Investigating the Mechanisms Underlying Enhanced Bioavailability of Artemisinin Delivered Orally as Dried Leaves of *Artemisia annua*

A Dissertation

Submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Biology and Biotechnology

By

Matthew R. Desrosiers

_____

5 May 2020

APPROVED BY:

_____

Pamela J. Weathers, PhD
Professor
Department of Biology and Biotechnology
Worcester Polytechnic Institute

_____

Jagan Srinivasan, PhD
Associate Professor
Department of Biology and Biotechnology
Worcester Polytechnic Institute

_____

José M. Argüello, PhD
Professor
Department of Chemistry and Biochemistry
Worcester Polytechnic Institute

_____

Scarlet Shell, PhD
Assistant Professor
Department of Biology and Biotechnology
Worcester Polytechnic Institute

Dedication

All of the work herein is dedicated to the memories of my brother, Brian Thomas Desrosiers, and my dad, Ronald Donald Desrosiers, both of whom passed away during my final two years of graduate school. While I will never get to hug them and hear them congratulate me upon my graduation, I do know two things: they will be with me always, and they would have been immeasurably proud.

Abstract

Malaria, a disease caused by parasites of the *Plasmodium* genus, infects over 220 million people annually, resulting in over 400,000 deaths. Most of these deaths occur in Africa in children < 5 years of age. *Artemisia annua* L., an ancient Chinese medicinal herb, is known for its foremost phytochemical constituent, artemisinin (AN). Semisynthetic derivatives of AN form the primary component of artemisinin combination therapies (ACTs), the frontline treatment for malaria worldwide. However, ACTs have several drawbacks including cost and availability. Thus, cheaper, more readily available antimalarials are needed. Recent clinical data suggested dried leaves of *A. annua* (DLA) administered orally as a tea infusion may be as efficacious as ACTs despite a significantly lower AN dose delivered. In mice, AN plasma concentration was improved when administered as DLA compared to pure AN. I therefore hypothesized that phytochemicals within DLA enhanced the oral bioavailability of AN. To investigate this hypothesis, here I examined the effects of DLA on the underlying mechanisms that govern oral bioavailability. Using an *in vitro* human digestion model, I showed that AN solubility was greater when delivered as DLA, largely due to essential oil in the plant. Furthermore, AN intestinal permeability was enhanced in a Caco-2 cell model of the intestinal epithelium. Extracts, teas, and phytochemicals produced by *Artemisia* also inhibited the activity of CYP2B6 and CYP3A4, the enzymes responsible for first-pass AN metabolism in the liver. Additionally, AN tissue distribution was improved when delivered as DLA and AN accumulation in tissues was higher in female vs. male rats. Finally, I showed that DLA was a more efficacious anti-inflammatory than pure AN in rats, potentially due to enhanced AN bioavailability. Taken together, these results shed light on the mechanisms behind enhanced oral bioavailability afforded by DLA and demonstrate the potential for DLA to be used as a therapeutic for malaria and other diseases.

Acknowledgements

A PhD is by no means one person's accomplishment. While my name is on the cover of this dissertation, I never could have accomplished any of this without such a wonderful community of people supporting me. I would like to first thank my outstanding advisor, Dr. Pamela Weathers. I still remember my initial interview in her office and ultimately it was Pam's passion for her science that made me choose WPI for my graduate schooling. She taught me how to think critically, how to approach a problem methodically, and how to write like a scientist. But Pam is so much more than a research mentor. She supports her students in all aspects of life and I'll always be grateful for the guidance she gave me after my brother and dad passed away. Throughout the past 6 years she has become like family to me. There are few people I respect and admire more.

I also would like to thank the rest of the members of the Weathers Lab, past and present, and especially Dr. Melissa Towler. From lab meetings to GC-MS analysis to flavonoid assays, she's helped me in nearly every aspect of my lab work at WPI. I am also appreciative of the current and former members of my graduate committee, Dr. José Argüello, Dr. Scarlet Shell, Dr. Jagan Srinivasan, and Dr. Jill Rulfs, for their insight and guidance throughout my time in graduate school.

I would like to say a special thank you to our colleagues in facilities, especially Andy Butler, Eric Sabacinski, and Daryl Johnson. Their efforts to keep our building and core equipment up and running properly are invaluable to the students and faculty performing research at WPI. Similarly, I'd like to thank Miguel Norden, Dr. Virender Chandna, Vicki Huntress, Elizabeth Diers, and the rest of the WPI IACUC and vivarium staff. Their help in training, handling, caring for, and ordering animals was instrumental to this work.

My sincere appreciation goes out to all the professors and administrative faculty of the WPI Biology and Biotechnology Department. Each of you have helped make me a better scientist and person.

I am especially appreciative of the Society for *In Vitro* Biology, the International Congress on Integrative Medicine and Health, the American Society of Pharmacognosy, and the Northeast Section of the American Society of Plant Biologists for providing me the opportunity to share my work and expand my professional network through their annual meetings. Thank you to the WPI Graduate Student Travel Fund, Dean Terri Camesano, and the WPI Biology and Biotechnology Department for generously providing funding for me to attend these annual meetings. It was through the ASP meeting that I was able to form a collaboration with Dr. Nadja Cech to better understand the components in *A. annua* that affect hepatic metabolism. Special thanks to Dr. Cech, her student Manead Khin, and the rest of the Cech Lab for making me feel welcome and teaching me so much in the two weeks I spent in their lab at University of North Carolina Greensboro this past Fall. Similarly, I am grateful to Dr. David Greenblatt and his student, Mia Angeli, of Tufts University School of Medicine who trained me in the use of human liver microsomes.

I'd also like to thank the National Center for Complementary and Integrative Health (NCCIH) and our program officer, Dr. Craig Hopp, for providing the funding for this research. Dr. Hopp was a great source of advice when we met at the ASP meeting in July 2019 and also helped connect me to Dr. Cech.

I'm so incredibly thankful for all the lifelong friends I've met at WPI, especially my roommates, Conor Herlihy, Travis Henchie, Kevin Keating, Dr. Josh Gershlak, Zach Goldblatt, and Meena Khayami who have made my time in graduate school way more fun than graduate school should ever be. Michelle McKee also deserves a special shout out for sitting next to me,

putting up with me, and making me laugh on a daily basis for the past several years. You have all made a lasting impact on my life and it has been a privilege to work, laugh, cry, and now celebrate alongside each and every one of you.

Finally, I am eternally grateful to my mother Marcie, sister Cara, and my lovely girlfriend Adrienne. Your love and support means the world to me and I never would've been able to do any of this work without you.

List of Publications

The following list of peer-reviewed articles were published during my time as a graduate student at WPI. Some are included partially or in full as part of this dissertation.

Desrosiers M. and Weathers P.J., 2016. Oral consumption of dried *Artemisia annua* leaves to treat malaria: leaf digestion and artemisinin solubility. Journal of Ethnopharmacology 190: 313-318. **(Chapter 3 of this dissertation)**.

Desrosiers M. and Weathers P.J., 2017. Artemisinin permeability via Caco-2 cells increases after simulated digestion of *Artemisia annua* leaves. Journal of Ethnopharmacology. 210: 254-259. **(Chapter 4 of this dissertation)**.

Desrosiers M, Towler M.J., Weathers P.J. 2019. Chapter 6: *Artemisia annua* and *A. afra* essential oils and their therapeutic potential. IN: Essential Oil Research – Trends in Biosynthesis, Analytics, Industrial Applications and Biotechnological Production. Ed. Sonia Malik, Springer International Publishing AG, Heidelberg, GDR. Pp. 197-209.

Desrosiers M., Mittelman A., Weathers P.J. 2020. Dried leaf *Artemisia annua* improves bioavailability of artemisinin via cytochrome P450 inhibition and enhances artemisinin efficacy downstream. Biomolecules. 10: 254. **(Chapter 5 of this dissertation)**.

Weathers P.J., Cambra H.M., **Desrosiers M.**, Rassias D., Towler M.J., 2016 Chapter 5: Artemisinin the Nobel molecule: from plant to patient. Studies in Natural Products Chemistry (Bioactive Natural Products) Volume 52, Ed. Atta-ur-Rahman Elsevier Science Publishers, The Netherlands. Pp 193-229.

Gruessner B.M., Cornet-Vernet L., **Desrosiers M.R.**, Lutgen P., Towler M.J., Weathers P.J. 2019. It is not just artemisinin: *Artemisia* sp. for treating diseases including malaria and schistosomiasis. Phytochemistry Reviews. 18:1509-1527.

Table of Contents

List of Tables

List of Figures

Chapter 1 Background and Introduction[a]

1.1 History of Malaria

Malaria, a disease caused by parasites of the *Plasmodium* genus, has wreaked havoc on humankind since time immemorial. Indeed, malaria as a disease was documented for several centuries by ancient civilizations prior to Charles Laveran's discovery of the *Plasmodium* parasite in 1880 (Institute of Medicine 2004). The Nei Ching, the Chinese Canon of Medicine and the oldest known medical text, makes the earliest known reference to a disease that was almost certainly malaria in 2700 BC (Shampo and Kyle 1989; Cox 2010; Institute of Medicine 1991) and *Plasmodium*-specific antigens were discovered in the remains of Egyptian mummies from as far back as 3200 BC (Miller et al. 1994). Additionally, there are references to malaria in cuneiform on clay tablets from Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC, and sixth century BC Hindu texts (Cox 2010). By the 19th century, malaria had spread to all 6 permanently inhabited continents and over half of the world's population was at risk (Carter and Mendis 2002). Without a formal cure or therapy, about 1 in 10 people affected by malaria died from the disease (Carter and Mendis 2002). It's estimated malaria killed 150-300 million people, roughly 2-5% of the total population, in the 20th century (Carter and Mendis 2002) and it is thought that malaria has killed more humans than any other disease (Butler, Khan, and Ferguson 2010). All of this is to say malaria is one of the most significant and deadly diseases in human history and thus its eradication would be a monumental achievement.

[a] Small portions of this chapter were published in Weathers et al. (2017).

1.2 History of Malaria Treatment: From Quinines to Artemisinins

The discovery and isolation of quinine from the bark of the Peruvian *Cinchona* tree in 1820 marked the first major breakthrough in treating malaria in the Western world (Butler, Khan, and Ferguson 2010). It eventually led to the development of synthetic quinine derivatives, mepacrine and chloroquine, which dominated malaria treatment in the 20th century (Butler, Khan, and Ferguson 2010). However, *Plasmodium* species quickly developed resistance to these treatments in the late 1960s and soon new drugs were severely needed (Tu 2016). Thus the Chinese government launched Project 523, a project enlisting over 500 scientists at over 60 institutions to examine ancient traditional Chinese medicine in search of a new therapy for malaria (Butler, Khan, and Ferguson 2010). The primary outcome of the project was the discovery of a powerful antimalarial molecule, artemisinin (Figure 1.1), isolated from the Chinese herb *Artemisia annua* by Dr. Youyou Tu for which she was later awarded the 2011 Lasker Award and the 2015 Nobel Prize in Physiology or Medicine (Tu 2016). Semisynthetic derivatives of artemisinin (Figure 1.1) were soon developed to combat the near insolubility of artemisinin in water to form the current malaria therapies used today (Butler, Khan, and Ferguson 2010).

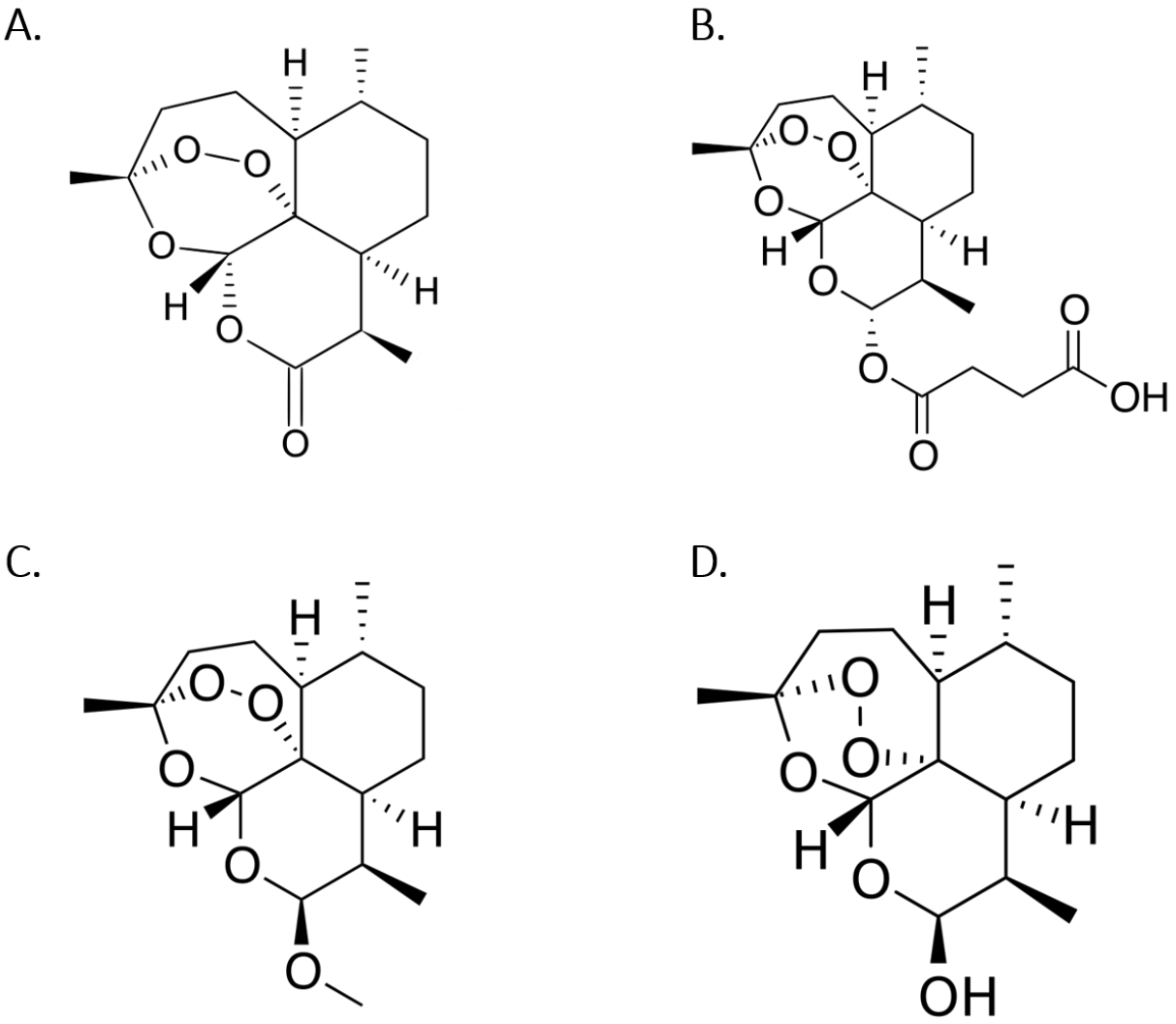

Figure 1.1 Chemical structures of artemisinin (A) and the semisynthetic artemisinin derivatives artesunate (B), artemether (C), and dihydroartemisinin (D).

1.3 Malaria Today

The discovery and widespread use of quinines and artemisinins in conjunction with global efforts to eradicate malaria drastically reduced the number of annual malaria deaths (Carter and Mendis 2002). However, there is still work to be done and the development of artemisinin resistance threatens to undo decades of progress made towards eradication (Butler, Khan, and Ferguson 2010). In 2018 , there were an estimated 228 million malaria cases worldwide resulting

in about 405,000 deaths (WHO 2019c). What is perhaps most disturbing about malaria is that it is largely a disease that kills children and toddlers. In fact, in 2018 children under the age of 5 accounted for 67% of all malaria deaths worldwide (WHO 2019c). In order to understand how to treat malaria, we must first understand the communities and demographics most affected by malaria. Of all malaria cases in 2018, 93% occurred in the World Health Organization (WHO) Africa Region and 94% of all global malaria deaths occurred in this same region (WHO 2019c). Even more specifically, 19 countries in sub-Saharan Africa were home to 85% of the global malaria burden (WHO 2019c).

Malaria is also intimately linked with poverty. The disproportionate effect felt by those in extreme poverty is illustrated by the fact that the highest malaria mortality rates occur in regions of the world with the highest proportion of people living on less than US$1.25/day (WHO 2012c). Causality is difficult to determine, however, it is generally thought that causality works in both directions (Teklehaimanot and Mejia 2008; Sachs and Malaney 2002). Extreme poverty sustains the conditions for malaria transmission and survival and in turn the tremendous burden of malaria stifles economic growth and keeps endemic communities impoverished (Teklehaimanot and Mejia 2008; Sachs and Malaney 2002). This repetitive cycle will likely only be broken by the introduction of low-cost malaria interventions that treat all stages of the disease to ease the disease burden in endemic communities.

1.4 Malaria Lifecycle

The parasites that cause malaria undergo their lifecycle in both female *Anopheles* mosquitoes and humans (Figure 1.2) (CDC 2019a). Infection in humans begins when a mosquito takes a blood meal and injects sporozoites into the human (Figure 1.2) (CDC 2019a). The

sporozoites travel to the liver where they divide and mature until they are released into the blood (Figure 1.2) (CDC 2019a). The erythrocytic stages of the malaria life cycle, where parasites rapidly divide and rupture red blood cells, are responsible for the symptoms usually associated with malaria (CDC 2019a). In most cases, uncomplicated malaria symptoms include fever, chills, sweats, head and body aches, nausea and vomiting, and general malaise (CDC 2019b). However, in certain cases patients develop severe malaria which can include cerebral malaria causing abnormal behavior, impairment of consciousness, seizures, coma, and other neurologic abnormalities (CDC 2019b). Severe malaria can also cause severe anemia, acute respiratory distress, and acute kidney injury among other serious symptoms (CDC 2019b). The malaria lifecycle stages that take place within the bloodstream include trophozoites and schizonts (Figure 1.2) (CDC 2019a). Importantly, some trophozoites differentiate into male and female gametocytes during initiation of the sexual cycle and gametocytogenesis (Figure 1.2) (CDC 2019a). These gametocytes are subsequently taken up by the mosquito when it draws a blood meal and male and female gametocytes mate in the gut of the mosquito (Figure 1.2) (CDC 2019a). The mated gametocytes mature and multiply in the mosquito forming sporozoites that travel to the salivary glands of the mosquito beginning the cycle anew (Figure 1.2) (CDC 2019a).

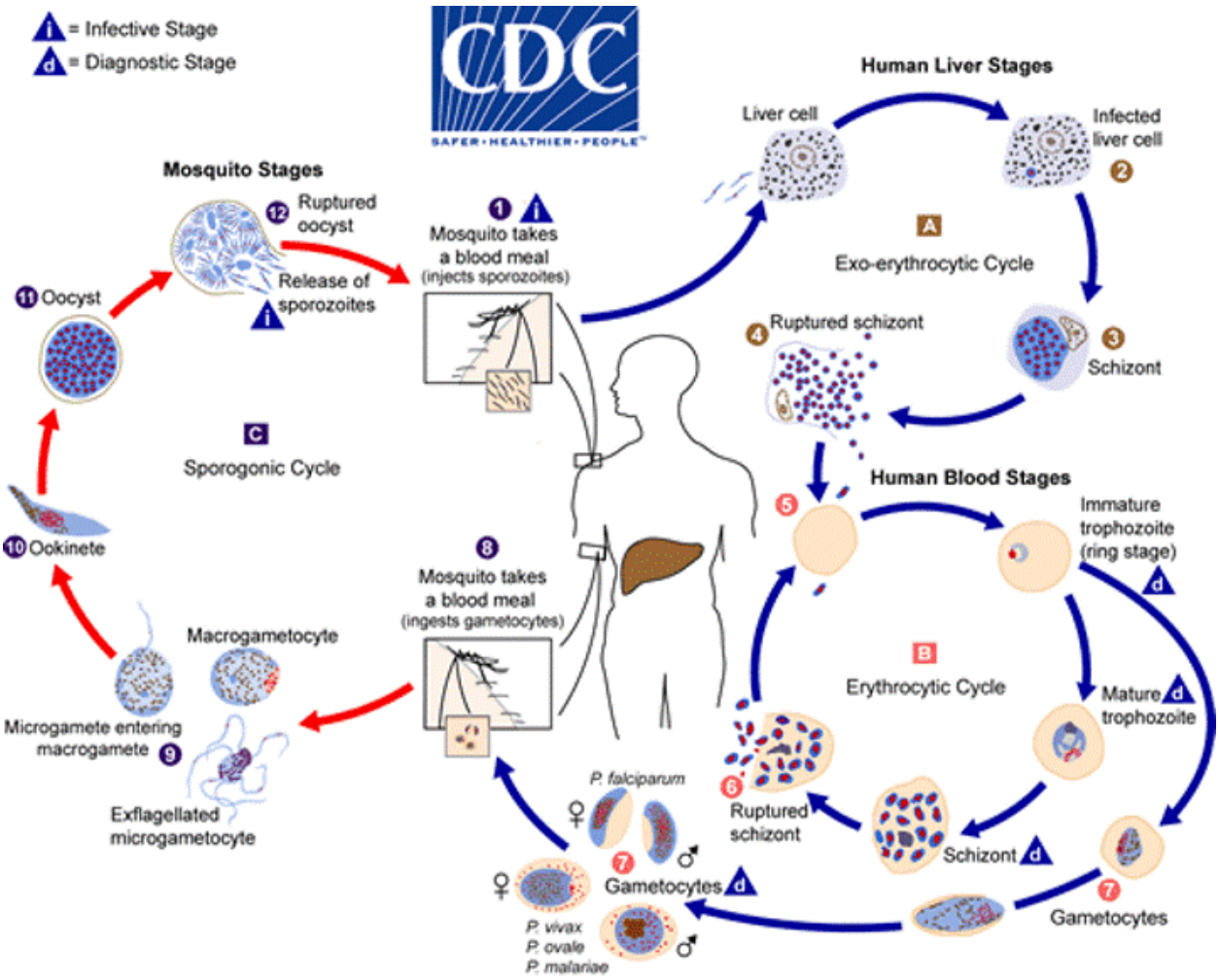

Figure 1.2 Malaria lifecycle in mosquitos and humans. Taken from Centers for Disease Control and Prevention (CDC 2019a).

1.5 Artemisinin Combination Therapies

Today, a commonly used antimalarial drug, chloroquine, is largely ineffective due to widespread resistance, so artemisinin combination therapies (ACTs) have emerged as the frontline treatment for malaria worldwide (CDC 2019c; WHO 2015). ACTs achieve their powerful antimalarial effectiveness by combining semisynthetic derivatives of artemisinin (Figure 1.1) with a secondary antimalarial, e.g. lumefantrine (see below), that remains in the blood for a longer duration to eliminate parasites that withstand the initial artemisinin challenge (WHO 2015;

Eastman and Fidock 2009; Tilley et al. 2016). Artemisinin is a sesquiterpene lactone produced in the glandular trichomes of the Chinese herb *A. annua* L. (Figure 1.3) (Tellez et al. 1999; Xiao, Tan, and Zhang 2016).

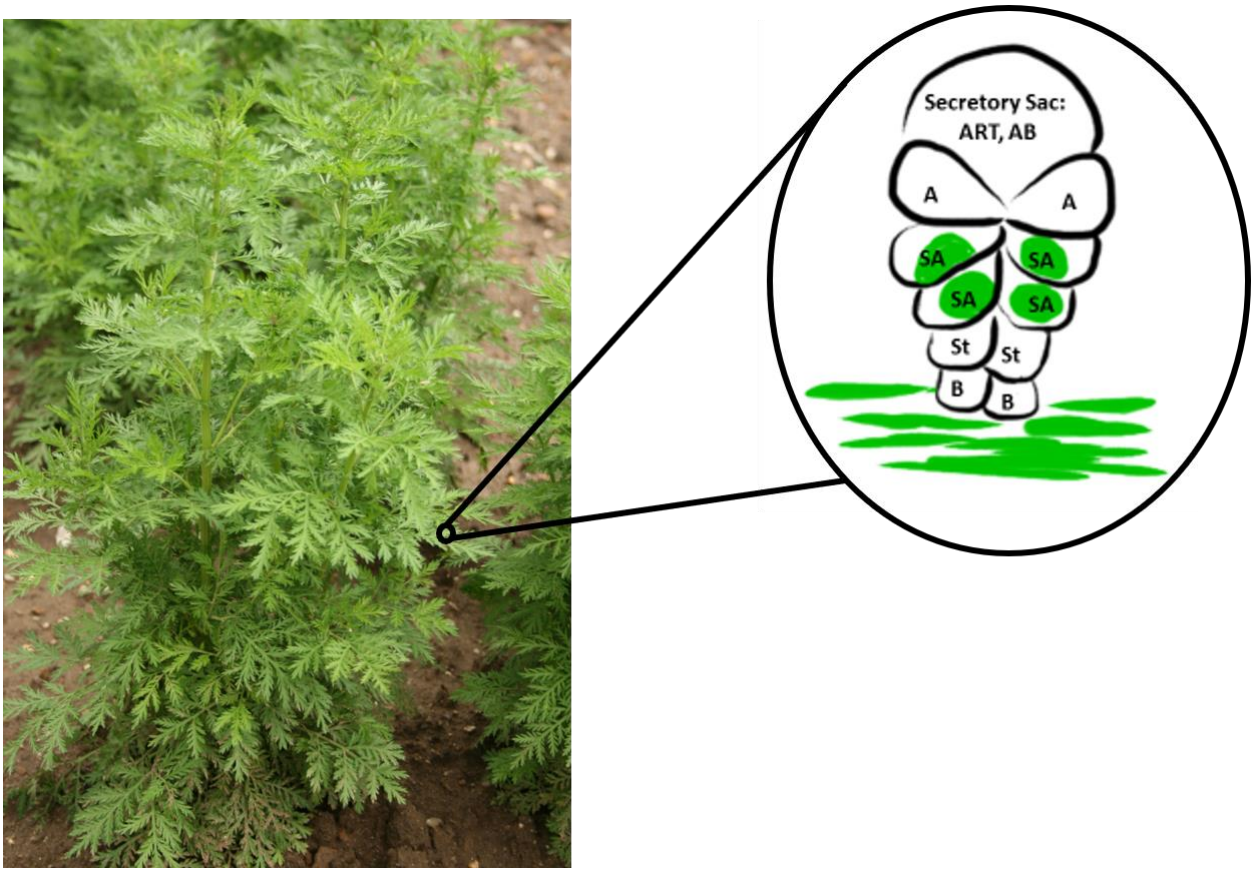

Figure 1.3 *A. annua* L. plant with a diagram of glandular trichome morphology (inset). A, apical cell; B, basal cell; SA, subapical cell; St, stalk cell; ART, artemisinin; AB, arteannuin B. Shaded cells contain chloroplasts. Adapted from Weathers et al. (2017).

Artemisinin's chemical structure contains an endoperoxide bridge that is rare in a natural product and affords artemisinin its primary potent antimalarial efficacy (Meunier and Robert 2010). Antimalarial derivatives of artemisinin such as artesunate, artemether, and dihydroartemisinin, all retain this endoperoxide bridge (Figure 1.1). However, artemisinic compounds, such as deoxyartemisinin (Figure 1.4), that lack this feature have no antimalarial

efficacy (Meunier and Robert 2010). As of 2015, the WHO recommended 5 ACTs for the treatment of malaria based on high quality evidence. These ACTs include artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, dihydroartemisinin + piperaquine, and artesunate + sulfadoxine-pyrimethamine (WHO 2015). One of the major drawbacks to ACTs is their cost. As mentioned in section 1.3, high death rates due to malaria occur mostly in parts of the world with high levels of extreme poverty.

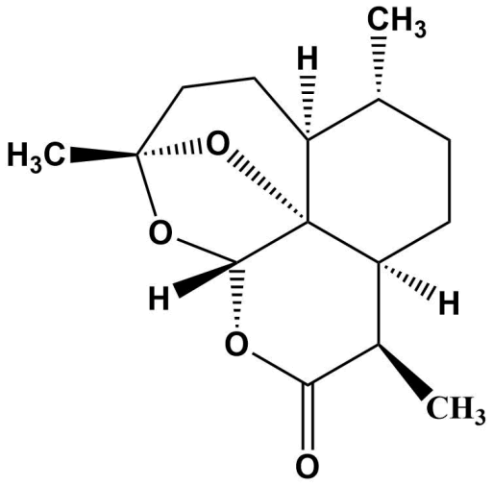

Figure 1.4 Chemical structure of deoxyartemisinin.

Whereas chloroquine costs about $0.10 per treatment course, ACTs cost upwards of 10 times that figure (Institute of Medicine 2004). In 2004, the cost per treatment for CoArtem® (artesunate + lumefantrine) was $2.40 wholesale before being marked up by pharmacies in Africa (Institute of Medicine 2004). While efforts have been made to reduce ACT costs, a 2014 study showed the median cost per malaria case averted was still $1.94 (Okell et al. 2014). This cost makes treatment with ACTs prohibitively difficult for those in extreme poverty to afford treating themselves and their children. Interestingly, ACTs are typically free or heavily subsidized in the public or not-for-profit sector, but most people in Africa seek malaria treatment in the private sector where ACTs are 10-20 times more expensive than non-artemisinin therapies (O'Connell et al. 2011; Littrell et al. 2011). A 2011 survey in 6 African countries asked about treatment seeking behavior of caregivers to children under 5 (Littrell et al. 2011). In 5 of the 6 countries, private sector outlets were the most common place for caregivers to seek treatment for fever (Littrell et al. 2011). However, in Benin, the Democratic Republic of the Congo, Madagascar, and Nigeria, ACTs were only given as treatment in 10% or fewer of cases. In Uganda and Zambia ACTs were given in 21%

of cases (Littrell et al. 2011). Public sector ACT administration was improved but still very low (Littrell et al. 2011). Part of the problem may be that caregivers do not know that ACTs are the first-line treatment for malaria. In 4 of 6 countries 10% or fewer caregivers named ACTs as the frontline treatment for malaria (Littrell et al. 2011). Since most caregivers seek treatment in the private sector, a private sector pilot initiative, the Affordable Medicines Facility for Malaria (AMFm), was established to lower costs. AMFm provided subsidized, quality-assured ACTs to the private at a target retail price of $0.50 per treatment course (O'Connell et al. 2011). This pilot initiative, carried out by the Global Fund to Fight AIDS, TB, and Malaria, was initially successful, reducing price and increasing availability of ACTs in 5 of 8 countries (Tougher et al. 2012). After the pilot ended in 2011, the subsidy program was continued using the private sector co-payment mechanism established in the pilot program (ACTwatch et al. 2017). While prices for ACTs improved, they still remained higher than $1.00 per adult treatment in most countries using the private sector co-payment mechanism in 2015 (ACTwatch et al. 2017).

Beyond the challenge of cost, availability is also a major barrier to the widespread use of ACTs. Rural areas are often isolated from Western style healthcare providers, do not have access to these life-saving drugs, and poor infrastructure further exacerbates travel ability. The effect of this combination of extreme poverty and inaccessibility of ACTs is that people, especially children, do not get treatment or do not get treatment in time to thwart the infection. In a 2017 Lancet article addressing coverage of ACTs in Africa, the authors wrote,

> "Although we documented noticeable increases in ACT coverage from 2003 to 2015, which correspond with increased investments in ACTs and RDTs, coverage remains unacceptably low across Africa: over three quarters (80.3%) of children with malaria did not get a potentially life-saving ACT in 2015. The two biggest drivers of this coverage gap are poor access to and delivery of health services." (Bennett et al. 2017).

This same study showed that children with malaria in rural areas whose household wealth index was below the national median were significantly less likely to receive an ACT than children living in urban areas with a household wealth index above the national median (Bennett et al. 2017). Thus, while ACTs are effective and important tools for combatting malaria, unless drastic changes are made to improve availability and affordability, their use alone will not be enough to eradicate malaria.

1.6 Historical Use of Dried Leaves of *A. annua* to Treat Malaria

While artemisinin was discovered to be the primary antimalarial compound produced by *A. annua* in the early 1970s, medicinal use of the plant dates back over 2000 years (Hsu 2006). In the fourth century CE, the Chinese philosopher Ge Hong described a method of soaking the whole *Artemisia* plant in water and then wringing out the juices. This resulting juice was ingested in its entirety for the treatment of "intermittent fevers." Since the *Plasmodium* parasite was not discovered until 1880 (Institute of Medicine 2004), it is impossible to know for sure if these fevers were due to malaria (Hsu 2006). There is considerable confusion as to which species of *Artemisia* was most commonly used. While *A. annua* is the better known, due to its importance as a producer of artemisinin, a careful examination of the history of traditional Chinese medicine reveals that *A. apiacea* was used as often, if not more often, than *A. annua* (Hsu 2006). In fact, the commonly used Chinese name for *A. annua*, "qing hao," was actually the name given to *A. apiacea* while the Chinese name for *A. annua* is actually "huang hua hao" (Hsu 2006). Nevertheless, the name "qing hao" is still commonly used to refer to *A. annua*. Notably, *A. apiacea* produces significantly less artemisinin than *A. annua* (Hsu 2006; Numonov et al. 2019; Mannan et al. 2010) so then the question remains: why would the ancient Chinese use *A. apiacea* over *A. annua*? Perhaps *A. apiacea* contained other antimalarial compounds or compounds that synergize with artemisinin

(Hsu 2006). There is much lost in translation over thousands of years and reputable information is scarce, so these questions are very difficult to answer with certainty. Regardless, researchers are now slowly discovering that artemisinin is likely not the only molecule responsible for the antimalarial efficacy of *Artemisia* species against malaria.

1.7 Use of Dried Leaves of *A. annua* Against Malaria *In Vitro*

A few studies have indeed tested various *A. annua* formulations against various *Plasmodium* strains *in vitro* to compare their antimalarial efficacy to pure artemisinin or other standard drugs. Wright et al. (2010) investigated pounded vs. wrung juices of fresh *A. annua* and determined the antiplasmodial efficacy was 6-18-fold better than pure artemisinin. De Donno et al. (2012) tested *A. annua* tea infusions against a chloroquine-sensitive and a chloroquine-resistant *P. falciparum* strain *in vitro* and showed the tea infusions to have stronger antimalarial efficacy in both strains compared to pure artemisinin, indicating there was likely synergism between artemisinin and secondary phytochemicals produced in the plant.

Indeed, several phytochemicals found in *Artemisia* species have now been identified as having either their own inherent antimalarial efficacy or synergistic antimalarial activity with artemisinin (Gruessner et al. 2019). For example, Liu et al. (1992) showed 6 flavonoids commonly found in *A. annua*, artemetin, casticin, chrysosplenetin, chrysosplenol-D, cirsilineol, and eupatorin have antimalarial activity as well as synergistic activity with artemisinin, albeit at orders of magnitude less potent than artemisinin. Suberu et al. (2013) found antimalarial activity of artemisinic acid, arteannuin B, dihydroartemisinic acid, chlorogenic acid, rosmarinic acid, and isovitexin as well as some strain-dependent additive and synergistic effects, but synergistic interaction levels were concentration dependent and thus difficult to explicitly define. Inherent antimalarial efficacy was also shown for the flavonoids apigenin, luteolin, kaempferol, myricetin,

quercetin, and rutin (Lehane and Saliba 2008; Ganesh et al. 2012; Penna-Coutinho, Aguiar, and Krettli 2018). Besides flavonoids, the monoterpenes α-pinene, 1,8-cineole (eucalyptol), limonene, and nerolidol, all found in *A. annua* also have antimalarial activity (Van Zyl et al. 2006; Goulart et al. 2004). Other phytochemicals from *A. annua* reported to have antiplasmodial activity include phytol and scopoletin (Grace et al. 2012; Zaid et al. 2016). It is important to note that of these antiplasmodial phytochemicals, none were as potent as artemisinin (Gruessner et al. 2019). In fact, all but 3 of the previously mentioned phytochemicals had IC_{50} values against *P. falciparum* 3 orders of magnitude higher than artemisinin (0.022-0.033 µM) and only 1, nerolidol, had an IC_{50} below 1 µM (0.76 µM) (Gruessner et al. 2019; Liu et al. 1992; Suberu et al. 2013; Goulart et al. 2004). These results are summarized in Table 1.1 (taken from (Gruessner et al. 2019)).

Table 1.1. Phytochemicals in *A. annua* with antimalarial activity. Taken from Gruessner et al. (2019).

Compound	Compound IC_{50} (µM)	Compound + artemisinin IC_{50} (µM)	References
Artemisinin	0.033	NA	(Liu et al. 1992)
	0.022, 0.023[a]	NA	(Suberu et al. 2013)
Artemisinic acid	77.8, 61.6[a]	varies with compound concentration	
Arteannuin B	3.2, 4.8[a]		
Dihydroartemisinic	21.1, 17.7[a]		
Chlorogenic acid	69.4, 61.4[a]		
Rosmarinic acid	65.1, 65.0[a]		
Isovitexin	72.5, 48.1[a]		
Artemetin	26.0	0.026	(Liu et al. 1992)
Casticin	24.0	0.026	
Cirsilineol	23.0	0.023	
chrysoplenol-D	32.0	0.015	
Chrysoplenetin	36.0	0.016	
Eupatorine	65.0	0.030	
Apigenin	20.0, 13.0[b]	not determined	(Lehane and Saliba 2008)
Luteolin	11.0, 12.0[b]		
Kaempferol	33.0, 25.0[b]		
Myricetin	40.0, 76.0[b]		
Quercetin	15.0, 14.0[b]		
	14.7, 4.1, 2.9[c]		(Ganesh et al. 2012)
	13.0[d,i]		(Penna-Coutinho, Aguiar, and
Rutin	7.1, 3.5, 10.4[c]		(Ganesh et al. 2012)
a-pinene	1.0[e]		(Van Zyl et al. 2006)
1,8-cineole	70.0[e]		
Limonene	533.0[e]		
Nerolidol	9.0[e]		
	0.76[f]		(Goulart et al. 2004)
Phytol	18.9[g]		(Grace et al. 2012)
Scopoletin	128.0, 121.5[h]		(Zaid et al. 2016)

NA, not applicable; CQ, chloroquine.
[a]CQ-sensitive HB3 and CQ-resistant Dd2 strains, respectively.
[b]CQ-sensitive 3D7 and CQ-resistant 7G8 strains, respectively.
[c]fresh blood isolates (Bangladesh), CQ-sensitive 3D7, and CQ-resistant K1 strains, respectively.
[d]CQ-resistant W2 strain.
[e]CQ-resistant FCR-3 strain.
[f]CQ-sensitive 3D7
[g]CQ-sensitive D10 strain.
[h]CQ-resistant K1 and CQ-sensitive 3D7 strains, respectively
[i]test *in vitro* and also *in vivo* in mice.

