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Full Length Research Paper

Propagation in culture of *Plasmodium falciparum* gametocytes clinical isolates and antiplasmodial activity of two medicinal plant extracts on parasitic growth

Beourou S[1]*,Okou OC[2], Tuo K[1], Kamenan KCD[2], Gnondjui AA[1], Kouakou L[1], Attemene DSD[3], Penali L[1], Ronan Jambou[1,5], Djaman AJ[1,4,6]

[1]Laboratory of Malariology, Department of Parasitology and Mycology, Institut Pasteur of (Côte d'Ivoire),01 PO Box 490 Abidjan 01.
[2]Biochemistry-Microbiology Pedagogy Unit, University of Jean Lorougnon Guédé of Daloa, PO Box 150 Daloa
[3]Laboratory of Nutrition and Pharmacology, Faculty of Biosciences, University of Felix Houphouet Boigny, Côte d'Ivoire. 22 PO Box 582 Abidjan 22 (Côte d'Ivoire).
[4]Laboratory of Biochemistry and Pharmacodynamy Faculty of Biosciences, University of Felix Houphouet Boigny, Côte d'Ivoire.22 PO Box 582 Abidjan 22 (Côte d'Ivoire).
[5]Institut Pasteur de Paris, Department of Parasites and Insect Vectors
[6]Laboratory of Fundamental Biochemistry, Institut Pasteur of Côte d'Ivoire, 01 PO Box 490 Abidjan 01.

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Gametocytes were produced from the asexual forms of clinical isolates cultured at 2.45% parasitaemia and 6% hematocrit using the candle jar method. Formation of gametocytes up to maturation (stage II to stage V) and a variable number of gametocytes going up to 1.26% for about two weeks with relatively high parasitaemia were observed. For the chemo-sensitivity tests, six crude extracts of two medicinal plants were tested on the clinical isolates and the standard strain using the SYBR Green method based on DNA fluorescence. Determination of the IC_{50}s of our extracts after reading the 96-well microplates was performed using the IVART software from WWARN. Results showed that the aqueous extracts (Isolate 1, IC_{50} = 13.27 µg / mL, Isolate 2, IC_{50} = 13.93 µg / mL and Isolate 3, IC_{50}= 14.63 µg / mL and standard strain K1, IC50 = 14.01 µg / mL) and Ethanolic extracts (Isolate1, IC_{50} = 13.43 µg / mL, K1 strain, IC_{50} = 10.14 µg / mL) of *Entandrophragma angolense* (Lokoba) were active whereas chloroquine used as standard molecule showed mixed activity on Isolates and on chloroquine-resistant strain K1 (IC_{50}> 50 nM). Ethanolic extracts of *Cocoa nucifera* were only active on isolate 3.

Key words: Gametocytes, *Plasmodium falciparum*, hematocrit, isolates, parasitaemia, antiplasmodial.

INTRODUCTION

Malaria is the most serious and widespread parasitic disease in the world with a 90% incidence in sub-Saharan Africa where it recorded 92% of the most deaths in 2015 (WHO, 2016).Malaria is still considered a public health problem and nearly half the world's population was at risk of contracting malaria in 2015 in 91 countries. The number of cases of malaria was estimated at 212 million and the number of deaths was

*Corresponding author E-mail: be_sylvain@yahoo.fr

evaluated to 429,000 (WHO, 2016). Malaria remains a major factor of mortality among children under five years of age; two thirds (70%) of deaths occur in this age range, a child dies of malaria every two minutes (Pierre et al., 2016).

Ivory Coast is facing this scourge, since the country is ranked among the top ten countries affected by malaria infected *Plasmodium falciparum*. According to the NMCP-CI (2004) report, this parasite accounts for 80% of the reasons for consultation and hospitalization and 33% of the causes of death. Gametocytes represent the only stage of continuous transmission of parasites to mosquito vectors (Fivelman et al., 2007). Most of the antimalarial drugs discovery was focused on asexual stages. The struggle for malaria eradication, through the development of a malaria vaccine, also requires a great interest in studying the sexual stages of *Plasmodium falciparum*, which remains the most deadly species of malaria. Thus, the introduction of in vitro gametocyte production tests would be useful to assess new molecules and the efficacy of antimalarial drugs coming from the Ivorian pharmacopoeia. The first objective of this study was to set up cultures propagating the sexual forms of the parasite obtained from clinical isolates, and to evaluate the antiplasmodial activity of natural compounds on all forms of the parasite resulting from clinical isolates notwithstanding the poor equipment.

