In vitro susceptibility of *Plasmodium falciparum* Welch field isolates to infusions prepared from *Artemisia annua* L. cultivated in the Brazilian Amazon

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Artemisinin is the active antimalarial compound obtained from the leaves of Artemisia annua *L. Artemisinin, and its semi-synthetic derivatives, are the main drugs used to treat multi-drug-resistant* Plasmodium falciparum *(one of the human malaria parasite species). The in vitro susceptibility of* P. falciparum *K1 and 3d7 strains and field isolates from the state of Amazonas, Brazil, to* A. annua *infusions (5 g dry leaves in 1 L of boiling water) and the drug standards chloroquine, quinine and artemisinin were evaluated. The* A. annua *used was cultivated in three Amazon ecosystems (*várzea, terra preta de índio *and* terra firme*) and in the city of Paulínia, state of São Paulo, Brazil. Artemisinin levels in the* A. annua *leaves used were 0.90-1.13% (m/m). The concentration of artemisinin in the infusions was 40-46 mg/L. Field* P. falciparum *isolates were resistant to chloroquine and sensitive to quinine and artemisinin. The average 50% inhibition concentration values for* A. annua *infusions against field isolates were 0.11-0.14 µL/mL (these infusions exhibited artemisinin concentrations of 4.7-5.6 ng/mL) and were active in vitro against* P. falciparum *due to their artemisinin concentration. No synergistic effect was observed for artemisinin in the infusions.*

Key words: *Artemisia annua* L. - infusion - antiplasmodial - in vitro - field isolate - Amazon Region

Malaria is an infectious disease that affects millions of people in tropical and sub-tropical regions of the world. According to the estimates of the World Health Organization (WHO), 40% of the world population, that is, 3.3 billion people, live at high risk of contracting this disease in the 106 countries where malaria is considered to be endemic. In 2009, 225 million cases of malaria were registered around the world and caused approximately one million deaths, of which 90% were among African children less than five years of age (WHO 2010). In the Brazilian Amazon, this disease is still a grave problem for public health authorities due to its high incidence and the debilitating effects on people suffering from this disease. This disease is believed to negatively influence the potential economic development of this region (Oliveira-Ferreira et al. 2010).

Resistance of the human malaria parasite *Plasmodium falciparum* Welch to antimalarial drugs has emerged as one of the greatest challenges to the control of malaria. Drug resistance has led to increased mortality in hyper and holoendemic areas and contributed to the appearance and spread of new focal areas of malaria caused by *P. falciparum*. Furthermore, resistance has been identified as a factor that economically compromises malaria control efforts (Trape et al. 2002). Great effort and time have been dedicated to the in vitro reproduction of the erythrocytic stages of the *Plasmodium* life cycle as a fundamental tool for studying the mechanisms of susceptibility and resistance to drugs as well as screening for the identification of new drugs (Girard et al. 2007).

Several natural products have been revealed as promising sources of antimalarial compounds. Traditional knowledge of the usefulness of medicinal plants has led to the isolation and identification of the antimalarial compounds quinine and artemisinin, which are currently in use and which are isolated from species of *Cinchona* L. (Rubiaceae) and the leaves of *Artemisia annua* L. (Asteraceae), respectively (Vale et al. 2005).

A. annua is a plant native to North China, mainly in the provinces of Chahar and Suiyuan, where it is known by the common name *qinghao* (blue-green hao) (Hsu 2006). This herb has been used in traditional Chinese medicine for more than 2,000 years as an infusion for the treatment of fever and malaria. The modern Chinese Pharmacopeia describes the dry leaves of this plant as a medicine for fever, including malaria (Mueller et al. 2000). Chinese researchers have studied more than 30 species belonging to the genus *Artemisia* in the search for potential antimalarial activity; however, only *A. annua* and *Artemisia apiacea* Hance have proven to be

effective against *P. falciparum* and *Plasmodium vivax* Grassi & Feletti (Rodrigues et al. 2006). Among the secondary metabolites present in *A. annua*, artemisinin is considered to be the principle antimalarial component. Artemisinin levels vary considerably depending upon the part of the plant, cultivation conditions and seasonal and geographical variations. High levels of artemisinin are found in the leaves and flowering parts, especially in the period immediately before flowering (0.01-1.40%) (Baraldi et al. 2008). Most of this variation is attributed to genetic factors; however, even high-artemisinin-yielding varieties may have their artemisinin production reduced due to the influence of environmental conditions. Altitude, latitude, soil nutrients and pH, the length of day (photoperiod), flowering time, drying time and storage conditions determine the artemisinin content in the plant (Wilcox et al. 2004, Ferreira et al. 2005).

