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Tea *Artemisia annua* inhibits *Plasmodium falciparum* isolates collected in Pikine, Senegal

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Malaria is a major scourge of most countries in Africa which continues to defy science and technology. Several medicinal plants are traditionally used for the treatment of malaria and other protozoa infections. We aimed to assess by the Double-site Enzyme-linked Lactate dehydrogenase enzyme Immuno-detection (DELI) test for the first time in Senegal, *Plasmodium falciparum* isolates *in vitro* susceptibility to Tea *Artemisia annua* (TAA). In total, 40 field isolates have been tested and the mean IC_{50} was 0.095 µg/ml, while the IC_{50} for the 3D7 and W2 laboratory adapted strains were 0.14 and 0.39 µg/ml, respectively. Tea *A. annua* sensitivity was not obtained for three isolates because of lack of growth. The results suggest that tea *A. annua* has potent antiplasmodial activity against *P. falciparum* strains collected in Pikine, Senegal.

Key words: *Artemisia annua*, enzyme-linked lactate dehydrogenase enzyme immuno-detection (DELI), *Plasmodium falciparum*, IC_{50}.

INTRODUCTION

The combination of tools such as long-lasting insecticidal nets, artemisinin-based combination therapies, indoor residual spraying and intermittent preventive treatment in pregnancy has had a great public health impact in terms of reduction of malaria-associated death and morbidity especially in sub-Saharan Africa. Despite this gain, the number of estimated malaria cases remains still important with 225 million with 781,000 deaths (World Malaria, 2010). Many of them have died for reasons such as being out of reach of health centers or because of the cost of malaria treatment. This situation renders urgent the need to find ways to mitigate this problem. It is estimated that 80% of the world's population use herbal remedies to treat many illnesses and ailments (Zihiri et al., 2005). Additional reports shows that in the US, more than 158 million Americans spent US$ 17 billion on herbal medicines, while more than 70% of Germans recognized using herbal products in the management of many health conditions (US Report, 2002; Tuffs et al., 2002). In developing countries, the figure is almost the same with 80%

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Abbreviations: TAA, Tea artemisia annua; **IC_{50},** 50% inhibitory concentration; **RDT,** rapid diagnostic test; **DELI test,** enzyme-linked lactate dehydrogenase enzyme immuno-detection.

of the people using herbal remedies (Verma and Singh, 2008). In Africa also, medicinal plants are used for the treatment of many ailments (Wafo et al., 1999; Nkeh et al., 2001; Noumi and Yomi, 2001; Dieye et al., 2008). In this line, one acknowledges that plants such as the neem in Africa and artemisinin in China have been used for many years as drugs against falciparum malaria. The interest on artemisinin extracted from *Artemisia annua* has grown up since resistance to most of the affordable antimalarials has spread around the world.

A. annua L. is a member of the Asteraceace family and has been used by Chinese since ancient times in traditional medicine against fever and malaria. Artemisinin is a cadinane-type sesquiterpene lactone with an endoperoxide bridge which is presently the most potent and efficacious compound against the late-stage ring parasites and trophozoites of *Plasmodium falciparum* (Brisibe, 2009), the causative agent of malaria. The plant produces a portfolio of bioactive compounds including flavonoids, coumarins, steroids, phenolics, purines, lipids, aliphatic compounds, monoterpenoids, triterpenoids and sesquiterpenoids. Thus far, the most important of the sesquiterpenoids seems to be artemisinin, dihydroartemisinic acid, artemisinic acid and arteannuin B which are stored in the glandular trichomes found in the leaves and inflorescence (Ferreira and Janick, 1995). The action of artemisinin derivatives is different from that of the other antimalarials. Artemisinins have a very fast action and parasite clearance times are much shorter than with other malarial drugs. Whereas, most of antimalarials work at the late trophozoites and schizont stages of the malaria parasite, artemisinin derivates also act already at early trophozoites and ring stages.