The specific objectives are:

Maintaining a continuous culture of clinical isolates from the field, propagating gametocytes from clinical isolates through in vitro culture, perform chemo-sensitivity tests on asexual forms to determine the antiplasmodial activity of each plant extract.

MATERIAL AND METHODS

Collection of samples (clinical isolates)

The blood of patients presenting a proven uncomplicated *Plasmodium falciparum* malaria was collected. These patients were recruited during clinical trials which took place at Community Health and Urban Training of Anonkoua-Kouté, Abobo, (Abidjan). Prior to the inclusion of a patient in the study population, informed consent was obtained, and blood samples were collected in vacuum tubes containing EDTA. These samples were then sent to the laboratory.

Culture of Plasmodium falciparum

Strains of *Plasmodium falciparum* were cultuvated *in vitro* according to the modified method of Trager and Jensen in 1976. The principle of the in vitro culture of Plasmodium falciparum is to put parasites in a conducive culture condition for the continuation of their multiplication. The Giemsa solution used for slides staining during culture was prepared on one tenth with distilled water; 1 mL of Giemsa solution (Avonchem brand) diluted in 9 mL of distilled water. RPMI 1640 (Rosewell Park Memorial Institute) is a nutrient medium used as a base medium for the cultivation of Plasmodium falciparum. The synthetic medium prepared for our study was a complete medium supplemented with 0.5 g / L of hypoxanthine; this medium was used for the maintenance of clinical isolates (gametocytes propagation) and also for chloroquine-resistant K1 strain

Propagation of *P. falciparum* gametocytes

Erythrocytic stages were cultivated in a 25 cm^2 cell culture flask with 2.45% parasitaemia the 1st day (D0). For gametocytes production, the methods of Akompong et al. in 2000 and (Fivelman et al., 2007; Looker et al., 2014) have been adapted. Briefly, the asexual forms of *P. falciparum* clinical isolates were cultivated in 6% hematocrit in a total volume of 8 mL of RPMI 1640 medium and confined in a candle jar at 5% CO2 and incubated at 37 ° C. When parasitaemia reached 3.9% the 3rd day (D3), it was diluted to 2% parasitaemia and the hematocrit was reduced to 3%. The medium used for this purpose was prepared with 0.5% of albumax II, supplemented with 0.5 g / L of hypoxanthine, representing the complete medium (CM). Parasitaemia was regularly checked and the culture gassed for 1 min before incubation. Before any change, the medium and the material used were previously heated at 37° C. From D4, 4 mL of the culture medium was regularly replaced with an equal volume (V / V) of the complete medium.

Evaluation of the antiplasmodial activity

In vitro chemo-sensitivity tests are based on the culture of Plasmodium falciparum isolates in the presence of a concentration range of the studied antimalarial drug during the life cycle or part of the cycle. They evaluate the parasitic response through an in vitro treatment by direct contact between the parasite and the active principle. The concentration of the antimalarial drug inhibiting 50% (IC_{50}) of the parasite growth is determined. The tests are carried out on 96-well microplates containing a volume of parasitized erythrocytes. The inhibition of erythrocytic schizogony (*P. falciparum*) is measured using the SYBR Green method (Smilkstein et al., 2004, Basco, 2007, Akala et al., 2011). parasitemia during these tests were fixed between 0.1-0.5% and the hematocrit at 1.5%. The extracts

to be tested at different concentrations were added in duplicate in themicroplates.

The Principle of SYBR Green method

This method is based on the DNA fluorescence (Smilkstein et al., 2004), the SYBR Green is a DNA intercalator. After 72 hours of parasites incubation in the presence of the drug and after the lysis of the red blood cells after thawing, the SYBR Green is added to the culture. It's intercalated between the DNA bases and emits a fluorescence which is proportional to the level of DNA in the medium which is itself proportional to the number of parasites. The fluorescence is therefore evaluated using a spectrofluorimeter to monitor parasitic growth.

Preparation of concentration range

10 mg of the extracts were dissolved in 10 mL of distilled water for the aqueous extract. For the ethanolic and ethyl acetate extracts, 10 mg of both extracts were dissolved in 1 mL of DMSO and 9 mL of distilled water. A stock solution of 1 mg / mL was then obtained. Using the stock solution, a twofold serial dilution of a concentration range was prepared. Each extract was tested with a final range of 7 concentrations ranging from 50 µg / ml to 0.78 µg / ml or from 1600 nM to 50 nM.