The global demand for artemisinin and its derivatives increased from 11.2 million treatments in 2005 to approximately 158 million in 2009. The cost-benefit relationship represents the greatest problem for the viability of the synthesis of artemisinin. The isolation of artemisinin from the leaves of *A. annua* is the best method for obtaining artemisinin, from which semi-synthetic derivatives such as artesunate, artemether and arteether are prepared (WHO 2010). Given the possible decrease in the supply of artemisinin caused by this increase in world demand, the cultivation of *A. annua*, especially in endemic areas, may be an important alternative for the production of artemisinin-based drugs (Mueller et al. 2000). This plant was introduced in Brazil in the 1980's and has been the subject of constant study and attempts at genetic improvement through traditional methods. These studies resulted in the selection of high-artemisinin-yielding genotypes for cultivation in intertropical regions (Magalhães et al. 1997). Although trials with some of these genotypes have already been conducted in the Brazilian Amazon Region, it is necessary to perform multidisciplinary research on the genetic selection and improvement of *A. annua* for cultivation in distinct Amazon ecosystems.

Infusion is the pharmaceutical form indicated in the Chinese Pharmacopeia for *A. annua* and is the most commonly used form today. The recommended dose varies from 4-9 g of dried leaves for each litre of boiling water (Ridder et al. 2008). In the most important clinical studies reported in the literature involving infusions of *A. annua*, 5-9 g of dry plant per litre of boiling water per day for five-seven days has been the usual course of treatment. In clinical studies, the therapeutic efficiency of *A. annua* infusions was up to 70%, but high recrudescence rates were reported, which led the authors of these clinical studies to not recommend the use of *A. annua* infusions as a monotherapy (Mueller et al. 2000, 2004, Räth et al. 2004).

At present, *A. annua* is being cultivated in Amazon ecosystems through the *Artemisia* Project, which is being financed by a grant from the Brazilian Government's National Malaria Network. The evaluation of the quality of these hybrids has been performed by monitoring biomass during the plant's life cycle, as well as the evaluation of artemisinin levels in the leaves. In the present study, the susceptibility of field isolates of *P. falciparum*

from the Brazilian Amazon to *A. annua* infusions was evaluated. The *A. annua* used in this study was cultivated in the *várzea*, *terra firme* and *terra preta de índio* ecosystems. An attempt was made to understand the interaction between the parasites and the infusions and ultimately to establish the antiplasmodial activity of the infusions against *P. falciparum* in vitro as a parameter in the evaluation of the potency of cultivated *A. annua* and the infusions made from the cultivated plant.

SUBJECTS, MATERIALS AND METHODS

Plant material - The *A. annua* L. used in this study was grown from seed in greenhouses and then cultivated in *várzea*, *terra firme* and *terra preta de índio* Amazonian ecosystems in the experimental areas of the Brazilian Agriculture and Animal Husbandry Research Company (Embrapa) in Manaus, state of Amazonas, Brazil. Plant collection was performed in June 2008 from these three Amazonian ecosystems. A hybrid of *A. annua* from the Multidisciplinary Centre for Chemical, Biological and Agricultural Research (CPQBA) of the State University of Campinas (UNICAMP), Paulínia, state of São Paulo, Brazil, was also used for reference and was collected in March 2008. Reference plant materials (vouchers) are available at CPQBA and Embrapa and live plant specimens are maintained in continuous culture on a yearly basis at these institutions. For this study, the aerial parts of the plants were harvested immediately before flowering commenced and dried in an oven at 40ºC.

Preparation of leaf infusions - Infusions of *A. annua* leaves from plants grown in *terra firme*, *várzea* and *terra preta de índio* areas at Embrapa in Manaus and at CPQBA in Paulínia were prepared by combining 5.0 g of dry, ground leaves and 1.0 L boiling distilled water. The mixture was covered and left for 15 min and then filtered hot.

Preparation of leaf extracts for quantification of artemisinin - Toluene (10.0 mL) was added to dried leaves (200 mg) and this mixture was subjected to extraction in an Ultra-Turrax homogeniser (IKA, Guankzhou, China) for 30 s. The resulting suspension was centrifuged for 6 min at 3,200 rpm (1,720 *g*). The supernatant solution was used in the analyses as described below.

