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Assessment of the in vitro activity and selectivity of *Artemisia afra* and *Artemisia annua* aqueous extracts against artemisinin-resistant *Plasmodium falciparum*

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Abstract

Background The recent emergence of artemisinin resistance in Africa is drawing scrutiny toward the use of alternative anti-malarial therapy based on *Artemisia annua* and *Artemisia afra* phytotherapies. This study aimed to determine if either *A. annua* and *A. afra* extracts are active against artemisinin-resistant *Plasmodium falciparum* isolates and determine the selectivity of inhibitory phytotherapies.

Methods *Artemisia* extracts were tested in vitro to mimic parasites exposure to extracts in population drinking *Artemisia* sp. teas. *Artemisia* extracts were tested in Ring Stage Survival Assays ($RSA^{0-3\ h}$) against Cambodian clinical isolates previously genetically and phenotypically characterized as artemisinin resistant or sensitive. Primary human hepatocytes and a human hepatoblastoma cell line (HepG2 cells) were used to assess the cytotoxicity of *Artemisia* extracts.

Results The study revealed a substantially decreased in vitro activity of *A. annua* extracts when tested on artemisinin-resistant parasites mutated in the *Pfkelch13* gene (RSA_{50} 0.137–2.56 $g.L^{-1}$) compared to artemisinin-sensitive parasites (RSA_{50} 0.080 $g.L^{-1}$). Conversely, the *A. afra* extracts have a similar activity on the isolates tested whether they are sensitive or resistant to artemisinin (RSA_{50} 0.537–0.758 $g.L^{-1}$) However, the selectivity index for *A. afra* extracts was much lower than for *A. annua* extracts (*A. afra:* 4.628, 4.305 and 6.076 vs *A. annua:* 387.625, 226.350 and 12.099, respectively for WT, C580Y and R539T).

Conclusions *Artemisia annua* activity is driven by artemisinin, implicating the same resistance profiles and concerns associated with semisynthetic artemisinin derivatives. *Artemisia afra* showed artemisinin-independent antiplasmodial

†Camille Roesch and Kutub Ashraf have contributed equally to this work. Author order was determined on the basis of contribution to the initial draft writing.

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activity. However, the molecular basis of this activity is unknown and may not present a sufficient selectivity, thus further characterization of *A. afra* is essential.

Keywords *Plasmodium falciparum*, *Artemisia* sp., Resistance, Antimalarial activity

Background

The emergence of Kelch13 (K13) mediated artemisinin resistance in *Plasmodium falciparum* is now largely documented in Africa [1–7]. Although this resistance is only partial and does not compromise artemisinin-based combined therapy (ACT) endpoint efficacy, it could be a first step in the acquisition of multiresistance. The genetic analysis of the artemisinin-resistant strains collected so far in Africa has revealed a local emergence as opposed to an importation of Asian-originated parasites [8]. Although the basis of this recent evolution is still debated, the off-label uses of artemisinin(s) monotherapies are suspected to trigger this emergence [9]. In parallel to the inadequate use of commercial treatments, several malaria control stakeholders have raised concerns about the relation between broad use of *Artemisia* sp. infusions in sub-Saharan Africa and the selection of resistant parasites [9]. This problematic is notably reinforced by the inherent unrealistic standardization and observance of a homemade therapy and by the lack of concrete in vivo efficacy data in the literature [9–11]. The two main *Artemisia* species used in Africa are *Artemisia annua* and *Artemisia afra.* Both species contain artemisinin, at relatively high concentration for *A. annua* but substantially lower for *A. afra* [12]. In the absence of clear characterization, it could then be assumed that both might participate to the selection of resistant parasites. Previous data that showed artemisinin can readily select K13 mutant parasites reinforce this possibility [13, 14]. These hypotheses are discussed by the proponents of these phytotherapies, who notably argue that *Artemisia* sp. infusions consist in a poly-chemotherapy that would limit the selection of drug resistant parasites [15]. This is notably justified by data from a rodent malaria model showing that oral consumption of *A. annua* dried leaves is more active than pure artemisinin. In addition, these studies have showed slower acquisition of artemisinin resistance [16], greater reduction of parasitaemia [16–19] and higher bioavailability of artemisinin compared to mice receiving the corresponding dose of artemisinin [20–22]. However, the mechanisms of resistance in rodent malaria parasites could present discrepancies with *P. falciparum* artemisinin resistance, such as K13-independent mechanisms [23].

