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Research Article
Antiplasmodial Activity of *Diospyros monbuttensis* and *Newbouldia laevis*, Two Ivorian Medicinal Plants

[1]Offianan André Toure, [1]Tuo Karim, [1]Beourou Sylvain, [1]Koffi David, [2,3]Djaman Allico Joseph and [3]Coulibaly Adama

[1]Department of Parasitology and Mycology, Institut Pasteur of Côte d'Ivoire, Côte d'Ivoire
[2]Department of Clinical and Basic Biochemistry, Institut Pasteur of Côte d'Ivoire, Côte d'Ivoire
[3]Laboratory of Pharmacodynamics-Biochemistry, Félix Houphouet-Boigny University, Côte d'Ivoire

Abstract

Background and Objective: Resistance of human malaria parasites to anti-malarial compounds has become a considerable concern. 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 two medicinal plants, *Diospyros monbuttensis* and *Newbouldia laevis*, used in traditional medicine in Côte d'Ivoire, for *in vitro* antiplasmodial activities. **Materials and Methods:** SYBR GREEN fluorescence method 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 anti-malarials in comparison to plant extracts. In addition, the haemolytic activity of extracts with good antiplasmodial activity was evaluated. The IC_{50} and the corresponding correlation coefficients were determined graphically using *in vitro* Analysis and Reporting Tool (IVART) software of WWARN. **Results:** Methanol crude extract of *Newbouldia laevis* showed promising activity against field isolates and reference parasites while *Diospyros monbuttensis* had moderate antiplasmodial activity. The liquid-liquid partition has significantly improved antiplasmodial activity with F3 fraction of *Newbouldia laevis*. There was less than 1% hemolysis at the concentration of 200 μg mL^{-1} of plant extracts. **Conclusion:** These results validate the reported traditional use of *Diospyros monbuttensis* and *Newbouldia laevis* for malaria treatment in Côte d'Ivoire.

Key words: *Plasmodium falciparum*, haemolytic, *in vitro*, *Newbouldia laevis*, antiplasmodial, malaria and *Diospyros monbuttensis*

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Corresponding Author: Tuo Karim, Department of Parasitology and Mycology, Institut Pasteur of Côte d'Ivoire, Côte d'Ivoire Tel: 00225 08355392

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Despite the aforementioned efforts, malaria remains the number one killer disease of public health importance in many sub-Saharan countries[1]. More than two billion people are likely to contract malaria worldwide[1].

Emergence and rapid extension of *Plasmodium falciparum* resistance to the usual anti-malarials such as chloroquine, sulfadoxine-pyrimethamine and recently to the derivatives of artemisinin make urgent the discovery of new anti-malarial compounds[2-4].

It is well known that most widely used curative anti-malarial drugs are plant products, quinine and artemisinin. In addition, many phytochemical compounds with anti-plasmodial activity were isolated from plants[5]. The success of quinine and artemisinin isolated, respectively, from *Cinchona* spp. and *Atermisia annua* has focused attention on plants as a source of antimalarial drugs[6].

About 75% of people in Africa do not have direct access to conventinal medicine for malaria treatment. But they do have access for medicinal plants to treat malaria. Natural products isolated from plants constitute a potential source of new antimalarial drugs.

In Côte d'Ivoire, malaria transmission is intense and perennial in major parts of the country. Malaria morbidity and mortality remain high despite control strategies implemented by the National Malaria Control Program for many years. These strategies are based on the use of long lasting insecticidal nets (LLINs), intermittent preventive treatment with sulfadoxine-pyrimethamine (IPT-SP) and environmental sanitation, prompt diagnosis and treatment of malaria cases with artemisinin-based combination therapy (ACT). Despite the implementation of modern treatment in most regions, the majority of the population still uses traditional medicines to treat malaria. The most frequently mentioned reasons are the high cost of treatment and often cultural factors.

Diospyros monbuttensis and *Newbouldia laevis* have been reported in such areas in Côte d'ivoire as sources of treatment for various diseases, including malaria and/or related symptoms.