Determination of artemisinin in toluene solutions and infusions of A. annua leaves - Artemisinin levels were determined in dry *A. annua* leaf samples and infusions using thin-layer chromatography (TLC)-photodensitometry as previously described (Marchese et al. 2001) with slight modifications. After the centrifugation step described above, the supernatant toluene solution (3.0 μL) was applied in duplicate to a commercial aluminium-backed silica gel TLC plate (Merck). On the same plate, four different volumes (1.5-3.0 μL) of a standard solution of artemisinin (0.25 mg/mL) in methanol were applied to the TLC plate as calibration points. The TLC plate was eluted with a 98.5:1.5 mixture of chloroform and methanol. The average elution times were 4 ± 1 min. The eluted TLC plate was sprayed with *p*-anisaldehyde solution [0.5 mL *p*-methoxybenzaldehyde, 10 mL glacial acetic acid, 85 mL ethanol and 5 mL concentrated sulphuric acid (95-97%)] and heated for 6 min at 100ºC.

Next, the TLC plate was scanned using a photodensitometer (GE Healthcare Image Scanner®). The optical densities of the artemisinin spots in the toluene solutions were compared to the optical densities of the standard samples of artemisinin (external calibration) using GE Image Quant TL® software (GE Healthcare, Uppsala, Sweden). To quantify artemisinin in the infusions, 100 mL of each infusion was totally evaporated under vacuum (*ca.* 25 mmHg) using a rotary evaporator and a bath temperature of 38ºC. The resulting dry extract was suspended in 10.0 mL of methanol, which was measured using a volumetric pipette. A total of 3.0 μL of this suspension was applied to a TLC plate using a gas-tight microsyringe and evaluated as described above for toluene solutions.

Continuous culture of P. falciparum - The strains used in this study were the antimalarial drug-susceptible 3D7 clone of the NF54 isolate (unknown origin) and the chloroquine-resistant, pyrimethamine-resistant and cycloguanil-resistant K1 strain (Thailand). Strains were acquired from MR4 (Malaria Research and Reference Reagent Resource Center, Manassas, VA, USA). Additionally, nine *P. falciparum* field isolates (representative genotypes from the Brazilian Amazon Region) were obtained from symptomatic malaria patients who presented themselves for diagnosis and treatment at the Heitor Vieira Dourado Tropical Medicine Foundation (FMT-HVD), Manaus. Whole blood samples were processed as described elsewhere (Vieira et al. 2004) and infected erythrocytes were directly added to complete RPMI-medium and put in plastic flasks for in vitro culture (without cryopreservation) under a low-oxygen atmosphere. Patients presenting clinical symptoms related to severe malaria were invited to be enrolled in this study after confirmation of *P. falciparum* mono infection by thick smear diagnosis and their signing of an informed consent form (FMT-HVD Ethics in Research Commission 1838). Parasites were maintained in continuous culture using the method of Träger and Jensen (1976) with modifications, as described in Andrade-Neto et al. (2007), using type A⁺ human erythrocytes in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% A⁺ human plasma (donated by blood banks). Cultures were maintained under an environment of 5% O_2, 5% CO_2 and 90% N_2 and incubated at 37ºC. When cultures attained a parasitaemia level of 4-5%, they were synchronised with 5% sorbitol (Lambros & Vanderberg 1979).

In vitro susceptibility test - Infusions of *A. annua*, as prepared above, were diluted by a factor of 1:10 and then serially diluted in RPMI-1640 culture medium by a factor of 1:5 to produce seven diluted samples with overall dilutions that ranged from 1:10-1:1.5 × 10⁵. These dilute samples were tested in vitro against field isolates and standard strains of *P. falciparum*. The commercial drug standards chloroquine diphosphate salt (Sigma-Aldrich, Steinheim, Westphalia, Germany), quinine sulphate salt (Sigma-Aldrich, Steinheim, Westphalia, Germany) and artemisinin (Sigma-Aldrich, Steinheim, Westphalia, Germany) were also tested in the concentration ranges of 2.5 × 10³-3.4, 5.0 × 10²-0.70 and 1.0 × 10²-6.4 × 10⁻³ ng/mL, respectively. The microtest was performed according to the method of Rieckmann et al. (1978) with modifications as described in Andrade-Neto et al. (2007). Briefly, the wells of a 96-well test plate were loaded with 180 μL of a parasitised red blood cell suspension in culture medium containing predominantly young trophozoites (ring forms), a 3% haematocrit and 1.5% initial parasitaemia. Then, 20 μL of a commercial drug solution or a dilute infusion was added to wells for a final volume of 200 μL per well. Each test was performed in triplicate. Wells containing only parasitised red blood cells in complete culture medium (RPMI-1640 plus 10% human serum) were used as negative controls of parasite growth. Each test plate was incubated for 48 h at 37ºC and at low O_2 tension in an acrylic, gas-tight chamber in an incubator. After incubation, thin blood smears were prepared from the contents of each well. Blood smears were dyed with Panótico® (Laborclin, Pinhais, Paraná, Brazil) and examined under an optical microscope. The number of parasites present in a total of 2,000 red blood cells was counted. Parasitaemia levels were expressed as a percentage.

