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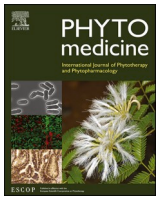

Original Article

In vivo antimalarial efficacy of *Artemisia afra* powder suspensions

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ABSTRACT

Background: A global death toll of 608,000 in 2022 and emerging parasite resistance to artemisinin, the mainstay of antimalarial chemotherapy derived from the Chinese herb *Artemisia annua*, urge the development of novel antimalarials. A clinical trial has found high antimalarial potency for aqueous extracts of *A. annua* as well as its African counterpart *Artemisia afra*, which contains only trace amounts of artemisinin. The artemisinin-independent antimalarial activity of *A. afra* points to the existence of other antimalarials present in the plant. However, the publication was retracted due to ethical and methodological concerns in the trial, so the only evidence for antimalarial activity of *A. afra* is built on *in vitro* studies reporting efficacy only in the microgram per milliliter range.
Hypothesis: Our study aims to shed more light on the controversy around the antimalarial activity of *A. afra* by assessing its efficacy in mice. In particular, we are testing the hypothesis that *A. afra* contains a pro-drug that is inactive *in vitro* but active *in vivo* after metabolization by the mammalian host.
Methods: Plasmodium berghei-infected mice were treated once or thrice (on three consecutive days) with various doses of *A. afra, A. annua*, or pure artemisinin.
Results: Aqueous powder suspensions of *A. annua* but not *A. afra* showed antimalarial activity in mice.
Conclusion: Our experiments conducted in mice do not support the pro-drug hypothesis.

Introduction

With a global death toll of 608,000 in 2022, malaria is still one of the deadliest infectious diseases worldwide. The overwhelming majority of the 249 million malaria cases reported in the same year affect African children below five (WHO, 2023). The World Health Organization (WHO) currently recommends combination therapies with artemisinin, a sesquiterpene lactone derived from the Chinese wormwood species *Artemisia annua* L., for the treatment of uncomplicated malaria (WHO, 2022). However, signs of resistance to both, artemisinin and its partner drugs, pose a threat to an effective treatment of malaria patients (WHO, 2023). Today, we thus find ourselves in a similar situation as in the 1960s, when resistance to chloroquine – the mainstay of malaria treatment at that time – started to spread (Rosenthal, 2001): The development of new antimalarials is of uttermost importance in order to prevent the death of millions.

A. afra Jacq., the African counterpart of *A. annua*, finds a wide range of applicability in African traditional medicine. Despite being mostly devoid of artemisinin, tea infusions of *A. afra* are commonly used to treat malaria (du Toit and van der Kooy, 2019; Maciuk et al., 2023). This has fueled the hope that there might be antimalarial constituents other than artemisinin in *A. afra* (du Toit and van der Kooy, 2019; Maciuk et al., 2023).

In vitro efficacy studies with crude *A. afra* extracts were performed with a variety of parasite strains using various plant parts and extraction protocols, and they have returned microgram *per* milliliter activity (Table S1) (Clarkson et al., 2004; Gruessner and Weathers, 2021; Kraft et al., 2003; Mokoka et al., 2011; Mouton et al., 2013; Moyo et al., 2016; 2019; Muthaura et al., 2015). Unlike *A. annua* (Liu et al., 2010), *A. afra* never reached antiplasmodial activity in the nanogram *per* milliliter range, even when fractionated (Liu et al., 2010; Moyo et al., 2019). The findings of these *in vitro* studies contrast with a clinical trial conducted in the Democratic Republic of the Congo in 2015, in which *A. annua* and *A. afra* aqueous infusions administered over seven days (3 × 333

Abbreviations: NSG, NOD/SCID/IL2Rγ$^{-/-}$; SEN, Senegal; TAN, Tanzania; WHO, World Health Organization.