Artemisinin acts on gametocyte development, resulting in decreased transmission in areas where artemisinin compounds are extensively used. Artemisinin is considered as the treatment of choice for malaria, and the WHO has called for an immediate halt to single-drug artemisinin preparations in favor of medications that combine artemisinin with another antimalarial, to reduce the risk of parasites developing resistance (WHO, 2006). Recently, the plant is introduced in Senegal and The Gambia and is grown in some areas where people have started to use it as herbal tea for malaria-like symptoms. To investigate whether the Tea *A. annua* has antiplasmodial effect, we studied its *in vitro* antimalarial activity on *P. falciparum* isolates collected from malaria patients at Pikine, Senegal. We used the DELI-microtest which is very sensitive allowing the measurement of drug response with very low parasite densities (0.005%) (Moreno et al., 2001).

MATERIALS AND METHODS

Study site and patients

The study samples were collected from malaria infected patients attending an outpatient clinic situated in Pikine (Figure 1) where malaria transmission is highly seasonal with an entomologic inoculation rate ranging from 0 to 16.8 infective bites per person per year (Pages et al., 2008). Patients with peripheral blood smears positive for *P. falciparum*, with non-complicated malaria, aged 5 years or greater were invited to participate in the study as previously reported (Thomas et al., 2002; Sarr et al., 2005). They were excluded in case of complicated malaria, pregnancy and recent history of antimalarial treatment.

Artemisia annua dried leaves

Leaves of *A. annua* were graciously provided by Dr Pierre Lutgen and colleagues from Iwerliewen Fir Bedreete Volleker (IFBV), Luxembourg. They were dried under shade in Luxembourg before being sent to Dakar in paper bags weighting 20 g. They were then kept at room temperature until analyses. Chloroquine (CQ) was obtained from Sigma Chemical Co. (St Louis, Mo, USA).

Extraction method

We used the method described by Rath (Rath et al., 2004) which yields 70 to 76% of artemisinin. Briefly, 10 g of dried leaves of *A. annua* are weighed and put in a glass container. Then 1 L of warm water is added and the mixture is stirred up for 10 min and covered by a lid which is not of iron because the latter reacts easily with artemisinin and may decrease the output of the extraction. The solution is then filtered to collect the tea *A. annua*.

Inclusion and exclusion criteria

We recruited study participants in an outpatient clinic in Pikine. The principal criterion of inclusion was uncomplicated *P. falciparum* malaria diagnosed by malaria Rapid Diagnostic Test (RDT). Patients were excluded in the presence of another *Plasmodium* species or any signs of severe malaria.

Socio-demographic characteristics of the study population

In total, 40 patients were recruited and 33 of them were male. The mean age was 25.3 years (12 to 60) (Table 1). The mean temperature and parasite density were 37.9°C and 15,955 parasites/µL respectively.

Consent

In case of positive RDT, we proposed to any patient to participate in the study. A written consent form is translated into wolof for any patient or his legal guardian in case he/she can not read. The form is signed by the patient and the investigator. To preserve the confidentiality of the participants, we have used a code. The study was approved by the Ethics Committee of the Ministry of Health and the Medical Prevention.

Blood collection

For each patient, 5 to 10 ml of whole blood is collected onto ethylen diamine tetracetic acid (EDTA) vacutainer tubes which are kept at room temperature until they are conveyed to the laboratory for analyses.

Parasite culture

To validate our study, we tested our TAA, using field strains as well as two known laboratory strains 3D7, susceptible to CQ and W2, chloroquinoresistant. 3D7 is a clone from NF54 which was isolated