Data analysis - The parasitaemia levels of the test wells were compared with those of the control wells to evaluate parasite growth in the presence of diluted *A. annua* infusion or drug standards. The inhibition of parasite growth was evaluated as the difference in the average level of parasitaemia of triplicate negative control wells and the average parasitaemia level of the triplicate wells of a given test sample and was expressed as a percentage (I%) by dividing this difference by the average negative control parasitaemia level [I% = (avg parasitaemia of control - avg parasitaemia of sample) ÷ avg parasitaemia of control]. The median inhibition concentration (IC_{50}) and the standard deviation with a 95% confidence interval were calculated based on linear regression analysis using Microcal Origin® software (OriginLab, Northampton, Massachusetts, USA).

RESULTS

The dried leaves of *A. annua* hybrids cultivated in the *várzea*, *terra preta de índio* and *terra firme* Amazonian ecosystems exhibited high levels of the antimalarial natural product artemisinin. The results from our TLC-photodensitometric analysis indicate that artemisinin levels in dry leaves ranged from 0.90 ± 0.10%-1.10 ± 0.07% (Table I). The dry leaves of the standardised plant cultivated in Paulínia exhibited artemisinin levels of 1.13 ± 0.05%. The seeds were obtained from these standardised plants, which in turn gave rise to the plants cultivated in the Amazon.

The concentration of artemisinin in infusions of leaves of cultivated *A. annua* ranged from 40-46 mg/L (Table I). Thus, we did not observe a large variation in the concentration of artemisinin in the infusions. Standardised *A. annua* cultivated in Paulínia exhibited higher levels of artemisinin in its leaves and in its leaf infusions. The efficiency of artemisinin extraction by the infusion of leaves in boiling water ranged from 78-91% for plants cultivated in *terra preta de índio* and *terra firme* plots. The efficiency of extraction described here refers to the percentage of the total artemisinin present in the leaf sample that was present in the final infusions.

The drug sensitivity profiles of the nine *P. falciparum* field isolates were evaluated against the commercial drugs chloroquine, quinine and artemisinin through the determination of their IC_{50} values (Table II). For chloroquine, the average IC_{50} value for the nine field isolates was 268 ± 129 nM. The lowest of the IC_{50} values for these parasites was 166 nM. Thus, all field isolates were considered resistant to chloroquine (WHO 2007). For quinine, the average IC_{50} value for the nine field isolates was 174 ± 40 nM (the absolute range of IC_{50} values was 110-259 nM), as shown in Table II. All *P. falciparum* field isolates were considered sensitive to quinine because their IC_{50} values were lower than the in vitro threshold values ($IC_{50} = 500$ nM) for quinine resistance established by the WHO (2007). Artemisinin was highly active against Amazonian field isolates of *P. falciparum*. The IC_{50} values obtained for artemisinin against Amazonian field isolates exhibited low variation (average $IC_{50} = 2.1 \pm 1.0$ nM). Given the number of samples studied, the levels of sensitivity of Amazonian *P. falciparum* field isolates to artemisinin are in agreement with results for other field isolates reported in the literature (Jambou et al. 2005, Ferreira et al. 2007, 2008, Gama et al. 2011). In Fig. 1, concentration-response curves are presented for the largest and smallest inhibition concentrations obtained for field isolates against chloroquine, quinine and artemisinin. These curves are generally representative of the experimental observations made on the rest of the field isolates.

