Is Artemisinin the Only Antiplasmodial Compound in the *Artemisia annua* Tea Infusion? An *in Vitro* Study

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Abstract

In our ongoing investigation into *Artemisia annua* for the treatment of malaria, we decided to study the possibility that synergism might enhance the efficacy of artemisinin. Our main objective was to test tea infusions and nonpolar extracts prepared from different *A. annua* varieties against *Plasmodium falciparum in vitro* in order to determine if synergism will increase the effectiveness of artemisinin in the samples as compared to pure artemisinin. We found that the IC_{50} of artemisinin in the tea and nonpolar extracts was not significantly different to the IC_{50} of pure artemisinin. We could show that the year and country of harvest or storage conditions did not have any influence on the activity and that it narrowly followed the concentration of artemisinin in all the extracts. In conclusion, based on these *in vitro* results, artemisinin seems to be the only active antiplasmodial compound in *A. annua*.

Key words

Artemisia annua L. · Asteraceae · artemisinin · tea infusion · *Plasmodium falciparum*

Artemisia annua L. (Asteraceae) contains the well-known antimalarial compound artemisinin (ART). The maceration of *A. annua* fresh plant material was used in China almost two thousand years before the isolation and identification of ART in the early 1970s, to treat fevers or chills [1]. It is of great importance to note that no resistance against ART has been reported in China despite its use over a very long period of time. Despite the commercialization of ART-based drugs and the use of ART combination therapies (ACTs), local populations of the most affected countries continue to drink tea infusions made from *A. annua* to treat malaria but also for the treatment of other diseases such as HIV [2]. The reason for this can be found in the social sciences – economics and trust. It is inexpensive and in a form that most third world country communities rely on. It is however claimed that synergy between ART and other components in the tea infusion will enhance the activity of ART and make the tea infusion more active than pure ART alone. If true, this could scientifically justify the traditional use. Some studies have already shown that other compounds can improve the antimalarial activity of ART [3] or exhibit some antimalarial activity alone [4].

Our study flows from the recently published WHO position statement on the effectiveness of non-pharmaceutical forms of *A. annua* against malaria. This statement contains three claims upon which the recommendation of the WHO is based that *A. annua* in any herbal formulation should not be used to treat malaria. In short the three claims are:

1. The content of ART is variable and overall too low in *A. annua*.
2. Patients are therefore underdosed which could lead to resistance.
3. Recrudescence is unacceptably high and indicates that potential synergism between ART and other compounds are negligibly low.

The final conclusion drawn from these claims is: "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 ART-resistant parasites" [5].

Our main objective in this study was to test the tea infusions prepared from different *A. annua* varieties against *Plasmodium falciparum in vitro* in order to determine if synergism will increase the effectiveness of ART in the tea infusions as compared to pure ART. Additionally, we also tested nonpolar extracts from *A. annua*. To determine if synergism plays a significant role, we prepared tea infusions and chloroform extracts from 16 plants of *A. annua* and two plants of *Artemisia afra* Jacq. *Ex* Willd. (Asteraceae) (**Table 1**) and tested their *in vitro* activity against *Plasmodium falciparum* (3D7). The inclusion of *A. afra* can be considered as a negative control because this plant does not contain ART but possesses other similar chemical constituents [6, 7], and it is being used in the treatment of malaria [8]. **Table 2** shows the IC_{50} of the tea infusions and chloroform extracts, the concentration of ART in each sample, and the calculated IC_{50} of ART in each sample. From **Table 2**, it can be seen that the *A. annua* tea samples and chloroform extracts with the lowest content of ART (samples 10, 11, and 12) are the least active against *P. falciparum* (IC_{50} > 1.5 µg/mL for the teas and IC_{50} > 0.500 µg/mL for the chloroform extracts). The *A. afra* teas were inactive at the highest concentration tested (IC_{50} = > 25 µg/mL), whereas the *A. annua* teas had an average IC_{50} of 0.75 µg/mL. In these samples, the IC_{50} of the calculated ART varies between 2.52 ng/mL and 6.61 ng/mL with an average of 4.97 ng/mL. This value is close to that found for pure ART (5.48 ng/mL). Taking into account the inherent variability of the bioassay and sample preparation, there appears to be no significant difference between the IC_{50} of pure ART and ART in the tea infusion except for sample 1 (IC_{50} = 2.52 ng/mL). However, the IC_{50} of pure ART varies between 3.27 ng/mL and 6.11 ng/mL from one test series to another. Based on this variation, we can conclude that the IC_{50} of ART in the tea infusions is similar to that of pure ART.