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ml/day) attained a strong antimalarial effect in 248 and 223 patients, respectively (Munyangi et al., 2020). However, the corresponding publication was soon retracted due to ethical and methodological concerns (André et al., 2022; Munyangi et al., 2020; WHO, 2019). The only *in vivo* support for this clinical trial comes from a study in *P. berghei*-infected mice reporting moderate antiplasmodial activity for *A. afra* lyophilisates (Gathirwa et al., 2008). But the lack of an artemisinin control (to ensure that the observed effect was not due to low concentrations of artemisinin potentially being present in the *A. afra* material) and of detailed information on the extraction protocol prevent reproduction and reliable conclusions.

The fact that *A. afra* displays only weak activity *in vitro*, but might be active *in vivo*, led to the hypothesis that *A. afra* may contain a pro-drug that unravels its antimalarial potential only after metabolization by a mammalian host (du Toit and van der Kooy, 2019). In the present study, we aimed at testing this pro-drug hypothesis by treating *P. berghei*-infected mice with suspensions of *A. afra* or *A. annua* plant powder, or with pure artemisinin.

Materials and methods

Plant material and drug preparation

We used two plant samples of *A. annua* and one of *A. afra*. The first *A. annua* sample was sourced from the cosmetics manufacturer Weleda AG (Arlesheim), arrived in powder form and had been harvested in September 1997 in Tanzania (*A. annua* TAN). The second *A. annua* sample (*A. annua* SEN, voucher ID: Yen-1–2018-AA, Le Relais Sénégal) and the *A. afra* sample (voucher ID: Yen-1–2018-AF, Le Relais Sénégal) were harvested on a plantation in Senegal (GPS: 114°56′19.8″N 16°51′00.0″W) run by Le Relais Sénégal. Both samples contained 65% leaves and 35% twigs, and were dried and coarsely shredded before being stored at room temperature in the dark. For *in vivo* application, shredded plant material was ground and both, shredded and powdered plant material, was sieved sequentially through sieves with 1 mm, 300 μm, and 120 μm mesh size to obtain a fine powder. On the day of treatment, the powder was resuspended in vehicle containing one volume of 70% Tween-80 (density = 1.08 g/ml) and 30% ethanol (density = 0.81 g/ml) and four volumes of water to yield a concentration of 0.14 g powder *per* ml vehicle. For the *A. annua* TAN, *A. annua* SEN and *A. afra* suspension, this corresponded to approximately 0.56 mg, 0.57 mg and <0.0071 mg artemisinin *per* milliliter, respectively.

Artemisinin powder was a gift from Holley Wuling Mountain Pharmaceutical Co, Ltd. (batch ID: 20061205X) and was prepared in the same vehicle as the *Artemisia* spp. suspensions.

Determination of artemisinin concentration in dry plant material of A. afra and A. annua

An amount of 2 mg shredded plant material ($n = 3$) was weighed in on a XP 26 microbalance (Mettler Toledo, OH, Columbus, USA) and extracted with 10 ml methanol. In addition, *A. afra* samples were extracted at a higher concentration of 1 mg/ml (10 mg in 10 ml methanol). The samples were agitated for 1–2 h on an orbital shaker and afterwards centrifuged for 30 min at 10 °C and 3220 g (5810 R, Eppendorf, Hamburg, Germany).

The artemisinin content of the plant extracts was determined by liquid chromatography (Shimadzu, Kyoto, Japan) tandem mass spectrometry (API 5000, AB Sciex, MA, USA). Artemisinin was detected by positive electrospray ionization in the multiple reaction monitoring mode. The mass transition *m/z* 283.1 → 247.1 was used as quantifier and the following transitions were used as qualifiers: *m/z* 283.1 → 209.1, *m/z* 283.1 → 265.0, *m/z* 283.1 → 151.2, *m/z* 283.1 → 105.1, *m/z* 283.1 → 133.1. The plant extract was separated on a Kinetex C18 analytical column (2.6 μm, 2.1 × 50 mm, Phenomenex, Torrance, CA, USA). Water and methanol were used as mobile phase A and B, respectively. Both mobile phases were supplemented with formic acid (0.1% v/v). Chromatographic separation was performed at 45 °C using a total flow rate of 0.5 ml/min. In the first 0.5 min of each run, the injected sample (2.5 μl) was diluted via a T-union with mobile phase A, which was installed in front of the analytical column. The detailed gradient program is given in supplementary table S2.

