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It is not just artemisinin: *Artemisia* sp. for treating diseases including malaria and schistosomiasis

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Abstract

Artemisia sp., especially *A. annua* and *A. afra*, have been used for centuries to treat many ailments. While artemisinin is the main therapeutically active component, emerging evidence demonstrates that the other phytochemicals in this genus are also therapeutically active. Those compounds include flavonoids, other terpenes, coumarins, and phenolic acids. *Artemisia* sp. phytochemicals also improve bioavailability of artemisinin and synergistically improve artemisinin therapeutic efficacy, especially when delivered as dried leaf *Artemisia* as a tea infusion or as powdered dry leaves in a capsule or compressed into a tablet. Here results from in vitro, and in vivo animal and human studies are summarized and critically discussed for mainly malaria, but also other diseases susceptible to artemisinin and *Artemisia* sp. including schistosomiasis, leishmaniasis, and trypanosomiasis.

Keywords

Artemisia annua and *Artemisia afra*; Artemisinin resistance; Bioavailability; Leishmaniasis; Malaria; Schistosomiasis; Trypanosomiasis

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Introduction

For centuries *Artemisia annua* L. and *Artemisia afra* Jacq. ex Willd. were used as medicinal herb infusions or powders to treat fever/malaria and worms (Liu et al. 2010; FAO 1986; van der Kooy and Sullivan 2013; Weathers et al. 2014b; Willcox et al. 2004). Both are commonly called “wormwoods”, and showed in vitro efficacy against *Plasmodium falciparum* (Gathirwa et al. 2008; Kraft et al. 2003; Liu et al. 2010; Moyo et al. 2016). Both species have long traditions in their respective ethnopharmacopeia of southeast Asia (A. *annua)* and southern Africa (A. *afra)*. While *A. annua* cultivars have been cultivated and selected for their high artemisinin content, *A. afra* is nearly devoid of this potent sesquiterpene lactone (van der Kooy and Sullivan 2013), having at most 0.05 mg/g DW (Munyangi et al. 2018, 2019) and, thus, this species also functions as a control herb for studies using artemisinin-rich *A. annua*.

A preliminary study suggested that ingestion of capsules containing leaf powder of either *A. afra* or *A. annua* were both effective in eliminating malaria parasites from patients (Tchandema and Lutgen 2016). Based on those encouraging results, two large-scale studies compared the efficacy of *Artemisia annua* and *Artemisia afra* tea infusions against the standard drugs for malaria and schistosomiasis. Current treatment for malaria is artemisinin combination therapy (ACT), and for schistosomiasis, praziquantel (PZQ). The ACTs contain usually either the artemisinin derivative, artemether or artesunate, and a co-drug such as lumefantrine or amodiaquine. Two common ACTs are artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ). ACTs are used to minimize emergence of artemisinin drug resistance (WHO 2014).

Doing human clinical trials using dried herb material is challenging. A good study should be double-blinded and include placebo and, if available, the current best therapy. Following recommendations of the WHO (2000), establishing a placebo for herbal clinical trials involved using very dilute *Artemisia* tea infusion and glucose tablets similar in appearance to the standard ACT or PZQ drugs. Thus, patients in both trials were treated with either a high dose tea infusion (5 g leaves + twigs/L) or a very low dose (0.2 g leaves + twigs/L, considered an appropriate amount for a tea infusion placebo), and each trial arm received placebo sugar tablets. All treatments included pre-weighed dried leaves + twigs, and tablets were placed in labeled envelopes prepared for each of the 7 days of treatment. All patients and investigators were double-blinded to the delivered treatments (Cornet-Vernet et al. 2019a, b; Munyangi et al. 2018, 2019).

Here summarized are the results of two superiority clinical trials conducted in 2015 in the Kalima district, Democratic Republic of Congo, comparing *A. annua* and *A. afra* tea infusions against the current standard drugs for treating malaria and schistosomiasis. Preliminary results for treating leishmaniasis and trypanosomiasis are also reviewed. In an effort to provide a scientific foundation for empirical in vivo observations, information about the mechanism of action of these complex herbal remedies follows.

Artemisia annua and *Artemisia afra* efficacy in clinical trials

Malaria

A double-blind malaria trial (Munyangi et al. 2019) was instigated following a number of smaller trials by others (summarized in Weathers et al. 2014a; Tchandema and Lutgen 2016) and had three treatment arms: 472 for ASAQ, 248 for *A. annua*, and 223 for *A. afra*. Figure 1 shows the mix of leaves and twigs used in the study; in the *A. annua* samples, the large white pieces are from the large thick stems characteristic of this species at harvest and that were chopped along with leaves to prepare the dried material for infusion. Artemisinin content of the *A. afra* and *A. annua* material was 0.036 and 1.34–1.70 mg/g DW, respectively. Patients receiving either *Artemisia* sp. drank 1 L/day of an *Artemisia* infusion, divided into 3, 330 mL aliquots per day (TID) for 7 days. Infusion was 5 g of dried plant material added to 1 L boiling water (0.2 g for placebo infusion), steeped for 10 min, and then strained through a sterilized 1 mm mesh to retain large particulates. Patients receiving *Artemisia* also received the artesunate-amodiaquine (ASAQ) sugar tablet placebo for all 7 days. Patients receiving ASAQ (Winthrop, Sanofi-Aventis) followed manufacturer's posology of 4 mg of artesunate-10 mg of amodiaquine/kg, once daily for 3 days, followed by ASAQ placebo tablets for the remaining 4 days to follow the 7 day treatment equal to that of *Artemisia* sp. ASAQ patients also received dilute (0.2 g/L) *Artemisia* tea infusion all 7 days to parallel the therapeutic *Artemisia* tea infusion treatments. All patients had regular follow-up to 28 days. Trophozoites and gametocytes in blood samples were micro-scopically counted over 28 days. Side effects were documented.

Despite the negligible artemisinin content of *A. afra*, its therapeutic response was similar to patients treated with *A. annua*; after 24 h, trophozoites cleared, but took up to 14 days to clear in ASAQ-treated patients. Day 28 cure rates (no detectable parasitemia) overall are shown in Table 1; for adults cure rates were 91, 100, and 30% for *A. afra, A. annua* and ASAQ, respectively (Table 1), with 82, 91, and 50% for pediatrics. Fever clearance took 24 h for *Artemisia*, but 48 h for ASAQ. Although from days 14 to 28 there were no detectable gametocytes in *Artemisia-treated* patients, gametocytes remained in 10 ASAQ-treated patients at day 28, suggesting that both A*rtemisia* sp. could break the cycle of malaria by inhibiting parasite transmission back to the mosquito vector. Eightfold more ASAQ patients reported adverse effects than Artemisia-treated patients, so *Artemisia* was more benign than ASAQ for sick patients. Although compelling, this trial recently received criticism from the scientific and medical establishment (Danis and Buisson 2019; Gillibert et al. 2019) to which a published rebuttal was made (Cornet-Vernet et al. 2019b). This trial corroborates the results of earlier tea trials (summarized in Weathers et al. 2014a) and also a set of recently published case studies where compassionate administration of dried *A. annua* leaf tablets successfully saved the lives of 18 patients with severe malaria who did not respond to therapy with ACT (artemether-lumefantrine) and *i.v.* artesunate (Daddy et al. 2017).

