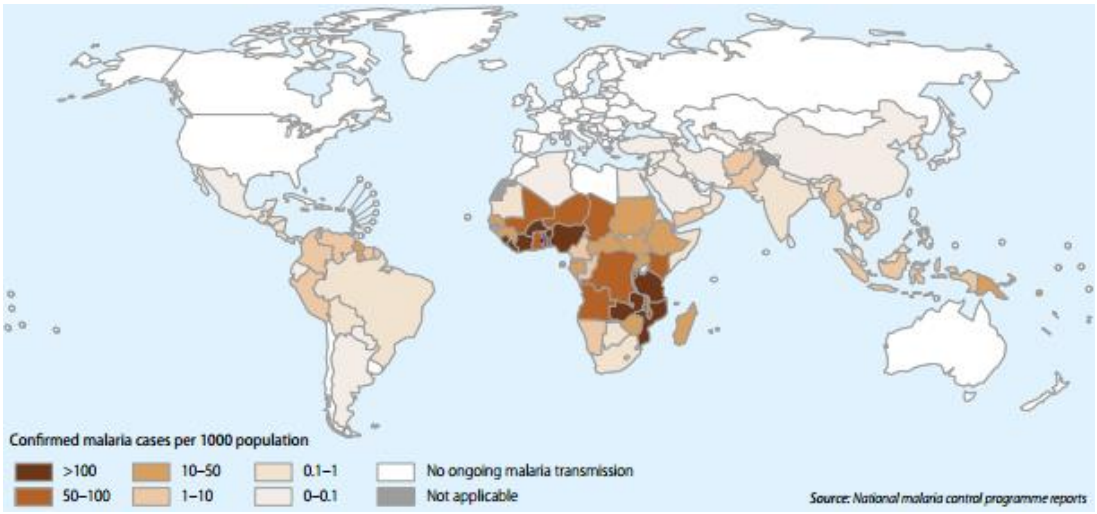


Figure 2: Countries with ongoing transmission of malaria, 2013 (World Health Organization, 2014)

1.1.1 Malaria Treatments and Parasite Drug Resistance

Many of the current malaria treatments are derived from plants that originated in several countries around the world. In Peru and Bolivia, cinchona tree bark was a treatment for fevers, a common symptom of malaria. Cinchona bark was brought to Europe and in 1820, the antimalarial, quinine, was extracted. Upon synthesizing derivatives of quinine, pharmaceutical chloroquine, a 4-aminoquinoline, was introduced to the public (Faurant, 2001; Petersen, et al., 2011). Chloroquine is absorbed efficiently when taken orally and has a relatively low safety margin in regards to dosage administration (World Health Organization, 2001).

In the past, pharmaceutical quinine derivatives, such as chloroquine and amodiaquine, were used to treat malaria. Although these drugs are currently used less because drug resistance has increased, they may still be used in some regions where patients have acquired partial immunity through recurrent malaria incidence, such as in Africa. Although chloroquine mechanisms of action are not fully understood, the drug is thought to accumulate in the parasite's digestive vacuole, inhibiting the parasite's degradation of hemoglobin. This allows free floating heme to poison the parasite, resulting in a permeable outer membrane and eventual parasite death (Petersen, et al., 2011).

Antimalarial drug resistance is defined as the ability of a parasite strain to survive and/or divide regardless of a specific drug's recommended dosage (World Health Organization, 2001). In the 1900s, *Plasmodium* parasites started becoming resistant to chloroquine, causing severe symptoms and high mortality rates among those infected (Petersen, et al., 2011; Gasasira et al., 2003). Initial overuse of chloroquine not only as an anti-malarial, but as a miracle drug to treat all afflictions, may have contributed to this resistance (Faurant, 2001). The drug's long half-life of 60 days is another factor possibly contributing to resistance, because it allows extended parasite exposure to sub-therapeutic drug concentrations. Specifically, mutations in the parasite's chloroquine-resistant transporter gene (MAL7P1.27), which encodes for a transmembrane protein in the digestive vacuole, prevents chloroquine from accumulating (Petersen, et al., 2011). Despite cases of resistance, chloroquine is still used in some regions to fight against *P. vivax* infections (World Health Organization, 2014).

Eventually, sulfadoxine-pyrimethamine was used as a replacement for chloroquine. A study in Uganda found that this drug was very effective in terms of parasite and clinical symptom clearance when combined with amodiaquine (Gasasira et al., 2003). Coulibaly et al. (2002) showed that sulfadoxine-pyrimethamine treated uncomplicated malaria with a greater than 99% efficacy, while chloroquine treatments resulted in 85-90% efficacy. The study further reiterated sulfadoxine-pyrimethamine as a dose-dependent drug that has been approved as a second-line treatment defense against malaria.

1.2 Current Malaria Treatments

Artemisinin, a sesquiterpene trioxane lactone, is a unique and highly effective antimalarial compound that was isolated in 1972 from the annual plant *Artemisia annua.* (Figure 3; Christen & Veuthey, 2001; van Agtmael et al., 1999). Originally native to China, *A. annua* has been used in Chinese traditional medicine for thousands of years in herbal tea infusions to treat fever and chills associated with malaria (World Health Organization, 2006; Mueller et al, 2004). Furthermore, artemisinin has been known to exhibit prophylaxis as reported by Ogwang et al.

(2011). Ugandans who ingested *A. annua* tea weekly saw an 80% decrease in malaria symptoms compared to those not regularly drinking the tea.

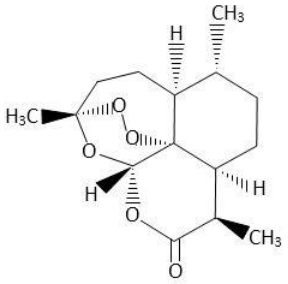


Figure 3: Artemisinin structure

Artemisinin, was publically introduced in 1979 (Carbonara el al., 2012; Li & Zhou, 2010). It has a very short half-life of 2-5 hours, indicative of low prophylactic properties as well as low resistance and high permeability across the blood-brain barrier; all of which makes it a good drug for treating cerebral malaria (de Vries & Dien, 1996; Petersen et al., 2011; Augustijns et al., 1996; Niu et al., 1985). Artemisinin derivatives are commonly used in place of artemisinin because they have increased solubility in either water or oil and thus, increased antimalarial properties (Golenser et al., 2006; World Health Organization, 2001). These artemisinin derivatives may include artether, artemether, artesunate, and dihydroartemisinin (Figure 4; World Health Organization, 2012).

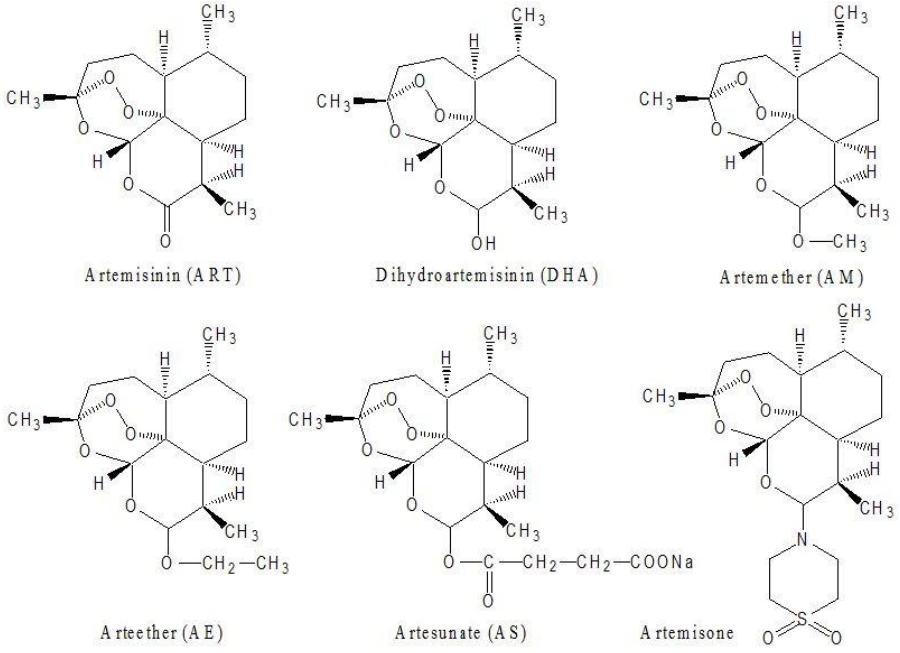


Figure 4: Artemisinin and derivatives (World Health Organization, 2012)

Structurally, artemisinin and its derivatives are similar, containing an endoperoxide bridge that plays an essential role in specific and non-specific mechanisms of action against the malarial parasite (Carbonara el al., 2012; Li & Zhou, 2010; World Health Organization 2006). The endoperoxide bridge is cleaved by iron in the parasite's digestive vacuole, producing carbon radicals, which have a negative effect on endocytosis of *P. falciparum*, as well as on parasite hemoglobin digestion (Li & Zhou, 2010; Eastman & Fidock, 2009).

Other mechanisms of action for artemisinin and its derivatives may include interference with parasite transport proteins, disruption of mitochondrial function, modulation of host immune function, and inhibition of angiogenesis (blood cell formation) (Golenser et al., 2006). Inhibition of nutrient uptake and prevention of parasite attack on red blood cells represent some of the artemisinin mechanisms of action against *P. falciparum* (Hoppe et al., 2004). Additionally, artemisinin inhibits the function of the Kelch 13 (K13) protein on the propeller domain in *P. falciparum*. This protein is important for interactions between protein sites and regulates an array of cellular function in the organism, such as protein degradation and responses to oxidative stress (Adams et al., 2000). Artemisinin disrupts these functions in the organism, allowing for rapid parasite clearance and recovery from malaria (Straimer et al., 2015). The relationship between this protein and artemisinin's mode of action was determined when K13 was identified as the molecular marker for emerging resistance to the drug (Ariey et al., 2014). Thapsigargin, similar in structure to artemisinin, but lacking an endoperoxide bridge, inhibits the sarcoplasmic/endoplasmic reticulum Ca2+ -ATPase (SERCA) within the parasite (PfATP6ase) (Eckstein-Ludwig et al., 2003; O'Neill et al., 2010). Without this specialized Ca2+ ATPase, calcium is not appropriately transported throughout the cells of the parasite, leading to problems with protein folding and signaling (Eckstein-Ludwig et al., 2003). After activation by iron, artemisinin binds to the PfATP6 protein in the parasite, inactivating the protein and causing the parasite to die (Jung et al., 2005; O'Neill et al., 2010).

Resistance to pure artemisinin malaria treatment as well as recrudescence, or recurrence, by reinfection poses a great risk (Golenser et al., 2006). Therefore, use of artemisinin-based combination therapy (ACTs) followed the spread of untreatable malaria. ACTs showed fast reduction in parasite biomass and overall clearance, as well as rapid elimination of patient

symptoms and diminution of gametocytes (World Health Organization, 2011). The short half-life of artemisinin derivatives requires coupling with longer half-life antimalarial drugs for enhanced potency and reduced chance for resistance. In 2005, the World Health Organization (WHO) advised that ACT be used as the primary treatment option for patients afflicted by the disease. Such treatment uses a combination of an artemisinin derivative in addition to another antimalarial medication that has proven effective. The artemisinin derivative mainly eliminates *P. falciparum* parasites, and the co-drug, commonly mefloquine amodiaquine, and piperaquine, is used to eliminate any artemisinin-resistant parasites (World Health Organization, 2011).

Unfortunately, however, artemisinin-resistance in *P. falciparum* now is found in Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand, and Vietnam (Figure 5; World Health Organization, 2014; Straimer et al., 2015). The Thailand-Cambodia border serves as regions of high artemisinin resistance. Based on a survey of patients from two different hospitals, Dondorp et al. (2009) discovered that malaria-stricken patients in western Cambodia took longer to recover from *P. falciparum* after ACT treatment than patients from Thailand.. In attempts to combat *P. falciparum* resistance in the Greater Mekong sub-region, the WHO recognized four priority areas of control: better intervention plan for all-risk groups, stricter case management, more publically-accessible information about resistance, and greater response and support (World Health Organization, 2013).

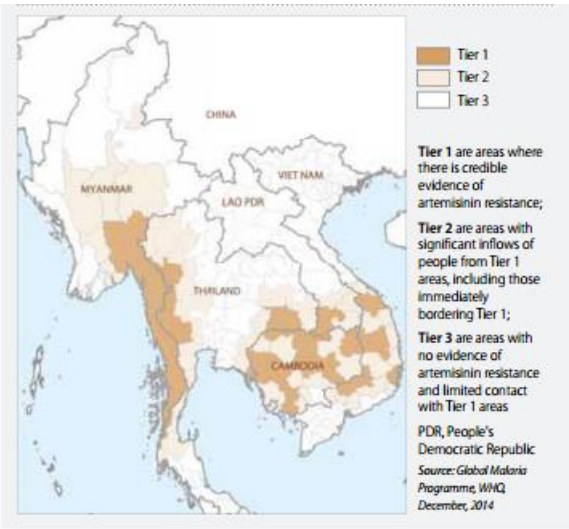


Figure 5: Areas of high artemisinin resistance in South-East Asia, December 2014 (World Health Organization, 2014)

Ashley et al. (2014) also observed that in regions of resistance, extending the ACT treatment for 6 days (as opposed to 3) allowed for a shorter parasite clearance half-life. Although ACT resistance occurred, prolonged treatment allowed for parasite clearance and gave hope for full patient recovery. These results suggested, however, that eventually additional mutations will likely occur in the parasite and the 6-day treatment plan will also fail (Ashley et al., 2014). Therefore, finding alternative treatment options is becoming important, especially for malaria endemic regions.

Recently, *P. falciparum* has shown some resistance to artemisinin due to mutations on chromosomes 5 and 13 in the parasite. When amplified, the *P. falciparum* multidrug resistance transporter 1 (PFE1150w) located on chromosome 5, reduces the parasite susceptibility to artemisinin (Petersen et al., 2011). More recent studies suggest that artemisinin resistance is caused by point mutations of the PF3D7_1343700 domain on chromosome 13 encoding a K13 (Kelch 13) protein, which regulates the parasite's propeller (Ariey et al., 2013; Takala-Harrision et al., 2014). Due to this developing artemisinin resistance, some ACTs (dihydroartemisinin–piperaquine, artemether–lumefantrine, artesunate–sulfadoxine–pyrimethamine, and artesunate–mefloquine) have shown low parasite clearance at the current WHO recommended dose (Ashley et al., 2014).

At specific sites in Asia and Africa, patients were given a dose of artesunate for either 1 or 3 days, followed by a 3-day course of ACT (Ashley et al., 2014). Blood samples were taken every 6 hours to determine parasite clearance half-lives, which ranged from 1.9 hours to 7.0 hours. In regions where *P. falciparum* had the kelch13 protein mutation, a longer parasite clearance half-life (length of time for 50% of peripheral blood parasite density to decrease) was observed (Ashley et al., 2014; Straimer et al., 2015). A long drug half-life is indicative of increased resistance, indicating the emergence of parasite resistance to ACT (Petersen et al., 2011).

Further research conducted by Elfawal et al. (2015) showed that consumption of dried *A. annua* leaves could be successful in prolonging artemisinin-resistant *Plasmodium* strains. When *P. chabaudi*-infected mice were given a single dose treatment of either pure artemisinin or powdered dried leaves of *A. annua* at a low (24 mg/kg) or high (120 mg/kg) concentration of

artemisinin, the percentage of parasitized red blood cells was found to be statistically greatest for the high-artemisinin whole plant treatment 16-48 hours post-treatment. Similar parasitemia reduction was seen in mice treated with either the high pure artemisinin or the low whole plant treatment, although lesser than high whole plant. Mice infected with *P. yoelli* and treated with high whole plant treatment for 9 consecutive days displayed complete parasite clearance 14 days post infection, whereas the high pure artemisinin treatment did not clear until 18 days post infection. Delayed artemisinin resistance was also shown in mice infected with *P. chabaudi* when whole plant was administered compared to pure artemisinin (Elfawal et al., 2015). This data gives suggestive evidence that whole *A. annua* leaves may be a feasible and more effective treatment in combating resilient malaria strains.

Viability of ACT has decreased in malaria endemic regions due to this emerging artemisinin resistance, as well as pressures of economic stability. Most people who contract malaria are impoverished, and current antimalarial treatments are not cost effective. In 2007 the WHO conducted a cost benefit analysis in Papa New Guinea evaluating one conventional malaria treatment and 3 ACTs. This study included costs of clinic visits, medications, and tests. The costs of 4 different therapies ranged from 3.93 to 5.19 US dollars (Davis et al., 2011). The average cost of any of the treatments listed amounted to more than 2 days wages according to the GNI data presented by World Bank (Papua, 2014), so for a family with several injections per year, malaria treatment is a major cost burden.

Current ACT therapies are relatively expensive and the populations most affected by malaria outbreaks are in areas that make drug delivery difficult. Additionally, parasite resistance to ACT has spread and the effectiveness of ACTs are likely to decrease. These two factors contribute to the idea that the current approved treatment is not ideal, so low cost, highly effective treatment options need to be explored.

1.3 Plant-Based Artemisinin Combination Therapy

A. annua contains over 100 secondary metabolites, some of which have reported antimalarial, antibacterial, antiviral, anti-inflammatory, and cytokine-like activity (Table 1; Carbonara et al.,

2012; de Magalhães et al., 2012). The plant is generally recognized as safe and has been consumed as a tea infusion for over 2,000 years (Duke, 2001). Oral consumption of dried *A. annua* leaves is also effective at reducing parasitemia in a rodent malaria model (Elfawal et al., 2012). Based on prior research, plant-based Artemisinin Combination Therapy (pACT) treatment of malaria can be achieved with dried leaves of *A. annua* (Figure 6; Weathers et al., 2011). Accumulating evidence suggests that pACT may be a viable alternative to ACT in the treatment of malaria (Weathers et al., 2014a).

Figure 6: *Artemisia annua* L. SAM cultivar vegetative form, and tablets from dried leaves.

Much disputed is use of *A. annua* tea; mainly of its inconsistent artemisinin composition and possible under dosage of patients. Some clinical studies have shown that the use of *A. annua* tea alone often provides a significantly lower dosage of the drug, artemisinin, than is suggested for proper eradication of the parasite in a patient (Mueller et al., 2004; Rath et al., 2004; World Health Organization, 2012b). Although, Kenyan human trials showed that lower artemisinin concentration can be efficacious (ICIPE, 2005). Additional factors such as growth, harvesting, storage, and processing have been shown to decrease the amount of artemisinin recovery from the plant. The possibility of sub-therapeutic doses and resistance has deterred the WHO from

accepting alternative *A. annua* malarial treatment methods that otherwise have shown promising results (World Health Organization, 2012b).

Table 1: Various phytochemicals found in *A. annua* SAM. These phytochemicals inhibit 50% growth in chloroquine sensitive and resistant strains of *P. falciparum* parasites.

