Original Research Article

*In vitro*antiplasmodial and hemolytic activities of *Trema orientalis, Cnestis ferruginea* and *Dialium dinklagei* used to treat malariain Côte d'Ivoire

Abstract

In 2019, approximately 229 million cases with 409,000 deaths attributable to malaria have been estimated over the world. Malaria constitutes one of the biggest health problems in tropical Africa due to the resistance of human malaria parasites to anti-malarial compounds. Investigating plants used in traditional medicine to treat malaria remains a credible option for new anti-malarial drug development.The aim of this study was to evaluate extracts from three medicinal plants, *Trema orientalis*, *Cnestis ferruginea* and *Dialium dinklagei*, used in traditional medicine in Côte d'Ivoire, for *in vitro* antiplasmodial activities. SYBR GREEN fluorescencemethod was used to evaluate the *in vitro* inhibitory activity of the extracts, chloroquine, artesunate and quinine against*Plasmodium falciparum* field isolates and two laboratory strains of *Plasmodium falciparum*: the chloroquine sensitive 3D7 and the chloroquine resistant Dd2. Chloroquine, quinine and artesunate have been selected as the reference antimalarials compared to plant extracts. In addidtion, the haemolytic activity of extracts showing good antiplasmodial activity was evaluated. The IC50 and the corresponding correlation coefficients were determined graphically, using *In Vitro* Analysis and Reporting Tool (IVART) software of WWARN. Results showed that no plant is active with the hexanolic extract. *Trema orientalis* had moderate activity with the methanolic extract with activities ranging from 14.46µg/mL to 28.32µg/mL. *Cnestis ferruginea* was active with the decoction extracts with activities ranging from 11.78µg/mL to 13.94µg/mL. *Dialium dinklagei* was active with both methanolic and aqueous extracts. There was less than 1% hemolysis at the concentration of 200 µg/mL of plant extracts. These results validate the reported traditional use of *Trema orientalis*, *Cnestis ferruginea* and *Dialium dinklagei* for malaria treatment in Côte d'Ivoire.

Keywords : *Plasmodium falciparum*, Haemolytic, *in vitro*, antiplasmodial, malaria

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1 Introduction

In 2019, approximately 229 million cases with 409,000 deaths attributable to malaria have been estimated [1]. These alarming statistics make malaria the world's leading endemic. Chemotherapy treatment of malaria has evolved over the past decade due to the spread of multidrug-resistant strains of Plasmodium falciparum. The World Health Organization (WHO) is currently promoting artemisinin-based combination therapy (ACT) as the reference drug for the management of uncomplicated falciparum malaria to reduce the risk of resistance [2, 3]. Appearance and rapid extension of *Plasmodium falciparum* resistance to the common antimalarials such as sulfadoxine-pyrimethamine, chloroquine and recently to the derivatives of artemisinin makes urgent the discovery of new antimalatrial compounds [2, 4, 5].

Plants have been and remain a good source of pharmacologically active compounds, including antiplasmodial agents, as shown by quinine, isolated from Cinchona sp. and artemisinin extracted from Artemisia annua.In addition, many phytochemical compounds with antiplasmodial activity were isolated from plants [6, 7].

Despite the implementation of modern treatment, the majority of the population still uses traditional medicines to treat malaria and others diseases. The most frequently mentioned reasons are the high cost of treatment and often cultural factors.

In Côte d'Ivoire, a large number of plant species have been identified as antimalarial medicinal plants. Pure products have been isolated from some of these plants including those with antimalarial activities comparable to or more active than chloroquine on susceptible and resistant strains of P. falciparum [8, 9]. It is therefore imperative to continue the development of antimalarial drugs, with these highly active products, through to pre-clinical testing, clinical trials and drug production.

What brought us interest to study *Cnestis ferruginea* Vahl, *Dialium dinklagei* and*Trema orientalis*, 3 plants traditionnally used in Cote d'ivoire to treat malaria.

Dialium dinklagei belongs to the *Fabaceae* family. It is a plant found in forest areas from Guinea to Congo. It is used in Côte d'Ivoire to treat malaria. Very little work has been done on this plant, but we note that Bouquet and Debray have described the presence of tannins in the leaves of this plant [10].