Schistosomiasis

Schistosomiasis, also known as bilharzia, affects millions globally with few cost-effective treatments (WHO 2018b). It is caused by members of the Schistosome family, e.g. *Schistosoma mansoni*. Based on an informal observation by Sister Elke Stienacher in

Degana, Senegal, people cured of their malaria by taking *Artemisia* tea also seemed cured of their bilharzia when they were co-infected. Malaria and schistosomiasis co-infection is common (Keiser and Utzinger 2012; Moriyasu et al. 2018), so in 2015 *A. annua* and *A. afra* tea infusions were compared to PZQ treatment in 800 patients in the Democratic Republic of Congo. There were three treatment arms in this double-blind trial: 400 for PZQ, 200 for *A. annua*, and 200 for *A. afra*; 780 completed the trial (Munyangi et al. 2018). The source of both *Artemisia* sp. was identical to that used for the above malaria trial. Patients receiving either *Artemisia* sp. drank 1 L/day of an *Artemisia* infusion, prepared and administered as described for the malaria trial. Patients receiving *Artemisia* also received sugar tablet placebo replacing PZQ for all 7 days. Patients receiving PZQ (Bayer) followed manufacturer's posology, receiving PZQ tablets (60 mg/kg daily) for three consecutive days, and PZQ placebo (sugar tablets) for four more days to follow the 7-day treatment equal to that of the *Artemisia* sp. PZQ patients also received dilute (0.2 g/L) *Artemisia* tea infusion all 7 days to parallel the therapeutic *Artemisia* tea infusion treatments. All patients had 28-day follow-up. All drugs were administered orally under investigator supervision who were blinded to identity of drug delivered via use of sealed envelopes. Side effects were documented every time patients visited the clinic to receive treatment.

Patients enrolled in the trial had an average of > 700 *S. mansoni* eggs per fecal sample on entry. At 14 days post treatment, there were no detectable eggs in fecal smears in any *Artemisia*-treated patients. In contrast it took > 21 days for eggs to fully disappear in all PZQ patients. There were significantly fewer adverse effects in the *Artemisia*-treated patients for both pediatrics and adults. Patients treated with either *Artemisia* sp. reported 95% fewer adverse events compared to those treated with PZQ, 69 versus 1411 events, respectively (Munyangi et al. 2018). As with the malaria trial, the schistosomiasis trial also received a published critique (Argemi et al. 2019) to which a full rebuttal was published (Cornet-Vernet et al. 2019a).

Leishmaniasis

Other diseases are also showing positive clinical responses to *Artemisia* leaf powders. Leishmaniasis is a neglected tropical disease endemic in 98 countries on 5 continents caused by > 20 species of an intracellular protozoan parasite, *Leishmania* sp., transmitted by sandflies. There are three clinical forms of the disease: cutaneous, mucosal, and visceral. About 12 million people are affected with 350 million at risk of infection (WHO 2018a). Cutaneous leishmania is most prevalent, and while the standard treatments (including a pentavalent antimonial, miltefosine, pen-tamidine isethionate, and amphotericin B) are still effective, these drugs have serious side effects, so safer effective new drugs are needed (den Boer et al. 2011).

One study encompassed using powdered *A. annua* leaves in in vitro and in vivo (hamsters) systems, and with two human subjects (Mesa et al. 2017). The leaves had an in vitro EC_{50} of 48.07 μg/mL against amastigotes of *L. (V) panamensis* with a therapeutic index of 8.73. Amphotericin B had a therapeutic index of 625 and an EC_{50} of 0.06 μg/mL. In vivo results using 500 mg/kg/day in hamsters showed an 83.3% cure by 30 days. After these studies, two human males were treated with powdered encapsulated *A. annua* (voucher MNHNL17732

Herbarium Luxembourg) containing about 0.1% artemisinin (Mesa et al. 2017). Using Giemsa-stained smears and PCR analysis, both patients were validated as positive for *L. (V) panamensis* leishmaniasis. One patient had 4 lesions ranging from 0.90 to 20.25 cm^2 and the other had a single lesion 20 cm^2. Both patients were given *A. annua* powder as follows: from days 1–3, 3 g/day; days 4–7, 2 g/day; days 8–20, 1 g/day. Total *A. annua* delivered was 30 g per patient over 20 days. By end of treatment, ulcers had shrunk 20–35% with complete closure of the ulcer 45 days after end of treatment. Patient ulcers remained healed 2 years post-treatment (Mesa et al. 2017). During and after treatment neither patient experienced any adverse reactions, suggesting that *Artemisia* leaf powder also could prove useful in treating leishmaniasis. Clearly larger human trials are required.

Trypanosomiasis

American trypanosomiasis *(Trypanosoma cruzi*; Chagas disease) and African trypanosomiasis *(T. brucei*; sleeping sickness) are two other major neglected tropical diseases that are transmitted through the triatomine bug that defecates after biting thereby transmitting disease, or the bite of the tsetse fly, respectively. Symptomatic treatment against both infections is restricted to drugs with severe side effects, mammalian toxicity, and limited efficacy, so better therapies are urgently needed (Naß and Efferth 2018).

Therapeutic herbal treatments are a possible alternative. In vitro and in vivo animal studies showed extracts (Efferth et al. 2011) or infusions (Berrizbeitia de Morgado et al. 2017; Naß and Efferth 2018) of *A. annua* to be effective in inhibiting parasite growth in both diseases. While there are to our knowledge no major human trials, *A. annua* infusions exerted dose-dependent antiproliferative effects on the *T. cruzi* epimastigotes, and results depended on the concentration of the infusions and time of exposure (Naß and Efferth 2018).

Other *Artemisia* species besides *A. annua* also have inhibitory activity towards trypanosomes (Naß and Efferth 2018). These species included, *A. absinthium, A. abyssinica, A. afra, A. douglasia, A. elegantissima, A. maciverae*, *A. mexicana*, *and A. roxburghiana*, which were active against *T. brucei*, *T. cruzi*, and *T. congolense*. This field of research is, however, still in its infancy and more research on pharmacokinetics, bioavailability, and synergism among *A. annua* phytochemicals and with current commercial drugs is needed to explore the full potential of diverse *Artemisia* species and their phytochemicals for eradication of trypanosomal infections. Given the previously described efficacy and safety of *A. annua* tea infusions against malaria, schistosomiasis and leish-maniaisis, human trials are also warranted.