Compound	IC_{50} values (µM)		Reference
	CQ sensitive	CQ resistant	
Artemisininic compounds			
Artemisinin	0.0226 ± 0.0007	.0212 ± 0023	Suberu et al. (2013)
dihydroartemisinic acid	21.1 ± 0.7	17.7 ± 4.2	
arteannuin B	3.2 ± 0.1	4.8 ± 0.4	
artemisinic acid	77.8 ± 1.5	61.6 ± 7.5	
Monoterpenes			
α-pinene	0.1	ND	Weathers & Towler(2014)
eucalyptol (1,8 cineole)	7.0	ND	
camphor	ND	ND	
Phenols			
chlorogenic acid	69.4 ± 6.4	61.4 ± 4.3	Suberu et al. (2013)
rosmarinic acid	65.1 ± 5.0	65.0 ± 7.0	
Coumarins			
scopoletin	ND	ND	
Flavonoids			
chrysoplenol-D	ND	32	Liu et al (1992)
chrysoplenetin	ND	23	
eupatorin	ND	65	
artemetin	ND	26	Liu et al (1992)
casticin	17.9 ± 4.7	12.2 ± 1.8	Suberu et al. (2013)
kaempferol	33 ± 7	25 ± 2	Lahane & Saliba (2008)
luteolin	11 ± 1	12 ± 1	
myricetin	40 ± 10	76 ± 23	
quercetin	15 ± 5	14 ± 1	

In a malaria infected mouse model, pACT effectively cleared parasitemia (Elfawal et al., 2012). The percent parasitemia in a murine model, infected with *P. chabaudi*, was measured post infection until 240-264 hours post-delivery of single oral dose treatments of varying levels of pure artemisinin or equal amounts of artemisinin delivered from *A. annua* whole plant leaves, dried and powdered. The data showed that the lower dose (24 mg/kg) of *A. annua* plant material eradicated the parasitemia in 88% of animals 30 hours post gavage. The higher dose (120 mg/kg) of pure artemisinin had similar parasite clearance to the lower dose of *A. annua.* Recrudescence was greatest in the low *A. annua* leaves dose than from either a high pure drug or *A. annua* dried leaf dose even though all had similar initial parasite clearance (Elfawal et al., 2012). These data implied that *A. annua* was just as effective as pure artemisinin at clearing malaria parasites in a murine model, but longer treatments are necessary to prevent recrudescence.

The therapeutic effects of *A. annua* compressed dried leaf tablets were tested in human malaria patients in Kenya (Table 2; ICIPE, 2005). The study used dried leaves containing 0.74% artemisinin. Each tablet contained 3.7 mg of artemisinin. Four different tablet doses were tested for 6 days in patients assumed to have *P. falciparum*. The highest *A. annua* dose (day 1 = 37 mg/day artemisinin, days 2-6 = 29.6 mg/day artemisinin) cleared parasites in 90.9% of patients by day 28.

In contrast, Giao et al. (2001) used a pure artemisinin treatment of 1,000 mg on the first day, and 500 mg on days 2-7, and only 76% of patients had parasite clearance on day 28 (Table 2; Giao et al., 2001). This percent clearance with artemisinin was similar to that of the lowest dried leaf dose in ICIPE (2005), containing 14.8 mg of artemisinin on day 1 and 7.4 mg on each subsequent day. Even with 27 to 67 fold higher doses of artemisinin, the pure drug showed less effective parasite clearance than the compressed *A. annua* tablets (Table 2).

In humans, Onimus et al. (2013) presented evidence that *A. annua* prevented malaria in post-operative patients. In preparation for surgery, 14 asymptomatic pediatric patients infected with *P. falciparum* were given 2 capsules of *A. annua* leaf powder containing between 0.4 and 0.5 mg of artemisinin per capsule. The first and second days post-surgery, these patients were given 2 more capsules each day, and the third morning after surgery, patients were given 1 capsule. In 11 other patients, the capsules were administered only after surgery in the same manner. Both the 60 and 36 hour treatments prevented the high temperature flare ups associated with malaria which were known to occur in asymptomatic patients during surgical recovery. The leaf powder also conveyed an added antinociceptive benefit.

Comparisons in Table 2 support a similar antimalarial effect of pACT treatment with *A. annua* whole leaf compressed tablets and ACT with artemisinin derivatives. Parasitemia clearance was slightly higher in patients treated with the ACT than with pACT. However, the dose of the artemisinin derivative was much greater in the ACT than the artemisinin in pACT treatment. This suggests that a higher dose of the whole plant compressed tablet may be comparable to the WHO recommended treatment of ACT with artemisinin derivatives. In further support of this,

ICIPE (2005) found, by varying pACT doses, that the clearance of parasites with pACT may be correlated with the dose of plant material.

Table 2: *Plasmodium falciparum* clearance with *Artemisia annua*, artemisinin, and various ACTs in human trials

Malaria treatment	Artemisinin/derivative dose (mg)		Leaf dry weight (g/day)		# of subj.	Total parasite clearance % (day)	Reference
Compressed whole leaf *A. annua* tablets	Day 1	Day 2-6	Day 1	Day 2-6			ICIPE (2005)
	7.4 × 2	3.7 × 2	2	1	12	75(28)	
	11.1 × 2	7.4 × 2	3	2	12	90.9(28)	
	14.8 × 2	11.1 × 2	4	3	12	83.3(28)	
	18.5 × 2	14.8 × 2	5	4	12	90.9(28)	
Pure artemisinin	Day 1	Days 2-7					Giao et al. (2001)
	500 × 2	500	N/A		227	76(28)	
ACTs	Standard WHO rec.		N/A		2741	95(28)	Zwang et al. (2009)
Artesunate +	600 + 750 mefloquine		N/A		51	97(28)	Congpuong et al. (2010)
Artesunate +	540 + 1350 amodiaquine		N/A		106	84(42)	Hasugian et al. (2007)
Dihydroartemisinin +	320 + 2430 piperaquine		N/A		114	95.2(42)	

Possibilities for increased antimalarial properties in pACT over ACT, as seen here, are currently being investigated. Weathers et al. (2011) measured artemisinin serum concentration in mice after oral gavage of powdered dried leaves from whole plant *A. annua* and showed that artemisinin was more effective at entering the serum than the pure drug alone. There was a greater than 40-fold increase of artemisinin (Weathers et al., 2011). More recently, Weathers et al. (2014a) showed that ingestion of plant material with artemisinin facilitated a higher artemisinin blood content (2.44 mg/L) than ingesting artemisinin alone (undetectable). *A. annua* artemisinin oral delivery doubled the blood serum level to 4.33 mg/L from that of artemisinin with vegetarian mouse chow, 2.44 mg/L. Those results suggested that the increased artemisinin

serum concentration in mice given oral *A. annua* may be due to synergistic chemicals that occur naturally in the plant and inhibit degradation and/or aid in the transport of artemisinin throughout the process of digestion (Weathers et al., 2014a). Synergistic chemicals have the ability to inhibit or enhance the transport of other compounds, so it is a matter of discovering which compounds facilitate maximal artemisinin bioavailability (Adithan, 2005).

1.4 Composition of *Artemisia annua*

Within organisms, there are various primary and secondary metabolites (intermediates and products of metabolism) that result from various internal and external cues, and are necessary for isolating amino acids and acquiring vaccines and antibiotics (Boundless, 2014). Primary metabolites are vital to growth, development, reproduction, and regulation of normal body functions of a given organism. Examples of primary metabolites include carbohydrates, lipids, and proteins. Secondary metabolites are organic compounds found necessary for defense, immune response, and pigmentation (Boundless, 2014). Secondary metabolites are grouped based on chemical structure, composition, solubility, or biosynthetic pathway (Crozier et al., 2006). The three main groups are terpenes, phenolics, and nitrogen-containing compounds (Crozier et al., 2006). Terpenes encompass monoterpenes, sesquiterpenes, diterpenes, triterpenoids, terpene polymers, and sterols. The class of phenolics includes phenolic acids, coumarins, lignans, flavonoids, tannins, and lignans (Humphrey & Beale, 2006). Alkaloids are common nitrogen-containing phenolic compounds.

A. annua is a green, aromatic plant rich in terpenes, phenols, acetylenes, coumarins and flavonoids (Carbonara et al., 2012). The wild type plants generally grow 30-100 cm, but cultivated plants can be as tall as 2 m (Figure 7; World Health Organization, 2006). The plant is ideal for drug production because *A. annua* has versatile growth requirements allowing for easy cultivation in many different types of environments including temperate, cool temperate, and subtropical regions (World Health Organization, 2006).

Figure 7: A young *Artemisia annua* plant viewed from the top. (taken in Mozambique by Ton Rulkens; Source: http://commons.wikimedia.org/wiki/File:Natural_anti-malarial_(4738072658).jpg)

Also known as annual or sweet wormwood and *qinghao*, *A. annua* has been recently distributed worldwide and naturalized in many countries (Ferreira & Janick, 1995; Willcox et al., 2004). These regions extend into Europe and North America (Klayman, 1993). The *Artemisia* genus belongs to the Compositae or Asteraceae family. The growth cycle of *A. annua* is as follows: seedling, branching, flower-budding, fruiting, and senescence; the length of each phase is greatly affected by cultivation techniques and location (World Health Organization, 2006). The biomass of plant material will continue to increase until just before flower-budding, at which point biomass will decrease until senesced. The optimal growing temperature is 20-25ºC and distribution occurs in a wide range from hillsides, forests edges, and wastelands (World Health Organization, 2006). *A. annua* grows well in soil with 4.5-8.5 pH, proper drainage, and optimal nitrogen, phosphate, and potassium. The plant is quite adaptable to both drought and flooding. The range of artemisinin content varies greatly from plant to plant, with the highest amount of artemisinin at 1-2% of dry weight of leaves (World Health Organization, 2006). To yield optimal levels of artemisinin in the plant, weather conditions, cultivation methods, and harvesting techniques must be evaluated.

Artemisinin is manufactured and stored in the glandular secretory trichomes present in *A. annua* (Duke et al., 1994). Concentrations of the antimalarial can vary depending on cultivar, growing conditions, and stage of development, but highest concentrations are just prior to flowering (Towler & Weathers, 2015; Ferraira & Janick, 1995). Glandular trichomes are located on various surfaces of plants, including *A. annua*, such as on the leaves, stems, and floral buds (Kelsey &

Shafizadeh, 1980; Slone & Kelsey, 1985; Ferreira & Janick, 1995). Artemisinin is not the only antimalarial compound present in *A. annua* (Table 1).

Although concentrations of chemicals found in *A. annua* cultivars vary depending on geographical origin and growing conditions (Ćavar et al., 2011), the range of compounds in *A. annua* includes at least 28 monoterpenes, 30 sesquiterpenes (including artemisinin), 12 triterpeniods/steroids, 36 flavonoids, 7 coumarins, 4 aromatic, 9 aliphatic compounds (Bhakuni et al., 2002). The plant is also rich in phenolics, mainly caffeic acids, that have been found to have a role in enhancing artemisinin solubility and extraction efficiency when used in water, as well as suspected to participate in a wide range of medicinal remedies including hemorrhoids and fevers (Carbonara et al., 2012; de Magalhães et al., 2012). In a study involving plant composition analysis of extracts from dried leaves of *A. annua* cultivars Bra-BRA, Bra-L, and Lux-L, four phenolic compounds were detected after a water extraction: chlorogenic acid (Bra-BRA: 61 µg/mL, Bra-L: 39 µg/mL, Lux-L: 34 µg/mL), isoquercetin (Bra-BRA: 2.5 µg/mL, Bra-L: 0.9 µg/mL, Lux-L: 1.2 µg/mL), scopoletin (Bra-BRA: 24 µg/mL, Bra-L: 18 µg/mL, Lux-L: 7.6 µg/mL), and rosmarinic acid (Bra-BRA: 566 µg/mL, Bra-L: 243 µg/mL, Lux-L: 73 µg/mL), where chlorogenic and rosmarinic acid (Figure 8) were found to be in the highest quantity (de Magalhães et al., 2012).

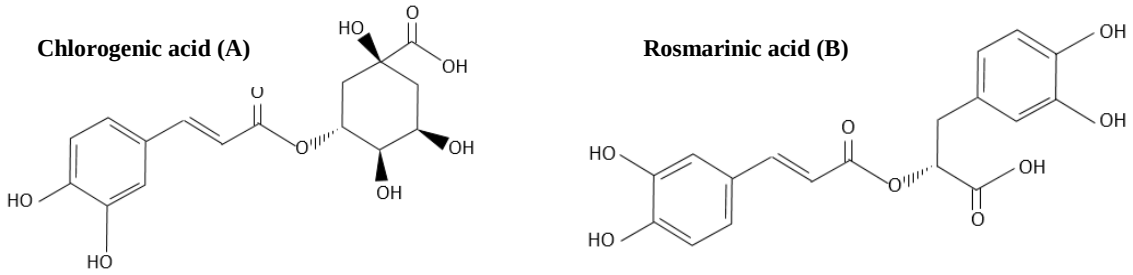


Figure 8: Two phenolic acids present in *A. annua*

Stressful environments, such as drought and pathogens, affect the production of phenolic compounds, so there could be significant variability in quantity among plants (de Magalhães et al., 2012). Weathers & Towler (2014) showed that the composition of plant material changes when dried and processed into tablets affecting the amount of phytochemicals present in the final product. The chlorogenic acid in fresh as well as dried plant material of *A. annua* SAM (both in the form of powder and tablets) revealed that chlorogenic acid was present in all forms of the

plant material, which indicates that if it assists transport, it would do so in all forms, at least in this cultivar (Weathers & Towler, 2014). When preparing dried compressed leaves into compressed tablets, chlorogenic acid initially comprised about 0.6% towards the dry weight of the mature leaves, lost about 90% of weight with drying, but nearly regains its original amount after the tablet is manufactured. Although the amount varied, chlorogenic acid measured in dried and fresh *A. annua* leaves was constant. In compressed dried leaf tablets there was about 5 mg/g dry weight (Weathers & Towler, 2014).

Suberu et al. (2013) studied the antiplasmodial activity of various hot water extracts of *A. annua* on chloroquine resistant and sensitive strains of *P. falciparum*. Rosmarinic acid seemed to synergize with artemisinin at a ratio of 1:3 and to have an IC_{50} (the value at which 50% of the parasite viability is decreased) of approximately 65 μM (Table 1). This was the 2nd and 3rd highest concentration of the phytochemicals present in the *A. annua* SAM cultivar, needed to inhibit growth of CQ resistant and sensitive *P. falciparum*, respectively (Table 1). Rosmarinic acid has low bioavailability as determined by low permeation rate (~0.2 μL/cm^2 after 40 min.) and is not susceptible to hydrolysis by intestinal cells, implying that it can stay at relatively high concentration in its native form (Konishi & Kobayashi, 2005; de Magalhães et al., 2012).

Chlorogenic acid, quinic acid (5-caffeoylquinic acid), is a phenolic compound that, when compared to other compounds, is found in relatively large quantities in *A. annua* (Rice-Evans et al., 1996). A relatively large portion of ingested chlorogenic acid (33%) was found to readily absorb into the bloodstream (Olthof et al., 2001). The amount that was not absorbed was found to be excreted mainly through the colon with less into the urine. Based on this information, it is likely that chlorogenic acid has biological effects throughout the body including high antioxidant properties (Olthof et al., 2001; Niggeweg et al., 2004). Chlorogenic acid has also been found to inhibit N-nitrosation reactions *in vitro* (Kono et al., 1995). Nitrosation refers to a reaction in which organic molecules are changed into compounds that are considered to be nitroso derivatives – a NO (nitroso) functional group attached to an organic group (Wang et al., 2002). These reactions typically produce mutations in tissue formation, resulting in cancerous cell accumulation. This research indicates that the presence of chlorogenic acid may also aid in the eradication of malaria, since it is found in *A. annua*. Whether this compound assists the transport

of artemisinin or enhances its antimalarial properties is still unknown. However, chlorogenic acid's powerful nature indicates that it should be explored closely when examining the therapeutic potential of this plant.

Rosmarinic acid has potent antioxidant activities and if present along with other compounds can account for the high antioxidant capacity of many plants (de Magalhães et al., 2012). Rosmarinic acid has inhibited liver damage from bacterial inflammation, inhibited nervous inflammation, and reduced oral inflammation (Qiang et al., 2011). Rosmarinic acid was only found in the budding stage of the *A. annua* SAM cultivar; it was not present in leaf powder or tablets which may indicate that it is burned off in the drying process (Weathers & Towler 2014).

Flavonoids are phytochemicals that contain two aromatic rings linked with a 3-carbon bridge (Figure 9). They function to protect the plant against pathogens and regulate growth. In humans, these compounds have been studied for their anti-inflammatory, antiviral, antioxidant, antitumor and antimicrobial properties (Feng et al., 2012; Wu et al., 2010; Bitis et al., 2010; Chang-Wi & Cheng-Bin, 2014; Xiaowei & D'Souza, 2013). More than 40 flavonoids have been identified in *A. annua* (Table 2; Ferreira et al., 2010). Of these 40, casticin (Figure 9) and artemetin were shown to decrease the parasite transport mechanism in human and murine malaria (Elford et al., 1987). Lehane & Saliba (2008) studied the antiplasmodial activity of 11 flavonoids found in various plants. Of these 11 flavonoids, kaempferol, myricetin, quercetin, and luteolin are also found in *A. annua* SAM (Figure 9; Towler & Weathers, 2015). Luteolin had an IC_{50} of 12 µM in chloroquine resistant *P. falciparum* (Table 1), which is a median concentration compared to the other phytochemicals present in *A. annua* SAM. Luteolin inhibits parasite growth by preventing the life cycle from being completed (Lehane & Saliba, 2008). The antimalarial mechanisms of kaempferol, myricetin, and quercetin have not been determined, but they do inhibit 50% of parasite growth at concentrations of 25 µM, 76 µM, and 14 µM, respectively, in chloroquine-resistant strains.