The susceptibilities of 11 samples of *P. falciparum* (2 standard and 9 field isolated samples) to infusions of *A. annua* leaves from three Amazonian ecosystems and Paulínia were evaluated herein. Immediately after preparation, each infusion was diluted and applied directly to the microtest plate without any prior treatment. The IC_{50} values were calculated in experimentally relevant units of microlitres of infusion per millilitre ($\mu L/mL$) of total solution in each microplate well. The average IC_{50} values of the *A. annua* leaf infusions against samples of *P. falciparum* were 0.11 ± 0.03, 0.11 ± 0.02, 0.13 ± 0.1 and 0.14 ± 0.04 $\mu L/mL$ for plants cultivated in Paulínia, *terra preta de índio*, *terra firme* and *várzea*, respectively. If the data from the TLC-photodensitometric analysis of the infusions are taken into account, these diluted infusions that produced the IC_{50} values contain 5.1, 4.7, 5.3 and 5.6 ng/mL of artemisinin, respectively. There was no significant difference in the IC_{50} values of plant infusions from different regions/ecosystems against individual field isolates of *P. falciparum*. Similarly, there was no significant difference in the susceptibility profiles among the field isolates to individual infusions. In Fig. 2, the concentration-response curves are presented for a field isolate against different infusions of *A. annua*. In general, these curves are representative of the behaviour profile of field isolates of *P. falciparum* in the presence of *A. annua* infusions. The biological responses of this field isolate to the leaf infusions of plants cultivated in *terra firme* and *várzea* areas were slightly delayed, which resulted in higher IC_{50} values.

DISCUSSION

The great importance attributed to *A. annua* after the discovery of the highly active antimalarial compound in the leaves of this plant led to the intensive collection and decimation of the plant in certain regions of the world.

TABLE I
Data on artemisinin in leaves and infusions

Plant origin	Artemisinin in dry leaves (m/m)	Concentration of artemisinin in infusions (mg/L)	Efficiency of extraction[a]
Paulínia	1.13 ± 0.05	46 ± 2	81
Terra preta	1.10 ± 0.07	43 ± 1	78
Terra firme	0.90 ± 0.10	41 ± 2	91
Várzea	0.90 ± 0.10	40 ± 1	90

a: percentage of artemisinin extracted from leaves into tea preparation. Artemisinin levels in dry leaves and infusions prepared from *Artemisia annua* cultivated in three Amazonian ecosystems (Manaus, state of Amazonas) and Paulínia (state of São Paulo) and artemisinin extraction efficiency of infusion procedure.

TABLE II
Antiplasmodial activity of drugs

	Median inhibition concentrations (IC_{50}) (nM)			
	Amazon field isolates		Standard strains	
Drug	Mean (n = 9)	Lowest/highest	K1	3D7
Chloroquine diphosphate	268	166/460	136 ± 10	60 ± 6
Quinine sulphate	174	110/259	160 ± 8	110 ± 10
Artemisinin	2.1	0.9/3.5	2.9 ± 1	1.7 ± 1

IC_{50} values for commercial drugs chloroquine, quinine and artemisinin against standard strains and Amazonian field isolates of *Plasmodium falciparum*.

As a response to the high demand for this plant, *A. annua* has been planted on a large scale in China, Vietnam, Turkey, Iran, Afghanistan and Australia, as well as some countries in Europe and the Americas (Bhakuni et al. 2001). Faced with a possible shortage in the supply of artemisinin caused by the increase in demand, the cultivation of *A. annua* in endemic areas should be considered a viable alternative that will guarantee an adequate supply of plant materials and production of the drug artemisinin (Ridder et al. 2008). The present study demonstrated that *A. annua* cultivated in three types of representative soils of the Amazon Region possesses a high level of artemisinin. This finding, together with agronomic and biomass data, support the notion that *A. annua* can be cultivated

in this rainy, humid equatorial region. The *A. annua* hybrid cultivated in Paulínia (UNICAMP/CPQBA) is considered stable because it has been cultivated for many generations and has had its ideal growth conditions established for some time. Previous studies have reported that leaves of this hybrid have artemisinin levels in the range 0.8-1.2% (Magalhães et al. 1997, Marchese et al. 2001). No large variation in the levels of artemisinin among the hybrids in the three Amazonian ecosystems was observed despite the rather large differences in the nutritional composition of soils of the *várzea*, *terra preta de índio* and *terra firme* areas (Sanchez et al. 1982). The levels of artemisinin found in these hybrids cultivated in the Amazon are similar to those found in the standardised and stabilised plants from Paulínia from which the Amazon hybrids are derived. The plants used in this study are the result of the first generation of *A. annua* cultivated in these Amazonian soils and these plants are still going through a process of adaptation to the climatic and geologic conditions of the Amazon Region.