While a host of secondary phytochemicals in *A. annua* have some antimalarial efficacy, artemisinin is by far the most important and there is debate over synergy as synergistic activity has not always been observed *in vitro*. A 2012 report testing *A. annua* infusions from 4 different locations in Brazil noted no synergy was found as each infusion had an IC_{50} similar to pure artemisinin (Silva et al. 2012). Another study tested tea infusions and chloroform extracts from 16 *A. annua* and 2 *A. afra* cultivars from 11 different countries and determined that there was no synergism as the IC_{50} values for the teas and extracts did not differ from that of pure artemisinin (Mouton et al. 2013). A recent 2019 report bolstered this argument by testing extracts from genetically modified *A. annua* plants (Czechowski et al. 2019). In that report, investigators compared extracts made from wild-type *A. annua* plants, *A. annua* plants with mutations to the *chalcone isomerase 1 (CHI1)* gene, plants with *cyp71av1-1* mutations, and plants treated with RNAi to silence amorpha-4,11-diene synthase (*AMS*). Wild-type plants produced normal amounts of artemisinin and flavonoids while *chi1-1* mutants produced undetectable levels of flavonoids and normal artemisinin levels. The *cyp71av1-1* mutants produced undetectable levels of artemisinin and normal flavonoids, while RNAi silenced *AMS* line produced normal flavonoid levels but 5% of the artemisinin found in the wild-type plants. Both wild-type and *chi1-1* mutants inhibited growth of *P. falciparum* at similar levels with indistinguishable differences in the rate of killing indicating that flavonoids in *A. annua* extracts likely do not contribute to antimalarial activity (Czechowski et al. 2019). Furthermore, *cyp71av1-1* homozygous mutants lacking artemisinin showed 300-fold reduction in antiplasmodial efficacy while *AMS* silenced lines produced 20-fold reduction in potency (Czechowski et al. 2019). It is thus highly unlikely that flavonoids contributed significantly to the antiplasmodial efficacy seen *in vitro*. However, that is not to say that flavonoids or other phytochemicals do not play a role *in vivo*.

1.8 Use of Dried Leaves of *A. annua* Against Malaria *In Vivo*

While there is some debate over *in vitro* data, there is strong evidence that *A. annua* is significantly more efficacious than pure artemisinin against malaria *in vivo.* A 2012 study showed that powdered dried leaves of *A. annua* (DLA) were more effective at reducing parasitemia in mice than pure artemisinin (Elfawal et al. 2012). Mice infected with *P. chabaudi* were given either pure artemisinin or powdered DLA at 2 equal artemisinin doses, 24 mg/kg and 120 mg/kg. At the low dose, pure artemisinin treatment showed no difference in parasitemia from the negative control while parasitemia was significantly reduced after DLA treatment (Elfawal et al. 2012). In fact, the 24 mg/kg dose of DLA was as efficacious at reducing parasitemia as the 120 mg/kg dose of pure artemisinin for the first 72 hours (Elfawal et al. 2012). Another study tested pounded juice of *A. annua* and found that two doses, corresponding to 9 mg/kg artemisinin each, given 12 hours apart were sufficient to reduce parasitemia by 95% in mice (Wright et al. 2010). This was markedly better than a single 30 mg/kg dose of pure artemisinin that reduced parasitemia by 88% (Wright et al. 2010). These studies suggested DLA could be a more effective antimalarial therapy than pure artemisinin however, clinical trials are the gold standard for evaluating efficacy of therapeutics in humans.

1.9 Clinical Trials of *A. annua* for Treatment of Malaria

A number of clinical trials have been performed to evaluate the efficacy of DLA against malaria with varying success (ICIPE 2005; Tchandema, Lubumbashi, and Lutgen 2016; Mueller et al. 2004; Munyangi et al. 2019; Wan, Zang, and Wang 1992; Chang and But 1986; Willcox et al. 2004; Mueller et al. 2000). Chang and But (1986) report on a trial using crude alcohol extracts of *A. annua* given to patients with *P. falciparum* and *P. vivax* malaria infections. The total dose

was 72 g crude extract over 3 days and a cure rate of 100% was observed in 485 cases of *vivax* malaria and 105 cases of *falciparum* malaria (Chang and But 1986; Willcox et al. 2004). However, the definition of cure was unclear and there was no follow-up at day 28 to assess recrudescence (Chang and But 1986; Willcox et al. 2004). Another smaller trial treated *vivax* malaria patients with two types of gelatin capsules containing a total of 128.8 g *A. annua* over 6 days of treatment (Wan, Zang, and Wang 1992; Willcox et al. 2004). The best performing capsule, containing oil to enhance artemisinin absorption, had faster parasite and fever clearance times than chloroquine, achieved 100% cure rate, and 8% recrudescence at day 28 (Willcox et al. 2004; Wan, Zang, and Wang 1992). Another proof-of-concept study was performed in Kenya in 2005 in which 4 cohorts of 11-12 malaria patients were given pressed tablets made from powdered DLA. Each cohort was given a different dosing regimen that totaled between 51.8 and 185 mg of artemisinin over the course of 6 days and parasitemia was assessed at day 7, 14, and 28 following the start of treatment. In each of the 4 cohorts only 1 of 11 (9%) or 2 of 12 (17%) patients had parasitemia at day 7 (ICIPE 2005). There were a few cases of recrudescence or reinfection but determining between the two was not possible without genotyping of the parasites (ICIPE 2005). Nevertheless, these early studies using extracts, capsules, or tablets made from *A. annua* suggested that DLA could be used as an antimalarial therapy.

Another later small trial using capsules was performed in the Democratic Republic of the Congo (DRC) in 3 cohorts of adults. Patients with uncomplicated *P. falciparum* malaria were treated with capsules of powdered *A. annua* leaves from Luxembourg or Burundi or powdered *A. afra* leaves. These three cohorts were 20, 37, and 25 patients, respectively. After 7 days of treatment, 85%, 76%, and 40% of patients were clear of parasites, respectively, indicating that the *A. annua* treatments were potentially efficacious (Tchandema, Lubumbashi, and Lutgen 2016).

This trial and the previously mentioned ICIPE trial have similar drawbacks. They were only done with small patient populations, had inadequate detail in their methods, and inadequate phytochemical analysis of the plant material used.

While extracts, capsules, and tablets have all been explored, the most traditional form of ingesting *A. annua* is via an aqueous infusion or tea. Preparation methods vary but most involve steeping a specified amount of dried leaf material in hot or boiling water. An early trial using this method reported a 93% cure rate in 254 patients given a 7-day course of treatment in the Democratic Republic of the Congo (Willcox et al. 2004). Follow-up of a subset of 31 patients indicated recrudescence was 13% after one month (Willcox et al. 2004). A smaller trial by Mueller et al. (2000) tested two preparation methods: a decoction where 5 g dried leaves were placed in 1 L water and boiled for 5 minutes then filtered; and an infusion where 1 L boiling water was added to 5 g dried leaves and allowed to cool for 15 minutes before filtering (Mueller et al. 2000). Both groups were dosed 250 mL 4 times per day, but the decoction group was treated for 4 days while the infusion group was treated for 5 days. The infusion group, with 5 total patients, achieved 100% cure rate while the decoction group, with 48 patients, achieved 92% cure rate (Mueller et al. 2000). Neither set was monitored for recrudescence, but the results justified a follow-up investigation.

Thus, this same group performed another small trial in the eastern Democratic Republic of the Congo in 2001 to assess the efficacy of aqueous *A. annua* infusions (teas) against malaria. Cohorts of 39, 33, and 43 patients with *P. falciparum* malaria were assigned to test the efficacy of a 5 g/L *A. annua* tea preparation (A5), 9 g/L *A. annua* tea preparation (A9), or quinine tablets (QN) respectively. On day 7 after beginning of treatment, the authors saw cure rates (defined as absence of parasitemia in thick Giemsa-stained blood smears) of 77%, 70%, and 91% for the A5, A9, and QN groups, respectively. However, cure rates at day 35 were markedly lower at 34%, 30%, and

79%, respectively. These results suggested there was more recrudescence in *Artemisia*-treated patients although without genotyping of parasites it is impossible to know whether lower cure rates are due to recrudescence or reinfection, which is common in areas with rampant malaria such as the DRC. Due to the perceived high recrudescence the authors concluded, "*Artemisia annua* can therefore not be recommended as a treatment option for malaria." (Mueller et al. 2004). However, this conclusion was premature as the trial did not adequately assess increasing doses of *A. annua* tea nor did it account for differences in efficacy between different cultivars of plant material. Indeed, another larger trial using plant material from a different source had significantly different results.

The largest and most rigorously run clinical trial assessing the efficacy of *Artemisia* species to treat malaria to date was performed in 2015 in the Kalima district of the Democratic Republic of the Congo (Munyangi et al. 2019). It was performed using a multi-center, randomized, double-blind design in children ≥ 5 years old and adults with uncomplicated *P. falciparum* malaria and enrolled 957 total patients from 5 different locations. The trial consisted of two arms: an artesunate-amodiaquine (ASAQ) arm of 472 patients and an *Artemisia* arm, which was split into two groups, *A. annua* and *A. afra*, containing 248 and 223 patients, respectively. ASAQ is a commonly used ACT recommended by WHO for that region of Africa, and treatment was for three days according to the standard posology followed by placebo tablets for 4 days. In addition, ASAQ patients received tea infusions made from 0.2 g of plant material per liter for the 7-day treatment period, a method recommended by WHO for herbal therapeutic investigations. In the *Artemisia* arm, patients were given 0.33 L of tea every 8 hours as well as placebo tablets for the 7-day treatment period. Tea was made by adding 5 grams of dried *A. annua* or *A. afra* plant material to 1 L of boiling water for 10 minutes, then steeped for 10 min before sieving out plant material. Day 28

cure rates, as defined by the absence of parasites in blood smears, in pediatric patients were 82%, 91%, and 50% for *A. afra*, *A. annua*, and ASAQ, respectively. For adult patients, the cure rates were 91%, 100%, and 30% respectively. Furthermore, fever cleared in 24 hours for *Artemisia* patients vs. 48 hours for ASAQ patients. Trophozoites cleared from the blood in 24 hours in *Artemisia* patients but took up to 14 days to clear in ASAQ patients. Both *Artemisia* treatments also eliminated gametocytes completely by day 14 and through day 28, while 10/472 ASAQ patients had gametocytes remaining in their blood at day 28 (Munyangi et al. 2019). The results of this trial indicated that *Artemisia* species, especially *A. annua*, could be used effectively as an antimalarial therapy. There was some criticism of this trial including speculation over the quality of the ASAQ tablets due to the abnormally low cure rate observed in that arm of the trial (WHO 2019a). However, authors countered that the ASAQ tablets were received directly from the manufacturer. Even if the ASAQ tablets were faulty, it would not affect the outcome of the *A. annua* arm of the trial that showed cure rates of 91% and 100% in children and adults, respectively. These rates are comparable to ACTs, which typically have a cure rate of 94-100% (WHO 2019a). Of particular note is that the amount of artemisinin delivered in the *Artemisia* arms was 3-1000 fold lower in *A. annua* and *A. afra*, respectively, than in the ASAQ arm (Munyangi et al. 2019). Importantly, no adverse effects attributable to DLA treatment were observed in any trials thus far (WHO 2019a).

To summarize, clinical trials involving the efficacy of *Artemisia*-based treatments for malaria have had varying degrees of success. Some smaller studies suggested *Artemisia* species provided an effective short-term treatment for malaria but often recrudescence occurred. However, the largest and most rigorously performed trial to date indicated that *A. annua* and, to a lesser extent, *A. afra* performed at equal or better efficacy levels than ASAQ, a frontline treatment for

malaria used worldwide. Although PCR was not done due to sample degradation, there was almost no microscopic recrudescence recorded in the Munyangi et al. (2019) trial. While the results of the trials vary, the underlying causes of this variation are largely unknown and speculative. The variation in trial results may be due to differences in the phytochemical composition of the plant material. Each trial was undoubtedly performed with plant material grown in different locations under different conditions and analyzed via different methods. Although for large scale use, some of these challenges can be nullified, they do remain as challenges inherent to working with plant-based therapies. Without the knowledge of the underlying mechanisms behind the efficacy of *Artemisia*-based treatments, it is difficult to determine the exact reasons why the results vary from trial to trial.

There are two prevailing hypotheses as to how whole-plant treatments may serve as effective antimalarials. Both rely on the largest inherent difference between whole-plant therapies and pure drugs: whole-plant therapies include a wide array of secondary phytochemicals that are not present in pure drug therapies. One hypothesis is that secondary phytochemicals present in *Artemisia* species have their own inherent antimalarial efficacy. This has been demonstrated *in vitro* as discussed in Section 1.7 and while the recent Czechowski study indicated flavonoids were not of importance to efficacy *in vitro* (Czechowski et al. 2019), it did not assess the importance of any other classes of phytochemicals such as monoterpenes and coumarins, which are found in abundance in *Artemisia* species. Furthermore, it does not prove that flavonoids do not have some effect *in vivo* such as modification of the immune system to better combat *Plasmodium* parasites, an effect that would not be detected *in vitro*. Thus, this hypothesis still needs more rigorous investigation.

The second prominent hypothesis deals with enhancement of artemisinin bioavailability by secondary phytochemicals present in whole-plant therapies. Notably, in each of the discussed trials, the delivered dose of artemisinin is much lower than the dose of artemisinic compounds delivered in ACTs. Even in the trials using the most *A. annua* plant material, the dose of artemisinin would be less than half the WHO-recommended dose (assuming 1% artemisinin concentration in the plant material) (Willcox et al. 2004). Pure artemisinin has notoriously low bioavailability (Birgersson et al. 2016) and thus, if artemisinin is the only antimalarial of merit in *A. annua*, its bioavailability is likely enhanced by some mechanism mediated by secondary phytochemicals to achieve similar therapeutic results as standard antimalarial treatments. This hypothesis and the mechanisms underlying enhanced bioavailability afforded by oral consumption of dried leaf *A. annua* treatment are the main focus of this dissertation.

1.10 Oral Bioavailability

Oral bioavailability is defined as the fractional extent of a drug dose, taken orally, that finally reaches the therapeutic site of action (Kim et al. 2014). In the case of malaria, the therapeutic site of action is mainly peripheral blood where *Plasmodium* parasites rapidly divide and mature, causing disease symptoms (CDC 2019b, 2019a; Delves et al. 2012). Bioavailability is a crucial determinant of whether a drug will be successful. In fact, many drugs perform well in efficacy testing *in vitro* and *in vivo* but fail during clinical testing due to poor pharmacokinetics or bioavailability. Indeed, poor pharmacokinetic properties and bioavailability is the third most common cause of drug attrition in Phase I clinical trials (Waring et al. 2015). Thus, the bioavailability of a compound is crucial to its overall utility as a therapeutic.

In some cases, poor oral bioavailability can be remedied by using alternate modes of delivery such as intravenous, intramuscular, or subcutaneous injection. However, the oral route of drug delivery is the preferred route due to advantages including ease of production and administration, flexibility in design of solid and liquid dosing forms, high patient compliance, cost-effectiveness, and fewer sterility constraints (Viswanathan, Muralidaran, and Ragavan 2017; Homayun, Lin, and Choi 2019). Many of these factors are even more important in the context of malaria. Since malaria largely exerts its deadly effects in impoverished parts of sub-Saharan Africa, drugs must be as simple as possible. Complicated delivery methods i.e. injections are borderline impossible to use in many parts of the world where malaria is most rampant. The logistics of keeping needles and drugs sterile are infeasible in rural areas that often lack electricity.

Another advantage of oral drugs is patient compliance. Compliance with oral drugs is much more feasible than injections as needles require medical professionals to administer. Additionally, Western based medicine is often distrusted in developing countries for a variety of reasons (van der Kooy and Sullivan 2013). The degree to which this affects human health outcomes is difficult to measure but should not be discounted and thus patient compliance is likely to be significantly higher in not only oral drugs but oral drugs of a traditional formulation such as decoctions or infusions made from plants (van der Kooy and Sullivan 2013).

Finally, injectable drugs are more costly as they must factor in the price of clean needles and syringes. As cost is already a major barrier in treating malaria (Section 1.5), it is clear why all first-line malaria drugs must be designed for oral administration. However, design of oral drugs presents a unique set of challenges that must be overcome in order to achieve effective therapeutic results. Chief among these challenges is designing efficacious drugs that can remain highly

bioavailable by surviving the barrage of obstacles presented to them in the stomach, gut, and liver before reaching systemic circulation (Figure 1.5) (Savjani, Gajjar, and Savjani 2012).

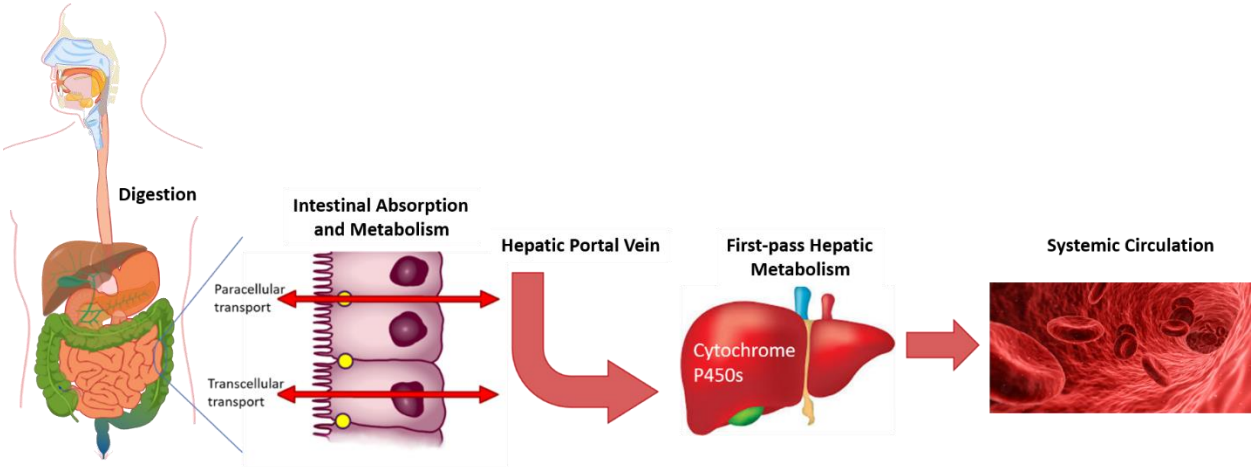

Figure 1.5 Schematic depicting the steps required for oral drugs to reach systemic circulation. Obstacles include degradation during digestion, absorption across the intestinal membrane, and metabolism by intestinal and hepatic cytochrome P450s. Adapted from Desrosiers, Towler, and Weathers (2019).

1.11 Oral Bioavailability of Artemisinin

Pure artemisinin is well known for having low oral bioavailability. In a study of oral bioavailability in healthy humans, the bioavailability of orally taken (*per os*, p.o.) artemisinin relative to intramuscularly (i.m.) injected suspension was only 32% indicating most p.o. artemisinin does not reach systemic circulation (Titulaer et al. 1990). In a study investigating the pharmacokinetics and absorption, distribution, metabolism, and excretion (ADME) of artemisinin in rats, a p.o. dose of 900 mg/kg had to be used because a 300 mg/kg dose was too low to reach detectable levels of artemisinin in the plasma (Xinyi et al. 1985).

While pure artemisinin has low bioavailability, some have speculated that whole-plant formulations of *A. annua* may enhance the bioavailability of artemisinin (Ferreira et al. 2010; van der Kooy and Sullivan 2013). A few studies done in both animals and humans supported this

hypothesis. In a small study, mice fed DLA at a total artemisinin dose of 30.7 µg achieved a maximum plasma concentration (C_{max}) of 0.087 mg L^{-1} (Weathers et al. 2011). However pure artemisinin-dosed animals needed about a 45-fold higher dose (1400 µg total artemisinin) to achieve a similar C_{max} (0.074 mg L^{-1}) (Weathers et al. 2011). In a follow-up pharmacokinetics study, no artemisinin was detected in the serum 1 hour after a 100 mg/kg pure artemisinin dose in healthy mice however, when the same artemisinin dose was given in the form of DLA, plasma artemisinin levels reached 4.33±0.40 mg L^{-1} (Weathers, Elfawal, et al. 2014). Interestingly, when pure artemisinin was combined with mouse chow, serum artemisinin levels rose to 2.44 mg L^{-1} one hour after dosing (Weathers, Elfawal, et al. 2014). These data suggested that mouse chow, largely made up of plant materials including soy, oats, wheat, alfalfa, beat pulp, corn etc., enhanced the pharmacokinetic properties of artemisinin (Weathers, Elfawal, et al. 2014). Another interesting finding was that disease state altered the pharmacokinetics of DLA-delivered artemisinin. Mice infected with *P. chabaudi* had a higher C_{max}, greater time to reach maximum plasma concentration (t_{max}), and a higher overall exposure to DLA-delivered artemisinin than healthy mice (Weathers, Elfawal, et al. 2014).

The only human pharmacokinetic study investigating a whole-plant *A. annua* formulation was published in 2004 (Räth et al. 2004). This study looked at solubility and extraction efficiency of artemisinin from a tea infusion as well as the pharmacokinetic properties of artemisinin when delivered as a tea infusion to healthy subjects (Räth et al. 2004). In this study, the average t_{max} was much shorter (0.5 hours) than what had been reported in previous studies looking at pure artemisinin (2.3 hours) (Räth et al. 2004). Interestingly, the overall bioavailability did not differ from previous studies with high doses of pure artemisinin (Räth et al. 2004). Importantly though, the plasma concentrations achieved in this study (240 ng mL^{-1}) far exceed the required

concentration required to inhibit *Plasmodium* growth (9 ng mL^{-1}) (Räth et al. 2004; Alin and Bjorkman 1994).

In summary, bioavailability and pharmacokinetic data for traditional formulations of *A. annua* are scarce. Only a few studies in mice have been done to show enhanced pharmacokinetics by dried leaf consumption and none have been done in humans. The only human data comes from the study examining *A. annua* tea infusions (Räth et al. 2004). While little pharmacokinetic data is available, there are some data examining the factors that influence oral artemisinin bioavailability such as solubility and extraction efficiency, intestinal permeability, and hepatic first-pass metabolism.

1.12 Artemisinin Solubility and Extraction Efficiency

Several factors influence the oral bioavailability of a drug. One of these major factors is the aqueous solubility of the drug (Song, Zhang, and Liu 2004). Solubility is critical because only dissolved drug is available for permeation across the intestinal mucosa and into the blood (Song, Zhang, and Liu 2004). Thus, drugs with low aqueous solubility often have poor oral bioavailability (Savjani, Gajjar, and Savjani 2012). It is estimated that 90% of preclinical drugs in the development pipeline have low aqueous solubility and subsequently will have pharmacokinetic and bioavailability problems (Kalepu and Nekkanti 2015). Indeed, one of the major reasons for developing ACTs was solubility of artemisinin (O'Neill and Posner 2004; Ploypradith 2004). Artemisinin itself has notoriously low bioavailability, which is partially due to low solubility in water and oil (Birgersson et al. 2016). In fact, artemisinin has two known crystal polymorphs: a triclinic form with an aqueous solubility of 48 mg/L and a dissolution rate of 4 hours; and an orthorhombic form with an aqueous solubility of 20 mg/L and a dissolution rate of 18 hours (Chan

et al. 1997). In one study, artemisinin oral bioavailability in healthy volunteers was 32% relative to i.m. injected artemisinin suspended in oil (Titulaer et al. 1990). Thus, artesunate and artemether, both semisynthetic derivatives of artemisinin, were developed for use in ACTs. Artesunate was developed as a more water soluble artemisinic compound while artemether was developed as an oil soluble artemisinic compound (Ploypradith 2004). The low aqueous solubility of artemisinin (20-50 mg/L), has led many to wonder how traditional formulations of *Artemisia* have been successful in treating malaria especially considering the low artemisinin doses delivered (van der Kooy and Sullivan 2013; van der Kooy and Verpoorte 2011). Some have hypothesized that other compounds in the plant material aid in solubilizing artemisinin in traditional preparations (van der Kooy and Sullivan 2013). There have been nearly 600 secondary metabolites identified in *A. annua*, so there are a wide array of compounds that could possibly alter its solubility and bioavailability (Brown 2010).

Several publications have investigated artemisinin solubility or extraction efficiency in traditional formulations of *A. annua*. The previously mentioned study by Räth et al. (2004) compared the artemisinin extraction efficiency of three different preparation methods for teas made from 5 or 9 grams of dried *A. annua*. The methods included were Method A: adding dried plant material to 1 liter of boiling distilled water and allowing the infusion to cool before filtering; Method B: adding dried plant material to 1 liter of boiling distilled water, boiling for 30 minutes, then allowing to cool before filtering; and Method C: adding dried plant material to 1 liter of boiling distilled water, covering and allowing to stand for 10 minutes, filtering and squeezing plant material gently to remove residual water, and allowing to cool (Räth et al. 2004). The measured extraction efficiencies varied by plant mass as well as extraction method with the 5 g/L tea using Method C having the highest extraction efficiency (86%) and the 9 g/L tea using Method B having

the lowest extraction efficiency (30%) (Räth et al. 2004). This wide range of extraction efficiencies correlated to a range of 94.5 – 37.8 mg/L artemisinin in various infusions and highlighted the importance of preparation methods on artemisinin extraction efficiency and dose delivered to patients (Räth et al. 2004). Similarly, other reports showed extraction efficiencies of 78-93% after boiling dried leaves (Silva et al. 2012; van der Kooy and Verpoorte 2011). An earlier study showed a lower extraction efficiency of 20-42% however this study used different plant material containing less than half the artemisinin content of the Räth et al. (2004) study and used a different harvesting and drying method (Räth et al. 2004; Mueller et al. 2000; van der Kooy and Sullivan 2013; de Ridder, van der Kooy, and Verpoorte 2008). Another study used the method most commonly used in traditional Chinese medicine of soaking the leaves and stems in water followed by wringing out (Wright et al. 2010). They found that the wrung juice of plant material soaked for 12 hours had the highest artemisinin concentration (72.6 mg/L) compared to plant material soaked for 2 hours (45.9 mg/L) and a dried leaf tea infusion (14.5 mg/L) (Wright et al. 2010). The authors suggest that wringing may damage glandular trichomes, releasing more artemisinin as well as monoterpenes and sesquiterpenes present in the essential oil that could aid in artemisinin solubilization (Wright et al. 2010). The extraction efficiency was however much higher in the dried leaf infusion (53.8%) compared to the 12 hour (14.1%) and 2 hour (9.1%) wrung juices indicating that heat may increase efficiency of extraction (Wright et al. 2010). Van der Kooy and Verpoorte (2011) showed that as temperature increased to 100 °C, extraction efficiency improved, topping out at 93% (van der Kooy and Verpoorte 2011). Extraction efficiency was best when plant material was kept in water at 100 °C for 5 minutes but decreased sharply after 1, 5, and 10 minutes at 115 °C (van der Kooy and Verpoorte 2011) and Weathers and Towler (2012) confirmed this study as well as the stability of artemisinin in tea infusions. Another group achieved 84% extraction efficiency using the same

method of boiling the dried leaves for 5 minutes (Debnath et al. 2011). This group also showed no difference in extraction efficiency between tap water and distilled water (Debnath et al. 2011; van der Kooy and Sullivan 2013). It is interesting to note that none of these studies evaluated the extraction efficiency of soaking *A. annua* in urine, which was actually a common preparation method in ancient China (van der Kooy and Sullivan 2013; Hsu 2006).

It is clear from this plethora of studies that extraction efficiency and artemisinin solubility is highly dependent on preparation method and likely the phytochemical content of the plant material. Thus, it is critical that studies document in detail the methods used to produce *Artemisia* teas or infusions if they are to be published in reputable academic journals. It is also often more relevant to publish solubility data as opposed to extraction efficiency as extraction efficiency is highly dependent on the original artemisinin content in the plant material, which is known to vary substantially.

1.13 Intestinal Permeability

Apart from solubility, intestinal permeability is another key factor in determining bioavailability of an oral drug (Song, Zhang, and Liu 2004). Even if a drug is soluble in intestinal fluid, it must be able to cross the membrane that makes up the small intestine to be absorbed into the blood. To understand this process, we must first become familiar with the cellular environment in which drug absorption occurs (Song, Zhang, and Liu 2004). The small intestine is the primary site of drug absorption after oral drug administration (Ozawa et al. 2015). The villi of the small intestine, which are responsible for its remarkable surface area and absorptive ability, are mostly lined with enterocytes (Ensari and Marsh 2018). These epithelial cells form a thin barrier between the intestinal lumen and the blood in the form of a cohesive monolayer that functions as a thin

membrane for absorption of exogenous substances into the blood (Ensari and Marsh 2018). Mature enterocytes are polarized tall columnar cells with an apical side in contact with the intestinal lumen and a basolateral side in contact with the basement membrane (Massey-Harroche 2000). The apical side contains microvilli approximately 2 μm in length that are closely packed together commonly referred to as a brush border (Massey-Harroche 2000).

Absorption across enterocyte monolayers occurs through either active transport or passive diffusion mechanisms (El-Kattan and Varma 2012). Passive diffusion mechanisms include paracellular and transcellular pathways (El-Kattan and Varma 2012). Drugs taking the paracellular pathway pass through aqueous pores in tight junctions between enterocytes (El-Kattan and Varma 2012). As they must pass through aqueous pores, drugs that take this route are mostly hydrophilic and must be small enough to fit through the small pores that range from 3-6 Å, usually limiting their molecular weight to ≤ 250 g/mol (El-Kattan and Varma 2012; Song, Zhang, and Liu 2004). This pathway is further limited by the fact that the space between enterocytes makes up < 0.01% of the intestinal surface area (El-Kattan and Varma 2012). The transcellular pathway requires drugs to pass through the apical cellular membrane of enterocytes, through the cytoplasm, and back out through the basolateral membrane through passive diffusion. For this reason, they are often more lipophilic than paracellularly diffused drugs and are not as limited by molecular size or weight. Transcellular passive diffusion is the most common route of absorption for drugs (El-Kattan and Varma 2012).

Active transport mechanisms of drugs include carrier-mediated active transport and endocytosis (El-Kattan and Varma 2012; Barthe, Woodley, and Houin 1999). Endocytosis is the mechanism by which large peptides and macromolecules are absorbed and is generally a slow process (Barthe, Woodley, and Houin 1999). Most active transport occurs through transporter

proteins such as those belonging to the ATP binding cassette (ABC) and solute carrier (SLC) superfamilies (El-Kattan and Varma 2012). Active transport proteins are usually localized to the apical or basolateral membrane and either function as mediators of uptake or efflux out of the cell (El-Kattan and Varma 2012). Thus, these proteins can function to increase or decrease the intestinal permeability, and in turn the bioavailability, of a drug depending on the drugs affinity for uptake transporters vs. efflux pumps (El-Kattan and Varma 2012).

Indeed, affinity for efflux pumps is often a major challenge in designing drugs with acceptable bioavailability (Thakkar 2015; Bohr et al. 2019). In the small intestinal epithelium, ABC efflux transporters include *P*-glycoprotein (P-gp, MDR1, *ABCB1*), the multi-drug resistance proteins (MRPs), of which there are 9 members, and breast cancer resistance protein (BCRP) (Murakami and Takano 2008). P-gp, MRP2 and BCRP are all expressed along the apical brush border of enterocytes and function to pump substrate drugs back into the intestinal lumen effectively limiting permeability (Murakami and Takano 2008).

Intestinal permeability and, as a result oral bioavailability, is also affected by drug metabolism via enzymatic degradation (Song, Zhang, and Liu 2004). The cytochrome P450 (CYP) superfamily, which makes up the major groups of enzymes involved in human drug metabolism, is mostly known for its activity in the liver, the primary site of drug elimination (Paine et al. 2006). However, CYPs are also expressed by mature enterocytes lining the villi of the small intestine and consequently, the intestinal mucosa is the most important site of drug metabolism outside of the liver (Barthe, Woodley, and Houin 1999; Song, Zhang, and Liu 2004; Paine et al. 2006). As a result, many drugs can be degraded as they pass through the intestine thus reducing their overall intestinal permeability (Barthe, Woodley, and Houin 1999; Song, Zhang, and Liu 2004). Of the CYP isoforms, CYP3A4 is by far the most abundant of the CYP enzymes present in the small

intestine making up anywhere from 50-70% of the total CYP content (Paine et al. 2006). In fact, for some drugs metabolized by CYP3A4, the small intestine contributes significantly and sometimes equally to the total first-pass metabolism of the liver (Paine et al. 2006; Fritz et al. 2019). While CYP3A4 levels in the intestine are high, most other CYPs have relatively low expression in the intestine (Paine et al. 2006). In a study that analyzed the CYP content of 31 human donor small intestines, the CYP3A family made up 82% of total intestinal CYP content, most of which was CYP3A4, followed by the CYP2C family at 16%, CYP2J2 at 1.4%, and CYP2D6 at 0.7% (Paine et al. 2006). The CYP isoforms 1A2, 2A6, 2B6, 2C8, and 2E1 were not detected or only faintly detected in any of the 31 donor samples (Paine et al. 2006). This intestinal CYP "pie" indicates a very different makeup of CYP content than that of the liver. Furthermore, while the CYP2C family made up the second most CYP content in the small intestine (16%), the content was an order of magnitude lower than what is found in the liver, and CYP2C isoforms also have 4-8 fold lower catalytic activity than hepatic CYP2C (Paine et al. 2006). Those data suggested that only drugs metabolized by CYP3A isoforms are likely to undergo any substantial first pass metabolism in the small intestine (Paine et al. 2006). It is also important to note that, similar to hepatic CYP content, there is also extreme inter-individual variation in the content of specific CYP isoforms in the small intestine (≥5-fold), so it is difficult to make accurate conclusions about intestinal drug metabolism on a population level (Paine et al. 2006).

Artemisinin is known to have low oral bioavailability, so Augustijns et al. (1996) wondered if intestinal permeability may play a role in the observed low bioavailability (Augustijns et al. 1996). They used Caco-2 cell monolayers seeded on semi-permeable transwell inserts in 6-well plates as an experimental model for the intestinal epithelium (Augustijns et al. 1996). Caco-2 cells are a unique cell line derived from human colon epithelial cancer that, upon confluence,

differentiate to form tight junctions between cells and express transporter proteins and efflux pumps found in human small intestinal tissue (van Breemen and Li 2005). These cells also grow in a polarized manner with the apical side forming a brush border and the basolateral side contacting the cell culture treated semi-permeable transwell inserts (van Breemen and Li 2005). Thus, test compounds can be added to the media on the apical or basolateral side and transport or permeability of the compound can be measured in either direction. In this way, Caco-2 cell monolayers mimic the human intestinal epithelium (van Breemen and Li 2005). Augustijns et al. (1996) tested the permeability of artemisinin in both the typical apical → basolateral direction and the basolateral → apical direction. In the apical → basolateral direction the apparent permeability (P_{app}), a common measure of intestinal permeability, was 30.4 x 10^6 cm s^{-1} while in the basolateral direction the P_{app} was similarly 30.9 x 10^6 cm s^{-1} (Augustijns et al. 1996). These very similar P_{app} values for each direction suggest artemisinin crosses the small intestine via passive diffusion. Active transport mechanisms usually occur only in one direction, so if an active transport mechanism were involved, artemisinin P_{app} values would be different in each direction indicating artemisinin is actively pumped in one direction or the other, but not both (Augustijns et al. 1996). To confirm this, the experiment was repeated in the apical → basolateral direction in the presence of sodium azide, an inhibitor of ATP generation and active transport mechanisms, and found no difference in the P_{app} (Augustijns et al. 1996). Those experiments confirmed that artemisinin is highly permeable to the intestinal membrane and crosses the small intestine by a passive diffusion mechanism (Augustijns et al. 1996). They also suggested that artemisinin is not a substrate of intestinal efflux pumps such as P-gp, a conclusion that was later confirmed *in vivo* in intestinal perfusion assays in rats (Svensson et al. 1999).

Although artemisinin is highly permeable via passive diffusion, delivery in combination with the complex milieu of phytochemicals found in *A. annua* may enhance its intestinal permeability. Indeed, there is precedence for this phenomenon in the literature and there are multiple synergistic mechanisms that can lead to enhanced intestinal permeability of plant constituents (Zhao et al. 2020). One mechanism by which one plant constituent can enhance intestinal permeability of another is by inhibition of intestinal efflux pumps (Zhao et al. 2020). For example, in a 2013 study, rats given an oral dose of crude extract of the twigs and leaves of *Taxus yunnanensis* had > 7 times the area under the curve (AUC) of paclitaxel, the major medicinal constituent in this plant, in their blood than rats given an equal dose of pure paclitaxel (Jin et al. 2013). Subsequent experiments using the Caco-2 transport model determined that secondary constituents found in the *T. yunnanensis* crude extract increased the intestinal permeability of paclitaxel by more than threefold (Jin et al. 2013). This effect was achieved via inhibition of the intestinal efflux pump P-gp, of which paclitaxel is a strong substrate; P-gp was inhibited by secondary constituents found in the extract (Jin et al. 2013). This mechanism is unlikely for artemisinin as it is not a strong substrate of P-gp (Svensson et al. 1999) however, other mechanisms have been described. These include inhibition of intestinal drug metabolizing enzymes, chiefly CYP3A4, by secondary plant constituents, increasing enterocyte permeability by amphiphilic molecules like saponins, "loosening" or opening of tight junctions by secondary plant constituents, and increased permeability mediated by naturally occurring nanoparticles (Zhao et al. 2020). Thus, it is plausible that secondary phytochemicals in *A. annua* may further enhance the intestinal permeability of artemisinin in turn leading to increased overall oral bioavailability.

1.14 Hepatic First-Pass Metabolism

Besides aqueous solubility and intestinal permeability, another major factor that influences the oral bioavailability of a drug is first-pass metabolism (Song, Zhang, and Liu 2004; Pond and Tozer 1984). While some metabolism occurs in the small intestine (Section 1.13), the liver is considered the major site of first-pass metabolism for orally administered drugs (Pond and Tozer 1984). The CYPs in the liver are the major enzymes involved in drug metabolism accounting for about 75% of all drug metabolizing reactions (Guengerich 2008). The CYP superfamily is made up of 57 enzymes identified by The Human Genome Project, but only 5 of these isoforms are responsible for 95% of drug metabolizing reactions performed by all CYPs (Guengerich 2008; Preissner et al. 2013). In fact, CYP3A4 alone is responsible for about 50% of drug metabolism (Singh and Zhao 2017). It therefore stands to reason that interactions between these enzymes and phytochemicals could affect the bioavailability of the drugs they metabolize. These interactions are commonly referred to as herb-drug interactions (Singh and Zhao 2017).

Hepatic herb-drug interactions can ultimately lead to an increase or decrease in bioavailability of the drug depending on the nature of the interaction. Herb-drug interactions usually involve either the inhibition of a CYP by phytochemicals produced in the herb, leading to increased bioavailability of the drug, or induction of the CYP gene by phytochemicals produced in the herb, leading to increased CYP expression, drug metabolism, and decreased bioavailability of the drug (Singh and Zhao 2017). Perhaps the best characterized herb-drug interaction occurs from *Hypericum perforatum*, or St. John's wort. St. John's wort is a widely distributed herbal dietary supplement ($570 million in annual sales worldwide) used by some to treat depression and anxiety (Russo et al. 2014). St. John's wort induces the expression of P-gp and several CYPs including CYP3A4, CYP2E1, and CYP2C19 (Russo et al. 2014; Di et al. 2008). As a result, at

least 35 drugs have shown clinical interactions with St. John's wort, most of which result in changes to the bioavailability of the drug (Russo et al. 2014; Di et al. 2008). While St. John's wort induces CYP expression, another top selling herbal supplement, goldenseal (*Hydrastis canadensis*), is a potent inhibitor of CYP3A4 and CYP2D6 (Gurley, Fifer, and Gardner 2012; Gurley et al. 2008). As a result it is strongly recommended to avoid taking goldenseal with most over-the-counter and prescription medications (Asher, Corbett, and Hawke 2017).