Diospyros monbuttensis Gürke is a plant species found in central Côte d'Ivoire. In this area, this plant is called "wood of God"[7]. It is considered as a good remedy for feverish aches, stomach pains, edema and leprosy[7,8]. The leaves are also used to treat chickenpox[8,9].

Newbouldia laevis is native to tropical Africa and thrives on moist, well-drained soils. It is also found in secondary forests extending from Senegal to Cameroon, Gabon, the Democratic Republic of Congo and Angola[10]. In Nigeria, the bark is chewed and swallowed for stomach pain, diarrhea and toothache[11]. This plant is reputed to be effective in the treatment of elephantiasis, dysentery, rheumatic swelling, syphilis, constipation and as a dewormer. It is also used for earaches, sore feet, chest pain, epilepsy and convulsions in children[12]. The leaf, stem and fruit are used as febrifuge and for the treatment of wounds and stomach ache[13]. The roots of *Newbouldia laevis* are used in Benin for the treatment of Buruli ulcer[14]. This plant is also used in cases of constipation, gastrointestinal pain and bronchial pneumonia. Externally, it is used to treat intercostal pain, rheumatic pain, neuralgia and toothache; it is also used to treat venereal diseases and known to facilitate childbirth[7,15,16].

Ethnobotanical information about anti-malarial activity of *Diospyros monbuttensis* and *Newbouldia laevis* is essential for further evaluation of the efficacy of plant anti-malarial remedies. Treatment with these remedies has suffered a number of deficiencies; identification of plant extracts may be insecure and the chemical content of extracts may vary considerably.

Lack of new antimalarial drugs in the pipeline and reduced sensitivity, highlight the urgent need to search for new antimalarial drugs to replace the currently used or to expand the antimalarial drug arsenal.

Natural products isolated from plants used in traditional medicine, which have potent anti-plasmodial action *in vitro*, represent potential sources of new anti-malarial drugs

This study was, therefore, designed to investigate *in vitro* antiplasmodial and haemolytic activities of extracts and fractions from leaves of *Diospyros monbuttensis* and *Newbouldia laevis* to provide scientific proof of the efficacies claimed by traditional healers.

MATERIALS AND METHODS

Plant materials: Plant samples consisted of the leaves of *Diospyros monbuttensis* and *Newbouldia laevis*. The leaves of *Diospyros monbuttensis* were harvested in the month of January 2013 at Talahini-Sokoura in the Department of Sandegué (North-East of Cote d'Ivoire). Those of *Newbouldia laevis*, were collected in March 2013 in the District of Abidjan. The timing of the plant harvest was the morning at 9 AM.

Biological materials: The biological samples consisted of blood samples of Group O with positive Rhesus (Rh+) for inoculum dilution with clinical and reference strains of *Plasmodium falciparum*.