The overall aim of this study is to determine to which extend artemisinin-resistant K13 mutant parasites present a better survivability upon *A. afra* and *A. annua* exposure, to evaluate the selection risk through in-population usage of these phytotherapies.

Methods

Plasmodium falciparum clinical isolate collection and culture

Clinical isolates were collected between 2014 and 2020 in Cambodia and adapted to in vitro continuous culture at 2% haematocrit (O + human blood, Centre de Transfusion Sanguine, Phnom Penh, Cambodia) in RPMI-1640 medium supplemented with 0.5% (w/v) albumax II, 2.5% (v/v) decomplemented human plasma (mixed serogroups) under an atmosphere of 5% CO_2 and 5% O_2 and kept at 37 °C. These isolates were previously characterized for their genotype (*k13* propeller domain sequence) [14] and their in vitro susceptibility to dihydroartemisinin (DHA) using the ring stage survival assay ($RSA^{0-3\,h}$) [24]. The selected sample set included 12 strains presenting a wildtype *k13* haplotype (WT group), 6 strains with the C580Y mutation (C580Y group), and 6 strains with the R539T mutation (R539T group) (Table 1).

Preparation of *Artemisia* extracts

Artemisia afra (voucher: LG0019528 Université de Liège) and *A. annua* (voucher: MNHNL 17733 Herbarium Luxembourg) were collected as leaves and twigs and preserved in their original packaging at room temperature. Both products were protected from sunlight until *Artemisia* extract preparation. The stock extract was prepared as follows: 5 g of *Artemisia* dried leaves and twigs were poured in 100 mL of pre-boiling molecular grade water and boiled for 5 min at 100 °C. The extract was allowed to cool for another 10 min at room temperature and then centrifuged at 3000 rpm for 10 min to pellet down the plant debris and fine solids. The supernatant was first filtered through a 40 µm cell strainer (Falcon, Corning Brand) and then through a 0.20 µm membrane filter (CA-Membrane), generating an extract stock (50 $g.L^{-1}$). The stocks of extracts were stored at −20 °C. The quantification method of artemisinin in extracts is described in Ashraf et al. [25]. The artemisinin concentration in 50 $g.L^{-1}$ extract stocks was 10.75 µM for *A. annua* and 251 nM for *A. afra*.

Evaluating anti-malarial activity of *Artemisia* extracts

Parasites susceptibility to *Artemisia* extracts was determined using $RSA^{0-3\,h}$ according to Witkowski et al. [24].

Table 1 Characterization of *Plasmodium falciparum* strains used

Group	Isolate ID	Year of collection	Province of provenance	*K13* genotype	RSA$^{0-3\,h}$ (%)	median RSA$^{0-3\,h}$ % (Standard deviation)
WT	1	2017	Rattanakiri	WT	0.01	0.00 (0.0029)
	2	2018	Rattanakiri		0.00	
	3	2018	Rattanakiri		0.00	
	4	2019	Rattanakiri		0.00	
	5	2019	Rattanakiri		0.00	
	6	2019	Rattanakiri		0.00	
	7	2019	Rattanakiri		0.00	
	8	2019	Rattanakiri		0.00	
	9	2020	Rattanakiri		0.00	
	10	2018	Kratie		0.00	
	11	2019	Kratie		0.00	
	12	2020	Stungtreng		0.00	
C580Y	13	2014	Mondulkiri	C580Y	19.31	14.57 (8.3094)
	14	2017	Mondulkiri		31.36	
	15	2017	Mondulkiri		9.27	
	16	2019	Kampong Speu		9.03	
	17	2019	Rattanakiri		15.36	
	18	2017	Pursat		13.78	
R539 T	19	2018	Rattanakiri	R539T	45.85	52.39 (22.4617)
	20	2020	Rattanakiri		44.13	
	21	2020	Rattanakiri		39.87	
	22	2020	Rattanakiri		88.79	
	23	2020	Rattanakiri		89.03	
	24	2016	Stung Treng		58.94	

Briefly, tightly synchronized parasites (rings 0–3 h post-invasion) were obtained by 75% Percoll centrifugation followed by sorbitol treatment and adjusted between 0.5 and 1% parasitaemia. Parasites were exposed to a range of increasing concentration of either *A. annua* or *A. afra* for 6 h, before washout. Twofold dilution series from *A. annua* and *A. afra* stock solutions (50 g.L^{-1}) were prepared in molecular grade water. Each concentration tested was diluted ten times when added to the culture media, making a 10% final extract concentration in the media. A carrier-control consisted of 10% molecular grade water in media. After 72 h, parasitaemia was measured by counting 1×10^5 red blood cells per sample condition via microscopy. The survival rate was determined as a ratio of parasitaemia in the drug-exposed condition versus the drug-free condition.