Cnestis ferruginea Vahl ex DC is a perennial shrub or tree belonging to the family *Conaraceae*. *Cnestis ferruginea* has a wide distribution in West Africa, particularly in Gambia, Ghana, Guinea-Bissau, Côte d'Ivoire, Liberia, Nigeria, Sierra Leone [11], Benin [12], Niger and Gabon, especially in semi-deciduous forests.

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According to Bouquet and Debray , the leaves are very active vermifuges against ascaris. These leaves are also used to treat scabies, asthenia, and would have purgative properties. Another therapeutic indication that the traditional pharmacopoeia recognizes to this plant is its use for the treatment of the ocular affections. Several other uses were described by certain authors [10].

C. ferruginea is well known in phytotherapy and certain literatures lend it diverse therapeutic uses such as the treatment of conjunctivitis, bronchitis, tuberculosis, migraines, sinusitis and oral infection. It is also used for the treatment of dysentery, syphilis, gonorrhea, cough, dysmenorrhea, ovarian and aphrodisiac disorders, abortion and constipation [13]. The leaves can be used as a laxative and against fever [14]. The roots and fruits have been considered as a remedy for snake bite. The decoction of the bark can be used to treat gum infections [11].

The fruits are also used in ocular pathologies such as conjunctivitis and against several other diseases like bronchitis or tuberculosis [14, 15]. Several works have proved the anti-inflammatory and analgesic activities [16, 17] and the anti-depressive and anxiolytic activities [18] of *Cnestis ferruginea*.

Tremaorientalis is an arborescent species belonging to the family Ulmaceae. In Africa, its geographical area extends from Senegal to Somalia, through the entire region of Central Africa.

In Ivory Coast, Tremaorientalis is very generally used to treat jaundice, broncho-pulmonary affections, fever, rheumatic pains and malaria. Administered orally, the plant is reported to have a purgative and diuretic action [10].

Bouquet and Debray noted the absence of alkaloids [10]. As for Bekro et al, they noted an absence of gall tannin, quinone and saponosides [19]. Kerharo and Adam showed that the leaves exert diuretic effects due to flavonoids and vermifuge effects due to polyterpenes [15]. Dimo et al showed that Tremaorientalis has anti-diabetic properties (hypoglycemic activity) and could be beneficial for diabetics with cardiovascular diseases [20]. Dijoux-Franca et al showed the presence of dihydrophenanthrene and phenyldihydroisocoumarin in Trema orientalis [21].

2 Material and methods

2.1 Plant material

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The plant material consists of the leaves of Dialium dinklagei, Cnestis ferruginea and Tremaorientalis. The leaves of these plants were collected in March 2013 in the District of Abidjan.

The timing of the plant harvest was the morning at 9 AM.

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2.2 Biological material

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Biological samples are constituted of group O blood samples with a positive rhesus (Rh+) for an innoculum dilution with clinical and reference strains of Plasmodium falciparum.

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Two ATCC reference strains were used: 3D7 chloroquino-sensitive was provided by Biochemistry and Molecular Department University of Legon, Ghana and Dd2 chloroquino-resistant was provided by MRA-156, lot N°58319486, MR4ATCC®Manassas, Virginia, USA.

3.3 Methods

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Ethnobotanical survey, bibliographic research and selection of studied plants

Popular pharmacopoeia methods were used to establish a list of plants used to treat malaria. Popular pharmacopoeia [22] consists of direct, collective or individual interviews with populations on the plants used in routine care.

Investigations were carried out in November 2012 in Abidjan and Bondoukou (city in the east of Côte d'Ivoire) by ethnobotanical approaches with 27 actors of traditional medicine[22]. Ethnobotanical data (local name, method of preparation, traditional use, combination of plants, indications, dosage, contraindications and side effects) are obtained through conversations with traditional healers.

Samples collected were identified at Centre National de Floristique (National Floristry Center) of Félix Houphouët-Boigny University, Abidjan, (Cote d'Ivoire) by Professeur Aké-Assi Laurent.

2.4 Extracts preparation

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The leaves were dried out of the sun for one week at room temperature before being reduced to fine powder using a mechanical grinder (Retsch M6951). From the powder obtained, the various crude extracts were prepared. Decoction of each plant was made as close as possible to the traditional healer's formula. Then, 3 successive extractions by solvents of increasing

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polarity (hexan, methanol and water), have been done according to the protocols made by Zirihi et al. et Bekro et al. [19, 23](Figures 1 et 2).