The observed low bioavailability of pure artemisinin is widely thought to be due to low aqueous solubility and high levels of first-pass metabolism in the liver (Titulaer et al. 1990; Medhi et al. 2009; Navaratnam et al. 2000). Artemisinin is primarily metabolized by CYP2B6 although there is thought to be a minor contribution by CYP3A4 in people with low CYP2B6 expression (Svensson and Ashton 1999). Importantly, metabolism of artemisinin in the liver results in 4 metabolites lacking the endoperoxide bridge rendering them biologically inactive against malaria: deoxyartemisinin, deoxydihydroartemisinin, 9,10-dihydrodeoxyartemisinin, and crystal-7 (Lee and Hufford 1990). This is not the case for the semisynthetic artemisinin derivatives artesunate, artemether, and arteether (Navaratnam et al. 2000). These compounds are metabolized predominantly by CYP2A6, CYP3A4, and CYP3A5 and all result in the formation of biologically active antimalarial dihydroartemisinin (Navaratnam et al. 2000).

Artemisinin is also an autoinducer (Gupta, Svensson, and Ashton 2001; Medhi et al. 2009). That is, it induces the expression of its own metabolizing enzymes CYP2B6 and CYP3A4 (Asimus et al. 2007; Simonsson et al. 2003). *In vitro* studies have since shown that artemisinin activates both the constitutive androstane receptor (CAR) and pregnane X receptor (PXR), two xenobiotic sensors that activate signaling pathways to upregulate the expression of CYPs, in hepatocytes thus further explaining the molecular mechanism of artemisinin autoinduction (Simonsson et al. 2006;

Burk et al. 2005). Interestingly, artemisinin also induces CYP2C19 expression (Mihara et al. 1999). As a result of the induction of CYP2B6 and CYP3A4, artemisinin plasma concentration usually declines after repeated dosing (Asimus et al. 2007; Simonsson et al. 2003; Ashton, Sy, et al. 1998; Ashton, Hai, et al. 1998). In one study, patients with uncomplicated *falciparum* malaria were treated with 500 mg/day oral artemisinin and artemisinin pharmacokinetics parameters were observed on day 1 and day 5. On day 5, artemisinin clearance rate had increased 5.4-fold, AUC had decreased 5.2-fold, and C_{max} had decreased 5.3-fold (Ashton, Sy, et al. 1998).

While much is known about artemisinin interactions with CYPs, less research has been done to determine how secondary phytochemicals from *A. annua* may affect the same CYPs. In fact, many classes of phytochemicals produced in *A. annua* are known to inhibit CYPs; these include flavonoids, monoterpenes, coumarins, and phenolic acids (Basheer and Kerem 2015; Foroozesh et al. 2019; Hodek, Trefil, and Stiborová 2002; Seo et al. 2008). There are nearly 40 flavonoids identified in *A. annua*, many of which have been implicated in CYP inhibition and induction (Ferreira et al. 2010; Hodek, Trefil, and Stiborová 2002). Some have posited that the enhanced bioavailability seen when artemisinin is delivered as DLA is due to inhibition of first-pass metabolism by DLA phytochemicals (Ferreira et al. 2010). In fact, a similar phenomenon has been seen with the artemisinin derivative artemether and grapefruit juice (van Agtmael et al. 1999). Grapefruit juice is well known for its interactions with drugs via CYP inhibition (Bailey, Dresser, and Arnold 2013). Phytochemicals called furanocoumarins are potent inhibitors of CYP3A4 in humans leading to interactions with over 85 drugs on the market (Bailey, Dresser, and Arnold 2013; Guo and Yamazoe 2004). The artemisinin peak plasma concentration of the semisynthetic derivative artemether more than doubled when administered with 350 mL grapefruit juice (van Agtmael et al. 1999).

Phytochemicals produced by DLA may inhibit CYP activity, thus enhancing artemisinin bioavailability. Melillo de Magalhães et al. (2012) tested teas made from 6 different *A. annua* cultivars for their ability to inhibit CYP3A4 in Caco-2 cells. Three cultivars significantly inhibited CYP3A4 activity with inhibition ranging from 63-45% compared to a negative control (Melillo de Magalhães et al. 2012). The authors also tested rosmarinic acid and chlorogenic acid, two phytochemicals found in their *A. annua* cultivars, but found no inhibitory activity for either compound (Melillo de Magalhães et al. 2012). Those data indicated that some compound present in *A. annua* inhibited intestinal CYP3A4, however no experiments were performed using liver microsomes or on CYP2B6 (Melillo de Magalhães et al. 2012).

Wei et al. (2015) investigated the efficacy of combinations of pure artemisinin and chrysosplenetin, a flavonoid commonly found in *A. annua*, at three different ratios against the mouse malaria parasite *P. berghei* (Wei et al. 2015). They determined that artemisinin plasma concentration AUC significantly increased in all combination treatments compared to pure artemisinin while the 1:1 and 1:2 artemisinin to chrysosplenetin ratio led to 1.64- and 1.65-fold increases in C_{max} relative to artemisinin alone (Wei et al. 2015). Furthermore, the 1:2 artemisinin to chrysosplenetin ratio led to a 1.59-fold reduction in parasitemia compared to artemisinin alone (Wei et al. 2015). Another group, Cai et al. (2017), showed that a methanolic extract of *A. annua* provided a 2.3-fold increase in exposure and 1.9-fold increase in the artemisinin plasma C_{max} compared to pure artemisinin (Cai et al. 2017). This increase was attributed in part to arteannuin B, a compound found in *A. annua* and their extracts, which inhibited CYP3A4 in their *in vitro* assays (Cai et al. 2017). Another recent study showed a 4-component therapy made up of compounds found in *A. annua* enhanced antiplasmodial activity as well as pharmacokinetics of artemisinin (Li et al. 2018). The authors combined artemisinin, arteannuin B, arteannuic acid, and

scopoletin at a 1:1:1:1 mass ratio and tested the efficacy of a 4-day treatment in *P. berghei* and *P. yoelii* infected mice (Li et al. 2018). Their results showed about 93% reduction in parasitemia for the 4-component therapy compared to about 31% reduction in the artemisinin control group, suggesting the artemisinin + phytochemicals combination therapy was much more efficacious (Li et al. 2018). Artemisinin exposure and plasma C_{max} also increased 3.78- and 3.47-fold in healthy mice and 2.62- and 1.82-fold in *P. yoelii* infected mice, respectively (Li et al. 2018). Combined, these results suggest that phytochemicals from *A. annua* help enhance artemisinin bioavailability through inhibition of CYPs leading to enhanced antimalarial efficacy.

1.15 Alternative Therapeutic Uses of Artemisia

While *A. annua* is mostly known as an antimalarial, the herb has several secondary purported activities and uses. Chief among these are anti-cancer, antiviral, antibacterial, anthelmintic, and anti-inflammatory. Anti-cancer activity of *A. annua* is mostly caused by artemisinin reacting with ferrous iron in cancer cells forming reactive oxygen species leading to DNA damage, cell cycle arrest, and cell death (Efferth 2015; Weathers et al. 2017). Artemisinin also inhibits neoangiogenesis in cancerous tumors (Weathers et al. 2017; Efferth 2015). *A. annua* has moderate to potent antiviral activity against HIV, human cytomegalovirus, herpes simplex virus type 1, Epstein-Barr virus, hepatitis B, Hepatitis C, bovine viral diarrhea virus, and SARS-associated coronaviruses (Lubbe et al. 2012; Efferth et al. 2008; Li et al. 2005). Antibacterial activity has been shown in a wide array of species (Juteau et al. 2002; Kim et al. 2015). Antiparasitic activity is not limited to *Plasmodium*. *A. annua* demonstrated activity against parasites of the *Trypanosoma* genus that cause trypanosomiasis (Naß and Efferth 2018) and potent anthelmintic activity in a clinical trial against schistosomiasis (Munyangi et al. 2018).

One of the best characterized bioactivities of *A. annua* is its anti-inflammatory activity (Kim et al. 2015; Hu et al. 2014). The inflammatory response in mammals is complex involving multiple signaling pathways (Newton and Dixit 2012). The major pathway controlling biosynthesis of inflammatory cytokines is the NF-κB signaling pathway (Newton and Dixit 2012; Lawrence 2009). This canonical pathway involves activation of a toll-like receptor (TLR) by a pathogen-associated molecular pattern (PAMP) such as lipopolysaccharide present in bacterial cell walls, thereby initiating the signaling cascade. This results in activation of the NF-κB transcription factor that regulates production of proinflammatory cytokines like TNF-α and IL-6 (Newton and Dixit 2012). This pathway is well studied and as a result there are many anti-inflammatory medications designed to interrupt this pathway (Nam 2006). In fact, artemisinins and several other plant-derived polyphenols are known to block this pathway, leading to their anti-inflammatory activity (Wang et al. 2006; Zhu et al. 2012). For example, artemisinin inhibited TNF-α and IL-6 release induced by lipopolysaccharide, heat-killed *Escherichia coli*, or CpG containing oligodeoxy-nucleotides from RAW264.7 macrophages in a time- and dose-dependent manner, and protected mice from lethal lipopolysaccharide doses. Anti-inflammatory effects were attributed to artemisinin blocking the NF-κB activation necessary for cytokine release, a finding consistent with other studies (Wang et al. 2006; Shakir et al. 2011; Zhu et al. 2012). Artemisinin has been increasingly studied recently for use in inflammatory diseases, i.e. inflammatory bowel disease (Hu et al. 2014). Using a mouse model for intestinal inflammation, investigators showed that artemisinin ameliorated the symptoms of dextran sulfate sodium-stimulated inflammation of the bowel (Hu et al. 2014). Several other studies showed the efficacy of artemisinins against inflammatory conditions and these are summarized in Table 1.2.

Table 1.2. Effect of artemisinins on components of the immune system. Adapted from Weathers et al. (2017).

Factor	Treatment	System	Response after AN treatment	Reference
Neutrophils	AS	Humans	Decreased number (stronger effect in HIV+ patients)	(Shakir et al. 2011)
	AS/AM	Humans	Slightly decreased number	(Shakir et al. 2011)
	DHA	Rats	Increased number after weekly treatments	(Shakir et al. 2011)
T cells	AN	Humans (HIV-)	No change in CD4 T cells	(Tatfeng et al. 2007)
	AM	Con-A induced splenocytes	Halted CD4 and CD8 T cell proliferation	(Wang et al. 2007)
	AS	Mice	No change in CD4 or CD8 T cells	(Ramacher, Umansky, and Efferth 2009)
Cytokines	AS	Human fibroblast synoviocytes	IL-1β, IL-6, IL-8, decreased	(Xu et al. 2007)
	SM905	RAW264.7 macrophages	IL-1β and TNF-α, dose decrease	(Wang et al. 2009)
	AM	Con-A induced splenocytes	IL-6 and NO decreased IL-2 dose decrease, IFN- γ decrease	(Wang et al. 2007)
	AS	Mice	IL-2 decreased, IL-4 increased	(Lee et al. 2014)
	AS	Mouse sepsis model	TNF-α and IL-6 dose decrease	(Li et al. 2008)
	AN	Mouse sepsis model and RAW 264.7 macrophage	TNF-α and IL-6 dose decrease	(Wang et al. 2006)
	AN	Rat primary microglia cells	TNF-α, IL-6, and MCP-1 dose decrease	(Zhu et al. 2012)
	AS	Con-A/LPS treated mouse splenocytes	IL-10, IFN- γ, and IL-12p40 dose decrease	(Gumede et al. 2009)
NO	AN	Human astrocytoma T67 cells	Decrease	(Aldieri et al. 2003)
	AN/AS/ SM905	RAW264.7 macrophages	Decrease	(Wang et al. 2009; Konkimalla et al. 2008)
NF-κB	AN	Human astrocytoma T67 cells	Activation decreased	(Aldieri et al. 2003)
	AN	Rat primary microglia cells	Dose decrease	(Zhu et al. 2012)

AN, artemisinin; **AS**, artesunate; **AM**, artemether; **DHA**, dihydroartemisinin; **SM905**, novel water-soluble artemisinin derivative; **LPS**, lipopolysaccharide; **NO**, nitric oxide

Besides artemisinin, *A. annua* produces a wide variety of phytochemicals including flavonoids, phenolic acids, monoterpenes, and coumarins, many of which have anti-inflammatory activity (Kumar and Pandey 2013; Ding et al. 2008; Hortelano 2009). Recently, two flavonoids found in *A. annua*, chrysosplenol D and casticin, were shown to decrease inflammation both *in vitro* and *in vivo* (Li et al. 2015). Further, rosmarinic acid, a phenolic acid found in DLA, was shown to decrease IL-6 and IL-8 expression *in vitro* (Melillo de Magalhães et al. 2012). Thus, *A. annua* may act as an anti-inflammatory herbal medicine via additive or synergistic anti-inflammatory activities of artemisinin and other phytochemicals produced *in planta*.

1.16 Summary

Artemisinin is a powerful and crucial antimalarial molecule that has been used for millennia in traditional formulations. While the modern formulations of ACTs have enhanced bioavailability and high antiplasmodial efficacy, there are still numerous problems relating to accessibility and affordability. In order to eradicate malaria, cheaper and more accessible therapeutics will be required to treat people who do not have the option of ACTs. Traditional formulations of *A. annua* may be able to bridge this gap. Teas made from the dried leaves of *A. annua* are effective clinically and *in vivo* testing suggests one reason for this efficacy may be due to enhanced bioavailability over pure artemisinin. The mechanisms underlying this enhanced bioavailability including increased solubility, intestinal permeability, and inhibition of hepatic first-pass metabolism by secondary phytochemicals are examined here through *in vitro* and *in vivo* studies. A better understanding of these mechanisms will shed light on how this medicinal herb achieves such strong antimalarial efficacy and hopefully this information will promote new thinking about how to design future plant-derived therapeutics.

Chapter 2 Hypothesis and Research Objectives

2.0 Hypothesis

A. annua has been used for millennia to treat fever, which was likely often malaria. Since the arrival of modern medicine and the "one drug, one target" drug design paradigm, medicinal plants have often been reduced to their most bioactive constituent to be used in medicine. *A. annua* has been no exception to this rule. Today artemisinin combination therapies (ACTs) represent the gold-standard therapy for malaria, however their cost and accessibility have been prohibitive obstacles to global eradication efforts. Therapies based around the use of dried leaves of *A. annua* would be significantly less expensive and likely more accessible as the plant is easily grown in a variety of climates and using dried leaves eliminates the extraction, purification, and chemical modification steps in ACT production that incur their high cost. In fact, *in vivo* and clinical studies suggest that *A. annua* therapies may be equal to or better than ACTs at treating malaria while being less susceptible to development of drug resistance. *In vivo* studies also indicate that artemisinin delivered as dried *A. annua* leaves reaches significantly higher concentrations in the blood than pure artemisinin. This phenomenon is likely one reason why dried *A. annua* leaves seem to be efficacious in treating malaria even at very low artemisinin doses.

Oral bioavailability is a complex process involving several underlying mechanisms at various locations within the human body. Drug bioavailability can be altered by breakdown or modification by amylases in saliva or low pH and proteases in the stomach. It can be influenced by physiochemical properties such as solubility that may differ from the oral cavity, to the stomach, and to the intestines. Absorption across the intestine can be altered by efflux pumps, tight junction modification, and inhibition or stimulation of active transporters. Furthermore, oral bioavailability

depends heavily upon first-pass metabolism in the liver. It is possible that any one of these processes is altered by phytochemicals present within *A. annua*. Thus, I hypothesize that the enhanced bioavailability of artemisinin from dried *A. annua* leaves seen *in vivo* is due to increased intestinal artemisinin solubility, increased intestinal permeability, and inhibition of hepatic first-pass metabolism afforded by secondary phytochemicals produced by *A. annua*.

2.1 Research Objectives

In order to investigate this hypothesis, there are several research objectives:

1. To investigate the changes in artemisinin solubility during digestion when delivered as pure drug vs. as dried leaves of *A. annua* and with or without various foods;

2. To investigate the intestinal permeability of artemisinin when delivered as dried leaves of *A. annua*. Using the Caco-2 cell model of the intestinal epithelium I will determine the apparent artemisinin permeability of pure artemisinin, digested dried leaves of *A. annua*, and artemisinin in combination with single phytochemicals produced by *A. annua* to determine which, if any, phytochemicals enhance intestinal permeability of artemisinin;

3. To study the ability of extracts, teas, and individual phytochemicals from *A. annua* to inhibit the enzymatic activity of CYP2B6 and CYP3A4, the cytochrome P450 isozymes responsible for artemisinin first-pass hepatic metabolism.

4. I also aimed to determine how artemisinin is distributed and eliminated based on delivery as pure drug vs. dried *A. annua* leaves and how delivery mode impacts downstream pathologies, e.g. inflammation, *in vivo*.

Through these objectives, I aimed to uncover some of the mechanisms that underlie the enhanced bioavailability of artemisinin when it is delivered orally as dried leaves of *A. annua* observed *in vivo.*

2.2 Thesis Organization

The work on understanding changes to artemisinin solubility based on delivery as pure drug vs. dried leaves of *A. annua* or combined digestion with food is described in Chapter 3. Chapter 4 examines the intestinal permeability of artemisinin when delivered as pure drug vs. as dried leaves of *A. annua* as well as combinations of single *A. annua* phytochemicals and artemisinin. In Chapter 5, the inhibitory activity of extracts, teas, and phytochemicals from *A. annua* on the CYP enzymes responsible for first-pass hepatic artemisinin metabolism is explored. I also determine how artemisinin distribution and elimination differs based on oral delivery as pure drug vs. dried leaves of *A. annua* as well as its impact on systemic inflammation *in vivo*. Chapters 3, 4, and 5 were each published individually in their entirety in either the Journal of Ethnopharmacology (Chapters 3 and 4) or Biomolecules (Chapter 5). Finally, Chapter 6 presents conclusions and prospective future studies for the continuation of this research project.

Chapter 3 Effect of Leaf Digestion and Artemisinin Solubility for Use in Oral Consumption of Dried *Artemisia annua* Leaves to Treat Malaria

Published as:

Desrosiers M. and Weathers P.J., 2016. Oral consumption of dried *Artemisia annua* leaves to treat malaria: leaf digestion and artemisinin solubility. Journal of Ethnopharmacology 190: 313-318.

3.1 Abstract

Ethnopharmacological relevance

Artemisia annua L. produces the antimalarial sesquiterpene lactone, artemisinin (AN), and was traditionally used by the Chinese to treat fever, which was often caused by malaria.

Aim of the study

To measure effects of plant-based and dietary components on release of artemisinin and flavonoids from *A. annua* dried leaves (DLA) after simulated digestion.

Materials and methods

Simulated digestion was performed on DLA in four types of capsules, or in conjunction with protein, and protein-based foods: dry milk, casein, bovine serum albumin, peanuts, peanut butter, Plumpy'nut®, and *A. annua* essential oils. Artemisinin and total flavonoids were measured in the liquid phase of the intestinal stage of digestion.

Results

After simulated digestion, peanuts and Plumpy'nut® lowered AN and flavonoids, respectively, recovered from the liquid digestate fraction. None of the compositions of the tested capsules altered AN or flavonoid release. Surprisingly, bovine serum albumin (BSA) increased both AN and flavonoids recovered from liquid simulated digestate fractions while casein had no effect. AN delivered as DLA was about 4 times more soluble in digestates than AN delivered as pure drug. Addition of a volume of essential oil equivalent to that found in a high essential oil producing *A. annua* cultivar also significantly increased AN solubility in simulated digestates.

Conclusion

These results indicate encapsulating DLA may provide a way to mask the taste of *A. annua* without altering bioavailability. Similarly, many peanut-based products can be used to mask the flavor with appropriate dosing. Finally, the essential oil fraction of *A. annua* contributes to the increased AN solubility in DLA after simulated digestion. Our results suggest that use of DLA in the treatment of malaria and other artemisinin susceptible diseases should be further tested in animals and humans.

Key Words: Key words: malaria, bioavailability, flavonoids, essential oils, sesquiterpenoids, artemisinin, digestion, pACT

Abbreviations: ACT, artemisinin combination therapy; AN, artemisinin; BSA, bovine serum albumin; DLAG, glandless (GLS) cultivar of *A. annua*; DLAS, SAM cultivar of *A. annua*; GC-MS, gas chromatography mass spectrometry; GRAS, generally recognized as safe; HPMC, hydroxypropyl methylcellulose; pACT, plant-based artemisinin combination therapy.

3.2 Introduction

In 2015, there were 214 million cases of malaria resulting in 438,000 deaths worldwide (WHO 2016). Of these deaths, 91% occurred in sub-Saharan Africa and 70% of the victims were children under 5 (WHO 2016). Since 2000, malaria incidence and death rates have decreased globally by 37% and 60%, respectively, however progress has been slower in sub-Saharan Africa (WHO 2016). The foremost therapeutic used to quell malaria worldwide is artemisinin (AN, Figure 3.1), but due to poor solubility, AN semisynthetic derivatives, e.g. artesunate, dihydroartemisinin and artemether, are the preferred drugs. AN is a naturally occurring

sesquiterpene lactone produced and stored in the glandular trichomes of the plant *Artemisia annua* L. (Ferreira and Janick 1995). AN derivatives are combined with other antimalarials, e.g. artemether + lumefantrine (Coartem®) to slow the evolution of AN resistance, and termed artemisinin combination therapies (ACTs), and are recommended by the WHO for treatment of malaria (WHO 2015). Although ACTs are accepted as the frontline treatment for malaria, they are often too expensive or unavailable to those in need (Davis et al. 2013; Kyaw et al. 2014; Yeung et al. 2008). Indeed the highest malaria mortality rates occur in regions of the world with the highest proportions of people living on < $1.25/day (WHO 2012a).

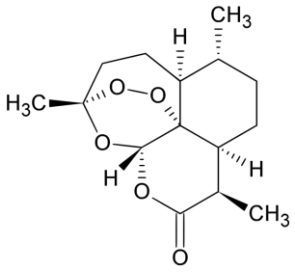

Figure 3.1. Structure of artemisinin (AN).

Recently, use of dried leaves of *A. annua* (DLA; aka pACT) to treat malaria has shown promise. This generally recognized as safe (GRAS) medicinal plant (Duke 2001) has been used since 168 BCE in traditional Chinese medicine to treat a variety of conditions including "fever", which was likely caused by malaria (Cui and Su 2009). Traditionally the plant was prepared as a tea infusion however, this mode of preparation is not recommended as it is difficult to control the many parameters, such as temperature and time, which dictate phytochemical extraction and stability (van der Kooy and Verpoorte 2011; Weathers and Towler 2012). Instead, *per os* (*p.o.*) consumption of DLA should be used to ensure administration of a consistent dose. In a rodent

study, *p.o.* DLA delivery of AN was compared to *p.o.* delivery of pure AN (Weathers et al. 2011), and DLA provided ~ 45 times more AN in the serum than delivered from similar doses of pure AN. Furthermore, delivery as DLA was five times more effective at reducing parasitemia than pure AN in mice infected with *Plasmodium chabaudi* (Elfawal et al. 2012) and three times more resilient against emerging AN drug resistance (Elfawal et al. 2015).

A. annua is also rich in a variety of other compounds including flavonoids, phenolic acids, terpenes, coumarins, saponins, and essential oils (Elford et al. 1987; Lehane and Saliba 2008; Suberu et al. 2013; Van Zyl et al. 2006; Ferreira et al. 2010). Many of these compounds have weak activity against malaria (Weathers and Towler 2014) and some have been shown to synergize with AN (Liu et al. 1992; Suberu et al. 2013). For these reasons, this plant-based artemisinin combination therapy (pACT) may provide an effective, inexpensive treatment option for those in extreme poverty.

One drawback to using DLA is the bitter taste associated with the dried leaves. Although preliminary data suggested about 61% of humans find the leaves distasteful, others actually like the taste (Appendix A, Table S1). Nevertheless, masking the unpalatable taste with readily available food items or by encapsulation is desirable, especially for pediatric patients. Encapsulation or alternative taste masking is only feasible, however, if the capsules or foodstuffs do not significantly alter the bioavailability of the therapeutic compounds. In this study, we use a simulated human digestion system to investigate how various food items, pure proteins, and capsules affected AN and flavonoid content of intestinal stage digestates. We also used simulated digestion to investigate the solubility of AN in intestinal stage digestates when digested as pure drug vs. DLA. Our results also suggest a possible partial mechanism for the increased bioavailability of AN when delivered as DLA vs. as pure drug.

3.3 Methods

3.3.1 Plant Material

Two *Artemisia annua* L. clonal cultivars propagated by rooted cuttings (Weathers and Towler 2012; Towler and Weathers 2015) were used in this study: SAM (DLAS) (voucher MASS 00317314), a high AN-producing cultivar (~1.4% w/w), and GLS (DLAG) (vouchers OR State Univ 171772 and 170353), a glandless AN-null mutant cultivar with no glandular trichomes that produces no AN (Duke et al. 1994) and 25% of the flavonoids found in SAM. SAM plant material used in protein and dietary constituent experiments was field grown in Stow, MA, harvested at floral budding stage, dried and processed as previously described (Weathers, Jordan, et al. 2014). SAM plant material used in solubility experiments was grown in the lab under glass-filtered sunlight, harvested at the vegetative stage, dried, and processed same as the field grown SAM. GLS, a gift from Dr. Stephen Duke at University of Mississippi, was grown in the lab, under glass-filtered sunlight, harvested in the vegetative stage, dried, and processed same as SAM.

3.3.2 Chemicals and Capsules

Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Toluene was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and *A. annua* essential oils from Bella Mira (Mannford, OK, USA). Vegetable capsules made from hydroxypropyl methylcellulose (HPMC) and water and gelatin capsules made from beef gelatin and water were purchased from Capsule Connection LLC (Prescott, AZ, USA). Vcaps®, Vcaps Plus®, and Plantcaps™ were a gift from Capsugel (Morristown, NJ, USA). Vcaps® are made of HPMC and a proprietary combination of gelling agents while Vcaps Plus® are made without the gelling agents. Plantcaps™ are made from pullulan, a polysaccharide polymer fermented from

tapioca. All capsules were size "00." Evaporated milk, smooth peanut butter, and plain unsalted peanuts were store brand purchased from a local Shaw's Supermarket (Stow, MA USA). Plumpy'nut®, made from peanut based paste, is a Ready to Use Therapeutic Food (RUTF; USAID 2015, https://www.usaid.gov/what-we-do/agriculture-and-food-security/food-assistance/resources/ready-use-therapeutic-food Accessed 12-28-15) for treating malnutrition. Plumpy'nut® is produced locally by Edesia (Providence, RI, USA) and was a gift from Maternova Inc. (Providence, RI, USA).

3.3.3 Simulated Digestion

Simulated digestion was performed according to Weathers et al. (2014a) (Figure 3.2). All digestions were taken to the intestinal stage before being vortexed and filtered through Whatman #1 chromatography paper (0.16 mm thickness, porosity < 10 µm) to separate solid and liquid digestate fractions. Liquid fractions were extracted in a sonicating water bath for 30 minutes with an equal volume of toluene to yield a clear two-phase separation to extract AN and flavonoids for analysis. Artemisinin but not all flavonoids are extracted by toluene. This solvent was required to obtain good phase separation especially for the oily materials, peanuts and Plumpy'nut®, thus we kept the extraction solvent constant for all experiments in the study. AN and flavonoids found in the liquid fraction were of interest because they were in solution and thus more likely to diffuse across the intestinal wall into the blood (Weathers, Jordan, et al. 2014). Extracts were dried under nitrogen gas and stored at -20°C until analysis. All digestions with capsules were performed with 2 capsules and all digestions with food items were performed with equal weights of food and plant material.

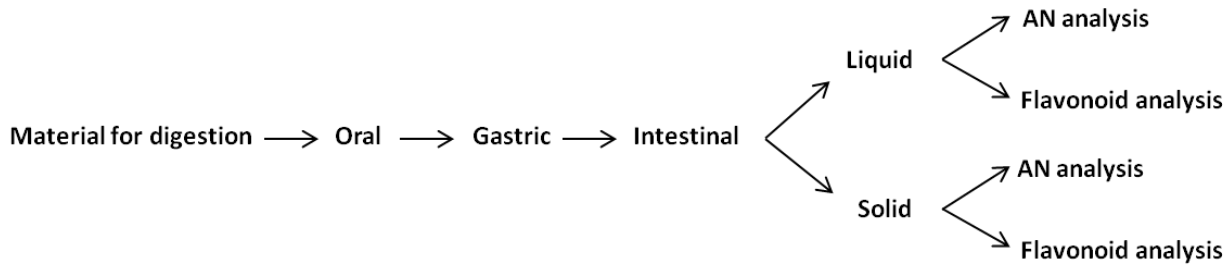

Figure 3.2. Schematic for simulated digestion method.

3.3.4 Solubility Experiments

To determine the difference in solubility when AN was delivered orally as pure drug vs. as DLA, we performed simulated digestions of AN and of DLA and then captured insolubilized AN on a filter and measured the soluble and insoluble fractions as subsequently described. A 0.18 g sample of sieved DLAS and an amount of AN equivalent to that measured in 0.18 g DLAS for that specific experiment were separately digested using the simulated digestion method of Weathers et al. (2014a). We also performed simulated digestions using a pure AN control and an equivalent amount of AN added to 0.18 g dried GLS leaves, which have no detectable AN. Finally, we performed simulated digestion of pure AN as a control and pure AN in combination with camphor (3.8 mg), quercetin (0.6 mg), or 0.55 and 7.2 µL of *A. annua* essential oil added directly to the simulated digestion. Camphor and quercetin amounts were chosen based on total amount of the monoterpene camphor or total flavonoids, respectively, as typically measured in DLAS. Essential oil values were chosen based on the reported extremes of essential oil in *A. annua* (Bilia et al. 2014). All simulated digestions were taken to the intestinal stage. After digestion, the digesta were filtered through Whatman #1 chromatography paper (0.16 mm thickness, porosity < 10 µm) to separate liquid and solid fractions. Both fractions were then extracted with toluene and the extracts dried under nitrogen gas and prepared for analysis. AN extracted from liquid fractions was

considered soluble and likely to be more bioavailable as particles <10 µm are absorbed more readily by intestinal epithelium (Desai et al. 1997). AN present in the solid fraction was considered insoluble or absorbed to residual plant solids and less likely to cross the intestinal wall. The percent AN dissolved was determined by dividing the amount of AN found in the liquid fraction by the total AN recovered. AN solubility (µg/mL) was calculated by dividing the total AN recovered from the liquid digestate fraction by the volume.

3.3.5 Artemisinin and Flavonoid Analysis

AN in extracts was quantified using GC-MS according to the method detailed in Towler and Weathers (2015). Flavonoids in extracts were quantified using the spectrophotometric $AlCl_3$ method (Arvouet-Grand et al. 1994) with quercetin as a standard. Flavonoid levels are expressed as quercetin equivalents.

3.3.6 Statistical Analysis

All experiments were performed in at least triplicate. Students T-tests and One-way ANOVA tests were used to determine statistical significance ($p < 0.05$) where appropriate and Kruskal-Wallis tests were used whenever there were unequal numbers of control and experimental samples. The statistical program GraphPad Prism 6 was used to perform all statistical analysis.

3.4 Theory

In malaria endemic countries, cheaper and readily available antimalarials are needed for people in extreme poverty. DLA may be able to fill this niche but masking the bitter taste would make delivery simpler for the mostly young children who need it. Using a simulated digestion

method, we tested masking agents and capsules for their effect on the levels of therapeutic compounds released into the intestinal digesta. This method also enabled measurement of AN solubility post simulated digestion, which will improve our understanding about DLA enhancement of AN bioavailability. Understanding the factors that dictate bioavailability of AN and flavonoids from DLA provides useful knowledge for clinicians.

3.5 Results and Discussion

3.5.1 Capsules Do Not Alter AN or Flavonoid Content of Digesta

When DLA was run through the simulated digestion with capsules, none of the tested capsules altered the amount of AN extracted from the intestinal liquid (Figure 3.3A). Previously we reported that vegetable and gelatin capsules negatively affected the release of AN from DLAS after simulated digestion (Weathers, Jordan, et al. 2014). That study used methylene chloride to extract the digestate and under some conditions there was not a well-defined phase separation. In this study, however, we used toluene as an extraction solvent instead of methylene chloride and obtained a well-defined two-phase separation. None of the tested capsules showed a significant difference in total flavonoid content of liquid digestate fractions (Figure 3.3B). Since capsules are designed to be chemically inert, these results should be expected. These results suggested that encapsulation may provide a simple means of masking the bitter taste of DLA without altering drug bioavailability. Capsule selection now can be based mainly on cost instead of performance.

3.5.2 Some Food Items Alter AN and Flavonoid Content of Digesta

Another option for masking the taste of DLA is to combine it with common dietary constituents found in sub-Saharan Africa. This serves a dual purpose by not only providing an

antimalarial therapeutic, but also a nutritious supplement. As malnutrition exacerbates malaria and other diseases, especially in low income countries (Caulfield, Richard, and Black 2004), this is desirable. Previously we showed that some dietary constituents commonly used in sub-Saharan Africa altered the AN and flavonoids released into liquid fractions of simulated digestions (Weathers, Jordan, et al. 2014). These food items were mainly simple and complex carbohydrates and various oils. Previously, however, there were reports that AN bound to serum proteins (Bian et al. 2006; Liu, Cheng, and Jiang 2014) and there was concern that protein in the diet may decrease AN bioavailability during digestion. Thus, we performed simulated digestions with several protein-rich dietary constituents to determine if they would alter AN content of the liquid digestate fraction. After simulated digestion of DLAS + peanuts, the AN content in the liquid digestate fraction decreased by about 23% (Figure 3.4A, p = 0.028). Dry powdered milk, smooth peanut butter, and Plumpy'nut®, however, had no effect on AN content of the liquid digestate fraction.

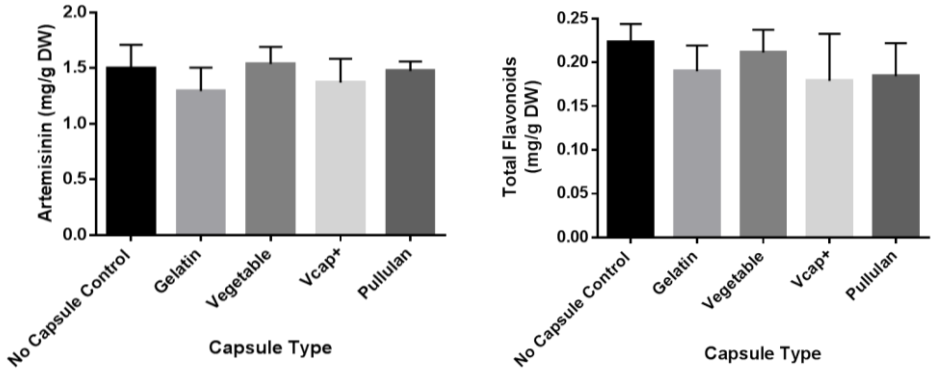

Figure 3.3. Artemisinin (A) and flavonoid (B) content in liquid fraction from intestinal stage of simulated digestions of DLAS ± various capsule types. N ≥ 3; *, $p \leq 0.05$ compared to AN control within each experiment.

There was also a significant increase in AN released after digestion of DLAS + bovine serum albumin (BSA) (Figure 3.4C p = 0.006). AN is known to bind BSA (Bian et al. 2006; Liu, Cheng,

and Jiang 2014) and is sometimes used in drug absorption studies to bind free drug and maintain sink conditions (Hubatsch, Ragnarsson, and Artursson 2007), so it is conceivable that the BSA interacted with free AN in the digestate solution allowing more AN to be extracted from the solid DLAS fraction into the liquid DLAS fraction of the digestate. Casein, the main protein found in milk, did not have any effect. It therefore seems unlikely that proteins are responsible for major decreases in AN content in liquid digestate fractions. The mechanism by which peanuts and not peanut butter decreased AN content is still unresolved.

Besides AN, we also tested the effects of these same dietary constituents on total flavonoid content in liquid digestate fractions. After digestion, total flavonoids decreased in the liquid fraction by about 24%, and only in the presence of Plumpy'nut® (Figure 3.4B, $p = 0.003$). However, in the presence of BSA, flavonoids increased by about 56% (Figure 3.4D, $p = 0.0015$) in the liquid digestate fraction. Similarly to AN, casein had no effect. BSA has been shown to also strongly bind flavonoids (Liu et al. 2014; Bi et al. 2012; Papadopoulou, Green, and Frazier 2005), so the observed increase in flavonoids is likely due to the same mechanism described for AN wherein BSA creates stronger sink conditions allowing more flavonoids to be extracted from the solid fraction DLAS into the liquid digestate. While the differences in AN and flavonoid content in the presence of peanuts and Plumpy'nut®, respectively, were statistically significant, neither therapeutic compound is decreased enough to discourage their use as a masking agent. Rather, if using either peanuts or Plumpy'nut® to mask DLA flavor, then the amount of DLA could be proportionately increased to ensure adequate delivery to yield a minimum therapeutically effective serum concentration of 9 $\mu g\ L^{-1}$ (Alin and Bjorkman 1994).