Artemisia annua as prophylaxis?

Ogwang et al. (2011, 2012) documented 80% reduction in malaria cases among Ugandan adults who drank a weekly dose of *A. annua* tea. Those individuals previously had malaria. Neither this review nor any of the authors' prior studies recommend or suggests prophylactic use of *A. annua* to thwart malaria. Indeed, there were two case studies where French travelers, with no medical history of malaria, used *A. annua* prophylactically to avoid malaria. They returned with active and severe cases of malaria (Lagarce et al. 2016). We urge caution, especially for those who are medically naïve to malaria.

What in these *Artemisia* sp. is therapeutically effective?

Most studies focused on antimalarial activity. Many other compounds found in *A. annua* besides artemisinin have therapeutic efficacy against *P. falciparum*, and some also improved the IC_{50} when used in conjunction with artemisinin, indicating a synergistic action (Liu et al. 1992; Suberu et al. 2013). Table 2 lists a number of phytochemicals along with their IC_{50} values alone and in combination with artemisinin, if known, against various *P. falciparum* strains. Flavonoids in particular have shown importance with respect to efficacy of whole-plant treatment regimens (dried leaves or tea infusion, for example). Since flavonoids can persist in the body for more than 5 days, they could be helpful in promoting prophylactic antimalarial action (Ogwang et al. 2011, 2012 and Table 2).

Artemisinin still seems to be the main antimalarial constituent in *A. annua* as shown recently by Czechowski et al. (2019). Artemisinin-null mutants that still had high flavonoid content were not as effective as the wild type against in vitro *P. falciparum*. Although compelling, their results contrasted with the previously discussed in vivo animal and human clinical results. They also seem to contradict the *A. afra* clinical results (Munyangi et al. 2019) that showed near equivalency with *A. annua*. Using the Rath et al. (2004) pharmacokinetic study of an *A. annua* tea infusion, however, we estimated that since a minimum of 9–10 μg artemisinin/L blood is needed to kill the parasite (Alin and Bjorkman 1994), there was not enough artemisinin at 51.6 μg/dose (TID) in *A. afra* to account for its reported efficacy. Clearly more studies are required of DLA and *A. annua* tea infusions to clarify in vitro and in vivo discrepancies.

Nevertheless, flavonoids have been shown to function as antimalarials by inhibiting various stages of the malaria growth cycle. While monoterpenes make up a large proportion of the essential oil content of *A. annua* (Desrosiers and Weathers 2018; Desrosiers et al. 2019), and some have been shown to have antimalarial potential (Table 2) or enhance the activity of other molecules, most are too volatile to persist after drying and processing the plant material for tablets (Weathers and Towler 2014). Like flavonoids, monoterpenes assist in treatment of malaria by arresting parasite development (Moura et al. 2001; Rodrigues-Goulart et al. 2004; Su et al. 2008; Weathers et al. 2017). Eucalyptol in particular also inhibits several proinflammatory cytokines (Juergens et al. 2004), and chlorogenic and rosmarinic acid exhibit similar effects in vitro (de Magalhães et al. 2012). For further details, see Weathers et al. (2017).

Table 3 shows artemisinin and total flavonoid contents of clonal *A. annua* (SAM, voucher MASS 00317314) over the past 7 years. While growth conditions and time of harvest affect artemisinin levels, the amount is relatively consistent at 1.28 ± 0.23% (w/w) and this clonal line propagated solely through rooted cuttings remains a high-artemisinin producer (average 1.28% DW), results consistent with Simonnet et al. 2009. Total flavonoid content is more sensitive to variations in processing of harvested leaves, but is still relatively predictable over time at 4.63 ± 0.90 mg/g DW (Table 3).

After harvesting and drying *Artemisia* leaves, the stability of the non-artemisinin phytochemicals is important if the leaves are to have future therapeutic efficacy. Similarly,

content stability should be verified after processing steps. Many of the essential oils (EOs) in *Artemisia* sp. are therapeutically active against malaria, albeit with a lower efficacy than artemisinin (Table 2). Although artemisinin is very stable over many years in harvested leaves (Simonnet et al. 2009), many of the EOs are volatile and decline with processing (Weathers and Towler 2014) and long-term storage. Total flavonoid content, however, of harvested dried *A. annua* was quite stable for at least 4.5 years (Fig. 2).

How does *Artemisia annua* work?

Bioavailability

Artemisinin, as a pure drug, has notoriously low bioavailability owed to its low aqueous solubility (van der Kooy and Verpoorte 2011; Jessing et al. 2009; Titulaer et al. 1990) and drove development of artemisinic derivatives such as artesunate and artemether, which solubilize more readily in water and oil, respectively. These derivatives are now commonly used in ACTs in the worldwide fight to eradicate malaria. However, if artemisinin's low bioavailability prevents its use as a pure drug to treat malarial infections, then how has *A. annua* been seemingly so effective at treating fever (associated with malaria) over the course of history? One likely reason is that artemisinin bioavailability is significantly increased when it is delivered in whole plant form as opposed to a purified singular drug (Weathers et al. 2011, 2014b).

In mice, to reach similar artemisinin levels in the blood, about 45 × more artemisinin had to be delivered as pure drug, compared to powdered *A. annua* leaves (dried leaf *Artemisia*, DLA) (Weathers et al. 2011). Furthermore, the pharmacokinetics of artemisinin in healthy and *Plasmodium chabaudi-infected* mice when given *A. annua* were significantly different (Weathers et al. 2014b). Artemisinin metabolism was significantly reduced in infected mice, indicating that the diseased state altered artemisinin pharmacology. The same study also confirmed higher bioavailability of artemisinin when delivered as plant material. Artemisinin delivered as pure drug at 100 mg/kg was undetectable in the serum at 1 h while artemisinin delivered as *A. annua* reached a concentration of 4.33 mg/L (Weathers et al. 2014b). Interestingly, when pure artemisinin at 100 mg/kg was delivered combined with mouse chow (~ 0.015 mg artemisinin/mg mouse chow, largely made up of plant material, including corn, soybean, beet, oats, molasses, alfalfa, and wheat), artemisinin was 2.44 mg/L in the serum at 1 h, demonstrating that the general plant matrix (i.e. phytochemicals, oils, fiber etc.) aided artemisinin absorption (Weathers et al. 2014b).

