Summer 2014

# Dried whole plant Artemisia annua as a novel antimalarial therapy

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**Dried whole plant *Artemisia annua* as a novel antimalarial therapy**

A Dissertation Presented
By

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

September 2014

Entomology

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**DEDICATION**

To my parents, my wife and my kids

## ACKNOWLEDGMENTS

I would like to thank my advisor, Stephen Rich, for his great guidance and support. I also thank Pamela Weathers and Doug Golenbock for their fruitful collaborations. Thanks are also due to Guang Xu for his sincere support. I would also like to thank the members of my committee, Anne Averill, John Burand and Michele Klingbeil for their helpful guidance during all stages of this project.

I want to thank UMass Center for Clinical and Translational Science for funding this research.

Many thanks are due to Evan Palmer-Young and all members of Laboratory of Medical Zoology for their help in this project. Thanks to my family and friends for their mutual support and encouragement to continue progressing during all stages of my academic study.

ABSTRACT

**DRIED WHOLE PLANT *ARTEMISIA ANNUA* AS A NOVEL ANTIMALARIAL THERAPY**

SEPTEMBER 2014

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Malaria is one of the worst vector-borne parasitic diseases in the developing world. The World Health Organization (WHO) estimated that 215 million cases of malaria occurred, with >655,000 deaths; half the world's population is at risk of contracting the disease. Drugs are primary weapons for reducing malaria in human populations. Successful drugs are highly efficacious and inexpensive to manufacture synthetically. However, emergence of resistant parasites has repeatedly curtailed the lifespan of each drug that is developed and deployed. Currently, the most effective anti-malarial is artemisinin, which is extracted from the leaves of *Artemisia annua*. Due to poor pharmacokinetic properties and prudent efforts to curtail resistance to monotherapies, artemisinin is prescribed only in combination with other anti-malarials composing an Artemisinin Combination Therapy (ACT). Low yield in the plant, and the added cost of secondary anti-malarials in the ACT, make artemisinin costly for the developing world. As an alternative, we compared the efficacy of oral delivery of whole plant (WP) *A. annua* to a comparable dose of pure artemisinin in a rodent malaria model. We found that WP reduces parasitemia at least five fold more effectively than a

comparable dose of purified drug, slows the evolution of malarial drug resistance in *Plasmodium chabaudi* infected mice, and is effective against already resistant *Plasmodium yoelii* (ART). This increased efficacy may result from the increase in the bioavailability of artemisinin in the blood of mice fed the whole plant, in comparison to those administered synthetic drug. When accompanied by plant material, more artemisinin enters the blood stream, demonstrating a beneficial effect of the plant matrix on the bioavailability of artemisinin. Increased efficacy and resilience against drug resistance may result from the synergistic benefits of other anti-malarial compounds in *A. annua,* such as flavonoids and terpenoids. Although effective against *Plasmodium,* neither WP nor artemisinin and artesunate are effective against *Babesia microti.* The differential response of *B. microti* and *Plasmodium* to artemisinins is likely the result of significant differences in their cell biology and metabolism of hemoglobin. Well-tolerated, and compatible with the public health imperative of forestalling evolution of drug resistance, inexpensive, locally grown and processed whole plant *A. annua* therapy might prove to be an effective addition to the global effort to reduce malaria morbidity and mortality.

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# GENERAL INTRODUCTION

For centuries, plant based therapies dominated medical practices in the ancient civilizations of Africa, Asia and South America and till today in many parts of the world where western medicine is scarce and herbal remedies are the only effective means to treat or prevent diseases (1). Modern, Western medicine dismisses these therapies for the most part as arcane remnants of a pre-enlightened era of modern drug design. From this predominant reductionist world view, therapies lacking precise explanation of the interaction of chemical moieties are not to be trusted. But like all reductionist reasoning, this view unnecessarily simplifies the complexity of the problem and ignores the context by which herbal remedies—not to mention the plants from which they are derived—have evolved. In the present study, we present empirical evidence of superior efficacy of an herbal remedy that outperformed it pseudo-pharmaceutical counterpart, which is the primary favored anti-malarial drug at this moment. We explain this observation in the proper evolutionary context, demonstrate the propensity for the herbal therapy's increased resilience, and outline a new paradigm that leverages rather than ignores cultural and evolutionary history.

Plants possess defensive physical barriers including their cuticle and cell walls that help them to avoid being overcome by herbivores and pathogens. Despite such physical barriers, some pathogens have developed invasion mechanisms to penetrate into plant's inner tissues through wounds and/or stomata. Parasitization puts selection pressure on these plants to evolve more advanced and complex chemical defenses to overcome infection. In turn, plant chemical defenses puts selection pressure on pathogens

to evolve counter-resistance mechanisms that circumvent plant defenses. This creates the circumstance of classical "arms race", and this particular interaction between plants and pathogens is one of the oldest evolutionary conflicts in nature. In plants, this has lead to evolution of complex array of chemical defenses.

Plants and animals have faced similar microbial enemies throughout their evolutionary histories and through convergence they have evolved similar systems of innate immunity whereby host cell receptors detect pathogen-associated molecular patterns and activate defense responses (2). Studies on plants, humans, mice and insects revealed evolutionary conservation in recognition of Pathogen-Associated Molecular Patterns (PAMPs) by extracellular Pattern Recognition Receptors (PRRs) across kingdoms (3, 4). The general elicitors of innate immunity in vertebrates—lipopolysaccharides, peptidoglycan, eubacterial flagellin, unmethylated bacterial DNA, glucans, chitins, and mannans—comprise the same set of molecules known to elicit plant defense responses (3-7).

To defend against pathogens, early land plants developed PRRs to recognize PAMPs and activate PAMP-Triggered Immunity (PTI). Through millions of years of interaction with pathogens, plants have been able to detect and recognize molecular patterns that are highly conserved across a broad range of microbes and essential to pathogen survival, such as lipopolysaccharide, peptidoglycan, flagellin (a flagellum protein), chitin, and fungal ergosterol (8). The recognition of PAMP's by PRR's activates PTI through a suite of activations and inductions that results in transcription of defense genes. The results of these transcriptional changes include physical effects such as callose deposition, cell death, stomatal closure (9, 10) as well as chemical effects including

production of reactive oxygen intermediates, antimicrobial proteins, and antimicrobial secondary metabolites.

PAMPs are conserved and indispensible epitopes for which mutations tend to be deleterious, hence microbes evolved resistance mechanisms by secreting effector proteins that suppress PTI in their host plants (4, 9, 10). Effector proteins are diversely produced by vast variety of microorganisms including bacteria, fungi, and viruses. Effector proteins are essential for pathogen invasion, growth and development since they enzymatically target plant cells to modify defense proteins leading to escape from host recognition. In response to effector proteins, plants have evolved specialized Effector Triggered Immunity (ETI) such as pathogen-Resistance (p*R*) proteins that recognize microbial effector proteins and/or detect pathogen-induced modification of self-proteins. p*R* proteins activate pathways that are ultimately responsible for a plant's pathogens-resistance phenotype, including production of further synthesis of a repertoire of antimicrobial secondary metabolites.

**The evolution of secondary metabolites**

The evolutionary rate in microorganisms is greater than in comparatively long-lived plants. How do plants overcome such a disadvantage in evolutionary rate? One means of overcoming fast evolving microorganisms is by the evolution of somatic broad-spectrum pR proteins that detect any damage or modification in PTI-associated proteins. Such indirect interaction with microorganisms enabled several pR proteins to provide protection against a diverse group of pathogens (2).

Another strategy that enables plants to compensate for their lower evolutionary rate is the continuous evolution, generation and retention of secondary metabolites. In

contrast to primary metabolism where high specificity of enzymes is favored to reduce costs associated with the production of non-essential end products (11), fitness advantages may be conferred for producing enzymes of secondary metabolism with lower specificity (10). To be effective, secondary metabolites must randomly intercalate into essential and conservative enzyme sites found in a broad range of microorganisms, hence the ability interact with multiple substrates is favored (12).

To offset costs of maintaining such diverse and non-specific secondary metabolism, plants have catalytic flexibility in which one enzyme is able to produce many products from one substrate (11). Such catalytic flexibility enabled plants to generate exceedingly diverse repertoires of secondary metabolites that play important roles in plant defense against microorganisms. Examples of such catalytic flexibility are found in sesquiterpene biosynthesis in which one enzyme produces 34 different products from a single substrate while in another instance the enzyme produces 52 products from a single precursor (11). Through evolution, plants have acquired a matrix of antimicrobial secondary metabolites marked by synergism among its components (13, 14). Natural selection favors the sequential increase of metabolic diversity and retention of secondary metabolites units into the chemical matrix until the marginal cost of producing additional metabolites outweighs their benefits (15).

Through their shared evolutionary history with microorganisms, plant and animal defense mechanisms have been refined against conserved and functionally indispensable components of microorganismal pathogens. Plant and animal immune systems evolved independently but in parallel given the commonality of the microbial pathogens in their aim. These parallels have resulted in some remarkable convergences, and it is therefore

not surprising that some of these defenses evolved in one kingdom should prove efficacious against microbial agents targeting the other kingdom. This conserved evolution is the basis and rational for a new approach to developing therapies to combat human pathogens. In particular, we assert that certain plant secondary metabolites, which evolved to protect against plant pathogens, provide the basis exploring novel therapies against human pathogens.

**Animals Self-medication**

Many animal species may exploit the antimicrobial properties of plant secondary metabolites for use against their own pathogens through evolved self-medication behavior. Self-medication is innate behavior in many insect species including the fruit fly, honeybee, wooly bear caterpillar and monarch butterfly (16). Using medicinal plants against microorganisms reduces the animal's immunity costs through reliance on the plant's matrix of antimicrobial secondary metabolites (16, 17). Self medication behavior in humans and non-human animals may be explained by two hypothesizes, social learning and natural selection (18).

Here we define self-medication in an infectious system as the circumstance where none species (self-medication host) is infected by a second species (pathogen) and ingests a third species (typically a plant) in direct response to that infection. To consider self-medication behavior as an adaptation arising by natural selection several conditions must be met.

1. Host must ingest a the material (eg. plant) in direct response to infection,
2. Ingestion should not occur in uninfected host individuals,
3. Ingestion must increase the fitness of infected hosts relative to infected host that do not ingest, and

4. Behavior must be innate and heritable; i.e. not learned through classical reinforcement or taught through social interactions.

The presumptive targets of this natural selection are the physiological aspects of sensory reception, eg. taste and olfaction. For example animals seek sodium containing substances using specific appetite for sodium that is immediate and requires no experience of previous deficiency. Such behavior appears innate and totally taste-guided (19). Analogous of salt-taste marker, medicinal plants generally have distinctive volatile fragrance, rough surface and bitter taste that may serve as cues for animals adapted to self-medicate (20, 21). Many plant antimicrobial secondary metabolites are often distasteful and bitter taste that also may be used as taste marker (18). Hart (2005) reviewed the taste of a range of medicinal plants and found the majority of them have bitter or astringent taste (18). Generally mammals dislike and reject bitter dietary foods, hence the shift in taste receptors to tolerate (or even prefer) bitterness upon infection, could be the specific adaptation responsible for self-medication. Examples of this stringent model of adaptation are limited, but there are tantalizing glimpses such as the observation that sick chimpanzees were unable to detect bitter substrate inserted into their food while healthy ones were able to detect the bitter taste and rejected such food (22).

Because, infection associated with disease causes dramatic changes in the physiology and/or the behavior of infected individual, natural selection could favor the condition in which sick individuals deviate from their regular diet if such dietary deviation gives a direct fitness advantage. This could occur either by behavioral changes that drive sick individuals to ingest non-regular diet or by physiological changes that thwart their sensory capability to discern and/or avoid bitter plants (18). This model

fulfills the first two requirements for demonstrating self medication behavior as adaptive. The actual conditions under which this would occur nonetheless require a balance of complex trade-offs since the aversion to bitter taste is presumably an adaptation itself to avoid toxicity. Therefore, subduing that protective behavior must lead to anti-pathogenic, anti inflammatory, immunomodulatory, or analgesic benefits that outweight risks of intoxication from the medicinal source or other non-medicinal, bitter tasting diet items.

Chimpanzees and bonobos use medicinal plants such as *Veronica spp*. to treat gastrointestinal illnesses and *Aspilia spp*., *Rubia spp.* and *Manniophyton spp*. to expel internal worms (16). Baboons use *Balanites spp*. to control schistosomiasis (23). Wood rats and starlings use *Umbellurlaria spp*. and *Agrimonia spp*. to kill nest-borne fleas and mites, respectively (24). Neanderthals used herbal remedies to treat diseases and to manipulate disease symptoms about 60,000 years ago (18).

From this view we may speculate that animals' anti-microbial defenses against microorganisms evolved in the presence of medicinal plants, so that animals lacking assistance from the matrix of plant secondary metabolites would not have optimal defenses. In this sense, use of single–compound pharmaceuticals against pathogenic microorganisms may actually relax challenges to those microorganisms trying to evolve ways of circumventing barriers to their proliferation.

To be certain, herbal remedies based upon plant secondary chemistries should not be accepted as efficacious without rigorous evaluation. However they also should not be discounted out-of-hand simply because they were not designed from a modern

biochemical perspective nor even when they may possess emergent properties that defy our specific ability to identify their mechanisms.

**Study Systems**

Parasitic protists in the phylum *Apicomplexa* are a large group specialized in parasitizing animal cells. They use the unique apical complex structure, for which the phylum is named, to penetrate host cells. Apicomplexan parasites are of great importance to society because they are the causative agents for many of human and domestic animal diseases. The phylum includes parasites of humans (*Plasmodium, Babesia, Toxoplasma, Cryptosporidium, Isospora*), and domesticated animals (*Theileria, Babesia, Sarcocystis*, *Neospora*, and *Eimeria*).

Among apicomplexan parasites, malaria is the worst vector-borne parasitic disease in the developing world. The World Health Organization (WHO) estimated that 215 million cases of malaria occur each year, with >655,000 deaths; and half of the world's population is at risk of contracting the disease (25). Only five species of *Plasmodium* parasites are known to infect humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Among the five species, *P. falciparum* is the most deadly and responsible for more than 90% of annual deaths, followed by *P. vivax*. Humans contract the disease after being bitten by female anopheles mosquitoes, which release the infectious sporozoites into circulating blood where they travel to the liver and invade hepatocytes. Within the hepatocytes, the parasite begins asexual replication (exo-erythrocytic schizogony), producing thousands of small merozoites that each invade a single red blood cell (RBC) and start the erythrocytic schizogony, producing about 20 small merozoites each. After maturation, the infected RBC's rupture, releasing the new

merozoites to invade new RBCs. These erythrocytic cycles result in the majority of malaria disease symptoms including anemia, fever, headache, splenomegaly, and hepatic and renal dysfunction. In falciparum malaria, the parasites export parasitic proteins to the surface of infected red blood cells, causing them to block small capillaries in the infected patient's brain, resulting in neurological symptoms, coma, and sudden death (26). Some merozoites develop into sexual gametocytes within RBC's. If the patient is bitten by another mosquito, these gametocytes are imbibed with the bloodmeal. Once in the mosquito's midgut, the male and female gametocytes fuse together, forming motile zygotes that penetrate the midgut wall and settle in the basal lamina, forming oocysts. Within the oocysts, the parasite undergoes asexual replication, producing thousands of sporozoites. Mature oocysts rupture, releasing thousands of sporozoites into the mosquito's hemolymph. From the hemolymph, sporozoites migrate to the insect's salivary glands, from which they infect human hosts when the mosquito next feeds.

Like *Plasmodium*, *Babesia* invades erythrocytes, has an invertebrate vector, and the symptoms of the two diseases are similar, including fever, flu-like symptoms, chills, headache, anemia, hemoglobinuria, and myalgia. In regions where malaria is endemic, co-infection with *Babesia* parasites have been observed, and babesiosis may be misdiagnosed as malaria (27). To date seven species of Babesia have been found to cause human babesiosis *Babesia microti*, *B. divergens*, *B. bovis*, *B. canis*, *B. duncani*, *B. venatorum* and *Babesia* KO1 (28). *Babesia* has infected increasing numbers of humans in recent years due to greater medical awareness and increased numbers of immunocompromised patients (28). Among all *Babesia spp., B. microti* causes most of human cases in United States (28).

**Antimalarial chemotherapy**

The fight against malaria predates the discovery of its causative agents. Periodic fevers associated with swamps and marches have been known since Hippocrates and were treated with herbal remedies. Two main herbal remedies, cinchona bark and qinghao (*Artemisia annua*), were used to treat malaria for hundreds of years. In western countries, cinchona bark has been known to treat swamp fever since 1640. In 1820, quinine was isolated from cinchona bark and used to treat malaria-like fever. Quinine was the first purified compound used against human disease (29). In the 1880's, the causative agent of malaria was discovered, although the role of the anopheles mosquito in malaria transmission was not discovered until the 1890's.