Cytotoxicity assessment of *Artemisia* extracts using primary human hepatocytes

Primary human hepatocytes (BioIVT, lot BGW) were thawed into InVitroGro™ CP Medium (BioIVT, Cat# Z99029) containing a 1 ×antibiotic mixture (PSN, Gibco Cat# 15,640,055 and Gentamicin, Gibco, Cat# 15,710,072). Cell viability was recorded using trypan blue exclusion on a haemocytometer, and 18,000 live cells were added to each well of a collagen-coated 384-well plate (Grenier, Cat# 781,956) and maintained at 37 °C and 5% CO_2. Primary human hepatocytes were treated with *Artemisia* extracts for 6 or 24 h at 7 days post-seeding. Twofold dilution starting from 5 g.L^{-1} were used to assess cytotoxic effect. Control consisted of 10% molecular grade water in media. After 66 (6 h treatment) or 48 (24 h treatment) additional culture hours following drug removal, hepatocytes were fixed for 1 h with 4% paraformaldehyde (PFA) (Thermo Fisher Scientific) in PBS (Gibco), washed twice with PBS, and stained with 1 µg mL^{-1} Hoechst 33,342 (Thermo Fisher Scientific, Cat# H3570) to detect hepatic nuclear DNA. Hepatic nuclei were imaged and quantified using a 4 ×objective on a Lionheart FX automated microscope (Biotek®).

Cytotoxicity assessment of *Artemisia* extracts using HepG2

The HepG2 human hepatocyte line from hepatocellular carcinoma (ATCC HC-8065) was cultured in rat collagen

I-coated (5 µg.cm^{-2}) flasks (Corning) at 20–90% confluence in DMEM without sugar (Lonza catalog 11,966–025) supplemented with 4.5 g.L^{-1} glucose (Lonza), 1 mM Sodium Pyruvate (Lonza), 2 mM L-glutamine (Gibco) and 10% fetal bovine serum (Hyclone) in a cell culture incubator at 37 °C and 5% CO_2. Cells were passed by treating with Trypsin LE (Gibco) for 7 min at 37 °C. Toxicity assays were performed in DMEM with glucose replaced with galactose to avoid false negative results due to potential Crabtree effect [26]. Cells were harvested from a flask, counted on a haemocytometer using trypan blue, diluted in DMEM prepared as above, but with 10 mM galactose instead of glucose, and seeded at a density of 2000 live cells in 40 µL per well into rat-tail collagen I-coated 384-well plates (Greiner Bio-one) using a Biomek NX (Beckman Coulter).

Twofold dilution series from *A. annua* and *A. afra* stock solutions (50 g.L^{-1}) were prepared in molecular grade water. Each concentration tested was diluted ten times when added to the parasite media, making a 10% final extract in the media. A carrier-control consisted of 10% molecular grade water in media. Assays were performed in two 72 h formats: one in which cells were treated for 24 h before a drug washout with media and 48 h of additional culture, and the other with treatment for the entire 72 h. At 72 h, media was removed and cells were fixed with 4% paraformaldehyde (Thermo Fisher Scientific) in PBS and then stained with 10 µg.mL^{-1} Hoechst 33,342 for 1 h. The entire culture area of each well of assay plates were imaged with a Lionheart FX automated microscope (Biotek®) with a 4 × objective, and net hepatic nuclei per well quantified.

Selectivity index determination

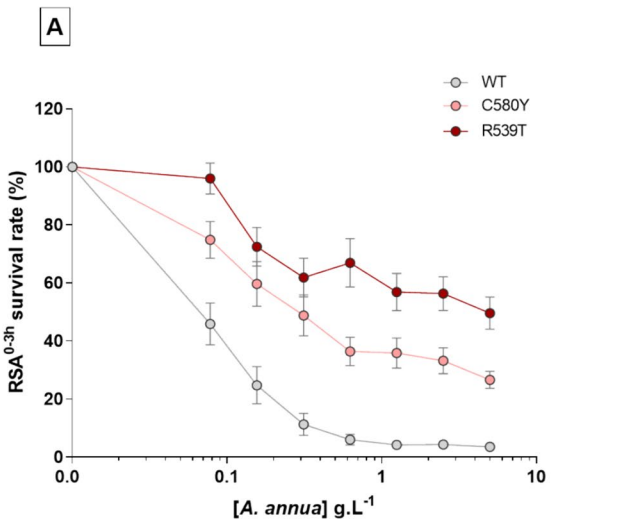
The selectivity index (SI) is the ratio that measures the cytotoxicity of the extracts and their anti-malarial activity. It was calculated for the 6 h treatment of *P. falciparum* clinical isolates with this formula SI = (PI_{50}_hepatocytes_6 h/RSA_{50}_parasites_6 h)) for *A. afra* and *A. annua* for the WT, C580Y and R539T groups.