3.5.3 Delivery Method Changes AN Solubility

AN has significantly higher bioavailability when delivered *p.o.* as DLA as opposed to pure AN (Weathers et al. 2011; Weathers, Elfawal, et al. 2014). However, the mechanism by which AN bioavailability is so greatly enhanced is yet to be uncovered. We posited that AN solvates better

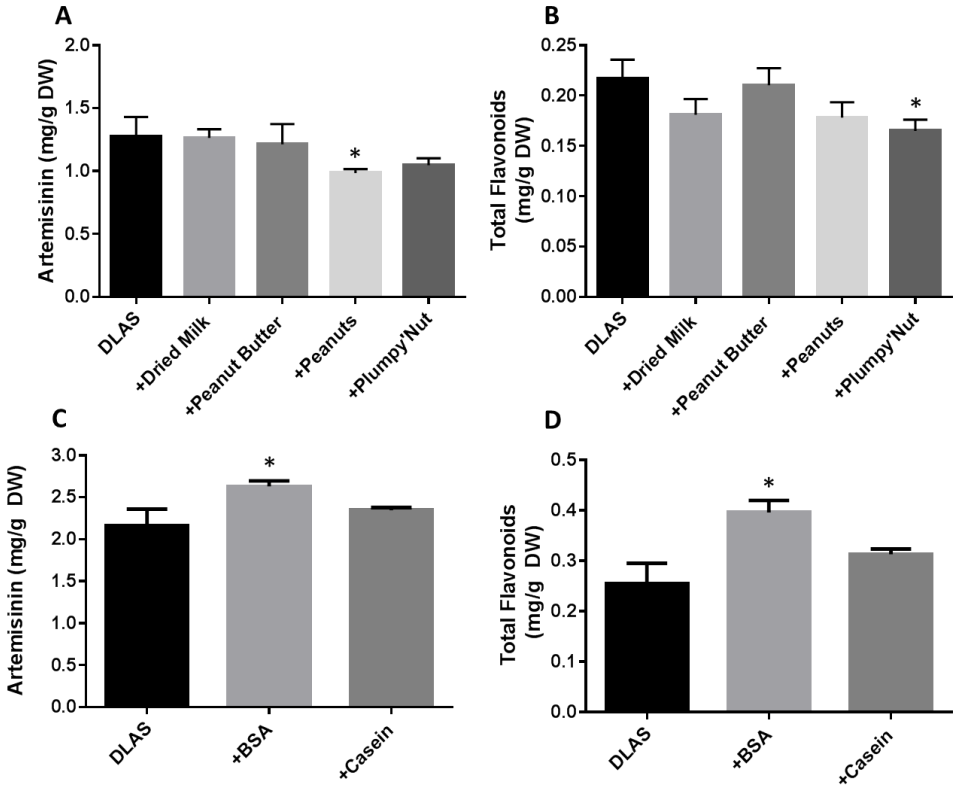

Figure 3.4. Artemisinin (A) and flavonoid (B) content in liquid fraction from intestinal stage of simulated digestions of DLAS ± various protein-rich dietary constituents. Artemisinin (C) and flavonoid (D) content in liquid fraction from intestinal stage of simulated digestions of DLAS ± BSA. $N \geq 3$; *, $p \leq 0.05$ compared to AN control within each experiment.

when combined with some other phytochemicals found in the plant material. To test this hypothesis, we carried out simulated digestions of either pure AN or an amount of DLAS containing the same amount of AN. After digestion, it was determined that the liquid digestate from DLAS contained about four times more AN than the pure AN liquid digestate fraction (Table

3.1, $p = 0.005$). This large increase in AN solubility partially explains the increased bioavailability seen when AN is delivered orally as DLAS in mice (Weathers et al. 2011). To query which compounds or groups of compounds were responsible for the increase in AN solubility we first performed simulated digestions with addition of pure AN and equal amounts of pure AN added to 0.18g DLA of the GLS cultivar (DLAG). GLS lacks glandular trichomes and thus produces no detectable AN, only 0.06% (w/w) essential oils (Tellez et al. 1999), and only 25% of the flavonoids of SAM, so we could determine if the increased AN solubility was due to general plant matrix or some specific groups of chemicals exclusive to one of the two cultivars. After simulated digestion of DLAG + AN and pure AN, there was no significant difference in the amount of AN in the liquid digestate fractions (Table 3.1). These results indicated that the compounds responsible for increasing AN solubility are present in DLAS but not in the GLS cultivar.

Table 3.1. Effects of different delivery methods on AN content in liquid simulated digestate fractions from four independent experiments.

Experiment	Treatment	AN fold change in digestate liquid fraction
Pure AN vs. DLAS	AN	1.00a
	DLA-S	4.08b
Pure AN vs. AN + DLAG	AN	1.00a
	AN + DLA-G	0.91a
Pure AN vs. AN + Essential Oils	AN	1.00a
	AN + 0.3% EO	0.95a
	AN + 4.0% EO	2.46b
Pure AN vs. AN + Camphor or Quercetin	AN	1.00a
	AN + Camphor	1.09a
	AN + Quercetin	0.89a

DLAS and DLAG, 0.18 g dry weight. Values normalized to AN control; $n \geq 3$; a,b letters show $p \leq 0.05$ compared to AN control within each experiment.

3.5.4 Essential Oils Increase AN Solubility

The essential oil composition of *A. annua* cultivars ranges from 0.3-4.0% (w/w) (Bilia et al. 2014). Since the essential oil content of DLAS is unknown, we tested both high and low levels of essential oil for its effect on AN solubility in digestates. Aliquots of *A. annua* essential oil equivalent to 0.3 and 4% of 0.18 g of DLAS dry mass were added to simulated digestions with pure AN. After the intestinal stage of digestion, there was about 2.5 times more AN dissolved in the liquid digestate fraction of the 4% essential oil group (Table 3.1, $p = 0.004$). No significant difference was found between the amount of solubilized AN in the pure AN control and 0.3% essential oil + AN groups (Table 3.1). Thus, essential oils in the plant are likely in part responsible for the increased solubility of AN when delivered *p.o.* as DLAS as opposed to pure AN. We also tested the hydrophobic flavonoid, quercetin, and a principal component of the hydrophobic essential oil, camphor, to determine if they played a role in increasing AN solubility. While both of these hydrophobic compounds have low aqueous solubility at the start of simulated digestion, the changes in temperature and pH, as well as the addition of bile salts in the intestinal phase likely allows some to become emulsified. Bile is a lipid emulsifier in digestion that allows the breakdown of lipids into micelles so they can be readily absorbed by the intestine. After digestion to the intestinal stage, quercetin did not alter AN solubility suggesting hydrophobic flavonoids play no role in increasing the solubility of AN delivered as DLAS. Interestingly, the hydrophobic monoterpene, camphor, also had no effect on AN solubility in digestates suggesting that it is likely other compounds present in the essential oil that lead to enhanced AN solubility.

3.6 Conclusions

Determining how digestion effects AN and flavonoid content in the intestine after oral drug delivery is paramount to ensuring proper dosing and understanding the differences between DLAS and pure AN. Using a simulated digestion method, we showed that peanuts decreased AN content in liquid digestate fractions by 23%. Although peanut butter was benign, Plumpy'nut®, a peanut-based RUTF used to treat malnutrition in sub-Saharan Africa, decreased flavonoid content by 24% in liquid digestate fractions. As these dietary constituents decrease the levels of therapeutic compounds delivered by DLA, those recommending DLA as a treatment for malaria should consider altered dosing if these food items are used to mask the bitter taste. Since none of the tested capsule types decreased either AN or flavonoid content, capsules offer an acceptable means of masking the unpalatable flavor of DLA.

Using this simulated digestion method, we were also able to partially explain the mechanism by which DLA enhances AN bioavailability in prior rodent studies. In simulated digestions AN delivered via DLAS yielded four times more AN in the liquid digestate fraction than pure AN, indicating some compounds in the SAM cultivar enhanced AN solubility. This was not true for AN delivered with dried leaves of the GLS cultivar, likely the result of the dearth of essential oils in GLS. Furthermore, AN delivered in combination with essential oil from *A. annua* at a volume consistent with a high essential oil producing plant showed a 2.5-fold increase in AN solubility suggesting essential oil plays a role in the increased solubility afforded by DLAS. Together these results provide insight into how DLAS might function and be used as an inexpensive yet still effective alternative to traditional ACT medication for malaria.

3.7 Acknowledgements

The authors would like to thank Capsugel for providing capsules, Dr. Melissa Towler of Worcester Polytechnic Institute for assistance in artemisinin and flavonoid analysis, Dr. Jill Rulfs, Dr. Natalie Farny, and Abbie White of Worcester Polytechnic Institute for running the taste tasting survey, and Worcester Polytechnic Institute for partially supporting Mr. Desrosiers. We are also grateful for Award Number NIH-R15AT008277-01 from the National Center for Complementary and Integrative Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Complementary and Integrative Health or the National Institutes of Health.

Chapter 4 Artemisinin permeability via Caco-2 cells increases after simulated digestion of *Artemisia annua* leaves

Published as:

Desrosiers M. and Weathers P.J., 2017. Artemisinin permeability via Caco-2 cells increases after simulated digestion of *Artemisia annua* leaves. Journal of Ethnopharmacology. 210: 254-259.

4.1 Abstract

Ethnopharmacological relevance

Artemisia annua has been used for > 2,000 years to treat fever and is more recently known for producing the important antimalarial drug, artemisinin.

Aim of the study

Artemisinin combination therapies (ACTs) are effective for treating malaria but are often unavailable to those in need. Dried leaves of *A. annua* (DLA) have recently been studied as a cost-effective alternative to traditional ACTs. DLA was shown to dramatically increase oral bioavailability compared to pure artemisinin, so more investigation into the mechanisms causing this increased bioavailability is needed.

Materials and Methods

In this study, we used a simulated digestion system coupled with Caco-2 cell permeability assays to investigate the intestinal permeability of DLA compared to pure artemisinin. We also determined the effects of different phytochemicals (7 flavonoids, 3 monoterpenes, 2 phenolic acids, scopoletin and inulin) and the cytochrome P450 isoform CYP3A4 on artemisinin intestinal permeability.

Results

Artemisinin permeability delivered as digested DLA significantly increased by 37% (P_{app} = $8.03x10^{-5}$ cm s^{-1}) compared to pure artemisinin (P_{app} = $5.03x10^{-5}$ cm s^{-1}). However, none of the phytochemicals tested or CYP3A4 had any significant effect on the intestinal permeability of

artemisinin. We also showed that essential oil derived from *A. annua* negatively affected the intestinal permeability of artemisinin, but only after simulated digestion. Finally, we showed that *A. annua* essential oil reduced the transepithelial electrical resistance of Caco-2 monolayers, but only in the presence of bile. Although also reduced by essential oils, artemisinin P_{app} subsequently recovered in the presence of plant matrix.

Conclusions

These results shed light on the mechanisms by which DLA enhances the oral bioavailability of artemisinin.

Key Words: antiprotozoal; drug transport; essential oils; flavonoids; malaria; terpenes

Abbreviations: AA, artemisinic acid; AB, arteannuin B; ACT, artemisinin combination therapy; AN, artemisinin; DLA, dry leaf Artemisia; DLAS, DLA *A. annua* cultivar with ~1.4% artemisinin; DLAG, DLA glandless; GLS, glandless *A. annua* cultivar that contains no artemisinin; TEER, trans-epithelial electrical resistance; VD3,1α,25-dihydroxyvitamin D3.

4.2 Introduction

Malaria, a disease caused by parasites of the *Plasmodium* genus, remains a major global health problem across the developing world. There are over 3 billion people at risk of contracting malaria, about half the world's population, and each year there are over 400,000 deaths due to the disease (WHO 2016). Although the vast majority of malaria infections can be treated effectively with artemisinin combination therapies (ACTs), there remains a large population, mostly in rural Africa, that does not have access or the financial resources to receive treatment. As a result, in

2015 about 90% of deaths due to malaria occurred in Sub-Saharan Africa. Of these deaths, about 70% were children under the age of 5 (WHO 2016).

Artemisia annua L., the plant that naturally produces artemisinin (AN) in its glandular trichomes, has been used traditionally in China to treat malaria dating back as far as the second century BCE (Hsu 2006). Recently, consumption of the dried leaves of *A. annua* (DLA) has been studied as a potential low-cost treatment option for people living in rural malaria endemic regions. In mouse studies, DLA was shown to be 5 times more effective than pure artemisinin at clearing *Plasmodium* parasites from the blood (Elfawal et al. 2012) and three times better at slowing the development of resistant parasites (Elfawal et al. 2015). In a small human trial in Kenya, patients treated twice daily for 6 days with tablets made from DLA achieved >90% parasite clearance at 28 days with <10% recrudescence, a result comparable with many ACTs (ICIPE 2005). More recently, DLA successfully treated 18 patients who had ACT and I.V. artesunate resistant malaria (Daddy et al. 2017). Furthermore, when oral delivery of DLA was compared to oral delivery of pure artemisinin in mice, there was about 45 times more artemisinin found in the serum of mice given DLA (Weathers et al. 2011). The mechanism causing this striking increase in drug bioavailability is yet to be fully determined. Our group recently showed through simulated digestion experiments that digestion of DLA results in about 4 times higher solubility of artemisinin in the resulting digestate and this is largely from essential oils found in the plant material (Desrosiers and Weathers 2016). Artemisinin has very low aqueous solubility, so its increased solubility from DLA partially explains the 45-fold increase in serum concentration, however, there are likely other mechanisms in play.

One potential mechanism for the increased artemisinin bioavailability afforded by DLA is modulation of the intestinal permeability of artemisinin. Several phytochemicals found in *A. annua*

have either increased the rate of transport of other drugs or inhibited key enzymes that mediate the first-pass metabolism of artemisinin. For example, De Magalhães et al. showed that tea infusions made from various *A. annua* cultivars inhibited CYP3A4 (Melillo de Magalhães et al. 2012), an enzyme present in the intestine involved in the metabolism of artemisinin (Svensson and Ashton 1999). Quercetin, a flavonoid found in *A. annua*, also increased the absorption of doxorubicin in rats (Choi, Piao, and Kang 2011), while tamarixetin, another flavonoid, increased absorption of fluvastatin in rats (Wang et al. 2014). Further, other studies showed that flavonoids found in *A. annua*, such as quercetin and rutin, inhibited CYP3A4 as well as other cytochrome P450 enzymes that mediate the metabolism of artemisinin (Wang et al. 2014).

In this study, we used the Caco-2 cell model of the intestinal epithelium to measure the intestinal permeability of artemisinin when delivered as pure drug or as DLA simulated digestate. We showed that delivery as digested DLA increased the rate of artemisinin transport across the intestinal epithelium. We also tested a wide variety of phytochemicals found in *A. annua* for their effects on artemisinin permeability and tested the role of CYP3A4 in this process by upregulating its expression and activity in Caco-2 cells.

4.3 Materials and Methods

4.3.1 Plant Material

We used two *Artemisia annua* L. cultivars in these studies. The first, SAM (DLAS) (voucher MASS 317314), is a high artemisinin and flavonoid producing cultivar, about 1.4% and 0.3% (w/w) respectively and was propagated clonally by rooted cuttings. DLAS was field grown in Stow, MA and harvested at the floral budding stage, dried and processed as detailed in (Weathers, Jordan, et al. 2014). The second cultivar used, GLS (DLAG) (vouchers OR State Univ

171772 and 170353), is a mutant cultivar lacking glandular trichomes and producing no artemisinin, 25% of the flavonoids of DLAS, and negligible levels of essential oils as measured by our lab (Appendix B, Table S1) and others (Tellez et al. 1999). DLAG was grown in the lab under glass-filtered sunlight, harvested during the vegetative stage, dried and processed as DLAS.

4.3.2 Chemicals and Reagents

All chemicals and reagents used were at least research grade from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated. *A. annua* essential oils were purchased from Bella Mira (Mannford, OK, USA) or Jiangxi Jinyuan Natural Perfume Company (Ji'an, Jiangxi, China). Rutin, eupatorin, casticin, and isovitexin were purchased from ChromaDex (Irvine, CA, USA).

4.3.3 Caco-2 Cell Culture

The Caco-2 cell line was purchased from the American Type Culture Collection (ATCC: HTB-37) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) containing 4.5 g/L D-glucose, 110 mg/L sodium pyruvate, 20% Fetal Bovine Serum (FBS) (Rocky Mountain Biologicals, Missoula, MT, USA), 1X GlutaMAX (Life Technologies, Carlsbad, CA, USA), and 1X penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) in a humidified incubator at 37° C and 5% CO_2. Cells were harvested using TrypLE (Life Technologies, Carlsbad, CA, USA), resuspended in culture medium, and seeded at a density of $2.6x10^5$ cells/cm^2 on 12 well polyethylene terephthalate transwell ThinCerts hanging well inserts (0.4 µm pore size, 1.13 cm^2 culture area) (Greiner Bio-One, Kremsmünster, Austria). Culture medium was changed every other day for 21-28 days and 24 hours before performing permeability experiments. All cells used for permeability experiments were between passages 32 and 52.

4.3.4 Transepithelial Electrical Resistance and Lucifer Yellow Assays

To ensure monolayer integrity throughout the experimental period, transepithelial electrical resistance (TEER) was measured before and after experiments using the EVOM2 epithelial voltmeter (World Precision Instruments, Sarasota, FL, USA). TEER values vary throughout the literature, so we set a TEER cutoff based on Lucifer yellow rejection. Lucifer yellow is a fluorescent dye that is only transported paracellularly and is thus used as a marker of Caco-2 tight junction integrity. To determine Lucifer yellow rejection rate, we performed a Lucifer yellow permeability assay using Caco-2 cells cultured in hanging wells. First, the TEER of Caco-2 monolayers was recorded and then 0.5 mL 100 µM Lucifer yellow in Hank's balanced salt solution (HBSS) was added to the apical side of the hanging wells. Hanging wells were then inserted into 12 well plates prefilled with 1.5 mL HBSS in each well and then stirred on a nutator (TCS Scientific, New Hope, PA, USA) at 24 RPM in a humidified, 37° C incubator for 1 hour. Afterwards samples were taken from the apical and basolateral sides of the hanging well and read on a fluorescent plate reader. The Lucifer yellow rejection value was calculated using the equation: LY % rejection = $100*(1-RFU_{basolateral}/RFU_{apical})$ where RFU is the relative fluorescent units recorded by the plate reader. It was determined that TEER values below 290 $\Omega*cm^2$ had Lucifer yellow rejection values below 95% and as a result, wells with a TEER value below 290 $\Omega*cm^2$ were not used for permeability assays.

4.3.5 Simulated Digestion

Simulated digestion was performed using the method described in Weathers et al. 2014 (Weathers, Jordan, et al. 2014). Either 0.36 g DLA or 2 mg pure artemisinin was digested in a 50

mL conical tube. Digestions were run through oral, gastric, and intestinal stages of digestion and then filtered through Whatman #1 chromatography paper (0.16 mm thickness, porosity < 10 µm) to separate liquid and solid fractions. Only the liquid fraction of the digestate was used for permeability experiments.

4.3.6 CYP3A4 Upregulation

Under normal culture conditions, the human cytochrome P450 isoform CYP3A4 is not expressed in Caco-2 cells. To better mimic the *in vivo* conditions, CYP3A4 expression was induced by adding 0.5 µM 1α,25-dihydroxyvitamin D_3 (VD3) to the culture media as described by Schmiedlin-Ren et al. 2001 (Schmiedlin-Ren et al. 2001). We performed RNA isolation and qPCR for CYP3A4 on cells cultured in this VD3 media to confirm the upregulation of CYP3A4 transcription. RNA isolation was performed using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Using qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA), cDNA was prepared from total RNA according to the manufacturer's instructions. Using PerfeCta SYBR Green FastMix, low ROX (Quanta Biosciences, Beverly, MA, USA), qPCR was performed according to the manufacturer's instructions with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qPCR: CYP3A4F (5'-CCTTACATATACACACCCTTTGGAAG-3'), CYP3A4R (5'-GGTTGAAGAAGTCCTCCTAAGCT-3') (Nowakowski-Gashaw et al. 2002), ActinF (5'-AGAGCTACGAGCTGCCTGAC-3'), ActinR (5'-GGATGCCACAGGACTCCA-3'). CYP3A4 upregulation was also verified at the level of enzymatic activity with a luciferin-based P450-Glo assay specific for CYP3A4 (Promega, Madison, WI, USA) following the manufacturer's

instructions. Caco-2 cells were cultured in monolayers on 12 well plates for 21-28 days in either standard or VD3 containing media prior to P450-Glo assays. Cells were then incubated for 2 hours with culture media containing 3 μM Luciferin-IPA, a CYP3A4-specific substrate that is converted to a luminogenic substrate by CYP3A4. After the 2 hour incubation, the medium was removed and combined with Luciferin Detection Reagent and luminescence measured on a PerkinElmer Victor3 plate reader (PerkinElmer, Waltham, MA, USA).

4.3.7 Permeability Experiments

Artemisinin permeability experiments were performed in 12 well plates with transwell inserts having a developed cell monolayer separating the wells into an apical and basolateral chamber. Culture medium was first decanted and cells on transwell inserts were washed 3 times in HBSS pre-warmed to 37° C. Inserts were then placed in a new 12 well plate pre-filled with 1.5 mL warm HBSS in each basolateral chamber and 0.5 mL warm HBSS was added to the apical chamber. The plate was placed in a humidified 37° C incubator on a nutator mixer and mixed at 24 RPM for 20 minutes to wash off excess media. During the 20-minute incubation, donor solutions were prepared and warmed to 37° C. Donor solutions were either the liquid digestate fraction from a simulated digestion or pure artemisinin with or without a pure test compound dissolved in 2.5% dimethyl sulfoxide (DMSO) in HBSS. The solubility of artemisinin in donor solutions was checked by filtering through a 0.45 μm nylon syringe filter. Filtered and unfiltered donor solutions had no significant difference in artemisinin content; artemisinin was fully soluble at 50 ug/mL in water, DLAG, and DLAS donor solutions. After incubation, the TEER of each well was recorded, HBSS was decanted from the apical chambers, 0.5 mL donor solution was added to the apical chamber and the plate was placed on the nutator in the incubator. Samples of the donor

solution were taken to determine the artemisinin content at time 0. Experiments were performed for 60 minutes. At each 15-minute interval, the transwell inserts were removed and placed into a new 12 well plate pre-filled with pre-warmed HBSS and placed immediately back into the incubator on the nutator. Samples were then taken from the basolateral chamber for extraction and analysis. After 60 minutes, the TEER of each well was recorded to validate integrity of the monolayer and a sample was taken from the apical chamber of each transwell insert for extraction and analysis.

4.3.8 Artemisinin and P_{app} Analysis

Artemisinin from samples was extracted by adding a 1:1 volume of methylene chloride, briefly vortexing, and sonicating in a sonication water bath for 30 minutes. The organic layer was then removed and dried under a mild stream of nitrogen gas, frozen, and stored at -20° C before analysis. Artemisinin analysis was by gas-chromatography mass-spectrometry (GC-MS) using the method detailed in Towler and Weathers 2015 (Towler and Weathers 2015). Apparent permeability (P_{app}) was calculated using the formula: $P_{app} = \left(\frac{\Delta Q}{\Delta t}\right)\left(\frac{1}{AC_o}\right) cm \cdot s^{-1}$ where $\Delta Q/\Delta t$ is the amount of drug transported per time, A is the surface area of the transwell insert, and C_o is the original concentration of drug in the donor chamber at time 0. This equation requires that sink conditions be maintained, thus transwell inserts were placed in new receiver chambers with pre-warmed HBSS at each 15-minute time point. Percent recovery of artemisinin was calculated by dividing the total artemisinin recovered in each well by the original amount of artemisinin in the donor solution and multiplying by 100. The percent recoveries of artemisinin for each experiment are shown in Appendix B, Table S2.

4.3.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Each experiment was the average of ≥3 wells and any experiment where a significant difference was observed between the control and experimental wells was subsequently repeated. One-way ANOVA and students T-tests were used where appropriate to determine statistical significance ($p<0.05$).

4.4 Theory

In animal studies artemisinin is significantly more bioavailable from per os delivery of DLA than from its pure form. Using the Caco-2 permeability assay and simulated digestion methods, we tested digestates of DLA and individual *A. annua* phytochemicals for their ability to enhance transport of artemisinin across intestinal cells. This approach improved our understanding of DLA enhancement of artemisinin bioavailability.

4.5 Results and Discussion

4.5.1 Digestate of *A. annua* Increases Intestinal Permeability of Artemisinin

To determine if digested DLA increased the intestinal permeability of artemisinin, we performed a Caco-2 permeability assay using simulated digestates of DLAS compared to a pure artemisinin control. When delivered as DLAS digestate, the intestinal permeability of artemisinin was significantly greater by 37% than artemisinin delivered as pure drug ($p = 0.013$) (Figure 4.1). This result suggested that one or more phytochemicals in *A. annua* may increase the intestinal absorption of artemisinin. Next, we performed a similar experiment using the glandless (DLAG) cultivar of *A. annua* that produces no artemisinin, very few flavonoids, and negligible levels of essential oils. We combined pure artemisinin with a digestate of DLAG and compared to a pure

artemisinin control in a Caco-2 assay. Interestingly, there was no change in intestinal permeability when artemisinin was combined with digestate from DLAG (Figure 4.1). These results suggested that one or more phytochemicals produced by DLAS, but not in DLAG, were responsible for the increased intestinal permeability afforded by the DLAS digestate. In addition to testing the P_{app} of artemisinin in the apical-to-basolateral direction, we also tested the P_{app} of artemisinin in the basolateral-to-apical direction. The P_{app} of artemisinin in the basolateral-to-apical direction was not significantly different from the P_{app} of artemisinin in the apical-to-basolateral direction suggesting active efflux does not play a role in artemisinin absorption. Unfortunately, permeability of artemisinin delivered as digestate from the basolateral-to-apical direction could not be determined because digestates significantly weakened the integrity of the tight junctions in the Caco-2 monolayers as determined by a sharp decrease in TEER.

4.5.2 Flavonoids and Other Phytochemicals Do Not Alter Artemisinin P_{app}

Flavonoids, a group of phytochemicals responsible for many of the pigments in plants, significantly enhanced the intestinal uptake of several drugs. For example oral administration of genistein increased the bioavailability of paclitaxel in rats (Li and Choi 2007). Furthermore, several flavonoids also increased intestinal absorption of ochratoxin A in Caco-2 cells at physiologically relevant concentrations (Sergent et al. 2005). *A. annua* is known for having high flavonoid content, so we hypothesized that the flavonoids present in DLAS may have enhanced the intestinal permeability of artemisinin. To test this hypothesis, we performed Caco-2 assays using pure artemisinin in combination with various pure flavonoids having different structural variations and known to be present in *A. annua* and compared their P_{app} to the P_{app} of pure

artemisinin. As shown in Table 4.1 below, none of the tested flavonoids significantly altered the P_{app} of artemisinin.

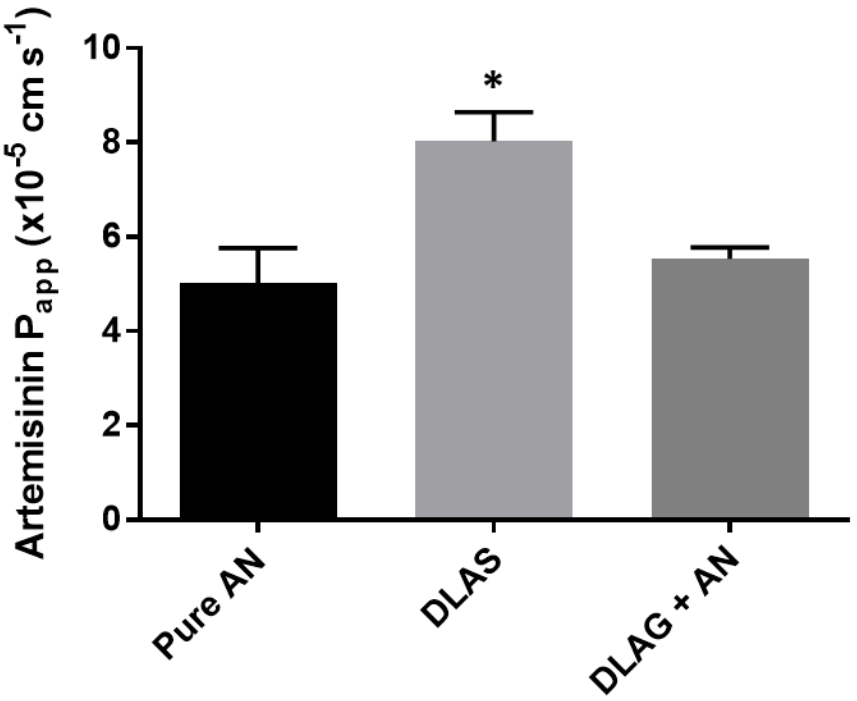

Figure 4.1. P_{app} of artemisinin in Caco-2 permeability assays when delivered as pure AN, DLAS, or DLAG+AN. $n \geq 3$; *, $p < 0.05$ compared to pure drug control.

Table 4.1. Flavonoid effects on P_{app} of AN

Flavonoid	P_{app} ($x10^{-5}$)($cm\ s^{-1}$) (±SD)
Pure AN	5.59 ± 0.94
+Quercetin	6.93 ± 1.21
+Rutin	7.18 ± 1.00
+Eupatorin	5.57 ± 0.27
+Kaempferol	5.39 ± 0.28
+Casticin	7.09 ± 0.92
+Isovitexin	6.45 ± 0.89
+Apigenin	5.98 ± 0.56

AN, artemisinin.

Other phytochemicals known to increase bioavailability of other xenobiotics or that were considered to also affect intestinal absorption of artemisinin were also tested. Although inulin, a polysaccharide produced by *A. annua*, increased intestinal absorption of magnesium, calcium, and other minerals (Scholz-Ahrens and Schrezenmeir 2007) it did not increase the intestinal permeability of artemisinin (Table 4.2). Recently, a combination of three phytochemicals found in *A. annua*, artemisinic acid (AA), arteannuin B (AB), and the coumarin scopoletin, increased oral bioavailability of artemisinin in mice (Zhang et al. 2016). This combination, however, did not increase the P_{app} of artemisinin in our Caco-2 assays (Table 4.2). Finally, chlorogenic acid and rosmarinic acid, two phenolic acids often found in *A. annua* (Zang et al. 2014) did not alter the P_{app} of artemisinin (Table 4.2). Together these results suggested that flavonoids were not responsible for the increased P_{app} of artemisinin afforded by DLAS. Furthermore, the results showed that neither inulin, phenolic acids, nor the combination of AA, AB and scopoletin altered artemisinin intestinal absorption. While these compounds do not alter artemisinin intestinal absorption, they may have affected artemisinin bioavailability via some combination of compounds or some other mechanism yet to be investigated, such as inhibition of artemisinin metabolism in the liver.

Table 4.2. Effects of other phytochemicals of interest on AN P_{app}

Phytochemical	P_{app} (x10-^{5})(cm s^{-1}) (±SD)
Pure AN	5.94 ± 0.69
+Chlorogenic Acid	6.17 ± 0.45
+Rosmarinic Acid	5.98 ± 0.75
+Inulin	6.56 ± 0.44
+AA+AB+Scopoletin	5.62 ± 1.22

AA, artemisinic acid; AB, arteannuin B; AN, artemisinin.

4.5.3 Intestinal CYP3A4 Does Not Play a Role in Increasing DLA Delivered Artemisinin

In humans, artemisinin is primarily metabolized in the liver by the cytochrome P450 isoform, CYP2B6. However, CYP3A4 also contributes in a minor role and is expressed in the liver as well as the small intestine. There are many phytochemicals found in *A. annua*, such as quercetin, luteolin, apigenin, and kaempferol, that have been shown to inhibit CYP450 enzymes (Basheer and Kerem 2015), so we hypothesized that inhibition of intestinal CYP3A4 by phytochemicals produced by *A. annua* would allow more artemisinin to pass through the intestine resulting in higher bioavailability. While CYP3A4 is expressed in the human intestine, it is not expressed in Caco-2 cells under normal culture conditions unless induced by VD3. Thus, to test our hypothesis, we first induced expression of CYP3A4 by including VD3 in the cell culture medium. CYP3A4 expression increased ~175 fold with VD3 treatment as determined by qPCR (Appendix B, Table S3). A luciferin-based CYP3A4 activity assay confirmed that CYP3A4 activity increased 183% in VD3 treated cells compared to untreated cells (p=0.012). Next we performed Caco-2 permeability assays using pure artemisinin, digested DLAS, or pure artemisinin combined with quercetin, a known CYP3A4 inhibitor found in DLAS, on cells treated with VD3 media. As expected, the P_{app} of DLAS +VD3 was significantly higher than the P_{app} of pure artemisinin +VD3 (p=0.02) (Table 4.3). However, addition of VD3 to media did not alter the P_{app} of artemisinin, regardless of whether it was delivered as pure drug, DLAS, or in combination with

quercetin. These results suggested that intestinal CYP3A4 does not play a major role in determining the bioavailability of artemisinin; this needs *in vivo* verification. Furthermore, the mechanism by which DLAS increased intestinal permeability of artemisinin seems independent of CYP3A4.

Table 4.3. P_{app} of artemisinin delivered as pure drug, DLA, or as pure drug + quercetin in media ± VD3

Donor Solution	P_{app} ($x10^{-5}$)($cm\ s^{-1}$) (±SD)
Pure AN –VD3	6.17 ±1.13
Pure AN +VD3	6.27 ± 1.66
DLAS Digestate –VD3	8.03 ± 1.23*
DLAS Digestate +VD3	9.40 ± 1.05*
Quercetin -VD3	6.93 ± 1.21
Quercetin +VD3	6.38 ± 0.89

AN, artemisinin; DLAS, dried leaf *Artemisia annua*; VD3, 1α,25-dihydroxyvitamin D3; *, $p \leq 0.05$ compared to pure AN control.

4.5.4 Digested *A. annua* Essential Oils Alter P_{app} and TEER of Caco-2 Cells

Recently our group showed that essential oil (EO) from *A. annua* increased the digestive solubility of artemisinin (Desrosiers and Weathers 2016). Since EO increased artemisinin solubility and contains >100 phytochemicals (Ćavar et al. 2012), we hypothesized that this fraction of *A. annua* would increase the intestinal permeability of artemisinin in Caco-2 assays. *A. annua* cultivars vary significantly in the amount and chemical profiles of EO, so we used EOs from two different sources (USA and China) and their relative contents are shown in Appendix B, Table S4. The range of total EOs in *A. annua* also varies between 0.3% and 4.0% (v/w) (Bilia et al. 2014). Thus, we performed Caco-2 permeability assays with digested EO from two sources at two different concentrations corresponding to these known ranges of EO produced by *A. annua* cultivars. Surprisingly, the digestate of EO from both sources and at both concentrations caused a

decrease in the P_{app} of artemisinin as well as a sharp decrease in the TEER suggesting a loss of the integrity of tight junctions (Table 4.4).

Table 4.4. Effects of 2 different digested *A. annua* essential oils at different concentrations on artemisinin Papp

Donor Solution	**P_{app} (x10-^{5})(cm s^{-1}) (±SD)**	**Loss of TEER**
Pure AN	5.22 ± 0.52	-
AN+US EO Digestate (4%)	3.39 ± 0.41*	+
AN+US EO Digestate (0.3%)	3.86 ± 0.53*	+
AN+Chinese EO Digestate (4%)	3.71 ± 0.66*	+
AN+Chinese EO Digestate (0.3%)	3.76 ± 0.60*	+
AN+DLAG+US EO Digestate (4%)	4.99 ± 0.57	+
AN+DLAG+US EO Digestate (0.3%)	5.06 ± 0.43	+

AN, artemisinin; EO, essential oil; TEER, transepithelial electrical resistance; US, United States; *, $p<0.05$ compared to pure AN control.

To confirm the drop in TEER was due to weakening of the tight junctions and not an artifact produced by the TEER measuring equipment, we performed a Lucifer yellow assay on the cells after the permeability experiment. Lucifer yellow is a fluorescent dye that only passes paracellularly and thus is only transported when tight junctions lose their integrity (Rastogi et al. 2013). After treatment with US and Chinese EO digestates, Caco-2 cells had Lucifer yellow rejection rates of 83.7% and 84.0%, respectively, compared with 97.6% for pure artemisinin. To determine which phytochemical in the EO caused the decreased P_{app} and TEER we tested three major components of *A. annua* EO, camphor (at 3 concentrations), eucalyptol, and caryophyllene in Caco-2 permeability assays. As shown in Table 4.5 below, none of the tested compounds altered the TEER or P_{app} of artemisinin. Interestingly, when digestate made with EO combined with artemisinin and DLAG plant material was used in Caco-2 assays, there was no observed decrease in P_{app} however, the TEER still dropped sharply suggesting that the *A. annua* plant matrix may

counteract the decreased intestinal permeability, but not the reduced tight junction integrity caused by EO, as indicated by the TEER loss.

Bile is an emulsifier that is an important component of lipid digestion and facilitates the formation of micelles from ingested lipids (Maldonado-Valderrama et al. 2011). To determine the role of digestion and bile in the digestate liquid on altering the P_{app} and TEER, we performed Caco-2 permeability assays with undigested EO and with bile extract added to EO without performing the simulated digestion process. Without digestion, neither of the tested EO's altered the artemisinin P_{app}. On the other hand, addition of bile extract to both EO sources resulted in a drop in the TEER after the experiment (Table 4.6).

Table 4.5. Effects of undigested pure compounds found in *A. annua* essential oil on artemisinin Papp and TEER

EO Phytochemical	P_{app} (x10-^{5})(cm s^{-1}) (±SD)	Loss of TEER?
Pure AN	5.45 ± 0.86	-
Camphor 1:1	5.84 ± 0.92	-
Camphor 1:2	6.20 ± 1.22	-
Camphor 1:10	4.74 ± 0.87	-
Eucalyptol	5.72 ± 0.34	-
Caryophyllene	6.13 ± 0.56	-

AN, artemisinin; EO, essential oil; TEER, transepithelial electrical resistance

Table 4.6. Effects of undigested essential oils ± bile on artemisinin P_{app} and TEER

Compound	Bile added?	P_{app} (x10^{-5})(cm s^{-1}) (±SD)	Loss of TEER?
Pure AN	-	**4.82 ± 0.60**	-
Pure AN	+	**4.12 ± 0.38**	-
AN+US EO (4%)	-	**4.85 ± 0.35**	-
AN+Chinese EO (4%)	-	**4.17 ± 0.10**	-
AN+US EO (4%)	+	**4.81 ± 0.51**	+
AN+Chinese EO (4%)	+	**5.67 ± 0.64**	+

AN, artemisinin; EO, essential oil; TEER, transepithelial electrical resistance; US, United States.

Taken together, these results suggested that EO from *A. annua* modulated the tight junctions of enterocytes, but only after digestion. Surprisingly, this decrease in tight junction integrity correlated with a decrease in P_{app}. It is possible that in EO digestates, some of the free artemisinin becomes sequestered in micelles formed by the bile emulsifier interacting with the EO. In this scenario, the free fraction of artemisinin is decreased reducing the rate of diffusion and thus the P_{app}. Although the tight junctions between enterocytes are more tenuous, there is a weaker driving force for the diffusion of lipophilic artemisinin across the membrane. A similar phenomenon was observed in several cases where surfactants were added to increase solubility of highly lipophilic drugs (Dahan and Miller 2012). Additionally, it is known that lipophilic drugs incorporate into lipid micelles in digestive fluid, especially when oil is present (Pullakhandam and Failla 2007). The drugs in solution are in an equilibrium state between micellarized and free drug states. While the free drug is capable of permeating the intestinal membrane, drug in the micellarized state is not and thus the overall permeability decreases with increased micelle concentration. This was demonstrated by Yano et al. who showed that the P_{app} of six different lipophilic drugs decreased with increasing micelle concentration in a Caco-2 system (Yano et al. 2010).

4.6 Conclusions

Understanding the mechanisms by which DLAS increases the bioavailability of artemisinin *in vivo* is important for the development of DLAS as a malaria therapeutic. While we previously showed that DLAS increased the solubility of artemisinin, solubility is only one factor that governs the overall bioavailability of a drug. Here, using Caco-2 cell permeability assays we showed that DLAS digestate also increased intestinal permeability of artemisinin when compared to pure drug.

This increase in permeability helps to but does not fully explain the >40 times increase in artemisinin bioavailability afforded by DLAS in murine studies.

There are several other known factors that drive bioavailability of artemisinin. For example, we previously showed artemisinin was significantly more soluble when delivered as DLAS (Desrosiers and Weathers 2016). Drug bioavailability is also largely dictated by the liver. Recently it was shown that chrysosplenetin, a polymethoxylated flavonoid found in *A. annua*, delivered orally to rats in conjunction with artemisinin increased the oral bioavailability of artemisinin and inhibited several CYP450 enzymes in the rat liver (Wei et al. 2015). It is therefore possible that phytochemicals found in *A. annua* inhibit the first pass metabolism of artemisinin by liver CYP450 enzymes resulting in higher levels of the drug in the blood. The liver is our next study target for explaining the enhanced bioavailability of artemisinin from DLA.