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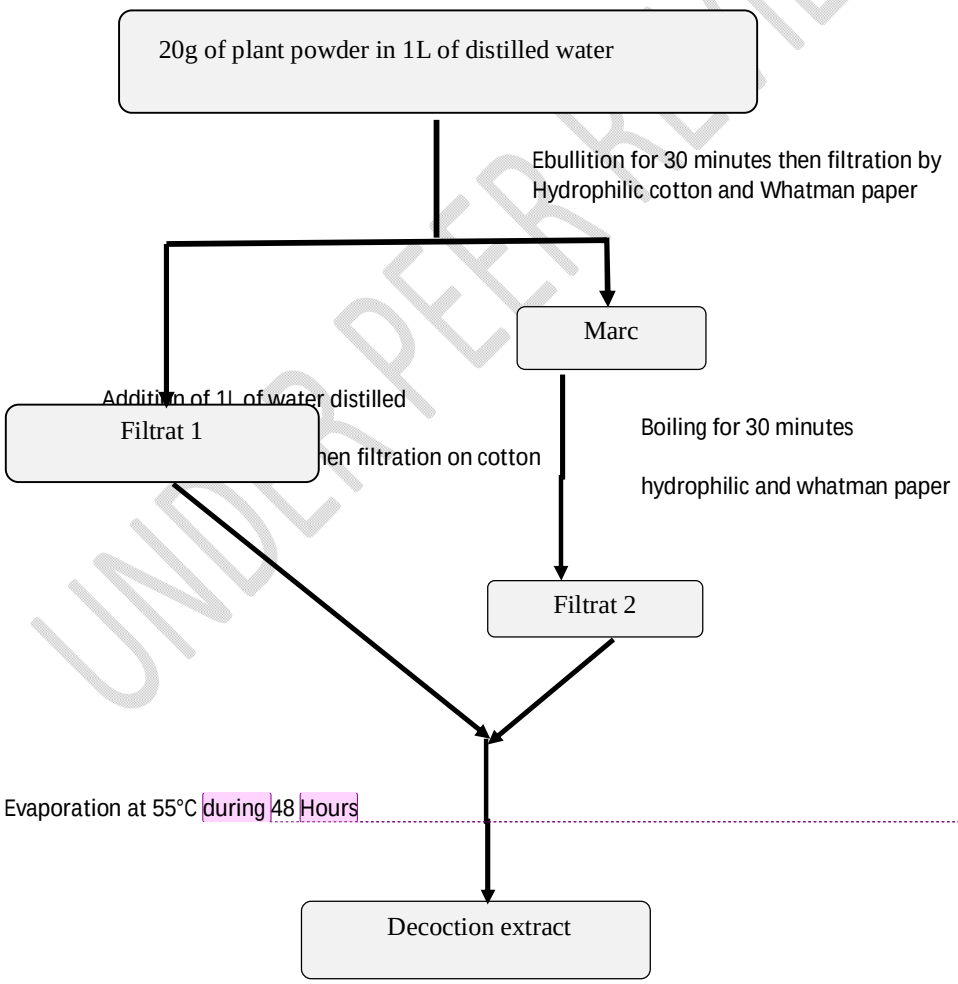

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Figure 1: Diagram showing obtention of decoction extract Procedure