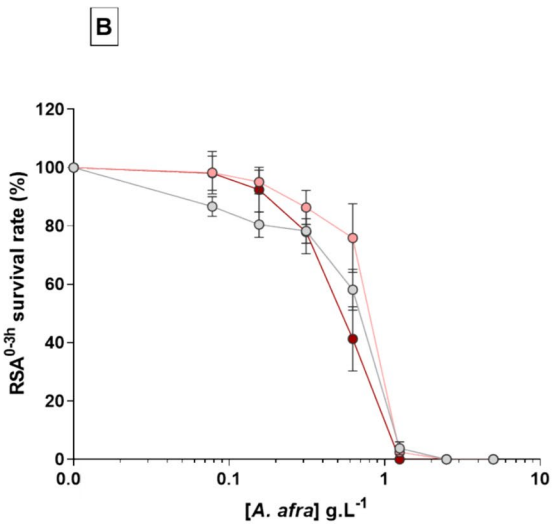
Statistical analysis

All analyses were performed using GraphPad Prism (v7.00). A p value < 0.05 was considered significant. $IC_{50\ s}$ were calculated using Quest Graph™ IC_{50} Calculator (https://www.aatbio.com/tools/ic50-calculator) or GraphPad Prism (v7.00).

Results

The in vitro activity of *A. annua* (Fig. 1A) and *A. afra* (Fig. 1B) extracts was measured against three groups of *P. falciparum* clinical isolates, designed out of their K13





Group	Median RSA_{50} *A. annua* g.L^{-1} (SD)	Comparison to WT group (Mann Whitney test - p value)
WT	0.080 (0.064)	not relevant
C580Y	0.137 (0.268)	**0,0289**
R539T	2.563 (2.678)	**0,0071**

Group	Median RSA_{50} *A. afra* g.L^{-1} (SD)	Comparison to WT group (Mann Whitney test - p value)
WT	0.705 (0.185)	not relevant
C580Y	0.758 (0.259)	0,7392
R539T	0.537 (0.155)	0,986

Fig. 1 Ring-stage survival assay ($RSA^{0-3\ h}$) following treatment with *Artemisia annua* and *Artemisia afra*. The susceptibility of clinical isolates (WT group, C580Y group and R539T group) to **A** *A. annua* and **B** *A. afra* measured with a $RSA^{0-3\ h}$. Each dot represents the mean survival of isolates at a given concentration. Error bars represent the SEM. Tables below graphs summarize the median $RSA_{50\ s}$ and the statistical analysis performed (Mann Whitney test)

profile (wild-type, C580Y and R539T), using $RSA^{0-3\ h}$. From the $RSA^{0-3\ h}$ values obtained for each concentration tested, an inhibitory concentration of 50% of the survival rate (RSA_{50}) after exposure was determined. The median RSA_{50} values (± standard deviation) of *A. annua* extracts were 0.080 $g.L^{-1}$ [± 0.064], 0.137 $g.L^{-1}$ [± 0.268] and 2.563 $g.L^{-1}$ [± 2.679] for parasites WT, C580Y and R539T, respectively. The RSA_{50} values from artemisinin-resistant parasites harbouring C580Y and R539T profile were significantly higher compared to wildtype artemisinin-sensitive parasites (WT) ($p = 0.0289$ and $p = 0.0071$ respectively for C580Y and R539T, Mann Whitney test, Fig. 1A). The concentration range of *A. annua* extracts used was insufficient to decrease parasitaemia to an undetectable level making impossible to determine a minimal inhibitory concentration (MIC) value.