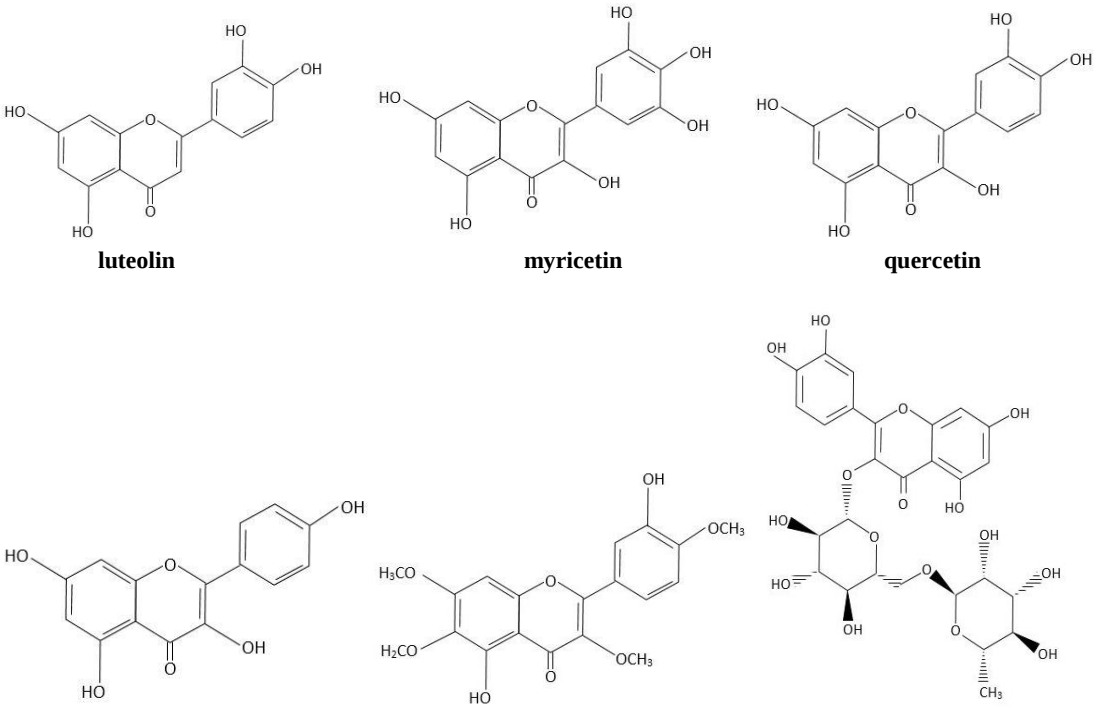


Figure 9: Structures of several flavonoids found in *A. annua*

Elford et al. (1987) used a chloroquine-resistant clone of late ring stage *P. falciparum* to study the antimalarial activity of artemisinin in combination with casticin. In concentrations greater than 10 µM, casticin alone had significant antimalarial activity. Artemisinin antimalarial activity was increased 3-5 fold when 5 µM of casticin was combined with artemisinin (Elford et al., 1987). Because the concentration of casticin added to artemisinin was less than the IC_{50} for casticin, the increased antimalarial activity of artemisinin is most likely due to synergism with casticin, not an addictive effect of casticin's antimalarial activity.

Artemisinic acid and arteannuin B both have antibacterial and antifungal properties and may be found in higher concentrations in some cultivars *A. annua* than artemisinin. Also, essential oils from different plants are similar in composition and are reported to contain antiplasmodial activity (Milhau et al., 1997; Fujisaki et al., 2012). The principle constituents of plant essential oils are hydrophobic and include monoterpenes, some of which are present in *A. annua* (Boyom et al., 2003). The oxygenated monoterpene, camphor, found in *A. annua,* may comprise as much as 43.5% of the chemical content of the essential oil (Juteau et al., 2002). Additionally, Cherneva

et al. (2012) studied the effects of camphor on thymocyte cultured cells finding that camphor increased thymocyte viability at a concentration of 50 μg/mL. The thymus is partially responsible for malaria immunity through the production of T-cells (Roberts et al., 1977), so increased thymocyte viability by camphor could reduce recrudescence in *A. annua* treated individuals. Because this monoterpene may comprise a large proportion of the essential oil in *A. annua*, camphor indirectly may contribute to the antimalarial activity of the plant. If camphor has a high bioavailability, as determined in transport through intestinal epithelial cells, low recrudescence rates in whole leaf *A. annua* treatment of malaria may be explained (Figure 10).

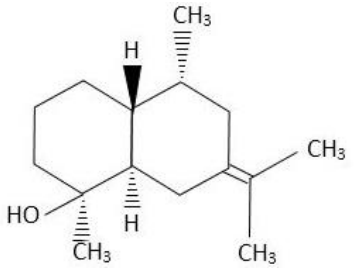


Figure 10: Structure of camphor

1.5 Digestion and Drug Metabolism

Artemisinin has low solubility in water and oil which requires oral or rectal administration (Golenser et al., 2006). Sometimes, oral administration becomes difficult for patients with severe malaria, requiring a water-soluble derivative (artesunate or artelinate) or an oil-soluble derivative (artemether and arteether) of artemisinin, often given through parenteral or intrarectal routes. Artemisinin bioavailability is thwarted by the metabolism of artemisinin in the human liver by the cytochrome P450 enzymes, CYP2C19, CYP3A4, and CYP2B6, where metabolism by CYP2B6 is most prevalent (Svensson & Ashton, 1999). This process is associated with first pass drug metabolism, and converts artemisinin into the secondary metabolites: deoxyartemisinin, deoxydihydroartemisinin, 9,10-dihydrodeoxyartemisinin, and crystal 7 (de Magalhães et al., 2012; Carbonara et al., 2012; Lee & Hufford, 1990). This information is pertinent in accounting for total artemisinin metabolism in the body.

During digestion, maximum absorption takes place in the small intestine. The wall of the small intestine is comprised of a single layer of epithelial cells (enterocytes), and is responsible for monitoring and limiting the absorption of nutrients and drugs (Hubatsch et al., 2007). There are four modes of transport of compounds across enterocytes into serum. These include: passive transcellular, passive intracellular, carrier-mediated, and transcytosis (Figure 2; Artursson et al., 1996). Passive transcellular transport is used by drugs that are hydrophilic and distribute readily into the cell membranes of the intestinal epithelium. Hydrophobic drugs have an incomplete and slow diffusion across intestinal epithelium, which necessitates passage through the water-filled pores of the passive intracellular transport (Artursson et al., 1996). When compounds mimic the structure of essential nutrients (e.g. amino acids, sugars, and peptides), the compound is actively transported using carrier-mediation by cellular carrier proteins (dipeptide carriers and P-glycoprotein). An example of carrier-mediation would be the sodium potassium pump. Transcytosis is also an active transport mechanism that usually occurs via membrane vesicles with macromolecules, such as insulin and transferrin.

In transport studies, an apparent permeability value (P_{app}) is often calculated in order to compare the results from different experiments. In 1996, Augustijns et al. conducted an experiment to measure the transport permeability of artemisinin, using a Caco-2 cell system, and obtained a P_{app} value of 30.4 X 10^6 cm/s at 37ºC. When sodium azide was added, artemisinin transport was not affected indicating that the transport mechanism was likely not active transport, but instead probably passive transcellular transport. Passive transcellular transport is the most common drug permeation route in the intestine, so the mode of transport for artemisinin is easily modeled in Caco-2 cells. This drug transport makes sense because artemisinin is hydrophobic, which easily allows permeation through the lipid bilayer of intestinal epithelial cells.

1.6 Caco-2 Model System for Transport Experiments

The human epithelial colorectal adenocarcinoma (Caco-2) continuous cell line is a commonly-used and FDA-approved immortalized cell model system employed to mimic the intestinal functions of the intestinal cells: absorption, metabolism, and bioavailability of nutrients and drugs (Hubatsch et al., 2007; Natoli et al., 2011). Caco-2 cells can differentiate spontaneously in

culture without the aid of differentiating factors, yet many functions of the small intestinal villi remain functional (Figure 11; Hubatsch et al., 2007). The intestinal properties of Caco-2 cells can vary, however, depending on the passage number, time in culture, origin of cell line, extracellular support, and cell culture media, so these variables should be minimized or otherwise controlled (Artursson et al., 1996).

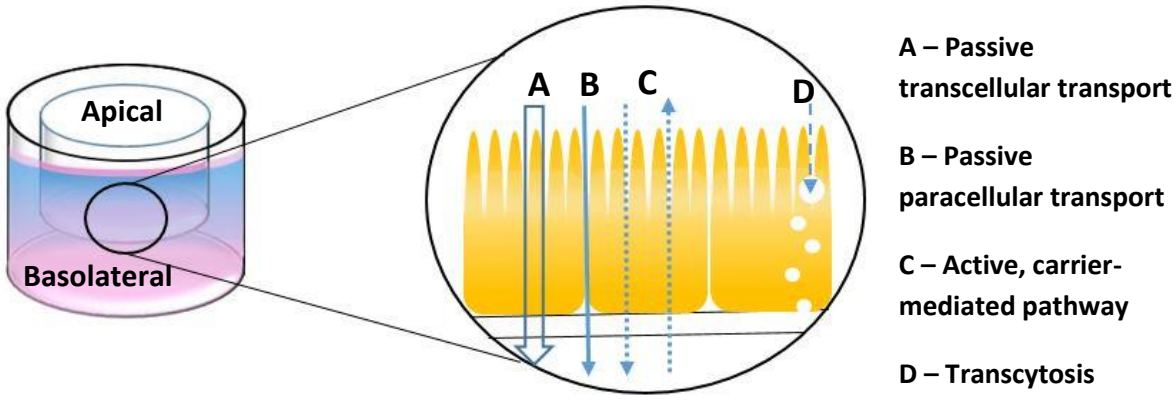


Figure 11: Transport mechanisms of Caco-2 cells

Caco-2 cells are adherent cells that attach and grow on semi-permeable filters forming a confluent monolayer with tight junctions to facilitate transport, and are thus considered the gold standard for *in vitro* simulation of monolayers (Figure 11; Hubatsch et al., 2007; Natoli et al., 2012). According to Artursson et al. (1996), Caco-2 cells are able to model all four types of transport across the intestinal epithelial layer making them a great choice for *in vitro* toxicology experiments (Figure 11). These cells also express most of the enzymes and carrier systems that intestinal mucosa have *in vitro*, including CYP3A4 (Augustijns et al., 1996). Therefore, they are ideal for studying small intestinal absorption.

For use in transport studies, Caco-2 cells are seeded on to transwell permeable filter hanging well inserts placed into a well in a multi-well plate. After a proper growing period on the filters, 21-29 days, the Caco-2 cells model the intestinal lumen with the apical side represented by the inner well and the basolateral side by the outer well (Figure 11; Hubatsch et al., 2007). In a transport study compounds of interest can be added to either the apical or basolateral side of the cells. Then the experiments are run for a designated amount of time during which the concentration of the compound is measured on both sides. By running transport experiments with this *in vitro*

model system, the P_{app} can be determined and then compared to that of other molecules (Augustijns et al., 1996).

1.7 Intestinal Transport of Flavonoids

Quercetin, considered toxic in concentrations higher than 200–1200 mg, may be conjugated to glucuronides during intestinal absorption to assist in detoxification (Harwood et al., 2007; Murota & Terao, 2003). However, quercetin glucosides are not absorbed intestinally and are first hydrolyzed to quercetin (Walle et al, 2000). Lactate phlorizin hydrolase is a common membrane bound enzyme in the intestinal lumen which has been shown to hydrolyze quercetin glucosides (Day et al., 2000). The flavonoid, rutin, was one quercetin glucoside that was not hydrolyzed by lactate phlorizin hydrolase (Day et al., 2000). Furthermore, rutin absorption may be inhibited by the p-glycoprotein and multidrug resistant proteins localized in apical and basolateral membranes of the intestine, as well as in Caco-2 differentiated cultures (Zhang et al., 2013). These transmembrane proteins may be responsible for the excretion of rutin from the serum supported by higher basolateral to apical than apical to basolateral transport (Zhang et al., 2013).

1.8 Intestinal Transport of Phenolic Acids

Konishi & Kobayashi (2004) reported that monocarboxylic acid transporters are responsible for the transport of some phenolic acids across Caco-2 cells. Most orally consumed chlorogenic acid reaches the colon fully intact (Konishi & Kobayashi, 2004); in the colon it is hydrolyzed into caffeic acid. Konishi & Kobayashi (2004) studied the transport of chlorogenic and caffeic acid in Caco-2 cells, and found that chlorogenic acids exhibit non-saturable transport across a Caco-2 cell monolayer through passive paracellular diffusion (Figure 11B). This suggested that once chlorogenic acid crosses the intestinal epithelial layer, it will not be transported back into the intestinal lumen.

Chlorogenic acid has been shown to upregulate CYP34A activity depending on concentration. At the lowest concentration (0.1 μM), chlorogenic acid showed the greatest CYP34A induction;

at moderate concentrations (1, 2, 10 μM) the induction was decreased, and at the highest concentration (20 μM) CYP34A activity increased slightly (Li et al., 2010). Konishi & Kobayashi (2005) studied transepithelial transport of rosmarinic acid. Similar to chlorogenic acid, when the proton gradient was set so that the apical side had a pH of 6 and the basolateral side a pH of 7.4, the transport in either direction across the cells was similar. Unlike chlorogenic acid, when the proton gradient was removed, rosmarinic acid still exhibited similar transport in both directions, apical to basolateral and the reverse, basolateral to apical. Because the cell transport of rosmarinic acid decreased as the tightness of intercellular junctions increased, it was thought to permeate by paracellular pathways (Figure 11B), similar to chlorogenic acid (Konishi & Kobayashi, 2005). Studies performed by Qiang et al. (2011) concluded that rosmarinic acid is transported by both passive transcellular (Figure 11A) and paracellular routes (Figure 11B).

Studying the role of phenolic acids in assisting artemisinin transport across the Caco-2 cell monolayer may provide insight into how these compounds function regarding artemisinin transport into the serum. Further experimentation with varying phenolic acid concentrations may yield different results, such that at one concentration artemisinin may be inhibited while at another it may be increased.

1.9 Intestinal Transport of Monoterpenes

Monoterpenes are organic compounds typically formed in plants (Hylemon & Harder, 1999). They can be toxic to cells of herbivores and serve as a defensive mechanism for the plant (Gershenzon & Croteau, 1992). This is often accomplished by the inhibition of digestive enzymes that prevent sufficient nutrient absorption, causing the animal to become weak and unable to properly function (DeGabriel et al., 2009). Additionally, monoterpenes have been found to inhibit acetylcholinesterase, which is involved in neurotransmission (Perry et al., 2000; Miyazawa et al., 1997). As a response, herbivores have developed their own defensive mechanisms to combat the harmful effects of monoterpenes and other plant secondary metabolites. Some of these mechanisms include stomach and intestinal pH regulation and the production of surfactants that prevent interactions between these metabolites and digestive

enzymes (Berenbaum, 1980; Martin & Martin, 1984). However, there is also research that suggests that >95% of consumed monoterpenes are absorbed through the wall of the intestines and therefore do not interact at all with these enzymes (Boyle et al., 1999; Foley et al., 1987; Sorensen et al., 2004; Shipley et al., 2012).

In a study by Kohl et al. (2015), the intestinal contents of Greater Sage-Grouse (avian herbivores) and chickens were examined for remains of monoterpenes and their effects on the inhibition of various digestive enzymes. Monoterpenes, including borneol, 1,8-cineole, and camphor, were found responsible for inhibiting the digestive enzymes of both species of birds. Specifically, the digestive enzyme, aminopeptidase-N (APN), was found to be inhibited by the presence of monoterpenes. APN is an important enzyme because it allows absorption of nutrients by cleaving the terminal amino acids that are attached to proteins during digestion (Sjöström et al., 2002). Contrary to this finding however, the monoterpene, β-pinene, increased digestive enzyme activity in chickens (Kohl et al., 2015). The larger implications of this study suggest that the presence of specific monoterpenes may inhibit or enhance the ability of proteins to be digested by birds (Kohl et al., 2015). Additionally, this research brings to question the effects that the presence of monoterpenes could have on intestinal transport of artemisinin using the Caco-2 model.

2.0 Aims and Hypotheses

Based on the evidence that orally consumed dried *A. annua* leaves were therapeutically successful in humans and animal models, our primary aim was to determine how many of the major phytochemicals in *A. annua* assisted in antimalarial activity by increasing the bioavailability of artemisinin, the primary plant antimalarial. We hypothesized that when rutin and quercetin were used in the transportation experiments, the results achieved would be the same as the previous MQP group's results with increased artemisinin transport. For the transport experiments with chlorogenic acid and rosmarinic acid, we hypothesized that the bioavailability of artemisinin would be increased. For the concentration series experiment with chlorogenic acid, we predicted that an increased amount of chlorogenic acid would increase the bioavailability of artemisinin.

Our major goals included:

1. Demonstrating reproducibility of the previous Caco-2 study that measured artemisinin permeability with or without quercetin or rutin.
2. Calculating the P_{app} of artemisinin with or without rosmarinic and chlorogenic acid.
3. Measuring the effect of varying concentrations of chlorogenic acid on artemisinin transport.
4. Calculating the P_{app} of artemisinin with or without camphor.
5. Measuring the effect of varying concentrations of camphor on artemisinin transport.

3.0 Methods

These methods were adapted from Natoli et al. (2012), Hubatsch et al. (2007), and developed also from an earlier study by Harten, et al. (2014). The drug transport study procedure has been adapted from Augustijns, et al. (1996) and Hubatsch, et al. (2007).

3.1 Materials

Caco-2 cultured cells were obtained from the American Type Culture Collection (passage number 18-49; Manassas, VA). Fetal Bovine Serum (FBS; cat #: S162H) was purchased from Biowest (Nuaillé, France). Hank's Balanced Salt Solution (HBSS; cat #: 14175-079), penicillin/streptomycin, and Dulbecco's Modified Eagle's Medium (DMEM + GlutaMAX, 4.5 g/L Glucose, 110 mg/L Sodium Pyruvate; cat #: 10569-010) were all obtained from Gibco. The 12-well plates were purchased from Corning (Corning, NY; cat #: 3512), and transwell inserts with 0.4 μm polycarbonate membranes were purchased from Greiner Bio-One (Monroe, NC; cat #: 665640). TrypLE (cat #: 12604-021) and trypan blue were obtained from Gibco (Grand Island, NY).

3.2 Caco-2 Cell Culture

Stock Caco-2 cells were grown in medium (containing 79% DMEM + GlutaMAX, 1% penicillin/streptomycin, and 20% FBS) up to 90% confluence at 37 ˚C in 5% CO_2. Medium was changed every 2 days for 3-5 passages. Cells were passaged at 50-90% confluence, ideal to minimize passages and cell line mutation. Passaging occurred 2-5 days after plating cells at an average known density of 2 X 10^6 per T75 (75mm^2) flask. Cells were split by treatment with 4mL of an HBSS wash (~1 minute at room temperature in the cell culture hood) and 4mL of TrypLE (15 minutes at 37 ˚C in 5% CO_2). All cells used for transport experiments were between 9 and 50 passages and were >95% viable as determined through a 1:1 (0.05 mL cell suspension, 0.05 mL trypan blue) trypan blue cell count, in which blue cells were dead. Cells were seeded at

a density of 1 X $10^5/cm^2$ in each of 12 transwell filters (diameter of 13.85 mm^2), and maintained by standard Caco-2 cell culture techniques, changing media every 1-2 days (Hubatsch et al., 2007).

3.3 Measuring Transepithelial Electrical Resistance

Caco-2 cells were grown on transwell inserts (0.4 μm pore size, Greiner Bio-One, Monroe, NC) for TEER measurement and transport experiments. Cells were seeded at a density of 1 X $10^5/cm^2$ and became confluent after about 21-29 days post inoculation. At about 21-29 days, cells formed tight junctions and differentiated into functional villi. Media were changed every 2 days. An $EVOM^2$ TEER probe (Worlds Precision Instruments, Sarasota, FL) was used according to the manufacturer's instructions to measure the tight junction integrity of the monolayer. A TEER >250 Ωcm^2 indicated that cell monolayers were ready for transport experiments (Equation 1).