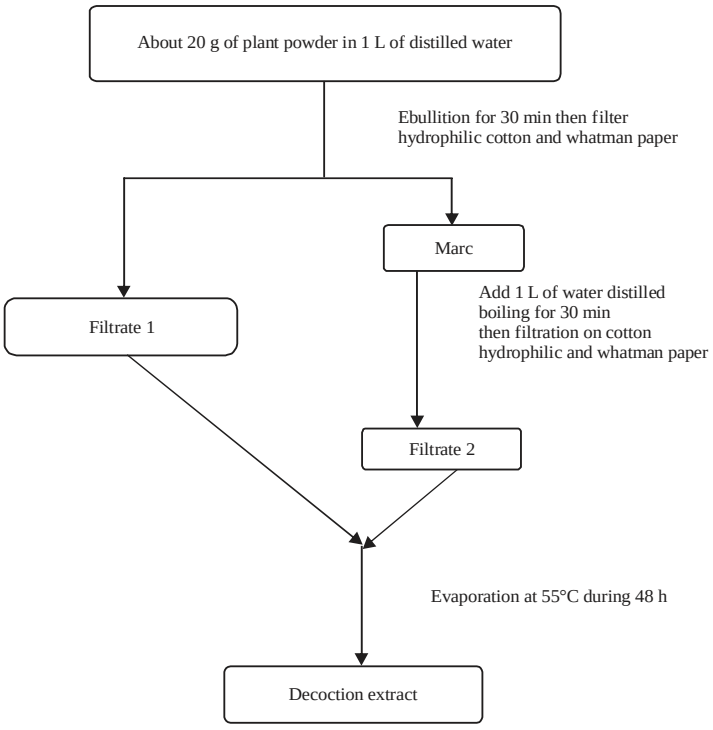

Fig. 1: Obtention of decoction extract

The ATCC reference strains: 3D7 chloroquino-sensitive was provided by the Biochemistry and Molecular Department, University of Legon, Ghana and Dd2 chloroquino-resistant was provided by MRA-156, lot N°58319486, MR4, ATCC® Manassas, Virginia, USA.

Ethnobotanical survey, bibliographic research and selection of studied plants: The investigations were carried out in November 2013 in Abidjan and Bondoukou (East Côte d'Ivoire) by ethnobotanical approaches with 27 actors of traditional medicine[17]. Ethnobotanical data (local name, method of preparation, traditional use, combination of plants, indications, dosage, contraindications and side effects) were obtained through conversations with traditional healers.

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

Extracts preparation: 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, 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 polarity (hexan, methanol and water) were done according to previous protocols by Zirihi *et al.*[18] and Bekro *et al.*[19] (Fig. 1, 2).

In order to improve anti-plasmodial activity, crude extracts were separated by partition chromatography using solvents of increasing polarity (diethyl ether, butanol and ethyl acetate) as previously[20-22] (Fig. 3).

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 and infected with *P. falciparum* malaria after informed consent was obtained. The samples were then transfered at 4°C to the Swiss Center for Scientific Research for *in vitro* test.

***In vitro* anti-plasmodial assay:** Anti-plasmodial activity was analyzed with the SYBR Green method. The assays were carried out on 96-well plates filled with infected red blood cells (IRBCs) in the following proportions of parasitaemia <0.3% and hematocrit 5%. The *in vitro P. falciparum* continuous culture used in our assays was derived from that developed by Trager and Jensen[23]. Inhibition of parasite growth was measured using the SYBR Green method[24-28].

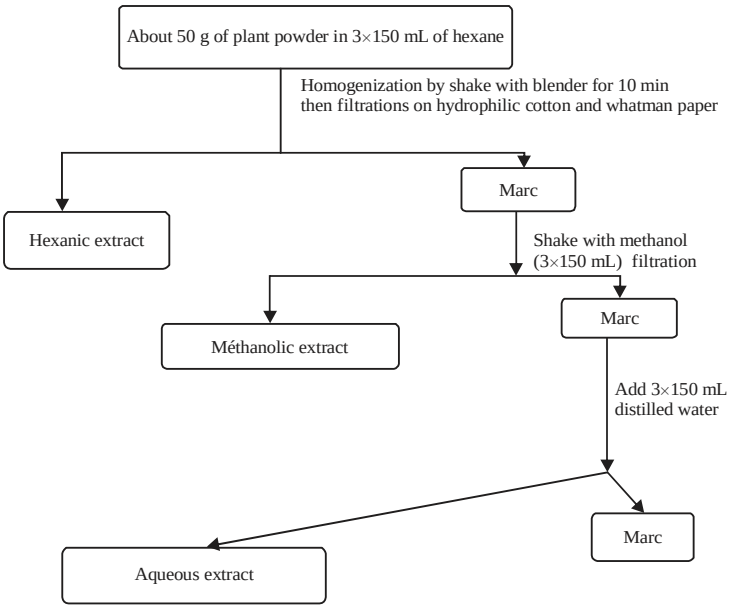

Fig. 2: Obtention of hexanic, methanolic and aqueous extracts[18,19]

The reading was done with the Spectra Max GEMINI XPS spectrofluorometer (Molecular Devices) at 535 nm after excitation at 485 nm. The IC_{50} were determined graphically, using *in vitro* Analysis and Reporting Tool (IVART) software of WWARN[25,27].