The *A. annua* chloroform extracts had an average IC_{50} of 0.18 µg/mL, and the calculated ART in these samples had an average IC_{50} of 5.27 ng/mL. The chloroform extracts therefore follow the same trend as was observed for the tea infusions, in that ART appears to be the only active compound. The *A. afra* chloroform extracts had an average IC_{50} of 11.02 µg/mL. Despite the fact that this plant does not contain ART, its chloroform extract does however show some antiplasmodial activity albeit in an order of magnitude lower than *A. annua*. This data confirms those previously described [7] and suggests that other compounds in *A. afra* also exhibit some *in vitro* antiplasmodial activity. According to these observations, it can be concluded that the *A. afra* tea is not active against *P. falciparum in vitro* and the *in vitro* activity of ART in the *A. annua* tea seems not to be improved by other compounds.

Table 1 Origin of *A. annua* and *A. afra** plant material used in this study.

Sample	Country of cultivation	Harvest period	Plant parts (dried)	Origin of seeds (breeding program)
1	South Africa	1999	leaves/flowers	Anamed
2	South Africa	2002	leaves/flowers	Anamed
3	South Africa	2006	leaves	Anamed
4	Tanzania	2006	leaves	Anamed
5	Cameroon	2007	leaves	Anamed
6	Germany	2007	leaves	Anamed
7	Mozambique	2007	leaves	Anamed
8	Germany	2009	leaves	Anamed
9	Germany	2010	leaves	Anamed
10	Belgium	2009	leaves	Téi vum Séi
11	Luxembourg	2011	leaves	Téi vum Séi
12	Luxembourg	2011	leaves/flowers	Téi vum Séi
13	Burundi	2010	leaves	Anamed
14*	Uganda	2009	leaves	Collected
15	Burundi	2010	leaves	Anamed
16	Cameroon	2009	leaves	CIPCRE
17	Congo	2010	leaves	Anamed
18*	South Africa	2011	leaves	Botanical garden, Univ. of Pretoria

Table 2 IC_{50} values of the extracts and ART in the different *A. annua and A. afra*† teas and chloroform extracts. Pure ART had an average IC_{50} of 5.48 ± 1.54 ng/mL.

Sample	Tea infusions			Chloroform extracts		
	Sample IC_{50} (µg/mL)	ART IC_{50} (ng/mL)	[ART] (µg/mL)	Sample IC_{50} (µg/mL)	ART IC_{50} (ng/mL)	[ART] (µg/mL)
1	0.53 ± 0.09	2.52 ± 0.32*	13.42	0.10 ± 0.01	5.74 ± 0.28	152.7
2	0.80 ± 0.03	5.11 ± 0.45	17.93	0.21 ± 0.01	6.80 ± 0.18	118.9
3	0.30 ± 0.01	4.74 ± 0.23	71.59	0.06 ± 0.00	7.93 ± 0.29*	356.2
4	0.53 ± 0.04	5.02 ± 0.25	38.39	0.13 ± 0.00	4.12 ± 0.03	179.6
5	0.36 ± 0.06	4.26 ± 1.01	48.78	0.06 ± 0.00	5.45 ± 0.06	234.5
6	0.38 ± 0.01	4.94 ± 0.36	64.31	0.03 ± 0.00	3.30 ± 0.29	320.7
7	0.55 ± 0.03	4.19 ± 0.20	32.15	0.10 ± 0.02	5.13 ± 0.76	177.2
8	0.32 ± 0.05	5.23 ± 0.26	58.24	0.05 ± 0.00	6.45 ± 0.37	417.4
9	0.39 ± 0.00	4.67 ± 0.30	44.82	0.06 ± 0.00	7.09 ± 0.30	263.1
10	2.69 ± 0.10	6.61 ± 2.64	8.44	0.79 ± 0.05	6.18 ± 0.41	21.18
11	2.32 ± 0.28	5.41 ± 0.41	8.36	0.52 ± 0.04	5.18 ± 0.38	22.82
12	1.70 ± 0.34	5.27 ± 0.46	11.14	0.50 ± 0.02	3.26 ± 0.10*	29.11
13	0.36 ± 0.01	4.85 ± 0.31	58.29	0.05 ± 0.00	3.55 ± 0.15	284.2
14†	> 25.00	nd	nd	11.66 ± 0.31	nd	nd
15	0.27 ± 0.00	5.20 ± 0.37	87.02	0.06 ± 0.01	4.62 ± 0.40	417.6
16	0.25 ± 0.00	6.15 ± 0.12	117.2	0.05 ± 0.00	4.07 ± 0.19	550.3
17	0.27 ± 0.04	5.30 ± 0.03	64.09	0.06 ± 0.00	5.44 ± 0.04	336.7
18†	> 25.00	nd	nd	10.37 ± 1.01	nd	nd

nd = not detected; * Denotes a significant difference ($p < 0.005$) with the IC_{50} value of pure ART