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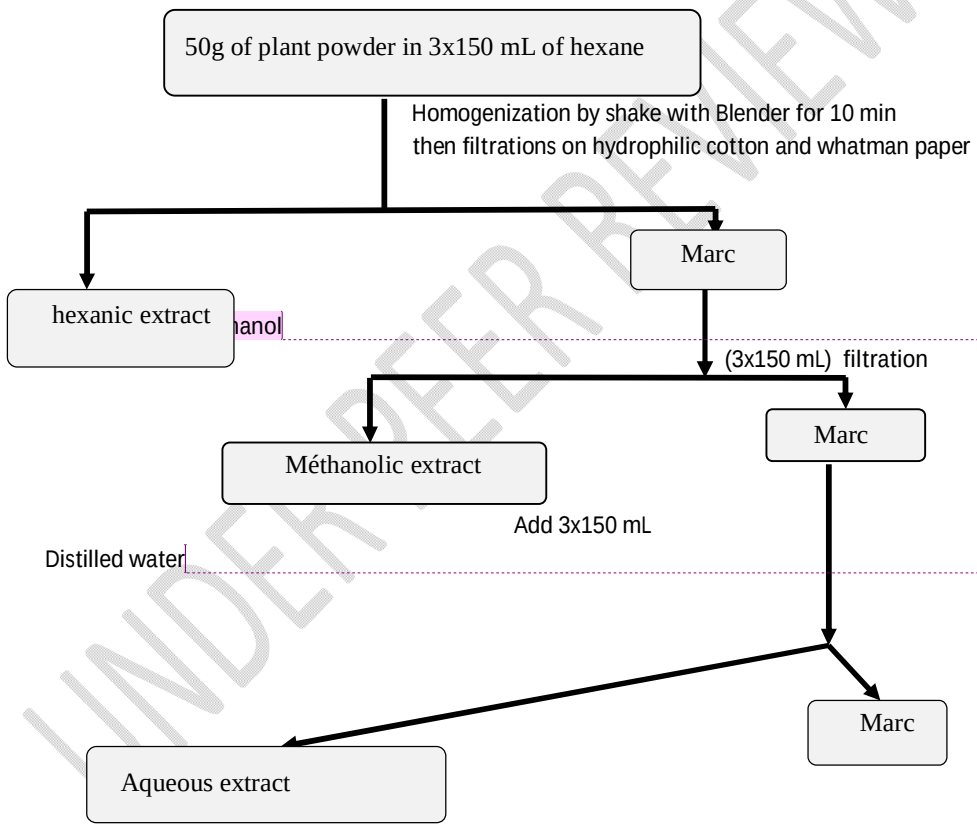

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Figure 2: Diagram showing obtention of Hexanic, Methanolic and Aqueous extracts Procedure [19, 23]

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2.5 Field isolates collection

Blood samples were collected at the health center of Wassakara (Abidjan Côte d'Ivoire) by venipuncture in heparinized tubes from patients older than 18 years with *P. falciparum* malaria after informed consent. The samples were then transfered at 4°C to the Swiss Center for Scientific Research for *in vitro* test.

2.6 *In vitro* antiplasmodial assay

Antiplasmodial activity was analyzed with SYBR Green method. The assays were carried out on 96-well plates filled with ~~a~~-infected red blood cells (IRBCs) in the following proportions of parasitaemia <0.3% and hematocrit 5%. The *in vitroP. falciparum* continuous culture used in our assays is derived from that developed by Trager et Jensen [24]. Inhibition of parasite growth was measured using the SYBR Green method [25–29].

The reading was done with the the Spectra Max GEMINI XPS spectrofluorometer (Molecular Devices) at 535 nm after excitation at 485 nm. The IC50 are determined graphically, using *In Vitro* Analysis and Reporting Tool (IVART) software of WWARN [26, 29].

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2.7 *In vitro* hemolysis assay

A stock solution of the samples was prepared in the appropriate solvent at concentrations of 100 µg/mL and 50 µg/mL, taking into account that the solvent volume must not be greater than 1% in the final solution.

To perform hemolysis assay, 10 µL of stock solution was placed in an Eppendorff microtube and mixed with 190 µL of RBC (10%) as controls. The negative control comprised 10 µL of PBS + 190 µL of 10% RBC and the positive control was prepared with 10 µL of 20% Triton X-100 + 190 µL of 10% RBC. Tubes were centrifuged for 5 minutes at 2200 rpm and 150 µL of supernatants were placed in a 96-well plate. The absorbance was read at 550 nm with a plate reader (Multiskan FC, Thermo Scientific).

The following formula was used to calculate the percentage of hemolysis: % hemolysis = [(Abs sample - Abs negative control) / (Abs positive control - Abs negative control)] x 100.

Abs= absorbance at 550 nm

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2.8 Ethical issues

The study was conducted in accordance with the local laws and regulations, and International Conference on Harmonization - Good Clinical Practice (ICH-GCP). The protocol was reviewed and approved by the National Ethical Committee for Research (03-2013 /MSLS/CNER-P). Written informed consent was obtained from participants for blood collection and from traditional healers. In case of an illiterate participant, his/her thumb impression and signature of an independent witness were sought.

3 Results

3.1 Antiplasmodial activity

The results showed that no plant is active with the hexanolic extract. *Trema orientalis* had moderate activity with the methanolic extract with activities ranging from 14.46µg/mL to 28.32µg/mL. *Cnestis ferruginea* was active with the decoction extracts with activities ranging from 11.78µg/mL to 13.94µg/mL. *Dialium dinklagei* was active with both methanolic and aqueous extracts.