Efficacy of A. afra and A. annua in P. berghei-infected mice

Animal experiments against *P. berghei* and *P. falciparum* were carried out at the Swiss Tropical and Public Health Institute and adhered to local and national regulations of laboratory animal welfare in Switzerland (awarded permission no. BS1731/BL519 and BL521). Protocols are regularly reviewed and revised following approval by the local authority (Veterinäramt Basel-Stadt and Basel-Land).

In vivo antimalarial activity was assessed in age-matched female NMRI mice, weighing between 22 g and 24 g, and ordered from the Charles River Laboratories, Sulzfeld, Germany. The numbers of mice *per* group and experiment were as follows: $n = 3$ mice *per* group except for the control group in experiment 1, where $n = 5$, and the group treated with 12.5 g/kg of *A. annua* TAN in experiment 2, where $n = 2$. On day 0, all mice were intravenously infected with 2×10^7 parasites of a GFP-transfected *P. berghei* ANKA strain (kindly donated by A. P. Waters and C. J. Janse, Leiden University Medical Center, Leiden, The Netherlands) from a donor mouse. The first treatment followed 2–4 h later (experiment 1 and 3) or on day 1 (experiment 2) and was administered *per os* (cannula specifications: 0.9 × 25 mm). Plant suspensions were prepared freshly at the day of treatment. Vehicle for controls and artemisinin solutions were prepared a week ahead, aliquoted and stored at −20 °C. In the multi-dose study (experiment 3), treatment was repeated on three consecutive days at intervals of approximately 24 h. In experiments 2 and 3, a fixed dose of *Artemisia* spp. suspensions was administered with 3.125 g/kg (*A. afra* and *A. annua* SEN) or 6.25 g/kg (*A. annua* TAN) for a low dose and 6.25 g/kg (*A. afra* and *A. annua* SEN) or 12.5 g/kg (*A. annua* TAN) for a high dose. Parasitemia was assessed microscopically from one day after the first treatment onwards. Blood smears were stained with Hemacolor® Rapid staining solutions (Sigma-Aldrich, St Louis, MO, USA, Ref: 111,956 and 111,957) and parasitemia was determined by counting up to ten fields containing approximately 3000 erythrocytes in total. The percentage activity on day 3 post infection was calculated using the following equation:

$$Activity = 100 - \left(\frac{\varnothing\ parsitemia\ treated}{\varnothing\ parasitemia\ control} \times 100 \right)$$

Mice were euthanized on day 3 in order to prevent death from infection.

The means between the different treatment groups was compared by one-way analysis of variance (ANOVA) in RStudio (version 2022.02.3).

Results

Determination of artemisinin concentration in dry plant material of A. afra and A. annua

LC-MS/MS was used to determine the artemisinin content in both *Artemisia* species. The artemisinin content in the methanol extract (2 mg/10 ml) of dry leaves and twigs of *A. annua* was 4.00 mg/g dry weight (95% CI: 3.33, 4.67 mg/g) for the sample cultivated and harvested at a Senegalese plantation (*A. annua* SEN) and 1.95 mg/g dry weight (95% CI: 1.89, 2.01 mg/g) for the commercial sample obtained from the cosmetics manufacturer Weleda and cultivated and harvested in Tanzania (*A. annua* TAN). In contrast, artemisinin was detectable neither in the 2 mg/10 ml nor the 10 mg/10 ml methanol extract of *A. afra* (Fig. 1), and hence its content was assumed to be <0.05 mg/g dry weight (*i.e.* the limit of quantification), which is at least 40- to 80-fold