The fight against malaria entered a new era in which synthetic compounds were used to combat parasites. In the1890's, Paul Ehrlich successfully treated two malaria patients using methylene blue and launched his "magic bullet" concept, the idea that a chemical compound could be made which selectively targeted a disease-causing organism. Methylene blue was the first synthetic compound to be used against human disease (29). Ehrlich's "magic bullet" concept was embraced in the search for new remedies for malaria. Some sixteen thousand synthetic compounds were tested against malaria parasites, but very few were effective against malaria (29). In 1934, chloroquine was developed and used in the fight against malaria. Chloroquine was both safe for humans and very effective against malaria parasites. Around that time, many purified and synthetic compounds were employed to treat human diseases, with herbal and natural remedies falling out of mainstream medical use.

In the 1950's the WHO launched the Global Malaria Eradication Program (GMEP), through which they hoped to eradicate malaria parasites using two main synthetic compounds, chloroquine as an antimalarial and Dichloro Diphenyl Trichloroethane (DDT) as an insecticide against mosquitos. Chloroquine was used on a wide scale as prophylactic treatment. However, although chloroquine was highly effective, it lacked resilience against drug resistance. The emergence and spread of chloroquine resistant malaria parasites and DDT resistant mosquitos were the main reasons for the demise of GMEP in the 1960's.

Malaria parasites were among the first pathogenic organisms to develop drug resistance against curative agents. After chloroquine's failure, many antimalarial compounds were deployed as monotherapy against malaria parasites; unfortunately, the effectiveness of each of these treatments was compromised by the emergence of drug-resistant parasites.

In the 1970's artemisinin was isolated from *A. annua* and found to be effective against malaria parasites resistant to chloroquine. In spite of its activity in fighting malaria parasites, artemisinin has low pharmacokinetic properties. To increase its pharmacodynamic and pharmacokinetic properties and to slow the evolution of drug resistance, the WHO recommended using artemisinin derivatives, mainly artesunate, in combination with other antimalarial drugs. The low yield in *A. annua* and the additive cost of partner drugs make the WHO-endorsed Artemisinin-based Combination Therapy (ACT) unaffordable for many people in developing countries. It was hoped that use of ACT would minimize risk of drug resistance. However, in 2005, the earliest evidence of

P. falciparum resistance to ACTs was found in Southeast Asia (30-34). The fight against malaria became critical again, as no affordable replacement for ACT was available.

**Hypothesis**

Plants have evolved complex matrices of secondary metabolites—produced as a response of PTI and ETI upon recognition by PAMPs and pR proteins respectively—that target conserved functions, and structural elements of invading microorganisms and animal herbivores. Such broad-spectrum defense is observed in many medicinal plant species for which the secondary metabolites matrix that has broad biological activity against multiple taxonomic groups. For example, in *A. annua* and artemisinin are known to have activity against bacteria, protozoa, viruses, arthropods, and other plant species (35-39). Synergism between the biological activities of artemisinin and other *A. annua* secondary metabolites results in a range of ecological effects, from allelopathic activity against other plants to antimalarial activity against animal disease.

*A. annua* is regarded as safe for human consumption and has been widely consumed in many parts of China for more than 2000 years (29) and was a known herbal treatment for fevers symptomatic of malaria (40). Recently, several reports have described synergism between artemisinin and other *A. annua* flavonoids. These include antimalarial activity of *A. annua* constituents other than artemisinin-- including other terpenes and flavonoids-- and increased bioavailability of artemisinin when delivered in plant form. Based on such reports, we hypothesized that the complex matrix of secondary metabolites present in the dried whole plant *A. annua* material may be used directly against malaria parasites, thereby saving the high cost associated with artemisinin isolation and purification. Because *A. annua* has been successfully used to treat malaria-

like fevers for more than 2000 years without development of resistance, and because of the multiple constituents in *A. annua* known to have antimalarial activity, we also hypothesized that dried whole plant *A. annua* may be used to treat malaria parasites resistant to artemisinin and to delay the evolution of drug resistance.

CHAPTER I

# DRIED WHOLE PLANT *ARTEMISIA ANNUA* AS ANTIMALARIAL THERAPY

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**Author contributions:** D.G., M.A.E., P.J.W., and S.M.R. designed research; M.A.E. performed the mouse-parasite experiments; M.J.T. and P.J.W. grew plants and conducted biochemical assays; M.A.E., N.G.R., and S.M.R. analyzed data; M.A.E., P.J.W., N.G.R., and S.M.R. wrote the paper (41).

## Introduction:

Malaria is among the most prevalent infectious diseases in the developing world, imposing a vast burden of mortality and perpetuating cycles of poverty. In 2009, the World Health Organization (WHO) estimated that 225 million cases of malaria occurred, with >780,000 deaths (42). In spite of recent advances in our understanding of this parasite, efforts to prevent transmission have remained largely unchanged for over a century. Though malaria vaccines hold future promise, vector control and chemotherapy remain the primary weapons for reducing the burden of disease in individuals and populations. Artemisinin, in the form of Artemisinin-based Combination Therapy (ACT), is currently the best treatment option against those malaria parasites that have evolved resistance to drugs such as chloroquine (43). Moreover, artemisenin (AN) and its derivatives have also been shown to have effects on a number of viruses, a variety of human cancer cell lines (35, 36), several neglected tropical parasitic diseases including

schistosomiasis (44), leishmaniasis (45, 46), New- and Old-World trypanosomiases (47), and many livestock diseases (48).

Current artemisinin production requires its extraction from the cultivated herb *Artemisia annua* L., which as an herb is "generally regarded as safe" (GRAS) for human consumption (49). However, considerable production costs and limited availability of artemisinin limit its present usefulness in the campaign against malaria (50). *De novo* chemical synthesis of artemisinin is neither practical nor cost-effective, and even the best plant cultivars yield only *ca.* 1.5% artemisinin, and agricultural yields seldom exceed 70 kg/ha (51). The drug is solvent-extracted from plant material, crystallized, and typically used for semi-synthesis of artemisinic derivatives. Although *A. annua* is relatively easy to grow in temperate climates, low yields of AN lead to relatively high costs for isolation and purification of the drug (52). Because of this shortcoming, plant scientists have focused their efforts on producing cultivars of *A. annua* with higher artemisinin crop yield (53). Transgenic production schemes are also underway (54, 55). Meanwhile, a worldwide shortage fails to meet the need to treat malaria, not to mention those other diseases against which artemisinin holds such promise (43).

One means of reducing the cost of production would be to limit the amount of post-harvest processing by using the whole plant (WP). We wondered whether omitting the extraction step, by using whole plant *A. annua* directly as the source of artemisinin, might prove efficacious in an experimental murine model. In a previous study, we showed that mice fed dried WP material had about 40 times more artemisinin in their bloodstream than mice that were fed a corresponding amount of pure drug (49). This amount exceeded by eight fold the minimum concentration of serum artemisinin required

for a single curative dose (10 μg/L) against *P. falciparum* (56). This suggested that the active ingredients were delivered faster, and in greater quantity, from whole plant treatments than from pure drug treatments. We further hypothesized that because of the combination of parasite-killing substances (artemisinin and flavonoids), perhaps augmented via synergism among the constituent compounds in this plant (artemisinin and the flavonoids: casticin, eupatorin, and chrysosplenetin) (57, 58), might render whole plant consumption as a form of ACT.

We thus sought to determine whether WP *A. annua* can kill malaria parasites *in vivo* using a rodent malaria model. *Plasmodium chabaudi* is an excellent model for the most deadly of the human parasites, *P. falciparum*, because both species demonstrate preference for mature erythrocytes as opposed to reticulocytes that are favored by other human and rodent malaria parasites (59, 60). Rodent malaria models are invaluable for studying modes and mechanisms of resistance to antimalarial compounds (61). Demonstrating parasite killing using this inexpensive and resilient treatment could be an important first step in establishing a novel therapy based on WP *A. annua* for treatment of human malaria. Furthermore, it may help to identify complementary and/or synergistic anti-malarial compounds within the plant.

## Materials and Methods

**Plant material.** *Artemisia annua* L. (SAM cultivar; voucher MASS 00317314) containing 1.48±0.06% AN (dry weight) was used in this study. To obtain adequate amounts of leafy biomass, plants were grown in soil either in greenhouses, culture rooms, or growth chambers under continuous light to maintain vegetative growth. Plants were propagated by cuttings to insure that the outcrossing characteristics of *A. annua* did not

result in genetic loss of AN content. When the plants reached about 0.5-1 m, they were harvested and dried in the light at 25°C for several days. Leaves were then stripped from stems and pulverized through a series of brass sieves ending with 600 μm meshed powder. All dry leafy biomass was pooled, homogenized and then assayed for AN.

AN was measured using GC-MS by extracting with pentane (~6 mg/mL) and sonicating for 30 min. Extract was decanted into glass test tubes and dried under nitrogen gas, then stored at -20°C until analysis. Samples were resuspended in pentane and transferred to a 1 mL vial with a 100 μL glass insert. A 1 μL injection into the GC-MS [GC, Agilent 7890A; MS, Agilent 5975C; column, Agilent HP-5MS (30 m x 0.25 mm x 0.25 μm)] used the following oven program: ion source temperature 280°C; inlet 250°C; initial temperature of 125°C for 1 min, then ramp to 300°C at 5°C/min, for a total time of 36 min. Ultrapure helium was the carrier gas at 1 mL $min^{-1}$. Identification was via NIST library and purchased AN standard (Sigma-Aldrich Chemical, St. Louis, MO).

**Parasite information.** *Plasmodium chabaudi* ASS (MRA-429) was obtained through the Malaria Research and Reference Reagent Resource Center (MR4) as a part of the BEI Resources Repository, NIAID, NIH. Tubes of blood collected from infected mice, were removed from liquid nitrogen storage and left at room temperature for 30 minutes. A 100μL aliquot of the parasite-infected blood was mixed with 500 μL Dulbecco's Phosphate Buffered Saline (DPBS). To recover parasite stocks, two C57BL/6 mice were injected intraperitoneally (i.p.) with 200 μL of the DPBS mixture. Percent parasitemia was determined in Giemsa-stained thin blood smears days 3-7 post-infection (p.i.). Seven days after infection, one mouse was euthanized and cardiac puncture was used to collect blood into lithium heparin tubes. Infected blood was volumetrically adjusted by dilution

in DPBS to create a 200 µL aliquot of $10^5$ infected erythrocytes for infection into two additional mice for a second round of recovery. The recovered parasites were used for subsequent challenge and drug study. Rodent malaria models showed differences in the degree of susceptibility among different strains of mice, as well as age related differences. We used two mice to draw the standard parasitemia curve in the C57BL/6, 8-12 weeks male mice. The two mice were inoculated i.p with $10^5$ infected erythrocytes and parasitemia was determined in Giemsa-stained thin blood smears from day 1 to day 11 p.i. (Fig. I.1). *P. chabaudi* produces a self-limiting infection in laboratory mice, such that parasites began to appear in the blood smears on day 4 p.i., reaching peak of the parasitemia on day 8 p.i. From the standard parasitemia curve we chose to treat mice on day 6 p.i. when the parasites commenced the log phase of growth.

**Mouse feeding and drug delivery details.** All mouse experiments used inbred male mice C57BL/6, 12 weeks of age. For each experimental replicate, an aliquot of $10^5$ infected erythrocytes was inoculated i.p. into each of 30 mice. For the first two replicates, mice were randomly divided into three groups of ten mice per group ($AN^{LO}$, $WP^{LO}$, and Control). For the third replicate, mice were randomly divided into five groups of six mice per group ($AN^{LO}$, $WP^{LO}$, $AN^{HI}$, $WP^{HI}$, and Control). Individual mice were identified by tail markings using permanent marker. Starting on day 3 p.i., percent parasitemia was determined in Giemsa-stained thin blood smears from a drop of peripheral blood obtained from the tail. Mice were observed twice daily for signs of disease stress. Food and water were introduced *ad libitum* for the first five days. On day five, food was withheld for 24 hours, but water was freely available.

Whole plant (WP) treatment consisted of dried *A. annua* plant powder ground and passed through a 0.3 mm sieve, mixed with water to a final volume of 0.5 mL. Two dosages were used: $WP^{LO}$ and $WP^{HI}$. $WP^{LO}$ consisted of 0.5 mL treatment slurry contained 40 mg dry weight (DW) of plant powder, containing 600 µg artemisinin and corresponding to 24 mg AN/kg live body weight. $WP^{HI}$ consisted of 0.75 mL treatment slurry contained 200 mg DW of plant powder, containing 3000 µg artemisinin and corresponding to 120 mg AN/kg live body weight.

Pure drug (AN) treatment consisted of artemisinin purchased from Sigma Aldrich freshly dissolved in DMSO and pulverized mouse chow. Two dosages were used, $AN^{LO}$ and $AN^{HI}$. $AN^{LO}$ consisted of a slurry containing 600 µg AN dissolved in 60 uL DMSO mixed with water and 40 mg powdered mouse chow to final volume of 0.5 mL. $AN^{LO}$ consisted of a slurry containing 3000 µg AN dissolved in 60 uL DMSO mixed with water and 200 mg powdered mouse chow to final volume of 0.75 mL.

Placebo control (CON) consisted of 60 µL DMSO, mixed with water and 40 mg powdered mouse chow to a final volume of 0.5 mL. Delivery of the appropriate 0.5 mL treatment/control was performed immediately after dose preparation by oral-gastric gavage into mice using a feeding needle (18G, curved, 2", and 2.25 pall diameter). Food and water were introduced *ad libitum* after gavage. Percent parasitemia was determined every 24 hours in Giemsa-stained thin blood smears from days 3-6 p.i., then every six hours for 48 hours post gavage, and again on 24 hour intervals for days 9-11 p.i.. All mice were euthanized on day 11 p.i. via asphyxiation in a $CO_2$ chamber followed by cervical dislocation. The experiment was repeated three times.

**Statistical analyses.** We fit linear mixed models to estimate and compare the average parasitemia for each treatment group at each measured time point. Including a random intercept for individual mice allowed us to adjust for repeated observations on the same mouse. The primary analysis compared CON, $WP^{LO}$ and $AN^{LO}$ treatment groups to assess statistically significant parasitemia differences in these groups at each time point (see Fig. I.2). For the primary analysis, data from all three replicates were used. A secondary dose-response analysis compared the CON, $WP^{LO}$, $WP^{HI}$, $AN^{LO}$, and $AN^{HI}$ treatment groups at all measured time points (see Fig. I.3). For the secondary analysis only data from the third replicate were used.

For each model, 10,000 Markov chain Monte Carlo (MCMC) samples were drawn from the posterior distributions of the average parasitemia levels for each treatment group at each time point. Then, 95% confidence interval endpoints for a particular parasitemia level were established at the $2.5^{th}$ and $97.5^{th}$ quantiles of the MCMC samples for that parameter. An estimated difference between two groups was declared "significant" if the 95% confidence interval for the difference did not cover zero. Analyses were conducted using the statistical software R v2.15(62) and the lme4 package(63), Graphics were produced using the ggplot2 package.

**Ethics Statement.** This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts (Protocol# 2011-0015). All efforts were made to minimize suffering of animals during experimental procedures.

**Results and Discussion**

We found conclusive evidence that whole plant *A. annua* kills malaria parasites more effectively than a comparable dose of pure drug. In our primary analysis, we compared parasitemia over time in mice treated with either low-dose, whole plant *A. annua* ($WP^{LO}$), low-dose, pure drug artemisinin ($AN^{LO}$), or placebo (CON). Only 24 hr after treatment, dead parasites (with condensed dark pigment) were observed in mice treated with $WP^{LO}$ ; 30 hours after treatment, parasitemia was < 0.001% (Fig. I.1). Mice treated with $WP^{LO}$ showed significantly lower parasitemia than those treated with $AN^{LO}$ from 12 to 72 hours post-treatment (Fig. I.2). Mice treated with $AN^{LO}$ did not show significant difference in parasitemia from those administered a placebo at any time point.

In a subsequent dose-response analysis, we added high-dose comparison groups, $AN^{HI}$ and $WP^{HI}$. Each of the four treatment groups experienced significantly lower parasitemia than the control mice (Fig. I.3). All mice treated with $WP^{LO}$ responded strongly in the first 24 hrs following gavage, showing the lowest level of parasitemia at 30 hours pos-gavage; only 3 of 26 $WP^{LO}$-treated mice (12%) had parasitemia of 3% or greater. However, 36 hours after gavage, 38% of the $WP^{LO}$-treated mice were above this threshold. Notably, treatment with $WP^{LO}$ was just as effective in reducing parasitemia as was treatment with $AN^{HI}$ for the first 72 hours post treatment (Fig. I.3). Thereafter, $WP^{LO}$-treated mice had significantly higher parasitemia. Although the suppression of parasitemia was significant in the three treatment groups after a single dose treatment, low dose $WP^{LO}$ resulted in faster recrudescence than either $WP^{HI}$ or $AN^{HI}$ (Fig. I.3), suggesting that multiple treatments at this dose would be necessary for a curative effect. Considering that a normal course of ACT treatment for human malaria currently requires

several doses, spread over several days, a similar regime may also be effective in the case of lower dose WP administration. Pharmacokinetics will be needed to determine serum levels of the drug. No differences between the $WP^{HI}$ and $AN^{HI}$ groups were detected after treatment. This is consistent with the short half-life seen in the pure drug in human patients and the prescribed need for multiple doses per day for several days (42).