Equation 1: Transepithelial Electrical Resistance

$$TEER = (R - RB) \times A$$

where R is resistance in Ω of filter and cells, RB is resistance in Ω of filter, and

A is growth area of the filter in cm^2.

3.4 Transport Donor Solutions

Two phenolic acids, rosmarinic acid (≥98%; Sigma Aldrich, cat#: R4033) and chlorogenic acid (≥95%; Sigma Aldrich, cat#: C3878) were investigated, as well as two flavonoids, quercetin (≥95%; Sigma Aldrich, cat#: 1001419342) and rutin, and monoterpene camphor (≥98%; Sigma Aldrich, cat#: 1001150215) to determine their effects on the bioavailability of artemisinin (≥98%; Sigma Aldrich, cat#: 1001496854) as it crosses the intestinal epithelium. Tables 3-6 display the concentrations that were used to create the donor solutions for all transport studies. These tables show the moles (μmol) molarity (μM) and mass (μg) added to each hanging well in the transwell plate. Molar ratios were chosen based on the relative concentrations found in the

SAM cultivar of *A. annua* (Table 3-6; Table A1, Appendix A). Volumes and artemisinin concentration were based on the information provided in the previous Caco-2 MQP from 2014 to enable experimental comparison. P_{app}s for artemisinin were calculated and compared once a line graph of concentration over time was plotted after the experimental results were received (Equation 2; Sample Calculation 1).

Equation 2: P_{app} Calculation. Where ΔQ is the cumulative change in µg of artemisinin from apical to basolateral side of monolayer, Δt is the change in time in seconds, C_0 is the initial concentration of artemisinin (µg/mL) in the donor solution, and A is surface area of the monolayer (cm^2).

$$P_{app} = \frac{\Delta Q}{\Delta t A C_0}$$

Sample Calculation 1: Calculation of P_{app} with the artemisinin sample 1 at 60 minutes as an example. The P_{app} is a measurement of rate of transport which takes into account culture growth surface area as well as the initial concentration of artemisinin. The units are cm/s because mL of water converts to cm^3which cancels out the cm^2, and the µg from initial concentration and the measured cumulative artemisinin at 60 minutes also cancel. Given that the transfer solution is mostly water, the units of P_{app} are cm/s.

$$P_{app} = \frac{\Delta Q}{\Delta t A C_0} \rightarrow \frac{\Delta Q}{\Delta t} \times \frac{1}{AC_0} \rightarrow \frac{\Delta Q}{\Delta t} / AC_0$$

$$\text{where } \frac{\Delta Q}{\Delta t} \text{ is the permeability rate at } \frac{\mu g}{sec}$$

$$A \text{ is the surface area of the monolayer in } cm^2$$

$$\text{and } C_0 \text{ is the initial concentration of AN at } \mu g/mL$$

$$\frac{\Delta Q}{\Delta t} = (4.763\ \mu g \text{ cumulative over 60 min.})/(3600 \text{ sec. of transport})$$

$$AC_0 = (1.12\ cm^2 \text{ surface area acording to corning}) \times (17.84\ \mu g/mL)$$

$$P_{app} = \frac{4.763\ \mu g}{3600 sec.} / (1.12\ cm^2) \times (17.84\ \mu g/mL) = 6.62 \times 10^{-5}\ cm/sec$$

Table 3: Experiment 1 Reagent amounts for artemisinin (AN) ± quercetin (Q) and rutin (R) per transwell												
Reagent	AN alone				AN + Q 2:1				AN + R 2:1			
	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM
AN	12.5	0.089	25	177	12.5	0.089	25	177	12.5	0.089	25	177
Q	-	-	-	-	12.5	0.049	15	99	-	-	-	-
R	-	-	-	-	-	-	-	-	12.5	0.049	30.1	98
EtOH (70%)	12.5	-	-	-	-	-	-	-	-	-	-	-
HBSS	475	-	-	-	475	-	-	-	475	-	-	-
Basolateral side always contained 1.5 mL HBSS. AN stock solution = 7.08 mM, Q stock solution = 3.97 mM, R stock solution = 3.95 mM												

Table 4: Experiment 2 Reagent amounts for AN ± chlorogenic acid (CA) and rosmarinic acid (RA) per transwell																
Reagent	AN alone				AN + CA 1:1.1				AN + RA 1:1.1				AN + (CA & RA) 1:1.1			
	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM
AN	12.5	0.089	25	177	12.5	0.089	25	177	12.5	0.089	25	177	12.5	0.089	25	177
CA	-	-	-	-	12.5	0.098	34.7	196	-	-	-	-	6.25	0.049	17.4	98
RA	-	-	-	-	-	-	-	-	12.5	0.099	35.7	198	6.25	0.049	17.8	99
EtOH (70%)	12.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HBSS	475	-	-	-	475	-	-	-	475	-	-	-	475	-	-	-
Basolateral side always contained 1.5 mL HBSS. AN stock solution = 7.08 mM, CA stock solution = 7.84 mM, RA stock solution = 7.92 mM																

Table 5: Experiment 3 Reagent amounts for AN ± CA concentration series per transwell																
Reagent	AN alone				AN + CA 1:1				AN + CA 2:1				AN + CA 3:1			
	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM
AN	12.5	0.089	24.7	175	12.5	0.089	24.7	175	12.5	0.089	24.7	175	12.5	0.089	24.7	175
CA	-	-	-	-	12.5	0.098	31	175	6.25	0.049	15.5	87.5	4.2	0.033	10.3	58.3
EtOH (70%)	12.5	-	-	-	-	-	-	-	6.25	-	-	-	8.30	-	-	-
HBSS	475	-	-	-	475	-	-	-	475	-	-	-	475	-	-	-
Basolateral side always contained 1.5 mL HBSS. AN stock solution = 7.0 mM, CA stock solution = 7.0 mM																

Table 6: Experiment 4 Reagent amounts for AN ± camphor (C) per transwell in concentration series																
Reagent	AN alone				AN + C 1:1				AN + C 2:1				AN + C 10:1			
	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM	µL stock	µmol	µg	µM
AN	12.5	0.089	25	177	12.5	0.089	25	177	12.5	0.089	25	177	12.5	0.089	25	177
C	-	-	-	-	12.5	0.088	13.32	177	6.25	0.044	6.66	88.5	1.26	0.009	1.35	17.7
EtOH (70%)	12.5	-	-	-	-	-	-	-	6.25	-	-	-	11.24	-	-	-
HBSS	475	-	-	-	475	-	-	-	475	-	-	-	475	-	-	-
Total volume in apical was 0.5 mL Basolateral side always contained 1.5 mL HBSS. AN stock solution = 7.0 mM, C stock solution = 7.0 mM																

3.5 Transepithelial Transport Experiments and Extractions

To measure the change in transport of artemisinin (apical to basolateral), a 1.5 mL solution of HBSS was added into the well of a 12-well transwell plate (basolateral side), and 0.5 mL of solution containing the appropriate phytochemical(s) was added into the transwell insert (apical side) (see Tables 3-6 for appropriate chemical concentrations added to each well for each experiment). The 12-well plate was incubated at 37°C for a total of 60 minutes; at every 15 minutes, the transwell inserts were transferred to a new well plate containing 1.5 mL HBSS [in basolateral compartment] in each well to maintain sink conditions. All basolateral solutions remaining in the wells were immediately extracted with equal parts methylene chloride. Remaining apical solutions were extracted only after the completion of the 60 minute transport experiment. To determine artemisinin content within the cell layer, the monolayer was trypsinized, incubated (30-60 min. at 37°C), and then scraped off of the inserts and extracted. These solutions were dried with N_2 and stored at 4°C until further analysis.

3.6 Analysis of Artemisinin and Deoxyartemisinin

After the transport experiment was complete and all apical and basolateral solutions were extracted, samples were prepared for measurement of artemisinin and deoxyartemisinin by gas chromatography/mass spectrometry (GC/MS). Prior to analysis, the samples were thawed from the freezer and resuspended in a known volume (100-200 µL) of methylene chloride. This quantity was then transferred to each GC/MS vial. Each sample was then air dried using a small hand dryer. Immediately before GC/MS analysis, each sample was re-suspended in 50 µL of pentane and placed into the GC/MS for analysis. An Agilent Technologies GC/MS was used for this experiment. Conditions of the GC/MS were as follows: GC, Agilent 7890B; MS, Agilent 5977A; column Agilent HP-5MS (30 m X 0.25 mm X 0.25 µm); carrier gas, Heat 1 mL/min; injection volume, 1 µL in splitless mode; ion source temperature, 280^{o}C; inlet, 150^{o}C; oven temperature, 125^{o}C help for 1 min and then increased to 300^{o}C at 5^{o}C/min (Towler & Weathers, 2015).

To compare the bioavailability of artemisinin from the transport studies, the concentration values calculated from the GC/MS chromatograms were compiled into a histogram showing the average basolateral concentrations over 15 minute time periods. An ANOVA (0.05 = Statistical significance) was used to compare the statistical significance between the concentrations of artemisinin in the basolateral layer among the artemisinin samples and combination artemisinin/phytochemical samples. This allowed for us to determine whether the tested compounds significantly contributed to the transport of artemisinin across the Caco-2 cells. Averaged artemisinin amounts were displayed for each time point, as well as initial and final apical amounts and cellular amounts. The data from each 15 minute interval were summed and compiled into a line graph of artemisinin accumulation over time on the basolateral side of the well. Slopes were calculated and artemisinin concentrations statistically compared.

3.7 Statistical Methods

To bring quantitative significance to the data, a statistical analysis must was performed. All conditions were replicated at least three times per experiment, data averaged, and means tested for standard deviation as well as significance using ANOVA and Tukey-Kramer test as well as a paired student's t-test for unequal variances when warranted.

4.0 Results

Artemisinin, synthesized and stored within the glandular trichomes of *Artemisia annua*, is recognized as a prominent and potent antimalarial treatment that in murine studies showed that ingestion of dried *A. annua* leaves yielded higher levels of artemisinin in the bloodstream than the pure drug. The exact cause of this difference is unknown, but it is speculated that other compounds within the plant are leading to the increased bioavailability of artemisinin. The goal of this project was to investigate specific compounds found in the plant that may enhance artemisinin bioavailability. These compounds were: two flavonoids, quercetin and rutin; two phenolic acids, chlorogenic and rosmarinic acids; and the monoterpene, camphor. Using the intestinal Caco-2 cell model, drug transport studies were conducted across the monolayer from apical to basolateral side.

4.1 Transepithelial Electrical Resistance Validation of Caco-2 Monolayer Integrity

Prior to beginning a transport study, the presence and integrity of the monolayer's tight junctions in each well was tested using transepithelial electrical resistance (TEER). The TEER value of a transwell insert, without cells, was recorded to be 94 Ω. By subtracting 94 Ω from the TEER value (obtained per well) and multiplying that value by the area of the filter, the actual resistance of the Caco-2 monolayer was calculated. Crucially, the resistance values must be 250 $\Omega \cdot cm^2$ or greater, which is necessary for proper assessment of permeability (Appendix C: 1-4). All subsequent transport data are calculated using wells where the TEER value was 250 $\Omega \cdot cm^2$ or greater; any below 250 $\Omega \cdot cm^2$ were discarded. TEER was measured immediately following the extractions to control for damages to the monolayer caused by exposure to ethanol or orbital shaker (Appendix C: 1-4). In most cases, there was a slight decrease in TEER from the before and after recordings, but in some cases there was an increase. The differences may have been attributed to uneven monolayers or an un-calibrated TEER instrument.

4.2 Effect of Flavonoids

The quercetin and rutin transport experiments with artemisinin conducted in early 2014 were replicated in this project to determine experimental reproducibility. The transport solutions were selected such that the concentration of compound per transwell (µM) was two parts artemisinin and one part either quercetin or rutin. Artemisinin stock was 7.08 mM, with 8.86 X 10^{-2} µmol of artemisinin (25 µg) in each well. The quercetin stock was 3.97 mM with 4.9 X 10^{-2} µmol (15 µg) in each well, while the rutin stock was 3.95 mM with 4.9 X 10^{-2} µmol (30.1 µg) in each well. Additionally, the experimental procedure was modified from last year's group in that the Caco-2 cells were lysed and extracted for any retained artemisinin following the 60 minute transport. Figure 12 displays the average basolateral concentrations of artemisinin for each experimental condition at every time point. In addition, after the 60 minute transport study, artemisinin from the apical side was extracted and an average of 1.99 µg across all treatments was measured. Content of artemisinin remaining in the cells was also measured after the transport experiment and a mean of 0.13 µg was obtained across all treatments.

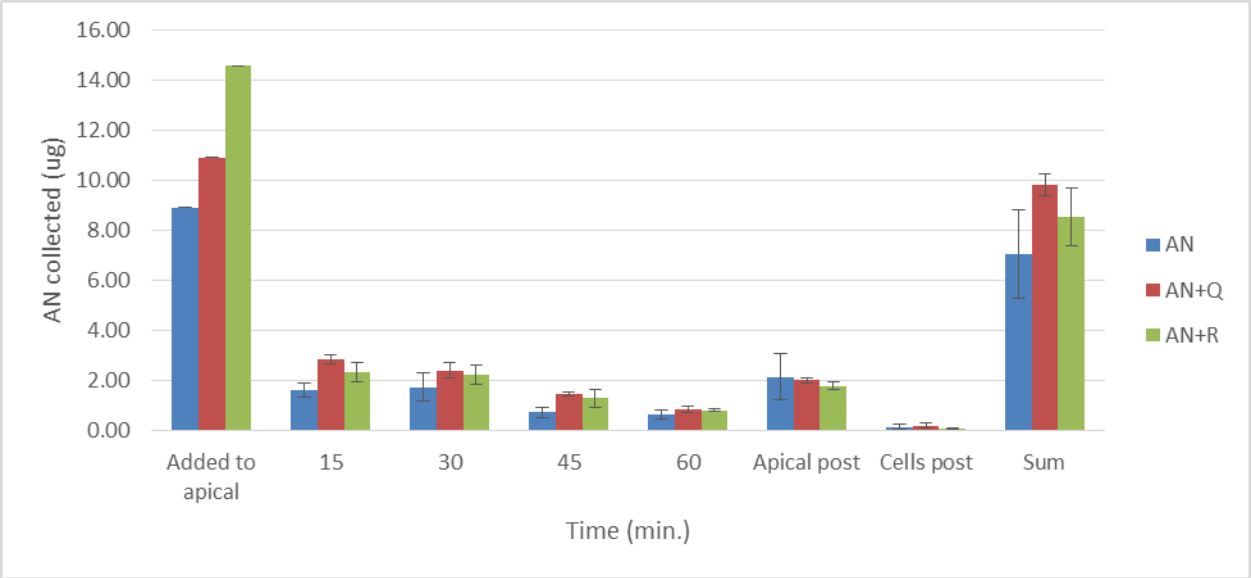


Figure 12: Average basolateral concentration of artemisinin over 60 minutes ± quercetin or rutin. The AN (artemisinin alone) treatment was not significantly different from either the AN + Q (artemisinin and quercetin) or AN + R (artemisinin and rutin) treatments (Table 5, Appendix C). Insignificant error bars in donor solution are due to one measurement. All other conditions have a 4 well replication. Sum refers to the total AN collected from 15-60 min as well as end point analysis of apical donor well (apical post) and cells (cells post).

Artemisinin transport was greatest after each of the first two 15 min transfers, declining by about 50% after the fourth transfer at 60 min (Figure 12). After 60 min the apical donor wells had about 12-25% of the original amount of donor well artemisinin. Extracted cells (cells post in Figure 12) had barely detectable amounts of artemisinin. When the cumulative amount of artemisinin transported was measured, it appeared that quercetin increased the transport of artemisinin, while rutin had no effect (Figure 13). However, the standard error was large in the artemisinin control as well as for artemisinin and rutin, suggesting that the values were not significantly different (Figure 13). Unfortunately, the initial artemisinin amounts added to the apical donor wells prior to the transport study were not equal (Figure 12), so comparisons between treatments is not possible. There was no significant artemisinin found in the Caco-2 cells at the end of the transport study demonstrating that they did not retain nor bind significant amounts of the drug (Figure 12).

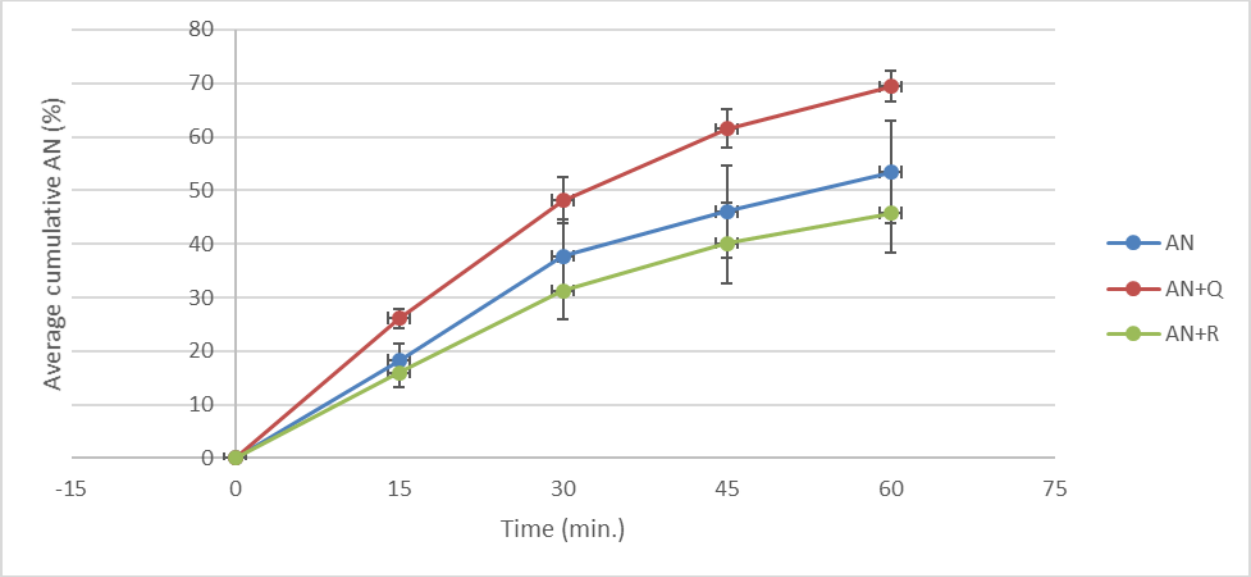


Figure 13: Cumulative artemisinin concentration at 15-minute time points for quercetin and rutin transport study. AN concentration is represented as a percentage of AN present in donor solution, added at time zero. Although the treatment of AN+Q has tight error bars (standard error) both one-way ANOVA and Student t-test with unequal variances show no statistical difference in the means of this treatment and AN control at the 60 minute completion (Table 5, Appendix C).