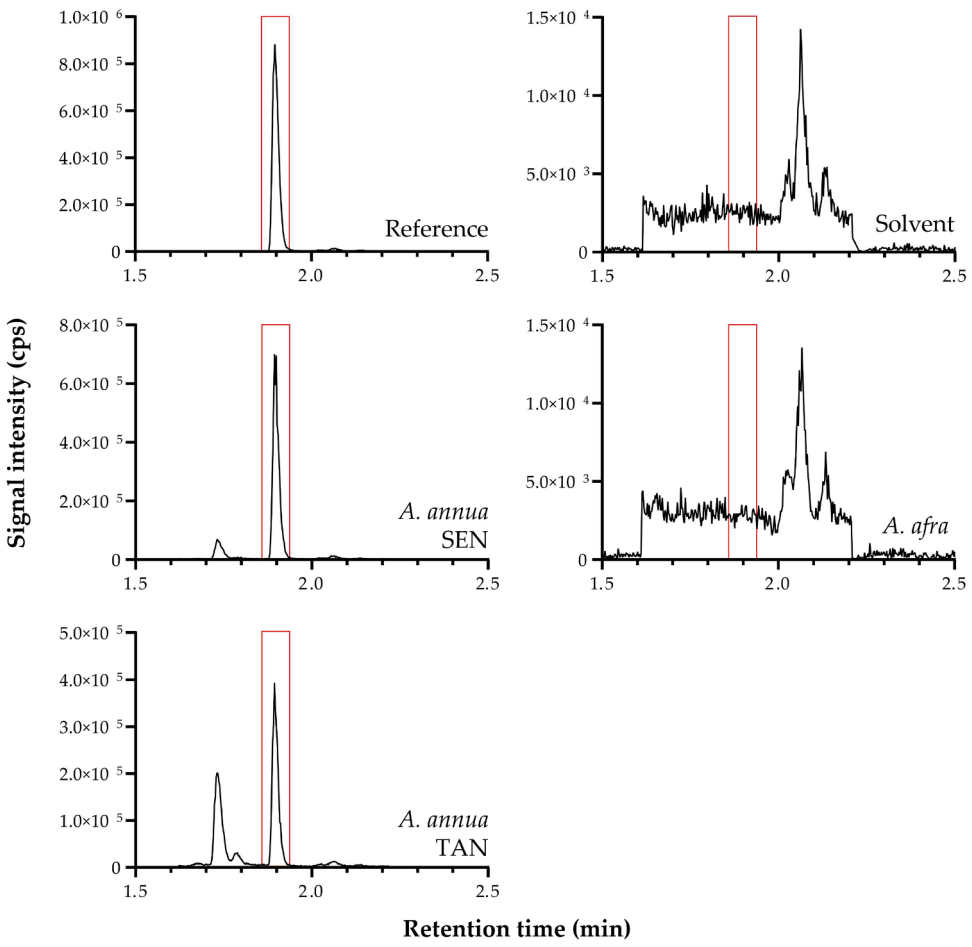

Fig. 1. Artemisinin (*m/z* 283.1 → 247.1) was analyzed using liquid chromatography tandem mass spectrometry in a solvent sample, reference standard (1 μg/ml), and three different *Artemisia* plant extracts. The expected retention time of artemisinin is highlighted in red in the chromatograms.

lower compared to the two tested *A. annua* cultivars. These results are in line with previously reported artemisinin contents for *A. afra* (Gruessner and Weathers, 2021; Martini et al., 2020; Snider and Weathers, 2021) and *A. annua* (De Donno et al., 2012; Mueller et al., 2000; Munyangi et al., 2020; Snider and Weathers, 2021), although the latter are rather at the lower end of the scale considering that *A. annua* can contain as much as 15.9 mg artemisinin *per* gram dry weight (Gruessner and Weathers, 2021).