Culture of isolates in the presence of extracts

Tests were performed with parasitaemia ranging from0.1 to 0.5%. Isolates with parasitaemia exceeding this percentage, was adjusted by diluting blood pellet with uninfected red blood cells. 100 µL of inoculum was added to all wells. Each well contained a final volume of 200 µL. Several controls were also prepared (growth control and negative control) and the hematocrit was maintained at 1.5%: The test well contains 100 µL of extract to be tested and 100 µL of parasitized red blood cells. The growth control contained100 µL of RPMI 1640 (without extract) and 100 µL of parasitized red blood cells. The negative control or background noise was prepared with 100 µL of RPMI 1640 and 100 µL of uninfected red blood cells.The incubation was carried out for 72 h at 37 ° C. in a humid atmosphere in a candle jar. After 72 h, the microplates were removed from the incubator and stored at -20 ° C

Evaluation of parasitaemia

Prior to evaluation, microplates were removed from the freezer and then exposed to room temperature for thawing. The parasitemia was evaluated using the SYBR Green method. Hemolysis of the culture was done through thawing and perfected with a lysis buffer prepared by dissolving 4.84 g of Tris and 700 µL of HCl in 1500 ml of distilled water. Then 3.72 g of EDTA, 160 mg of saponin and 1600 µL of Triton X-100 were added. The volume was adjusted to 2000 mL with distilled water. The pH of the solution was adjusted to 7 by adding a volume of HCl solution. Then a mixture containing 5 µL of SYBR Green and 25 mL of lysis buffer was prepared. 100 µL of the parasitic suspension was transferred to a virgin microplate. To these 100 µL of parasitic suspension, 100 µL of the mix were added. The microplate was then put for one hour at room temperature in the darkness. The reading was performed using the FLx100 spectrofluorimeter (BioTek) at 535 nm after an excitation at 485 nm.

Determination of IC_{50} and Test Interpretation

The IC_{50s} (extract concentration that inhibits parasitic growth by 50%) and the corresponding correlation coefficients were determined graphically using the WWARN *in vitro* Analysis and Reporting Tool (IVART) software (Basco, 2007; Le Nagard et al., 2011). It is a non-linear regression of a sigmoid type; the concentration-inhibition model applied to the data is based on approaches established by (Le Nagard et al., 2011). The responses obtained varied because the effect of the parasite is different depending on the drug and the isolate.
The antiplasmodial activity of extracts and pure compounds was classified by (Bero et al., 2009, Jansen et al., 2012).

RESULTS

In vitro* culture and gametocyte production of *Plasmodium falciparum

Initial parasitemia of isolates during the production of gametocytes reached a maximum and decrease from day 4. After 6 days of culture, stage II gametocytes were visible. Stages III were visible on day 8 and stage IV were visible on day 9. Gametocyte density was observed as well as the different stages from day 4 to day 12 (Table 1 and figure 1).

Chemo-Sensitivity Test

The IC_{50s} of our extracts and chloroquine the standard molecule were recorded in Table2after data analysis. According to(Bero et al.,2009; Jansen et al., 2012)classification tothe antiplasmodial activities of extracts and isolated pure compounds, we can argue that the aqueous extract of Lokoba with an IC_{50} ranging between 5 and 15 µg / mL was active on clinical isolates

Table 1. Parasitic and gametocyte densities of asexual and sexual forms for 12 days of culture.

	D_0	D_1 - D_3	D_4 - D_6	D_7 - D_8	D_9 - D_{10}	D_{11}- D_{12}
Parasitemia asexual forms (%)	2.45	3.9	5.56	2.63	1.66	0.96
Gametocyte density (%)	0	0	0.12	0.95	0.98	1.26