Using an in vitro digestion system in which pure artemisinin or DLA was digested through the intestinal stage, the resulting intestinal digestate liquid phase of the leaves contained about fourfold more solubilized artemisinin than from pure digested artemisinin. This was partially explained by the essential oils (EOs) in the plant. When digested with EOs extracted from *A. annua*, pure artemisinin was 250% more soluble (Desrosiers and Weathers 2016). These results were unsurprising as artemisinin is stored in the glandular trichomes of *A. annua* in solution with the volatile oils produced by the plant (Duke and Paul 1993; Ferreira and Janick 1995; Tellez et al. 1999). For a more complete review of the therapeutic effects of EOs from *Artemisia* species, see Desrosiers et al. (2019). TheEO enhanced solubility of artemisinin partially explains the greater bioavailability afforded by delivery as

DLA than as a pure drug; artemisinin is better absorbed through the intestine when in solution.

Independent of solubility, artemisinin delivered as DLA crosses the intestinal wall about 37% more efficiently than pure artemisinin (Desrosiers and Weathers 2018). This enhanced bioavailability also impacts different organs. In rats, artemisinin delivered as DLA vs. as pure artemisinin distributes in higher concentrations to many internal organs (results as yet unpublished from Weathers lab). A combination of artemisinin, arteannuin B, arteannuic acid, and scopoletin, all common components of *A. annua*, also increased the pharmacological properties of artemisinin (Li et al. 2018). This combination increased the area under the curve ($AUC_{0\rightarrow\infty}$), C_{max}, and t1/2 of artemisinin by 3.78-, 3.47-, and 1.13-fold respectively in healthy mice, while these same values increased by 2.62-, 1.82-, 1.22-fold in *Plasmodium yoelii-infected* mice, respectively, confirming the earlier studies concluding that infection affects artemisinin pharmacokinetics (Weathers et al. 2014b). The next steps in understanding how DLA alters bioavailability of artemisinin will involve the role of the liver and how DLA and its diverse phytochemicals impact hepatic cytochrome P450 (CYP450) enzymes in the liver. CYP3A4 and CYP2B6 are the major artemisinin-metabolizing enzymes present in the liver (Svensson and Ashton 1999). It is posited that *A. annua* small molecule phytochemicals such as flavonoids, coumarins, and terpenes may inhibit one or both of these hepatic P450 s, thereby increasing the amount of artemisinin that passes into the bloodstream. For example, one flavonoid found in DLA, chrysoplenetin, inhibited CYP3A activity and enhanced bioavailability of artemisinin in vivo (Wei et al. 2015).

Therapeutic activity of DLA secondary compounds

While some secondary compounds in DLA enhance bioavailability of artemisinin, others have synergistic or additive effects against the *Plasmodium* parasite. For example, the same combination of secondary compounds shown by Li et al. (2018) to enhance artemisinin bioavailability also showed a 93% reduction in parasitemia compared to 31% reduction in mice given pure artemisinin, indicating that artemisinin is not the only important component in *A. annua*. Chrysoplenetin, when given in combination with artemisinin, reduced parasitemia of *Plasmodium* berghei-infected mice more effectively than artemisinin alone (Wei et al. 2015). Besides these two examples, several secondary compounds found in DLA have their own inherent antimalarial efficacy or synergize with artemisinin to enhance its antiplasmodial affect. A comprehensive review and table of these phytochemicals is available in Weathers et al. 2017 (Artemisinin the Nobel Molecule Book Chapter); see also a review by Ferreira et al. (2010). In addition to treating malaria, DLA was shown recently to be efficacious against schistosomiasis (Munyangi et al. 2018). Interestingly, *A. annua*, which contains artemisinin, but also *A. afra* with negligible artemisinin, cured all patients significantly faster than praziquantel, the current standard therapy (Munyangi et al. 2018). This strongly suggested that artemisinin was not the only therapeutic compound found in the *Artemisia* species and these plants should be further studied to discover more therapeutic compounds.

Anti-inflammatory properties of *Artemisia*

Inflammation is a response to many infectious diseases (He et al. 2015) including malaria (Clark et al. 2006; Erdman et al. 2008). Besides its powerful antimalarial role, artemisinin and several other phytochemicals produced by the *Artemisia* species have immunomodulatory effects. Artemisinin alone has been extensively studied for its potent anti-inflammatory activity (Kim et al. 2015; Shi et al. 2015; Wang et al. 2017; Zhu et al. 2012). This anti-inflammatory capacity is owed to artemisinin's ability to inhibit the expression of pro-inflammatory cytokines such as TNF-α and IL-6 by blocking the NF-κB and MAPK signaling cascades (Fig. 3) (Wang et al. 2017; Zhu et al. 2012). Artemisinin is a well-studied anti-inflammatory agent and the *Artemisia* species produce numerous secondary phytochemicals with anti-inflammatory efficacy. For example, several flavonoids reduced inflammation in both in vitro and in vivo experiments. Two flavonoids produced by *A. annua*, casticin and chrysoplenol-D, reduced inflammatory cytokine production in vitro and in a mouse model of systemic inflammatory response syndrome by inhibiting NF-κB and MAPK signaling pathways (Li et al. 2015).

There are several good reviews that outline the anti-inflammatory effects and mechanisms of action of flavonoids from a variety of plant species, not just *A. annua* (Rathee et al. 2009; Serafini et al. 2010; Spagnuolo et al. 2018). Along with flavonoids, some monoterpenes produced by the *Artemisia* species have anti-inflammatory ability. As an example, 1,8-cineole (eucalyptol) inhibits the in vitro production of several pro-inflammatory cytokines including TNF-α, IL-1β, IL-4, IL-5, IL-6, and IL-8 (Juergens et al. 2004). A more comprehensive review of the effects of monoterpenes on inflammation is available (de Cássia da Silveira e Sá et al. 2013). Other phytochemicals found in *Artemisia* species with anti-inflammatory activities include rosmarinic acid, chlorogenic acid, and scopoletin (de Magalhães et al. 2012; Malik et al. 2011). Many *Artemisia* phytochemicals have anti-inflammatory activity, so extracts and teas have been tested for their overall anti-inflammatory activity. *A. annua* teas reduced IL-6 and IL-8 in activated Caco-2 cells (de Magalhães et al. 2012). Kim et al. (2015) showed extracts of *A. annua* reduced nitric oxide, prostaglandin E_2, IL-1β, IL-6, and IL-10 compared to induced controls (Kim et al. 2015). In another study, leaf extracts made from *A. argyi* and *A. princeps* reduced inflammation in a mouse model of contact dermatitis and inhibited expression of inducible nitric oxide synthase (iNOS), nitric oxide (NO), cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE_2) in vitro (Yun et al. 2016). To our knowledge, however, the anti-inflammatory efficacy of DLA from *A. annua* and *A. afra* has not yet been studied in vivo. In an as yet unpublished study, we recently showed in male rats that *A. annua* DLA treatment reduced production of TNF-α and IL-6 in comparison to control animals; pure artemisinin had no significant effect. Results were different in female rats. While DLA significantly reduced TNF-α production in female rats, so did pure artemisinin. Neither DLA nor pure artemisinin had an effect on IL-6 production, suggesting that gender plays a role in determining the efficacy of artemisinin and DLA on inflammation.