Efficacy of A. afra and A. annua in Plasmodium-infected mice

Before testing the efficacy of *A. afra* in *P. berghei*-infected mice, we first established a protocol for drug preparation and administration using *A. annua* TAN powder as positive control.

The artemisinin (or overall constituents) content in an extract can vary substantially depending on the solvent used for extraction (Liu et al., 2010; Mueller et al., 2000; Räth et al., 2004; van der Kooy and Verpoorte, 2011; Wright et al., 2010), and extract activity can easily be

Table 1
Antimalarial activity of pure artemisinin and *Artemisia* spp. suspensions in *P. berghei*-infected mice at varying dosing regimens. Parasitemia of untreated control mice is given for each experiment as indicator for the *in vivo* inter-assay variability. Data were generated in three independent experiments, each with = 3 mice *per* group except for the control group in experiment 1, where $n = 5$, and the group treated with a high dose of *A. annua* TAN in experiment 2, where $n = 2$.

exp. no.	dose	% activity (95% CI) on day 3 post infection				parasitemia (%) of control (95% CI)
		artemisinin	*A. annua* TAN	*A. annua* SEN	*A. afra*	
1	untreated					34 (27, 41)
	1 × high*	89 (89, 90)	97 (94, 99)			
	1 × low**	54 (47, 60)	93 (91, 94)			
2	untreated					51 (48, 54)
	1 × high*		97 (97, 97)	98 (98, 99)	28 (16, 40)	
	1 × low**		96 (95, 98)	95 (92, 99)	21 (−10, 51)	
3	vehicle					47 (42, 52)
	3 × high*				−4.6 (−26, 17)	
	3 × 0.3 mg/kg	4.6 (−0.5, 10)				

* A high dose corresponded to 6.25 g/kg of *A. annua* SEN, 12.5 g/kg of *A. annua* TAN (both containing 25 mg/kg artemisinin), to 6.25 g/kg A. afra (containing a maximum of 0.3mg/kg artemisinin), or to 25mg/kg pure artemisinin. Dose in g dry weight per kg mouse.

** A low dose corresponded to 3.125 g/kg of *A. annua* SEN, 6.25 g/kg of *A. annua* TAN (both containing 12.5 mg/kg artemisinin), to 3.125 g/kg A. afra (containing a maximum of 0.15mg/kg artemisinin), or to 12.5mg/kg pure artemisinin. Dose in g dry weight per kg mouse.

lost upon fractionation (Caesar and Cech, 2019). Therefore, we decided to deliver the pure plant powder in form of a slurry suspension to ensure that the mice ingested all plant constituents potentially active against the malaria parasite. During the preparation process, all ingredients were kept at room temperature. In a first experiment (experiment 1, Table 1), infected mice were treated once with varying doses of pure artemisinin or with a corresponding dose of *A. annua* TAN suspensions containing the same amount of artemisinin (12.5 or 25 mg/kg, as determined before by LC/MS-MS). At day 3 post infection, the antimalarial activity of pure artemisinin ranged from 89% to 54% for 25 and 12.5 mg/kg, respectively. In the case of *A. annua* TAN, both the single high and single low dose containing the same amount of artemisinin (25 and 12.5 mg/kg) displayed activities of 97% and 93%, respectively.

In a second experiment (experiment 2, Table 1), we tested a single low and high dose of *A. annua* TAN and *A. annua* SEN (12.5 and 25 mg/kg artemisinin) and *A. afra* (using equal volumes as for *A. annua* SEN, *i.e.* containing a calculated maximum dose of 0.3 mg/kg artemisinin). Larger doses could not be tested, since the required application volumes would have exceeded the maximal application volume recommended for mice (Diehl et al., 2001).

Although there had been thirteen years between the first and the second experiment, we found similar activities for *A. annua* TAN in both experiments (Table 1), indicating that the artemisinin content had remained stable for more than a decade. The *A. annua* SEN and TAN cultivars displayed similar activities (95–98%) at the tested doses, irrespective of their origin. The *A. afra* suspension, in contrast, had only weak – if any – antimalarial activity, ranging between 21% and 28%. From many years of experience with this mouse model we know that an activity below 30% should be taken with caution and has most likely to do with the variability of parasite growth *in vivo* (Table 1, right column).