The RSA_{50} values of *A. afra* extracts were 0.705 $g.L^{-1}$ [± 0.185], 0.758 $g.L^{-1}$ [± 0.259] and 0.537 $g.L^{-1}$ [± 0.155] for WT, C580Y and R539T parasite groups respectively (Fig. 1B). No significant differences were observed between wildtype versus K13-mutant parasites ($p = 0.7392$ and $p = 0.9860$ respectively for C580Y and R539T, Mann Whitney test; Fig. 1B). The concentration range for *A. afra* extracts resulted in a drop in parasitaemia to an undetectable level for all the strains tested and, therefore, allowed for a MIC determination ranging between 1.25 to 2.5 $g.L^{-1}$.

Individual RSA_{50} values were then used to evaluate the cross resistance of *Artemisia* extracts with DHA. The results showed a positive correlation between *A. annua* extract RSA_{50} and $RSA^{0-3\ h}$ values ($r = 0.4112$, $p = 0.0459$, Pearson correlation test; Fig. 2A) while no correlation was observed for *A. afra* extracts ($r = -0.1975$, $p = 0.3549$, Spearman correlation test; Fig. 2B).

The hepatotoxicity of the *Artemisia* extracts was evaluated by treating HepG2 cells, gold standard for hepatotoxicity assessment, in both a 24 h washout and 72 h assay (Fig. 3). Regardless of assay mode, the results showed a similar level of cytotoxicity between *A. annua* and *A. afra*. The concentration causing 50% of proliferation inhibition (PI), PI_{50}'s for *A. afra* extracts from 24 and 72 h assays were 2.025 $g.L^{-1}$ and 1.855 $g.L^{-1}$, respectively, while the PI_{50}'s for *A. annua* were 2.406 $g.L^{-1}$ and 2.260 $g.L^{-1}$ for 24 and 72 h assays, respectively. In addition, no significant difference between the PI_{50} of *A. annua* and *A. afra* for the 24 or 72 h treatment was observed (24 h, $p = 0.4395$ and 72 h, $p = 0.4890$, Unpaired-t test).

The selectivity of the *Artemisia* extracts against *P. falciparum* was then evaluated by treating primary human hepatocytes cultures with extracts for 6 h and 24 h. The results showed that exposure to *A. annua* extracts presented low cytotoxicity at the highest concentrations for the exposure duration tested. The PI_{50} was determined to be greater than 5 $g.L^{-1}$, with an estimated to be 31.010 $g.L^{-1}$ and 26.900 $g.L^{-1}$ for 6- and 24-h exposure, respectively (Fig. 4A). In contrast, *A. afra* extracts showed cytotoxicity at higher test concentrations, resulting in PI_{50}'s of 3.263 $g.L^{-1}$ and 2.124 $g.L^{-1}$ for 6 and 24 h exposure, respectively (Fig. 4B). The selectivity index (SI) for the 6 h treatment of *P. falciparum* clinical