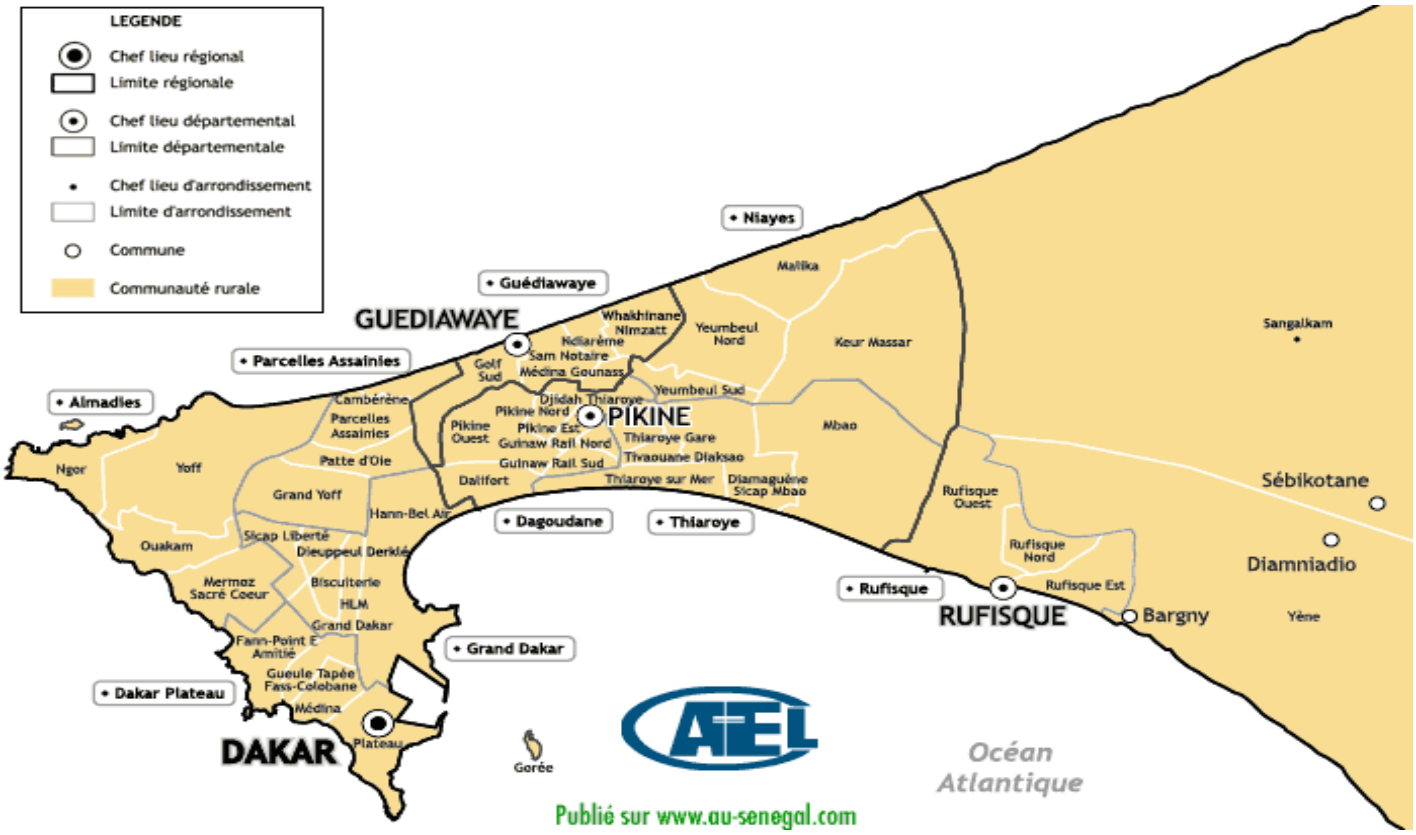

Figure 1. Administrative division of the region of Dakar, Senegal.

Table 1. Parasite density distribution based on the different age groups of our study population.

Different age groups (years)	Parasite density means (p/µL)
[12-15]	10739
[15-30]	18332.9
[30-60]	7390.1

from a patient who lived near Schipol Airport, Amsterdam, The Netherlands (Miller et al., 1993) while W2 was isolated from Indochina. Both strains are maintained in continuous culture in our laboratory.

When the isolates arrived in the laboratory, the parasitized blood sample is centrifuged at 2,000 rpm for 10 min. The plasma is collected in cryotubes and kept at –20°C. The pellet is washed twice with RPMI 1640 and the parasite load is adjusted between 0.5 and 1% with non-parasitized red blood cells. The last wash is done with supplemented RPMI 1640. The sample is put into 96-well microplates with supplemented RPMI 1640 along with tea *A. annua* at decreasing concentrations from 13.552 to 0.052 µg/ml. Two wells without tea *A. annua* are used as control. Each isolate is tested in duplicate. Parasites are allowed to grow during 48 h at 37°C in a candle jar after which, plates are taken out and frozen at –20°C until the DELI-test is performed.