In summary,decoction extract of *Cnestis ferruginea and Dialium dinklagei* showed good activity againts fields and references parasites. The methanolic extract of *Trema orientalis* had moderate antiplasmodial activity.The four field isolates tested were CQ sensible. Quinine and Artesunate whowed good activity againts field isolates (Table 1).

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Table 1 : antiplasmodial activity of crude extracts

			Strains tested /CI_{50} (µg/mL)					
			Clinical isolates				Reference strains	
Plants	**Extracts**	**Extraction yield (%)**	**W536**	**W539**	**W552**	**ANK02**	**3D7**	**Dd2**
Dialium dinklagei	Déc	18,5	13,19±3,17	13,74±4,12	12.8 ±2.4	12.9 ±3.01	14,35±0,79	13,82 ±2.7
	Hex	1	>50	>50	>50	>50	>50	>50
	Met	4,5	17,66±2,65	15,76±5,63	19,15±3,27	15,29±3,76	14,97±2,59	15,11±2,2
	Aq	4	18,34±3,01	15,43±2,24	17,33±4,21	21,67±3,29	19,54±1,58	17,47±1,2
Cnestis ferruginea	Déc	17,95	12,85±2,29	13,1±3,19	11,96±1,62	13,94±3,23	11,78±2,21	11,85±1,43
	Hex	1	>50	>50	>50	>50	>50	>50
	Met	5,4	>50	>50	>50	>50	>50	>50
	Aq	5,8	13,68±2,76	13,74±2,72	12, 35±1,44	13,56±3,1	12,15±1,91	12,35±4,21
Trema orientalis	Déc	20	>50	>50	>50	>50	>50	>50
	Hex	1,2	>50	>50	>50	>50	>50	>50
	Met	7,2	14,46±2,55	22,54±5,67	19,65±3,29	28,32±2,34	19,57±1,28	22,65±3,39
	Aq	7,6	>50	>50	>50	>50	>50	>50
Chloroquine (nM)			33,01±0,92 (0,01µg/mL)	42,71±1,32 (0,013µg/mL)	37,31±3,24 (0,011µg/mL)	35,38±4,92 (0,011µg/mL)	51,07±2,23 (0,016µg/mL)	116,71±5,11 (0,037µg/mL)
Quinine (nM)			5,76±0,95 (0,0019 µg/mL)	23,87±1,12 (0,008 µg/mL)	44,37±2,15 (0,015 µg/mL)			
Artesunate (nM)			3,43±0,49 (0,0013 µg/mL)	2,22±0,16 (0,0008µg/mL)	6,31±3,01 (0,0024µg/mL)			

Dec : Décotion ; Hex :Hexanique ; Met : Méthanolique ; Aq : Aqueux

3.2 Hemolytic activity

No extract was found to exhibit significant red blood cells lysis activity with a percentage of haemolysis <1% for all tested extracts (conc = 100 µg/mL and 200 µg/mL). This indicates that anti-plasmodial activity is not correlated with haemolysis of red blood cells but with a real action against the parasite.

4 Discussion

The study of the anti-plasmodial activity of a plant requires many analytical tools. In the case of this study, the effectiveness of a plant is demonstrated by the *in vitro* activity of one of its extracts against *P. falciparum*. We investigated the *in vitro* activity of extracts of 3 plants on

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Plasmodium falciparum reference strains (Dd2 and 3D7) and clinical strains (W536, W539, W552, ANK02). This work is justified by the emergence and spread of *P. falciuparum* resistance to current antimalarial drugs. Screening of plants used in traditional medicine for antiplasmodial activity is one way to discover new effective agents [9, 30].

In vitro inhibitory activity of aqueous, methanolic and hexanic extracts of *Dialium dinklagei, Cnestis ferruginea and Trema orientalis* leaves on chloroquine sensitive and chloroquine resistant laboratory strains and field isolates *P. falciparum* were tested.

The determination of antimalarial activity on reference strains of P. falciparum also raises the problem of the validity of in vitro tests. Indeed, these different strains used are adapted to laboratory culture and may not match the reality of the environment. This is why it appeared useful to evaluate the activity of the extracts on isolates from malaria patients in endemic areas. In brief, our results indicate that decoction extract of *Cnestis ferrugineaand Dialium dinklagei* showed good activity againts fields and references parasites. The methanolic extract of *Trema orientalis* had moderate antiplasmodial activity.