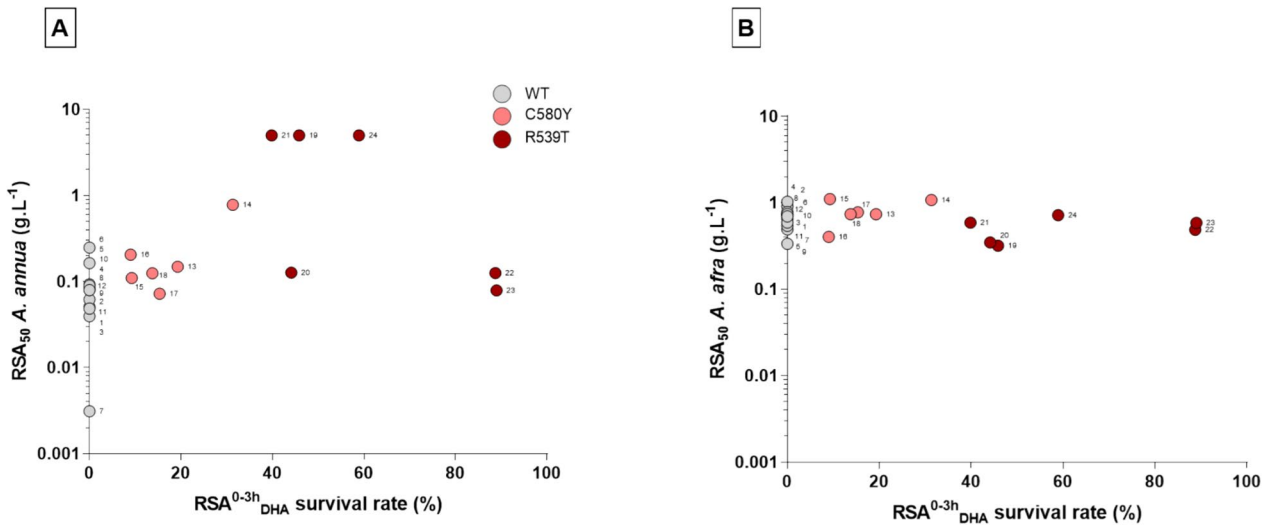


Fig. 2 In vitro activities of *Artemisia annua* and *Artemisia afra* extracts correlate with survival to dihydroartemisinin treatment. $RSA_{50\ s}$ of clinical isolates (WT group, C580Y group and R539T group) extrapolated from their survival during $RSA^{0-3\ h}$ with *A. annua* and *A. afra* correlate with the survival percentage in an $RSA^{0-3\ h}$ with a DHA treatment of 700 nM. **A** *A. annua* $RSA_{50\ s}$ show a positive moderate correlation with $RSA^{0-3\ h}{}_{DHA}$ values ($r = 0.4112$, $p = 0.0459$, Pearson correlation test), **B** *A. afra* $RSA_{50\ s}$ do not correlate with $RSA^{0-3\ h}{}_{DHA}$ values ($r = -0.1975$, $p = 0.3549$, Spearman correlation test). Each point was labelled according to Table 1 on both panel A and panel B

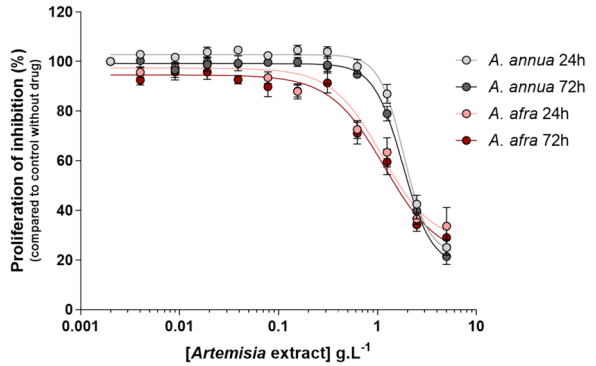


Fig. 3 Cytotoxicity studies of *Artemisia afra* and *Artemisia annua* tea extracts on HepG2 cells. Percentage of proliferation inhibition (PI) compared to non-treated condition after 72 h of culture with 24 or 72 h of drug exposure on HepG2 cells after 24 h seeding. N = 2 independent biological replicates and N = 2 technical replicates for each condition and biological replicate. $PI_{50\,s}$ for *A. afra* extracts were 2.025 $g.L^{-1}$ and 1.855 $g.L^{-1}$ for 24 and 72 h exposure, respectively. $PI_{50\,s}$ for *A. annua* extracts were 2.406 $g.L^{-1}$ and 2.260 $g.L^{-1}$ for 24 and 72 h exposure, respectively. No significant difference between the PI_{50} of *A. annua* and *A. afra* for the 24 or 72 h treatment was observed (24 h, p = 0.4395 and 72 h, p = 0.4890, Unpaired-t test). Similar results after 24 and 72 h of treatment show that the compounds are cidal. Each dot represents the mean proliferation inhibition percentage of isolates at a given concentration. Error bars represent the SEM

isolates (using the formula SI = (PI_{50}_hepatocytes_6 h/RSA_{50}_parasites_6 h)) for *A. afra* was 4.628, 4.305 and 6.076 for WT, C580Y and R539T groups, respectively, whereas for *A. annua* it was higher, with 387.625, 226.350 and 12.099 for WT, C580Y and R539T groups, respectively (Table 2).

Discussion

The results obtained here showed that the *A. annua* extract is largely less effective against the artemisinin-resistant *P. falciparum* isolates tested here compared to the artemisinin-sensitive isolates. Additionally, a cross resistance with DHA was observed. These data suggest the in vitro activity of *A. annua* extracts is mainly driven by its content in artemisinin and, therefore, might trigger the selection of resistant parasites.

This finding is consistent with previous studies [27–29] and notably Czechowski et al. [27] that showed that *A. annua* with a knocked-out artemisinin synthesis pathway lost its anti-malarial activity. Consequently, there is a non-negligeable risk in the use of *A. annua* as anti-malarial treatment surrogate leading to artemisinin resistance selection. Furthermore, these in vitro data do not suggest pharmacological benefits of *A. annua* phytotherapies over ACT. Several studies on animal models suggest that the therapeutic interest of *A. annua* lies in increased artemisinin plasmatic concentration due to co-present molecules, such as chrysosplenetin [15, 30]. However, this potential advantage should be put in perspective with human clinical data showing that neither susceptible nor resistant parasites clearances were achieved by higher artemisinin derivative concentrations [31].