The method used to determine the levels of artemisinin in the dry leaves and infusions prepared from these leaves is semi-quantitative and is based on TLC-photodensitometry, which has a high correlation with the method of choice, namely, high-performance liquid chromatography (Marchese et al. 2001). TLC-photodensitometry is rapid because 3-4 samples and calibration samples are simultaneously eluted and quantified by the software, which has as its input the high-resolution scan of the eluted TLC plate. Furthermore, TLC-photodensitometry is very low cost and is highly adaptable to field conditions, where it is useful in the processing of a large number samples during the process of plant selection for genetic improvements. This method was also used to measure artemisinin levels in previous studies that evaluated the biological activity of *A. annua* infusions (Atemnkeng et al. 2009).
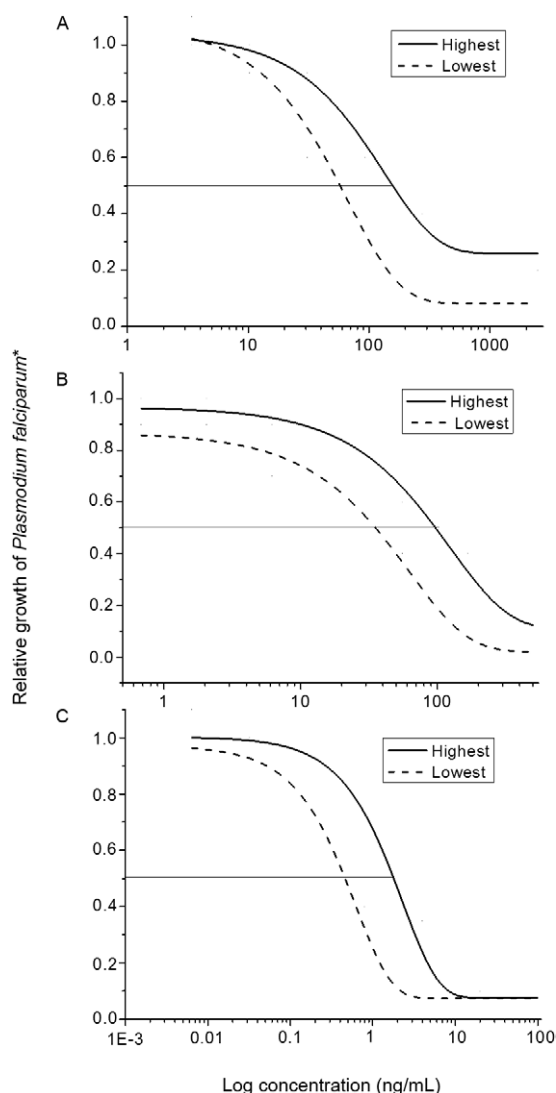

Fig. 1: concentration-response curves for field isolates of *P. falciparum* in the presence of various concentrations of commercial drug standards (A: chloroquine; B: quinine; C: artemisinin) (lowest and highest IC_{50} values). Asterisk means relative growth of *P. falciparum* = avg % parasitaemia of test (sample) wells/avg % parasitaemia of untreated control wells (95% confidence interval). Statistical analysis among additional assays: standard error = 0.0007; p = 0.10.
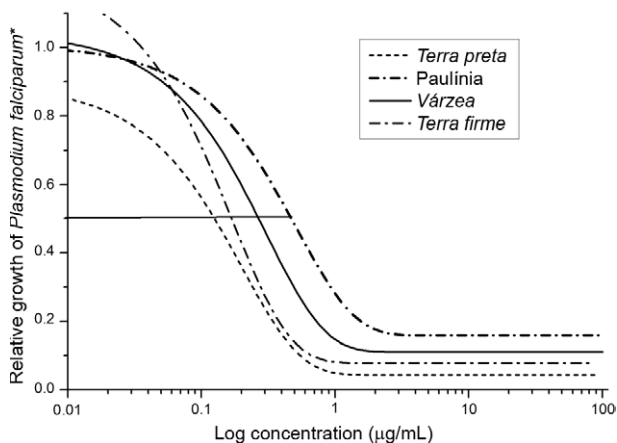

Fig. 2: concentration-response curves for a field isolate of *P. falciparum* in the presence of various concentrations of infusions of *Artemisia annua* from different ecosystems/regions. Asterisk means relative growth of *P. falciparum* = avg % parasitaemia of test (sample) wells/avg % parasitaemia of untreated control wells (95% confidence interval). Statistical analysis among additional assays: standard error = 0.0006; p = 0.10.