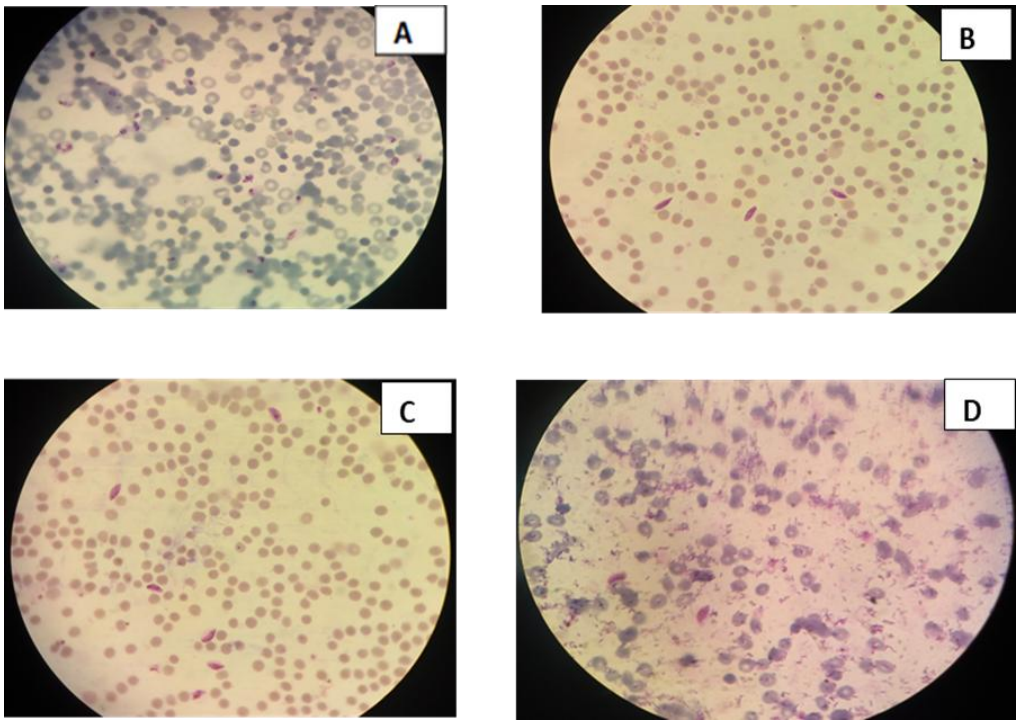

Figure 1. Gametocytes various stages Gametocytes (Stage II): 4-6 days (A) ; Gametocytes (Stage III): 7-8 days (B) ; Gametocytes (Stage IV): 9-10 days (C) ; Gametocyte Stage V: 11-12 days (D).

and standard strain. The ethanolic extract showed moderate activity on isolates 2 and 3 and was active on isolate1 and K1. However, for the same plant, the ethyl acetate extract was practically inactive on the clinical isolates with IC_{50}> 50 µg / mL. All extracts of the plant Ngbogbaleka had a moderate activity on some clinical isolates and were practically inactive on K1. As for chloroquine the standard molecule, it was a little bit active on the clinical isolates and inactive on K1 which is a chloroquine-resistant strain (Table2).Plant extracts and chloroquine tested on isolates and K1 (Table2) allowed us to plot the below histograms (Figure 2; 3; 4)

DISCUSSION

Cultivating conditions of clinical isolates and the standard strain according to the method described by Trager and Jensen in 1976 are based on a rigorous principle. The *in vitro* culture of *Plasmodium falciparum* in the blood stage is based on the fact that parasites are put in a conducive culture condition for pursuing their multiplication. The method of the in vitro gametocytogenesis induction used, lies on the fact that parasites could produce sexual forms through stress or suffering. Studies carried out in 1979 showed that in the in vitro culture the conversion rate of blood asexual stages of *Plasmodium falciparum* into gametocytes was directly linked to the conditions of the culture medium (Carter and Miller, 1979). Several factors are involved in the production of gametocytes in vitro. The genetic factors of the producing strain. Some strains produce more gametocytes than others (Graves et al., 1984). Certainly our clinical isolate genetically produces gametocytes. From the analysis of parasitemia recorded in Table1, we notice that they reached a maximum before the appearance of sexual forms. We can say that production of gametocytes is linked to the

Table 2. IC_{50} of extracts and chloroquine on 3 clinical isolates and K1.