Motivated by the fact that *Artemisia* teas had been taken on seven consecutive days in the clinical trial (Munyangi et al., 2020), and because we wanted to better understand the data from the previous single-dose experiment, we next explored whether multiple doses of *A. afra* would result in a more substantial parasite suppression (experiment 3, Table 1 and Fig. 2). In addition to an experimental group treated with a high dose of *A. afra*, we also included a vehicle control group and an artemisinin control group that was treated with an artemisinin dose of only 0.3 mg/kg, corresponding to the highest possible content of artemisinin in the *A. afra* cultivar at this dosing volume according to the limit of quantitation of the LC-MS/MS detection method. We found that neither multiple doses of low-dose artemisinin (0.3 mg/kg) nor high doses of *A. afra* were active in *P. berghei*-infected mice (activity <5%). Indeed, the parasitemia profile between the experimental group and the two control groups did not differ over the whole treatment course (Fig. 2), and all mice were euthanized at day 3 due to a high parasite burden.

In order to account for potential enzymatic differences between distinct parasite species, we also assessed the antimalarial activity of *A. afra* suspensions and pure artemisinin in *P. falciparum*-infected mice (immunodeficient NOD/SCID/IL2R$\gamma^{-/-}$ (NSG) mice engrafted with human erythrocytes). In contrast to the NMRI mice in the *P. berghei* model, it was found that the majority of the NSG mice did not tolerate the required high application volumes. The only valid data obtained stems from $n = 1$ *P. falciparum*-infected NSG mouse treated on four consecutive days with *A. afra* and $n = 2$ *P. falciparum*-infected NSG mice treated on four consecutive days with pure artemisinin. The experimental outcome showed a pattern similar to that observed in the *P. berghei*-infected mice (no activity and 96% activity, respectively).

Discussion

In the present study, we have assessed the efficacy of *Artemisia* spp. suspensions in two malaria mouse models. The two *A. annua* cultivars tested displayed high antimalarial activity after a single oral dose of plant suspension with determined artemisinin content of 12.5 or 25 mg/kg, respectively. Given the artemisinin content in the plant material, this is not unexpected. Indeed, *in vivo* antimalarial activity of *A. annua* preparations has been demonstrated many times before, both in mice (Elfawal et al., 2012; Wright et al., 2010) and in humans (Blanke et al., 2008; Daddy et al., 2017; Mueller et al., 2000; Räth et al., 2004; Zime-Diawara et al., 2015).

The *A. annua* TAN suspension containing artemisinin of 12.5 mg/kg showed comparable activity against *P. berghei* as the pure artemisinin tested at 25 mg/kg (93% and 89%, respectively). This shows that *A. annua* powder was about twice as potent as the pure compound under