This study also determined that intestinal CYP3A4 is an unlikely contributor to the bioavailability of artemisinin and showed that flavonoids and other phytochemicals of varying structure are not sufficient to alter artemisinin intestinal permeability. Although there were some deleterious effects of digested *A. annua* EO on tight junctions and a drop in artemisinin P_{app}, the plant matrix appeared to counteract the P_{app} decline. While the observed increases in solubility and intestinal permeability help explain the increased bioavailability of artemisinin from DLAS, there may still be other mechanisms at play. These mechanisms should be investigated to better understand the factors that govern artemisinin oral bioavailability when delivered via DLA.

4.7 Acknowledgements

The authors thank Dr. Melissa Towler, Dr. Andy Butler, Daryl Johnson, Dr. Sarah Hernandez, and Prof. Jill Rulfs of Worcester Polytechnic Institute for technical assistance. We are

also grateful for Award Number NIH-R15AT008277-01 from the National Center for Complementary and Integrative Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Complementary and Integrative Health or the National Institutes of Health.

Chapter 5 Dried Leaf *Artemisia Annua* Improves Bioavailability of Artemisinin via Cytochrome P450 Inhibition and Enhances Artemisinin Efficacy Downstream

Published as:

Desrosiers M., Mittelman A., Weathers P.J. 2020. Dried leaf *Artemisia annua* improves bioavailability of artemisinin via cytochrome P450 inhibition and enhances artemisinin efficacy downstream. Biomolecules. 10: 254. doi: 10.3390/biom10020254.

5.1 Abstract

Artemisia annua L. and artemisinin, have been used for millennia to treat malaria. We used human liver microsomes (HLM) and rats to compare hepatic metabolism, tissue distribution, and inflammation attenuation by dried leaves of *A. annua* (DLA) and pure artemisinin. For HLM assays, extracts, teas, and phytochemicals from DLA were tested and IC_{50} values for CYP2B6 and CYP3A4 were measured. For tissue distribution studies, artemisinin or DLA was orally delivered to rats, tissues harvested at 1 hour, and blood, urine and feces over 8 hours; all were analyzed for artemisinin and deoxyartemisinin by GC-MS. For inflammation, rats received an intraperitoneal injection of water or lipopolysaccharide (LPS) and 70 mg/kg oral artemisinin as pure drug or DLA. Serum was collected over 8 hours and analyzed by ELISA for TNF-α, IL-6, and IL-10. DLA-delivered artemisinin distributed to tissues in higher concentrations *in vivo*, but elimination remained mostly unchanged. This seemed to be due to inhibition of first-pass metabolism by DLA phytochemicals, as demonstrated by HLM assays of DLA extracts, teas and phytochemicals. DLA was more effective than artemisinin in males at attenuating proinflammatory cytokine production; the data were less conclusive in females. These results suggest that the oral consumption of artemisinin as DLA enhances the bioavailability and anti-inflammatory potency of artemisinin.

Key Words: *Artemisia annua*; artemisinin; cytochrome P450 inhibition; tissue distribution; inflammation

Abbreviations: ACT, artemisinin combination therapy; ADME absorption, distribution, metabolism, and excretion; AN, artemisinin; AUC, area under the curve; CYP, cytochrome P450; DLA, dried leaves of *A. annua*; ELISA, enzyme-linked immunosorbent assay; GC-MS, gas

chromatography mass spectrometry; HLMs, human liver microsomes; IACUC, Institutional Animal Care and Use Committee; IL-6, interleukin 6; IL-10, interleukin 10; LPS, lipopolysaccharide; SAM; high artemisinin producing cultivar of *A.* annua; SEN, cultivar of *A. afra* from Senegal; TLC, thin-layer chromatography; TNF-α, tumor necrosis factor α.

5.2 Introduction

In 2017, there were 219 million cases and 435,000 deaths from malaria according to the World Health Organization (WHO 2019b). The frontline treatment is artemisinin combination therapy (ACT) that relies on semisynthetic derivatives of the drug artemisinin plus a codrug to eliminate patient parasites (WHO 2015). While effective, ACTs are often not financially feasible to those most in need in Africa, where 93% of malaria deaths occur (WHO 2019b). Thus, there is great need for a more cost-effective alternative to ACTs that can treat malaria among the poorest of the world.

The Chinese herb, *Artemisia annua* L., naturally produces and stores artemisinin (Figure 5.1A) in the glandular trichomes on its leaves, stems, and flowers (Olsson et al. 2009). Used for

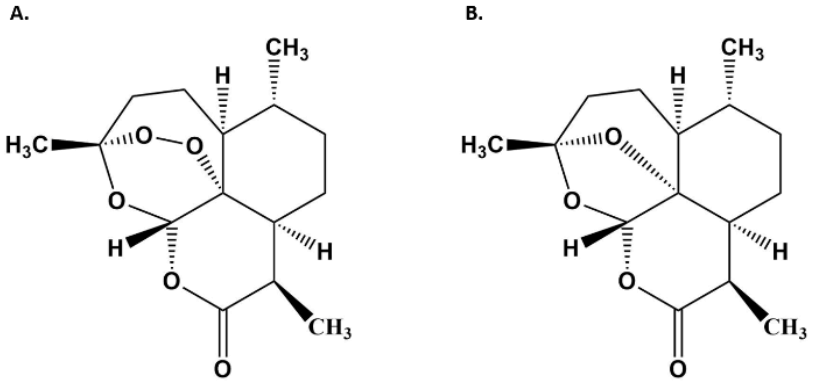

Figure 5.1. Chemical structures of artemisinin (**A**) and deoxyartemisinin (**B**).

centuries as a tea infusion to treat a variety of illnesses including malaria (Hsu 2006), recent

clinical data suggested that carefully prepared tea infusions made from the plant were as effective or better at eliminating malaria parasites as ACTs (Munyangi et al. 2019; ICIPE 2005). Furthermore, artemisinin delivered as a tea infusion or as dried leaves of *A. annua* (DLA) is significantly more bioavailable, crosses the intestine more efficiently, and has about four-fold greater solubility than pure artemisinin (Desrosiers and Weathers 2016, 2018; Weathers et al. 2011). DLA also does not require extraction and purification, making it an affordable alternative to ACTs.

Artemisinin is metabolized by liver cytochrome P450s (CYPs), mainly CYP2B6 with a minor contribution from CYP3A4, to yield deoxyartemisinin (Figure 5.1B), crystal 7, deoxydihydroartemisinin, and 9,10 dihydrodeoxyartemisinin (Svensson and Ashton 1999; Lee and Hufford 1990), all of which are therapeutically inactive. While the enhanced bioavailability of artemisinin afforded by DLA is partially attributed to increased solubility and intestinal transport (Desrosiers and Weathers 2016, 2018), we posit that inhibition of CYP2B6 and/or CYP3A4 by DLA phytochemicals also plays a role in significantly increasing artemisinin bioavailability because more artemisinin would pass through the liver unmetabolized. In turn, that should also increase artemisinin distribution in tissues and organs with greater impact on biological responses, e.g., effects on inflammation.

Despite significant bioavailability and pharmacokinetic differences between pure artemisinin and DLA-delivered artemisinin (Weathers, Elfawal, et al. 2014; Weathers et al. 2011; Räth et al. 2004), to our knowledge, no studies have been done to determine absorption, distribution, metabolism and excretion (ADME) of the drug *in vivo* when delivered as DLA. There is only one report that determined tissue distribution of orally delivered pure artemisinin; it used a semiquantitative thin-layer chromatography (TLC) densitometric method to quantify artemisinin

(Xinyi et al. 1985). We are also not aware of any studies to determine *in vivo* gender differences in ADME of DLA-delivered artemisinin (Ashton 1999). One study did show that the area under the curve (AUC) for pure artemisinin was two-fold higher in female rats compared to males after intraperitoneal administration. That same study showed artemisinin disappearance was 3.9-fold higher in liver microsomes from male vs. female livers, suggesting that artemisinin metabolism is gender dependent (Ashton 1999).

Artemisinin and other *A. annua* phytochemicals also have anti-inflammatory properties and are being investigated as therapeutics for several inflammatory diseases (Wang et al. 2017; Kim et al. 2015; Rathee et al. 2009; de Cássia da Silveira e Sá, Andrade, and de Sousa 2013). Artemisinin attenuates inflammation by blocking NF-κB and MAPK signaling pathways that lead to inflammatory cytokine production (Wang et al. 2017). *A. annua* also produces other anti-inflammatory phytochemicals, including flavonoids and monoterpenes. The flavonoids, casticin and chrysosplenol-D, reduced *in vitro* and *in vivo* inflammation (Li et al. 2015), and the monoterpene, 1,8-cineol (eucalyptol) inhibited the production of six inflammatory cytokines *in vitro* (Juergens et al. 2004). *A. annua* also produces rosmarinic and chlorogenic acids, each of which has anti-inflammatory activity (Melillo de Magalhães et al. 2012). With this rich mixture of anti-inflammatory phytochemicals, it was expected that *A. annua* could provide an alternative cost-effective therapeutic for inflammatory diseases. However, few *in vivo* studies have investigated whole plant *A. annua* treatment for inflammation.

The present study shows that artemisinin consumed orally as DLA is more bioavailable from inhibition of the important artemisinin-metabolizing enzymes, CYP2B6 and CYP3A4, by compounds produced in the plant. Downstream of this first pass metabolism, DLA-delivered artemisinin is greater in tissues and organs and inflammation is decreased compared to pure

artemisinin. We also show significant differences in gender with female rats absorbing higher amounts of artemisinin in several tissues regardless of delivery as DLA or pure drug. Together, these results better explain how *per os* DLA-delivered artemisinin bioavailability is greater than that from *per os* pure artemisinin.

5.3 Materials and Methods

5.3.1 Plant Material

We used dried leaves of the SAM cultivar of *Artemisia annua* L. (voucher MASS 317314) and *Artemisia afra* (SEN) (voucher LG0019529). SAM and SEN contained artemisinin between 1.0–1.2% and 0.02%, and flavonoid contents of 0.93–0.63% and 0.71% (w/w), respectively. *A. annua* was grown and processed as detailed in Weathers, Jordan, et al. (2014). *A. afra* was grown in Senegal and dried leaves were provided as a gift from Guy Mergaei, Université de Liège, Belgium. Artemisinin content was determined via GC-MS as detailed in Weathers and Towler (2012). Total flavonoid content was measured by the $AlCl_3$ method of Arvouet-Grand et al. (1994). The *A. annua* used remains consistent in its phytochemical content over time as detailed in Weathers and Towler (2014) and Gruessner et al. (2019). The *A. afra* used was from one batch of dried leaves for all experiments and was thus phytochemically consistent. Extensive phytochemical profiles of the *A. annua* and *A. afra* dried leaf materials used in this study are detailed in Weathers and Towler (2014) and Munyangi et al. (2018), respectively.

5.3.2 Chemicals and Reagents

Chemicals and reagents were from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated. Artemisinin was from Cayman Chemical (Ann Arbor, MI, USA); deoxyartemisinin from

Toronto Research Chemicals (North York, ON, Canada). Lipopolysaccharide was *Escherichia coli* serotype O111:B4 and dissolved in sterile water at 1 mg/mL before storage at −20°C. LEGEND MAX™ Enzyme-linked immunosorbent assay (ELISA) kits for rat TNF-α and IL-6 were from BioLegend (San Diego, CA, USA). ELISA kits for rat IL-10 were from Thermo Fisher Scientific (Waltham, MA, USA). P450-Glo CYP2B6 and CYP3A4 kits were from Promega (Madison, WI, USA). Human liver microsomes (HLMs) from a 200-donor pool of male and female donors were from Sekisui XenoTech (Kansas City, KS, USA).

5.3.3 *In Vitro* P450 Inhibition

Promega P450-Glo kits for CYP2B6 or CYP3A4 were used with pooled HLMs (200 subjects) to determine an IC_{50} for each extract, tea, or phytochemical. Test concentrations ranged from 0.01–600 µM for each extract, tea, or phytochemical. *A. annua* extracts and teas were classified by their artemisinin content. For example, a 600 µM DLA extract is an extract with artemisinin at a 600 µM concentration. *A. afra* extracts and teas were made with a leaf dry weight equivalent to that used for *A. annua* extracts and teas such that a 600 µM *A. afra* extract was from the same leaf dry weight as a 600 µM extract of *A. annua*. Assays followed manufacturer instructions and plates were read on a luminescence plate reader. Methanol, acetonitrile, or acetone were cosolvent vehicles at ≤1% (v/v); a vehicle control was used for each comparison. IC_{50} values were determined through nonlinear regression using GraphPad Prism 7 (San Diego, CA, USA). All experiments had technical duplicates. Extracts, teas, and phytochemicals with an IC_{50} below 50 µM were repeated in biological triplicate.

5.3.4 Animals

Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) between 200–300 g (age 5–8 weeks for males and 6–9 weeks for females) were used for all animal studies and housed at the Worcester Polytechnic Institute Vivarium on a 14 hour light, 10 hour dark cycle with ad libitum food and water until the day before an experiment. Food was withheld 14 hours before experiments as animal chow has been shown to alter artemisinin bioavailability (Weathers, Elfawal, et al. 2014). All animal work was approved by the Worcester Polytechnic Institute Institutional Animal Care and Use Committee (IACUC Protocol 15–69 and 18–105) and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

5.3.5 *In Vivo* Artemisinin Tissue Distribution

Artemisinin was delivered to rats *po* as either a slurry of powdered dried *A. annua* in water (DLA) or pure artemisinin in water with 12% DMSO. Artemisinin is essentially insoluble in water, so DMSO was used to enhance artemisinin solubility and achieve more consistent, replicable data. DLA-treated animals received 85 mg/kg dose of artemisinin in DLA slurry, the highest achievable dose that could be delivered while still conforming to animal welfare regulations. Initially we aimed to use the same dose of artemisinin in DLA and pure artemisinin-dosed animals, but preliminary experiments with a DLA-dosed rat and an artemisinin-dosed rat at 85 mg/kg showed no artemisinin was detectable in artemisinin treated animals. This was not surprising because pure artemisinin has very low oral bioavailability (Titulaer et al. 1990); a previous study had to dose at 900 mg/kg of artemisinin to yield detectable artemisinin levels in the tissues (Xinyi et al. 1985). Consequently, we increased the artemisinin dose in the control animals to 500 mg/kg and normalized amounts recovered in tissues, serum, urine, and feces to the delivered dose. After 1

hour, animals were euthanized by CO_2 inhalation; tissues and blood were harvested immediately after death confirmation. For longer experiments, rats housed in metabolic cages for the duration of the 8-hour time course had urine, feces, and 0.4 mL blood samples collected 1, 2, 4 and 8 hours after gavage to track artemisinin and one of its liver metabolites, deoxyartemisinin. Tissues were collected and flash frozen on liquid nitrogen 8 hours after gavage. Blood was collected in heparin-free serum collection tubes, allowed to clot 20 min before centrifugation at 1,500 g for 10 min, and then stored at −80°C until extraction.

5.3.6 Tissue Extraction

Artemisinin and deoxyartemisinin were extracted from harvested tissues including heart, lungs, liver, spleen, kidneys, muscle, testes/ovaries, and brain. Briefly, a tissue sample was weighed, minced and then transferred to a Potter-Elvehjem tissue homogenizer. Water was added to yield a thick slurry and then homogenized. An aliquot was transferred into a new test tube and dichloromethane added at a 1:1 volume ratio. Samples were sonicated in a sonicating water bath for 30 min at ambient temperature and the organic layer decanted. The tissue residue was extracted twice again. Extracts were pooled, dried under nitrogen gas at ambient temperature, and stored at −20°C until analysis.

5.3.7 Serum and Urine Extractions

Serum and urine were thawed before extraction, aliquot transferred into a new tube, and combined with dichloromethane at a 2:1 ratio. Each sample was vortexed, placed in the sonicating water bath for 30 min, the dichloromethane layer decanted, and extraction twice repeated. Extracts were pooled, dried under nitrogen, and stored at −20°C until analysis.

5.3.8 Fecal Extractions

Fecal samples collected at 1, 2, 4 and 8 hours were stored at −80℃ until extraction. Before extraction, samples were vacuum dried for 72 hours, ground with a mortar and pestle, wrapped in a tared Kimwipe, and weighed. Four milliliters dichloromethane was added to each sample in a test tube and then sonicated for 30 min and dried as described. Dried extracts were resuspended in 2 mL pentane and filtered through a 0.45 µm PVDF syringe filter into a new test tube to remove particulates. After a second extraction, pooled extracts were filtered and stored at −20℃ until analysis.

5.3.9 Preparation of DLA Extracts and Teas

Briefly, 1 g DLA (*A. annua* or *A. afra*) was twice extracted with 10 mL methanol, sonicated and filtered as previously described. The process was repeated with the same plant material and the extract was dried under a mild stream of nitrogen before artemisinin content was analyzed by GC-MS. Tea at 5 g/L was prepared as described by Munyangi et al. (2018) (Munyangi et al. 2018).

5.3.10 Artemisinin and Deoxyartemisinin Analyses

Artemisinin and deoxyartemisinin were analyzed by gas chromatography mass spectrometry (GC-MS) as detailed previously (Towler and Weathers 2015). Quantitation was based on standard curves from authenticated standards.

5.3.11 Attenuation of Inflammation I*n Vivo*

A rat model of systemic inflammation was modified from Bison et al. 2009 (Bison et al. 2009) with four test groups: Injection control, LPS control, LPS+AN, and LPS+DLA. Injection

controls received a single intraperitoneal injection of sterile water as vehicle control for LPS. LPS controls received an intraperitoneal injection of LPS in water at 2.5 mg/kg to induce a systemic inflammatory response. LPS+AN, and LPS+DLA animals received a 2.5 mg/kg intraperitoneal LPS dose, followed immediately by oral gavage of pure artemisinin or DLA at a 70 mg/kg artemisinin dose, respectively; 70 mg/kg was the maximum artemisinin dose deliverable as DLA. Pure artemisinin was delivered with 12% DMSO to enhance solubility. Blood samples (0.4 mL) were taken via tail snip at 0 (taken immediately before injection), 1, 2, 4 and 8 hours, clotted for 20 min at room temperature, and then centrifuged at 1,500 × G for 10 min to isolate serum. Serum was aliquoted and stored at −80°C before ELISA analysis performed according to manufacturer.

5.3.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7. IC_{50} values were generated for CYP inhibition studies using nonlinear regression analysis in GraphPad Prism 7. Experiments were done in technical duplicate; three biological replicates were performed. IC_{50} values were compared to pure artemisinin by ANOVA. Each *in vivo* tissue distribution experiment compared the averages of three rats per group as permitted by the WPI IACUC. Students T-tests, used where appropriate, compared pure artemisinin to DLA dosed rats to determine statistical significance ($p < 0.05$). Each *in vivo* artemisinin elimination study compared the average of eight rats per group, as permitted by the WPI IACUC, and used students T-tests to compare DLA-dosed rats to rats dosed with pure artemisinin. Each *in vivo* inflammation experiment compared the averages of 5–6 rats per group except for the injection control in which there were three rats as was permitted by the WPI IACUC. ANOVA was used where appropriate to compare pure artemisinin- and DLA-dosed rats to LPS controls in inflammation studies.

5.4 Results

5.4.1 Inhibition of CYP2B6 and CYP3A4 by DLA

Extracts (DLA_{me} and A. $afra_{me}$) and teas (DLA_{tea} and A. $afra_{tea}$) of both *A. annua* and *A. afra* inhibited both CYP2B6 and CYP3A4 activity (Table 5.1). All extracts and teas had a lower IC_{50} than that for artemisinin alone, indicating that compounds other than artemisinin from DLA inhibited CYP2B6 and CYP3A4 activity. In addition to extracts and teas, some individually tested phytochemicals had inhibitory activity, but few were as strong as the whole extract (Table 5.1)

Table 5.1. Inhibition of CYP2B6 and CYP3A4 by DLA extracts, teas and phytochemicals.

Phytochemical or Extract	CYP2B6 IC_{50} (µM)	CYP3A4 IC_{50} (µM)
DLA_{me}[a]	6.07**	4.93[#]
DLA_{tea}[a]	2.31**	5.67[#]
A. $afra_{me}$[b]	7.89**	5.18[#]
A. $afra_{tea}$[b]	11.27**	5.59[#]
Arteannuin B	9.36**	12.33[#]
Quercetin	19.86*	4.59[#]
Artemisinin	27.75	>600
Chrysosplenol-D	52.06	22.96[#]
Chrysosplenetin	74.67	21.68[#]
Kaempferol	>600	33.84[#]
Deoxyartemisinin	110.86	>600
Camphor (+)	119.01	>600
Artemisinic Acid	122.83	239.5[#]
Camphor (-)	141.93	>600
Scopoletin	>600	410.19[#]
Chlorogenic Acid	>600	>600

* IC_{50} significantly lower than artemisinin; $p \leq 0.05$; ** $p \leq 0.0001$; $n = 3$.
[#] IC_{50} lower than artemisinin but no statistical difference determined because the artemisinin IC_{50} is higher than the highest tested concentration (600 µM).
[a] Concentrations of DLA are equivalent to artemisinin concentration in extract or tea.
[b] *A. afra* contains negligible artemisinin so extracts and teas were made with equivalent plant biomass as DLA. Thus, IC_{50} values for *A. afra* are what artemisinin concentration would be in DLA for comparison.

5.4.2 Artemisinin Delivered as DLA Increased Bioavailability in Some Tissues

To determine how DLA alters distribution of artemisinin to tissues, we orally gavaged rats with DLA or pure artemisinin and measured artemisinin content. Compared to artemisinin-dosed males artemisinin in DLA-dosed rats at 1 hour had significantly more artemisinin in the heart, muscle and serum (Figure 5.2A), while DLA-dosed females had significantly more artemisinin in the heart, lungs, liver, muscle, brain tissue, and serum (Figure 5.2B). In males, artemisinin accumulated mostly in serum followed by lungs, heart, liver, brain, spleen, muscle, and kidneys (Figure 5.2A). DLA-delivered artemisinin has greater bioavailability (Desrosiers and Weathers 2016, 2018; Weathers, Elfawal, et al. 2014), so we expected more artemisinin in DLA-treated tissues; this was confirmed, as shown in Figure 5.2.

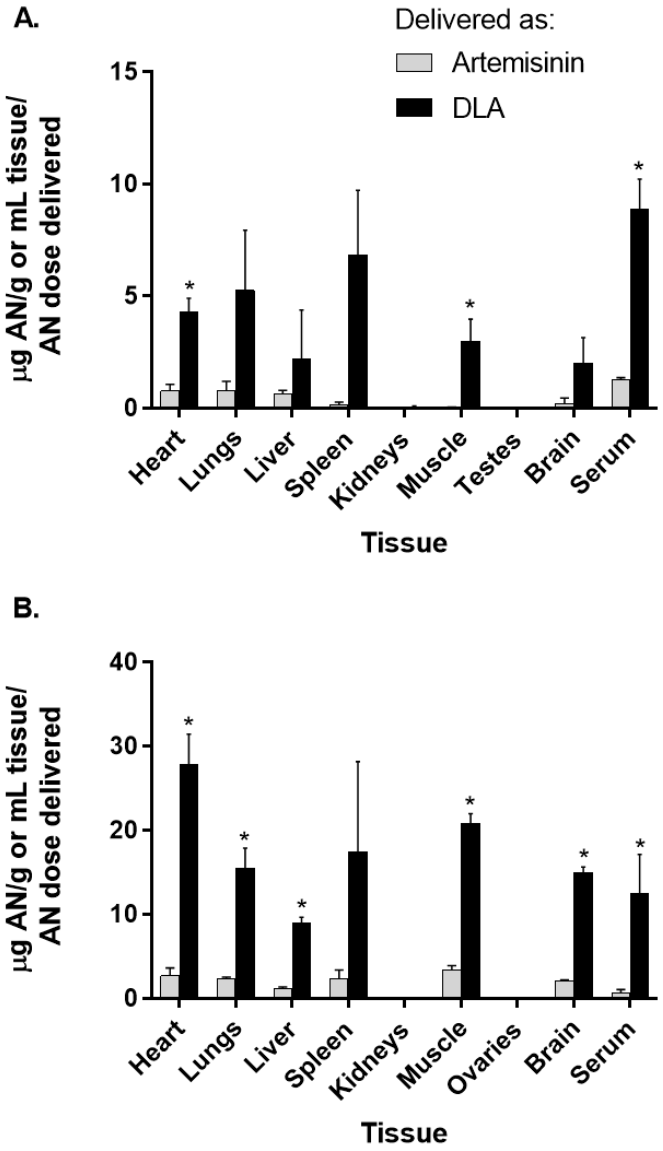

Figure 5.2. Artemisinin distribution in tissues and serum of male (**A**) and female (**B**) rats after oral delivery of artemisinin as dried leaf *A. annua* (DLA) or pure artemisinin. $n = 3$, *; $p \leq 0.05$; error bars = SEM.

While artemisinin distributed well to most tissues, it cleared by 8 hours. Neither artemisinin nor deoxyartemisinin was detectable in any tissues or serum 8 hours post-gavage. Serum was also collected at 2- and 4-hours post-gavage; however, neither artemisinin nor deoxyartemisinin was detectable in either gender. In male rats, heart, liver, and muscle had a significant difference in deoxyartemisinin distribution between DLA and pure artemisinin-treatments (Figure 5.3A). In

female rats, however, there was a significant difference in deoxyartemisinin distribution to heart, lungs, spleen, muscle, brain, and serum (Figure 5.3B). DLA contains a small amount of deoxyartemisinin (7.5 mg/kg dose), so deoxyartemisinin detected in animals treated with DLA is likely not all from liver metabolism. Animals given pure artemisinin, however, did not receive any deoxyartemisinin, so any deoxyartemisinin detected in their tissues was due solely to artemisinin liver metabolism.

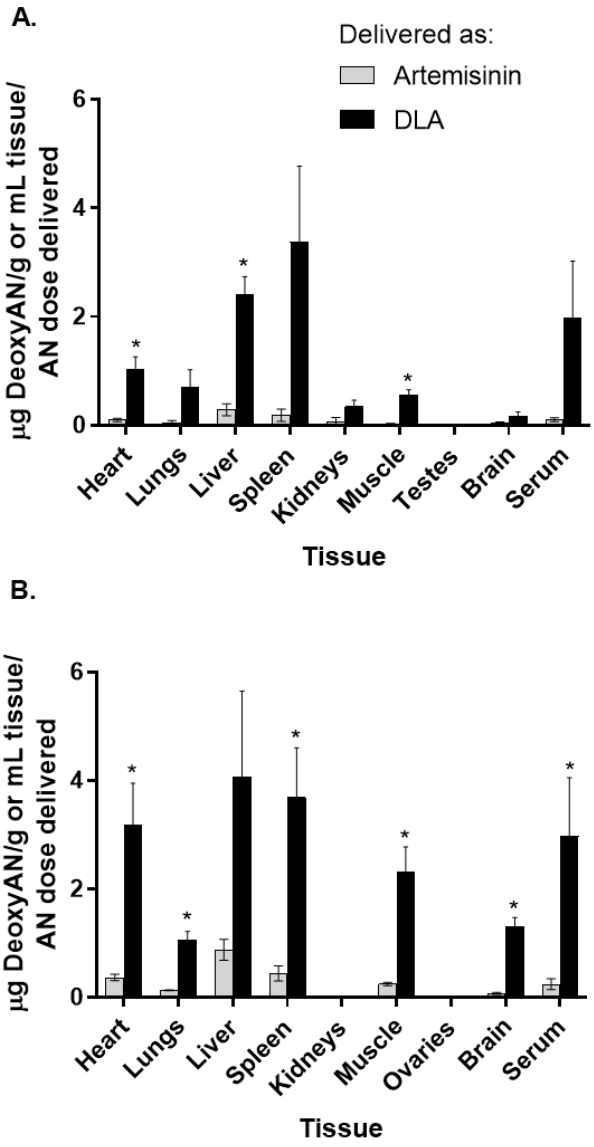

Figure 5.3. Deoxyartemisinin (deoxyAN) distribution in tissues and serum of male (**A**) and female (**B**) rats after oral delivery of artemisinin as dried leaf *A. annua* (DLA) or pure artemisinin. $n = 3$, *; $p \leq 0.05$; error bars = SEM.

Distribution of artemisinin was significantly different in several tissues between males and females regardless of delivery mode (Table 5.2). At 1 hour, females gavaged with pure artemisinin had more artemisinin in lungs, liver, muscle, and brain tissue than males (Table 5.2). Similarly, females gavaged with DLA had more artemisinin in the heart, lungs, liver, muscle, and brain tissue at 1 hour compared to males (Table 5.2). In almost all tissues, although not always statistically

significant, artemisinin and deoxyartemisinin were more abundant in females than males at 1 hour. These data suggest females absorb and distribute artemisinin throughout the body more efficiently than males. There was, however, no difference in serum artemisinin levels at 1 hour between female and male rats given either pure artemisinin or DLA (Table 5.2), suggesting that tissue distribution of artemisinin—but not absorption into the blood—is gender dependent.

Table 5.2. Gender-specific differences in artemisinin and deoxyartemisinin distribution to tissues in artemisinin- and DLA-treated rats.

	Artemisinin Treated				DLA Treated			
	Artemisinin (µg AN/g tissue/g AN delivered ± SD)		Deoxyartemisinin (µg DeoxyAN/g tissue/g AN delivered ± SD)		Artemisinin (µg AN/g tissue/g AN delivered ± SD)		Deoxyartemisinin (µg DeoxyAN/g tissue/g AN delivered ± SD)	
Tissue	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Heart	0.77 ± 0.49	2.70 ± 1.87	0.097 ± 0.050	0.37 ± 0.12*	4.30 ± 1.06	27.81 ± 7.25*	1.02 ± 0.42	3.18 ± 1.54
Lungs	0.79 ± 0.69	2.37 ± 0.26*	0.055 ± 0.054	0.13 ± 0.018*	5.26 ± 4.64	15.56 ± 4.63*	0.70 ± 0.65	1.06 ± 0.31
Liver	0.62 ± 0.30	1.23 ± 0.26*	0.29 ± 0.19	0.88 ± 0.39	2.19 ± 3.79	8.96 ± 1.40*	2.42 ± 0.55	4.06 ± 3.18
Spleen	0.14 ± 0.24	2.37 ± 2.05	0.19 ± 0.20	0.44 ± 0.28	6.82 ± 5.03	17.49 ± 21.37	3.38 ± 2.79	3.69 ± 1.83
Kidneys	0.00 ± 0.00	0.00 ± 0.00	0.074 ± 0.12	0.00 ± 0.00	0.048 ± 0.096	0.00 ± 0.00	0.35 ± 0.23	0.00 ± 0.00*
Muscle	0.019 ± 0.034	3.41 ± 0.85*	0.025 ± 0.018	0.25 ± 0.049*	2.99 ± 1.68	20.84 ± 2.26*	0.57 ± 0.15	2.31 ± 0.93*
Testes/Ovaries	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Brain	0.22 ± 0.38	2.05 ± 0.35*	0.038 ± 0.043	0.075 ± 0.034	2.02 ± 1.96	15.00 ± 1.31*	0.18 ± 0.12	1.30 ± 0.36*
Serum	1.29 ± 0.11	0.67 ± 0.75	0.10 ± 0.061	0.24 ± 0.20	8.90 ± 2.63	12.49 ± 9.34	1.97 ± 2.10	2.97 ± 2.17

AN, artemisinin; deoxyAN, deoxyartemisinin; SD, standard deviation; $n = 3$; *, $p \leq 0.05$ when compared to opposite gender.

5.4.3 Artemisinin Elimination Through the Urine is Gender-Dependent

To compare artemisinin elimination from DLA vs. pure artemisinin, urine collected over 8 hours from female rats showed significantly more artemisinin elimination than males (Figure 5.4C). Because there is some deoxyartemisinin naturally present in DLA, those animals received 7.5 mg/kg of deoxyartemisinin in the DLA gavage. Animals treated with pure artemisinin did not

receive any deoxyartemisinin. When comparing genders, females had more artemisinin in urine than males when given DLA. Females also had more deoxyartemisinin in urine than males, regardless of how artemisinin was delivered (Figure 5.5).

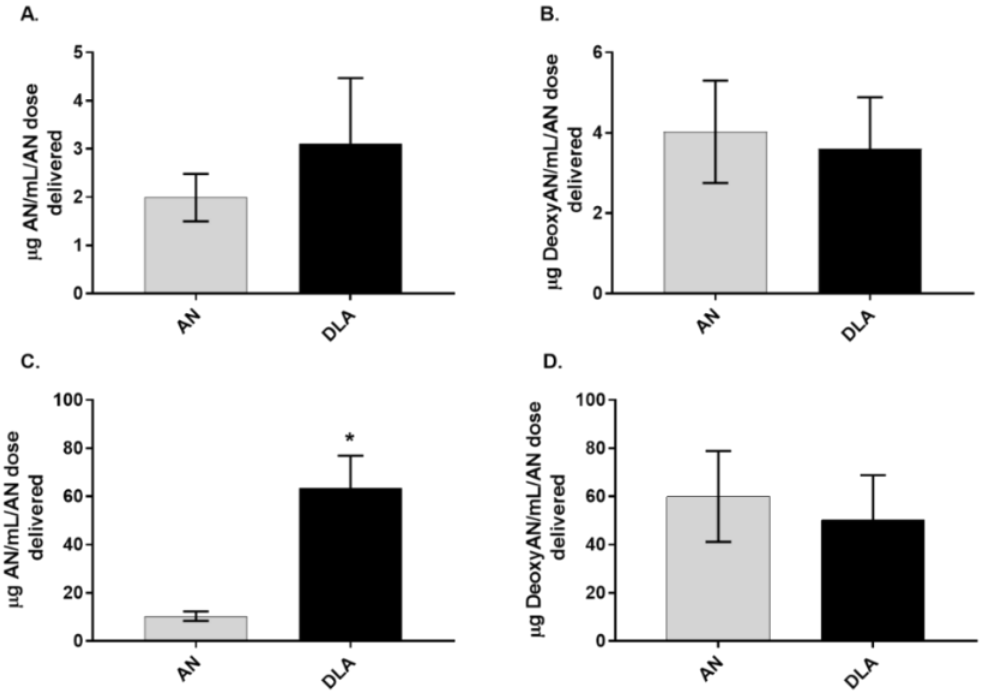

Figure 5.4. Artemisinin (AN) and deoxyartemisinin (DeoxyAN) accumulation in urine of male (**A**, **B**) and female (**C**, **D**) rats after oral delivery of artemisinin as dried leaf *A. annua* (DLA) or pure artemisinin. $n = 8$, *; $p \leq 0.05$; error bars = SEM.

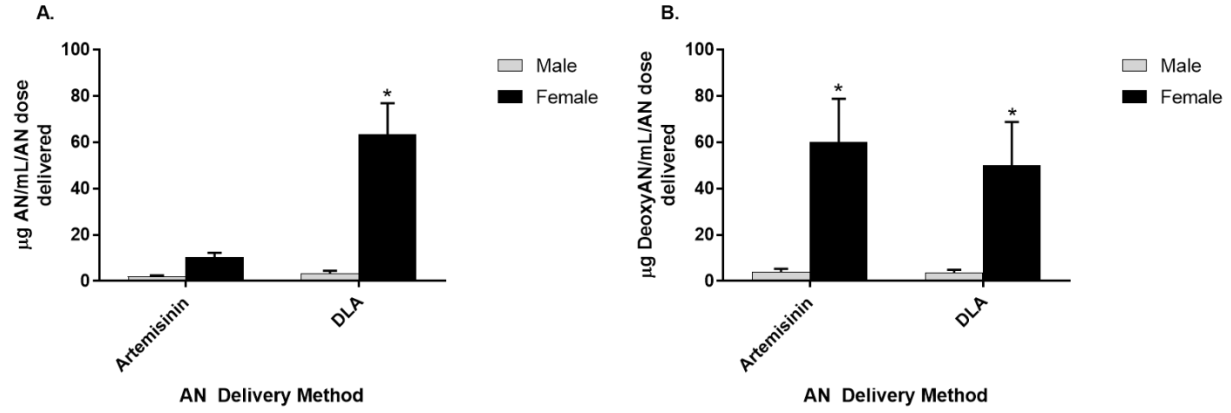

Figure 5.5. Artemisinin (**A**) and deoxyartemisinin (**B**) found in the urine of male and female rats orally gavaged with either pure artemisinin or dried leaves of *A. annua* (DLA). $n = 8$; * $p \leq 0.05$; error bars = SEM.

5.4.4 Artemisinin Elimination Through Feces

Artemisinin and deoxyartemisinin was measured in the feces of rats up to 8 hours after oral gavage with DLA or pure artemisinin. As expected, most artemisinin and deoxyartemisinin was eliminated through feces, and was detected at later time points. Although total artemisinin and deoxyartemisinin was summed from the feces over 8 hours, there were no significant differences in either artemisinin or deoxyartemisinin between DLA and pure artemisinin dosed animals (Appendix C, Figure S1). We also measured fecal microbiome samples and found neither artemisinin nor DLA treatment affected the microbial population (Appendix C, Table S1).

5.4.5 Attenuation of Inflammation by Artemisinin and DLA

As expected, both male and female LPS controls responded to LPS challenge with a systemic inflammatory response. Serum TNF-α levels spiked quickly and peaked at about 4,600 pg/mL for males (Figure 5.6A) and 5,300 pg/mL for females (Figure 5.6B) at 1 hour after LPS injection. The spike in TNF-α was followed by a spike in serum IL-6 at 2 hours after LPS injection with males peaking at about 15,400 pg/mL (Figure 5.6C) and females peaking at about 25,100 pg/mL (Figure 5.6D). Interestingly, only DLA was effective at reducing serum TNF-α levels in males and only at the 1-hour time point, while both DLA and pure artemisinin significantly reduced serum TNF-α levels at 1 and 2 hours post-injection in females (Figure 5.6). Although artemisinin and DLA were effective at reducing TNF-α production in females, neither treatment had any effect on IL-6 serum levels in females. In males, however, DLA reduced serum IL-6 levels at 2 and 4 hours post-injection. Overall, DLA was more effective at reducing inflammatory cytokine

production in both genders, as expected based on the diversity of anti-inflammatory phytochemicals in DLA.