Will *Artemisia* sp. consumption induce artemisinin drug resistance?

Artemisinin drug resistance—current evidence

Artemisinin drug resistance was first observed in Southeast Asia as a prolonged parasite clearance half-time in patients (Dondorp et al. 2009). Artemisinin drug resistance likely was hastened by the conditions in Southeast Asia, including a susceptible subpopulation of migrant workers, an abundance of counterfeit medicines that amounted to insufficient and single-drug therapies, and a relatively high level of selective pressure from artemisinin monotherapy (Eastman and Fidock 2009; Packard 2014). A search for the physical mechanisms of activity soon followed. Early ring stages of resistant patient isolates and laboratory strains cultured under continuous artemisinin pressure enter a dormant state when under drug pressure (Witkowski et al. 2010, 2013). Eventually, Genome-Wide Association Studies identified a region of chromosome 13 as the region most closely associated with resistance in patient isolates (Cheeseman et al. 2012). Further studies revealed that single-nucleotide polymorphisms in the *kelch13* gene were present in resistant isolates and were sufficient to induce artemisinin drug resistance in vitro (Ariey et al. 2014; Ménard et al. 2016; Straimer et al. 2015). Currently, in vitro artemisinin resistance is most commonly assessed using the Ring-Stage Survival Assay (RSA), wherein the degree of survival of a given isolate of *P. falciparum* is assessed by comparing parasite growth in a solvent control to growth following a brief, intense treatment with endoperoxides. Most commonly, this procedure uses 700 nM dihydroartemisinin for 6 h followed by 66 h of recovery and growth (Ariey et al. 2014; Mbengue et al. 2015; Rocamora et al. 2018; Straimer et al. 2015; Witkowski et al. 2013). Generally, these experiments find that mutant *kelch13* strains have RSA survival rates greater than an order of magnitude higher than wild-type (Straimer et al. 2015).

The *kelch13* protein appears to be an ubiquitin-ligase adaptor for the phosphatidylinositol-3-kinase protein (PI3 K). When mutated, *kelch13* less effectively ubiquitinates PI3 K, reducing its rate of degradation that compensates for reduction of active PI3 K when the intracellular protein pool is targeted by artemisinins (Fig. 4) (Mbengue et al. 2015; Wang et al. 2015). The cellular synthesis of phosphatidylinositol-3-phosphate subsequently increases. Increased PI3P activates protein kinase B (Akt) in humans, reducing cell growth and division while increasing survival mechanisms such as autophagy and the unfolded protein response (UPR) (Blaustein et al. 2013). Evidence of PI3P-induced Akt activation in *P. falciparum* is not definitive. However, overexpression of Akt induces some artemisinin resistance in plasmodia, implicating it as a potential downstream effector of artemisinin resistance (Blaustein et al. 2013; Mbengue et al. 2015). Whether through Akt-dependent or-independent mechanisms, UPR and antioxidant pathways are upregulated in plasmodia placed under artesunate stress and are especially upregulated in clinical isolates of resistant *P.* falciparum (Mok et al. 2015; Natalang et al. 2008). However, transgenic upregulation of genes implicated in this *P. falciparum* stress response did not induce levels of artemisinin resistance high enough to account for the survival seen in *kelch13* mutants (Rocamora et al. 2018). Instead, they suggest a marginal role in resistance for the UPR and ROS responses.

An alternate pathway that appears to be involved in artemisinin resistance is the PK4-eIF2α pathway. Under this model, the *kelch13* mutant leads to a constitutive phosphorylation and deactivation of eIF2α in the early ring stage of the life cycle, likely by PK4 kinase. Because the primary function of eIF2α is tRNA trafficking, this leads to a global decrease in translation, thereby allowing the parasite to better maintain proteostasis under stress conditions (Zhang et al. 2017). This model better explains the ultimate downstream effects of *kelch13* mutants, as phosphorylation status of eIF2α is affected by *kelch13* mutation and pharmacological modulation of eIF2α phosphorylation appears to powerfully alter the rate of recrudescence in *P. berghei*-infected mice treated with artemisinins (Zhang et al. 2017). The intermediate effectors between *kelch13* function and eIF2α phosphorylation have not been experimentally determined, but based on the homologous human pathway, the PK4-binding protein BiP has been conjectured to play a role (Paloque et al. 2016).

In humans, both the UPR/stress response pathways and the PK4-eIF2α pathways are interlinked, wherein the UPR ultimately enhances phosphorylation of eIF2α. However, in *P.* berghei-infected mice, *kelch13*-mutant plasmodia did not increase phosphorylation of eIF2α in the face of artemisinin stress, but instead maintained a steady moderate level of phosphorylation (Zhang et al. 2017). This suggested that the conventional stress response is separate from the global downregulation of translation in artemisinin-treated plasmodia. The major strategies used by mutant *P. falciparum* to resist artemisinin treatment are known, but the entire response pathway has not yet been experimentally elucidated. Unfortunately, artemisinin resistance now has spread well beyond Southeast Asia and into Africa (Naß and Efferth 2019).

Artemisia annua and artemisinin drug resistance

The current most common treatment for malaria is with artemisinin combination therapies (ACTs), which combine an artemisinin-based derivative with a second antimalarial that persists in the patient (Eastman and Fidock 2009; WHO 2012, 2017). ACTs include, but are not limited to, artemether + lumefantrine, artesunate + amodiaquine, dihydroartemisinin + piperaquine (Gbotosho et al. 2012; Lwin et al. 2012; Niaré et al. 2016; Sowunmi et al. 2017), and several other drug combinations. The rationale behind this treatment is to combine the fast parasite-clearing activity of an artemisinin with the long-term activity of a partner drug, but still kill parasites resistant to either of the two drugs. This strategy was expected to prevent the emergence and spread of resistant strains (Eastman and Fidock 2009). For these reasons, the use of artemisinin monotherapies is contraindicated. The World Health Organization officially extended this policy to use of *A. annua*-based therapies (WHO 2012). The WHO instituted this policy based on the findings that *Artemisia* therapies contain less artemisinin than pharmaceutical therapies and that weak artemisinin monotherapies would lead to high rates of recrudescence in patients and potentiate the development of plasmodia artemisinin resistance. In its 2012 white paper, the WHO assumed *A. annua* therapy was a monotherapy. It is not. As documented in Table 2, *A. annua* contains a plethora of antimalarial phytochemicals. The WHO (2012) assumed there was too little artemisinin delivered from *per os A. annua* (DLA). That is not the case. Weathers et al. (2011) showed that > 40 × more artemisinin entered the serum from *per os A. annua* than from *per os* artemisinin. Rath et al. 2004 also showed that 5 g DW of *A. annua* leaves (1%

artemisinin) administered as a tea in humans resulted in 240 μg/L of artemisinin in the bloodstream. *A. annua* therefore delivers a large amount of artemisinin into the blood and acts as a polytherapy.