***In vitro* hemolysis assay:** A stock solution of the samples was prepared in the appropriate solvent at the concentrations of 100 and 50 µg mL^{-1}, 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. The positive control was prepared with 10 µL of 20% Triton X-100+190 µL of 10% RBC. The tubes were centrifuged for 5 min at 2200 rpm and 150 µL of supernatant 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:

$$\text{Hemolysis (\%)} = \frac{\text{Abs sample-Abs negative control}}{\text{Abs positive control-Abs negative control}} \times 100$$

where, Abs is the absorbance at 550 nm.

Ethical issues: The study was conducted in accordance with the local laws and regulations and the 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.

RESULTS

Anti-plasmodial activity: The methanol crude extract of *Newbouldia laevis* showed good activity against fields and reference parasites while the methanolic extract of *Diospyros monbuttensis* had moderate anti-plasmodial activity.

The four field isolates tested were CQ sensitive. Quinine and artesunate showed good activity against the field isolates (Table 1).

The highest selective anti-plasmodial activity was found with *Newbouldia laevis* acetate fraction. Methanolic and aqueous fractions also had a good activity on field isolates and Dd2 strain. All of the results were presented in Table 2.

Hemolytic activity: No *Newbouldia laevis* fraction extract was found to exhibit significant red blood cells lysis activity with a percentage of haemolysis <1% for all tested extracts (conc. = 100 and 200 µg mL^{-1}). Therefore, this anti-plasmodial activity was not associated with haemolysis of red blood cells but with a real effect on the parasite (Table 3).

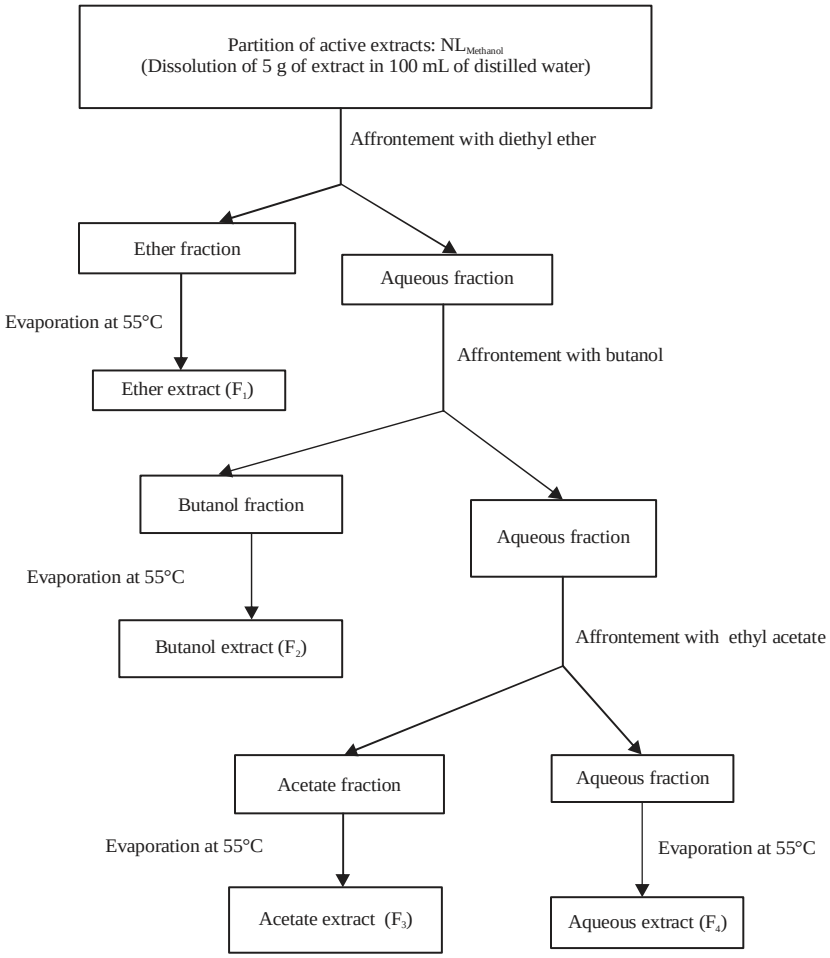

Fig. 3: Successive liquid-liquid using diethyl-ether, butanol and acetate solvents[20-22]