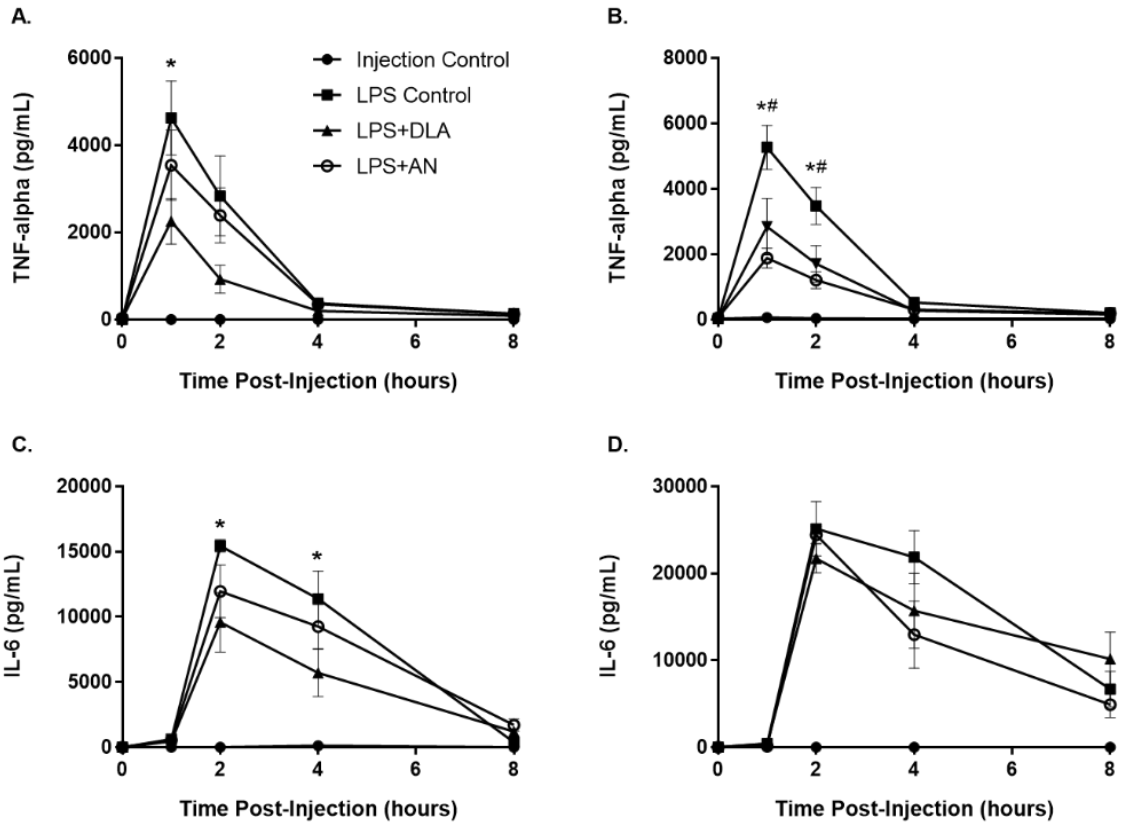

Figure 5.6. Production of proinflammatory cytokines TNF-α and IL-6 in male (**A**, **C**) and female (**B**, **D**) rats after LPS challenge and treatment with either pure artemisinin (AN) or DLA (equal artemisinin doses). n = 5–6 for experimental conditions, n = 3 for injection control; error bars = SEM; *, $p \leq 0.05$ when comparing LPS+DLA to LPS Control; #, $p \leq 0.05$ when comparing LPS+AN to LPS Control.

We also measured the effects of DLA and pure artemisinin treatment on the anti-inflammatory cytokine IL-10. However, there were no significant differences in serum IL-10 levels, regardless of treatment (Appendix C, Figure S2).

5.5 Discussion

A. annua produces many phytochemicals including kaempferol, chrysosplenetin, and quercetin (Weathers and Towler 2014; Ferreira et al. 2010) that have already been shown to inhibit either cytochrome P450 isoform 3A4 or 2B6, which are responsible for artemisinin metabolism in humans (Lau and Chang 2009; Wei et al. 2015; Sergent et al. 2009; Choi, Piao, and Kang 2011; Svensson and Ashton 1999). We hypothesized that the increased bioavailability of artemisinin afforded by DLA-delivery (Weathers et al. 2011) is partly due to inhibition of hepatic metabolism by phytochemicals produced by DLA. The data presented in Section 3.1 confirm that phytochemicals present in DLA do inhibit liver P450s and this inhibition is part of the mechanism that leads to the previously reported increased bioavailability of DLA-delivered artemisinin (Weathers et al. 2011). Furthermore, since no individually tested phytochemicals produced stronger inhibition than the DLA extract, this inhibition is not solely due to any of the measured phytochemicals. It could, however, be from other as yet unidentified phytochemicals or a complex mixture of phytochemicals with moderate inhibitory activity. Future experiments will involve bioassay-guided fractionation of DLA extract to determine if there are any singular phytochemicals with potent inhibitory P450 activity.

In mice, artemisinin reaches peak serum concentration at 1 hour post-gavage (Weathers, Elfawal, et al. 2014). Using TLC, Xinyi et al. (1985) showed orally delivered artemisinin accumulated mostly in the liver followed by brain, plasma, lung, kidney, muscle, heart, and spleen in male rats after 1 hour (Xinyi et al. 1985). However, it is unclear whether their TLC method could resolve artemisinin from deoxyartemisinin, an important liver metabolite. Here, using GC-MS analysis, we expanded on those results by determining differences when artemisinin was instead delivered as DLA and by comparing gender differences. DLA-delivered artemisinin

distributed in higher quantities to many tissues in males and females. This was not surprising, given that DLA-delivered artemisinin was known to have increased bioavailability (Weathers et al. 2011; Weathers, Elfawal, et al. 2014; Räth et al. 2004). Interestingly, female rats had both better absorption of artemisinin into several tissues (Table 5.2) and higher amounts of artemisinin in their urine when given DLA (Figure 5.5). These data are consistent with a previous report showing females to have significantly decreased artemisinin metabolism compared to males *in vitro* (Ashton 1999).

The enhanced tissue distribution seen here and increased bioavailability afforded by DLA shown in the literature (Weathers et al. 2011; Weathers, Elfawal, et al. 2014; Räth et al. 2004) led us to hypothesize DLA would be a more potent anti-inflammatory therapeutic than pure artemisinin. Indeed, the results shown in Figure 5.6 confirm this hypothesis. The enhanced efficacy against inflammatory cytokine production was likely due to increased bioavailability of artemisinin afforded by the inhibition of CYP2B6 and CYP3A4 by DLA phytochemicals. However, it is also plausible some DLA phytochemicals have their own inherent anti-inflammatory activity, and thus act additively with artemisinin to suppress inflammatory cytokine production. It is also possible that both mechanisms are in play. In either case, the benefit of the whole plant-based treatment is evident. While gender differences were expected, it was surprising that IL-6 production was affected neither by DLA nor pure artemisinin treatment in females, especially considering artemisinin is better absorbed and less efficiently metabolized in females compared to males (Ashton 1999).

We also posited that if DLA reduced inflammation, the reduction could occur by increasing anti-inflammatory cytokine production, e.g., IL-10. However, the results shown in Appendix C, Figure S2 suggest that is unlikely. More likely is that DLA phytochemicals interact with molecular

pathways responsible for inflammatory cytokine production, e.g., NF-κB and MAPK signaling, and should be further investigated *in vitro* with DLA extracts and individual phytochemicals.

Taken together, these results should be considered for potential drug–drug interactions with other medications taken by patients consuming *A. annua*. Consistent with the human pharmacokinetic study of Räth et al. (2004), our results also indicate that delivery of artemisinin via *per os* consumption of *A. annua* can exceed the minimum antimalarial threshold of 9–10 µg/L blood (Alin and Bjorkman 1994) and should therefore obviate some concerns expressed by WHO (WHO 2012b, 2019a) regarding inadequate delivery of artemisinin from *per os* consumption of *A. annua*.

5.6 Conclusions

Although DLA extracts, teas, and phytochemicals inhibited CYP2B6 and CYP3A4, thereby showing that phytochemicals in DLA inhibit CYP450 enzymes that metabolize artemisinin, almost nothing is known about its tissue distribution and elimination. While the ADME of pure artemisinin was briefly explored previously by TLC (Xinyi et al. 1985), it was not compared to artemisinin delivered as DLA. Here, we showed that distribution of artemisinin to several tissues and serum significantly increased when delivered as DLA in male and female rats. Furthermore, the data suggest that artemisinin is differentially eliminated in males compared to females and differentially metabolized from DLA vs. pure artemisinin. More artemisinin therefore passes into the serum. Although we showed DLA is an effective anti-inflammatory agent *in vivo*, reducing TNF-α and IL-6 in males, in females, results were not as definitive because neither DLA nor pure artemisinin reduced IL-6 production, but did reduce TNF-α. Finally, neither artemisinin nor DLA affected IL-10 production, indicating that DLA and artemisinin likely reduce inflammation by inhibiting molecular signaling pathways responsible for proinflammatory

cytokine production. Inhibition of the NF-κB and MAPK signaling cascade pathways by DLA extracts and phytochemicals require further investigation. Overall, these results enhance our understanding of how artemisinin delivered from *A. annua* is more bioavailable than when delivered as a pure drug.

5.7 Funding

This research was funded by The National Center for Complementary and Integrative Health, award number NIH-2R15AT008277-02. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Complementary and Integrative Health or the National Institutes of Health.

5.8 Acknowledgments

The authors thank Miguel Norden and Dr. Virender Chandna of Worcester Polytechnic Institute for training Mr. Desrosiers in all procedures with rats as well as assistance with intraperitoneal injections. We also thank vivarium staff for help with animal welfare and upkeep. We thank Dr. David Greenblatt and Mia Angeli of Tufts University School of Medicine for training in liver microsome studies. We thank Drs. M. Andrea Azcarate-Peril, Jakub Kwintkiewicz, and Kshipra Chandrashekhar of the University of North Carolina Chapel Hill Microbiome Core Facility for help with 16s rRNA sequencing. We also thank Dr. Melissa Towler of Worcester Polytechnic Institute for phytochemical analysis of DLA.

5.9 Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Chapter 6 Conclusions and Future Work

6.1 Conclusions

The major conclusions of this study explain how *per os* dried leaf *A. annua* L. affects bioavailability of artemisinin in its digestive solubility, intestinal permeability, and hepatic first-pass metabolism. Specifically, dried leaves of *A. annua* increase serum artemisinin levels at 1-hour post-gavage in rats compared to pure artemisinin. Although there were published reports indicating this occurred (Weathers, Elfawal, et al. 2014; Weathers et al. 2011), the data from Chapter 5 provides confirmation. Both male and female rats given DLA had significantly greater artemisinin concentrations in their serum at 1-hour post-gavage compared to rats given pure artemisinin (Figure 5.2).

Artemisinin solubility also was about 4-fold greater when delivered as DLA compared to pure artemisinin. Essential oils present in the leaves were largely responsible for this increase as evinced by the observation that digestive artemisinin solubility increased almost 2.5-fold when combined with the "high" level of *A. annua* essential oil (Table 3.1).

Digestion of peanuts in combination with DLA decreased artemisinin detected in digestive fluid by 23% while digestion with Plumpy'nut®, a ready to use therapeutic food used to treat malnutrition, decreased flavonoids detected in digestive fluid by 24% (Figure 3.4). Considering that children are the largest group of malaria victims, these results have potentially important clinical relevance. If peanuts or Plumpy'nut® decreased artemisinin or flavonoid bioavailability, it could impact serum bioavailability and therefore delivered dose. However, this should be confirmed *in vivo* before any dosage recommendations are made. Artemisinin from digested DLA has about 37% higher intestinal permeability than digested pure artemisinin. The explanation for

this increase, however, remains unclear as none of the tested individual DLA components similarly increased intestinal permeability (Figure 4.1).

Importantly, four different types of capsules for encapsulating DLA were tested and none affected the amount of artemisinin or flavonoids detected in the digestive liquid phase (Figure 3.3). Capsules may offer a simple, low-tech means of delivery of DLA in rural communities that will deliver the full spectrum of phytochemicals that may not be delivered in a tea infusion. Encapsulated DLA has also been used as a low-tech rectal suppository to treat malaria successfully in neonates (Pierre Lutgen, unpublished).

Extracts and teas made from both *A. annua* and *A. afra* inhibited CYP2B6 and CYP3A4 activity, the hepatic enzymes responsible for artemisinin metabolism, suggesting that some phytochemicals produced by *Artemisia* species are CYP inhibitors. Although some individual phytochemicals typically found in *A. annua* inhibited CYP2B6 and CYP3A4, none was as potent as the whole extract or tea (Table 5.1). Thus, it is still unclear which specific phytochemicals or combinations of phytochemicals are most important in the inhibition of artemisinin-metabolizing CYPs.

Artemisinin distributes to tissues in rats in greater quantities when delivered as DLA vs. as pure artemisinin; amounts were similar to those observed in serum (Figure 5.2). Artemisinin accumulated in higher concentrations in many tissues, but not in the serum, of female rats compared to male rats regardless of delivery as DLA or pure artemisinin. These results suggested a gender bias in artemisinin metabolism and therefore in tissue accumulation (Table 5.2). Indeed, a gender bias in artemisinin metabolism was demonstrated previously using *ex vivo* liver slices from male and female rats (Xinyi et al. 1985). Together these results may inform possible dosage

differences between genders in the future, but more work should be completed in gender-specific human liver microsomes and humans before a recommendation is made.

In males, DLA had stronger anti-inflammatory efficacy than pure artemisinin while in females, results were less clear. IL-6 was unaffected by DLA or pure artemisinin while, TNF-α production was reduced by both (Figure 5.6).

Together these results shed light on the underlying mechanisms behind enhanced artemisinin bioavailability afforded by DLA and suggested DLA may have better efficacy on some afflictions such as inflammation compared to pure artemisinin. While many questions have been answered by these studies, the results have prompted additional questions that deserve further investigation.

6.2 Future Work

Following are some new research questions that should be studied in the future to better understand the differences between DLA treatment and pure artemisinin or ACTs.

6.2.1 Essential Oil Yield and Content of Weathers Lab *Artemisia* Plants

This study showed that the essential oil of the plant can have beneficial effects including enhancing solubility of artemisinin. In the solubility studies one source of essential oil was sourced from the United States, however, for Caco-2 cell studies, we used essential oil that was ordered from two different sources, one in the United States and one in China. Both were produced putatively from *A. annua* but qualitatively had different properties i.e. color and smell. While they did not produce different results in Caco-2 studies, their monoterpene content was analyzed via GC-MS. As expected, the oils had substantially different composition with large differences in the

major constituents camphor, thujone, α-pinene, and 1,8-cineole (eucalyptol) (Desrosiers, Towler, and Weathers 2019) (Table 6.1). Thujone, a monoterpene produced by some *Artemisia* species, is not found in *A. annua*, so the oil sourced from the U.S. was likely contaminated with other thujone-producing plant material (Abad et al. 2012; Bilia et al. 2014; Tzenkova et al. 2010).

Table 6.1. Relative abundance of some phytochemicals identified by GC-MS in *A. annua* essential oil from U.S. and Chinese sources. Adapted from (Desrosiers and Weathers 2018).

Phytochemical	**U.S. EO Source % of Total Peak Area**	**Chinese EO Source % of Total Peak Area**
1,8-cineole	16.5	27.4
α-pinene	0.3	17.5
β-pinene	2.5	4.7
Borneol	3.7	1.8
Camphor	30.3	14.1
Camphene	13.2	5.5
Caryophyllene	1.0	6.2
Caryophyllene oxide	0.9	Nd
Carene	Nd	2.2
Copaene	Nd	1.1
Humulene	Nd	5.5
Limonene	Nd	5.2
Myrcene	0.1	Nd
Phellandrene	Nd	8.5
Santolina triene	0.1	0.2
Stigmasterol	0.1	Nd
Terpineol	0.2	Nd
Thujone	30.9	Nd

EO, essential oil; Nd, not detected.

The content of essential oil can vary widely even within the same plant species and in *A. annua* can range from 0.3-4.0% (Bilia et al. 2014). It is therefore important that the yield and contents of the essential oil be identified in studies. Doing so would provide a better understanding of the phytochemical profile of different *A. annua* plant cultivars and may help explain variability in results obtained outside and within our lab using differing plant material.

The variability in phytochemical content between plants is an inherent problem when discussing the possible use of plant-based therapies for disease. In one study the *A. annua* "Bra" cultivar grown in Brazil showed 45% inhibition of CYP3A4 activity, but when the same cultivar was grown under different environmental conditions in Luxembourg, no inhibition was observed (Melillo de Magalhães et al. 2012). Their phytochemical makeup also differs by a wide margin with the Brazil-grown "Bra" cultivar having more than double the artemisinin, rosmarinic acid, and isoquercitrin of the Luxembourg-grown "Bra" cultivar (Melillo de Magalhães et al. 2012). These data illustrate a pertinent and common critique of whole-plant therapies: the phytochemical content of plants is very difficult to control, and even plants of the same genotype can display vastly different phytochemical profiles when propagated under differing environmental conditions. It is thus critical that quality control measures are put in place for any whole-plant product intended for use in medicine. Importantly, this problem can be overcome. In our lab, *Artemisia* cultivars are clonally propagated and growth conditions are monitored and standardized. Plants are harvested at precise developmental ages and plant material is dried under controlled conditions. As a result, the artemisinin and total flavonoid contents of our *A. annua* SAM cultivar has remained stable for many years (Weathers and Towler 2014; Gruessner et al. 2019).

6.2.2 Bioassay-Guided Fractionation of *A. annua* to Putatively Identify CYP Inhibitors

Results from Chapter 5 indicated that *A. annua* and *A. afra* extracts and teas contain phytochemicals that inhibit CYP2B6 and CYP3A4 activity. While these extracts and teas had strong inhibitory activity, none of the tested individual phytochemicals had equal or greater inhibitory activity on CYP2B6, the main artemisinin-metabolizing enzyme, as the *A. annua* extract or tea (Table 5.1). Although quercetin had a similar IC_{50} to the *Artemisia* extracts and teas in CYP3A4, it is likely not the only contributor as although quercetin has been detected in *A. annua*,

it is not present in detectable levels in our SAM cultivar using our GC-MS method used to analyze the extracts and teas from these studies (Weathers and Towler 2014; Ferreira et al. 2010). There have been almost 600 secondary metabolites identified in *A. annua*, many of which are not available in pure form or are prohibitively expensive, making it nearly impossible to test every one for inhibitory activity. Thus, a more directed and rational approach must be taken.

Bioassay-guided fractionation is the process of separating a complex crude extract into less complex fractions using organic solvent partitioning and various chromatography techniques. This method uses biological activity assays to guide the fractionation meaning that after each fractionation step, an activity assay is performed on each fraction to identify the most bioactive fractions. The most bioactive fractions are then further fractionated until pure compounds with potent bioactivity can be isolated. Several well-known drugs such as paclitaxel and camptothecin were discovered using this technique (Wall and Wani 1996). This same approach could be used to isolate strong CYP2B6 and CYP3A4 inhibitors produced by *A. annua*. In fact, preliminary work has already been completed towards this goal.

Our lab has formed a collaboration with Dr. Nadja Cech's group at the University of North Carolina at Greensboro. Dr. Cech's group specializes in bioassay-guided fractionation and allowed me to work in their lab for 2 weeks, producing crude methanolic extract from 1.125 kg of dried *A. annua* leaves from our lab. This methanolic extract was subsequently fractionated through solvent partitioning with hexane, then ethyl acetate, then saline. This resulted in four partitions containing hexane-soluble, ethyl acetate-soluble, methanol-soluble, and saline-soluble compounds. This first fractionation step resulted in fractions that are still highly complex, but provide a good starting point for separating compounds based on polarity; the relative polarities of these solvents are hexane> ethyl acetate>methanol>water (Reichardt and Welton 2011). The ethyl acetate partition

was then further fractionated using 2 different methods of flash chromatography on a Teledyne ISCO CombiFlash HPLC chromatography unit resulting in 20 fractions. The fractions were then dried and returned to WPI for analysis. I first analyzed the crude extract, hexane partition, and ethyl acetate partition for artemisinin content and inhibitory activity on CYP2B6 and CYP3A4 using already described methods (Desrosiers, Mittelman, and Weathers 2020) (Table 6.2).

Table 6.2. Inhibition of CYP2B6 and CYP3A4 by *A. annua* crude extract and organic partitions

Partition or Extract	**CYP2B6 IC_{50} (mg/mL)**	**Artemisinin Concentration in CYP2B6 IC_{50} (µM)**	**CYP3A4 IC_{50} (mg/mL)**	**Artemisinin Concentration in CYP3A4 IC_{50} (µM)**
Crude Extract	0.0294	2.91	0.0161	1.60
Ethyl Acetate Partition	0.0121	6.17	0.0131	6.66
Hexane Partition	0.0096	3.32	0.0048	1.65

Initial experiments indicated that IC_{50} values of the ethyl acetate partition were around 20 and 10 ug/mL for CYP3A4 and CYP2B6, respectively. Thus, we screened flash chromatography fractions at 20 and 10 ug/mL against CYP3A4 and CYP2B6 respectively and fractions that produced <50% activity vs. vehicle control were considered of interest. The flash chromatography fractions were also assayed for total flavonoid content (Table 6.3).

Table 6.3. CYP2B6 and CYP3A4 inhibition by flash chromatography fractions of *A. annua* ethyl acetate partition

Flash Chromatography Fraction ID	Artemisinin Present (Based on GC-MS)?	Flavonoid Content (mg/G)	% CYP3A4 Activity Relative to Vehicle Control (20 ug/mL of fraction)	% CYP2B6 Activity Relative to Vehicle Control (10 ug/mL of fraction)
ISCO-2-(1-8)	No	5.20	61.29	76.12
ISCO-2-(9-11)	No	9.36	65.80	58.27
ISCO-2-(12-17)	Yes	6.24	84.03	**23.13**
ISCO-2-(18-21)	Yes	80.04	**34.06**	**33.45**
ISCO-2-(22-25)	Yes	190.23	**25.05**	65.01
ISCO-2-(26-36)	Yes	60.29	**22.77**	63.95
ISCO-2-(37-39)	Yes	172.56	**9.69**	**41.92**
ISCO-2-(40-46)	No	81.08	65.24	52.82
ISCO-2-(47-50)	No	61.33	60.73	51.58
ISCO-2-(51-58)	No	63.41	69.26	77.46
ISCO-2-(59-65)	No	33.26	69.52	86.92
ISCO-3-(1-6)	No	17.67	64.37	**28.20**
ISCO-3-(7-30)	Yes	25.99	90.44	**28.22**
ISCO-3-(31-35)	Yes	21.83	78.45	62.44
ISCO-3-(36-39)	Yes	224.53	**8.53**	**21.98**
ISCO-3-(40-44)	No	343.04	**11.53**	**46.00**
ISCO-3-(45-63)	Yes	119.54	**18.59**	57.45
ISCO-3-(64-66)	No	106.03	**10.83**	**39.97**
ISCO-3-(67-B5)	No	135.14	**48.38**	**42.94**
ISCO-3-(B6-B18)	No	124.74	66.37	63.50

While most fractions did not produce >50% inhibition of both CYP2B6 and CYP3A4, several fractions did. These fractions should be investigated further as they contain compounds that likely inhibit the metabolizing enzymes of artemisinin. These fractions may be used to identify known or even undescribed phytochemicals that enhance the bioavailability of artemisinin when delivered as dried *A. annua* leaves through inhibition of first-pass metabolism.

6.2.3 Induction of Cytochrome P450s by *A. annua*

One topic that has not yet been addressed is the induction of CYP2B6 and CYP3A4 by artemisinin. Artemisinin is an autoinducing drug; it induces the expression of its own metabolizing enzymes (Xing et al. 2012). Artemisinin achieves this induction through activation of the pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which in turn translocate to the nucleus where they bind promoters to increased expression of CYP2B6 and CYP3A4 (Xing et al. 2012; Timsit and Negishi 2007). However, little is known about how other phytochemicals in *A. annua* may alter this autoinduction. *A. annua* is rich in flavonoids, many of which, including quercetin, kaempferol, naringin, wogonin, and baicalin, either increase or decrease expression of many CYPs (Cermak 2008; Hodek, Trefil, and Stiborová 2002). It is thus possible that dried leaves of *A. annua* may alter the autoinduction of CYP2B6 and CYP3A4 by artemisinin. Indeed, we have begun preliminary studies in our lab using HepaRG cells to test extracts of *A. annua* and *A. afra* for their ability to alter induction of CYP2B6 and CYP3A4. These specialized liver cells retain the CYP induction pathways that most other cell lines and human liver microsomes do not. I treated HepaRG cells with pure artemisinin and extracts from *A. annua* and *A. afra* at artemisinin concentrations ranging from 1-50 µM for three days and then measured the enzymatic activity of CYP3A4 and CYP2B6 with their respective P450-Glo assays and compared to a vehicle control. At 1-20 µM artemisinin, SAM, our high-artemisinin producing *A. annua* cultivar, had little effect on CYP3A4 activity but at 50 µM CYP3A4 activity decreased substantially to < 2% of the vehicle control (Appendix D, Figure S1). In contrast, when treated with *A. afra* CYP3A4 activity increased with increasing extract concentration before leveling off around 700% relative to vehicle control (Appendix D, Figure S1). CYP2B6 activity was similarly increased by *A. afra* albeit not as strongly (Appendix D, Figure S2). Pure artemisinin did not alter induction of CYP3A4 activity relative to

vehicle control, which is in contrast to what was previously observed by others (Appendix D, Figure S1) (Xing et al. 2012). In my analysis, *A. annua* extract decreased CYP2B6 activity in a concentration-dependent manner ultimately achieving about 29% activity relative to vehicle control at 50 µM (Appendix D, Figure S2). Pure artemisinin treatment increased activity at 1-5 µM but decreased activity at 10-50 µM (Appendix D, Figure S2). Importantly, all of these experiments were performed once, albeit in technical triplicate, so they must be repeated to confirm results. Furthermore, while these assays tested enzymatic activity, future CYP induction assays should also evaluate mRNA transcript levels through qPCR to determine if either transcription, translation, or both, are affected.

6.2.4 Human Pharmacokinetics of *A. annua* Delivered as Tea vs. Capsules

Another study that should be completed is a human pharmacokinetic study using teas and capsules made from dried *A. annua* leaves. To date, there is virtually no information regarding the pharmacokinetics of artemisinin delivered via DLA. There are several trials on efficacy against malaria and other diseases, but no data exists to confirm in humans what was observed in rodents: artemisinin delivered as DLA reaches significantly higher plasma concentrations compared to pure artemisinin. Such studies are needed to determine if what is observed in rodents correlates to humans and to determine whether teas or capsules provide higher artemisinin bioavailability.

6.2.5 *In Vitro* Inflammation Studies with Extracts, Extract Fractions, and Pure Phytochemicals

While the data from Chapter 5 indicate that *A. annua* may work to combat inflammatory cytokine production during systemic inflammation, little is known about the mechanism of this action. The inflammatory response in mammals is complex involving multiple signaling pathways

(Newton and Dixit 2012). The major pathway controlling biosynthesis of inflammatory cytokines is the NF-κB signaling pathway (Newton and Dixit 2012; Lawrence 2009). This canonical pathway involves activation of a toll-like receptor (TLR) by a pathogen-associated molecular pattern (PAMP) such as lipopolysaccharide, thereby initiating the signaling cascade. This eventually results in activation of the NF-κB transcription factor that regulates production of proinflammatory cytokines like TNF-α and IL-6 (Newton and Dixit 2012). This pathway is well studied and as a result there are many anti-inflammatory medications designed to interrupt this pathway (Nam 2006). In fact, AN and many other plant-derived polyphenols are known to block this pathway, leading to their anti-inflammatory activity (Figure 6.1) (Wang et al. 2006; Zhu et al. 2012; Gruessner et al. 2019).

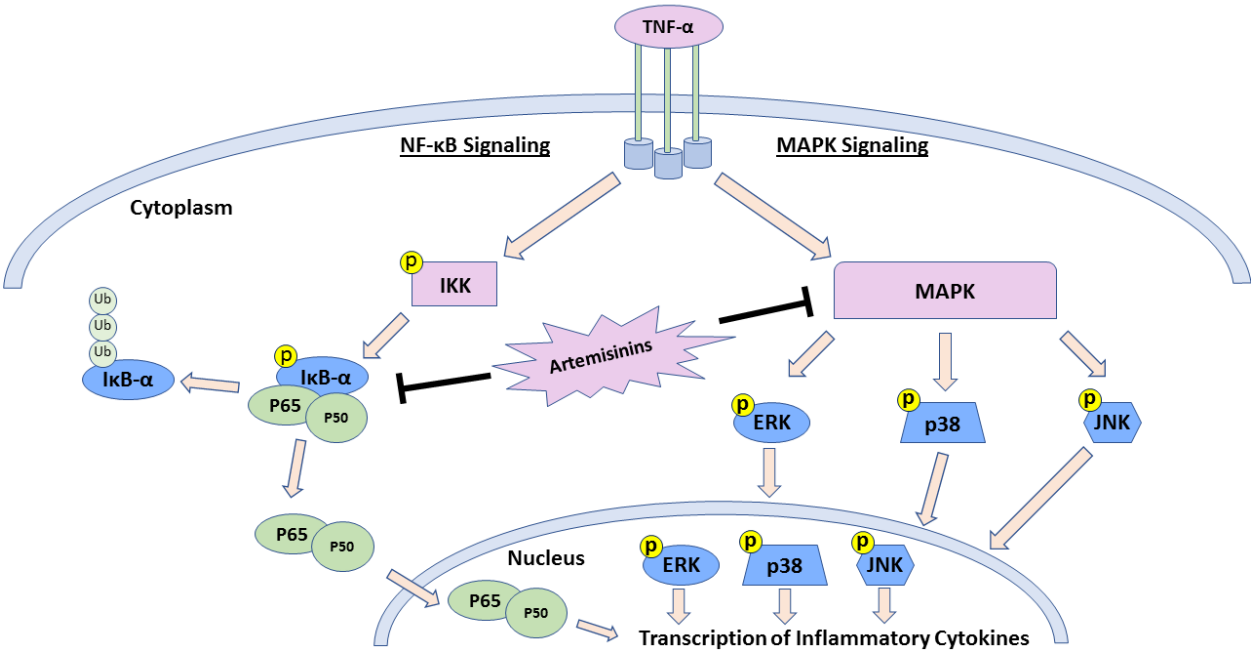

Figure 6.1. Artemisinin inhibition of inflammation through NF-κB and MAPK signaling cascades. Abbreviations: TNF-α – tumor necrosis factor alpha; NF-kB – nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK – mitogen-activated protein kinases; IKK – I kappa B kinase; IkB-alpha – inhibitor of kappa B alpha; Ub – ubiquitin; Yellow p's indicate phosphorylation of protein; ERK – extracellular signal-regulated kinases; JNK – c-Jun N-terminal kinases. Figure adapted from (Gruessner et al. 2019).

There is also another key pathway that plays a role in inflammatory cytokine production, namely the MAPK signaling pathway (de Souza et al. 2014; Newton and Dixit 2012; Otterbein et al. 2000). MAPK signaling is also initiated by TLR activation, is implicated in the inflammatory response, and also can regulate cytokine production similar to NF-κB (Newton and Dixit 2012). Artemisinin also interferes with this pathway (Wang et al. 2017).

While *in vivo* studies showed that dried *A. annua* leaves decreased inflammatory cytokine production (Figure 5.6) more than pure artemisinin, the mechanisms are unknown. These mechanisms should be studied *in vitro* in using LPS stimulated RAW 264.7 macrophages to determine if enhanced anti-inflammatory activity of DLA is simply due to increased artemisinin bioavailability or other phytochemicals in the plant directly impacting cytokine production. This could be investigated using AN, crude extracts or fractionated extracts of DLA, and pure phytochemicals. Using Western blotting and ELISA assays, proteins from the NF-κB and MAPK pathways could be probed to determine how DLA and its phytochemicals affect each pathway. Investigation of *A. annua* and its phytochemicals effects on these pathways would further our understanding of the utility of *A. annua* as an anti-inflammatory therapeutic.

6.3 Summary

A. annua is the main source of artemisinin and ACTs, the frontline drug protecting over 1 billion people from malaria in high risk regions worldwide (WHO 2019c). Here, I showed that delivery of artemisinin as dried leaves of *A. annua* leads to enhanced artemisinin bioavailability. This enhanced bioavailability is afforded through multiple mechanisms including increased digestive solubility via *A. annua* essential oils, increased intestinal permeability, and inhibition of

first-pass artemisinin metabolism. This enhanced bioavailability may be responsible, at least in part, for the efficacy of dried leaf *A. annua* treatments in recent clinical trials on malaria and schistosomiasis and may also enhance therapeutic outcomes in inflammatory diseases. More work is needed, especially in humans, to confirm these results but the studies presented here provide a better understanding of the potential benefits of dried leaf *A. annua* as a treatment for malaria and inflammatory diseases.

References

Abad, María José, Luis Miguel Bedoya, Luis Apaza, and Paulina Bermejo. 2012. 'The artemisia L. Genus: a review of bioactive essential oils', *Molecules (Basel, Switzerland)*, 17: 2542-66.

ACTwatch, Group, Sarah Tougher, Kara Hanson, and Catherine Goodman. 2017. 'What happened to anti-malarial markets after the Affordable Medicines Facility-malaria pilot? Trends in ACT availability, price and market share from five African countries under continuation of the private sector co-payment mechanism', *Malaria Journal*, 16: 173-73.

Aldieri, Elisabetta, Daniela Atragene, Loredana Bergandi, Chiara Riganti, Costanzo Costamagna, Amalia Bosia, and Dario Ghigo. 2003. 'Artemisinin inhibits inducible nitric oxide synthase and nuclear factor NF-kB activation', *FEBS Letters*, 552: 141-44.

Alin, M. Hassan, and Anders Bjorkman. 1994. 'Concentration and time dependency of artemisinin efficacy against Plasmodium falciparum in vitro', *The American journal of tropical medicine and hygiene*, 50: 771-76.

Arvouet-Grand, A, B Vennat, A Pourrat, and P Legret. 1994. 'Standardization of propolis extract and identification of principal constituents', *Journal de pharmacie de Belgique*, 49: 462-68.

Asher, Gary N., Amanda H. Corbett, and Roy L. Hawke. 2017. 'Common herbal dietary supplement—drug interactions', *Am Fam Physician*, 96: 101-07.

Ashton, Michael. 1999. 'Quantitative in vivo and in vitro sex differences in artemisinin metabolism in rat', *Xenobiotica*, 29: 195-204.

Ashton, Michael, T. N. Hai, N. D. Sy, D. X. Huong, N. Van Huong, N. T. Niêu, and L. D. Công. 1998. 'Artemisinin pharmacokinetics is time-dependent during repeated oral administration in healthy male adults', *Drug Metab Dispos*, 26.

Ashton, Michael, Nguyen Duy Sy, Nguyen Van Huong, Toufigh Gordi, Trinh Ngoc Hai, Dinh Xuan Huong, Nguyen Thi Niêu, and Le Dinh Công. 1998. 'Artemisinin kinetics and dynamics during oral and rectal treatment of uncomplicated malaria', *Clinical Pharmacology & Therapeutics*, 63: 482-93.

Asimus, Sara, Doaa Elsherbiny, Trinh N. Hai, Britt Jansson, Nguyen V. Huong, Max G. Petzold, Ulrika S. H. Simonsson, and Michael Ashton. 2007. 'Artemisinin antimalarials moderately affect cytochrome P450 enzyme activity in healthy subjects', *Fundamental & Clinical Pharmacology*, 21: 307-16.

Augustijns, P., A. D'Hulst, J. Van Daele, and R. Kinget. 1996. 'Transport of Artemisinin and Sodium Artesunate in Caco-2 Intestinal Epithelial Cells', *Journal of Pharmaceutical Sciences*, 85: 577-79.

Bailey, David G., George Dresser, and J. Malcolm O. Arnold. 2013. 'Grapefruit-medication interactions: forbidden fruit or avoidable consequences?', *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne*, 185: 309-16.

Barthe, Laurence, John Woodley, and Georges Houin. 1999. 'Gastrointestinal absorption of drugs: methods and studies', *Fundamental & Clinical Pharmacology*, 13: 154-68.

Basheer, Loai, and Zohar Kerem. 2015. 'Interactions between CYP3A4 and dietary polyphenols', *Oxidative medicine and cellular longevity*, 2015.

Bennett, Adam, Donal Bisanzio, Joshua O. Yukich, Bonnie Mappin, Cristin A. Fergus, Michael Lynch, Richard E. Cibulskis, Samir Bhatt, Daniel J. Weiss, Ewan Cameron, Peter W. Gething, and Thomas P. Eisele. 2017. 'Population coverage of artemisinin-based combination treatment in children younger than 5 years with fever and Plasmodium falciparum infection in Africa, 2003–2015: a modelling study using data from national surveys', *The Lancet Global Health*, 5: e418-e27.

Bi, Shuyun, Lili Yan, Bo Pang, and Yu Wang. 2012. 'Investigation of three flavonoids binding to bovine serum albumin using molecular fluorescence technique', *Journal of Luminescence*, 132: 132-40.

Bian, Hedong, Mei Li, Qing Yu, Zhenfeng Chen, Jianniao Tian, and Hong Liang. 2006. 'Study of the interaction of artemisinin with bovine serum albumin', *International journal of biological macromolecules*, 39: 291-97.

Bilia, Anna Rita, Francesca Santomauro, Cristiana Sacco, Maria Camilla Bergonzi, and Rosa Donato. 2014. 'Essential oil of Artemisia annua L.: an extraordinary component with numerous antimicrobial properties', *Evidence-Based Complementary and Alternative Medicine*, 2014.

Birgersson, Sofia, Pham Van Toi, Nguyen Thanh Truong, Nguyen Thi Dung, Michael Ashton, Tran Tinh Hien, Angela Abelö, and Joel Tarning. 2016. 'Population pharmacokinetic properties of artemisinin in healthy male Vietnamese volunteers', *Malaria Journal*, 15: 90-90.

Bison, Silvia, L. Carboni, R. Arban, S. Bate, P. A. Gerrard, and M. Razzoli. 2009. 'Differential behavioral, physiological, and hormonal sensitivity to LPS challenge in rats', *International Journal of Interferon, Cytokine and Mediator Research*, 1: 1-13.

Bohr, Adam, Thais Leite Nascimento, Necati Harmankaya, Johan Juhl Weisser, Yingya Wang, Holger Grohganz, Thomas Rades, and Korbinian Löbmann. 2019. 'Efflux Inhibitor Bicalutamide Increases Oral Bioavailability of the Poorly Soluble Efflux Substrate Docetaxel in Co-Amorphous Anti-Cancer Combination Therapy', *Molecules (Basel, Switzerland)*, 24: 266.

Brown, Geoffrey D. 2010. 'The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of Artemisia annua L.(Qinghao)', *Molecules*, 15: 7603-98.

Burk, Oliver, Katja A. Arnold, Andreas K. Nussler, Elke Schaeffeler, Ekaterina Efimova, Bonnie A. Avery, Mitchell A. Avery, Martin F. Fromm, and Michel Eichelbaum. 2005. 'Antimalarial Artemisinin Drugs Induce Cytochrome P450 and MDR1 Expression by Activation of Xenosensors Pregnane X Receptor and Constitutive Androstane Receptor', *Molecular Pharmacology*, 67: 1954.

Butler, A. R., S. Khan, and E. Ferguson. 2010. 'A brief history of malaria chemotherapy', *The journal of the Royal College of Physicians of Edinburgh*, 40: 172-77.