Despite the WHO's official policy, there is evidence that *A. annua* dried leaves (DLA) and/or tea infusions would be an effective antimalarial therapy. In mouse models of malaria, *A. annua* more effectively reduced *P. chabaudi* parasitemia than artemisinin (Elfawal et al. 2012). *A. annua* as DLA also treated artemisinin-resistant mouse malaria *(P. yoellii)* while artemisinin did not (Elfawal et al. 2015). Most importantly, when cultured under artemisinin or DLA drug pressure, DLA proved at least three times more resilient against the evolution of artemisinin drug resistance in *P. chabaudi* (Elfawal et al. 2015). In case studies of patients with severe malaria, treatment with *A. annua* DLA also reversed *P. falciparum* infections that had not responded to ACTs or intravenous artesunate (Daddy et al. 2017). Together these studies suggest that *A. annua* DLA treatment is a promising solution to the challenges of artemisinin resistant *P. falciparum* containment and treatment. Based on all the evidence published since the 2012 WHO whitepaper, there is now a growing body of in vitro, animal, and clinical evidence arguing for the WHO to reconsider its position on use of *A. annua* as a malaria therapy.

Non-artemisinin phytochemical activity against artemisinin-resistant malaria and other microbes

As described above, there is empirical evidence that there is antimalarial activity in *A. annua* leaves beyond their artemisinin content. Artemisinin content fails to explain its efficacy as an antimalarial prophylactic well beyond the persistence time of artemisinin in the patient (Birgersson et al. 2016; Gordi et al. 2000; Hien et al. 2011; Ogwang et al. 2012). That conclusion is further supported by the antimalarial efficacy of *A. afra*, which contains at best traces of artemisinin (Munyangi et al. 2018). The most reasonable explanation for this phenomenon is that, like in ACTs, there are other antimalarial agents present in the plants that persist in the host. Examples include quercetin, which has a biological half-life of over 20 h when consumed from some fruits (Hollman et al. 1997), and a-pinene with a biological half-life of about 10 h (Kohlert et al. 2000).

Flavonoids present in *A. annua* are implicated as potential inhibitors of malaria. In an in vitro study, many flavonoids, including fisetin, kaempferol, luteolin, myricetin, and quercetin, inhibited FabI, FabZ, and FabG enzymes (Tasdemir et al. 2006). The Fab enzymes are important in parasite fatty acid synthesis. Those flavonoids inhibited parasite growth with an IC_{50} in the μM range (Tasdemir et al. 2006). Some flavonoids also inhibit hemoglobin digestion. For example, myricetin and fisetin inhibit both plas-mepsins and falcipains in vitro, while a variety of other flavonoids have a lesser activity towards the enzymes (Jin et al. 2014).

Other nonartemisinic terpenes within the plant also have antimalarial activity. For example, nerolidol, found in *A. annua*, has a sub-micromolar IC_{50} against *P. falciparum* (Rodrigues-Goulart et al. 2004), inhibiting *Plasmodium* isoprene synthesis. Apart from being a necessary component of a variety of biological compounds, isoprene may help protect against reactive oxygen species (ROS). Isoprene, for example, protects against ozone

oxidative stress in tobacco plants, possibly through direct action as an antioxidant (Vickers et al. 2009). Inhibition of isoprene synthesis may benefit antimalarial drugs whose action is ROS-dependent.

Polyphenolic acids present in the extracts of *A. annua* also play a role in parasite inhibition. Chlorogenic acid inhibits drug efflux pump activity in *Staphylococcus aureus* (Fiamegos et al. 2011; Suberu et al. 2013). It is unclear, however, whether chlorogenic acid affects the *P. falciparum* multi-drug resistance transporter that confers resistance to many antimalarials (Petersen et al. 2011).

A. annua and *A. afra* teas also inhibited HIV in vitro (Lubbe et al. 2012). *A. annua* efficacy was not entirely explained by its artemisinin content, and *A. afra* efficacy was comparable to *A. annua* despite a much lower artemisinin content. That response was similar to results of recent clinical trials with these two *Artemisia* species against schistosomiasis (Munyangi et al. 2018) and malaria (Munyangi et al. 2019). While the specific chemicals are not yet known, caffeoylquinic acids in *Artemisia* species have antiretro-viral activity (Lubbe et al. 2012; McDougall et al. 1998). These positive effects suggest that DLA is an effective therapy for infectious diseases and most likely contain multiple active phytochemical components.

Taken together there is evidence that many compounds in *Artemisia* sp. inhibit diverse parasitic pathways thereby providing additional antimalarial activity that could add to or synergize with the effects of the artemisinin content in the plant. In vitro studies have probed the question of whether tea preparations of *A. annua* have more potent antimalarial activity than would be expected based solely on their artemisinin content. In some cases, the apparent IC_{50} of the tea was threefold lower or less than the equivalent concentration of pure artemisinin (De Donno et al. 2012; Suberu et al. 2013); this finding, however, is not consistent across the literature (Mouton et al. 2013; Silva et al. 2012). Such discrepancies may be attributed to use of different cultivars with different phytochemical content. Further study is needed to resolve these discrepancies and parse out the critical factors that would make DLA a robust combination therapy and a powerful tool in the treatment and containment of drug-resistant malaria and other diseases.

Conclusions

In 2012 WHO published a white paper stating that

> WHO does not recommend the use *A. annua* plant material, in any form, including tea, for the treatment or the prevention of malaria. … extensive fundamental and clinical research would be required to demonstrate that non-pharmaceutical forms of *A. annua*, including tea bag, are safe and effective to treat malaria and that their dissemination would not promote the development of artemisinin-resistant parasites.
>
> (WHO 2012)

Despite the absence of artemisinin in *A. afra*, it has proved nearly as effective as *A. annua* in treating malaria and equally effective in treating schistosomiasis. Accumulating studies

show that, with respect to artemisinin, *A. annua* and *A. afra* infusions are not monotherapies, but instead polytherapies with better outcomes than the disease standard drugs against malaria and schistosomiasis and merit further investigation for possible inclusion of *A. annua and A. afra* preparations as a global alternative for fighting and eradicating malaria, schistosomiasis and other *Artemisia*-susceptible diseases.