Although the precise mechanisms of its anti-malarial activity remain unproven, artimisinin is suspected (like other drugs, including chloroquine) to interfere with heme detoxification, a crucial requirement for parasite survival in erythrocytes. *Plasmodium* parasites digest hemoglobin, producing heme as a byproduct. Free heme molecules are toxic, so parasites sequester it in the form of hemozoin polymers in unique digestive vacuoles. Artemisinin is a sesquiterpene lactone with a crucial endoperoxide bridge (64), and in the presence of heme, this bridge is broken, thereby releasing free radicals harmful to the parasite (65).

The empirical evidence for increased anti-malarial activity of WP relative to AN might be explained by differential bioavailability of the artemisinic compounds. Weathers *et al* (2011) showed that mice treated with a WP equivalent of 1.2 mg/kg AN reached their highest concentration of artemisinin in the blood (87 μg /L) 30 min after gavage, whereas mice treated with AN did not reach their the maximum concentration (74 μg /L) until much later ( $\geq$ 60 min) (49). Moreover, poor solubility and high metabolic breakdown of artemisinin by hepatic and intestinal cytochrome P enzymes (CYP P450 and CYP3A4) may reduce its bioavailability when administered in pure form (66). Infusions made from whole plant *A. annua* showed marked inhibition of the

intestinal and hepatic CYP enzymes by flavonoids and/or other compounds (48). Hence, inhibition of metabolic enzymes correlates with greater bioavailability of artemisinin, which is consistent with our findings that WP demonstrates greater parasite killing activity than a comparable pure drug treatment.

Whole plant (WP) *A. annua* may also have enhanced antimalarial activity due to synergism among particular plant compounds and artemisinin (14, 48, 67). Among these compounds are many flavonoids, of which at least six (artemetin, casticin, chrysosplenetin, chrysosplenol-D, cirsilineol, and eupatorin) are of interest for their antimalarial roles. The synergism between artemisinin and these flavonoids may be due to their ability to potentiate the activity of artemisinin. When each of these six flavonoids was combined individually with artemisinin, the $IC_{50}$ of AN against *P. falciparum* decreased by 20-50%, demonstrating an apparent synergy between the sesquiterpene lactone, artemisinin, and those six methoxylated flavonoids (57, 58). The precise mechanism of flavonoids in activating artemisinin is not fully understood, however it has been reported that *A. annua* methoxylated flavonoids enhance the formation of the artemisinin-heme complex (68), which increases the release of free radicals.

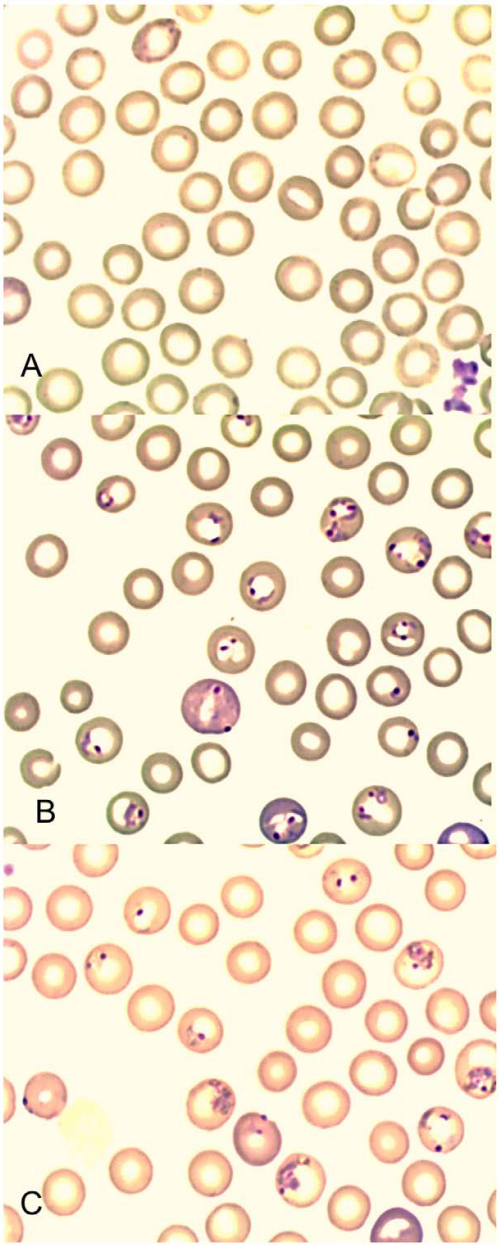


**Fig. I.1.** Giemsa stained blood smears from mice showing erythrocytes infected with *P. chabaudi,* 30 hours after treatment with (A) $WP^{LO}$ (24 mg/kg whole plant delivered artemisinin), (B) $AN^{LO}$ (24 mg/kg pure drug artemisinin), and (C) Placebo control.

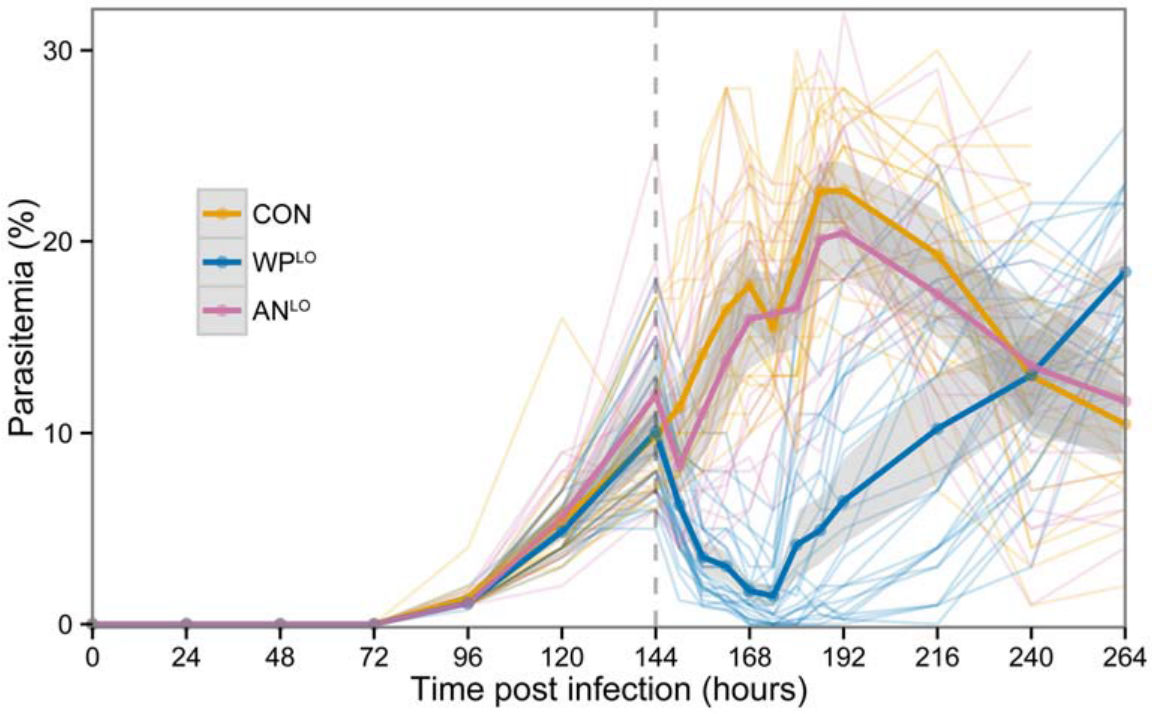


**Fig. I.2.** *P. chabaudi* parasitemia of three treatment groups across all experimental replicates: low-dose whole plant *A. annua* ($WP^{LO}$, N=26 containing 24mg/kg *in planta* artemisinin, and low-dose pure drug ($AN^{LO}$, N=20) containing 24mg/kg pure artemisinin, and placebo control (CON, N=23) which received only mouse chow. Dark lines indicate average parasitemia by treatment type. Lighter lines show individual mouse trajectories. Shaded regions indicate the 95% confidence interval for the means calculated based on normality assumptions. Dashed vertical line indicates time of treatment.

Two other major *A. annua* flavonoids, myricetin and quercetin, are known to inhibit mammalian thioredoxin reductase, which is critical for cellular redox control (69). Thioredoxin reductase is also essential for the *P. falciparum* erythrocytic stage (70); therefore, inhibition of this parasite enzyme by myricetin and quercetin may work in synergy with artemisinin against *P. falciparum* (71).

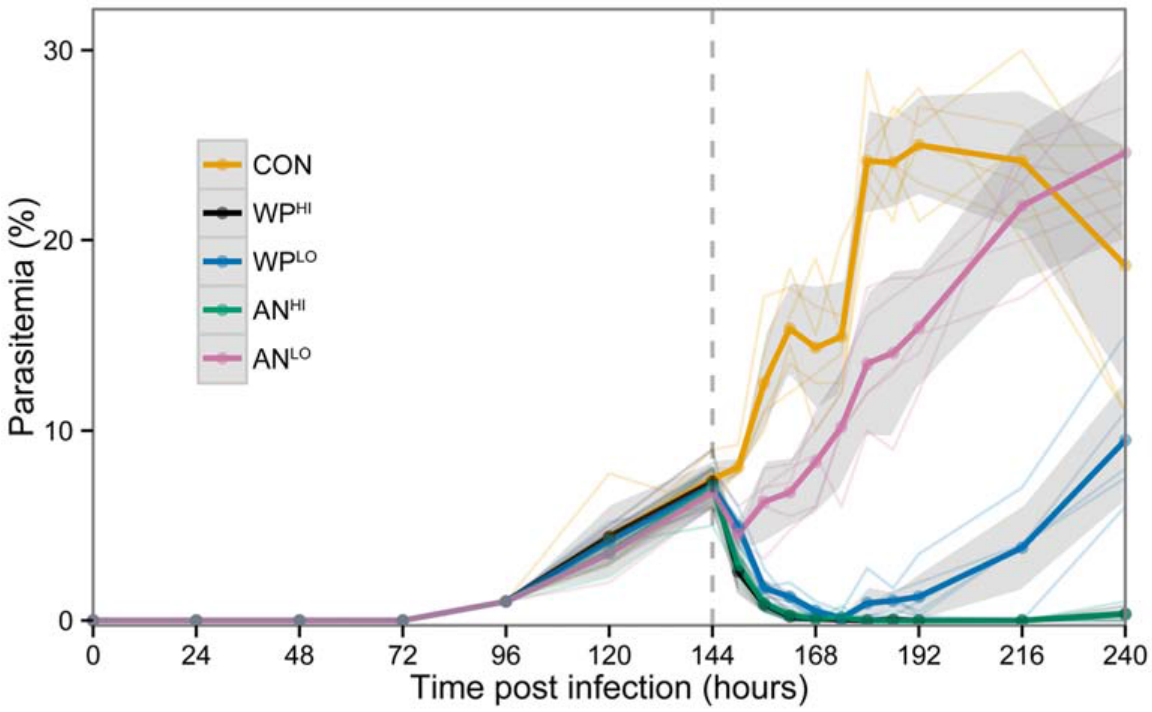


**Fig. I.3.** Dose comparisons of WP and AN treatments from the third replicate experiment: $WP^{LO}$ (N=6) and $WP^{HI}$ (N=6) received 24 mg/kg and 120 mg/kg *in planta* artemisinin, respectively; $AN^{LO}$ (N=5) and $AN^{HI}$ (N=6) received 24 mg/kg and 120 mg/kg pure artemisinin, respectively. Placebo control (CON, N=6) received only mouse chow. Dark lines indicate average parasitemia by treatment type. Lighter lines show individual mouse trajectories. Shaded regions indicate the 95% confidence interval for the means calculated based on normality assumptions. Dashed vertical line indicates time of treatment.

In addition to the bioavailability and potentiation attributes of WP, there are other compounds in *A. annua* that may act to reduce parasitemia independent of artemisinin. Liu *et al* (1992) reported the antimalarial activity of several *A. annua* flavonoids delivered *in vitro* as isolated compounds, in the absence of artemisinin (58). Moreover, antimalarial activity has been documented for related plant species that do not produce artemisinin (72). Among the compounds in *A. annua* not yet fully investigated are more than a dozen other sesquiterpenes, some of which have shown promise for killing parasites in rodent models (67).

Determining the mechanisms for increased efficacy of WP will require further investigation, but it seems certain that the constituent compounds contained within *A. annua* comprise a complex set of interactions and synergies yet to be described. Given the complex nature of the plant and its many components, WP may not necessarily be considered a simple monotherapy. While the temptation might be to consider WP as merely an alternative delivery mechanism for artemisinin, our results strongly indicate that WP is unique and may represent an innovative combination therapy. We refer to this as a *plant Artemisinin Combination Therapy* (pACT). A pACT can be distinguished from other combinational therapies where the drug components do not necessarily work synergistically because their combinations are artificially contrived. The pACT comprises a biologically complex entity, in which the combinations are the result of evolutionary processes that would have attributes of redundancy and resiliency that make combination therapies selectively advantageous to simple monotherapies. Refinements of these combinations by evolutionary processes ensures they are robust.

The novelty of the WP pACT cannot be overemphasized as there is a common misconception that this therapy has been tested previously. It has not. The WP therapy tested in the present study should not be confused with tea or infusion therapy. Whole plant A. annua (WP) tested here against murine malaria, utilizes the plant in its entirety, dried under controlled conditions and ingested by the host. Such a preparation of *A. annua* has never been tested against malaria parasites (in humans, mice or otherwise).

Because *A. annua* has long been used to make tea to treat fever in Asia (73), several investigators have proposed to re-establish the use of *A. annua* tea for rapid treatment of malaria (43, 74). These teas have major shortcomings. Firstly, large volumes

of tea must be consumed over short periods to ensure adequate ingestion of drug, a nontrivial matter considering the bitter taste of the tea, especially for pediatric patients. Moreover, while a 5 min boiling water extraction yields about 90% of the plant's artemisinin (74), this is not an effective process for extracting key flavonoids (58). Analysis of hot water tea extracts following the optimized protocol described by van der Kooy and Verpoorte (74), showed loss of about 99% of the original flavonoids that reportedly synergize with artemisinin (75).

Not only does WP differ from teas and infusions in terms of its efficacy and pharmaceutical properties, but because of its preparation, it can be carefully controlled and preserved. We was previously proposed development of a new form of anti-malarial therapy based on dried, encapsulated *A. annua* leaves as an inexpensive, dose-controlled, rapid delivery of the drug to treat uncomplicated cases of malaria and other neglected tropical diseases for which artemisinin has been shown to be effective (49). Dosage can be controlled because dried WP *A. annua* can be homogenized and assayed for artemisinin content prior to encapsulation. Capsule size and number can be adjusted based on artemisinin content and patient weight.

Our purpose in the present study was to determine whether this "generally regarded as safe" (GRAS) herb (76) is effective in killing malaria parasites *in vivo*. Extrapolating appropriate human dosage from experimental evidence in mouse models will require additional investigation, however it is generally understood that this extrapolation will not scale linearly with respect to body mass. Better indicators that allow for allometry include use of total body surface area (77) and/or take full consideration of physiochemical properties of the drugs and species involved (78). As an

example of this non-linear relationship, we can look to the results from $AN^{LO}$ treatment administered to mice in this study. $AN^{LO}$ mice received a dose of 24 mg/kg which exceeds the current WHO single therapeutic dose (20 mg/kg) for treating human malaria, however, in our study this dose of pure drug had very little effect against mouse malaria parasites. This is most likely due to metabolic differences and rates of uptake of oral drug between mice and humans.

Single oral dosages of AN proven effective against human *P. falciparum* malaria range from 100-500 mg (79), which corresponds to 6-33 grams of WP assuming a 1.5% artemisinin content. Assuming an average tablet size of 1 gram, delivery of a comparable dose of WP seems plausible. However, our data suggest that WP requires a smaller overall amount of artemisinin since even the $WP^{LO}$ effectively reduced malaria after just a single dose. Moreover, it is important to bear in mind that while $WP^{LO}$ was the lowest concentration in our study, it does not necessarily represent a minimum effective dose.