Cai, Tian-Yu, Yun-Rui Zhang, Jian-Bo Ji, and Jie Xing. 2017. 'Investigation of the component in Artemisia annua L. leading to enhanced antiplasmodial potency of artemisinin via regulation of its metabolism', *Journal of Ethnopharmacology*, 207: 86-91.

Carter, Richard, and Kamini N. Mendis. 2002. 'Evolutionary and Historical Aspects of the Burden of Malaria', 15: 564-94.

Caulfield, Laura E., Stephanie A. Richard, and Robert E. Black. 2004. 'Undernutrition as an underlying cause of malaria morbidity and mortality in children less than five years old', *The American journal of tropical medicine and hygiene*, 71: 55-63.

Ćavar, Sanja, Milka Maksimović, Danijela Vidic, and Adisa Parić. 2012. 'Chemical composition and antioxidant and antimicrobial activity of essential oil of Artemisia annua L. from Bosnia', *Industrial Crops and Products*, 37: 479-85.

CDC. 2019a. 'Malaria - About Malaria - Biology'. https://www.cdc.gov/malaria/about/biology/index.html.

CDC. 2019b. 'Malaria - About Malaria - Disease'. https://www.cdc.gov/malaria/about/disease.html.

CDC. 2019c. 'Malaria - Malaria Worldwide - How Can Malaria Cases and Deaths Be Reduced? - Drug resistance in the Malaria Endemic World'. https://www.cdc.gov/malaria/malaria_worldwide/reduction/drug_resistance.html.

Cermak, Rainer. 2008. 'Effect of dietary flavonoids on pathways involved in drug metabolism', *Expert opinion on drug metabolism & toxicology*, 4: 17-35.

Chan, Kit-Lam, Kah-Hay Yuen, Hiroaki Takayanagi, Sunil Janadasa, and Kok-Khiang Peh. 1997. 'Polymorphism of artemisinin from Artemisia annua', *Phytochemistry*, 46: 1209-14.

Chang, Hson-Mou, and Paul Pui-Hay But. 1986. *Pharmacology and Applications of Chinese Materia Medica* (World Scientific Publishing: Singapore).

Choi, Jun-Shik, Yong-Ji Piao, and Keon Wook Kang. 2011. 'Effects of quercetin on the bioavailability of doxorubicin in rats: Role of CYP3A4 and P-gp inhibition by quercetin', *Archives of Pharmacal Research*, 34: 607-13.

Cox, Francis E. G. 2010. 'History of the discovery of the malaria parasites and their vectors', *Parasites & vectors*, 3: 5.

Cui, Liwang, and Xin-zhuan Su. 2009. 'Discovery, mechanisms of action and combination therapy of artemisinin'.

Czechowski, Tomasz, Mauro A. Rinaldi, Mufuliat Toyin Famodimu, Maria Van Veelen, Tony R. Larson, Thilo Winzer, Deborah A. Rathbone, David Harvey, Paul Horrocks, and Ian A. Graham. 2019. 'Flavonoid Versus Artemisinin Anti-malarial Activity in Artemisia annua Whole-Leaf Extracts', *Frontiers in plant science*, 10: 984-84.

Daddy, Nsengiyumva Bati, Luc Malemo Kalisya, Pascal Gisenya Bagire, Robert L. Watt, Melissa J. Towler, and Pamela J. Weathers. 2017. 'Artemisia annua dried leaf tablets treated malaria resistant to ACT and iv artesunate: case reports', *Phytomedicine*.

Dahan, Arik, and Jonathan M. Miller. 2012. 'The Solubility–Permeability Interplay and Its Implications in Formulation Design and Development for Poorly Soluble Drugs', *The AAPS Journal*, 14: 244-51.

Davis, Ben, Joel Ladner, Kelley Sams, Ebru Tekinturhan, Donald de Korte, and Joseph Saba. 2013. 'Artemisinin-based combination therapy availability and use in the private sector of five AMFm phase 1 countries', *Malar J*, 12: 10-1186.

de Cássia da Silveira e Sá, Rita, Luciana Nalone Andrade, and Damião Pergentino de Sousa. 2013. 'A review on anti-inflammatory activity of monoterpenes', *Molecules*, 18: 1227-54.

De Donno, Antonella, Tiziana Grassi, Adele Idolo, Marcello Guido, Paride Papadia, Alessandro Caccioppola, Luciano Villanova, Alessandro Merendino, Francesco Bagordo, and Francesco Paolo Fanizzi. 2012. 'First-time comparison of the in vitro antimalarial activity of Artemisia annua herbal tea and artemisinin', *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 106: 696-700.

de Ridder, Sanne, Frank van der Kooy, and Robert Verpoorte. 2008. 'Artemisia annua as a self-reliant treatment for malaria in developing countries', *Journal of Ethnopharmacology*, 120: 302-14.

de Souza, Andréia Pacheco, Vera Lúcia Costa Vale, Marcos da Costa Silva, Inara Barbosa de Oliveira Araújo, Soraya Castro Trindade, Lília Ferreira de Moura-Costa, Gabriele Costa Rodrigues, Tatiane Santana Sales, Heidiane Alves dos Santos, and Paulo Cirino de Carvalho-Filho. 2014. 'MAPK involvement in cytokine production in response to Corynebacterium pseudotuberculosis infection', *BMC microbiology*, 14: 230.

Debnath, Chhanda, Andrea Dobernig, Pijus Saha, and Astrid Ortner. 2011. 'Electrochemical Determination of Artemisinin in Artemisia annua L Herbal Tea Preparation and Optimization of Tea Making Approach', *Journal of the Korean Chemical Society*, 55: 57-62.

Delves, Michael, David Plouffe, Christian Scheurer, Stephan Meister, Sergio Wittlin, Elizabeth A. Winzeler, Robert E. Sinden, and Didier Leroy. 2012. 'The activities of current antimalarial drugs on the life cycle stages of Plasmodium: a comparative study with human and rodent parasites', *PLoS medicine*, 9: e1001169-e69.

Desai, Manisha P., Vinod Labhasetwar, Elke Walter, Robert J. Levy, and Gordon L. Amidon. 1997. 'The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent', *Pharmaceutical research*, 14: 1568-73.

Desrosiers, Matthew R., Alexis Mittelman, and Pamela J. Weathers. 2020. 'Dried Leaf Artemisia Annua Improves Bioavailability of Artemisinin via Cytochrome P450 Inhibition and Enhances Artemisinin Efficacy Downstream', *Biomolecules*, 10: 254.

Desrosiers, Matthew R., Melissa J. Towler, and Pamela J. Weathers. 2019. 'Artemisia annua and Artemisia afra Essential Oils and Their Therapeutic Potential.' in Sonia Malik (ed.), *Essential Oil*

Research: Trends in Biosynthesis, Analytics, Industrial Applications and Biotechnological Production (Springer International Publishing: Cham).

Desrosiers, Matthew R., and Pamela J. Weathers. 2016. 'Effect of leaf digestion and artemisinin solubility for use in oral consumption of dried Artemisia annua leaves to treat malaria', *Journal of Ethnopharmacology*, 190: 313-18.

Desrosiers, Matthew R., and Pamela J. Weathers. 2018. 'Artemisinin permeability via Caco-2 cells increases after simulated digestion of Artemisia annua leaves', *Journal of Ethnopharmacology*, 210: 254-59.

Di, Yuan M., Chun Guang Li, Charlie Changli Xue, and Shu-Feng Zhou. 2008. 'Clinical Drugs that Interact with St. John's Wort and mplication in Drug Development', *Current pharmaceutical design*, 14: 1723-42.

Ding, Zuoqi, Yue Dai, Haiping Hao, Rong Pan, Xiujuan Yao, and Zhengtao Wang. 2008. 'Anti-inflammatory effects of scopoletin and underlying mechanisms', *Pharmaceutical biology*, 46: 854-60.

Duke, James A. 2001. *Handbook of phytochemical constituents of GRAS herbs and other economic plants* (CRC Press LLC: Boca Raton, FL).

Duke, M.V., R.N. Paul, H.N. Elsohly, G. Sturtz, and S.O. Duke. 1994. 'Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L.', *International Journal of Plant Sciences*, 155: 365-72.

Eastman, Richard T., and David A. Fidock. 2009. 'Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria', *Nature reviews. Microbiology*, 7: 864-74.

Efferth, Thomas. 2015. 'Artemisinin—second career as anticancer drug', *World J Tradit Chin Med*, 1: 2-25.

Efferth, Thomas, Marta R. Romero, Dana G. Wolf, Thomas Stamminger, Jose J. G. Marin, and Manfred Marschall. 2008. 'The Antiviral Activities of Artemisinin and Artesunate', *Clinical Infectious Diseases*, 47: 804-11.

El-Kattan, Ayman, and Manthena Varma. 2012. 'Oral absorption, intestinal metabolism and human oral bioavailability', *Topics on drug metabolism*, 10: 31087.

Elfawal, Mostafa A., Melissa J. Towler, Nicholas G. Reich, Douglas Golenbock, Pamela J. Weathers, and Stephen M. Rich. 2012. 'Dried whole plant Artemisia annua as an antimalarial therapy', *PLOS one*, 7: e52746.

Elfawal, Mostafa A., Melissa J. Towler, Nicholas G. Reich, Pamela J. Weathers, and Stephen M. Rich. 2015. 'Dried whole-plant Artemisia annua slows evolution of malaria drug resistance and overcomes resistance to artemisinin', *Proceedings of the National Academy of Sciences*, 112: 821-26.

Elford, Barry C., Margaret F. Roberts, J. David Phillipson, and Robert J. M. Wilson. 1987. 'Potentiation of the antimalarial activity of qinghaosu by methoxylated flavones', *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 81: 434-36.

Ensari, Arzu, and Michael N. Marsh. 2018. 'Exploring the villus', *Gastroenterology and hepatology from bed to bench*, 11: 181-90.

Ferreira, Jorge F. S., and Jules Janick. 1995. 'Floral morphology of Artemisia annua with special reference to trichomes', *International Journal of Plant Sciences*: 807-15.

Ferreira, Jorge F. S., Devanand L. Luthria, Tomikazu Sasaki, and Arne Heyerick. 2010. 'Flavonoids from Artemisia annua L. as antioxidants and their potential synergism with artemisinin against malaria and cancer', *Molecules*, 15: 3135-70.

Foroozesh, Maryam, Jayalakshmi Sridhar, Navneet Goyal, and Jiawang Liu. 2019. 'Coumarins and P450s, Studies Reported to-Date', *Molecules (Basel, Switzerland)*, 24: 1620.

Fritz, Anja, Diana Busch, Joanna Lapczuk, Marek Ostrowski, Marek Drozdzik, and Stefan Oswald. 2019. 'Expression of clinically relevant drug-metabolizing enzymes along the human intestine and their

correlation to drug transporters and nuclear receptors: An intra-subject analysis', *Basic & Clinical Pharmacology & Toxicology*, 124: 245-55.

Ganesh, Deepa, Hans-Peter Fuehrer, Peter Starzengrüber, Paul Swoboda, Wasif Ali Khan, Johannes A. B. Reismann, Milena S. K. Mueller, Peter Chiba, and Harald Noedl. 2012. 'Antiplasmodial activity of flavonol quercetin and its analogues in Plasmodium falciparum: evidence from clinical isolates in Bangladesh and standardized parasite clones', *Parasitology Research*, 110: 2289-95.

Goulart, Herbert Rodrigues, Emília A. Kimura, Valnice J. Peres, Alicia S. Couto, Fulgencio A. Aquino Duarte, and Alejandro M. Katzin. 2004. 'Terpenes arrest parasite development and inhibit biosynthesis of isoprenoids in Plasmodium falciparum', *Antimicrobial Agents and Chemotherapy*, 48: 2502-09.

Grace, Mary H., Carmen Lategan, Rocky Graziose, Peter J. Smith, Ilya Raskin, and Mary Ann Lila. 2012. 'Antiplasmodial activity of the ethnobotanical plant Cassia fistula', *Natural Product Communications*, 7: 1934578X1200701002.

Gruessner, Brian M., Lucille. Cornet-Vernet, Matthew. R. Desrosiers, Pierre. Lutgen, Melissa J. Towler, and Pamela J. Weathers. 2019. 'It is not just artemisinin: Artemisia sp. for treating diseases including malaria and schistosomiasis', *Phytochemistry Reviews*.

Guengerich, F. Peter. 2008. 'Cytochrome P450 and Chemical Toxicology', *Chemical Research in Toxicology*, 21: 70-83.

Gumede, B., P. J. Waako, P. I. Folb, and B. Ryffel. 2009. 'Modulation of IFN-γ TNF-α, IL-10 and IL-12 production by Artesunate in mitogen treated splenocytes', *Int J Trop Med*, 4: 65-69.

Guo, Lian-Qing, and Yasushi Yamazoe. 2004. 'Inhibition of cytochrome P450 by furanocoumarins in grapefruit juice and herbal medicines', *Acta Pharmacologica Sinica*, 25: 129-36.

Gupta, S., U. S. Svensson, and M. Ashton. 2001. 'In vitro evidence for auto-induction of artemisinin metabolism in the rat', *Eur J Drug Metab Pharmacokinet*, 26.

Gurley, Bill J., Espero Kim Fifer, and Zoë Gardner. 2012. 'Pharmacokinetic herb-drug interactions (part 2): drug interactions involving popular botanical dietary supplements and their clinical relevance', *Planta Medica*, 78: 1490-514.

Gurley, Bill J., Ashley Swain, Martha A. Hubbard, D. Keith Williams, Gary Barone, Faith Hartsfield, Yudong Tong, Danielle J. Carrier, Shreekar Cheboyina, and Sunil K. Battu. 2008. 'Clinical assessment of CYP2D6-mediated herb–drug interactions in humans: Effects of milk thistle, black cohosh, goldenseal, kava kava, St. John's wort, and Echinacea', *Molecular nutrition & food research*, 52: 755-63.

Hodek, Petr, Pavel Trefil, and Marie Stiborová. 2002. 'Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450', *Chemico-Biological Interactions*, 139: 1-21.

Homayun, Bahman, Xueting Lin, and Hyo-Jick Choi. 2019. 'Challenges and Recent Progress in Oral Drug Delivery Systems for Biopharmaceuticals', *Pharmaceutics*, 11: 129.

Hortelano, Sonsoles. 2009. 'Molecular basis of the anti-inflammatory effects of terpenoids', *Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy)*, 8: 28-39.

Hsu, Elisabeth. 2006. 'The history of qing hao in the Chinese materia medica', *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 100: 505-08.

Hu, Donghua, Yuguang Wang, Zhiwu Chen, Zengchun Ma, Qing You, Xianxie Zhang, Tao Zhou, Yong Xiao, Qiande Liang, Hongling Tan, Chengrong Xiao, Xianglin Tang, Boli Zhang, and Yue Gao. 2014. 'Artemisinin protects against dextran sulfate-sodium-induced inflammatory bowel disease, which is associated with activation of the pregnane X receptor', *European Journal of Pharmacology*, 738: 273-84.

Hubatsch, Ina, Eva G. E. Ragnarsson, and Per Artursson. 2007. 'Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers', *Nat. Protocols*, 2: 2111-19.

ICIPE. 2005. "Whole-leaf Artemisia annua-based antimalarial drug: report on proof-of-concepts studies." In *Nairobi, Kenya.* Nairobi, Kenya.

Institute of Medicine. 1991. *Malaria: Obstacles and Opportunities* (The National Academies Press: Washington, DC).

Institute of Medicine. 2004. *Saving Lives, Buying Time: Economics of Malaria Drugs in an Age of Resistance* (The National Academies Press: Washington, DC).

Jin, Jing, Dake Cai, Huichang Bi, Guoping Zhong, Hang Zeng, Lianquan Gu, Zhiying Huang, and Min Huang. 2013. 'Comparative pharmacokinetics of paclitaxel after oral administration of Taxus yunnanensis extract and pure paclitaxel to rats', *Fitoterapia*, 90: 1-9.

Juergens, Uwe R., Tanja Engelen, Kurt Racké, Meinolf Stöber, Adrian Gillissen, and Hans Vetter. 2004. 'Inhibitory activity of 1, 8-cineol (eucalyptol) on cytokine production in cultured human lymphocytes and monocytes', *Pulmonary pharmacology & therapeutics*, 17: 281-87.

Juteau, Fabien, Veronique Masotti, Jean Marie Bessière, Michel Dherbomez, and Josette Viano. 2002. 'Antibacterial and antioxidant activities of Artemisia annua essential oil', *Fitoterapia*, 73: 532-35.

Kalepu, Sandeep, and Vijaykumar Nekkanti. 2015. 'Insoluble drug delivery strategies: review of recent advances and business prospects', *Acta pharmaceutica Sinica. B*, 5: 442-53.

Kim, Marlene T., Alexander Sedykh, Suman K. Chakravarti, Roustem D. Saiakhov, and Hao Zhu. 2014. 'Critical evaluation of human oral bioavailability for pharmaceutical drugs by using various cheminformatics approaches', *Pharmaceutical research*, 31: 1002-14.

Kim, Wan-Su, Woo Jin Choi, Sunwoo Lee, Woo Joong Kim, Dong Chae Lee, Uy Dong Sohn, Hyoung-Shik Shin, and Wonyong Kim. 2015. 'Anti-inflammatory, Antioxidant and Antimicrobial Effects of Artemisinin Extracts from Artemisia annua L', *The Korean Journal of Physiology & Pharmacology*, 19: 21-27.

Konkimalla, V. Badireenath, Martina Blunder, Bernhard Korn, Shahid A. Soomro, Herwig Jansen, Wonsuk Chang, Gary H. Posner, Rudolf Bauer, and Thomas Efferth. 2008. 'Effect of artemisinins and other endoperoxides on nitric oxide-related signaling pathway in RAW 264.7 mouse macrophage cells', *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society*, 19: 184-91.

Kumar, Shashank, and Abhay K. Pandey. 2013. 'Chemistry and Biological Activities of Flavonoids: An Overview', *The Scientific World Journal*, 2013: 162750.

Kyaw, Shwe Sin, Tom Drake, Ronatrai Ruangveerayuth, Wirongrong Chierakul, Nicholas J. White, Paul N. Newton, and Yoel Lubell. 2014. 'Cost of treating inpatient falciparum malaria on the Thai-Myanmar border', *Malaria Journal*, 13: 416.

Lau, Aik Jiang, and Thomas K. H. Chang. 2009. 'Inhibition of Human CYP2B6-Catalyzed Bupropion Hydroxylation by Ginkgo biloba Extract: Effect of Terpene Trilactones and Flavonols', *Drug Metabolism and Disposition*, 37: 1931.

Lawrence, Toby. 2009. 'The nuclear factor NF-κB pathway in inflammation', *Cold Spring Harbor perspectives in biology*, 1: a001651.

Lee, Ik-Soo, and Charles D. Hufford. 1990. 'Metabolism of antimalarial sesquiterpene lactones', *PHARMACOLOGY & THERAPEUTICS*, 48: 345-55.

Lee, Sung Ho, Young-Chang Cho, Kyung Hee Kim, Ik-Soo Lee, Hyun Jin Choi, and Bok Yun Kang. 2014. 'Artesunate inhibits proliferation of naïve CD4+ T cells but enhances function of effector T cells', *Archives of Pharmacal Research*: 1-9.

Lehane, Adele M, and Kevin J Saliba. 2008. 'Common dietary flavonoids inhibit the growth of the intraerythrocytic malaria parasite', *BMC Research Notes*, 1: 26.

Li, Bin, Rong Zhang, Jun Li, Lezhi Zhang, Guofu Ding, Ping Luo, Shengqi He, Yan Dong, Weiwei Jiang, Yongling Lu, Hongwei Cao, Jiang Zheng, and Hong Zhou. 2008. 'Antimalarial artesunate protects sepsis model mice against heat-killed Escherichia coli challenge by decreasing TLR4, TLR9 mRNA

expressions and transcription factor NF-κB activation', *International Immunopharmacology*, 8: 379-89.

Li, Jing, Chao Zhang, Muxin Gong, and Manyuan Wang. 2018. 'Combination of artemisinin-based natural compounds from Artemisia annua L. for the treatment of malaria: Pharmacodynamic and pharmacokinetic studies', *Phytotherapy Research*, 32: 1415-20.

Li, Shi-You, Cong Chen, Hai-qing Zhang, Hai-yan Guo, Hui Wang, Lin Wang, Xiang Zhang, Shi-neng Hua, Jun Yu, Pei-gen Xiao, Rong-song Li, and Xuehai Tan. 2005. 'Identification of natural compounds with antiviral activities against SARS-associated coronavirus', *Antiviral Research*, 67: 18-23.

Li, Xiuguo, and Jun-Shik Choi. 2007. 'Effect of genistein on the pharmacokinetics of paclitaxel administered orally or intravenously in rats', *International Journal of Pharmaceutics*, 337: 188-93.

Li, Yu-Jie, Yan Guo, Qing Yang, Xiao-Gang Weng, Lan Yang, Ya-Jie Wang, Ying Chen, Dong Zhang, Qi Li, Xu-Cen Liu, Xiao-Xi Kan, Xi Chen, Xiao-Xin Zhu, Eva Kmoníèková, and Zdenìk Zídek. 2015. 'Flavonoids casticin and chrysosplenol D from Artemisia annua L. inhibit inflammation in vitro and in vivo', *Toxicology and Applied Pharmacology*, 286: 151-58.

Littrell, Megan, Hellen Gatakaa, Illah Evance, Stephen Poyer, Julius Njogu, Tsione Solomon, Erik Munroe, Steven Chapman, Catherine Goodman, Kara Hanson, Cyprien Zinsou, Louis Akulayi, Jacky Raharinjatovo, Ekundayo Arogundade, Peter Buyungo, Felton Mpasela, Cherifatou Bello Adjibabi, Jean Angbalu Agbango, Benjamin Fanomezana Ramarosandratana, Babajide Coker, Denis Rubahika, Busiku Hamainza, Tanya Shewchuk, Desmond Chavasse, and Kathryn A. O'Connell. 2011. 'Monitoring fever treatment behaviour and equitable access to effective medicines in the context of initiatives to improve ACT access: baseline results and implications for programming in six African countries', *Malaria Journal*, 10: 327-27.

Liu, K. Chiung-Sheue Chen, Shi-Lin Yang, M. F. Roberts, B. C. Elford, and J. D. Phillipson. 1992. 'Antimalarial activity of Artemisia annua flavonoids from whole plants and cell cultures', *Plant Cell Reports*, 11: 637-40.

Liu, Rong, Zhengjun Cheng, and Xiaohui Jiang. 2014. 'Comparative studies on the interactions of dihydroartemisinin and artemisinin with bovine serum albumin using spectroscopic methods', *Luminescence*, 29: 1033-46.

Liu, Shuqing, Chunmei Guo, Yimeng Guo, Hongshan Yu, Frederick Greenaway, and Ming-Zhong Sun. 2014. 'Comparative Binding Affinities of Flavonoid Phytochemicals with Bovine Serum Albumin', *Iranian journal of pharmaceutical research: IJPR*, 13: 1019.

Lubbe, Andrea, Isabell Seibert, Thomas Klimkait, and Frank van der Kooy. 2012. 'Ethnopharmacology in overdrive: The remarkable anti-HIV activity of Artemisia annua', *Journal of Ethnopharmacology*, 141: 854-59.

Maldonado-Valderrama, Julia, Pete Wilde, Adam Macierzanka, and Alan Mackie. 2011. 'The role of bile salts in digestion', *Advances in Colloid and Interface Science*, 165: 36-46.

Mannan, Abdul, Ibrar Ahmed, Waheed Arshad, Muhammad F. Asim, Rizwana A. Qureshi, Izhar Hussain, and Bushra Mirza. 2010. 'Survey of artemisinin production by diverse Artemisia species in northern Pakistan', *Malaria Journal*, 9: 310-10.

Massey-Harroche, Dominique. 2000. 'Epithelial cell polarity as reflected in enterocytes', *Microscopy Research and Technique*, 49: 353-62.

Medhi, B., S. Patyar, R. S. Rao, P. Byrav Ds, and A. Prakash. 2009. 'Pharmacokinetic and Toxicological Profile of Artemisinin Compounds: An Update', *Pharmacology*, 84: 323-32.

Melillo de Magalhães, Pedro, Isabelle Dupont, Aurélie Hendrickx, Aurélie Joly, Thomas Raas, Stéphanie Dessy, Thérèse Sergent, and Yves-Jacques Schneider. 2012. 'Anti-inflammatory effect and modulation of cytochrome P450 activities by Artemisia annua tea infusions in human intestinal Caco-2 cells', *Food Chemistry*, 134: 864-71.

Meunier, Bernard, and Anne Robert. 2010. 'Heme as Trigger and Target for Trioxane-Containing Antimalarial Drugs', *Accounts of Chemical Research*, 43: 1444-51.
Mihara, Kazuo, Ulrika S. H. Svensson, Gunnel Tybring, Trinh Ngoc Hai, Leif Bertilsson, and Michael Ashton. 1999. 'Stereospecific analysis of omeprazole supports artemisinin as a potent inducer of CYP2C19', *Fundamental & Clinical Pharmacology*, 13: 671-75.
Miller, R. L., S. Ikram, G. J. Armelagos, R. Walker, W. B. Harer, C. J. Shiff, D. Baggett, M. Carrigan, and S. M. Maret. 1994. 'Diagnosis of Plasmodium falciparum infections in mummies using the rapid manual ParaSight™-F test', *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 88: 31-32.
Mouton, Julia, Olivia Jansen, Michel Frédérich, and Frank van der Kooy. 2013. 'Is artemisinin the only antiplasmodial compound in the Artemisia annua tea infusion? An in vitro study', *Planta Medica*, 79: 468-70.
Mueller, Markus S., I. B. Karhagomba, H. M. Hirt, and E. Wemakor. 2000. 'The potential of Artemisia annua L. as a locally produced remedy for malaria in the tropics: agricultural, chemical and clinical aspects', *Journal of Ethnopharmacology*, 73: 487-93.
Mueller, Markus S., Nyabuhanga Runyambo, Irmela Wagner, Steffen Borrmann, Klaus Dietz, and Lutz Heide. 2004. 'Randomized controlled trial of a traditional preparation of Artemisia annua L. (Annual Wormwood) in the treatment of malaria', *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 98: 318-21.
Munyangi, Jérôme, Lucile Cornet-Vernet, Michel Idumbo, Chen Lu, Pierre Lutgen, Christian Perronne, Nadège Ngombe, Jacques Bianga, Bavon Mupenda, Paul Lalukala, Guy Mergeai, Dieudonné Mumba, Melissa Towler, and Pamela J. Weathers. 2019. 'Artemisia annua and Artemisia afra tea infusions vs. artesunate-amodiaquine (ASAQ) in treating Plasmodium falciparum malaria in a large scale, double blind, randomized clinical trial', *Phytomedicine*, 57: 49-56.
Munyangi, Jérôme, Lucile Cornet-Vernet, Michel Idumbo, Chen Lu, Pierre Lutgen, Christian Perronne, Nadège Ngombe, Jacques Bianga, Bavon Mupenda, Paula Lalukala, Guy Mergeai, Dieudonné Mumba, Melissa Towler, and Pamela Weathers. 2018. 'Effect of Artemisia annua and Artemisia afra tea infusions on schistosomiasis in a large clinical trial', *Phytomedicine*, 51: 233-40.
Murakami, Teruo, and Mikihisa Takano. 2008. 'Intestinal efflux transporters and drug absorption', *Expert opinion on drug metabolism & toxicology*, 4: 923-39.
Nam, Nguyen-Hai. 2006. 'Naturally occurring NF-κB inhibitors', *Mini reviews in medicinal chemistry*, 6: 945-51.
Naß, Janine, and Thomas Efferth. 2018. 'The activity of Artemisia spp. and their constituents against Trypanosomiasis', *Phytomedicine*, 47: 184-91.
Navaratnam, Visweswaran, Sharif Mahsufi Mansor, Nam-Weng Sit, James Grace, Qigui Li, and Piero Olliaro. 2000. 'Pharmacokinetics of artemisinin-type compounds', *Clinical pharmacokinetics*, 39: 255-70.
Newton, Kim, and Vishva M. Dixit. 2012. 'Signaling in innate immunity and inflammation', *Cold Spring Harbor perspectives in biology*, 4: a006049.
Nowakowski-Gashaw, Isabella, Przemyslaw M. Mrozikiewicz, Ivar Roots, and Jürgen Brockmöller. 2002. 'Rapid Quantification of CYP3A4 Expression in Human Leukocytes by Real-Time Reverse Transcription-PCR', *Clinical Chemistry*, 48: 366.
Numonov, Sodik, Farukh Sharopov, Aminjon Salimov, Parviz Sukhrobov, Sunbula Atolikshoeva, Ramazon Safarzoda, Maidina Habasi, and Haji Akber Aisa. 2019. 'Assessment of Artemisinin Contents in Selected Artemisia Species from Tajikistan (Central Asia)', *Medicines (Basel, Switzerland)*, 6: 23.
O'Connell, Kathryn A., Hellen Gatakaa, Stephen Poyer, Julius Njogu, Illah Evance, Erik Munroe, Tsione Solomon, Catherine Goodman, Kara Hanson, Cyprien Zinsou, Louis Akulayi, Jacky Raharinjatovo, Ekundayo Arogundade, Peter Buyungo, Felton Mpasela, Chérifatou Bello Adjibabi, Jean Angbalu

Agbango, Benjamin Fanomezana Ramarosandratana, Babajide Coker, Denis Rubahika, Busiku Hamainza, Steven Chapman, Tanya Shewchuk, and Desmond Chavasse. 2011. 'Got ACTs? Availability, price, market share and provider knowledge of anti-malarial medicines in public and private sector outlets in six malaria-endemic countries', *Malaria Journal*, 10: 326-26.

O'Neill, Paul M., and Gary H. Posner. 2004. 'A Medicinal Chemistry Perspective on Artemisinin and Related Endoperoxides', *Journal of Medicinal Chemistry*, 47: 2945-64.

Okell, Lucy C., Matthew Cairns, Jamie T. Griffin, Neil M. Ferguson, Joel Tarning, George Jagoe, Pierre Hugo, Mark Baker, Umberto D'Alessandro, Teun Bousema, David Ubben, and Azra C. Ghani. 2014. 'Contrasting benefits of different artemisinin combination therapies as first-line malaria treatments using model-based cost-effectiveness analysis', *Nature Communications*, 5: 5606.

Olsson, Mikael E., Linda M. Olofsson, Ann-Louise Lindahl, Anneli Lundgren, Maria Brodelius, and Peter E. Brodelius. 2009. 'Localization of enzymes of artemisinin biosynthesis to the apical cells of glandular secretory trichomes of Artemisia annua L', *Phytochemistry*, 70: 1123-28.

Otterbein, Leo E., Fritz H. Bach, Jawed Alam, Miguel Soares, Hong Tao Lu, Mark Wysk, Roger J. Davis, Richard A. Flavell, and Augustine M. K. Choi. 2000. 'Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway', *Nature medicine*, 6: 422.

Ozawa, Tatsuya, Kazuo Takayama, Ryota Okamoto, Ryosuke Negoro, Fuminori Sakurai, Masashi Tachibana, Kenji Kawabata, and Hiroyuki Mizuguchi. 2015. 'Generation of enterocyte-like cells from human induced pluripotent stem cells for drug absorption and metabolism studies in human small intestine', *Scientific Reports*, 5: 16479-79.

Paine, Mary F., Heather L. Hart, Shana S. Ludington, Robert L. Haining, Allan E. Rettie, and Darryl C. Zeldin. 2006. 'The human intestinal cytochrome P450 "pie"', *Drug metabolism and disposition: the biological fate of chemicals*, 34: 880-86.

Papadopoulou, Athina, Rebecca J. Green, and Richard A. Frazier. 2005. 'Interaction of flavonoids with bovine serum albumin: a fluorescence quenching study', *Journal of agricultural and food chemistry*, 53: 158-63.

Penna-Coutinho, Julia, Anna Cc Aguiar, and Antoniana Ursine Krettli. 2018. 'Commercial drugs containing flavonoids are active in mice with malaria and in vitro against chloroquine-resistant Plasmodium falciparum', *Memorias do Instituto Oswaldo Cruz*, 113: e180279-e79.

Ploypradith, Poonsakdi. 2004. 'Development of artemisinin and its structurally simplified trioxane derivatives as antimalarial drugs', *Acta Tropica*, 89: 329-42.

Pond, Susan M., and Thomas N. Tozer. 1984. 'First-Pass Elimination Basic Concepts and Clinical Consequences', *Clinical pharmacokinetics*, 9: 1-25.

Preissner, Sarah C., Michael F. Hoffmann, Robert Preissner, Mathias Dunkel, Andreas Gewiess, and Saskia Preissner. 2013. 'Polymorphic cytochrome P450 enzymes (CYPs) and their role in personalized therapy', *PLOS one*, 8: e82562-e62.

Pullakhandam, Raghu, and Mark L. Failla. 2007. 'Micellarization and intestinal cell uptake of β-carotene and lutein from drumstick (Moringa oleifera) leaves', *Journal of medicinal food*, 10: 252-57.

Ramacher, Marcel, Viktor Umansky, and Thomas Efferth. 2009. 'Effect of artesunate on immune cells in ret-transgenic mouse melanoma model', *Anti-cancer drugs*, 20: 910-17.

Rastogi, Himanshu, Jakir Pinjari, Pradnya Honrao, Suneel Praband, and Ramesh Somani. 2013. 'The impact of permeability enhancers on assessment for monolayer of colon adenocarcinoma cell line (CACO-2) used in in vitro permeability assay', *Journal of Drug Delivery and Therapeutics*, 3: 20-29.

Räth, Karin, Katja Taxis, Gitta Walz, Christoph H. Gleiter, Shu-Ming Li, and Lutz Heide. 2004. 'Pharmacokinetic study of artemisinin after oral intake of a traditional preparation of Artemisia annua L.(annual wormwood)', *The American journal of tropical medicine and hygiene*, 70: 128-32.

Rathee, Permender, Hema Chaudhary, Sushila Rathee, Dharmender Rathee, Vikash Kumar, and Kanchan Kohli. 2009. 'Mechanism of action of flavonoids as anti-inflammatory agents: a review', *Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy)*, 8: 229-35.

Reichardt, Christian, and Thomas Welton. 2011. *Solvents and solvent effects in organic chemistry* (John Wiley & Sons).

Russo, Emilio, Francesca Scicchitano, Benjamin J. Whalley, Carmela Mazzitello, Miriam Ciriaco, Stefania Esposito, Marinella Patanè, Roy Upton, Michela Pugliese, Serafina Chimirri, Maria Mammì, Caterina Palleria, and Giovambattista De Sarro. 2014. 'Hypericum perforatum: Pharmacokinetic, Mechanism of Action, Tolerability, and Clinical Drug–Drug Interactions', *Phytotherapy Research*, 28: 643-55.

Sachs, Jeffrey, and Pia Malaney. 2002. 'The economic and social burden of malaria', *Nature*, 415: 680-85.

Savjani, Ketan T., Anuradha K. Gajjar, and Jignasa K. Savjani. 2012. 'Drug solubility: importance and enhancement techniques', *ISRN pharmaceutics*, 2012: 195727-27.

Schmiedlin-Ren, Phyllissa, Kenneth E. Thummel, Jeannine M. Fisher, Mary F. Paine, and Paul B. Watkins. 2001. 'Induction of CYP3A4 by 1α,25-Dihydroxyvitamin D3 Is Human Cell Line-Specific and Is Unlikely to Involve Pregnane X Receptor', *Drug Metabolism and Disposition*, 29: 1446-53.

Scholz-Ahrens, Katharina E., and Jürgen Schrezenmeir. 2007. 'Inulin and Oligofructose and Mineral Metabolism: The Evidence from Animal Trials', *The Journal of Nutrition*, 137: 2513S-23S.

Seo, Kyung-Ah, Hyunmi Kim, Hei-Young Ku, Hee-Jeong Ahn, Soo-Jin Park, Soo Kyung Bae, Jae-Gook Shin, and Kwang-Hyeon Liu. 2008. 'The monoterpenoids citral and geraniol are moderate inhibitors of CYP2B6 hydroxylase activity', *Chemico-Biological Interactions*, 174: 141-46.

Sergent, Thérèse, Isabelle Dupont, Edwige Van Der Heiden, Marie-Louise Scippo, Luc Pussemier, Yvan Larondelle, and Yves-Jacques Schneider. 2009. 'CYP1A1 and CYP3A4 modulation by dietary flavonoids in human intestinal Caco-2 cells', *Toxicology Letters*, 191: 216-22.

Sergent, Thérèse, Serge Garsou, Annelore Schaut, Sarah De Saeger, Luc Pussemier, Carlos Van Peteghem, Yvan Larondelle, and Yves-Jacques Schneider. 2005. 'Differential modulation of ochratoxin A absorption across Caco-2 cells by dietary polyphenols, used at realistic intestinal concentrations', *Toxicology Letters*, 159: 60-70.

Shakir, Lubna, Muzammal Hussain, Aqeel Javeed, Muhammad Ashraf, and Amjad Riaz. 2011. 'Artemisinins and immune system', *European Journal of Pharmacology*, 668: 6-14.

Shampo, Marc A., and Robert A. Kyle. 1989. 'Nei Ching—Oldest Known Medical Book', *Mayo Clinic Proceedings*, 64: 134.

Silva, Luiz Francisco Rocha e, Pedro Melillo de Magalhães, Mônica Regina Farias Costa, Maria das Graças Costa Alecrim, Francisco Célio Maia Chaves, Ari de Freitas Hidalgo, Adrian Martin Pohlit, and Pedro Paulo Ribeiro Vieira. 2012. 'In vitro susceptibility of Plasmodium falciparum Welch field isolates to infusions prepared from Artemisia annua L. cultivated in the Brazilian Amazon', *Memórias do Instituto Oswaldo Cruz*, 107: 859-66.

Simonsson, Ulrika S. H., Britt Jansson, Trinh Ngoc Hai, Dinh Xuan Huong, Gunnel Tybring, and Michael Ashton. 2003. 'Artemisinin autoinduction is caused by involvement of cytochrome P450 2B6 but not 2C9', *Clinical Pharmacology & Therapeutics*, 74: 32-43.

Simonsson, Ulrika S. H., M. Lindell, F. Raffalli-Mathieu, A. Lannerbro, P. Honkakoski, and M. A. Lang. 2006. 'In vivo and mechanistic evidence of nuclear receptor CAR induction by artemisinin', *European Journal of Clinical Investigation*, 36: 647-53.

Singh, Amrinder, and Kaicun Zhao. 2017. 'Chapter Nine - Herb–Drug Interactions of Commonly Used Chinese Medicinal Herbs.' in Bai-Yun Zeng and Kaicun Zhao (eds.), *International Review of Neurobiology* (Academic Press).

Song, Nai-Ning, Shao-Yu Zhang, and Chang-Xiao Liu. 2004. 'Overview of factors affecting oral drug absorption', *Asian J Drug Metab Pharmacokinet*, 4: 167-76.

Suberu, John O., Alexander P. Gorka, Lauren Jacobs, Paul D. Roepe, Neil Sullivan, Guy C. Barker, and Alexei A. Lapkin. 2013. 'Anti-Plasmodial Polyvalent Interactions in Artemisia annua L. Aqueous Extract – Possible Synergistic and Resistance Mechanisms', *PLOS one*, 8: e80790.