Pure artemisinin has poor solubility in water and, as was shown above, infusions of the dry leaves of *A. annua* prepared as recommended in the Chinese Pharmacopeia contain a substantial quantity of artemisinin (Table I). These findings are similar to those found by Räth et al. (2004), who performed a pharmacokinetic study utilising an infusion of *A. annua* to determine the plasma concentration of artemisinin after the oral administration of the infusion. In the study by Räth et al. (2004), the leaf infusion was prepared in a way that was analogous to that used in the present study starting from dry leaves with 1.39% artemisinin and yielding infusions with 57.5 mg artemisinin/L. Atemnkeng et al. (2009) used this procedure to prepare infusions containing 34 mg artemisinin/L from dry leaves that contained 1.12% artemisinin. Despite the low solubility of artemisinin in water, the large quantities of the drug in infusions is believed to be due to the presence of other extracted constituents of the plant that have amphiphilic properties (*e.g.*, flavonoids and/or saponins), which increase the solubility of artemisinin in water (Ridder et al. 2008).

During the adaptation of medicinal plants to new climatic and geologic conditions, it is important to accompany the process with techniques that will guarantee the quality of the plant material obtained, such as the monitoring of the levels of active compounds which is a common practice. For the quality control of cultivated *A. annua*, the determination of artemisinin levels in the leaves is crucial (Celeghini et al. 2006). In the present study, the quality of the *A. annua* hybrids cultivated in the Brazilian Amazon was monitored to establish artemisinin levels in the leaves through extraction with the low-polarity solvent toluene. The leaves of the hybrids cultivated in the Amazonian ecosystems contained acceptable levels of artemisinin: however, the standardised *A. annua* hybrids from CPQBA (Paulínia) had the highest levels of artemisinin. The quality of the infusions prepared from the leaves of the Amazonian hybrids was also monitored and compared with the standardised plant from Paulínia through the levels of artemisinin in the infusions and by testing these infusions for antimalarial activity against *P. falciparum* in vitro. There are reports in the literature on the in vivo antimalarial activity, pharmacology, efficacy and safety of *A. annua* infusions in human clinical trials (Mueller et al. 2000, 2004, Räth et al. 2004) and in vivo in animals using rodent malaria parasite-based models (Atemnkeng et al. 2009). However, we could find no previous report on the in vitro antiplasmodial activity of *A. annua* infusions. In a recent study, the cytotoxicity of *A. annua* infusions was assessed by determining cellular viability using the (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide assay. In that study, the viability of cells in the presence of different concentrations of *A. annua* infusion was above 72% even when the highest concentrations were tested (Oliveira et al. 2009). These data are consistent with the generally low cytotoxicity of *A. annua* infusions.

The stabilisation of geographically specific populations of *P. falciparum* in continuous culture permits the evaluation of the actual susceptibility profiles to drugs and to natural products of the parasites that are in circulation in the human (and insect) populations in a given region (Andrade-Neto et al. 2007). In the present study, it was possible to stabilise, in continuous culture, nine (16%) of 55 field isolates of *P. falciparum*. The increase in the therapeutic failure rates of many antimalarial drugs in most malarious regions requires epidemiologic monitoring and monitoring of the dynamics of drug resistance. Information on the epidemiology of resistance can be applied to the monitoring and control of the appearance and propagation of chemoresistance in populations of natural parasites. This monitoring can be performed with drugs in use in a particular location or with new drug alternatives, especially when the parasite exhibits cross-resistance to drugs (Ferreira et al. 2007, Chiyaka et al. 2009). The stabilised field isolates developed and examined in the present study exhibited similar sensitivity profiles to chloroquine, quinine and artemisinin. The resistance of Amazonian *P. falciparum* to chloroquine in vitro was expected given that this parasite is generally known to be resistant to this drug throughout the Amazon and many other parts of the world. In general, chloroquine is no longer recommended for the treatment of *P. falciparum* infections due to the high levels of resistance observed in the clinical setting (WHO 2005). Although there are reports on the in vivo and in vitro resistance to quinine in the Amazon Region (Alecrim 1981), in the present work resistance to quinine was not detected. However, two samples revealed high IC_{50} values (224 and 259 nM) that were lower than the in vitro threshold value for quinine resistance (500 nM) established by the WHO (2007). The levels of field isolate parasite inhibition ($IC_{50} = 2.1 \pm 1.0$ nM) by artemisinin are generally in agreement with data from other parts of South America and Brazil (Jambou et al. 2005, Ferreira et al. 2007, 2008). The drug susceptibility of field isolates was evaluated as a means to characterise the resistance profiles of the samples of Amazonian parasites utilised to evaluate their susceptibility to infusions.