Notwithstanding challenges to be overcome and further research needed, our preliminary investigations hold great promise for easing the burden of high cost and limited availability currently confronting use of artemisinin-based semi-synthetic derivatives. While production costs for pharmaceuticals are not generally made publically available, it is possible to estimate potential savings associated with using WP vs. AN by looking to the estimated efficiencies of processing AN from the plant. Kindermans et al (80) estimate the yield for extraction and purification of drug from *A. annua* to be 50-80% efficient. This is consistent with general understanding of downstream processing costs wherein product losses increase with the number of unit operations (unit ops) (81). All things being equal (eg. artemisinin content for a given cultivar), this would suggest a

20-50% savings realized by forgoing the extraction and purification steps, as would be the case for production of WP. An example of the steps involved in production of AN from whole plant is provided by de Vries *et al* (1999), and compared with steps for producing WP (82) (Fig. I.4). Current production of AN by extraction from *A. annua* has seven more unit ops than the single unit op associated with direct use of the whole plant, and this net loss in efficiency translates to higher costs per product unit (Fig. I.4). Given the multiplicative principle of downstream processing, even if each processing step has relatively high efficiency, the overall efficiency will be less. For example, even if each of the seven additional steps for production of AN could be made 95% efficient, the overall efficiency would be merely 69.8%, which means less than three quarters of the starting material is realized as drug. Such a loss means a higher cost for the delivered drug. This simple analysis does not even consider the additional costs for reagents, labor, and energy that are required for processing the extract (Fig. I.4), which de Vries *et al* (1999) estimated may comprise up to 22.9% of the total manufacturing costs(82). This 22.9% represents additional cost savings for the use of WP because those inputs are no longer required.

And while our data are far from representing a clinical trial, they do provide preliminary indications that the WP therapy requires a far smaller dose than the corresponding amount of pure artemisinin. Indeed, our experiments indicate that a dose of WP has a five-fold increase in anti-malarial activity over that of the corresponding amount of AN. This increased activity per unit of plant mass not only affects the dosing regimen but also has profound economic impacts if the WP approach should prove useful on a large scale to treat human malaria.

**AN process:**
**Dried leaves** → Milling and homogenization → Extraction at 30-40°C → Crystallization → Filtration/washing → Decolorization → Crystallization → Filtration → Drying at 55-60°C →AN → Assay

**WP process:**
**Dried leaves** → Milling and homogenization → AN →Assay

**Fig. I.4.** Comparison of production processes for AN and WP demonstrated reduction in unit operations associated with use of whole plant as therapy. (Based on de Vries *et al* (1999)(82).

Much work remains to determine feasibility and efficiency of bringing whole plant *A. annua* into the arsenal in the fight against malaria. Among the challenges to be faced are some botanical obstacles, not the least of which is that this plant readily outcrosses, making it difficult to maintain high artemisinin content in the plant using seed saving methods which are standard agricultural practice in the developing world. Moreover, to date all efforts at improving the *A. annua* crop have focused on plant breeding and growth methods to maximize artemisinin content and in so doing to increase efficiencies and drive down costs. The use of the whole plant as therapy may represent a paradigm shift in this regard, since it may well be the case that effectiveness of WP is not wholly dependent on artemisinin content. New plant breeding strategies would have to be considered to optimize plant performance and maximize efficacy. But the potential for an inexpensive malaria therapy that by its very nature possesses great resilience to parasite resistance, makes this investment of effort well worth while.

## Conclusions

We have demonstrated that orally delivered WP *A. annua* is an effective means of killing malaria parasites in a mouse model. An edible WP *A. annua* treatment approach could significantly increase the number of patients treated and at significantly less cost. In fact, our results suggest that the WP treatment is a more efficient delivery mechanism than the purified drug, which is both costly and inefficient. Because AN has such broad potential therapeutic power against many infectious agents (35), our approach would dramatically reduce the cost of healthcare not only in developing countries, but also in more developed nations. Furthermore, use of *A. annua* could be implemented locally: a plan for plant cultivation, processing, and drug content validation was described in an earlier report (49). This, in turn, could provide a broad socioeconomic stimulus for developing countries while making them less dependent on the West.

## Chapter II

# PHARMACOKINETICS OF ARTEMISININ DELIVERED BY ORAL CONSUMPTION OF *ARTEMISIA ANNUA* DRIED LEAVES IS IN DIFFERENT FOR HEALTHY VS. *PLASMODIUM CHABAUDI*-INFECTED MICE.

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***Contributed equally to the work**
**Author contributions:** M.A.E., P.J.W., and S.M.R. designed research; M.A.E. performed the mouse-parasite experiments; M.J.T. and P.J.W. grew plants and conducted all biochemical analyses; P.J.W. and G.A-M. analyzed data; **M.A.E., P.J.W., M.J.T., G.A-M. and S.M.R. wrote the paper** (83).

## Introduction

Malaria is one of the worst vector-borne infectious diseases in the developing world and the World Health Organization (WHO) estimated that 215 million cases of malaria occurred, with >655,000 deaths; half the world's population is at risk of contracting the disease (25). Artemisinin, delivered in combination with an alternate drug as Artemisinin-based Combination Therapy (ACT), is currently the preferred treatment against the disease in order to prevent emergence of drug resistance.

Artemisinin (AN; Fig. II.1) is produced, extracted, and purified from the "generally regarded as safe" GRAS; (40) plant *Artemisia annua* L. (Asteraceae). The drug also has shown promise against a wide variety of human (84) and livestock diseases (85); many are common to the developing world. Although new sources of the drug are emerging from chemical synthesis (86) and engineered microbes (87), artemisinin is currently only

commercially available from *A. annua* with insufficient supply to treat malaria, let alone other artemisinin-susceptible diseases. To obviate emergence of drug resistance, ACT co-drugs are also needed, which usually increases cost (88).

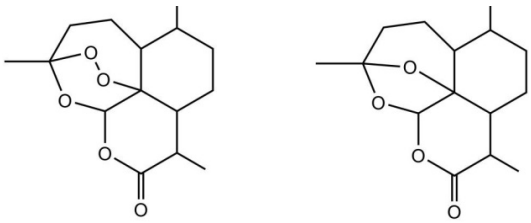


Fig. II.1. Artemisinin (left) and deoxyartemisinin (right).

In an effort to improve the efficacy of artemisinin therapy and lower cost, we recently showed that when delivered orally to *Plasmodium chabaudi*-infected mice, a single dose of dried leaves of *A. annua* quelled parasitemia at least fivefold more than an equal amount of the pure drug (41). Beyond the abnormalities associated with infection, analysis of blood toxicology showed no toxicity, results consistent with some human trials using dried *A. annua* leaves (89, 90). It was also previously showed that healthy mice fed dried *A. annua* leaves had > 40 times more artemisinin in their bloodstream than mice fed a corresponding amount of pure drug (49). The measured serum levels exceeded by eight fold the minimum concentration of artemisinin (~10 μg $L^{-1}$) required for lethality against *P. falciparum* (56). Together these results suggested that more artemisinin was delivered from whole plant treatments than from the pure drug treatment. Indeed, in a recent simulated digestion study, > 50% of dry leaf-delivered AN was still available in the intestinal digestate (91). It is thought that besides artemisinin, the combination of other parasite-killing substances normally present in the plant (flavonoids, monoterpenes

etc.; (92); (57); (93); (94) may be responsible for the observed responses either by improving bioavailability and/or improving therapeutic efficacy. In short, the plant may itself be providing endogenous combination drugs with its artemisinin. We thus termed this a plant-based artemisinin combination therapy, hereafter referred to as pACT.

To better assess the potential of pACT, we conducted a longer pharmacokinetic study to answer two main questions: were the pharmacokinetics of pACT different between healthy and infected mice, and was the plant matrix critical to the appearance of artemisinin in the blood? These results will further our understanding of how the drug moves into the blood when orally delivered as dried leaves of the whole plant.

**Materials and Methods**

**Plant material.** *Artemisia annua* L. (SAM clonal cultivar; voucher MASS 00317314; vernacular names: annual wormwood; *qīnghāo;* sweet annie, sweet wormwood) containing 1.48 ± 0.06% artemisinin (dry weight) as determined by GCMS was used in this study. Dried leaves of the SAM cultivar also contain monoterpenes (e.g., 0.21% (w/w) camphor, 0.007% eucalyptol, 0.037% α-pinene) and flavonoids (0.37% total). Plants were grown under controlled conditions, harvested, dried, and leaves sieved and pulverized as previously described (41). Homogenized dried leaf biomass was assayed for artemisinin and deoxyartemisinin using GCMS as described in Elfawal et al. (2012). Identification was via NIST library and purchased standards of artemisinin (Sigma-Aldrich Chemical, St. Louis, MO) and deoxyartemisinin (Toronto Research Chemicals Inc.).

**Mouse infection, feeding and drug delivery.** *Plasmodium chabaudi* ASS (MRA-429) was obtained through the Malaria Research and Reference Reagent Resource Center (MR4) as a part of the BEI Resources Repository, NIAID, NIH. Infection of 20 inbred male C57BL/6 mice weighing an average of 23 g was established by intraperitoneal (i.p.) injection with $10^7$ *P. chabaudi*-infected red blood cells as detailed in Elfawal et al. (2012). A second group of 20 of these same mice served as healthy controls. Infected (n=20) and healthy (n=20) mice received oral-gastric gavage of pACT treatment consisting of dried, powdered *A. annua* leaves in a water slurry, prepared fresh, and administered 72 hours post-infection (when parasitemia reached 2-3%) at an amount corresponding to 100 mg AN $kg^{-1}$ live body weight as detailed in Elfalwal et al. (2012). Blood was collected by cardiac puncture from five infected and five healthy mice at each of 4 time intervals (15, 30, 60, and 120 min post-gavage). For additional serum comparisons, two groups of three healthy mice were individually treated with 100 mg $kg^{-1}$ of pure AN in DMSO and water per kg live body weight with or without mouse chow (Elfawal et al., 2012) and blood collected at 60 min post gavage. Because a preliminary study showed that serum AN was at best barely detectable when mice were fed the pure drug, blood from each group of mice was pooled prior to extraction and analysis.

**Blood extraction and analysis.** Serum and red blood cells were separated by centrifugation at 5,000 x *g* for 10 min and both fractions were immediately stored at -80°C until extraction. Samples were extracted overnight at room temperature with an equal volume of methylene chloride. Where necessary, extracts were filtered through a

0.45 μm filter to remove particulates. Extracts were dried under nitrogen gas at room temperature and the dried residue resuspended in a known volume of methylene chloride for analysis of artemisinin and deoxyartemisinin by GCMS. Analysis and identification using authentic standards was as detailed above for analysis of plant extracts.

**Statistical analyses.** The locally weighted scatterplot smoothing (LOWESS) function was employed to generate a polynomial regression trend line for each of the kinetics plots (95). Similar results were obtained using the R PK package (96), which was used to compute the area under the serum concentration versus time curve (AUC). Specifically, functions relevant to the serial sampling design employed in this study were applied. Based on mean concentrations at the various time points, the method of residuals was used to estimate a first order elimination rate constant (and half-life) as well as a first order absorption rate constant.

**Ethics Statement.** This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts (Protocol# 2011-0015). All efforts were made to minimize suffering of animals during experimental procedures.

**Theory**

Oral consumption of dried leaves of *A. annua* is showing therapeutic promise (41, 89, 90). In contrast to what is known for pure artemisinin, little is known about how the drug moves from orally ingested plant leaves (e.g. pACT) into the blood stream. Comparing

the pharmacokinetics of the plant-delivered drug and one of its most common metabolic products in the serum of healthy and infected animals will improve our understanding of artemisinin metabolism and help clarify how such a therapeutic could eventually be implemented.

**Results and Discussion**

Similar to a preliminary study that compared oral gavage of either pure artemisinin or dried *A. annua* leaves (pACT) in healthy mice (49), we fed pACT to healthy or *P. chabaudi*-infected mice and measured artemisinin and deoxyartemisinin in the serum for two hours post gavage (Fig. II.2). The same data are replotted in Fig. II.3 to depict the independent kinetics of artemisinin and deoxyartemisinin in healthy vs. infected mice. The first order elimination rate constant for artemisinin in pACT-treated healthy mice was estimated to be 0.80 $hr^{-1}$, which corresponds to an elimination half-life ($T_{½}$) of 51.6 min as determined by the method of residuals (45 ± 5.75 via the PK package of Jaki and Wolfsegger, 2011), similar to that observed in rats by Niu et al. (1985)(97). The first order absorption rate constant was estimated at 1.39 $hr^{-1}$. $C_{max}$ and $T_{max}$ were 4.33 mg $L^{-1}$ and 60 min, respectively (Table II.1). The area under the curve (AUC) for the length of this study was 299.5 μg·min $mL^{-1}$. In contrast, the AUC for pACT-treated infected mice was significantly greater at 435.6 mg·min $L^{-1}$ (Table II.1).

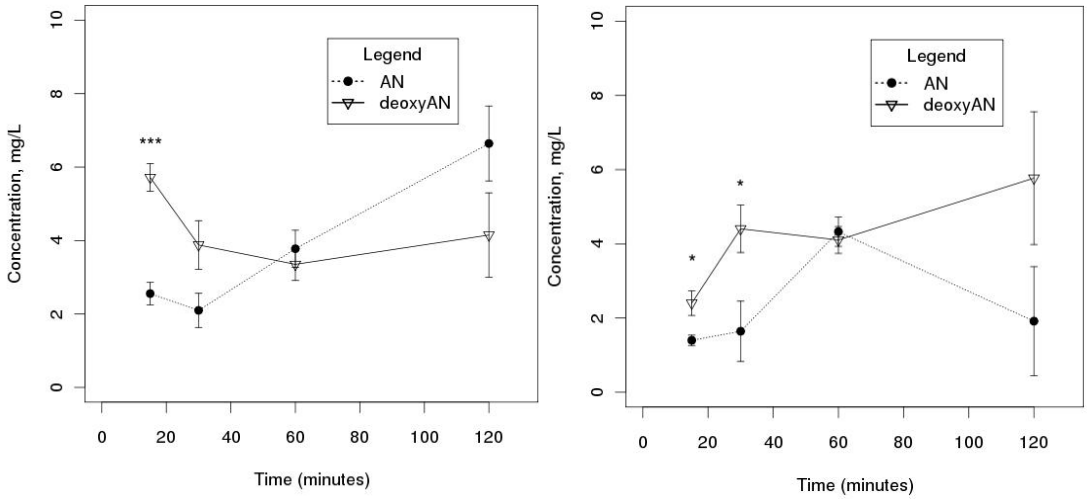


Fig. II.2. Pharmacokinetics of artemisinin and one of it metabolites, deoxyartemisinin in serum of healthy (right) and *P. chabaudi*-infected (left) mice. Mice (n = 4 - 6 per time point per treatment group) were orally gavaged with dried leaves of *A. annua* to deliver 100 mg $kg^{-1}$ body weight dose of artemisinin; bars show SE and * $p < 0.05$, *** $p < 0.001$ via Student's t- test.

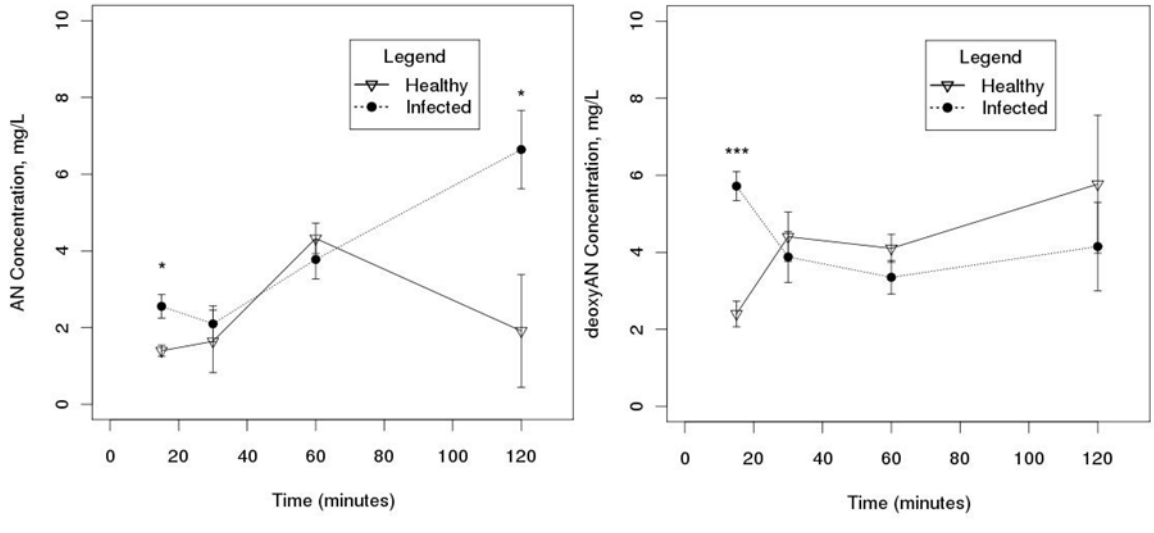


Fig. II.3: Pharmacokinetics of artemisinin (left) and one of it metabolites, deoxyartemisinin (right) in serum of healthy and *P. chabaudi*-infected mice. Mice (n = 4 - 6 per time point per treatment group) were orally gavaged with dried leaves of *A. annua* to deliver 100 mg $kg^{-1}$ body weight dose of artemisinin; bars show SE and * $p < 0.05$, *** $p < 0.001$ via Student's t- test.