4.3 Effect of Phenolic Acids

Rosmarinic and chlorogenic acids were chosen as the second set of experimental phytochemicals. These phenolic acids have similar structures, and are both secondary metabolites typically found in *A. annua* with rosmarinic and chlorogenic acids of particular interest. A 1:1.1 molar ratio of phenolic acid to artemisinin was used for this experiment (Table 4). Artemisinin content in apical sides of each well was theoretically 0.089 µmoles (25 µg), and phenolic acid content was 0.098 µmoles (~35 µg) per well (Table 4). Four treatments were compared: artemisinin alone (control), artemisinin and chlorogenic acid, artemisinin and rosmarinic acid, and artemisinin, chlorogenic acid, and rosmarinic acid. Artemisinin transport was greatest after the first 15 min of the transport experiment, declining by ~75% at 60 min (Figure 14).

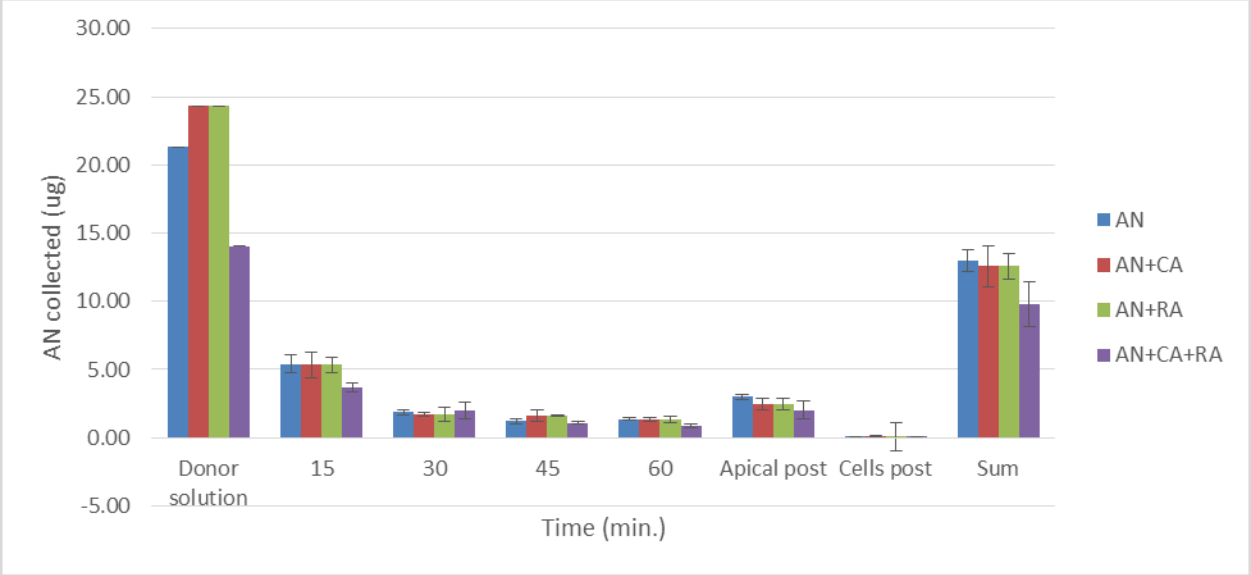


Figure 14: Average basolateral concentration of artemisinin when combined with phenolic acids over 60 minutes. Artemisinin alone (AN), artemisinin with chlorogenic acid (AN + CA), artemisinin with rosmarinic acid (AN + RA), and artemisinin with both chlorogenic and rosmarinic acid (AN + CA + RA) showed little difference in artemisinin transport in each time period (Table 6, Appendix C). Error bars represent the standard error of the mean. All means were taken from 3 well replicates except donor solutions in which only 1 aliquot was taken. Sum refers to the total AN collected from 15-60 min as well as end point analysis of apical donor well (apical post) and cells (cells post) samples.

In all treatments, the cumulative amount of artemisinin transported across the membrane increased with each 15 minute interval (Figure 15). Although Figure 15 shows almost 25% greater artemisinin transport with both phenolic acids than with either one alone, this transport is displayed as a percentage of the artemisinin measured in the donor solution. The total amount of

artemisinin transported, with artemisinin alone or combined with either phenolic acid averaged between 9.5-10 µg/mL. However, when artemisinin was combined with both phenolic acids, the total artemisinin amount was only about 7.5 µg/mL. Unfortunately donor solutions differed in artemisinin concentration between theoretical (25µg) and measured amounts of artemisinin in the control and that in both phenolic acid treatments (Figure 14). Therefore, the only two conditions that could be compared were artemisinin with chlorogenic acid, and artemisinin with rosmarinic acid which began with approximately 24.5 µg of artemisinin.

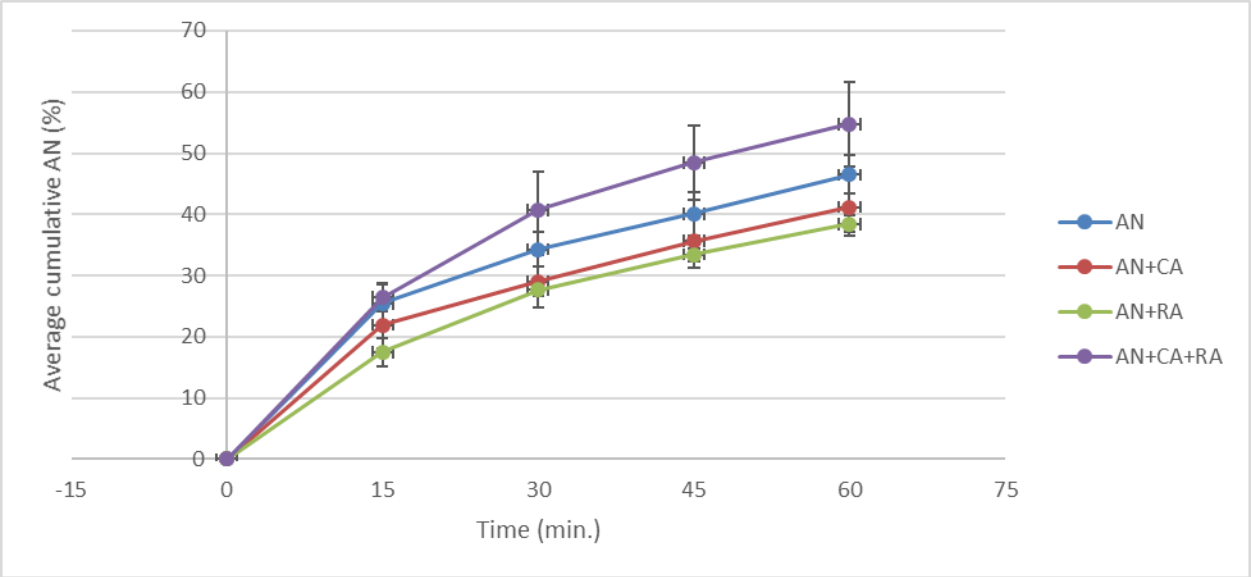


Figure 15: Percentage of artemisinin transport across Caco-2 cells when adding chlorogenic and rosmarinic acid. Cumulative artemisinin (AN) concentration at 15-minute time points for AN + chlorogenic acid (CA) and AN + rosmarinic acid (RA) transport studies are represented by a percentage of the donor solution added at time zero to the apical side of cells due to differences in measured artemisinin in donor solutions (Figure 6, Appendix C).

The standard error for these values indicated that at 60 min the transport of artemisinin was not significantly different across conditions (Figure 15). Differences in proportion and amount of artemisinin transported in the combined phenolic acid treatment may be due to inconsistent artemisinin concentration in donor solutions (Figure 14 and 15; Table 6, Appendix C).

4.3.1 Chlorogenic Acid Concentration Series

Results thus far suggested that chlorogenic acid, at a molar ratio of artemisinin:chlorogenic acid of 1:1.1 did not enhance artemisinin transport (Figures 14 and 15). To determine if alteration of that ratio affected artemisinin transport, a concentration series was tested with artemisinin:chlorogenic acid ratios of 1:0, 1:1, 2:1, and 3:1. Although after 15 min it appeared that the 2:1 ratio enhanced artemisinin transport, results at 60 min were not statistically significant and dropped to about half measured at the earlier time points (Figure 16). Cumulative artemisinin transport showed a similar, but also insignificant result (Figure 17).

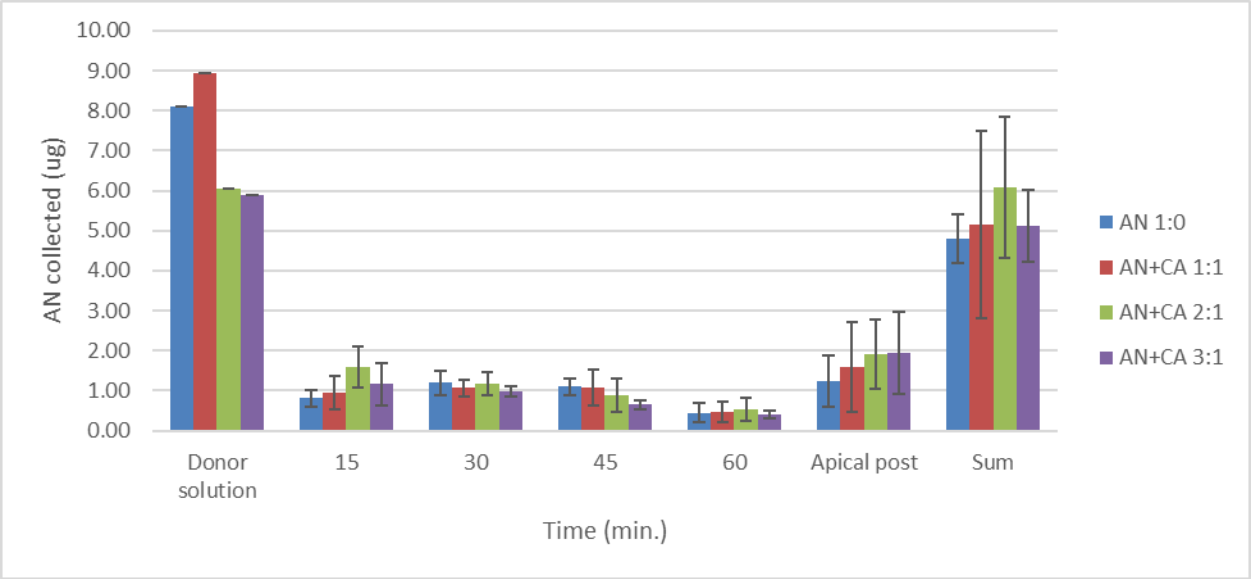


Figure 16: Average basolateral concentration of artemisinin over 60 minutes with varying concentration treatments with chlorogenic acid. The AN (artemisinin alone) treatment was not significantly different from any of the CA (chlorogenic acid) concentration treatments (Table 7, Appendix C). All conditions have a 3 well replication. Sum refers to the total AN collected from 15-60 min as well as apical post samples. The cells were not lysed in this experiment nor those that followed due to minute artemisinin content found in the cells from the previous experiments.

Despite all donor well solutions supposedly having an artemisinin concentration of 25 µg/well (Figure 16), donor well artemisinin concentrations were again measured lower, at 6-10 µg/well. The treatments with similar artemisinin donor solutions, artemisinin alone and artemisinin with chlorogenic acid at a 1:1 concentration, as well as artemisinin with chlorogenic acid at 2:1 and 3:1 concentrations, can be compared pairwise but not with the others. Some of the artemisinin was unaccounted for as shown by the much lower sum data for the 1:0 and 1:1 ratios (Figure 16).

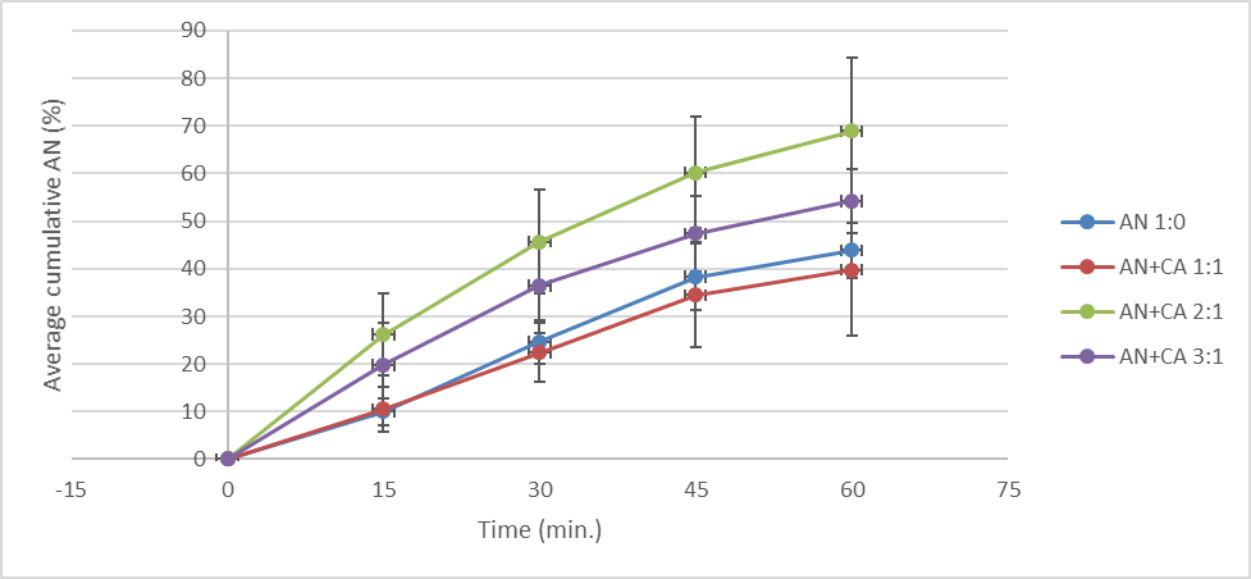


Figure 17: Percentage of artemisinin transport across Caco-2 cells when adding varying chlorogenic acid concentrations. Cumulative AN concentration at 15-minute time points for AN + CA transport study is represented by a percentage of the donor solution added at time zero to the apical side of cells due to differences in measured artemisinin in donor solutions (Figure 7, Appendix C).

4.4 Effect of Monoterpenes

Because of camphor abundance in *A. annua*, a concentration series was performed to determine if this phytochemical increased transport of artemisinin. To accomplish this, a concentration series was conducted with artemisinin and camphor at 1:0, 1:1, 2:1, and 10:1 artemisinin:camphor molar ratios with the 10:1 correlating with the measured in dried *A. annua* leaf tablets. The amount of artemisinin collected for all samples at each time points is illustrated in Figure 18.

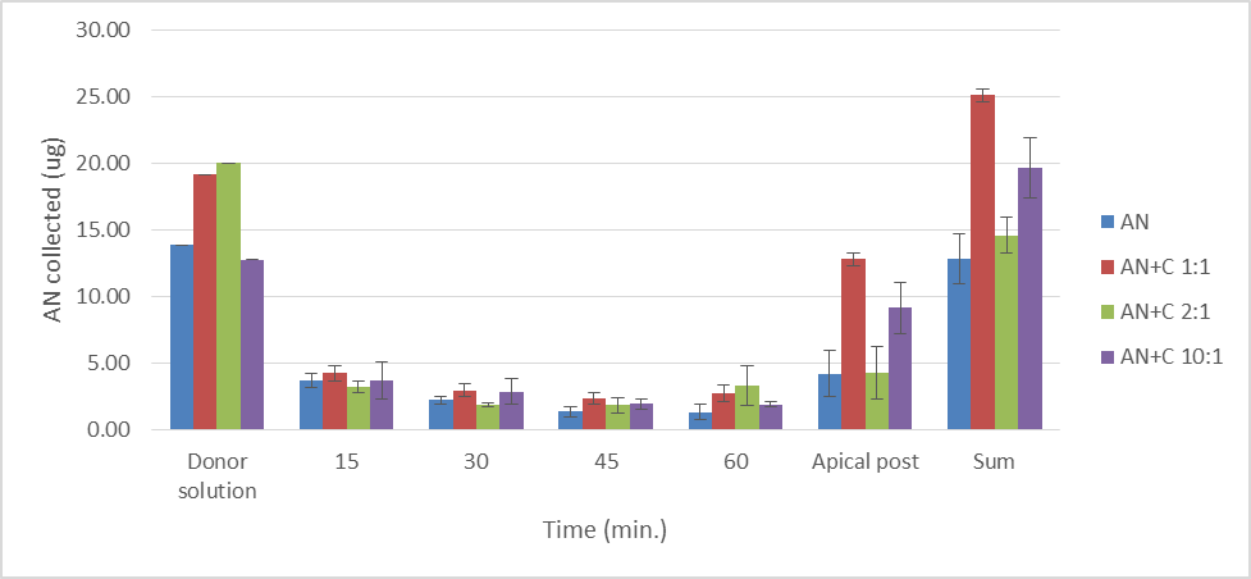


Figure 18: **Average basolateral concentration of artemisinin over 60 minutes with varying concentration treatments with camphor.** The AN (artemisinin alone) treatment was significantly different for the 2:1 C (camphor) concentration treatment at 45 minutes (Table 8, Appendix C). All conditions have a 3 well replication. Sum refers to the total AN collected from 15-60 min as well as apical post samples.

The donor well solutions varied from 12.5 to 20 µg of starting artemisinin, clearly different again from the theoretical starting amount of 25 µg. The amount of artemisinin collected at each 15 min interval was about 2-4 µg (Figure 18). The 1:1 artemisinin to camphor concentration and 10:1 concentration showed elevated amounts of artemisinin on the apical side post transport experiment; this ultimately resulted in a greater amount of summed artemisinin than the measured amount originally put into the wells (Figure 18). The artemisinin and camphor ratio of 10:1 showed the greatest amount of artemisinin transport with about 80% crossing form the apical to basal side after 60 minutes (Figure 19), which was the only statistically significant result.