Compound	Extracts (E)	IC_{50} of clinical isolates and K1 standard strain			
		Isolate 1	Isolate 2	Isolate 3	K1
Nbobaleka *(Cocoa nucifera)* (µg/mL)	E_{aq}	>50	40.14	17.18	> 50
	E_{AcoE}	40	40.25	26.5	> 50
	E_{ETOH}	> 50	> 50	12.09	> 50
Lokoba *(Entandrophragma angolense)* (µg/mL)	E_{aq}	13.27	13.93	14.63	14.01
	E_{AcoE}	> 50	> 50	16.92	> 50
	E_{ETOH}	13.41	19.73	18.89	10.14
chloroquine (nM)	CQ	25.24	24.38	22.42	> 50

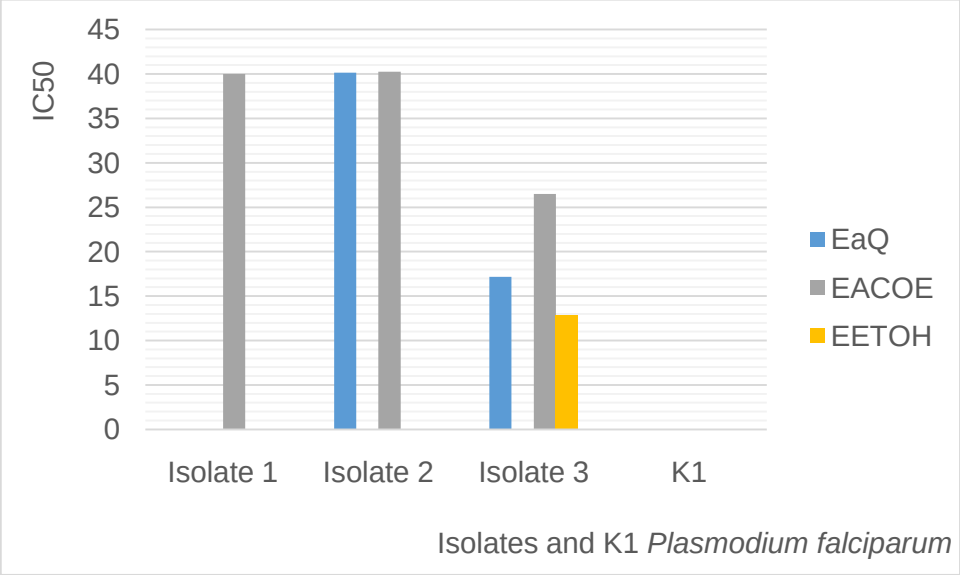

Figure 2. IC_{50} of three extracts of Nbobaleka (*Cocoa nucifera*).

increase in asexual parasitaemia (Alano and Carter, 1990). Furthermore, it should be noticed that this outbreak could be associated with a parasitemia limit likely to trigger off the gametogenesis of clinical isolates in culture. In the conditions of rapid growth of asexual parasites, their conversion rate into gametocytes remains low. They increase in low growth conditions (Carter and Miller, 1979). The waste medium preserved during medium change might have factors that played a key role in promoting and increasing propagation of gametocytes in culture.

The efficacy of a plant is revealed by the activity of its extracts against *P. falciparum*. However, some extracts may be inactive during test but may be active in the traditional treatment of malaria. Because traditional methods are not reproduced in laboratory. Potential degradation of active ingredient (s) during extraction, the efficacy linked to plant associations, do not have direct action on the parasite (Soh and Benoit-Vical, 2007, Eze et al., 2013). That was the case of *Cocoa nucifera* extracts (Nbobaleka) which showed their limit in chemosensitivity tests but demonstrated their efficacy

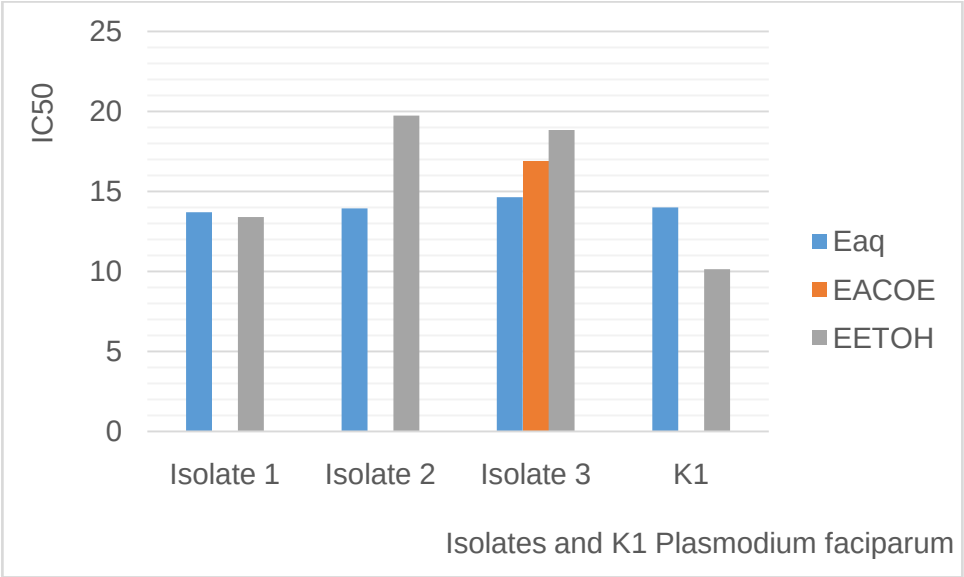

Figure 3: IC_{50} of three extracts from Lokoba (*Entandrophragma angolense*).