Serum levels of artemisinin in the infected mice continued to rise over the 120 min of the study period (Fig. II.2), and as a result, the $T_{½}$ was not determined; $C_{max}$ and $T_{max}$ were estimated at ≥ 6.64 mg $L^{-1}$ and ≥ 120 min, respectively. The key pharmacokinetic descriptors are shown in Table II.1 and are consistent with those of an earlier preliminary study using only healthy mice (49) and with other studies (97, 98) using rats fed pure artemisinin.

| Dose per animal (mg AN $kg^{-1}$) | Disease status | # of subj. | Artemisinin | | | |
|---|---|---|---|---|---|---|
| | | | $C_{max}$ (mg $L^{-1}$) | $T_{max}$ (min) | $T_{½}$ (min) | AUC (mg·min $L^{-1}$) |
| 100 | healthy | 4-6 | 4.33 ± 0.40 | 60 | 51.6 | 299.5a |
| 100 | infected | 5 | ≥ 6.64 | ≥ 120 | nd | 435.6b |

Table II.1. Pharmacokinetic parameters for artemisinin after oral gavage of healthy or *P. chabaudi*-infected mice with pACT. Animals were fed *A. annua* dried leaves (pACT) to deliver 100 mg AN $kg^{-1}$; average body weight was 23 g; nd, not determined. Letters a,b after AUC values indicate statistical difference at $p \leq 0.05$; values are ± SE.

Batty et al. (2008) compared the pharmacokinetics of dihydroartemisinic acid (DHA) in healthy and *Plasmodium*-infected mice that were administered a single intraperitoneal dose of DHA (100 mg $kg^{-1}$) and found no significant difference in elimination half-lives. Since we was unable to determine $T_{½}$ for infected mice in our experiment, we cannot compare it to healthy mice(99). Although a direct comparison cannot be made between the pharmacokinetics of DHA and artemisinin, in contrast to Batty *et al* (2008), healthy mice in our study had a significantly lower AUC than infected mice.

Because it is one of several liver metabolic products of artemisinin, we also measured deoxyartemisinin (66). Like many other drugs, artemisinin is metabolized in the liver by cytochrome P450s including CYP2B6 and CYP3A4 (66), yielding a number of compounds including deoxyartemisinin, deoxydihydroartemisinin, 9,10-dihydrodeoxyartemisinin, and a metabolite named crystal 7 that are found in human urine (100). Each of these degradation products lacks the endoperoxide moiety present in artemisinin, and is therefore therapeutically inactive (100). In contrast to its metabolism in healthy mice (Fig. II.2), metabolism of artemisinin to deoxyartemisinin appeared to be suppressed in the infected mice (Fig. II.2); infection retarded the capacity of the mice to form deoxyartemisinin from artemisinin over the two-hour study period. Overall, artemisinin concentrations decreased with a concomitant rise in deoxyartemisinin levels, but only in healthy subjects. In contrast, in infected mice artemisinin levels continued to rise over the study period whilst deoxyartemisinin levels fell and then remained constant. *A. annua* contains a diverse group of compounds (101) including flavonoids and monoterpenes that have been shown to inhibit *P. falciparum* (57, 92-94); many also have been shown to inhibit CYP3A4 (102-104).

Results showed an initial dip in artemisinin levels at 15 to 30 minutes in healthy subjects, which we attributed at least partially to conversion of artemisinin to deoxyartemisinin (Fig. II.2). The metabolism of artemisinin to deoxyartemisinin appeared to be biphasic, declining slightly between 30 and 60 minutes, and then rising thereafter. The rising arm of the artemisinin pharmacokinetic profile in healthy mice occurred during this period of decreased metabolism to deoxyartemisinin; here, absorption

exceeded elimination. When Du *et al* (2012) measured deoxyartemisinin in healthy rats given a single oral dose of pure artemisinin (40 mg $kg^{-1}$), the $T_{max}$ of deoxyartemisinin was 30-60 min longer than that of artemisinin (60 min)(98). Their artemisinin metabolism kinetics were the opposite of our results for a single oral dose of pACT where the peak of artemisinin was later than the first peak for deoxyartemisinin, but earlier than the second deoxyartemisinin peak (Fig. II.2). The pharmacokinetics in rats is not necessarily the same as in mice, so this may account for the differential response between our two sets of results.

We also examined the effects of mouse chow on pure artemisinin entry into the serum. Mouse chow contains a variety of plant materials including soy, oats, wheat, alfalfa, beet pulp, corn, etc. No artemisinin was detected in the serum of mice fed 100 mg AN $kg^{-1}$ body weight (Table II.2), a result consistent with the reported poor availability of the pure drug (105). When plant material was present either as mouse chow or *A. annua* pACT, the level of artemisinin in the serum rose to 2.44 and 4.32 mg $L^{-1}$, respectively, indicating that the presence of plant material, even that in mouse chow, had a positive impact on the appearance of artemisinin in the blood (Table II.2). These results were consistent with preliminary study (49). *A. annua* leaves can contain as much as 1.4% (w/w) essential oils (101), which may enhance solubility and thus bioavailability of the nonpolar artemisinin molecule.

For a human comparison, Giao et al. (2001) showed that when humans were given 500 mg oral doses of pure artemisinin (twice on day 1, then once thereafter for days 2-7), the level of recrudescence was 25%(106). In a summary of early human trials,

oral delivery of at least 600 mg per patient for 5 days was required to achieve ≤ 10% recrudescence (105). More recently a Kenyan clinical trial with four cohorts (4 x 12 subjects) of malaria patients (90) showed that patients receiving dried *A. annua* leaf tablets containing 11.1 mg artemisinin (twice on day 1, so 22.1 mg $d^{-1}$) followed by 7.4 mg artemisinin (twice a day for days 2-6, so 14.8 mg $d^{-1}$) had 9.1% recrudescence (107).

| Mouse treatment | Artemisinin (mg $L^{-1}$) |
|---|---|
| Healthy untreated (3 pooled mice) [a] | 0 |
| Healthy + 100 mg AN $kg^{-1}$ (3 pooled mice) [a] | 0 |
| Healthy + chow + 100 mg AN $kg^{-1}$ (3 pooled mice) [a] | 2.44 |
| Healthy + pACT at 100 mg AN $kg^{-1}$ (n = 5) | 4.33 ± 0.40 [b] |
| Infected + pACT at 100 mg AN $kg^{-1}$ (n = 5) | 3.78 ± 0.50 [b] |

Table II.2. Artemisinin in serum 60 min post pACT or pure artemisinin gavage.
[a] These mice were treated in groups of three; blood was harvested and pooled prior to extraction and measurement of artemisinin, so SE was not calculated.
[b] Values are ± SE.

Furthermore, increasing the amount of dried leaf tablets did not improve therapeutic response, suggesting that maximum effective dose had been achieved. Thus, although the patients treated with dried leaf tablets received about 3-4% the equivalent amount of pure artemisinin used in earlier studies (105, 106), they had an equal or better therapeutic outcome than patients treated with pure artemisinin. Those results are consistent with the results of this study.

## Conclusions

To our knowledge this is the first comparison between healthy and diseased animals of the pharmacokinetics of artemisinin and one of its liver metabolites, deoxyartemisinin, from orally delivered dried leaves of *A. annua.* When accompanied by plant material, more artemisinin enters the blood stream, demonstrating a beneficial effect of the plant matrix on the bioavailability of artemisinin. These results have implications for possible therapeutic use of pACT in treating malaria and other artemisinin-susceptible diseases.

Chapter III

# DRIED WHOLE PLANT *ARTEMISIA ANNUA* SLOWS EVOLUTION OF MALARIA DRUG RESISTANCE AND OVERCOMES RESISTANCE TO ARTEMISININ

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**Author contributions:** D.G., M.A.E., P.J.W., and S.M.R. designed research; M.A.E. performed the mouse-parasite experiments; M.J.T. and P.J.W. grew plants and conducted biochemical assays; M.A.E., N.G.R., and S.M.R. analyzed data; M.A.E., P.J.W., N.G.R., and S.M.R. wrote the paper.

## Introduction

The fight against malaria predates the discovery of its causative agent. For centuries malaria-associated fever was treated using herbal remedies. In the west, quinine (Cinchona bark extract) was the only affordable treatment against malaria until Paul Ehrlich's magic bullet concept was adopted and thousands of synthetic compounds were tested against malaria parasites. Very few of these compounds were effective and safe for human use, but in the 1930's chloroquine rose to ascendancy as a miracle cure for malaria (108). In the late 1950s, chloroquine was the main weapon when the World Health Organization launched the Global Malaria Eradication Program (GMEP). Development of drug resistant parasites and concomitant failure of chloroquine as the drug of choice lead to the eventual demise of GMEP by the close of the 1960s. Following

chloroquine's failure, various antimalarial compounds were serially deployed one after another, and each unfortunately faced the same demise leaving millions of malaria patients without affordable treatment.

In 1970s artemisinin was discovered as a pure drug extract from *Artemisia annua*. In wide scale clinical trials, artemisinin showed poor pharmacokinetic properties but nonetheless demonstrated potent antimalarial activity with a high safety profile (109). It was determined that AN was best when used in combination with other antimalarial drugs, mainly mefloquine, which became known as Artemisinin Combination Therapy (ACT) (110). It was hoped that use of ACTs would minimize risk of drug resistance. However, in 2005 evidence arose of *P. falciparum* resistant to ACTs in South East Asia (30-34, 111). The fight against malaria again entered another critical phase trying to avoid ACT from following chloroquine's path towards obsolescence with no affordable replacement in sight.

WHO has cautioned against use of non-pharmaceutical sources of artemisinin because of the risk of delivering sub-therapeutic doses that could exacerbate the resistance problem (112). This warning is valid given the low artemisinin content of most extractions and tea/infusions utilized for most non-pharmaceutical plant based therapies. However, in 2012 we demonstrated the efficacy of the whole plant—not tea, not infusion—as a malaria therapy and found it to be more effective than a comparable dose of pure artemisinin in a rodent malaria model (41). We contend that Whole Plant (WP) *A. annua* therapy could provide a more abundant and affordable source of artemsinin-based therapy by eliminating need for artemisinin extraction during manufacture.

WP is more effective than monotherapeutic artemisinin because WP constitutes a naturally occurring combination therapy that augments artemisinin delivery and synergizes its activity. This plant Artemisinin Combination Therapy (pACT) is the result of evolutionary refinement of the plant's secondary metabolic products into a resilient and multi-component defense system. As has been demonstrated for other combination therapies, we hypothesize that WP-based pACT will: (1) overcome existing resistance to monotherapeutic pharmaceutical artemisinin, and (2) increase the longevity of this therapy by delaying the onset of parasite resistance among wild types. Here we've tested these two hypotheses in two mouse malaria models, artemisinin-resistant *Plasmodium yoelii* (ART) and artemisinin-sensitive *Plasmodium chabudi chabaudi* (ASS).

**Materials and methods**

**Rodent Malaria Parasites:** All experiments were performed using an appropriate rodent malaria model, obtained through the Malaria Research and Reference Reagent Resource Center (MR4) as a part of the BEI Resources Repository, NIAID, NIH. For long-term selection experiments, we chose *P. chabaudi* (ASS; MRA-429) because of its known susceptibility to WP and artemisinin treatment. *P. yoelii* (ART; MRA-421) was chosen as available an artemisinin-resistant strain (ART).

***Plasmodium chabaudi* (ASS):** Tubes of blood collected from infected mice, were removed from liquid nitrogen storage and left at room temperature for 30 minutes. To activate parasite stocks, two C57BL/6 mice were injected intraperitoneally (i.p.) with 200 μL of infected blood. Percent parasitemia was determined in Giemsa-stained thin blood smears days 3-7 post-infection (p.i.). Seven days after inoculation, one mouse was

euthanized and cardiac puncture was used to collect blood into lithium heparin tubes. Infected blood was volumetrically adjusted by dilution in DPBS to create a 200 µL aliquot of $10^5$ infected erythrocytes for infection into two additional mice for a second round of activation. The activated parasites were used in subsequent drug selection and resilience tests.

***Plasmodium yoelii* (ART):** To revive parasites after long-term storage in liquid nitrogen, two C57BL/6 mice were injected (i.p.) with 200 µL of infected blood. Seven days after infection to build up parasitemia, one mouse was euthanized and cardiac puncture was used to collect blood into lithium heparin tubes. Infected blood was volumetrically adjusted by dilution in DPBS to create a 200 µL aliquot of $10^7$ infected erythrocytes for infection into four additional mice. Two mice were immediately treated with a dose of 150 mg/kg AN recommended by the provider to activate and maintain resistant phenotype, while the other two mice were left untreated as controls. Seven days post inoculation, infected blood of one mouse, which first reaches 2% parasitemia from each group, was used in the next passage. Activation for AN resistant phenotype was repeated over 10 passages until the treated and untreated mice reached 2% parasitemia at the same time. The activated parasites were used in the subsequent challenge.

**Anti-malarial treatments.** *Artemisia annua* L. (SAM cultivar; voucher MASS 00317314) containing 1.48±0.06% AN (dry weight) was used in this study. Detailed information about plant material and AN analysis was reported in our previous work (41, 83). To test dose dependent effects, we used two doses of each treatment such that the amount of artemisinin was equivalent in the two low ($WP^{LO}$ and $AN^{LO}$) and the two high ($WP^{HI}$ and $AN^{HI}$) treatment groups.

**Mouse feeding and drug delivery details:** Each mouse in the $WP^{LO}$ and $WP^{HI}$ groups received 40 mg and 200 mg of dried *A. annua* plant powder corresponding to 24 mg AN/kg and 120mg AN/kg live body weight respectively. Plant powder was mixed with water to a final volume of 0.5 mL ($WP^{LO}$) and 0.75 mL ($WP^{HI}$). Each mouse in the artemisinin $AN^{LO}$ and $AN^{HI}$ groups received 600 µg or 3000 µg of AN (Sigma-Aldrich Chemical, St. Louis, MO) corresponding to 24 mg AN/kg and 120mg AN/kg, respectively, which was freshly dissolved in 60 µL of DMSO mixed with water and 40mg or 200mg of powdered sieved mouse chow to a final volume of 0.5 or 0.75 mL, respectively. Placebo control (CON) consisted of 60 µL DMSO, mixed with water and 40 mg powdered sieved mouse chow to a final volume of 0.5 mL. Delivery of the appropriate treatment/control was performed immediately after dose preparation by oral-gastric gavage into each mouse using a feeding needle (18G, curved, 2", and 2.25 pall diameter). Individual mice were identified by tail markings using permanent marker. Percent parasitemia was determined in Giemsa-stained thin blood smears from a drop of peripheral blood obtained from the tail. Mice were observed twice daily for signs of disease or stress.

**Artemisinin resistant *P. yoelii* single dose response:** An aliquot of $10^6$ *Plasmodium yoelii (ART)* infected erythrocytes was inoculated i.p. into each of 30 C57BL6, 12 week old male mice weighing an average of 25 g, and were randomly divided into five groups ($WP^{HI}$ and $AN^{HI}$, $WP^{LO}$, $AN^{LO}$, and CON) of six mice per group. Food and water were provided *ad libitum* for the first four days. On day four, food was withheld for 24 hours, but water was freely available. Mice were treated on day 5-post inoculation. Percent parasitemia was determined every 24 hours in Giemsa-stained thin blood smears for the

first four days post inoculation, then every eight hours for 48 hours post gavage, and again on 24 hour intervals for days 8-13 p.i. All mice were euthanized on day 13 p.i. via asphyxiation in a $CO_2$ chamber followed by cervical dislocation.

**Artemisinin resistant *P. yoelii* curative treatment:** An aliquot of $10^7$ *Plasmodium yoelii (ART)* infected erythrocytes was inoculated i.p. into each of 12 DBA/2, 12 week old male mice, which were randomly divided into two groups ($WP^{HI}$, $AN^{HI}$) of six mice per group. Starting on day 2 post-inoculation, each mouse in the $WP^{HI}$ and $AN^{HI}$ groups received 9 daily dosing of 200 mg plant material (corresponding to 120mg AN/kg) and 3000 µg AN (corresponding to 120mg AN/kg) respectively. Percent of parasitemia was determined every 24 hours in Giemsa-stained thin blood smears from days 1-24 p.i., then every 72 hours for days 27-42 p.i. All mice were euthanized on day 42 p.i. via asphyxiation in a $CO_2$ chamber followed by cervical dislocation.