The results showed that no plant is active with the hexanic extract.

Cnestis ferruginea was active with the decoction extracts with activities ranging from 11.78µg/mL to 13.94µg/mL. *Cnestis ferruginea* has not yet been tested for its antimalarial activity, however it is well known for antibacterial property, and has been used traditionally to treat several infectious diseases [31]. Aqueous extracts of *C. ferruginea* leaves showed antimicrobial activity due to the presence of hydroquinone and caffeic acid methyl ester [32]. The antiplasmodial activity observed in our study could be due to the presence of these chemical compounds.

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A review found that many *Connaraceae* species with associated pharmacological potential have traditional medicinal use in tropical areas worldwide. The traditional uses reported in the different ethnobotanical studies include a wide range of therapeutic functions, which can guide research for actions that have not been confirmed yet by the scientific community [33].

Trema orientalis had moderate activity with the methanolic extract with activities ranging from 14.46µg/mL to 28.32µg/mL. However, no activity was observed with hexanic and aqueous extracts. Very little work has been done in vitro, however several studies have been done on the *in vivo* antiplasmodial activity. In fact, the other study indicates that the aqueous leaf and bark extracts of *T. orientalis* have good antiplasmodial activity, with varying degree and/or differential effect on the meassured parameters [33–35]. According to these results, the acetone leaf extract of *T. orientalis* has antiplasmodial activities and dosage of 800 mg/kg/day is the most effective dose [36–38]. In the prophylactic experiment, dichloromethane, methanol

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fraction and extract showed significant chemopreventive effects against *P. berghei* invasion of the red blood cells when compared with both Sulfadoxine-Pyrimethamine and untreated controls [39]. In addition, these study provides evidence of the claims by the traditional healers of the efficacy of aqueous extracts of *T. orientalis*.

Dialium dinklagei was active with both methanolic and aqueous extracts with activities ranging from 12.80µg/mL to 21.67µg/mL. According to Akoué et al, the abundance of bioactive compounds would explain the therapeutic effects observed and the use of *Dialium dinklagei* in traditional medicine. Abundance in secondary metabolites of *Dialium dinklagei* can justifies their use in ethnotherapy by populations [40].

All plants species exhibited *in vitro* antiplamodial activity against reference strain of *Plasmodium falciparum*. Some activities are less than 15 µg/mL and are considered promising according toBero et al [41, 42]**.** The four field isolates tested were Chloroquine sensible. Chloroquine has been withdrawn from malaria treatment guidelines since 2003. It seemed that this molecule became currently active on falciparum isolates. However, this reversion of chloroquine resistance should be confirmed by future *in vitro* and *in vivo* and molecular studies. Quinine and Artesunate whowed good activity againts field isolates.

Haemolytic activity represents a useful starting point as it provides the primary information on the interaction between molecules and biological entities at cellular level. Haemolytic activity of any compounds is an indicator of general cytotoxicity towards normal healthy cell. The results of this study indicated the absence of hemolytic activity. This indicates that anti-plasmodial activity was not due to haemolysis of red blood cells but with a real effect of the extracts against the parasite. Therefore, we can conclude that the results obtained during the antiplasmodial activity are not influenced by this weak haemolytic action [43].

4 Conclusion

Our experimental approach allowed to select extracts with good antiplamodial activities and to validate their using of the traditional Ivorian pharmacopoeia in the treatment of malaria. Overall, the *in vitro* activities of these plant extracts are compatible with their use as traditional remedies for malaria. Although the traditional healers used some plants to treat malaria symptoms these had no antiplasmodial activity *in vitro*. It should be noted that we only tested antiplasmodial activity on the asexual erythrocytic stage of *Plasmodium falciparum*, and the extracts that we found inactive might possibly inhibit other parasite stages. Alternatively, some plants without *in vitro* antiplasmodial activity may stimulate the immune response. In addition, the haemolytic activity tests of the extracts did not reveal

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hemolytic activity which could interfere with the antimalarial activity. This work could be a starting point for the development of traditionally improved drugs in the treatment of malaria, after deepening aspects of this study and conducting clinical trials.

Compliance with ethical standards

Conflict of interest : The authors declare no conflict of interest.

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