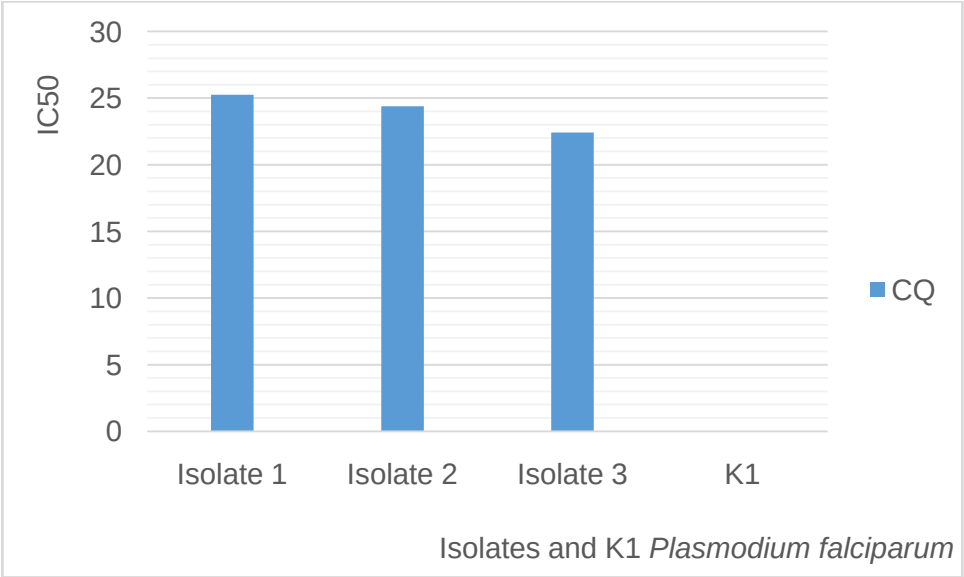

Figure 4: IC_{50} of Chloroquine.

in traditional treatment. In contrast, the aqueous and ethanolic extracts of *Entandrophragma angolense* (Lokoba) on chloroquine-resistant strain (K1) and on clinical isolates showed a good antiplasmodial activity. The chloroquine-resistant strain seemed to be more sensitive to this plant extracts. This is indeed encouraging and would allow us to hope for a plant that could overcome the problem of chloroquine resistance. The limited antiplasmodial activity of chloroquine, a standard generic molecule on clinical isolates, revealed the reasons for its non-acceptance as a given drug in Côte d'Ivoire today. Chloroquine was the first line drug for the National Malaria Control Program (NMCP) for uncomplicated cases in Côte d'Ivoire for many years (Adou-Bryn et al., 1999). Unfortunately, P. falciparum resistance to chloroquine has reached high levels in Africa. This led several countries, including Cote d'Ivoire, to withdraw it as a first line treatment

against uncomplicated malaria (Henry et al., 2002).

CONCLUSION

The *in vitro* culture of Plasmodium remains one of the useful methods for the determination of resistant phenotype and the monitoring of the efficacy of an antimalarial. Immunology, molecular biology and pathogenicity studies of *Plasmodium falciparum* often require the adaptation of naive clinical isolates to culture. At the end of this study, it seems that the maintenance and adaptation of clinical isolates as well as the propagation of gametocytes inculture were a success despite the lack of suitable equipment. This work paved the way for studying various aspects of the parasite biology. The chemo-sensitivity tests on the asexual forms of the parasite demonstrated the richness of the Ivorian pharmacopoeia. They revealed that some plant extracts such as the aqueous and ethanolic extracts of Lokoba barks were active on all the clinical isolates cultivated. These extracts could constitute a natural source for antimalarial molecules and allow us to understand its usage in the Ivorian pharmacopoeia to treat malaria. Therefore, it could be interestingto test the active identified extracts on the parasitic biomass.

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Conflict of interest

The authors declare no competing interest.

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