**Selection for drug resistance and resilience test:** Parasites were selected for resistance following the 2% relapse technique (113-115), in which a single treatment dose is given immediately after parasite inoculation to each mouse in the treated group, while a control group is inoculated and left untreated. The delay in reaching a 2% parasitemia in the treated group compared to the time required to reach the same parasitemia in the control group is the 2% delay time (113, 115). In each selection passage three groups of two mice were inoculated i.p. with $10^7$ red blood cells infected with AN-sensitive *Plasmodium chabaudi chabaudi* (ASS). Mice used in selection for AN resistance received single dose of 2500 µg of AN corresponding to100mg/kg, on the other hand mice used in selection for WP resistance received 167 mg of WP material corresponding to100mg/kg AN in WP, while mice use as control received placebo. Gavaged materials were prepared as

previously mentioned. Inoculations to the next passage occur on day 7 when all groups passed 2% parasitemia and build up enough parasitemia for the next passage. Seven days post inoculation, blood of one mouse, which first reaches 2% parasitemia from each group, was used in the following passage. On passage number 17 AN dose was doubled in the AN group after three consistent passages of zero delay time (AN treated group reachs 2% parasitemia at the same time as the control group). Parasites were considered resistant to a given dose if no delay time for three consequent passages. Selection for WP resistance ended on passage number 48.

**Statistical analysis.** We fit linear mixed models to estimate and compare the average parasitemia for each treatment group at each measured time point. Including a random intercept for individual mice allowed us to adjust for repeated observations on the same mouse. Single dose analysis compared the CON, $WP^{LO}$, $WP^{HI}$, $AN^{LO}$, and $AN^{HI}$ treatment groups at all measured time points.

For each model, 10,000 Markov chain Monte Carlo (MCMC) samples were drawn from the posterior distributions of the average parasitemia levels for each treatment group at each time point. Then, 95% confidence interval endpoints for a particular parasitemia level were established at the $2.5^{th}$ and $97.5^{th}$ quantiles of the MCMC samples for that parameter. An estimated difference between two groups was declared "significant" if the 95% confidence interval for the difference did not cover zero. Analyses were conducted using the statistical software R v2.15(62) and the lme4 package(63), fig. III.1 was produced using the ggplot2 package.

**Ethics Statement.** This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the

National Institutes of Health. All efforts were made to minimize suffering of animals during experimental procedures.

**Results and Discussion**

Previously, we found that dried whole plant *A. annua* kills *P. chabaudi* malaria parasites more effectively than a comparable dose of pure pharmaceutical artemisinin (AN). Here, we show that dried whole plant *A. annua* effectively kills malaria parasites resistant to artemisinin. Mice treated with single dose of $WP^{HI}$ (corresponding to 120 mg/kg AN) showed significantly greater reduction in parasitemia than those treated with a single comparable dose of $AN^{HI}$ (120mg/kg) from 16 to 168 hours post-treatment (Fig. III.1). Mice treated with $AN^{LO}$ did not differ in parasitemia from those administered a placebo at any time point. Treatment with $WP^{LO}$ (corresponding to 24 mg/kg AN) was as effective as $AN^{HI}$, despite the fact that $AN^{HI}$ dose contains 5 times more AN/kg (Fig. III.1). In these single dose studies, all parasite populations recrudesced around day 5 post-treatment, which is typical of this parasite model.

To test whether WP can completely clear infection, we administered multiple dose daily treatment of $WP^{HI}$ corresponding to 120 mg AN/Kg/day for 9 consecutive days. Multi-dose treatment reduced *P. yoelii* ART parasitemia below 2% for seven days post inoculation and to less than 1% from day 8 until complete clearance of parasitemia on day 14-post inoculation. Mice treated from both WP treatment groups were normal and did not show any signs of sickness. Infected groups of mice treated with comparable multiple doses of pharmaceutical AN (120mg AN/kg/day) for 9 days had mean parasitemia of 6% through day 7 with complete clearance on day 18-post inoculation

(Fig. III.2). Cured mice in WP and AN groups were monitored by Giemsa stained thin blood smears taken every 72 hours from day 24 until mice were euthanized on day 42 and no recrudescence was observed.

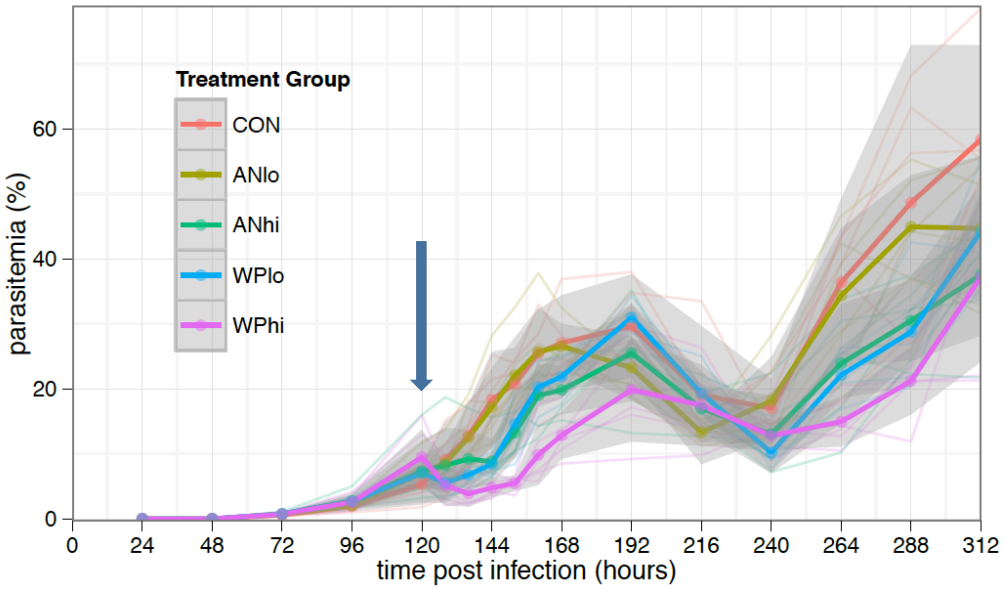


**Fig. III.1.** AN-resistant *P. yoelii* (ART) single dose comparisons of WP and AN treatments: $WP^{LO}$ (N=6) and $WP^{HI}$ (N=6) received *in planta* artemisinin at 24 mg/kg and 120 mg/kg, respectively; $AN^{LO}$ (N=6) and $AN^{HI}$ (N=6) received pure artemisinin at 24 mg/kg and 120 mg/kg, respectively. Placebo control (CON, N=5) received only mouse chow. Dark lines indicate average parasitemia by treatment type. Lighter lines show individual mouse trajectories. Shaded regions indicate the 95% confidence interval for the means calculated based on normality assumptions. Arrow indicates time of treatment

Having demonstrated that WP is effective against artemisinin-resistant parasites, we conducted an artificial evolution experiment to compare rates at which resistance to these two modalities arose among wild-type parasite lines. Using the 2%-relapse technique for long-term selection of drug resistance in *Plasmodium chabaudi* parasites, we achieved stable resistance to 100mg/kg AN at passages 13-16. On passage number 17, the dose was increased to 200mg/kg AN. Parasites acquired tolerance to this double dose after 40 passages with constant delay time less than 25% of the first passage (Fig. III.3).

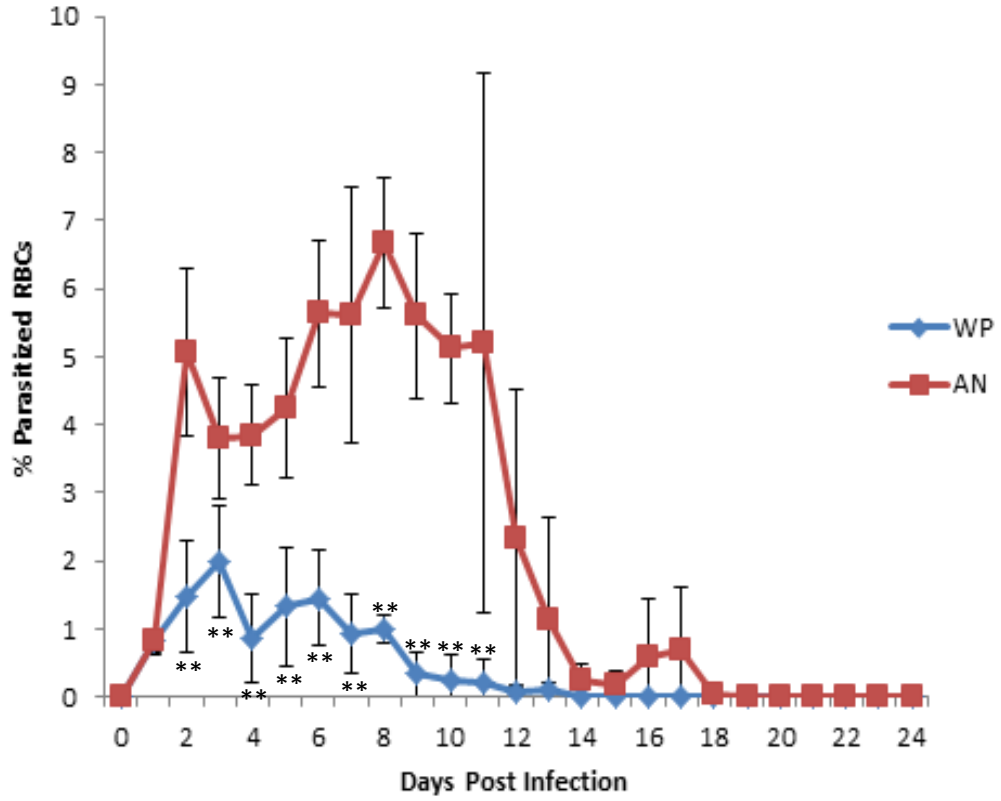


**Fig. III.2**: AN-resistant *P. yoelii* (ART) curative treatment: Mice were infected with *P. yoelii* (ART) and treated with either pure artemisinin (N=6) at 120 mg/kg, or WP *in planta* artemisinin (N=6) at 120 mg/kg. Mice were treated for 9 consecutive days starting on day 2 post-infection. ** = $P < 0.01$. Error bars represent SD.

The comparable dose of WP (100mg/kg AN) was more resilient such that resistance was achieved after only 45 passages. The WP treatment of 100mg/kg was even more resilient than the doubled dose (200mg/kg) of pure AN in the second phase of selection. *P. chabaudi* generally develops resistance to antimalarial drugs faster than other rodent malaria species (113). In a similar selection study using a combination therapy of artesunate 30mg/kg/day + mefloquine 5mg/kg/day- given over three days, resistance was achieved after 27 passages (116). Just as ACT treatment is more resilient

than a purified AN (117), we contend that WP treatment is more resilient than a pharmaceutical AN because WP treatment contains multiple and complementary compounds effective in killing malaria parasites including those resistant to pharmaceutical AN.

*A. annua* extracts have a number of different practical uses in agriculture and public health. These include repellency against stored product beetles (118), larvicidal activity against *Anopheles stephensi* (119), insecticidal activity against elm leaf beetle (120), and use as an acaricide (121). Moreover, AN and its derivatives have also shown to have effects on a number of viruses replication, a variety of human cancer cell lines (35, 36), several neglected tropical parasitic diseases including schistosomiasis (44), leishmaniasis (45, 46), New- and Old-World trypanosomiases (47), and many livestock diseases (48).

To better understand the reasons for efficacy of phytochemicals in killing mammalian parasites, we must consider carefully plant biology and evolution. Like all plants, *A. annua* lacks an immune system that can adapt somatically to its natural enemies including viruses, bacteria, fungus, parasites, insects and other animals. Instead of an acquired adaptive immune response, plants possess a complex repertoire of chemical defenses against a wide range of organisms. Evolutionarily successful plant species such as *A. annua* possess large arrays of bioactive compounds providing resilient protection against micro and macro threats (122) (123). The bioactive secondary metabolites of *A. annua* are synthesized and stored in glandular trichomes, which are small cusps of epidermal origin consisting of differentiated basal, stalk, and apical cells. Trichomes are

specialized to synthesize, store, and secrete large amounts of phytochemicals including artemisinin, flavonoids, phenolics and terpenoids (123-125).

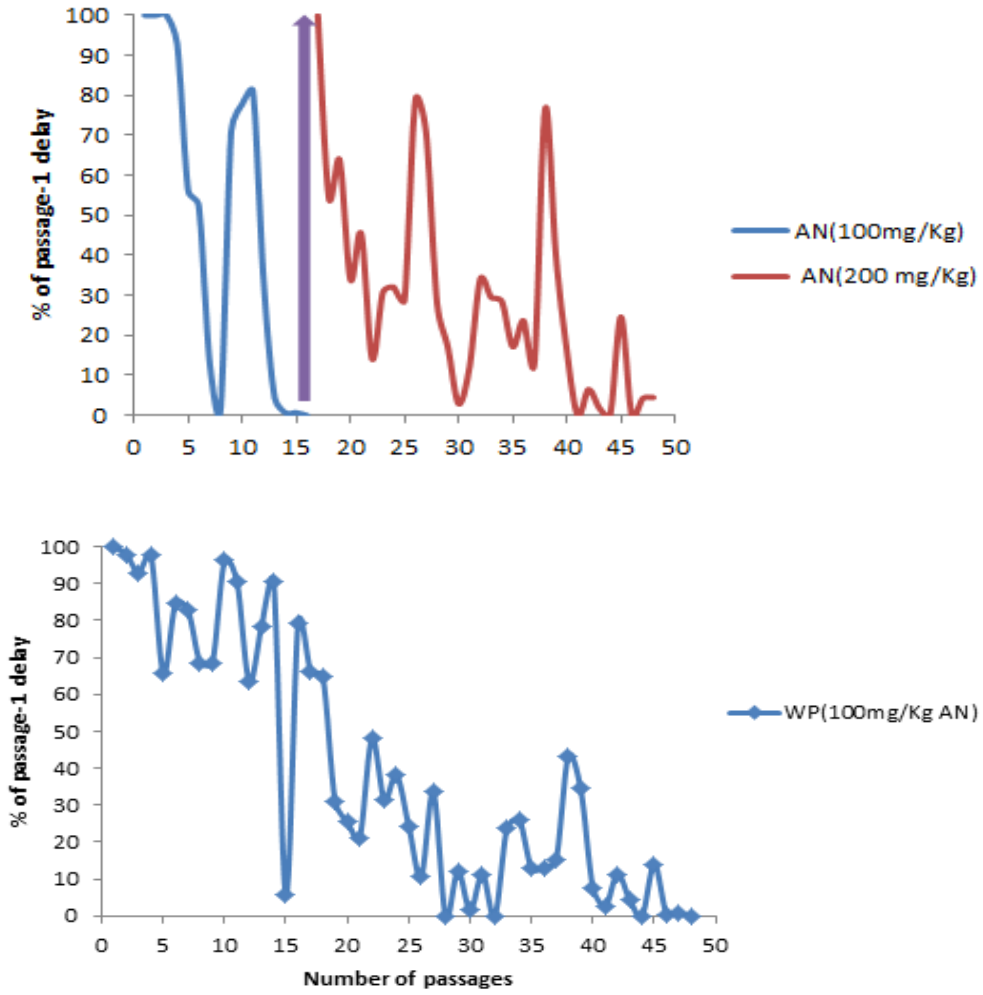


**Fig. III.3.** Evolution of resistance in *P. chabaudi* (ASS) exposed initial dose of 100 mg/kg AN (upper blue line), then increased (Arrow) to 200 mg/kg AN (upper red line) and WP 100 mg/kg *in planta* artemisinin ( lower blue line) , following the 2% relapse technique.

Components of the *A. annua* chemical defense include allelopathic factors that inhibit neighboring plants from germinating or growing. Artemisinin is known to have potent herbicidal activity acting on the electron transport in chloroplast membrane (124)

and plant cell membrane (39, 126). That these defenses are also effective at killing a mammalian blood-borne parasite seems at first coincidental, however there is a plausible evolutionary explanation for this cross species protection. Malaria parasite cells contain apicoplasts, which were derived initially from the same ancestral cyanobacterial endosymbiont that gave rise to chloroplasts in plants (127). This vestigial organelle in *Plasmodium* is believed to be an essential target of the *A. annua* allelopathy defense mechanisms (38).

It is well known that the bacterial origin of apicoplasts renders malaria parasites susceptible to antibiotics, such as doxycycline, which have known to inhibit transcription and translation of prokaryotes by blocking its replication and malaria parasite cell death (127, 128). Likewise, the presence of plant-like organelles and pathways in parasites that are absent in the human host present intriguing prospects for co-opting herbicidal compounds as medicaments(129). Various plant extracted herbicides already have been found to have specific parasite killing activity against apicomplexan parasites including *P. falciparum*, *Cryptosporidium parvum* and *Toxoplasma gondii* (38). These herbicides may be synthetic or, as with the WP therapy discussed here, derived from naturally occurring plant forms.