Svensson, Ulrika S. H., and Michael Ashton. 1999. 'Identification of the human cytochrome P450 enzymes involved in the in vitro metabolism of artemisinin', *British Journal of Clinical Pharmacology*, 48: 528-35.

Svensson, Ulrika S. H., Rikard Sandström, Örjan Carlborg, Hans Lennernäs, and Michael Ashton. 1999. 'High In Situ Rat Intestinal Permeability of Artemisinin Unaffected by Multiple Dosing and with No Evidence of P-glycoprotein Involvement', *Drug Metabolism and Disposition*, 27: 227.

Tatfeng, Y. M., J. C. Ihongbe, M. Okodua, F. Oviasogie, J. Isibor, S. Tchougang, E. Tambo, and T. Otegbeye. 2007. 'CD4 count, viral load and parasite density of HIV positive individuals undergoing malaria treatment with dihydroartemisinin in Benin City, Edo state, Nigeria', *Journal of vector borne diseases*, 44: 111.

Tchandema, Constant Kansongo, RDCongo Lubumbashi, and Pierre Lutgen. 2016. 'In vivo trials on the therapeutic effects of encapsulated Artemisia annua and Artemisia afra', *Global J Res Anal*, 5: 228-34.

Teklehaimanot, Awash, and Paola Mejia. 2008. 'Malaria and Poverty', *Annals of the New York Academy of Sciences*, 1136: 32-37.

Tellez, Mario R., Camilo Canel, Agnes M. Rimando, and Stephen O. Duke. 1999. 'Differential accumulation of isoprenoids in glanded and glandless *Artemisia annua* L', *Phytochemistry*, 52: 1035-40.

Thakkar, P. Hetal. 2015. 'Influence of excipients on drug absorption via modulation of intestinal transporters activity', *Asian Journal of Pharmaceutics (AJP): Free full text articles from Asian J Pharm*, 9: 69-82.

Tilley, Leann, Judith Straimer, Nina F. Gnädig, Stuart A. Ralph, and David A. Fidock. 2016. 'Artemisinin Action and Resistance in Plasmodium falciparum', *Trends in Parasitology*, 32: 682-96.

Timsit, Yoav E., and Masahiko Negishi. 2007. 'CAR and PXR: the xenobiotic-sensing receptors', *Steroids*, 72: 231-46.

Titulaer, H. A. C., J. Zuidema, P. A. Kager, Jcfm Wetsteyn, Ch B. Lugt, and Fwhm Merkus. 1990. 'The pharmacokinetics of artemisinin after oral, intramuscular and rectal administration to volunteers', *Journal of Pharmacy and Pharmacology*, 42: 810-13.

Tougher, Sarah, Yazoume Ye, John H. Amuasi, Idrissa A. Kourgueni, Rebecca Thomson, Catherine Goodman, Andrea G. Mann, Ruilin Ren, Barbara A. Willey, Catherine A. Adegoke, Abdinasir Amin, Daniel Ansong, Katia Bruxvoort, Diadier A. Diallo, Graciela Diap, Charles Festo, Boniface Johanes, Elizabeth Juma, Admirabilis Kalolella, Oumarou Malam, Blessing Mberu, Salif Ndiaye, Samuel B. Nguah, Moctar Seydou, Mark Taylor, Sergio Torres Rueda, Marilyn Wamukoya, Fred Arnold, and Kara Hanson. 2012. 'Effect of the Affordable Medicines Facility—malaria (AMFm) on the availability, price, and market share of quality-assured artemisinin-based combination therapies in seven countries: a before-and-after analysis of outlet survey data', *The Lancet*, 380: 1916-26.

Towler, Melissa J., and Pamela J. Weathers. 2015. 'Variations in key artemisinic and other metabolites throughout plant development in Artemisia annua L. for potential therapeutic use', *Industrial Crops and Products*, 67: 185-91.

Tu, Youyou. 2016. 'Artemisinin—A Gift from Traditional Chinese Medicine to the World (Nobel Lecture)', *Angewandte Chemie International Edition*, 55: 10210-26.

Tzenkova, R., Z. Kamenarska, A. Draganov, and A. Atanassov. 2010. 'Composition of Artemisia Annua Essential Oil Obtained from Species Growing Wild in Bulgaria', *Biotechnology & Biotechnological Equipment*, 24: 1833-35.

van Agtmael, M. A., V. Gupta, T. H. van der Wösten, J. P. B. Rutten, and C. J. van Boxtel. 1999. 'Grapefruit juice increases the bioavailability of artemether', *European Journal of Clinical Pharmacology*, 55: 405-10.

van Breemen, Richard B., and Yongmei Li. 2005. 'Caco-2 cell permeability assays to measure drug absorption', *Expert opinion on drug metabolism & toxicology*, 1: 175-85.

van der Kooy, Frank, and Shaun Edward Sullivan. 2013. 'The complexity of medicinal plants: The traditional Artemisia annua formulation, current status and future perspectives', *Journal of Ethnopharmacology*, 150: 1-13.

van der Kooy, Frank, and Robert Verpoorte. 2011. 'The Content of Artemisinin in the Artemisia annua Tea Infusion', *Planta Med*, 77: 1754-56.

Van Zyl, Robyn L., Sammy T. Seatlholo, Sandy F. Van Vuuren, and Alvaro M. Viljoen. 2006. 'The biological activities of 20 nature identical essential oil constituents', *The Journal of Essential Oil Research*, 18: 129-33.

Viswanathan, Pragasam, Yuvashree Muralidaran, and Gokulakannan Ragavan. 2017. 'Chapter 7 - Challenges in oral drug delivery: a nano-based strategy to overcome.' in Ecaterina Andronescu and Alexandru Mihai Grumezescu (eds.), *Nanostructures for Oral Medicine* (Elsevier).

Wall, Monroe E., and Mansukh C. Wani. 1996. 'Camptothecin and taxol: from discovery to clinic', *Journal of Ethnopharmacology*, 51: 239-54.

Wan, Yao-De, Qi-Zhong Zang, and Jie-Sheng Wang. 1992. 'Studies on the antimalarial action of gelatin capsule of Artemisia annua', *Chinese journal of parasitology & parasitic diseases*, 10: 290-94.

Wang, Hong-Jaan, Li-Heng Pao, Cheng-Huei Hsiong, Tung-Yuan Shih, Meei-Shyuan Lee, and Oliver Yoa-Pu Hu. 2014. 'Dietary Flavonoids Modulate CYP2C to Improve Drug Oral Bioavailability and Their Qualitative/Quantitative Structure–Activity Relationship', *The AAPS Journal*, 16: 258-68.

Wang, Jun-Xia, Li-fei Hou, Yang Yang, Wei Tang, Ying Li, and Jian-ping Zuo. 2009. 'SM905, an artemisinin derivative, inhibited NO and pro-inflammatory cytokine production by suppressing MAPK and NF-[kappa]B pathways in RAW 264.7 macrophages', *Acta Pharmacol Sin*, 30: 1428-35.

Wang, Jun-Xia, W. Tang, L. P. Shi, J. Wan, R. Zhou, J. Ni, Y. F. Fu, Y. F. Yang, Y. Li, and J. P. Zuo. 2007. 'Investigation of the immunosuppressive activity of artemether on T-cell activation and proliferation', *British Journal of Pharmacology*, 150: 652-61.

Wang, Jun, Hong Zhou, Jiang Zheng, Juan Cheng, Wei Liu, Guofu Ding, Liangxi Wang, Ping Luo, Yongling Lu, Hongwei Cao, Shuangjiang Yu, Bin Li, and Lezhi Zhang. 2006. 'The Antimalarial Artemisinin Synergizes with Antibiotics To Protect against Lethal Live Escherichia coli Challenge by Decreasing Proinflammatory Cytokine Release', *Antimicrobial Agents and Chemotherapy*, 50: 2420-27.

Wang, Ke Si, Junbo Li, Zhe Wang, Chunliu Mi, Juan Ma, Lian Xun Piao, Guang Hua Xu, Xuezheng Li, and Xuejun Jin. 2017. 'Artemisinin inhibits inflammatory response via regulating NF-κB and MAPK signaling pathways', *Immunopharmacology and immunotoxicology*, 39: 28-36.

Waring, Michael J., John Arrowsmith, Andrew R. Leach, Paul D. Leeson, Sam Mandrell, Robert M. Owen, Garry Pairaudeau, William D. Pennie, Stephen D. Pickett, Jibo Wang, Owen Wallace, and Alex Weir. 2015. 'An analysis of the attrition of drug candidates from four major pharmaceutical companies', *Nature Reviews Drug Discovery*, 14: 475-86.

Weathers, Pamela J., Patrick R. Arsenault, Patrick S. Covello, Anthony McMickle, Keat H. Teoh, and Darwin W. Reed. 2011. 'Artemisinin production in Artemisia annua: studies in planta and results of a novel delivery method for treating malaria and other neglected diseases', *Phytochemistry Reviews*, 10: 173-83.

Weathers, Pamela J., Hailey M. Cambra, Matthew R. Desrosiers, Dina Rassias, and Melissa J. Towler. 2017. 'Artemisinin the Nobel Molecule: From Plant to Patient', *Studies in Natural Products Chemistry*, 52: 193-229.

Weathers, Pamela J., Mostafa A. Elfawal, Melissa J. Towler, George K. Acquaah-Mensah, and Stephen M. Rich. 2014. 'Pharmacokinetics of artemisinin delivered by oral consumption of Artemisia annua dried leaves in healthy vs. Plasmodium chabaudi-infected mice', *Journal of Ethnopharmacology*, 153: 732-36.

Weathers, Pamela J., Nikole J. Jordan, Praphapan Lasin, and Melissa J. Towler. 2014. 'Simulated digestion of dried leaves of Artemisia annua consumed as a treatment (pACT) for malaria', *Journal of Ethnopharmacology*, 151: 858-63.

Weathers, Pamela J., and Melissa J. Towler. 2012. 'The flavonoids casticin and artemetin are poorly extracted and are unstable in an Artemisia annua tea infusion', *Planta Medica*, 78: 1024-26.

Weathers, Pamela J., and Melissa J. Towler. 2014. 'Changes in key constituents of clonally propagated Artemisia annua L. during preparation of compressed leaf tablets for possible therapeutic use', *Industrial Crops and Products*, 62: 173-78.

Wei, Shijie, Hongyan Ji, Bei Yang, Liping Ma, Zhuchun Bei, Xiang Li, Hongwan Dang, Xiaoying Yang, Cheng Liu, Xiuli Wu, and Jing Chen. 2015. 'Impact of chrysosplenetin on the pharmacokinetics and anti-malarial efficacy of artemisinin against Plasmodium berghei as well as in vitro CYP450 enzymatic activities in rat liver microsome', *Malaria Journal*, 14: 432.

WHO. 2012a. 'Factsheet on the World Malaria Report 2012', World Health Organization. http://www.who.int/malaria/media/world_malaria_report_2012_facts/en/.

WHO. 2012b. 'WHO Position Statement. Effectiveness of Non-Pharmaceutical Forms of *Artemisia annua* L. against malaria'. https://www.who.int/malaria/diagnosis_treatment/position_statement_herbal_remedy_artemisia_annua_l.pdf?ua=1.

WHO. 2012c. "World malaria report 2012." In. Geneva.

WHO. 2015. "Guidelines for the treatment of malaria." In. Geneva, Switzerland: World Health Organization.

WHO. 2016. 'Malaria Fact Sheet', World Health Organization. http://www.who.int/mediacentre/factsheets/fs094/en/.

WHO. 2019a. "The use of non-pharmaceutical forms of Artemisia." In.: World Health Organization.

WHO. 2019b. 'WHO | Malaria', World Health Organization. http://www.who.int/mediacentre/factsheets/fs094/en/.

WHO. 2019c. "World malaria report 2019." In. Geneva.

Willcox, Merlin, Gerard Bodeker, Philippe Rasoanaivo, and Jonathan Addae-Kyereme. 2004. *Traditional medicinal plants and malaria* (CRC press).

Wright, Colin W., Peter A. Linley, Reto Brun, Sergio Wittlin, and Elisabeth Hsu. 2010. 'Ancient Chinese methods are remarkably effective for the preparation of artemisinin-rich extracts of Qing Hao with potent antimalarial activity', *Molecules*, 15: 804-12.

Xiao, Ling, Hexin Tan, and Lei Zhang. 2016. 'Artemisia annua glandular secretory trichomes: the biofactory of antimalarial agent artemisinin', *Science Bulletin*, 61: 26-36.

Xing, Jie, Brian J. Kirby, Dale Whittington, Yakun Wan, and David R. Goodlett. 2012. 'Evaluation of P450 Inhibition and Induction by Artemisinin Antimalarials in Human Liver Microsomes and Primary Human Hepatocytes', *Drug Metabolism and Disposition*, 40: 1757.

Xinyi, Niu, H. O. Liyi, Ren Zhihong, and Song Zhenyu. 1985. 'Metabolic fate of Qinghaosu in rats; a new TLC densitometric method for its determination in biological material', *European Journal of Drug Metabolism and Pharmacokinetics*, 10: 55-59.

Xu, H., Y. He, X. Yang, L. Liang, Z. Zhan, Y. Ye, F. Lian, and L. Sun. 2007. 'Anti-malarial agent artesunate inhibits TNF-α-induced production of proinflammatory cytokines via inhibition of NF-κB and PI3 kinase/Akt signal pathway in human rheumatoid arthritis fibroblast-like synoviocytes', *Rheumatology*, 46: 920-26.

Yano, Koji, Yoshie Masaoka, Makoto Kataoka, Shinji Sakuma, and Shinji Yamashita. 2010. 'Mechanisms of Membrane Transport of Poorly Soluble Drugs: Role of Micelles in Oral Absorption Processes', *Journal of Pharmaceutical Sciences*, 99: 1336-45.

Yeung, Shunmay, Wim Van Damme, Duong Socheat, Nicholas J. White, and Anne Mills. 2008. 'Cost of increasing access to artemisinin combination therapy: the Cambodian experience', *Malaria Journal*, 7: 84.

Zaid, O. I., R. Abd Majid, M. S. Hasidah, M. N. Sabariah, Rahi Sattar, and R. Basir. 2016. 'Antiplasmodial and chloroquine chemosensitizing and resistance reversal effects of coumarin derivatives against Plasmodium falciparum 3D7 and K1', *Tropical Biomedicine*, 33: 14-26.

Zang, Meitong, Fanping Zhu, Xinxiu Li, Aijuan Yang, and Jie Xing. 2014. 'Auto-induction of phase I and phase II metabolism of artemisinin in healthy Chinese subjects after oral administration of a new artemisinin-piperaquine fixed combination', *Malaria Journal*, 13: 214.

Zhang, Chao, Mu-Xin Gong, Feng Qiu, Jing Li, and Man-Yuan Wang. 2016. 'Effects of arteannuin B, arteannuic acid and scopoletin on pharmacokinetics of artemisinin in mice', *Asian Pacific Journal of Tropical Medicine*, 9: 677-81.

Zhao, Qing, Xin Luan, Min Zheng, Xin-Hui Tian, Jing Zhao, Wei-Dong Zhang, and Bing-Liang Ma. 2020. 'Synergistic Mechanisms of Constituents in Herbal Extracts during Intestinal Absorption: Focus on Natural Occurring Nanoparticles', *Pharmaceutics*, 12: 128.

Zhu, Cansheng, Zhaojun Xiong, Xiaohong Chen, Fuhua Peng, Xueqiang Hu, Yanming Chen, and Qing Wang. 2012. 'Artemisinin Attenuates Lipopolysaccharide-Stimulated Proinflammatory Responses by Inhibiting NF-κB Pathway in Microglia Cells', *PLOS one*, 7: e35125.

Appendix A: Supplementary Material for Chapter 3

Published in:

Desrosiers M. and Weathers P.J., 2016. Oral consumption of dried *Artemisia annua* leaves to treat malaria: leaf digestion and artemisinin solubility. Journal of Ethnopharmacology 190: 313-318.

Taste test of *Artemisia annua* (SAM cultivar) tea or leaves by visiting adults and children. Outreach activity was held at a Touch Tomorrow event at WPI (Worcester, MA, USA) on June 13, 2015. Visitors were invited to either taste a piece of fresh leaves of the plant or sip a small cup of tea brewed from the dried leaves. Afterwards they placed a green sticky circle above the facial expression,  ,  ,  , on a large segment of papered wall (see photo below) that best exemplified their response to the taste of the leaves or tea. Comments were encouraged and written on the large page to the far right of the facial span. Although not statistically tracked, there was no apparent difference between children and adults, nor linkage between related individuals, e.g. parents and children. Table S1 shows that about 23% of the tasters actually thought the plant tasted good, while 61% thought the taste was either unpleasant or terrible. About 15% were indifferent.

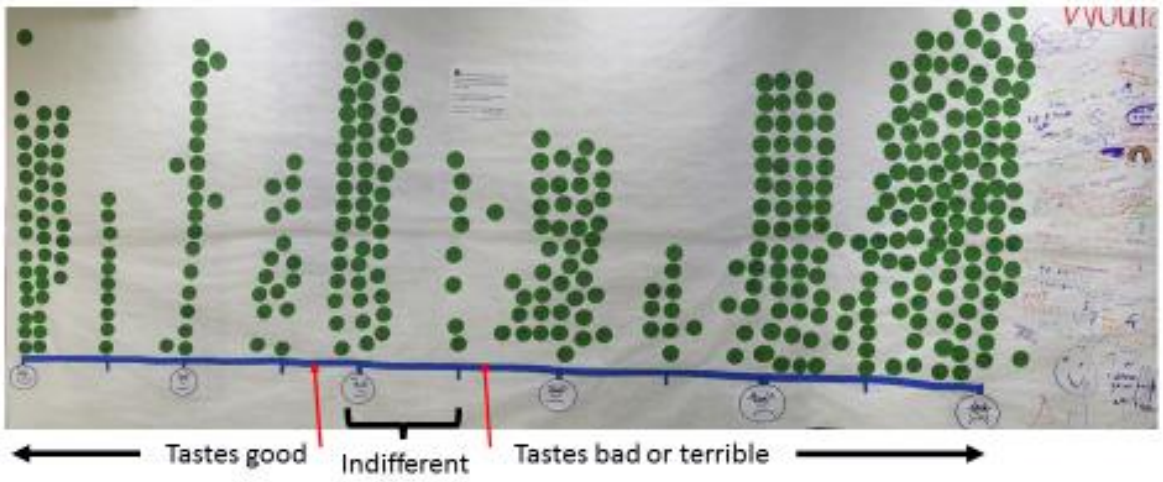

Figure S1. Picture of responses to *A. annua* taste test at Touch Tomorrow 2015 at WPI.

Table S1. Tasters vs. nontasters of *A. annua* tea.

Response	Number of respondents	% of total
Tastes good	88	23.3
Indifferent	58	15.3
Tastes bad	232	61.4

Appendix B: Supplementary Material for Chapter 4

Published in:

Desrosiers M. and Weathers P.J., 2017. Artemisinin permeability via Caco-2 cells increases after simulated digestion of *Artemisia annua* leaves. Journal of Ethnopharmacology. 210: 254-259.

Table S1. Phytochemical comparison between dried leaves of the two *Artemisia annua* cultivars, DLAS and DLAG, used in this study

Compound	(µg g^{-1} DW)	
	DLAG	DLAS
Artemisinin	nd	15,897
deoxyartemisinin	nd	nd
dihydroartemisinic acid	nd	1,857
arteannuin B	nd	2,323
artemisinic acid	nd	367
α-pinene	nd	nd
eucalyptol (1,8 cineole)	nd	261
camphor	nd	21,018
chlorogenic acid	269.71	673
rosmarinic acid	nd	2,261
scopoletin	nd	36
artemetin	nd	nd
casticin	nd	nd
chrysoplenol-D	nd	413
chrysoplenetin	nd	154
eupatorin	nd	nd
kaempferol	nd	nd
luteolin	nd	207
myricetin	nd	nd
quercetin	nd	nd
total flavonoids	209.85	2,783

nd, not detectable; nm, not measured.

Table S2. Percent artemisinin recovered in Caco-2 permeability experiments

Experiment Treatment	% Artemisinin Recovered
AN+Digestates	
AN	79.0
DLAS Digestate	92.4
DLAG Digestate	84.7
AN+Flavonoids	
AN	81.4
Quercetin	75.5
Rutin	74.3
Eupatorin	80.0
Kaempferol	80.6
Casticin	88.4
Isovitexin	82.3
Apigenin	87.8
AN+Other Phytochemicals	
AN	80.7
Chlorogenic Acid	74.8
Rosmarinic Acid	72.4
Inulin	88.7
AA+AB+Scopoletin	81.1
VD3 Media	
AN	80.2
DLAS Digestate	84.9
Quercetin	87.7
AN+EO Digestates	
AN	81.2
US EO Digestate (4%)	79.8
US EO Digestate (0.3%)	77.6
Chinese EO Digestate (4%)	82.0
Chinese EO Digestate (0.3%)	87.1
DLAG+US EO Digestate (4%)	84.4
DLAG+US EO Digestate (0.3%)	86.5
AN+EO Components	
AN	74.4
Camphor (1:1)	72.1
Camphor (1:2)	73.4
Camphor (1:10)	74.3
Eucalyptol	72.1
Caryophyllene	72.9
AN+EO Undigested	
AN	73.4
AN+Bile	75.5
US EO	77.3
US EO+Bile	79.9

Chinese EO	75.2
Chinese EO+Bile	76.5

AN, artemisinin; DLAS, dried leaf *Artemisia annua* SAM cultivar; DLAG, dried leaf *Artemisia annua* GLS cultivar; AA, artemisinic acid; AB, arteannuin B; EO, essential oil.

Table S3. Fold change in CYP3A4 transcript expression with VD3 media measured by qPCR

Target	**Ct Mean**	**Ct SE**	**ΔΔCt**	**Fold Change**
B-Actin (control) – VD3	14.743	0.258	Nd	Nd
B-Actin (control) + VD3	14.778	0.147	Nd	Nd
CYP3A4 – VD3 (experimental)	33.157	0.949	Nd	Nd
CYP3A4 + VD3 (experimental)	25.741	0.177	-7.4509	**174.96**

Table S4. Relative abundance of phytochemicals identified by GCMS in both U.S. and Chinese essential oil sources.

Phytochemical	United States EO Source % of Total Peak Area	Chinese EO Source % of Total Peak Area
Thujone	30.9	Nd
Camphor	30.3	14.1
Eucalyptol	16.5	27.4
Camphene	13.2	5.5
Borneol	3.7	1.8
β-pinene	2.5	4.7
Caryophyllene	1.0	6.2
Caryophyllene oxide	0.9	Nd
α-pinene	0.3	17.5
Terpineol	0.2	Nd
Santolina triene	0.1	0.2
Stigmasterol	0.1	Nd
Myrcene	0.1	Nd
Phellandrene	Nd	8.5
Humulene	Nd	5.5
Limonene	Nd	5.2
Carene	Nd	2.2
Copaene	Nd	1.1

EO, essential oil; Nd, not detected.

Appendix C: Supplementary Material for Chapter 5

Published in:

Desrosiers M., Mittelman A., Weathers P.J. 2020. Dried leaf *Artemisia annua* improves bioavailability of artemisinin via cytochrome P450 inhibition and enhances artemisinin efficacy downstream. Biomolecules. 10: 254.

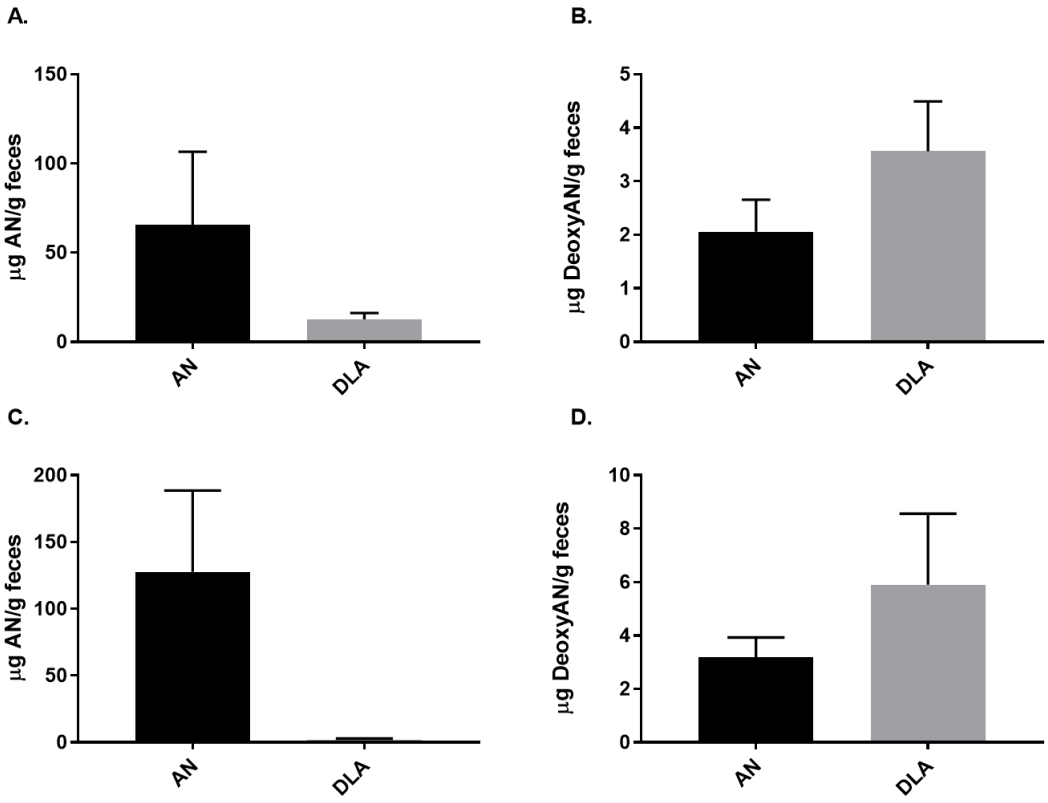

Figure S1. Artemisinin (AN) and deoxyartemisinin (DeoxyAN) accumulation in feces of male (**A**, **B**) and female (**C**, **D**) rats after oral delivery of artemisinin as dried leaf *A. annua* (DLA) or pure artemisinin. n = 7–8, *; $p < 0.05$; error bars = SEM.

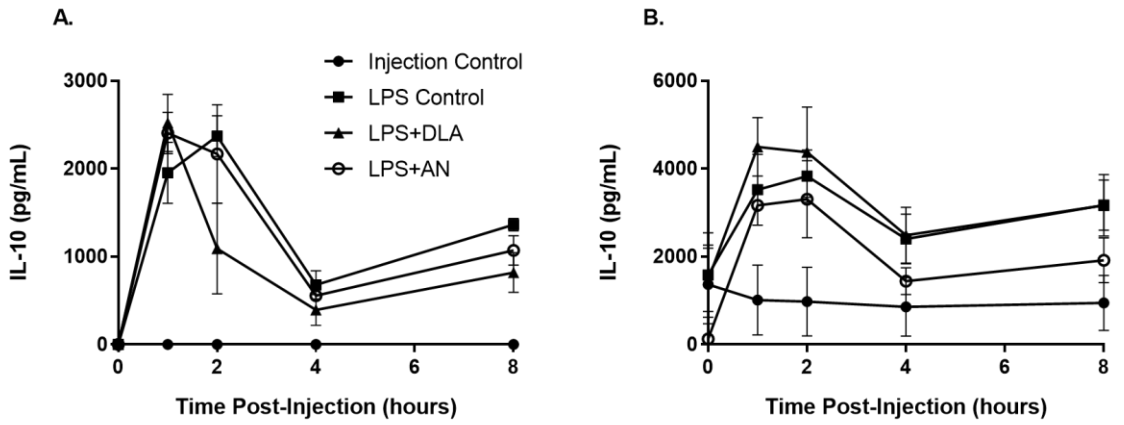

Figure S2. Production of anti-inflammatory cytokine IL-10 in male (**A**) and female (**B**) rats after LPS challenge and treatment with either pure artemisinin (AN) or DLA (equal artemisinin doses). *n* = 5–6 for experimental conditions, *n* = 3 for injection control; error bars = SEM.

Fecal Microbiome Analysis

We also examined the microbial populations present in fecal samples from male rats following administration of artemisinin or DLA to determine if either treatment affected the microbiome. Fecal samples from four DLA-treated and four artemisinin treated rats were collected at 1 and 8 hours post-gavage and stored at −80℃ until analysis. Samples were sent on dry ice to the University of North Carolina Microbiome Core Facility (Chapel Hill, NC, USA) for 16S rRNA sequencing. The sequencing results showed that neither treatment altered the microbiome of the animals at either time point (Supplementary Table 1). These results suggested DLA and artemisinin would likely not alter the microbiome of patients, however, studies should be performed over a longer time course to confirm these results.

Table S1. Microbiome population by Phylum 1 hour and 8 hours after treatment with DLA or pure artemisinin in male rats.

	Artemisinin Treated		DLA Treated	
Timepoint	**1-hour**	**8-hour**	**1-hour**	**8-hour**
Phylum	**Average % Microbial Population ± SD**	**Average % Microbial Population ± SD**	**Average % Microbial Population ± SD**	**Average % Microbial Population ± SD**
Unassigned	0.23 ± 0.10%	0.15 ± 0.06%	0.15 ± 0.10%	0.18 ± 0.10%
Actinobacteria	0.35 ± 0.21%	0.80 ± 0.50%	0.28 ± 0.22%	0.58 ± 0.36%
Bacteroidetes	49.53 ± 9.38%	61.58 ± 7.62%	48.13 ± 6.78%	51.70 ± 8.58%
Cyanobacteria	0.13 ± 0.13%	0.38 ± 0.22%	0.00 ± 0.00%	0.45 ± 0.21%
Deferribacteres	0.03 ± 0.05%	0.00 ± 0.00%	0.00 ± 0.00%	0.00 ± 0.00%
Firmicutes	32.83 ± 8.99%	29.00 ± 8.89%	31.38 ± 17.17%	30.75 ± 20.26%
Lentisphaerae	0.00 ± 0.00%	0.00 ± 0.00%	0.00 ± 0.00%	0.00 ± 0.00%
Proteobacteria	3.85 ± 2.04%	2.28 ± 0.57	2.63 ± 1.99%	4.13 ± 1.82%
Synergistetes	0.00 ± 0.00%	0.00 ± 0.00%	0.00 ± 0.00%	0.00 ± 0.00%
TM7	0.33 ± 0.26%	0.30 ± 0.32%	0.63 ± 0.95%	0.23 ± 0.32%
Tenericutes	0.68 ± 0.57%	0.75 ± 1.17%	0.43 ± 0.17%	0.43 ± 0.10%
Verrucomicrobia	12.15 ± 10.64%	4.80 ± 6.54%	16.38 ± 10.95%	11.65 ± 11.70%

SD, Standard Deviation.

Appendix D: CYP Induction Methods and Figures

Methods

HepaRG Culture

HepaRG cells from Lonza (Basel, Switzerland) were thawed and plated into white-walled, clear-bottom 96-well collagen coated plates according to the manufacturer's provided protocol. The outside wells were not seeded with cells but filled with 100 µL media to prevent evaporation. Cells were cultured for 72 hours, changing media daily based on the protocol provided. After 72 hours of culture, media was replaced with induction media containing either artemisinin, *A. annua* methanolic extract, *A. afra* methanolic extract, or rifampicin at various concentrations (1-50 µM for artemisinin and extracts, 10-50 µM for rifampicin). Negative control wells were prepared with induction media alone and vehicle control wells were prepared with induction media containing 0.2% DMSO as this concentration of DMSO was needed to solubilize artemisinin and extracts in induction media. *A. annua* extract concentration was the artemisinin concentration. *A. afra* contains negligible artemisinin so equal masses of dried plant material to *A. annua* were used to produce extracts. Media containing artemisinin, extracts, or rifampicin was replaced daily for 72 hours. After 72 hours in induction medias, media was removed, and cells were washed 3 times with DPBS to remove any remaining artemisinin, extract, or rifampicin that could interfere with activity assays.

CYP3A4 and CYP2B6 Activity Assays

Luminescent Promega (Madison, WI, USA) P450-Glo assays were used to assess the activity of both CYP3A4 and CYP2B6 after induction. P450-Glo assays were performed using the "Nonlytic P450-Glo Assays Using Cultured Cells in Monolayers" protocol provided by Promega.

These assays measure specific P450 activity by using a luminescent probe that produces light when combined with luciferase after cleavage by a specific P450. After recording luminescence, Promega CellTiter-Glo luminescent cell viability assays were performed according to the manufacturer's instructions in each well to normalize P450 activity to the number of viable cells in each well.

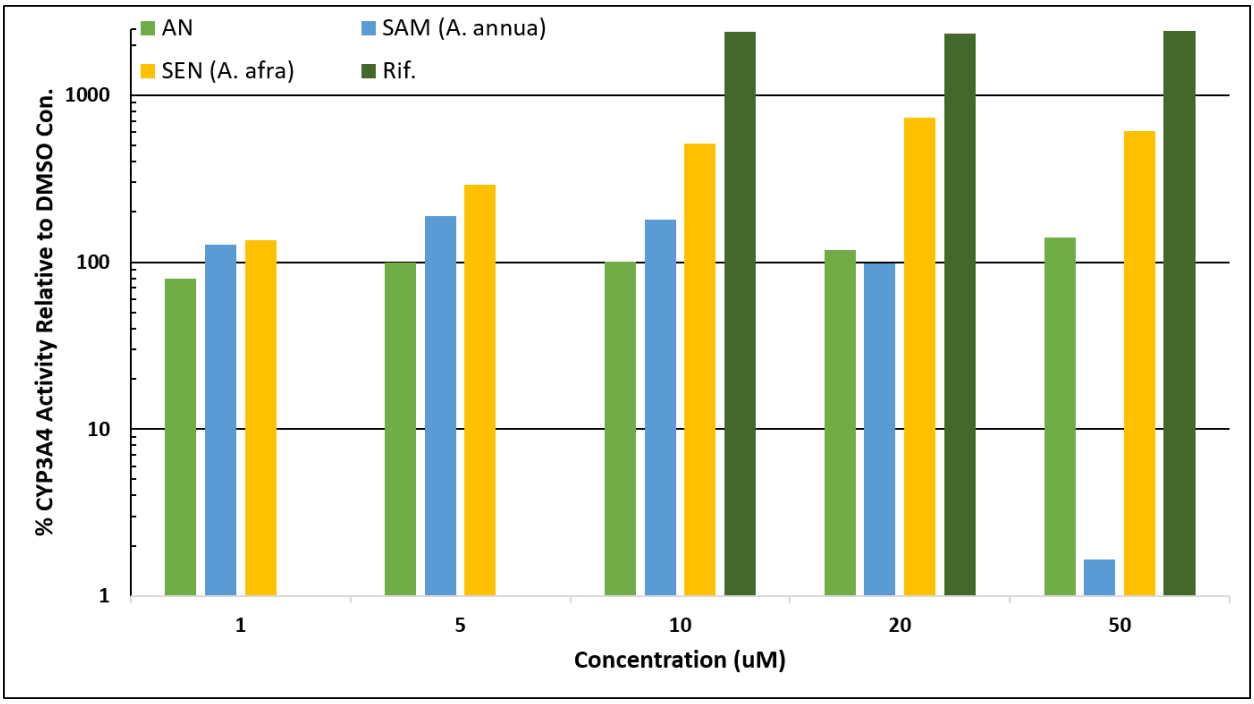

Figure S1. % Activity of CYP3A4 relative to DMSO vehicle control after 72 hour treatment with artemisinin (AN), methanolic extract of *A. annua*, methanolic extract of *A. afra*, or rifampicin (positive control). Concentration of *A. annua* = that of the pure artemisinin concentration. *A. afra* contains negligible artemisinin so extracts were made with equivalent mass of dried leaves as *A. annua* extracts. n=1.

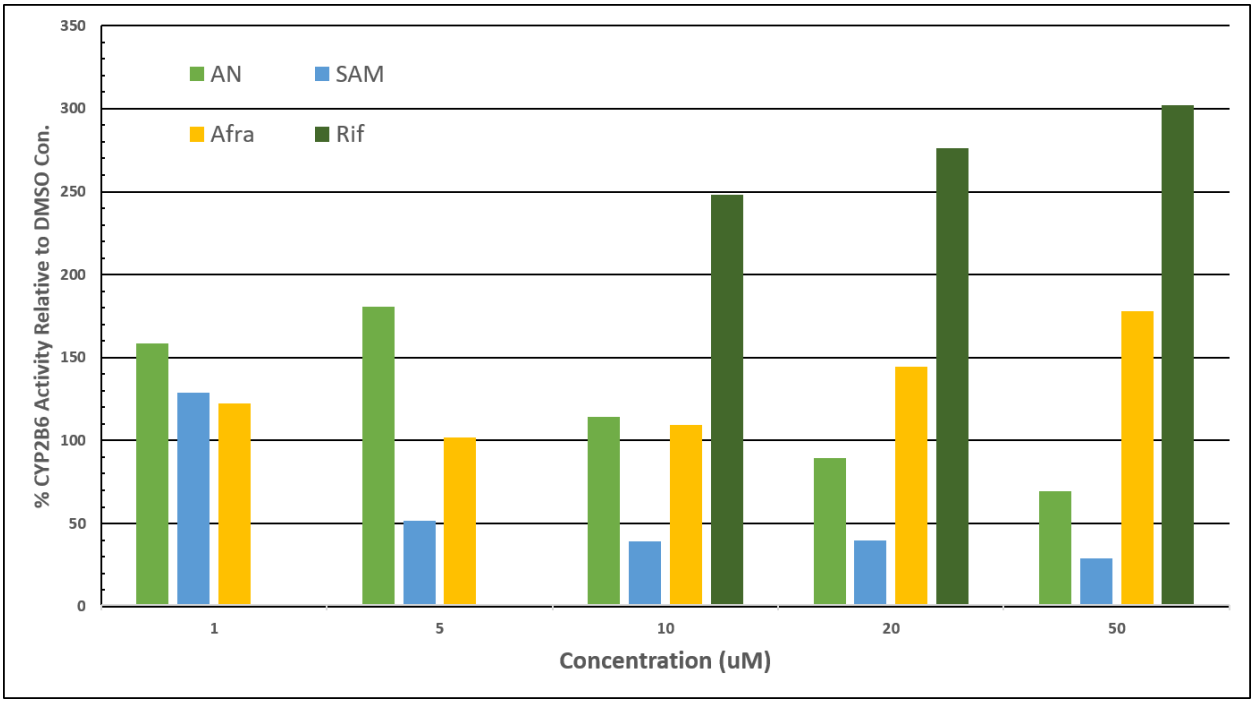

Figure S2. % Activity of CYP2B6 relative to DMSO vehicle control after 72 hour treatment with artemisinin (AN), methanolic extract of *A. annua*, methanolic extract of *A. afra*, or rifampicin (positive control). Concentration of *A. annua* = that of the pure artemisinin concentration. *A. afra* contains negligible artemisinin so extracts were made with equivalent mass of dried leaves as *A. annua* extracts. n=1.