The DELI-test

The test measures the amount of lactate dehydrogenase (LDH) produced by the parasite. The level of *pLDH* is proportional to the growth of *P. falciparum in vitro*. This rate is measured by means of immuno-enzymatic technique in double sandwich as described by Druilhe (Druilhe et al., 2001). The technique uses two monoclonal antibodies Mab 17EA and Mab 19G7 directed against an epitop of *pLDH.*

Coating plates

The 96-wells plates were coated with Mab 17 at 1 µg/L diluted in phosphat buffered saline (PBS) pH 7.4 for 12 h at 4°C and then washed twice with PBS-BSA (bovine serum albumin) at 1%. Thereafter, 300 µl of PBS-BSA at 1% are distributed into the wells and left for 4 h at room temperature. Finally, the plates are emptied and sealed in aluminum foil for a maximum of 8 days.

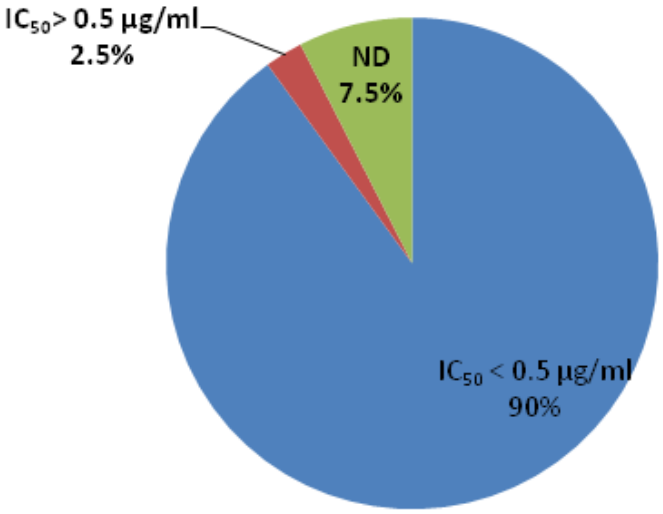

Figure 2. Representative curve of the different IC_{50} highlighting the distribution of isolates. In total, 7.5% assays were unsuccessful while 2.5% of the isolates were resistant, and 90% sensitive to the TAA.

The DELI-Test

The 96-well plates are thawed and frozen 3 times to lyze the red blood cells. One deposits 100 µL of supernatant of culture in the plates with dimensions and saturated. After incubation at 37°C for 1 h, they are washed 3 times with PBS-BSA with 1% and supplemented with biotinylated anti-pLDH Mab 19G7 antibody. After 1 h of incubation at 37°C, the plates are washed 3 times with PBS-BSA then a streptavidin-peroxidase solution is added to the wells followed by incubation for 30 min at room temperature. They are washed 3 times with PBS-BSA 1%. Peroxidase substrate, 3,3', 5,5'-tétraméthylbenzidine and hydrogen peroxide to 0.02% are added to the mixture. The enzymatic reaction is stopped after 5 min per addition of 100 µL 1M phosphoric acid per well. The intensity of the yellow color obtained is measured at 450 nm using a spectrophotometer (StatFax-2100, Awareness Technology Inc). The test is interpretable when the optical density of the well without tea *A. annua* lies between 0.4 and 1.5. The interpretation of the results is based on the optical density which is proportional to the quantity of *pLDH*. The results of the tests are expressed in 50% inhibitory concentration (IC_{50}), which is the tea *A. annua* concentration inhibiting 50% of the parasite growth. The IC_{50} are calculated starting from the curve dose/OD (optic density) by the software GraphPad PRISM 4.

RESULTS

Forty fresh samples collected from the study participants were tested and 37 *P. falciparum* isolates adapted to the *in vitro* growth. The cut-off value for resistance to TAA is 0.5 µg/ml. The geometric mean IC_{50} was 0.095 µg/ml.

The IC_{50} values were lower than 0.5 µg/ml for 36 samples and only one exhibited an IC_{50} > 0.5 µg/ml. The IC_{50} were undetermined for 3 isolates for unknown reasons. The 3D7 and W2 laboratory adapted strains exhibited IC_{50} of 0.146 and 0.394 µg/mL, respectively (Figure 2). The *in vitro* susceptibility of those isolates was also determined for CQ. The cut-off value for CQ resistance was set at 100 nM (Le Bras et al., 1990). The prevalence of isolates which exhibited resistance to CQ is 18.9%. Linear regression shows quite a weak association between TAA and CQ (R-squared = 0.0034). The geometric mean TAA IC_{50} in samples resistant and susceptible to CQ was 0.144 and 0.141 µg/ml, respectively (Table 2).