The data obtained with *A. afra* are contrasting and do not support a potential role for artemisinin resistance

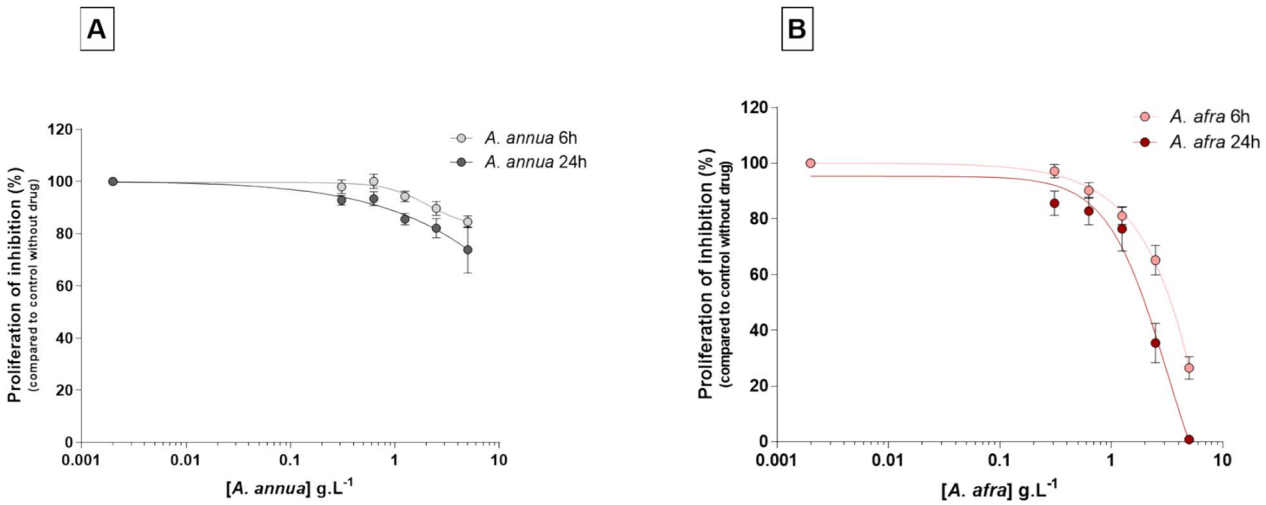


Fig. 4 Cytotoxicity studies of *Artemisia annua* and *Artemisia afra* tea extracts on primary human hepatocytes. Percentage of proliferation inhibition (PI) compared to non-treated condition after 72 h of culture with 6 or 24 h of drug exposure on hepatocytes 7 days after seeding. N = 4 independent biological replicates for *A. annua* and N = 5 independent biological replicates for *A. afra*. For each biological replicate, 3 to 6 technical replicates were made. Means of proliferation inhibition percentage of hepatocytes with SEM are represented on the graphs. $PI_{50\,s}$ for *A. afra* extracts were 3.263 $g.L^{-1}$ and 2.124 $g.L^{-1}$ for 6 and 24 h exposure, respectively. $PI_{50\,s}$ for *A. annua* were undetermined ($PI_{50} > 5$ $g.L^{-1}$), but were estimated by Graphpad prism7 to 31.010 $g.L^{-1}$ and 26.900 g L^{-1} for 6 and 24 h exposure, respectively

Table 2 Selectivity index (SI) for 6 h *Artemisia annua* or *Artemisia afra* exposure of *Plasmodium falciparum* clinical isolates

A. afra				
	PI_{50} hepatocytes	**RSA_{50} WT**	**RSA_{50} C580Y**	**RSA_{50} R539T**
$g.L^{-1}$	3.263	0.705	0.758	0.537
Selectivity Index		4.628	0.930	1.412
A. annua				
	PI_{50} hepatocytes	RSA_{50} WT	RSA_{50} C580Y	RSA_{50} R539T
$g.L^{-1}$	31.010	0.080	0.137	2.563
Selectivity index		387.625	226.350	12.099