$NL_{Methanol}$: Methanolic extract of *Newboudia laevis*

Table 1: Antiplasmodial activity of crude extracts

			Strains/CI_{50} (µg mL^{-1})					
			Isolates				Reference strains	
Plants	Extracts	Extraction yield (%)	W536	W539	W552	ANK02	3D7	Dd2
Diospyros monbuttensis	Dec	18.0	35.43±6.11	39.32±4.53	47.57±2.59	41.23±2.63	38.26±3.33	43.56±5.32
	Hex	1.6	>50	>50	>50	>50	>50	>50
	Met	17.8	29.21±1.78	23.67±4.35	27.89±3.61	30.78±2.22	23.51±3.26	25.41±4.76
	Aq	7.6	>50	>50	>50	>50	>50	>50
Newbouldia laevis	Dec	10.9	>50	>50	>50	>50	>50	>50
	Hex	1.2	>50	>50	>50	>50	>50	>50
	Met	4.4	11.51±0.43	11.45±0.36	12.85±0.74	10.21±3.22	13.35±1.46	10.79±2.15
	Aq	5.0	>50	>50	>50	>50	>50	>50
Chloroquine (nM)			33.01±0.92 (0.01µg mL^{-1})	42.71±1.32 (0.013 µg mL^{-1})	37.31±3.24 (0.011 µg mL^{-1})	35.38±4.92 (0.011 µg mL^{-1})	51.07±2.23 (0.016 µg mL^{-1})	116.71±5.11 (0.037 µg mL^{-1})
Quinine (nM)			5.76±0.95 (0.0019 µg mL^{-1})	23.87±1,12 (0.008 µg mL^{-1})	44.37±2.15 (0.015 µg mL^{-1})			
Artesunate (nM)			3.43±0.49 (0.0013 µg mL^{-1})	2.22±0.16 (0.0008 µg mL^{-1})	6.31±3.01 (0.0024 µg mL^{-1})			

Dec: Decotion, Hex: Hexanic, Met: Methanolic, Aq: Aqueous

Table 2: Anti-plasmodial activity of *Newbouldia laevis* chromatography fractions

		Isolates		Reference strains
Extracts	Yield (%)	W639	A149	Dd2
$F1_{Methanol}$	52.0	9.63±2.21	9.39±2.83	13.29±2.71
$F2_{butanol}$	16.5	9.26±1.74	22.50±1	19.36±3.05
$F3_{acetate}$	5.2	6.11±1.3	12.62±1	6.32±1.7
$F4_{aqueous}$	23.8	8.00±0.9	7.32±1.18	17.51±2.31
		26.49±2.33 nM	52.49±3.82 nM	129±7.32 nM
		(0.008 μg mL^{-1})	(0.016 μg mL^{-1})	(0.041 μg mL^{-1})
		1.59±0.46 nM	3.16±0.38 nM	1.36±0.56 nM
		(0.0006 μg mL^{-1})	(0.0012 μg mL^{-1})	(0.0005 μg mL^{-1})

F_1: Ether fraction, F_2: Butanol fraction, F_3: Acetate fraction and F_4: Aqueous fraction

Table 3: Haemolytic activity of *Newbouldia laevis* extracts

Extract	Concentration (μg mL^{-1})	Haemolytic activity (%)
$NL_{Methanol}$	200	1.04
	100	0.97
$F1_{ether}$	200	0.85
	100	0.70
$F2_{butanol}$	200	1.09
	100	0.77
$F3_{acetate}$	200	1.04
	100	0.95
$F4_{aqueous}$	200	0.82
	100	0.63
Negative control (PBS)	-	0.00
Positive control (Triton X100)	-	100

DISCUSSION

The spread of *P. falciuparum* resistance to anti-malarial drugs is a major reason for research and discovery of new effective antimalarial agents[29]. Screening of plants used in traditional medicine for anti-plasmodial activity is one way to discover promising drugs/compounds[30-32].