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Abbreviations

ACT	Artemisinin combination therapy
ASAQ	Artesunate and amodiaquine
CQ	Chloroquine
DLA	Dried leaf *Artemisia*
EIF2α	Eukaryotic translation initiation factor 2α
ERK	Extracellular signal-regulated kinases
EOs	Essential oils
IC_{50}	Half-maximal inhibitory concentration
IkB-alpha	Inhibitor of kappa B alpha
IKK	I kappa B kinase
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinases
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PI3P	Phosphatidylinositol triphosphate
PI3K	Phosphatidylinositol-3-kinase
PK4	Protein kinase 4
PZQ	Praziquantel
RSA	Ring-stage survival assay
TID	*ter in die*, three times/day
TNF-α	Tumor necrosis factor alpha

Ub	Ubiquitin
UPR	Unfolded protein response
WHO	World Health Organization

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Fig. 1.

A. annua (left) and A. *afra* (right) leaves and twigs as prepared for therapeutic tea infusions

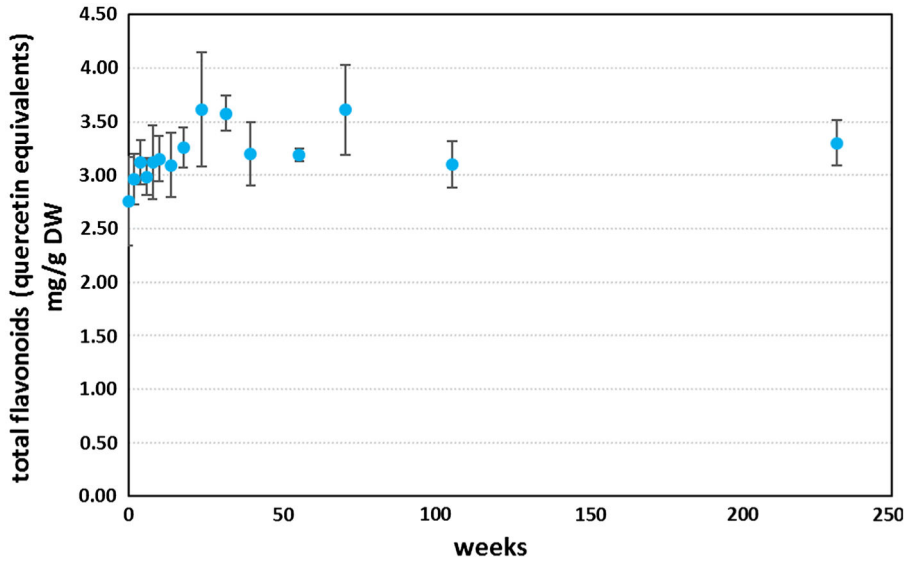

Fig. 2.

Long-term stability of total flavonoid content of dried *A. annua* leaves kept in a sealed clear plastic bag at room temperature under dim light for nearly 4.5 years

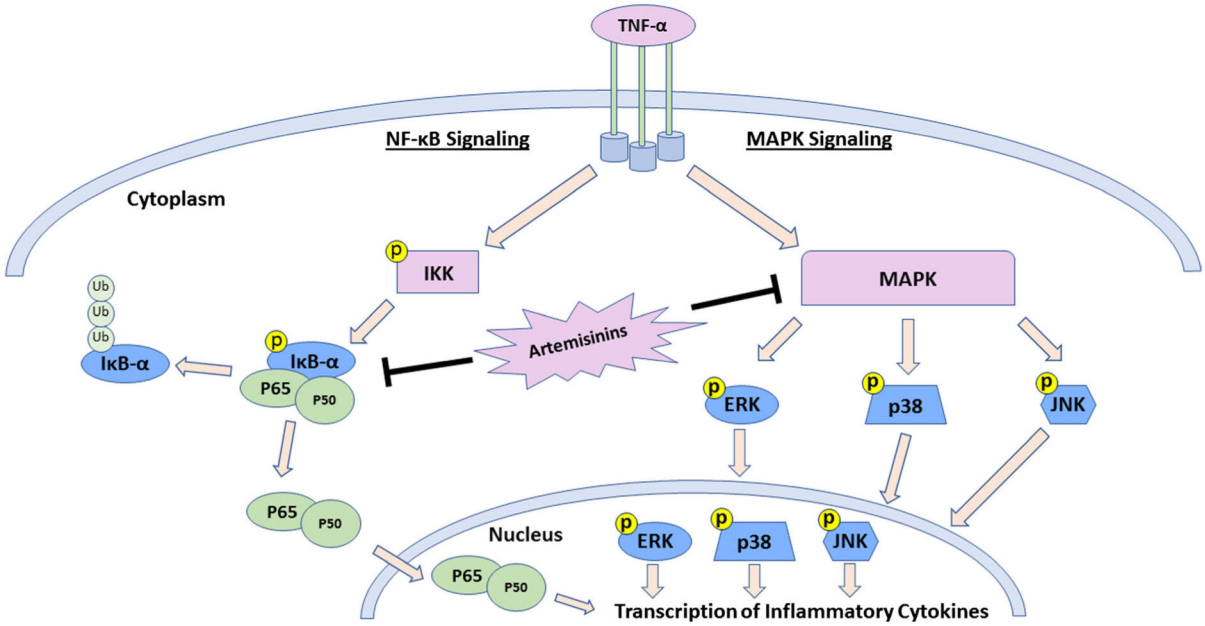

Fig. 3.

Artemisinin inhibition of inflammation through NF-κ B and MAPK signaling cascades. Abbreviations: *TNF-α* tumor necrosis factor alpha, *NF-kB* nuclear factor kappa-light-chain-enhancer of activated B cells, *MAPK* mitogen-activated protein kinases, *IKK* I kappa B kinase, *IkB-alpha* inhibitor of kappa B alpha, *Ub* ubiquitin, Yellow p's indicate phosphorylation of protein, *ERK* extracellular signal-regulated kinases, *JNK* c-Jun N-terminal kinases