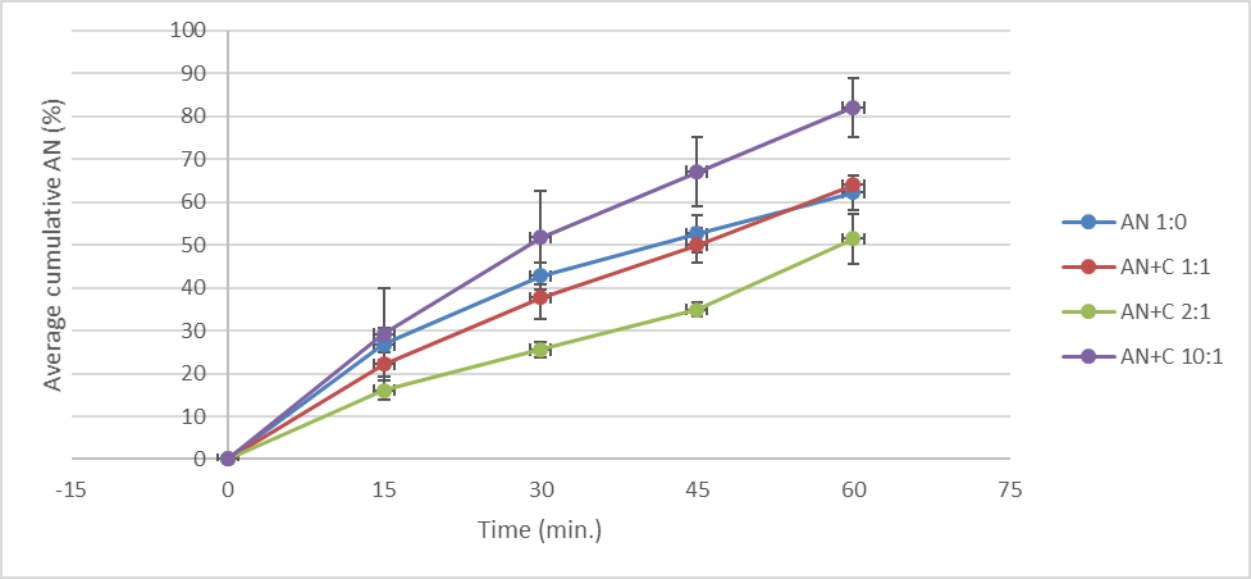
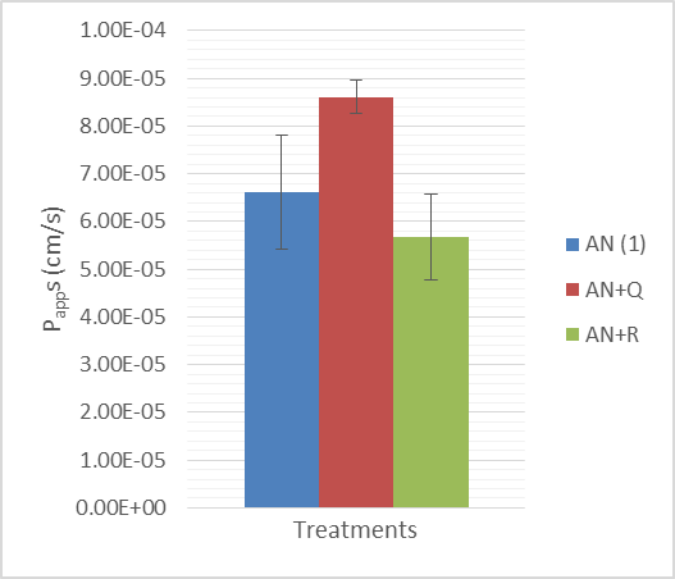


Figure 19: Percentage of artemisinin transport across Caco-2 cells when adding varying camphor concentrations. Cumulative AN concentration at 15-minute time points for AN + C transport study is represented by a percentage of the donor solution added at time zero to the apical side of cells due to differences in measured artemisinin in donor solutions (Figure 8, Appendix C).

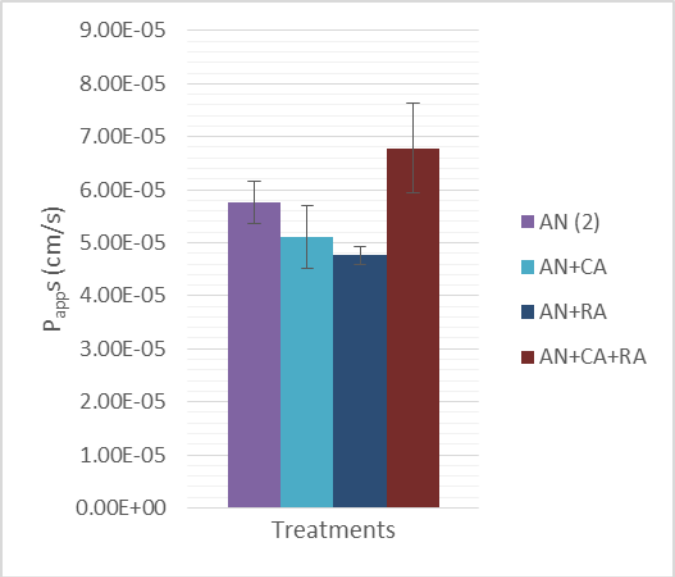
4.5 Apparent permeability value

In transport studies, P_{app} is commonly calculated to account for differences in donor well concentration, cell growth area, and experiment duration. P_{app} was calculated using the average cumulative artemisinin at 60 minutes from all replicate transport experiments. Except for one condition within the camphor experiment, the average P_{app} from each condition in each experiment was not statistically significant from its respective artemisinin control (Figures 20-23). The 10:1 artemisinin to camphor treatment yielded significant differences, and showed an increase compared to the control (Figure 23).



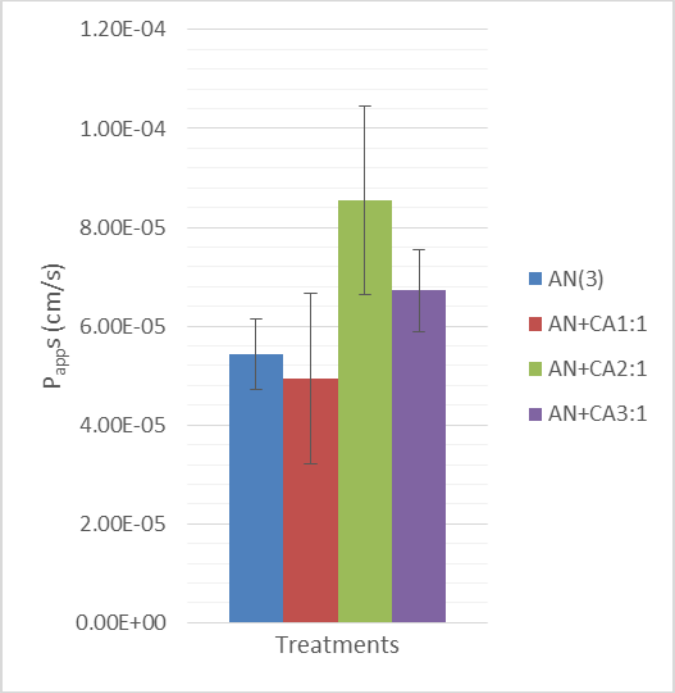
Treatment	P_{app}	St. error
AN	$6.62x10^{-5}$	$1.2x10^{-5}$
AN+Q 2:1	$8.61x10^{-5}$	$3.52x10^{-6}$
AN+R 2:1	$5.67x10^{-5}$	$9.04x10^{-6}$

Figure 20: Apparent permeability value (P_{app}) of flavonoid treatments. Error bars represent standard error for all means presented. Flavonoids tested, quercetin (Q) and rutin (R) were replicated 3 times for a total of 4 wells. All molar ratios were 2 parts AN to 1 part flavonoid. Note that AN donor solutions contained 9 µg, AN+Q 11 µg, and the AN+R treatment contained 14 µg AN.



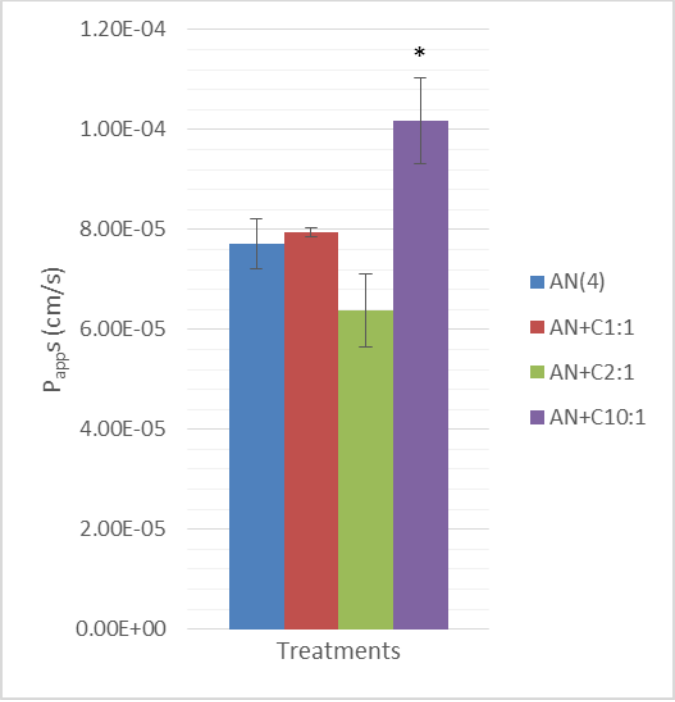
Treatment	P_{app}	St. error
AN	$5.77x10^{-5}$	$3.94x10^{-6}$
AN+CA 1:1	$5.11x10^{-5}$	$5.87x10^{-6}$
AN+RA 1:1	$4.76x10^{-5}$	$1.66x10^{-6}$
AN+(CA+RA) 1:1	$6.79x10^{-5}$	$8.53x10^{-6}$

Figure 21: Apparent permeability value (P_{app}) of artemisinin in phenolic acid treatments. Error bars represent standard error for all means presented. Phenolic acids tested, chlorogenic acid (CA) and rosmarinic acid (RA) were replicated twice for a total of 3 wells. All molar ratios were 1 part AN to 1 part phenolic acid. Note that AN donor solutions contained 21 µg, AN+CA and AN+RA contained ~24.5, & AN+CA+RA treatment contained 14 µg AN.



Treatment	P_{app}	St. error
AN	$5.44x10^{-5}$	$7.13x10^{-6}$
AN+CA 1:1	$4.93x10^{-5}$	$1.72x10^{-5}$
AN+CA 2:1	$8.55x10^{-5}$	$1.91x10^{-5}$
AN+CA 3:1	$6.72x10^{-5}$	$8.33x10^{-6}$

Figure 22: Apparent permeability value (P_{app}) of artemisinin in the chlorogenic acid concentration series. Error bars represent standard error for all means presented. Chlorogenic acid (CA) concentrations were tested with 2 replicates for a total of 3 wells for each treatment. Molar ratios are denoted. Note that AN concentration remained the same, and CA concentration was altered accordingly in each treatment. Note that AN & 1:1 donor solutions contained 8 µg AN and 2:1 & 3:1 treatments contained 6 µg AN in their donor solutions.



Treatment	P_{app}	St. error
AN	$7.72x10^{-5}$	$5.03x10^{-6}$
AN+C 1:1	$7.94x10^{-5}$	$9.56x10^{-7}$
AN+C 2:1	$6.38x10^{-5}$	$7.32x10^{-6}$
AN+C 10:1	$1.02x10^{-4}$	$8.6x10^{-6}$

Figure 23: Apparent permeability value (P_{app}) of artemisinin in the camphor concentration series. Error bars represent standard error for all means presented. Camphor (C) treatments were tested with 2 replicates for a total of 3 wells per treatment. Molar ratios of AN to C are denoted, but note that the concentration of AN remained the same while C was altered accordingly. *, statistical significance at $p \leq 0.05$ when compared to AN alone (t-Test: Paired Two Sample for Means). Note that the AN and 10:1 donor solutions contained ~13µg of AN, 1:1 and 2:1 donor solutions contained ~20 µg of AN.

4.6 Analysis of Artemisinin Donor Well Solutions

Some confusion arose as to the artemisinin differences between the applied and measured levels in donor wells for each treatment (Tables 3-6; Figures 12, 14, 16, 18). The same volume of artemisinin stock solution was added to each well in each experiment (12.5µL), but the measured artemisinin concentrations in donor solutions were all different. Consequently, comparisons could only be made between those treatments having the same starting artemisinin concentration. While this made it invalid to compare P_{app} across all conditions, it did permit us to compare the effect of artemisinin concentration in the donor well with artemisinin P_{app} *per se*. To determine if the P_{app} was dependent on artemisinin content in the donor well, each P_{app} for artemisinin treatments was plotted against the amount of artemisinin actually measured in the donor well (Figure 24). Intermediate amounts of artemisinin resulted in the highest rate of artemisinin transport. A lower P_{app} was seen at both the high and low artemisinin donor well concentrations. To our knowledge, this comparison had not yet been studied.

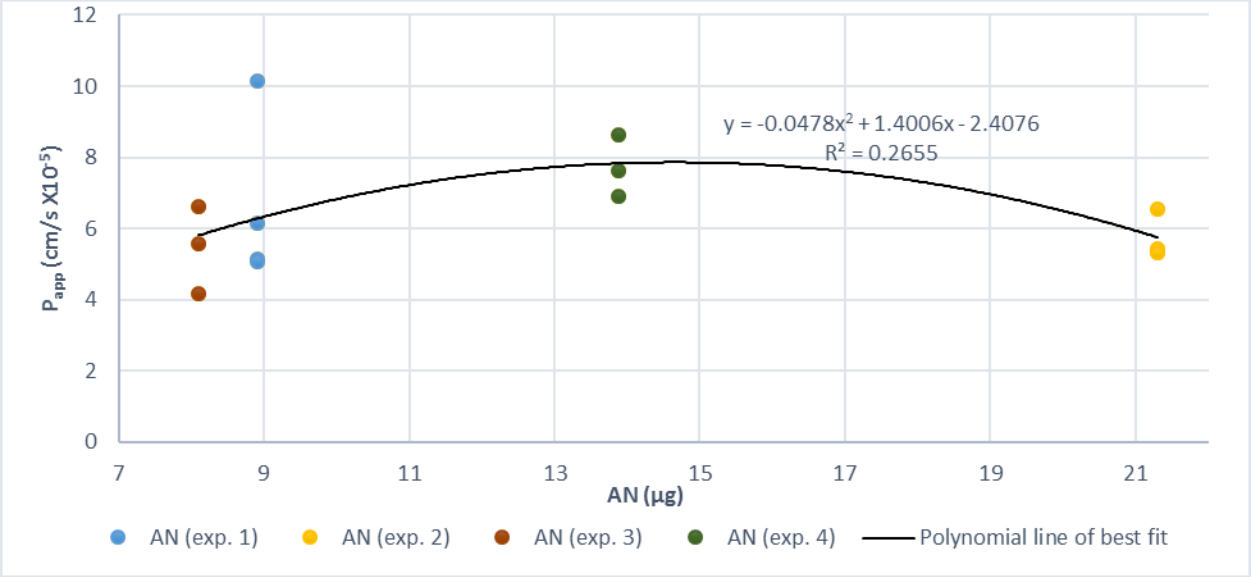


Figure 24: P_{app} with varied artemisinin in donor well. From each transport experiment, the AN in donor solutions varied. To determine the role of AN on rate of transport, all control (AN only) P_{app}s were plotted. The polynomial curve fit the points best with a low R^2 of 0.2655.

5.0 Discussion

The overarching goal of this project was to understand how the ingestion of dried leaves of *A. annua* (pACT) allows for a greater bioavailability of artemisinin than just the drug alone. Elfawal et al. (2012) showed that a single dose dried whole plant *A. annua* (24mg/kg) cleared parasitemia in 88% of the animals used. A later pharmacokinetic study showed that even plant material used in rodent chow increased artemisinin movement into the serum. Those studies suggested that something about the presence of artemisinin in plants allowed for a larger parasite clearance in the murine model. Therefore, it was proposed that other phytochemicals that are present in *A. annua* may facilitate the transport of artemisinin across the intestinal barrier, thus allowing for a greater amount of the drug to be present in the blood and available to combat malaria parasites.

To test whether artemisinin transport changed with the addition of phytochemicals from *A. annua*, an intestinal Caco-2 model system was used to measure the transport of artemisinin. The model system was influenced by an artemisinin transport study performed by Augustijns et al. (1996) among other transport studies. In the 60 minute control transport study (artemisinin alone), a P_{app} of artemisinin was expected to be similar to 30.4 X 10^{-6} cm/s at 37^{o}C, as measured by Augustijns et al. (1996). Although the culture growth cross-sectional area in this experiment was smaller, 1.22 cm^2, than the 4.67 cm^2 in the study by Augustijns et al. (1996), P_{app} standardizes for culture growth area and allows comparison between the results of both studies. The actual permeability of artemisinin in each treatment in our study is considered "high" (P_{app} > 20 X 10^{-6} cm/s) for P_{app} values (Cerep, 2013). Previously Harten et al. (2014) showed a more modest permeability (P_{app} = 2 X 10^{-6} cm/s to 20 X 10^{-6} cm/s) for artemisinin alone, compared to our study which showed high permeability for all conditions (Figures 20-24).

Additionally, the differences in artemisinin transport rate may be due to differences in artemisinin donor solution concentration. In plotting all control P_{app}s, in which varied concentrations of artemisinin was added to the apical side, a parabolic trend showed that rate of transport is dependent on artemisinin in donor solutions. Intermediate amounts of artemisinin (14

µg) gave the greatest P_{app}s when both higher (21 µg) and lower (8-9 µg) amounts resulted in lower transport. Augustijns et al. (1996) studied the transport of artemisinin at 100 µM donor concentrations which, based on the curve in Figure C 1, should have given a high P_{app} of approximately 8.0 X 10^{-5} (derived from the equation of the curve: -1 X 10^{-8}(100 µM)^2 + 2 X 10^{-6}(100 µM) – 2 X 10^{-5}). However, Caco-2 cells are heterogeneous and may evolve due to selective pressures in culture resulting in experimental differences between transport studies (Vachon & Beaulieu, 1992). In addition, artemisinin detection techniques varied between the two studies. Augustijns et al. (1996) used HPLC and our study used GC/MS. Based on the conflicting findings, more research should cover the effect of artemisinin donor solution concentration on the transport of artemisinin.

The purpose of repeating the flavonoid quercetin and rutin study conducted by Harten et al. (2014) was to verify reproducibility of those results Reproducibility is important in making comparisons between different studies that use cells at different passages. Unfortunately, there was no true statistical difference between the treatments. This may be due to a small replication of 4 wells. To truly see a significant difference the flavonoid treatments would have to be replicated across multiple plates with a large number of wells to compare. This is because the difference between treatments can be small relative to the variation within treatments (McDonald, 2014). With that in mind, the increase of artemisinin transport in the artemisinin and quercetin treatment may be significantly different from the control with multiple replications. This conflicts with the results of Harten et al. (2014) where the artemisinin and rutin treatment showed the greater increase in artemisinin transport. However, Harten et al. (2014) only performed each treatment on 2 wells with the control in only 1 well. High standard error in the artemisinin control for the flavonoid experiment (std. error = 0.18-0.56) could have resulted in a low statistical significance (p=0.110) in the mean difference between the quercetin/artemisinin treatment and the artemisinin control.