In vitro inhibitory activity of aqueous, methanolic and ethanolic extracts of *Newbouldia laevis* and *Diospyros monbuttens* leaves on chloroquine sensitive and chloroquine resistant laboratory strains and field isolates *P. falciparum* were tested. Results showed a strong anti-plasmodial activity on Dd2 and 3D7 laboratory strains with methanolic crude extract of *Newbouldia laevis* leaves. In contrast, the hexanic, decoction and aqueous crude extracts of the same plant were inactive on both strains. The aqueous and hexanic crude extract of *Diospyros monbuttensis* were also inactive. Meanwhile, decoction and methanolic crude extracts had a moderate anti-plasmodial activity against all strains, both from the fields and laboratory. The liquid-liquid partition had significantly improved anti-plasmodial activity. The highest selective anti-plasmodial activity was found with *Newbouldia laevis* acetate fraction with 6.11±1.3 μg mL^{-1} of IC_{50} on Dd2 strains. This interesting anti-plasmodial activity of *Newbouldia laevis* was confirmed by previous studies[33-35]. The anti-plasmodial activity of *Newbouldia laevis* leaves could be due to its chemical compounds. Previous studies showed that the leaves of *Newbouldia laevis* contain plenty of saponins and alkaloids, moderate flavonoids, polyphenols, anthocyanins, tannins and in low quantities, sterols, polyterpenes, coumarins, quinones and leucoanthocyanins[36,37].

For anti-plasmodial activity of *Diospyros monbuttensis*, similar results were observed by Olasehinde *et al.*[6] with an IC_{50} of 32 μg mL^{-1} with the methanolic extract.

In this study, chloroquine was active against the field isolates and the Dd2 strain. The values of IC_{50} obtained were lower compared to those found in previous studies. 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*, *in vivo* and molecular studies.

Haemolytic activity represents a useful starting point as it provides the primary information of the interaction between molecules and biological entities at cellular level. The haemolytic activity of a compounds is an indicator of general cytotoxicity towards normal healthy red blood cells. The results of this study indicated that the majority of *Newbouldia laevis* extracts have low hemolytic activity suggesting that the anti-plasmodial activity of the extracts of this plant was not due to haemolysis of the red blood cells but with a real effect of the extracts against the parasite. Therefore, it was concluded that the results obtained during the anti-plasmodial activity tests were not influenced by this weak haemolytic action[35].

These data suggested that the non-haemolytic effect of *Newbouldia laevis* extracts makes it suitable for the preparation of drugs in the treatment of malaria.

While synthetic pharmaceutical agents continue to dominate research, attention had increasingly been directed to natural products. In previous works saponins, flavonoid, alkaloids and cardiac glycoside were found to be present in

the *Newbouldia laevis* plants while polyphenols, quiniones and saponins were found in *Diospyros monbuttensis*[36,37]. These results will be helpful to phytochemists and pharmacologists for identification of new active compounds from plants.

The identification and isolation of the bioactive components of *Newbouldia laevis* will serve as a basis for in-depth pharmacological evaluation of bioactive phytochemicals for anti-malarial drugs development. Further works will also focused on identification of single chemical entity responsible for anti-plasmodial activity from the leaves of *Newbouldia laevis*, using ethyl acetate fraction.

CONCLUSION

Current experimental approach allowed to identify plant extracts with good anti-plasmodial activities and to validate their use in the traditional Ivorian pharmacopoeia for the treatment of malaria. In addition, the haemolytic activity tests of the extracts did not reveal haemolytic 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 identifying the mechanism of action and completing further pre-clinical and clinical studies.

SIGNIFICANCE STATEMENT

Screening of plants used in traditional medicine for anti-plasmodial activity is one way to discover promising drugs/compounds. This study is a contribution to the evaluation of ethnomedicinal plants for search of new chemical compounds with known anti-plasmodial properties. Current findings increase database of *Diospyros monbuttensis* and *Newbouldia laevis* whose extracts are active against *P. falciparum* parasite and confirm the use of the 2 plants in malaria treatment by traditional healers.

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