The SI was determined using the formula SI = PI_{50}_hepatocytes_6 h/RSA_{50}_parasites_6 h

selection. *A. afra* extracts presented a notable in vitro activity regardless of the K13 genotypes tested and showed no cross resistance with dihydroartemisinin. These findings are in accordance with the very low artemisinin content in *A. afra* and rather suggest the presence of potent, non-endoperoxide anti-malarial pharmacophores absent in *A. annua*. However, these results only reflect an activity based on an aqueous extraction and might not represent the whole extent of the anti-malarial activity. These likely differences were evidenced by Kane and colleagues showing the level of phytochemical compounds of *A. afra* is modulated by the origin of the plant but also to the extraction solvent chosen and subsequent the intrinsic anti-malarial activity as well [10]. Several studies have assumed a possible anti-malarial activity of *Artemisia* sp. related to flavonoids [15], but the nature of the anti-malarial activity of *A. afra* aqueous extracts remains an open question. Moreover, the in vitro anti-malarial activity showed here occurred at high doses unlikely to be reached out of reported tea consumption [9, 11, 32].

In addition, the absence of published data on the therapeutic efficacy of *A. afra* does not enable discussing this point further. This important in vitro anti-malarial activity of *A. afra* could be explained by pharmacophore(s), but that lack specificity. HepG2 cells, considered as the gold-standard in vitro model for hepatotoxicity, were used to verify the toxicity level of *Artemisia* extracts. A cytotoxic effect of both *A. annua* and *A. afra* extracts was observed. However, it is relevant to note that at least the toxicity of *A. annua* was expected since HepG2 cells have already been described as artemisinin sensitive [33]. Therefore, a second model was chosen to assess the selectivity of *Artemisia* extracts. Thus, cytotoxicity data on primary human hepatocytes showed a much more marked cytotoxicity of *A. afra* aqueous extracts than those prepared with *A. annua,* regardless of the duration of treatment. Discrepancies with *A. annua* on primary human hepatocytes might be due to the anti-tumoral activity of artemisinin as previously observed [34, 35].

The cytotoxicity of these *A. annua* and *A. afra* extracts was previously tested on primary human and monkey hepatocytes [25]. In this report, these extracts presented low toxicity, contrasting with the results in this study. These differences might be explained by inter-donor variability within both studies [36]. Either way, these data highlight a toxicity concern, that might be different when observed in vivo after *per os* consumption of *Artemisia* than was observed in vitro on direct hepatocytes exposure. However, toxicity has already been noted in some case reports describing adverse events related to *Artemisia* tea consumption [37, 38]. This toxicity could be due to the same compounds found in other *Asteraceae* such as *Artemisia absinthium* or *Artemisia dracunculus* that are notoriously hepatotoxic at high doses [39]. At this stage it is still unclear if the molecule(s) responsible for *A. afra* anti-malarial activity are the same that exert cytotoxicity, but the narrow in vitro selectivity indices (4.048 to 6.423) question the safety of its use. These observations advocate for conducting proper pharmacovigilance studies within population using *A. afra*.

Conclusion

In summary, this study raised several concerns regarding *Artemisia* phytotherapies. The use of *A. annua* as anti-malarial surrogate could trigger the selection and facilitate the emergence of artemisinin resistant parasites. Further, based on these in vitro results, *A. afra* exhibits toxicity issues, despite a possible content in anti-malarial pharmacophores that are unlikely to trigger selection of artemisinin resistance. All these aspects associated with the absence of factual efficacy data emphasize the need for additional investigations, but also lead to renewed reservation about anti-malarial phytotherapies.

Abbreviations

RSA	Ring-stage survival assay
HepG2	Hepatoblastoma cell line
PI_{50}	50% of proliferation inhibition

k13	Kelch 13 gene
ACT	Artemisinin-based combination therapy
RPMI	Roswell Park Memorial Institute
DMEM	Dulbecco's modified eagle medium
WT	Wildtype
MIC	Minimum inhibitory concentration
SI	Selectivity Index
PFA	Paraformadehyde
RSA_{50}	50% of ring-stage survival assay
DHA	Dihydroartemisinin

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Author contributions
CR and BW conceptualized, designed the study and wrote the first draft of the manuscript. CR, KA, NK, SK, AAM and AV were involved in data acquisition. CR, HTMV, KA, JFF and DM participated in the data analysis and interpretation. CR, BW and SPM contributed to statistical analysis. AV, JFF, SPM and DM participated in manuscript edition and rewieving. All authors reviewed and approved the final manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The research was conducted in accordance with the Declaration of Helsinki and national and institutional standards. Samples have been collected in the frame of therapeutic efficacy studies upon protocol acceptance from Cambodia National Ethics Committee, under references NECHR #0273, #0188, #099, #087, #0102, #082, #0120 and #0290.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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