Rosmarinic and chlorogenic acids were chosen due to their structural similarities, role in immune response, and abundance in many *A. annua* cultivars (Boundless, 2014; de Magalhães et al., 2012). Other Caco-2 intestinal transport studies showed these phenolic acids possessed IC_{50} values about 65 µmol/L, significantly thwarted activity of the CYP3A4 P450 cytochromes,

and greatly reduced inflammation via limiting IL-6 and IL-8 cytokine secretions (Svensson & Ashton, 1999; de Magalhães et al., 2012). Figures 14 and 15 showed that the highest proportion of artemisinin was transported when the drug was combined with both rosmarinic and chlorogenic acid, although there was no statistically significant differences. Greater artemisinin transport occurred when the two phenolic acids were combined together with artemisinin than with either single phenolic acid. The lowest amounts of artemisinin transported resulted from transport with chlorogenic acid and rosmarinic acid individually. This differed from Suberu et al. (2013) findings, where rosmarinic acid exemplified a synergistic relationship with artemisinin (1:3 parts artemisinin to rosmarinic acid) in chloroquine sensitive *Plasmodium* strains. Although instead using a breast cancer cell line to test the toxicity of artemisinin, Suberu et al. (2014) determined that there may actually be a decreased artemisinin potency with increased chlorogenic acid concentrations. Additionally, rosmarinic and chlorogenic acid use passive transport just as artemisinin, perhaps the phenolic acids are competing with artemisinin for the same passage routes through the tight junctions (Konishi & Kobayashi, 2004; Konishi & Kobayashi, 2005). Indeed Suberu et al. (2014) showed there was a concentration effect on therapeutic response when artemisinin was combined with some of the individual phytochemicals found in *A. annua*.

The treatment that combined artemisinin with both chlorogenic acid and rosmarinic acid showed an increase of artemisinin transport compared to artemisinin alone. However, when each chemical was combined individually with artemisinin, a lower percentage was transported across the membrane. These results suggested that a combination of phytochemicals may be more effective than any single phytochemical, as proposed by the study that demonstrated effective parasite clearance when using pACT in a murine model (Elfawal et al., 2012). Also, the fact that rutin, chlorogenic acid, and rosmarinic acid transported a lower percentage of artemisinin, than just artemisinin alone, suggested that some phytochemicals may have an inhibiting effect on the drug. Conducting further transport studies and replication of all thirteen phytochemicals would allow for further characterization of those that promote transport and those that inhibit it.

A camphor concentration series was also performed because this monoterpene is often abundant in the essential oil of *A. annua* (Juteau et al., 2002). Camphor also affects thymocyte viability

(Cherneva et al. 2012). Three different ratios of artemisinin to camphor were tested (1:1, 2:1, and 10:1). Because of variability in donor solution artemisinin concentration, the artemisinin treatment can only be compared with the 10:1 treatment of artemisinin to camphor. Additionally, the 2:1 ratio can only be compared with the 1:1 ratio of artemisinin to camphor. The results of this experiment showed that an increase in the percentage of transported artemisinin occurred with the 10:1 ratio (artemisinin:camphor) compared to artemisinin alone. The P_{app} values for all camphor transports also showed a high level of permeability, and the 10:1 artemisinin to camphor treatment significantly (p=0.024) increased the artemisinin P_{app} compared with the artemisinin control.

Camphor, borneol, and 1,8-cineole (or eucalyptol) were found to inhibit the digestive enzyme, aminopeptidase-N, when consumed by both sage-grouse and chickens (Kohl et al., 2015). The sage-grouse used in that study was known to primarily consume sagebrush, which is a common name for many plants in the *Artemisia* genus. Therefore, the contents of their digestive system contained plant material that was most likely of this genus, although not likely to include *A. annua*. However, many of the monoterpenes present in the digested material of the birds were the same or similar to those present in the *A. annua* cultivar used in this study. Although Kohl et al. (2015) primarily focused on the activity of the bird's digestive enzymes, when taken together with the results of this study, it appeared that camphor had both inhibitory and enhancing effects with respect to digestion and absorption. Furthermore, β-pinene increased digestive activity in chickens without showing this same effect in sage-grouse (Kohl et al., 2015). Although both are avian species, their digestive systems are different enough that β-pinene did not have the same effect in both. Thus it is possible that in different digestive systems, camphor interacts differently with the enzymes that are present. Human intestinal cells are different than avian intestinal cells, which could explain the difference in the effect of camphor. Indeed, other phytochemicals may show similar variations and may show an increase or decrease in digestive activity and transport, depending on the enzymes and makeup of the organism's digestive system and body.

Elfawal et al. (2012) showed that parasite clearance in mice diminished significantly with one dose, thus leading to the conclusion that a larger amount of artemisinin was transported across the epithelial membrane (Elfawal et al., 2012). However, the amount of artemisinin present in the

bloodstream was unknown. It was not until a later pharmacokinetic study that the artemisinin concentration was quantified in healthy and *Plasmodium chabaudi*-infected mice after a 100 mg kg^{-1} dose of artemisinin given orally from either a pure drug or pACT treatment (Weathers et al. 2014). The results showed that when accompanied by plant material, more artemisinin passed into the serum, with as much as 6.6 mg/L of artemisinin found in the infected mice. Therefore, there is still the possibility that other phytochemicals in *A. annua,* such as camphor, have antimalarial properties or that the plant matrix itself aided in the eradication of the parasite in these murine models. If this prospect were to be considered, individually testing phytochemicals that showed antimalarial capability in murine models, could be one way to obtain evidence to why artemisinin oral consumption via pACT eradicated malarial parasites more effectively than pure artemisinin.

Of all the phytochemicals tested, only camphor at a molar ratio of 10:1 [artemisinin:camphor] showed a significant increase in artemisinin transport, which was indicative of an increase in artemisinin in the serum. More artemisinin in the serum could aid in parasite clearance, as demonstrated in pACT parasite clearance in the study by Elfawal et al. (2012). This study therefore, may suggest that certain phytochemicals in *A. annua* facilitate the transport of artemisinin, but only at specific ratios relative to artemisinin.

Information regarding the transport of artemisinin in combination with phytochemicals found in *A.annua* is important in understanding the mechanism by which artemisinin serum concentration is increased after pACT treatment, relative to pure artemisinin. Our data show that quercetin, rutin, chlorogenic acid, and rosmarinic acid, did not significantly alter artemisinin serum concentration. However, the rate of transport was increased significantly by camphor at a 10:1 molar concentration [artemisinin:camphor] possibly allowing a faster *in vivo* absorption, and with multiple doses of pACT, a higher overall concentration of artemisinin in the blood. Since the majority of artemisinin is degraded and eliminated through the liver, the activity of the enzymes responsible, e.g. CYP2B6 and CYP3A4 (Niu et al., 1985; Svensson & Ashton, 1999), should be measured in response to various concentrations of different *A. annua* phytochemicals. As previously mentioned, phenolic acids may inhibit such enzymes, so this research would solidify previous findings (Suberu et al. 2014). Also, because artemisinin has been found to

break down into four metabolites (deoxy-dihydroartemisinin, dihydroxyartemisinin, deoxyartemisinin, and crystal-7), it is possible that other metabolites still remain unknown (Lee & Hufford, 1990). Understanding how artemisinin is degraded by the body could give further insight about why consumption of whole plant *A. annua* clears parasitemia more effectively than artemisinin alone.

6.0 Conclusions and Future work

First and foremost, the artemisinin transport experiments of this study collectively focused on only five of the thirteen major phytochemicals that have been measured in the *A. annua* SAM cultivar. The goal was to determine if one specific phytochemical was responsible for a noticeable increase in artemisinin. Our concentration studies revealed that artemisinin transport was affected, not only by certain phytochemicals, but also by the amount of phytochemical relative to artemisinin. Camphor was shown to significantly increase artemisinin transport at a molar ratio of 10:1 [artemisinin:camphor]. To determine if any other phytochemicals are indeed responsible for the increased bioavailability of artemisinin, all of the measured and as of yet unmeasured phytochemicals should be tested using the same transport study, but also at various molar ratios to artemisinin. Because there are multiple phytochemicals in *A. annua*, a combination effect on artemisinin transport could also be occurring during treatment. If any combination experiments were conducted, it would make sense to test combinations of the phytochemicals that showed the most promising results when individually combined with artemisinin. An alternative option would be to chemically block various chemistries of *A. annua* plant material, such as proteins, to gain insight on the deleterious effects in transport of artemisinin.

The culmination of our camphor (monoterpene) results, as well as data collected indicative of increased digestive enzyme activity in chickens as a result of the present of β-pinene, should also be considered when continuing with artemisinin transport studies (Kohl et al., 2015). Pinene is a monoterpene that has two isomers, α and β (Simonsen, 1957). Although, β-pinene is not found in *A. annua*, its isomer, α-pinene, is present (Weathers & Towler 2014). Since β-pinene showed promising results for increased enzymatic activity in chickens, it is recommended that α-pinene be tested in combination with artemisinin to see if an increase in bioavailability results. Artemisinin concentration should also be explored as a determinant of optimal transport. Based on our results, P_{app} appears to have a parabolic trend with artemisinin concentration in the donor well suggesting that there may an optimum concentration of artemisinin for optimal transport.

Thus, a more extensive artemisinin concentration series should be tested to determine if indeed there is an optimum artemisinin concentration for the donor well.

7.0 References

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8.0 Appendices

Appendix A: Additional Introductory and Background Material

Table A 1: Key *A. annua* compounds from SAM leaves harvest (Weathers & Towler, 2014)

Compound	Freshly extracted leaves (μg g^{-1} DW)*		Dry extracted leaves (μg g^{-1} DW)		
	Shoot tips	Mature leaves	2 mm sieved	Powdered leaves	
				Leaf powder	Tablets
Artemisinin	8,188.92 ± 2,817.70a	11,380.32 ± 2,587.92b	15,897.23 ± 1,040.55x	17,306.24 ± 1,397.72y	17,180.86 ± 782.00y
Dihydroartemisinic acid	40,210.40 ± 6,414.62	nd	1,857.43 ± 737.22x	1,286.23 ± 205.24x	369.04 ± 32.00y
Arteannuin B	nd	96.76 ± 183.52	2,323.12 ± 246.97x	2,460.68 ± 246.86x	2,450.95 ± 202.63x
Artemisinic acid	2,209.12 ± 449.88	nd	367.39 ± 45.81x	345.30 ± 57.04x	1,328.84 ± 546.39y
α-Pinene	nd	nd	nd	nd	nd
Eucalyptol (1,8 cineole)	1,101.64 ± 106.39a	301.72 ± 51.52b	261.16 ± 42.35x	278.31 ± 44.34x	nd
Camphor	10,121.24 ± 1,141.62a	3,569.88 ± 318.76b	21,017.68 ± 1,600.64x	16,671.84 ± 1,260.17y	1,870.23 ± 214.98z
Chlorogenic acid	10,839.65 ± 3,004.50a	6,473.12 ± 981.72b	673.37 ± 138.64x	2,735.68 ± 487.12y	5,217.48 ± 528.44z
Rosmarinic acid#	nd	nd	2,261.19 ± 3,414.68#	nd	nd
Scopoletin	5,516.05 ± 905.12a	1,319.44 ± 454.96b	36.08 ± 16.43x	84.44 ± 47.58x,y	139.27 ± 47.22y
Artemetin	nd	nd	nd	nd	nd
Casticin	10.67 ± 28.92	nd	nd	nd	nd
Chrysoplenol-D	703.48 ± 201.90a	211.063 ± 99.44b	413.43 ± 56.59x	402.35 ± 30.55x	323.21 ± 28.61y
Chrysoplenetin	310.10 ± 76.13a	83.62 ± 40.67b	153.88 ± 23.95x	154.69 ± 13.41x	153.87 ± 11.26x
Eupatorin	nd	nd	nd	nd	nd
Kaempferol	nd	nd	nd	nd	nd
Luteolin#	nd	nd	206.61 ± 294.38#	128.39 ± 183.09#	nd
Myricetin	nd	nd	nd	nd	nd
Quercetin	nd	nd	nd	8.64 ± 0.55x	7.13 ± 4.25x
Total flavonoids ($AlCl_3$ method)	6,058.77 ± 419.96a	1,554.28 ± 439.60b	2,782.51 ± 274.19x	5,047.18 ± 671.02y	10,967.78 ± 656.35z

Each value in the table is the mean ± standard deviation (n = 4–6). Letters within a row indicate statistically significant data with a,b for fresh leaves and x,y,z for sieved/ground/tableted; $p \leq 0.05$; nd, not detectable; *DW calculated using DW/FW ratio of 0.25; #, only two of six replicates had rosmarinic acid or luteolin; shown is average of all 6 samples. DW, dry weight.

Table A 2: Molecular weight and MSDS links for compounds used in transport experiments

Compound	MW (g/mol)	MSDS from Sigma-Aldrich
Artemisinin	282.33	http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=361593&brand=SIGMA&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2F361593%3Flang%3Den
Quercetin	302.24	http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=Q4951&brand=SIGMA&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Fq4951%3Flang%3Den
Rutin	610.52	http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=R5143&brand=SIGMA&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Fr5143%3Flang%3Den
Chlorogenic Acid	354.31	http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=C3878&brand=ALDRICH&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Faldrich%2Fc3878%3Flang%3Den
Rosmarinic Acid	360.31	http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=536954&brand=ALDRICH&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Faldrich%2F536954%3Flang%3Den
Camphor	152.23	http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=148075&brand=ALDRICH&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Faldrich%2F148075%3Flang%3Den

Appendix B: Methods

Standard Operating Procedure for Caco-2 Transwell Plate

This SOP was adapted from the Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers available from Hubatsch et al. (2007) and Thawing, Propagating, and Cryopreserving Protocol from the American Type Culture Collection (2012). Caco-2 cells work well in absorption systems because, when fully confluent, they have the ability to form tight junctions. The following steps are used to prepare a transwell transport plate by seeding the appropriate concentration of Caco-2 cells.

A. Maintaining stock cells

1. Place complete media in 37°C water bath until warm
2. Remove T75 flask of stock cells from 37°C incubator
3. Aspirate media from flask
4. Add 12 mL of warmed, complete media into the flask

B. Seeding cells on transwell inserts

1. Obtain T75 flask of stock cells when ~90% confluent and aspirate media
2. Remove any remaining media by washing flask with 3-5 mL of HBSS; then aspirate
3. Add 3-5 mL of Tryp-LE to flask and incubate at 37°C for 15-30 minutes, or until the cell layer dissociates from the flask bottom
4. Immediately stop trypsinization by adding 8-10 mL of complete media to flask
5. Transfer the suspension to a 15 mL conical tube and centrifuge on 1,500 rpms for 3 minutes
6. Aspirate off media until just above the cell pellet and resuspend in 2 mL of new media
7. Perform a cell and viability count (must be at least 95% viable) using hemacytometer
8. Calculate volume of cell suspension and complete media needed to obtain a concentration of $6.0 * 10^5$ cells/mL and mix together
    a. Example: You do a cell count and find you have 7.0×10^5 cells/mL, in a total of 2 mL of suspension. You want to seed 3 wells (0.5 mL/well), but calculate for 4 wells to account for mistakes (2 mL). The final cell concentration you are aiming

for is 6.0 x 10^5 cells/mL. This way you will have 3 x 10^5 cells in each well (the density called for in the Nature Methods paper). Given the equation, $C_1V_1=C_2V_2$: (7.0 x 10^5 cells/mL)(V_1) = (6.0 x 10^5 cells/mL)(2 mL). V_1 = 0.58 mL, so you need 0.58 mL of cell suspension and 1.42 mL of media to make 2 mL of cell suspension at a concentration of 6.0 x 10^5 cells/mL for seeding.

9. Place transwell filter inserts into wells of 24-well plate
10. Wet each filter with 0.1 mL of complete media for ~2 minutes
11. Pipette 0.5 mL of cell suspension in each well, such that there is 300,000 cells/well.

    * Be sure to appropriately resuspend cells in medium by pipetting media against the bottom surface of the tube – visually check tube to ensure no cells are still attached. Try to
12. Fill each basolateral compartment with 1.5 mL of complete media
13. Cover and incubate for 6-16 hours (no longer)

C. Maintaining cells on transwell filter inserts

1. After 6-10 hours post seeding, aspirate basolateral media (lower chamber)
2. Aspirate media from apical media (upper chamber)
3. Replace apical side with 0.5 mL of complete media and then basolateral side with 1.5 mL of complete media

   * Important this is done to remove dead cells, debris, and aggregates that may cause multilayered growth

4. Change media every 2-3 days by aspirating basolateral then apical; then adding 0.5 and 1.5 mL of complete media to apical then basolateral side
5. Repeat Step 4, until cells ready for transport experiment, 21-29 days

Standard Operation Procedure for Transport Experiment

This SOP was modified from the Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers, available from Hubatsch et al., (2007). To complete all parts of the transport experiment and GC/MS preparation, it takes about 6 hours.