The samples of *P. falciparum* evaluated in this study exhibited a high degree of susceptibility to infusions prepared from the dry leaves of *A. annua* cultivated in all ecosystems. Even highly dilute infusions (1:6.2 × 10^3; effective concentrations of artemisinin of *ca.* 6.4 ng/mL) inhibited up to 100% of parasite growth in almost all of the field isolates (data not shown). The IC_{50} values of the infusions were as low as 0.11 ± 0.02 μL/mL. Based on our TLC-photodensitometry studies, this value corresponds to an effective artemisinin concentration in the experimental test wells of 4.7 ng/mL. These IC_{50} values were observed for infusions prepared from the leaves of *A. annua* cultivated in the *terra preta* ecosystem. IC_{50} values obtained in the in vitro tests involving infusions were comparable (Table III). Similar levels of artemisinin in the *A. annua* plants cultivated in the different soils of the Amazon and Paulínia (Table I) were found. Also, similar concentrations of artemisinin were detected in the infusions prepared from the leaves

of *A. annua*. In addition, pure artemisinin was generally quite active in vitro against all parasites used in this study, as evidenced by the IC$_{50}$ values (Table II). It is important to develop high-artemisinin-yielding genotypes of *A. annua,* especially in endemic areas, where such plants may be a means by which to guarantee a constant supply of plant material for artemisinin production. However, according to previous in vitro studies on the active components of *A. annua* against *P. falciparum*, artemisinin is not the only active substance present in the plant. It has been demonstrated previously that flavonoids present in *A. annua* are also involved in the in vitro antiplasmodial effect of artemisinin. These flavonoids have little antiplasmodial activity themselves, but are capable of acting in synergy with artemisinin (Willcox et al. 2004, Bilia et al. 2006). This result indicates that the evaluation of the in vitro antiplasmodial activity of infusions prepared from plants whose adaptation is underway can be an important tool to monitor and guarantee the activity and stability of *A. annua*. It is noteworthy that in the data generated herein, there was no evidence for in vitro synergistic effects.

As stated above, the plants in the present study are first-generation *A. annua* cultivated in the Amazon. Despite the relatively high yield of artemisinin detected in leaves and the high antiplasmodial activity detected in the in vitro assay (as evidenced by the low IC$_{50}$ values of all infusions against all *P. falciparum* strains tested), these plants require agronomic improvement through selection to increase biomass and genetic homogeneity for growth in the Amazonian ecosystems. In vitro testing for antiplasmodial activity is an important tool for quality control because the inhibition of *P. falciparum* by *A. annua* infusions is related, in general, to high levels of artemisinin and other antimalarial components to which these parasites are sensitive.

TABLE III

Antiplasmodial activity of *Artemisia annua* infusions

Origin of *A. annua*	Median inhibition concentrations (IC$_{50}$) (µL/mL)			
	Field isolates		Standard strains	
	Mean[a] (n = 9)	Lowest/highest	K1	3D7
Paulínia	0.11 (5.1)	0.08/0.18	0.09	0.10
Terra preta	0.11 (4.7)	0.08/0.15	0.10	0.09
Terra firme	0.13 (5.3)	0.07/0.36	0.08	0.11
Várzea	0.14 (5.6)	0.09/0.20	0.10	0.13

a: in parentheses are the IC$_{50}$ values if one assumes only artemisinin as the active component, based on TLC-photodensitometry results (Table I). IC$_{50}$ values of infusions prepared from the leaves of *A. annua* from three Amazonian ecosystems (Manaus, state of Amazonas) and Paulínia (state of São Paulo) against standard strains and isolated field samples of *Plasmodium falciparum* from the Brazilian Amazon.

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