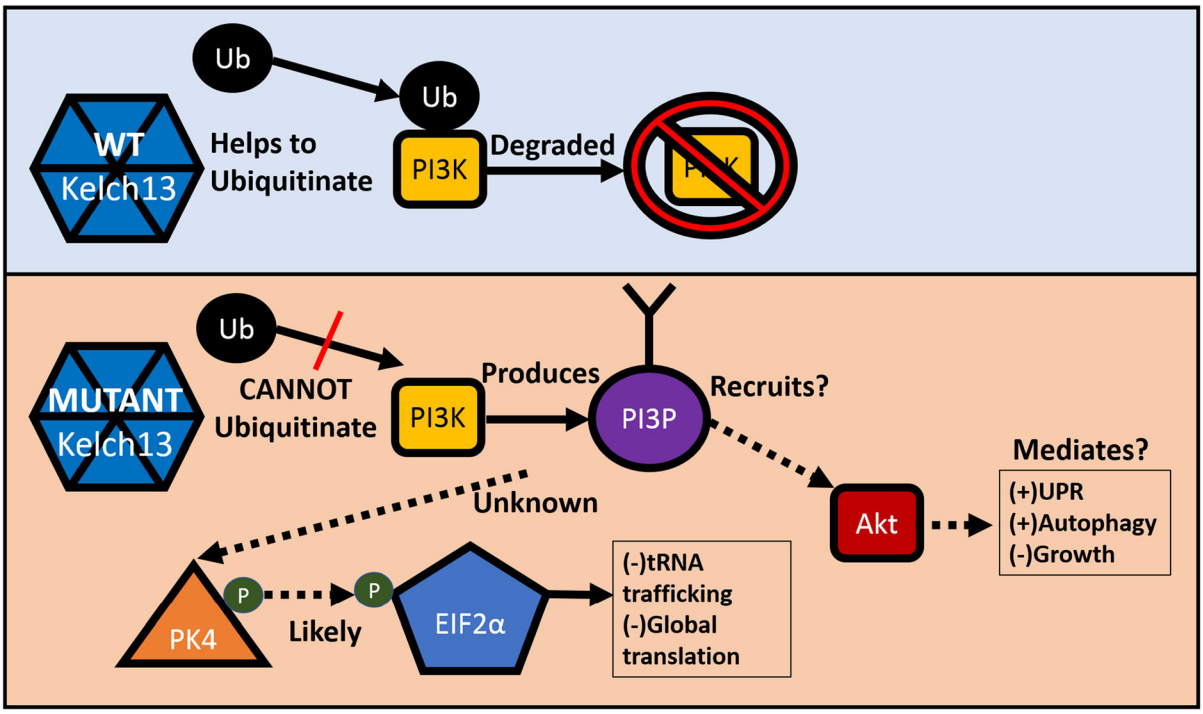

Fig. 4.

Proposed molecular mechanism of artemisinin resistance. Solid lines represent mechanisms with experimental evidence, while dashed lines represent postulated mechanisms. Abbreviations: *PI3P* phosphatidylinositol triphosphate, *PI3 K* phosphatidylinositol-3-kinase, *Ub* ubiquitin, *UPR* unfolded protein response

Table 1

Comparative antimalarial results among *A. annua, A. afra* and ASAQ

Treatment	Average parasite clearance (day)	Artemisinin dose (mg/day)	Total artemisinin delivered in 7 days (mg)	% Cure[c]	% Gametocyte carriage at day 28[d]
A. annua	1	6.7–8.5[b]	33.5–59.5[b]	96.4	0
A. afra	1	0.18	1.26	88.8	0
ASAQ[a]	≤ 14	80–696	240–2088	34.3	2

[a]Dose was 4 mg artesunate/kg per manufacturer posology

[b]There were two different cultivars of *A. annua* used with different levels of artemisinin in the trial at each of the five test sites

[c]Based on parasitemia in the blood

[d]Based on microscopic analysis

Table 2

Phytochemicals in *A. annua* with antimalarial activity

Compound	Compound IC_{50} (μM)	Compound + artemisinin IC_{50} (μM)	References
Artemisinin	0.033	na	Liu et al. (1992)
	0.022, 0.023[a]	na	Suberu et al. (2013)
Artemisinic acid	77.8, 61.6[a]	Varies with compound concentration	
Arteannuin B	3.2, 4.8[a]		
Dihydroartemisinic acid	21.1, 17.7[a]		
Chlorogenic acid	69.4, 61.4[a]		
Rosmarinic acid	65.1, 65.0[a]		
Isovitexin	72.5, 48.1[a]		
Artemetin	26.0	0.026	Liu et al. (1992)
Casticin	24.0	0.026	
Cirsilineol	23.0	0.023	
Chrysoplenol-D	32.0	0.015	
Chrysoplenetin	36.0	0.016	
Eupatorin	65.0	0.030	
Apigenin	20.0, 13.0[b]	Not determined	Lehane and Saliba (2008)
Luteolin	11.0, 12.0[b]		
Kaempferol	33.0, 25.0[b]		
Myricetin	40.0, 76.0[b]		
Quercetin	15.0, 14.0[b]		
	14.7, 4.1, 2.9[c]		Ganesh et al. (2012)
	13.0[d]		Penna-Coutinho et al. (2018)[i]
Rutin	7.1, 3.5, 10.4[c]		Ganesh et al. (2012)
a-Pinene	1.0[e]		Van Zyl et al. (2006)

Compound	Compound IC_{50} (μM)	Compound + artemisinin IC_{50} (μM)	References
1,8-Cineole (eucalyptol)	70.0[e]		
Limonene	533.0[e]		
Nerolidol	9.0[e]		
	0.76[f]		Rodriguez-Rodrigues-Goulart et al. (2004)
Phytol	18.9[g]		Grace et al. (2012)
Scopoletin	128.0, 121.5[h]		Zaid et al. (2016)

Na not applicable, *CQ* chloroquine

[a] CQ-sensitive HB3 and CQ-resistant Dd2 strains, respectively

[b] CQ-sensitive 3D7 and CQ-resistant 7G8 strains, respectively

[c] Fresh blood isolates (Bangladesh), CQ-sensitive 3D7, and CQ-resistant K1 strains, respectively

[d] CQ-resistant W2 strain

[e] CQ-resistant FCR-3 strain

[f] CQ-sensitive 3D7

[g] CQ-sensitive D10 strain

[h] CQ-resistant K1 and CQ-sensitive 3D7 strains, respectively

[i] Test in vitro and also in vivo in mice

Table 3

Artemisinin and total flavonoid content of clonal crops of *A. annua*

Lab versus field/garden cultivation	Harvest date	Artemisinin (mg/g DW)	Total flavonoids[a] (mg/g DW)
Lab 2011	4/2011	14.55	5.75
Lab May 2011	5/2011	11.92	3.72
Lab December 2011	12/2011	8.71	3.05
Lab February 2012	2/2012	13.91	5.34
Lab March 2012	3/2012	14.79	4.35
Field 2012	7/2012	13.96	4.17
Garden 2012	9/2012	11.96	4.16
Lab 2012	12/2012	15.78	5.44
Lab 2013	1/2013	14.19	5.89
Lab July 2013	7/2013	15.13	4.82
Field 2013	9/2013	11.23	3.02
Garden 2013	9/2013	13.70	4.82
Lab 2014	6/2014	9.44	3.59
Garden 2015	9/2015	8.33	5.24
Garden 2016	9/2016	10.80	na
Garden 2017	9/2017	15.12	5.34
Garden 2018	9/2018	13.46	5.39
Average over 7 years		12.76 ± 2.28	4.63 ± 0.90

na not available

[a]Quercetin equivalents