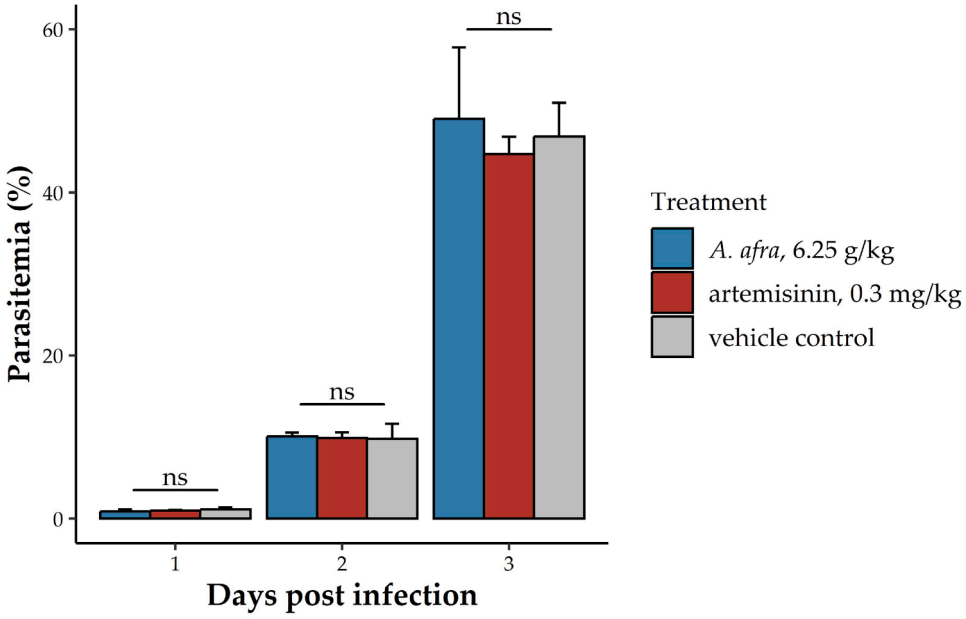

Fig. 2. Antimalarial activity of *A. afra* suspensions and pure artemisinin compound in *P. berghei*-infected mice. The doses were 6.25 g/kg *A. afra* (dry weight/kg mouse) and 0.3 mg/kg artemisinin (corresponding to the highest possible content of artemisinin in the *A. afra* cultivar at this dosing volume), and were administered on day 0, 1, and 2. The vehicle was one volume of 70% Tween-80 and 30% ethanol, and four volumes of water. Error bars are standard deviations for $n = 3$ mice. ns indicates *P*-values > 0.05 (one-way ANOVA).

those experimental conditions. This is in line with experiments showing that *A. annua* delivered as powder-water suspension (Elfawal et al., 2012) or as pounded juice (Wright et al., 2010) was more active than comparable doses of pure artemisinin in *P. chabaudi*- and *P. berghei*-infected mice, respectively. A range of potential explanations was suggested for this phenomenon, including synergistic drug-drug interactions between the plant constituents (De Donno et al., 2012; Li et al., 2018), better artemisinin bioavailability (Weathers et al., 2014), and improved solubility of artemisinin in *A. annua* preparations due to the presence of flavonoids and other amphiphilic constituents in the herb (de Ridder et al., 2008; Mueller et al., 2000; Räth et al., 2004).

Moreover, the efficacy of *A. annua* TAN was comparably high in two independent experiments, even though there were thirteen years between those two experiments. The plant powder, which had been prepared from a cultivar harvested more than 20 years ago, has been stored at room temperature and protected from sunlight. Under these conditions, artemisinin was stable in dried plant material. This is in line with a study by Lee et al. (2022), showing that dry material of *A. annua* harvested in 1999 and 2002 contained stable amounts of artemisinin when comparing measurements from 2009, 2012, and 2021 (Lee et al., 2022).

In contrast to the *A. annua* suspensions, neither *A. afra* suspensions nor the corresponding low-dose artemisinin control (0.3 mg/kg) displayed antimalarial activity in our mouse models. While antimalarial activity of *A. afra* suspensions would have confirmed the pro-drug hypothesis, the observed lack of activity is not sufficient to discard it: the host (mouse *versus* human), the plant material (origin, harvesting conditions, processing), the delivery form (cold suspension *versus* tea infusion), and the dosing regimen (single dose *per* day *versus* multiple doses across the day) might have an impact on the efficacy of the herb. From an empirical point of view, the best way to find out whether *A. afra* tea infusions are active in malaria patients or not would be to assess its efficacy in a small confirmatory clinical trial, such as a volunteer infection study (VIS) in an endemic area.

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CRediT authorship contribution statement

Annabelle Walz: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ursula Lehmann:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Urs Duthaler:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis, Data curation. **Pascal Mäser:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Sergio Wittlin:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.155644.

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