DISCUSSION

As the use of *A. annua* in Chinese population has been documented for many centuries against different ailments and malaria-like symptoms, we found it relevant to assess the *in vitro* antiplasmodial effect of TAA in *P. falciparum* strains. The known reference laboratory adapted strains 3D7 and W2 were tested in parallel as a control. The tea showed a strong antiplasmodial effect against the chloroquine sensitive 3D7 (0.14 µg/ml) and chloroquine resistant W2 (0.39 µg/ml) strains. The latter is considerably lower than the one obtained in Brazil, where a chloroquine resistant strain showed an IC_{50} of 6.1 µg/ml (De Mesquita et al., 2007). This difference is likely due to many parameters as the origin of the leaves of *A. annua*. The Brasilian leaves were grown locally and the local environment in which the plants are cultivated is of importance in the concentration of different chemical groups that are present in the leaves. However, the mean IC_{50} obtained in our study (0.095 µg/ml) is comparable to values obtained in Tanzania by Malebo and colleagues (Malebo et al., 2009) who showed a strong antiplasmodial activity of the tea *A. annua* with IC_{50} less than 5 µg/ml. The Senegalese and Tanzanian TAA have a strong antiplasmodial activity, though they are not as rich in artemisinin as the one from Brazil. This suggests that the antiplasmodial activity of the tea is caused by not only the artemisinin, but the combined effect of other substances including coumarine, scopoletines, ployphenols, flavonoids, camphors and microelements. This hypothesis is supported by other studies from Europe, where *Artemisia absinthium* and *Artemisia abrotanum* have demonstrated antiplasmodial properties, though they do not contain artemisinin (Cubukcu et al., 1990; Deans and Kenedy, 2002). Additionally, Valécha showed anti-plasmodial activity in plants of the genus *Artemisia*, though they also do not contain artemisinin (Valecha et al., 1994).

A. Afra extracts are effective against *P. falciparum in vitro*, and this activity is attribuate to a complex mixture of flavonoids and sesquiterpene lactones, rather than to a single compound (Kraft et al., 2003). Furthermore, the potential synergistic effects of artemisinin and flavonoids

Table 2. Correlation between parasite density and the IC_{50}. P represents the statiscal significance of the test (*p-value*) whereas N represents the number of analyzed isolates.

		Parasite density (parasites /µL)	IC_{50} (µg/ml)
Parasite density (parasites /µL)	Correlation coefficient	1	0.004
	p	-	0.981> 0.05
	N	40	37
IC_{50} (µg/ml)	Correlation coefficient	0.004	1
	p	0.981> 0.05	-
	N	37	37

were described in 1987 (Elford et al., 1987). *A. annua* produces at least 36 flavonoids (Liu et al., 1992). Five of these have been shown selectively to potentiate the *in vitro* activity of artemisinin against *P. falciparum* (Liu et al., 1992). A study shows also that flavonoids present in TAA have shown a variety of biological activities and may synergize the effects of artemisinin against malaria (Ferreira et al., 2010). *Coumarin*, in addition to its role in the immune system and on schizonts, is also known as a metal chelator, notably of iron, a chief element of malaria and other infectious diseases (Yang et al., 1992). In Bolivia, a serie of plants showed an IC_{50} of 9 µg/ml for *Amburana osarensis* against *P. falciparum* isolates essentially due to the coumarin (Bravo, 2003). *Curcumin*, likewise, is an excellent iron chelator, in addition to its numerous therapeutic properties (Means, 2009).

In conclusion, tea *A. annua* has shown strong *in vitro* antiplasmodial activities in *P. falciparum* isolates collected in Pikine. Additional investigations including *A. annua* of different origins are needed to determine which plants have the best antimalarial effects. In addition, more investigations should be carried out to assess the cytotoxicity levels when TAA is used daily by local populations to avoid adverse events and if necessary set a reporting system of those adverse reactions.

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