We do however need to be very careful in interpreting these findings. This analysis excluded the possibility that there are any obvious or easy to find compounds with direct activity against a specific life cycle phase of *P. falciparum*. There are however numerous points to consider, for example:

1. Due to the availability of *A. annua* we used relatively old plant material (> 1 year) as opposed to the recommendation that fresh plant material should be used. Antiplasmodial compounds such as pyrethrins [9] which are known to be present in *A. annua* [10] and are known to be photosensitive would be degraded in the material that we used. Upon testing all the samples for the presence of these compounds, we could not detect any (data not shown).
2. Our bioassay targets the erythrocyte phase of the parasite, hence any potentially active compounds against other phases of the life cycle will not be detected.
3. The analysis does not take into account that the tea infusion may contain "prodrugs" – compounds that become active only after metabolization.

In conclusion we investigated the claim that synergism enhances the activity of *Artemisia* tea. Our results indicate that in the bioassay used, ART appears to be the only antiplasmodial compound in the tea and the chloroform extracts. Very recently, two other studies were published reporting on the activity of the *A. annua* tea infusion against *P. falciparum*. In the first one [11], they found that the tea infusions were up to three times more active compared to ART alone against chloroquine-sensitive (D10) and -resistant (W2) strains, while in the second one [12], they found that no synergism occurred and that the infusion was equally active to ART alone against field isolates of *P. falciparum* (chloroquine-resistant). Our study confirms the finding in [12] and expands on it by the inclusion of 16 different *A. annua* samples and testing of

their tea infusions and nonpolar extracts against chloroquine-sensitive strains of *P. falciparum*. Although all three studies ([11, 12], current study) show similarities (tea infusions being tested) and differences (different *P. falciparum* strains used, different quantification techniques used to quantify ART in tea infusions), the overall results are remarkably similar with one study leaning towards synergism [11], while the other two ([12], current study) lean towards the tea infusion having no or little synergistic effect. However, before we can claim that there is (no) synergism occurring in the tea infusion, we have to test the above-mentioned points, and particularly, *in vivo* assays have to be conducted.

Materials and Methods

Table 1 gives the origin of all the plant material used in this study. All plant material obtained from Anamed was identified by Dr. Hans-Martin Hirt (Anamed International). Samples 10–12 were developed by a commercial gardening center in Luxemburg, Téi vum Séi, and was certified by the Ministry of Agriculture of Luxemburg. Sample 16 was certified by CIPCRE as *A. annua*. *A. afra* (sample 14) was collected in Uganda in 2009, and sample 18 in the Botanical garden of the University of Pretoria, South Africa in 2011.

The tea samples were prepared according to the method previously described [13]. The chloroform extracts were prepared by extracting 200 mg of dried plant material with 5 mL of chloroform. These samples were sonicated for 30 min, filtered and transferred into HPLC vials (duplicate). The chloroform was evaporated under nitrogen gas. All dried extracts were weighed, and one sample of each duplicate was evaluated for the antiplasmodial activity testing. The duplicate samples were kept for ART quantification which took place on the same day as the antiplasmodial testing.

The ART concentration was determined as previously described using HPLC-ELSD analysis [14]. The antiplasmodial bioassays were performed on the erythrocyte phase of the parasite *Plasmodium falciparum* 3D7 (chloroquine-sensitive strain) as previously described [7]. Pure ART (Sigma-Aldrich, 98% purity, cat. nm. 361593–100MG) was used as a positive control. The medium containing parasitized red blood cells was used as a positive growth control and the medium alone as a negative growth control. The samples were dissolved in DMSO (for the chloroform extracts) or in 10% ethanol (for the tea infusions) in order to obtain a stock concentration of 5 mg/mL. References and samples were then diluted with medium in a series of twofold dilutions (tea) or fourfold dilutions (chloroform extract).

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Conflict of Interest

The authors declare no conflicts of interest.

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