Among the phytochemical repertoire that is synthesized and located in the trichome are the terpenoids, the essential oil constituents of *A. annua* giving the plant its fragrance (101). Terpenoids are compositions of component isoperenes with extensive branching and cyclization ability. *Plasmodium* spp. possesses its own terpenoid pathways present with the apicoplast which is essential for the growth and replication of intra and extra erythrocytic stages (130) (128). Due to similarity among the intermediates of plant

and parasite pathways, some of the plant derived terpenoids have been shown to disrupt function of the apicoplast leading to parasite killing (131). Among the anti-parasitic terpenoids is nerolidol that inhibits the biosynthesis of the isoprenic chain (132) and limonene that inhibits the protein isopenylation in the erythrocytic stages of *P. falciparum* (133). Nerolidol, linalool, limonene and farnesol have $IC^{50}$ against *P. falciparum* of 760 nM, 0.28mM, 1.22mM and 64uM, respectively (134). Monterpenes isolated from eucalyptus oil (80% cineole) inhibit chloroquine-resistant and chloroquine-sensitive *P. falciparum* by mechanisms believe to be connected to membrane disruption (135). Indirect evidence of other non-AN antimalarial activity in *A. annua* is provided the documented parasite killing using related species of *Artemisia* that do not produce artemisinin (67). Moreover, several other sesquiterpenes in *A. annua* have shown activity against malaria parasites (67). The ability of plants to produce a virtually unlimited diversity of terpenoids by elongations, cyclizations, and rearrangements gives potential for redundant and resilient anti-parasite killing activity (11, 136)

As in the challenge experiments against malaria parasites, the allelopathic activity is higher for the whole plant than a comparable dose of its pure extract AN when incorporated into soil (137). The higher allelopathic activity of whole plant *A. annua* than its pure extract AN is attributable to the synergism among *A. annua* constituents (137) and/or the presence of other constituents of *A. annua* that have potent herbicidal activity (37). This same synergism between *A. annua* constituents has been reported to potentiate the antimalarial activity against *P. falciparum* (14, 41, 48, 67).

The fact that antimalarial activity of WP is better than artemisinin can possibly be explained by enhanced bioavailability of artemisinin due to inhibitory effects of some *A.*

*annua* flavonoids on the hepatic and intestinal cytochrome P450 enzymes that metabolize artemisinin *(66, 83)*. Plant material seemed to enhance by >40 fold the amount of artemisinin that entered the blood stream (49). The presence of plant material in mouse chow significantly increased the amount of artemisinin that appeared in the serum (83).

Whole plant *A. annua* may also have increased activity against malaria parasites due to the synergism among some key flavonoids and artemisinin (14, 41, 48, 67) or by the antimalarial activity of other *A. annua* constituents independent from AN. Certain *A. annua* flavonoids (artemetin, casticin, chrysosplenetin, chrysosplenol-D, cirsilineol, and eupatorin) showed antimalarial activity against *P. falciparum* in the absence of AN (138). Similarly, four dietary flavonoids—kaempferol, quercetin, apigenin and luteolin—that are present in *A. annua* (101) were tested against chloroquine-sensitive *P. falciparum* (strain 3D7) and showed 50% Inhibitory Concentrations ($IC^{50}$) of 33uM, 15uM, 20uM and 11uM, respectively. Also, these four flavonoids were effective against chloroquine-resistant *P. falciparum* (strain 7G8) giving $IC^{50}$ of 25uM, 14uM, 13uM and 12uM, respectively. Similarity of $IC^{50}$ values for each of these four flavonoids regardless of their chloroquine sensitivity suggests that the flavonoids are acting on targets distinct from those of chloroquine.

The likelihood of developing resistance is greater for a single antimalarial compound than a combination therapy. Increased resilience of WP relative to a comparable dose of pharmaceutical AN is consistent with this pattern. Resilience of WP can be explained by the presence of a large phytochemical repertoire of small molecules that target many active enzymatic sites essential for malaria parasite survival and growth,

making it more difficult for parasites to accumulate necessary resistance mutations in the ensemble of genes responsible for encoding those essential target sites.

The 19th century conceptualization of "magic bullets" led us to the serial use of monotherapies that blindly select for resistant micro- and macro-organisms throughout the 20th century. The benefits realized in roughly the first half of that period in medicine and agriculture were undone in the latter half by proliferation of resistance to antibiotics, antimalarials, insecticides and herbicides. A renewed appreciation for evolution and the adaptive potential of the targeted organisms has lead to more sustainable approaches using combinations of control agents. The success of the combinatorial approach is evidenced throughout nature, and *A. annua* is a prime example with its repertoire of several hundred compounds comprising that plant's defense system. If plants had followed the pharmaceutical model of serial production of single component protection against its enemy, they would gone extinct long ago (14). The WP anti-malarial therapy serves as a case study of how those resilient naturally occurring systems might be co-opted for human use. While much work remains, the clear evidence of the efficacy of WP as a naturally occurring combination therapy pACT warrants its further consideration to explore how we might develop inexpensive, abundant and resilient malaria therapies from a non-pharmaceutical product.

**Conclusions**

Emergence of artemisinin-resistant malaria parasites raises urgent need for alternative treatment that is affordable, resilient and effective against resistant parasites. In our previous study we reported that antimalarial activity of whole plant *Artemisia annua* was at least 5 times more effective than an equivalent dose of its pure extract AN

(41). Here we also show that dried whole plant material of *A. annu* is more effective in killing malaria parasites resistant to AN. We've also shown that WP is more resilient than pharmaceutical AN and may actually delay the onset of resistance. While the exact mechanisms still need to be identified, the antimalarial activity of WP against artemisinin-resistant parasites can be explained by increased bioavailability of AN, synergism among AN and some *A. annua* constituents and/or the presence of other compounds in *A. annua* that may have antimalarial activity independent from AN. The efficacy and resilience of *A. annua*-based WP treatment against rodent malaria provides compelling reasons to further evaluate the role of non-pharmaceutical forms of AN to treat human malaria.

## Chapter IV

# *BABESIA MICROTI* IS NOT AFFECTED BY ARTEMISININ, ARTESUNATE AND WHOLE PLANT *ARTEMISIA ANNUA L.* BASED THERAPY

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## **Introduction**:

Babesiosis is a tick-borne disease caused by haemotropic apicomplexan protozoa of the genus *Babesia*, which are transmitted mostly by *Ixodes* ticks but also by blood transfusion and prenatally(28, 139-143). After trypanosomes, *Babesia* is the most common mammalian blood parasite (140). Like *Plasmodium*, *Babesia* invades erythrocytes, has an invertebrate vector, and the symptoms of the two diseases are similar, including fever, flu-like symptoms, chills, headache, anemia, hemoglobinuria, and myalgia. In regions where malaria is endemic, co-infection with *Babesia* parasites has been observed, and babesiosis may be misdiagnosed as malaria (27).

The life cycle of *Babesia* requires a tick vector for sexual reproduction and transmission to the vertebrate host. After gametogenesis in the tick midgut, the motile embryo replicates in ovaries and salivary glands. Sporozoites in the salivary glands are transmitted to vertebrate hosts parasitized by ticks, while ookinetes in ovarian cells are transmitted to the tick's offspring (28). Unlike *Plasmodium* and *Theileria*, babesial

sporozoites directly invade erythrocytes without the need for replication in hepatocytes (*Plasmodium*) or lymphocytes (*Theileria*).

To date, seven species of Babesia have been found to cause human babesiosis: *Babesia microti*, *B. divergens*, *B. bovis*, *B. canis*, *B. duncani*, *B. venatorum* and *Babesia* KO1 (28). *Babesia* has been diagnosed in increasing numbers of humans in recent years due to greater medical awareness and increased numbers of immunocompromised patients (28). Because *Babesia* has historically been a disease of domestic animals, current antibabesial pharmaceuticals may be licensed only for veterinary, but not human use (28). The lack of routine treatment for *Babesia* and the similarity between *Babesia* and *Plasmodium* triggered drug development investigators to test antimalarial drugs against different species of *Babesia* (144). The combination of quinine and clindamycin was effective in treating human and animal cases. However *B. microti* infections persisted after treatment and adverse reactions to treatment were reported in about 25% of all treated cases (145) (146, 147). Immunocompromised patients are usually treated with a combination of atovaquone and azithromycin, but relapses after prolonged treatment suggested that *B. microti* readily evolved resistance to these compounds (148). Resistance against atovaquone, another common antimalarial, was reported in hamster *B. microti* (149). The lack of effective treatment for human babesiosis, particularly in immunocompromised patients, creates an urgent need for new antibabesial therapeutics. The difficulty of distinguishing malarial and babesial infections and the frequent co-infections with the two diseases create the need for cost-effective treatments that combat both diseases.

Artemisinin, extracted from the herb *Artemisia annua,* is currently the preferred treatment against malaria. To delay the onset of drug resistance, the World Health Organization (WHO) recommended using artemisinin or its derivative, artesunate, in combination with other antimalarial drugs, lumefantrine or mefloquine, in a treatment known as Artemisinin Combination Therapy (ACT)(42). Beside antimalarial activity, artemisinin and its derivatives have also shown efficacy against a number of viruses, human cancers (35, 36), schistosomiasis (44), leishmaniasis (45, 46), trypanosomiases (47) and possibly babesiosis (150).

At concentrations ≥ 2.6 μM, Goo et al. (2010) showed that, artesunate inhibited the *in vitro* growth of *B. bovis* the causative agent of bovine babesiosis, but to inhibit *B. gibsoni* the causative agent canine babesiosis, the concentration had to be raised 100-fold to 260 μM (150). In contrast in mice infected with *B. microti,* an intramuscular treatment of 50 mg $kg^{-1}$ artesunate delayed, but did not completely quell parasitemia.

Recently, using a rodent malaria model *P. chabaudi*, we showed that oral consumption of powdered dried leaves of *A. annua* (100 mg artemisinin $kg^{-1}$ body weight) inhibited parasitemia at least five-fold more than pure artemisinin (41). Plant material seemed to enhance by >40 fold the amount of artemisinin that entered the blood stream (49). There are many other compounds in the plant that may account for the enhanced efficacy of orally consumed dried *A. annua* leaves and thus, we have called this treatment plant Artemisinin Combination Therapy, pACT. Such a therapy would also be more cost effective than current ACTs saving the cost of artemisinin isolation and purification. Beside malaria, pACT represents a candidate treatment against many other diseases susceptible to artemisinin and its derivatives.

In light of the earlier study by Goo et al. (2010) and the potent activity of pACT against malaria (150), we hypothesized that pACT may also have enhanced activity against *B. microti*, the cause of most of human cases in the United States. The objective of this work was to compare the therapeutic effects of pACT to those of the pure compounds, artemisinin and artesunate, in a murine model of *B. microti* infection. We hypothesized that the increased bioavailability of artemisinin from the whole-plant material, combined with the possible additive and synergistic effects of other plant compounds, could make whole-plant therapy more effective against babesiosis than treatment with individual compounds.

**Materials and Methods:**

**Treatments:** *Artemisia annua* L. (SAM cultivar; voucher MASS 00317314) containing 1.48±0.06% artemisinin, 0.37% flavonoids, monoterpenes (e.g., 0.21% (w/w) camphor, 0.007% eucalyptol, 0.037% α-pinene) as determined by GC-MS was used in this study. Plants were grown, harvested, dried, and leaves sieved and pulverized as previously described (41). Artemisinin (AN) and artesunate (ART) were purchased from Sigma Aldrich.

**Parasite information:** Cryopreserved isolates of *Babesia microti* were kindly provided by Samuel Telford (Tufts University). To revive the parasites, cells were intraperitoneally injected into two inbred male DBA/2 mice. The revived parasites were used in subsequent experiments.

**Mouse infection and feeding details:** To test the effects of artemisinin intake on parasitemia, inbred male DBA/2 mice 12 weeks old were intraperitoneally injected with

$10^7$ infected red blood cells and randomly divided into four treatment groups: AN, ART, pACT and CON (control) with eight mice in each group. Beginning two days after parasite inoculation, mice were treated daily for six days via oral gastric gavage with 100mg/kg mixed with a water slurry of mouse chow containing either AN or ART and water slurry of dried ground *A. annua* leaves (pACT) corresponding to 100mg AN /kg body weight. Mice in the CON group were gavaged a placebo consisting of water slurry of mouse chow only. Detailed information about mouse feeding, drug preparation and parasitemia count can be found in Elfawal el al (2012)(41). Parasitemia was measured daily on Giemsa-stained thin blood smears and mice were euthanized on day17 post inoculation (Fig. IV.1).

**Ethics Statement:** This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts (Protocol# 2011-0072). All efforts were made to minimize suffering of animals during experimental procedures.

**Results and discussion**

We compared the antibabesial activity of a whole-plant (pACT) to that of pure compounds used in conventional malaria treatment (AN and ART). Parasites were found in blood of infected mice 48 hours after inoculation. Days 2-7 post-inoculation, parasitemia increased, peaking on day 11; this was 4 days after cessation of treatments in all groups. Parasitemia declined on days 12-14 post inoculation, then increased again on day15 post inoculation (Fig. IV.1). Mice were euthanized 17 days post-inoculation when

parasites were found in about 15% of erythrocytes. The course of infection was similar to that previously observed in *Babesia microti*-infected mice (150, 151). None of the treatment groups showed significant reduction in parasitemia in comparison to controls treated with water slurry of mouse chow (Fig. IV.1).

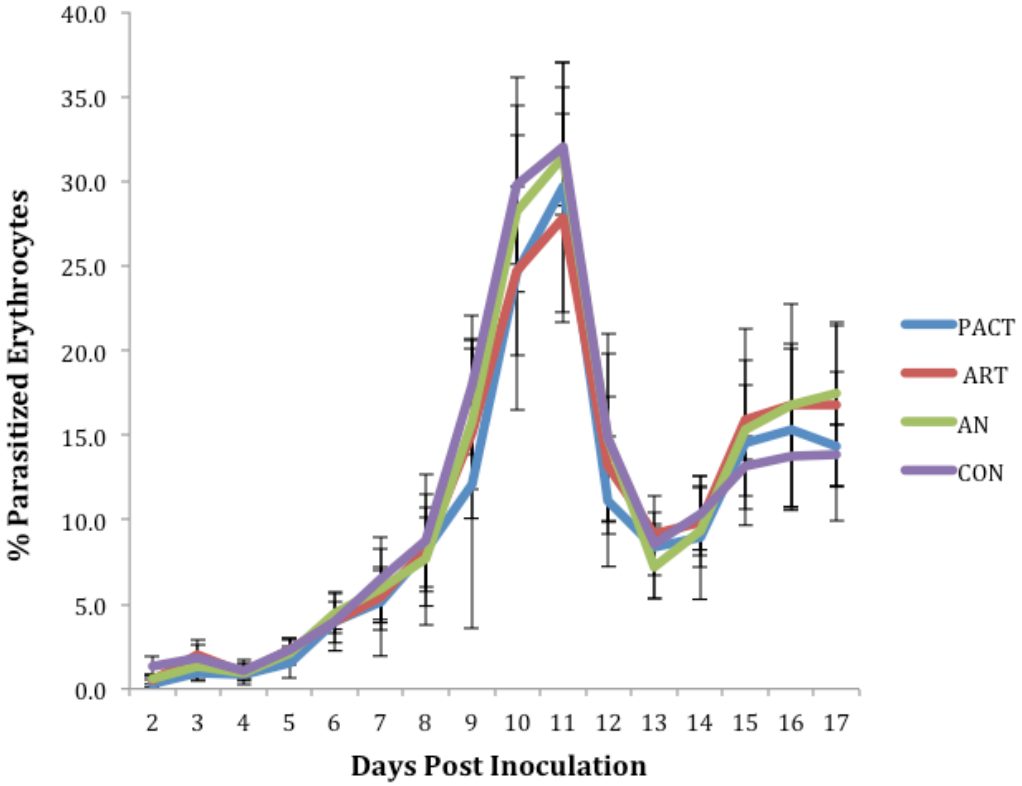


Fig. IV.1: *B. microti* mean of daily parasitemia of four treatment groups: whole plant *A. annua* corresponding to 100mg/kg (pACT), artemisinin 100mg/kg (AN), artesunate 100mg/kg (ART) and placebo control (CON). Mice were treated once daily for six days via oral gastric gavage starting two days post intraperitoneally infection with $10^7$ parasite-infected red blood cells. Means of daily parasitemia were analyzed for statistical significance by analysis of variance. P >0.05 was considered to be statistically not significant.

The lack of effective antibabesial drugs and the similarity between *Babesia* and *Plasmodium* in biology and disease symptoms have led to the testing of antimalarial compounds against different species of *Babesia* (144). Chloroquine was the first

antimalarial drug tested against human babesiosis without success (145, 152). Following chloroquine many antimalarial compounds were tested and found to be ineffective against *Babesia microti* including artesunate, mefloquine and halofantrine (144, 149, 153). In contrast, artesunate was found to be partially effective in controlling *Babesia microti* and to delay the peak of parasitemia during treatment days, but it failed to eradicate the disease or to control parasitemia levels that reached 19% and 24% only 5 days after the last treatment of 10mg/kg and 50mg/kg, respectively (150).