A. Final Caco-2 monolayer preparation

1. 12-24 hours before the experiment, change the culture medium
   * No longer than this period to avoid adaption into a more starved phenotype
2. Aspirate basolateral then apical side
3. Add 0.5 and 1.5 mL of complete to apical then basolateral side
4. Prior to beginning transport, wash the transwell filters by transferring monolayers into new 12-well plate containing 1.5 mL of HBSS in the basolateral compartments and carefully transfer 0.5 mL of HBSS into the apical sides
5. Cover and incubate transwell plate under gentle shaking for 15-20 min at 37°C
6. Set aside until needed for transport

B. TEER Protocol

1. Place the transwell inserts in new 12-well plate containing 1.5 mL of HBSS in the basolateral compartments and carefully transfer 0.5 mL of HBSS into the apical sides
2. Place TEER meter probe into each well: short probe in apical side and long probe in the basolateralside
3. Record reading in (ohms)
4. Separately, record reading of transwell insert without cells, for use in calculating TEER value

C. Apical-to-basolateral transport experiment

1. Remove washing solution from transwell plate by decanting and transfer inserts into new 12-well plate with 1.5 mL of HBSS in the basolateral compartment

2. Add 0.5 mL of donor solution to the corresponding apical compartment (t=0), as well as put 0.5 mL aside in a vial
3. Cover plate and incubate at 37°C on an orbital shaker at 300-400 r.p.m. to minimize the effects of unstirred water
4. After 15 min remove plate from incubator and transfer the transwell inserts into new 12-well plate with 1.5 mL of HBSS in the basolateral compartment to maintain sink conditions
5. Return covered plate to incubator at 37°C on an orbital shaker at 300-400 r.p.m
6. Use Pasteur pipet to transfer contents of basolateral compartment into a vial
7. Repeat Steps 4-6 every 15 min until 60 min time point is reached
8. At 60 min, use Pasteur pipet to transfer contents of apical compartment into a vial, as well as transfer contents of the basolateral compartment into a separate vial

D. Lysing Cells

1. After measuring the TEER value, add 0.25 mL of Tryp-LE to the apical compartment and cover
2. Incubate the plate at 37°C on an orbital shaker at 300-400 r.p.m. for 15-30 min or until cell layer becomes dissociated from the filter
3. Remove from incubator
4. Add 0.25 mL of HBSS to apical side and 1 mL to basolateral side
5. Use Pasteur pipet to transfer contents of apical and basolateral into the same vial

E. Extraction of artemisinin

1. To each vial add equal parts methylene chloride and vortex sample
2. Cover with Dura Seal and sonicate in a sonicating water bath for 30 min
3. Pipette the methylene chloride and artemisinin layer (bottom layer) into a new vial
4. Nitrogen dry samples to remove any HBSS
5. Resuspend samples in 100-200 µL of methylene chloride and transfer contents into GC/MS tube
6. Resuspend sample in 50 µL of pentane prior to insertion into GC/MS

Appendix C: Supplemental Results, Figures and Tables

Table C 1: TEER values for Caco-2 monolayers prior and after quercetin and rutin transport experiment

	Prior to Tranport Experiment			After Transport Experiment		
Sample	R (Ω)[1]	R – R_b (Ω)[2]	TEER value (Ω*cm^2)[3]	R (Ω)	R – R_b (Ω)	TEER value (Ω*cm^2)
AN1	477	383	428.96	252	158	176.96
AN2	511	417	467.04	500	406	454.72
AN3	470	376	421.12	435	341	381.92
AN4	475	381	426.72	477	383	428.96
AN+Q1	506	412	461.44	556	462	517.44
AN+Q2	513	419	469.28	526	432	483.84
AN+Q3	511	417	467.04	505	411	460.32
AN+Q4	508	414	463.68	541	447	500.64
AN+R1	478	384	430.08	518	424	474.88
AN+R2	491	397	444.64	400	306	342.72
AN+R3	472	378	423.36	330	236	264.32
AN+R4	483	389	435.68	363	269	301.28

[1] R is the raw voltage value of the sample obtained from the TEER instrument
[2] R_b is the raw voltage value of the transwell insert without any cells [obtained from the TEER instrument]
[3] TEER value = (R-R_b) * A , where A is the area of the filter

Table C 2: TEER values for Caco-2 monolayers prior to chlorogenic and rosmarinic acid transport experiment

	Prior to Tranport Experiment		
Sample	R (Ω)[1]	R – R_b (Ω)[2]	TEER value (Ω*cm^2)[3]
AN1	482	388	434.56
AN2	471	377	422.24
AN3	502	408	456.96
AN+CA1	489	395	442.4
AN+CA2	490	396	443.52
AN+CA3	499	405	453.6
AN+RA1	492	398	445.76
AN+RA2	470	376	421.12
AN+RA3	505	411	460.32
AN+CA+RA1	478	384	430.08
AN+CA+RA2	454	360	403.2
AN+CA+RA3	492	398	445.76

[1] R is the raw voltage value of the sample obtained from the TEER instrument
[2] R_b is the raw voltage value of the transwell insert without any cells [obtained from the TEER instrument]
[3] TEER value = (R-R_b) * A , where A is the area of the filter
*Note that TEER was not taken after this experiment.

Table C 3: TEER values for Caco-2 monolayers prior to chlorogenic acid concentration series transport experiment

	Prior to Transport Experiment		
Sample	R (Ω)[1]	$R - R_b$ (Ω)[2]	TEER value ($\Omega*cm^2$)[3]
AN1	337	243	272.16
AN2	344	250	280
AN3	373	279	312.48
AN1:CA1-1	348	254	284.48
AN1:CA1-2	356	262	293.44
AN1:CA1-3	364	270	302.4
AN2:CA1-1	374	280	313.6
AN2:CA1-2	359	265	296.8
AN2:CA1-3	347	253	283.36
AN3:CA1-1	330	236	264.32
AN3:CA1-2	342	248	277.76
AN3:CA1-3	367	273	305.76

[1] R is the raw voltage value of the sample obtained from the TEER instrument
[2] R_b is the raw voltage value of the transwell insert without any cells [obtained from the TEER instrument]
[3] TEER value = $(R-R_b) * A$, where A is the area of the filter
*Note that TEER was not taken after this experiment.

Table C 4: TEER values for Caco-2 monolayers prior to camphor concentration series transport experiment

	Prior to Transport Experiment			After Transport Experiment		
Sample	R (Ω)[1]	$R - R_b$ (Ω)[2]	TEER value ($\Omega*cm^2$)[3]	R (Ω)	$R - R_b$ (Ω)	TEER value ($\Omega*cm^2$)
AN1	348	254	284.48	322	228	255.36
AN2	353	259	290.08	290	196	219.52
AN3	362	268	300.16	320	226	253.12
AN1:C1-1	340	246	275.52	328	234	262.08
AN1:C1-2	355	261	292.32	375	281	314.72
AN1:C1-3	309	215	240.8	300	206	230.72
AN1:C0.05-1	361	267	299.04	314	220	246.4
AN1:C0.05-2	348	254	284.48	338	244	273.28
AN1:C0.05-3	359	265	296.8	335	241	269.92
AN1:C0.01-1	324	230	257.6	330	236	264.32
AN1:C0.01-2	335	241	269.92	322	228	255.36
AN1:C0.01-3	349	255	285.6	331	237	265.44

[1] R is the raw voltage value of the sample obtained from the TEER instrument
[2] R_b is the raw voltage value of the transwell insert without any cells [obtained from the TEER instrument]
[3] TEER value = $(R-R_b) * A$, where A is the area of the filter

Table C 5: Artemisinin content (µg) per well from quercetin and rutin experiment

AN content per sample (µg)							
Sample	Row A	Row B	Row C	Row D	Ave.	STD.	STD. Error
AN							
Donor Solution	8.92	8.92	8.92	8.92	8.92	0.00	0.00
15 min	1.23	1.16	1.81	2.33	1.63	0.55	0.27
30 min	1.02	1.84	3.27	0.82	1.74	1.11	0.56
45 min	0.56	0.82	1.26	0.32	0.74	0.40	0.20
60 min	0.90	0.60	0.95	0.17	0.66	0.36	0.18
Apical post	1.67	1.76	4.77	0.38	2.15	1.86	0.93
Cells post	0.40	0.08	0.11	0.03	0.16	0.17	0.08
Total collected	5.78	6.26	12.17	4.05	7.07	3.53	1.77
AN+Q							
Donor Solution	10.94	10.94	10.94	10.94	10.94	0.00	0.00
15 min	2.35	2.88	2.91	3.30	2.86	0.39	0.20
30 min	1.58	2.44	2.98	2.63	2.41	0.60	0.30
45 min	1.66	1.52	1.25	1.41	1.46	0.17	0.09
60 min	1.14	0.75	0.97	0.61	0.87	0.24	0.12
Apical post	1.76	2.18	2.14	1.98	2.01	0.19	0.09
Cells post	0.12	0.09	0.46	0.18	0.21	0.17	0.08
Total collected	8.60	9.85	10.70	10.11	9.82	0.88	0.44
AN+R							
Donor Solution	14.59	14.59	14.59	14.59	14.59	0.00	0.00
15 min	1.64	1.80	2.54	3.33	2.33	0.77	0.39
30 min	1.59	1.73	2.27	3.36	2.24	0.81	0.40
45 min	1.07	1.00	0.81	2.31	1.30	0.68	0.34
60 min	0.92	0.85	0.71	0.78	0.82	0.09	0.05
Apical post	1.98	1.41	1.80	2.01	1.80	0.28	0.14
Cells post	0.10	0.06	0.06	0.11	0.08	0.03	0.01
Total collected	7.30	6.84	8.19	11.90	8.56	2.30	1.15

Table C 6: Artemisinin content (µg) per well from chlorogenic and rosmarinic acid experiment

AN content per sample (µg)						
Sample	Row A	Row B	Row C	Ave.	STD.	STD. Error
AN						
Donor Solution	21.31	21.31	21.31	21.31	0.00	0.00
15 min	6.82	4.65	4.80	5.42	1.21	0.70
30 min	1.64	2.25	1.76	1.88	0.32	0.18
45 min	1.60	0.98	1.12	1.23	0.33	0.19
60 min	1.19	1.47	1.45	1.37	0.16	0.09
Apical post	3.18	2.62	3.18	2.99	0.32	0.19
Cells post	0.07	0.06	0.07	0.06	0.01	0.00
Total collected	14.50	12.01	12.37	12.96	1.35	0.78
AN+CA						
Donor Solution	24.31	24.31	24.31	24.31	0.00	0.00
15 min	7.02	5.17	3.82	5.34	1.61	0.93
30 min	1.77	1.98	1.42	1.72	0.28	0.16
45 min	2.02	0.76	2.04	1.61	0.74	0.43
60 min	1.48	1.13	1.42	1.34	0.19	0.11
Apical post	3.17	1.76	2.41	2.45	0.71	0.41
Cells post	0.08	0.10	0.13	0.10	0.03	0.02
Total collected	15.56	10.90	11.24	12.57	2.60	1.50
AN+RA						
Donor Solution	25.09	25.09	25.09	25.09	0.00	0.00
15 min	3.53	4.12	5.52	4.39	1.02	0.59
30 min	3.53	2.35	1.80	2.56	0.88	0.51
45 min	1.51	1.41	1.43	1.45	0.05	0.03
60 min	1.66	1.18	0.90	1.25	0.38	0.22
Apical post	1.07	2.18	2.43	1.90	0.72	0.42
Cells post	3.20	0.06	0.12	1.13	1.80	1.04
Total collected	14.49	11.29	12.21	12.66	1.65	0.95
AN+CA+RA						
Donor Solution	14.03	14.03	14.03	14.03	0.00	0.00
15 min	3.91	4.12	3.10	3.71	0.54	0.31
30 min	1.48	3.23	1.30	2.00	1.06	0.61
45 min	1.32	0.96	0.97	1.08	0.21	0.12
60 min	0.73	1.15	0.77	0.88	0.23	0.13
Apical post	1.94	3.26	0.88	2.03	1.19	0.69
Cells post	0.10	0.07	0.05	0.07	0.02	0.01
Total collected	9.49	12.78	7.07	9.78	2.87	1.66

Table C 7: Artemisinin content (µg) per well from chlorogenic acid concentration series experiment

AN content per sample (µg)						
Sample	Row A	Row B	Row C	Ave.	STD.	STD. Error
AN						
Donor Solution	8.11	8.11	8.11	8.11	0.00	0.00
15 min	1.05	0.37	1.01	0.81	0.38	0.22
30 min	1.69	1.20	0.68	1.19	0.51	0.29
45 min	1.49	0.78	1.05	1.11	0.36	0.21
60 min	0.09	0.36	0.90	0.45	0.41	0.24
Apical post	0.09	1.28	2.34	1.24	1.13	0.65
Total collected	4.41	3.99	5.98	4.80	1.05	0.60
AN+CA 1:1						
Donor Solution	8.95	8.95	8.95	8.95	0.00	0.00
15 min	0.35	0.71	1.76	0.94	0.73	0.42
30 min	1.19	0.67	1.34	1.06	0.35	0.20
45 min	0.76	0.53	1.94	1.08	0.76	0.44
60 min	0.16	0.27	1.00	0.48	0.45	0.26
Apical post	0.43	0.52	3.82	1.59	1.93	1.12
Total collected	2.90	2.70	9.86	5.15	4.08	2.36
AN+CA 2:1						
Donor Solution	6.04	6.04	6.04	6.04	0.00	0.00
15 min	0.93	1.21	2.60	1.58	0.90	0.52
30 min	0.66	1.62	1.26	1.18	0.49	0.28
45 min	0.64	1.68	0.30	0.87	0.72	0.42
60 min	0.07	0.47	1.04	0.53	0.49	0.28
Apical post	0.38	2.01	3.35	1.91	1.49	0.86
Total collected	2.68	7.00	8.55	6.08	3.04	1.76
AN+CA 3:1						
Donor Solution	5.89	5.89	5.89	5.89	0.00	0.00
15 min	2.21	0.73	0.56	1.16	0.91	0.52
30 min	1.10	0.72	1.13	0.98	0.23	0.13
45 min	0.42	0.78	0.74	0.65	0.20	0.11
60 min	0.23	0.41	0.56	0.40	0.17	0.10
Apical post	0.51	1.38	3.93	1.94	1.78	1.03
Total collected	4.47	4.01	6.92	5.13	1.56	0.90

Table C 8: Artemisinin content (µg) per well from camphor concentration series experiment

AN content per sample (µg)						
Sample	Row A	Row B	Row C	Ave.	STD.	STD. Error
AN						
Donor Solution	13.89	13.89	13.89	13.89	0.00	0.00
15 min	3.44	3.00	4.72	3.72	0.89	0.52
30 min	2.74	2.11	1.83	2.23	0.47	0.27
45 min	2.13	1.13	0.84	1.37	0.68	0.39
60 min	1.36	2.29	0.34	1.33	0.98	0.56
Apical post	3.40	7.50	1.77	4.23	2.95	1.71
Total collected	13.08	16.04	9.50	12.87	3.27	1.89
AN+C 1:1						
Donor Solution	19.20	19.20	19.20	19.20	0.00	0.00
15 min	4.07	5.29	3.42	4.26	0.95	0.55
30 min	3.70	3.26	1.99	2.98	0.89	0.51
45 min	1.53	2.47	3.03	2.35	0.76	0.44
60 min	2.87	1.57	3.70	2.71	1.07	0.62
Apical post	11.98	12.80	13.72	12.84	0.87	0.50
Total collected	24.15	25.40	25.86	25.14	0.88	0.51
AN+C 2:1						
Donor Solution	20.04	20.04	20.04	20.04	0.00	0.00
15 min	3.65	3.74	2.30	3.23	0.80	0.46
30 min	1.59	1.96	2.15	1.90	0.28	0.16
45 min	2.29	0.72	2.60	1.87	1.01	0.58
60 min	1.71	6.26	1.98	3.31	2.55	1.47
Apical post	7.96	1.25	3.72	4.31	3.40	1.96
Total collected	17.20	13.92	12.75	14.63	2.31	1.33
AN+C 10:1						
Donor Solution	12.79	12.79	12.79	12.79	0.00	0.00
15 min	6.38	1.74	3.07	3.73	2.39	1.38
30 min	2.81	4.57	1.30	2.89	1.64	0.95
45 min	1.21	2.18	2.46	1.95	0.66	0.38
60 min	1.50	2.23	2.02	1.92	0.37	0.22
Apical post	7.61	12.99	6.95	9.18	3.31	1.91
Total collected	19.51	23.71	15.81	19.68	3.96	2.28

Table C 9: Student t-test: paired two sample comparing means of AN with AN:C of 10:1

	AN	*AN+C 10:1*
Mean	52.16145913	65.78048
Variance	17.50902152	84.93522
Observations	3	3
Pearson Correlation	0.987379258	
Hypothesized Mean Difference	0	
df	2	
t Stat	-4.60048336	
P(T<=t) one-tail	0.022071974	
t Critical one-tail	2.91998558	
P(T<=t) two-tail	0.044143948	
t Critical two-tail	4.30265273	

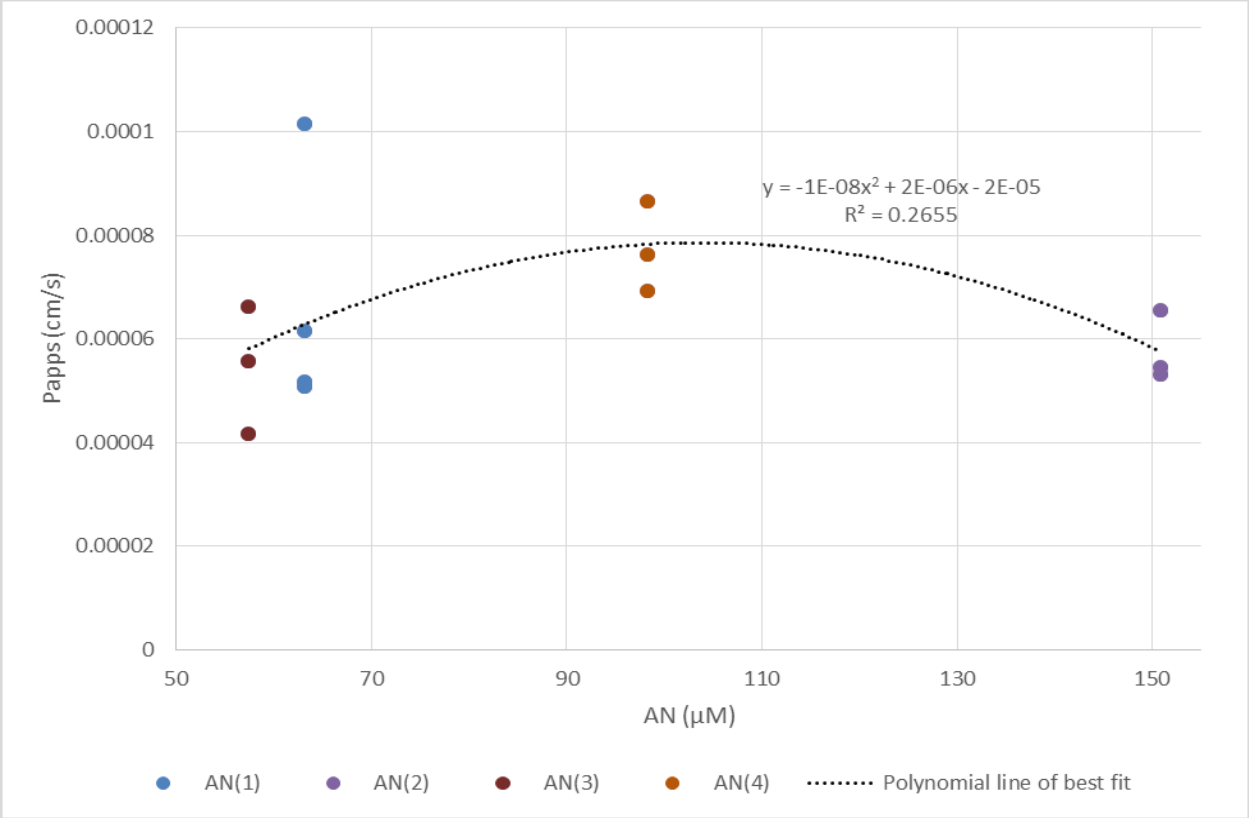

Figure C 1: P_{app} with varied artemisinin concentration in donor solutions. From each transport experiment, the AN concentration in donor solutions varied. To determine the role of AN concentration on rate of transport, all control (AN only) P_{app}s were plotted. The polynomial curve fit the points best with a low R^2 of 0.2655. From this figure, the concentration of AN can be used to approximate the rate of transport.

Table C 10: ANOVA single factor between P_{app} from all control transport experiments as plotted in Figure C 1.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
63.17269	3	0.000164	5.45E-05	3.54E-11
150.9236	3	0.000173	5.77E-05	4.65E-11
57.43839	3	0.000163	5.44E-05	1.53E-10
98.38355	3	0.000232	7.72E-05	7.59E-11

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.08E-09	3	3.6E-10	4.635748	0.036788	4.066181
Within Groups	6.21E-10	8	7.76E-11			
Total	1.7E-09	11				