Artemisinin is a sesquiterpene lactone with a peroxide bridge that is responsible for its antimalarial activity (154-156). The mode of action of artemisinin is under considerable discussion with several proposed modes of action. One is interference with digestion of hemoglobin by the parasite. Hemoglobin digestion yields toxic heme, but the parasite forms crystalline polymers of insoluble nontoxic hemazoin in the digestive vacuole. This mode of action, however, conflicts with reported activity of artemisinin against younger stages of the malaria parasite and other parasites that lack hemazoin synthesis and a digestive vacuole (157). A second possible mechanism for artemisinin mode of action is through cleavage of its peroxide bridge by heme, producing a carbon-centered free radical that alkylates, modifies and inhibits essential parasite molecules thus resulting in parasite death (154-156). A third alternative proposes direct interaction between artemisinin and the SERCA-like $Ca^{+2}$ –PfATP6 calcium pump present in *Plasmodium*; mutation in the PfATP6 gene is associated with artemisinin resistance (156, 158, 159). Iron-containing molecules are essential for enhancing artemisinin-PfATP6 binding and the heme-artemisinin complex interacts well with PfATP6 causing modification in its molecular structure preventing calcium binding, leading to disruption

of the SERCA-like pump (156). Iron containing molecules known to enhance artemisinin activity by enhancing PfATP6 activity and other free radicals include heme (154, 160-163), hemin (68, 162), denatured hemoglobin in the digestive vacuole (154) and inorganic iron (164). When these four iron sources were tested for their ability to activate artemisinin, heme was the best; inorganic iron was ineffective (154). In malaria heme is produced *de novo* in the apicoplast, the plastid-like organelle exclusive to apicomplexan parasites and involved in biosynthesis of heme, fatty acids, and isoprenoid precursors. The antimalarial activity of artemisinin may be increased additively by the *de novo* synthesized heme.

The intracellular parasite *B. microti* has a single membrane without a parasitopherous vacuole. The importance of the second membrane and the parasitopherous vacuole seems to be in host parasite interaction and forming the digestive vacuole, the main target for many antimalarial drugs, where hemoglobin digestion takes place. It has been suggested that in contrast to malaria, *B. microti* invades erythrocytes using a mechanism other than membrane invagination (165). Although in *Babesia* the double membrane is absent in mature trophozoites, it is present in the young merozoites shortly after invasion thereby confirming that *B. microti* invades erythrocytes by invagination like *Plasmodium,* but with rapid degradation of the second host membrane shortly after penetration. A similar phenomena was observed in *Eimeria manga* (165). Such a rapid degradation of the second membrane suggests a unique interaction with host cells in obtaining nutrients and hemoglobin digestion (165, 166).

In further contrast to *Plasmodium*, *B. microti* has a highly polymorphic intracellular stage with pseudopods forming host cytoplasm invaginations. Such

invaginations were believed to be a digestive vacuole. However liberated parasites from host cells were found to have vacuole-like structures lacking host cytoplasm (165, 166). The absence of a digestive vacuole was confirmed using electronic microscope imaging of different sections of the intracellular parasites showing invagination of the host cytoplasm forming what is named pseud vacuole (165, 166).

The lack of a parasitopherous vacuole, a digestive vacuole, and hemazoin formation in *Babesia* parasites has led to contrasting conclusions about the ability of *Babesia* to digest hemoglobin. One opinion suggested that *Babesia* may completely digest hemoglobin extracellularly without hemazoin formation and by unknown mechanisms (167). On the other hand, it was also suggested that *Babesia* parasites may not even digest hemoglobin (165, 166). The latter conclusion was recently supported by sequencing the whole genome of *B. microti* and surprisingly, among all apicomplexan parasites *B. microti* has only three nuclear chromosomes, its overall genome size is about 72% smaller than *P. falciparum,* and it lacks hemoglobin proteases (139).

In a molecular phylogenetic study using the full sequence of 316 genes, *B. microti* was placed as paraphyletic group that was a root for all Babesiidae including both *Babesia* and *Theileria*. Such a result confirmed the distinction between *B. microti* and other piroplasms and suggested the need for creating a new genus for *B. microti* (139); (168).

The increased surface area of the parasite with intra-erythrocytic polymorphic shapes (pseudopods, whirls, and pseudo vacuole), the absence of the double membrane, the absence of parasitopherous vacuole, the absence of hemoglobin proteases, and the low metabolism of this parasite suggest that *B. microti* is highly dependent on the host

red blood cell for obtaining nutrients by diffusion through its single membrane (165, 166). Dependence on host cells in obtaining essential amino acids is not a feature unique to *Babesia; P. falciparum* also depends on host cells to obtain essential amino acids unavailable from hemoglobin digestion (169). In contrast to apicoplast function in *Plasmodium* that also synthesizes heme and fatty acids, apicoplast function in *B. microti* is limited to synthesis of isoprenoid precursors and lacks heme biosynthesis (139). The absence of the digestive vacuole and heme biosynthesis in *Babesia* may explain the lack of activity of artemisinin or artesunate against *B. microti*. However, although *Toxoplasma gondi* lacks hemazoin, digestive vacuole and hemoglobin digestion, it is still susceptible to artemisinin. The anti-parasitic activity of artemisinin against *T. gondi* may be explained by the *de novo* heme synthesized in its apicoplast. On the other hand, artemisinin and its derivatives failed to control infection of *Cryptosporidium parvum* in neonatal mice (170) and in in vitro culture (171). Among all apicomplexan parasites, only *C. parvum* lacks the apicoplast. *C. parvum* also lacks the digestive vacuole and hemoglobin digestion. The lack of artemisinin activity against *C. parvum* may be explained by the lack of enough heme concentration either from hemoglobin digestion (as in *Plasmodium*) or de novo synthesized (as in *Plasmodium* and *Toxoplasma*). Despite *B. microti* harboring the apicoplast, that function is limited to isoprenoid synthesis, it lacks both sources of heme. Thus, artemisinin and its derivatives failed to control infection in *B. microti* similarly to *C. parvum*. In conclusion, the anti-parasitic activity of artemisinin is dependent on enough heme concentration within the parasite.

The antimalarial activity of pACT is better than artemisinin and is possibly explained by enhanced bioavailability of artemisinin due to inhibitory effects of some *A. annua* flavonoids on the hepatic and intestinal cytochrome P450 enzymes that metabolize artemisinin (83). In contrast, enhanced generation of heme does not seem to occur in *B. microti*, so greater bioavailability of artemisinin from pACT is moot.

Enhanced antimalarial activity of pACT is also potentially explained by the possible synergism between artemisinin and one or more other constituents in *A. annua* including terpenes, phenolic acids and flavonoids, (14, 48, 67, 172). For example, flavonoids were shown to reduce the $IC_{50}$ of artemisinin against *P. falciparum* (41, 57, 58). Although not fully understood, methoxylated flavonoids enhanced formation of the artemisinin-heme complex, which reportedly enhances artemisinin binding to SERCA-like calcium pump and cleavage of the peroxide bridge releasing free radicals (68). The absence of anti-babesial effects by *Artemisia annua* and its chemical components may reflect the differences in artemisinin drug targets between *Babesia* and *Plasmodium.*

**Conclusion:**

Human babesiosis is an emerging disease caused by a parasite distinct from all apicomplexans. The disease particularly affects immunocompromised people in developing nations where malaria often co-infects and new treatments are urgently needed. Although reported to be effective for other *Babesia* sp., and in contrast to *Plasmodium* sp., none of the three forms of artemisinins tested here seem effective against *B. microti.* The differential response of *Babeisa* sp. to artemisinins is likely the result of significant differences in their cell biology and metabolism of hemoglobin. The

apicoplast of both *Plasmodium* and *Babesia* has the nonmevalonate isoprenoid anabolic pathway, which is susceptible to fosmidomycin, so this antibiotic could be used to treat patients with malaria-*Babesia* co-infections. Although effective against malaria, some *Babesia* species, and a number of other apicomplexans, neither pACT nor artemisinin and artesunate are effective against *B. microti*, so the search for new therapeutics against this debilitating disease must continue.

## FINAL CONCLUSIONS

Plant antimicrobial secondary metabolites are complex multicomponent matrices with synergy among their components and biological activity against a broad spectrum of microbes through a variety of mechanisms (13, 14, 173). During their own coevolution with pathogens, animals evolved self-medication behavior relying on direct ingestion of plant matrices of antimicrobial secondary metabolites which, when directly ingested, could treat or prevent diseases. Plants have limitless ability to synthesize secondary metabolites, which serve as plant defense against predation by microorganisms, insects, and herbivores (174). Secondary metabolites give plants their odor, flavor and pigment. Plants generate several categories of secondary metabolites: phenolics (simple phenolics, quinones, flavonoids, tannins, coumarins), terpenoids, alkaloids and polypeptides (174). The medicinal properties of plants come from the interactions of multiple constituents, and its overall properties may never be completely achieved by using their component parts (14). These interactions in medicinal plants are known as synergy, when the effect of the combination is greater than the sum of the individual effects (14). Crude extracts from many medicinal plants were found to be effective against viruses (175-179), bacteria (180-185), protozoa (186, 187), helminths (188-190) and fungi (180, 182, 191). Such antimicrobial properties are made of small molecules that fit into sites in enzymes or receptors essential for pathogens survival. Because pathogens tend to develop resistance, many of the plant's secondary metabolites are developed to inhibit resistance mechanisms (192).

About 25% of prescribed drugs and about half of newly discovered drugs are from plant origin (193), and about 80% of the world population relay on plant-derived

medicines (173, 194). But current pharmaceutical industry strives to isolate single active compounds (195), and thus may overlook many useful complex secondary metabolites with potential synergistic antimicrobial activity.

Use of single-target monotherapeutic drugs tends more often to select for micro- and macro-organisms resistance, whether that resistance is to antibiotics (196), antiparasitic (110), insecticides (197), and herbicides (198). Prolonged use of antimicrobial monotherapy leads to the evolution of drug resistance in microorganisms that initially were sensitive but gradually adapt and develop resistance. Repeated use of antimicrobial agent selects for resistant and select against sensitive microorganisms. Microorganisms achieve drug resistance through number of mechanisms, including reduction in drug uptake through efflux pumps, avoiding drug activity by drug degradation or alteration, increased expression of drug target, decrease binding ability of drug due to modification in its target (196, 199-201). Drug resistance can be innate to microorganisms or acquired through mutation and horizontal gene transfer (196, 200). Resistance-associated mutations are likely to be deleterious because they result in metabolic pathway modification (200). The development of new mutation depends on mutation rate and fitness cost, thus resistance-associated mutations are less favored or costly in absence of the selective drug. If resistance phenotype requires two mutations, the initial frequency of organisms harbor these two mutations is much lower than the frequency of organisms harbor only one mutation. Thus the likelihood of developing drug resistance to a combination therapy is much lower than developing resistance to monotherapy (202). Similarly the likelihood of developing resistance to single compound that require more than one mutation is much lower than those require single mutation.

Combination therapy may delay the evolution of drug resistance more than monotherapy (202), however if multiple compounds are actively target the same site, single mutation may lead to resistance to combination of these compounds (110).

Herbal remedies have been used for several thousands of years, but resistance does not seem to have appeared. In contrast isolated or synthetic compounds have suffered from the appearance of resistant microorganisms. Matrices of secondary metabolites are evolutionary designed to be effective against microorganisms using hundreds of small molecules that target sites of multiple enzymes and receptors essential for microorganisms (14). Such strategy of using synergic multicomponent defense system enabled plants to thwart microorganism's ability to develop resistance. Using complex matrix of secondary metabolites by plants, microorganisms fall easy target for such complexity, and to develop resistance against single component microorganisms will be more vulnerable against others. Synergic activities of secondary metabolites were found to enhance activity against resistant pathogens including bacteria (185, 203, 204) and protozoa (14, 41, 48, 205).

Because it is almost impossible to generate hundreds of resistance-associated mutations at once, against multiple repertoires of secondary metabolites, microorganisms had to develop multidrug resistance involving protein pump located on cell membrane throwing foreign molecules out of the cell. Facing similar matrixes of secondary metabolites many microorganisms convergently develop MDR including bacteria (196), protozoa (110), fungi (206), and viruses (207). In response to MDR, plants co-evolve and produce secondary metabolites that are specialized to disrupt MDR. Phytochemicals from many plant species were found to be effective against multidrug resistant pathogens.

Crude leave extracts from *Lantana camara L.* were found to be effective against three multidrug resistant Gram-positive bacteria: Methicillin-Resistant-*Staphylococcus aureus* (MRSA), *Streptococcus pyrogenes*, and Vancomycin-Resistant-*Enterococcus faecalis* (VRE); and five B-lactamase producing-Gram-negative bacteria: *Acinetobacter baumannii*, *Citrobacter freundii*, *Proteus mirabilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. Dichloromethane extract had the most antibacterial activity against MRSA with lowest Minimum Inhibitory Concentration (MIC), followed by methanol extract. On the other hand the lowest antibacterial activity with highest MIC was observed in water extract (208). Phytochemical analysis of dichloromethane extract approved the presence of alkaloids, glycoside, terpenoids, saponins, flavonoids, tannins, and steroids. On the other hand methanol extract lack tannins and glycosides. It was concluded that the more secondary metabolites in extract the more antibacterial activity against bacteria (208). Similarly extracts from *Lygodium flexuosum L.* were found to be effective against MDR-MRSA and –VER (209). Alkaloids from *Meconopsis simplicifolia* were found to be effective against MDR-K1CB1 *P. falciparum* (210). Extracts from *Mangifera indica* leaves and bark were found to be effective against MDR-Dd2 *P. falciparum* (205).

Here we found dried whole plant (WP) *Artemisia annua* is at least five times more effective than purified compounds in killing malarial parasites. Moreover WP overcomes artemisinin-resistant malaria parasites. Moreover, whole plant *A. annua* was at least three times more resilient against the evolution of drug resistance than purified artemisinin.

Enhanced pharmacokinetic and pharmacodynamic properties of whole plant *A. annua* compared to purified artemisinin may be explained by the presence of a complex matrix of secondary metabolites, some of which increases the bioavailability of artemisinin by

inhibiting hepatic and intestinal enzymes that degrade artemisinin. This was supported by our observation that plant material such as that found in mouse chow was critical to the appearance of artemisinin in the blood of orally-treated mice. The pharmacokinetic properties of artemisinin delivered *in planta* were also different between uninfected and infected mice. Enhanced antimalarial activity of *A. annua* over artemisinin can be explained by the presence of *A. annua* constituents with antimalarial activity independent from artemisinin, including flavonoids and terpenes other than artemisinin. Alternatively, non-artemisinin *A. annua* constituents with antimalarial activity may depend on the synergistic effects of artemisinin to realize their parasite-killing potential.

Use of dried whole plant *A. annua* directly against malaria could dramatically decrease the cost of therapy, giving hope to millions of people living in malaria endemic regions. Because chemical synergism in the plant chemical matrix is common in medicinal plants, we speculate that many whole plants material will show increased bioactivity compared to compounds purified from them. Using plants in their whole form will decrease drug production costs and eventually reduce healthcare costs.

Creating greater opportunity for use of whole plant therapy to fight human diseases, no less than three fundamental paradigm shifts must occur in how the pharmaceutical enterprise approaches use of whole plants in drug development.

The first shift must be to alter the objective for using plant derived compounds themselves. Currently, the convention is to use purified compounds, thus relying on very costly drug purification processes (194). Because we showed that whole plant *A. annua* is more effective than a purified compound, we suggest testing the clinical efficacy of whole plants, not simply their purified extracts. Using the whole plant directly, thereby

maintaining its matrix of secondary metabolites, may have the added benefit of delaying evolution of drug resistance as we've found here, thus saving many drugs from premature obsolescence.

Second will be new approaches to plant breeding programs. Currently, breeding programs are designed to create crops that yield increased concentration of a single compound without regard to beneficial effects of many other plant constituents (211). We suggest an alternative breeding strategy that selects plants for their optimal biologically active phenotype. The goals would be to maintain the complex and diverse repertoire of secondary metabolites and buffer associated synergies that require their co-isolation.

The third paradigm shift would be in the field of drug discovery. Current drug discovery strategies most often follow simplistic models, testing activity of individual purified compounds isolated from medicinal plants but ignoring synergism between plant constituents(194). This archetypal approach neglects the effects of compounds that may not show any activity when tested individually. Simple drug discover models were exclude many important compounds from recognition because often the therapeutic does exceed the toxicity dose of those single compounds. We suggest an alternative approach that uses advanced computational and laboratory tools-- high-throughput screening to test millions of combinations for potential antimicrobial activity-- to build complex combinatorial models incorporating synergisms, antagonisms and other interactions among plant components. More research is needed on the pharmacodynamics and pharmacokinetics synergy, resistance reversal, and immunomodulation properties of medicinal whole plants including toxicity studies and clinical research.

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