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(RESEARCH ARTICLE)

Antiplasmodial activity of *Anthocleista djalonensis* leaves extracts against clinical isolates of *Plasmodium falciparum* and multidrug resistant K1 strains

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Abstract

Information collected from nine (09) traditional healers in the Moronou village in the Department of Toumodi revealed that *Anthocleista djanlonensis* is regularly used by the population for primary health care in the processing of malaria. Evaluation of the *In vitro* antiplasmodial activity showed that the aqueous extracts inhibit growth of clinical isolates and chloroquinoresistant strains (K1) with IC_{50} of 8.29 µg/mL and 10.23 µg/mL while the ethanolic extracts had IC_{50} of 37.65 µg/mL and 46.07 µg/mL on the same strains respectively. Results of the *In vitro* antimalarial bioassay showed that aqueous extracts have *promising antiplasmodial* effects on clinical isolates and on *Plasmodium falciparum* multidrug resistant K1 strain (3 µg/mL $< IC_{50} <$ 15 µg/mL). Phytochemical screening revealed that the extracts contain mainly alkaloids, polyphenols, polyterpenes and flavonoids

Keywords: Medicinal plants; Toumodi, malaria; Phytochemical screening; Côte d'Ivoire; *Anthocleista djalonensis*

1. Introduction

According to the World Health Organization's Malaria Report, there were 228 million cases of malaria in 2018. The estimated number of malaria deaths stood at 405,000 in the same year [1]. The absence to date of an effective vaccine leaves us only chemotherapy to fight against *Plasmodium falciparum* infection, the most virulent Plasmodium species that infects humans. One of the problems of malaria control is the emergence and spread of *P. falciparum* strains that become resistant to almost all drugs available. Chloroquine (CQ) was one one of the used molecules in the fight against malaria because of its cost-effectiveness, but just some years after being placed on the market, the first cases of chloroquine resistance emerged in Southeast Asia, then in Latin America before spreading to all endemic areas [2]. Later, this phenomenon was repeated for the other available drugs (proguanil, sulfadoxine–pyrimethamine, halofantrine, mefloquine). To prevent, or at least delay the onset of new resistant strains, WHO recommended in 2001 the use of drugs association and that one of the drugs be an artemisinin derivative [3]. At that time, no artemisinin resistance had been identified yet and many hopes were placed onto artemisinins. Unfortunately, in 2008 Noedl et al. reported evidence of artemisinin resistance in Western Cambodia [4]. To date, artemisinin resistance has spread to Thailand, Myanmar, Laos and Vietnam [5, 6, 7]. Constituting a promising source of new drugs, medicinal plants have been given a priority interest worldwide in the search of safe and effective antiplasmodial agents from plants [8]. Artemisinin derivatives and cinchona alkaloids, such as quinine, are quite exemplary of such assertions. Studies conducted on several traditionally claimed Ivorian medicinal plants confirmed their antimalarial activities [9, 10]. Accordingly, the present study aimed at investigating the *In vitro* antimalarial activity of *Anthocleista djolonensis*, an important medicinal plant used in the Toumodi region for the treatment of malaria [11]. *Anthocleista djalonensis* is a

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woody plant 8-15 m high, growing on river banks. The leaves are 9-35 cm or even 1m long, opposite, petiolate, with oval or elliptical blades. The flowers are grouped in long corymbs. Fruits are elliptic berries [12]. This study evaluated the *In vitro* antimalarial activity of aqueous extracts and 70% ethanol of A. *djalonensis leaves against* chloroquine-sensitive (CQ-S) NF54 and chloroquine-resistant (CQ-R) K1 strains.

2. Material and methods

2.1. Collection and preparation of plant extract

Fresh leaves of *Anthocleista djalonensis* A.Chev were collected in the Moronou village at the Department of Toumodi (Central Côte d'Ivoire) between July and September 2014. After identification at the National Floristic Center and a sample deposit at the herbarium, plant samples were air dried in shade at room temperature and ground into powder. Two times 100g of the powder were macerated respectively in 1L of distilled water and 1L of 70% ethanol hydroalcoholic solvent using a blender. Macerates were filtered twice on hydrophilic cotton and once on Whatmann filter paper. Filtrates were evaporated through rotary vacuum evaporator and dried in an oven at 45 ^{o}C for 48 h to obtain aqueous and hydroethanolic extracts which were stored at 4 ^{o}C for further use [13].

2.2. Malaria parasites

Informed consent was obtained from all patients in this study prior to clinical isolates collection. Four fresh clinical isolates of *Plasmodium falciparum* such as W6622, W6708, W6743 and W7177 were obtained from symptomatic patients at the Urban Health Unit (FSU-COM) of Wassakara in the district of Yopougon (Abidjan). Moreover, *Plasmodium falciparum* multidrug resistant K1 strain and *Plasmodium falciparum* chloroquine sensitive NF54 strains obtained from Medicine for Malaria Venture (MMV) were used for this study. The parasites were cultivated and maintained continuously in a human type O positive erythrocytes according to the method described by Trager and Jensen [14].

2.3. *In vitro* antiplasmodial assay

Culture medium was consisted of RPMI 1640 medium [supplemented with 12.60 mL HEPES (25 mM), 100 mL hypoxanthine, 312.5 lL gentamycin (40 mg/mL) and glucose (20 g/L, Wagtech)]. Symptomatic blood samples of patients collected in EDTA collecting tubes were centrifuged at 3000 rpm for 5 min, then blood serum and buffy coat were removed and blood pellet washed thrice in RPMI 1640 medium (Gibco USA) and diluted with uninfected human type O positive red blood cells to reach a parasitemia of 0.24% at 1.5% hematocrit. Thawing of *Plasmodium falciparum* K1 strain was performed according to the method described by Witkowski et al. (2013) [15]. After withdrawing the cryovial from the nitrogen liquid, it was left thawing inside the Biosafety hood Class II (STERILGUARD) and transferred in a Falcon tube (15 mL) and then centrifuged at 3000 rpm for 5 min. The supernatant was removed, an equal volume of NaCl (3.5%) was added dropwise to blood pellet and slowly stirred. The tube was left resting for 1 min, then 12 mL of RPMI 1640 washing medium preheated at 37 ^{o}C was added and centrifuged at 3000 rpm for 5 min and the supernatant was removed. Then 50 µL of the blood pellet was suspended in 8 mL of complete medium in a culture flask cells (25 mL, Nunc WVR) and a volume of 110 µL of uninfected human type O positive red blood cells were added at 2% hematocrit. Daily, the infected blood pellets were transferred into fresh complete medium to propagate the culture. The stock solution of both crude extracts and Chloroquine were dissolved separately, 10 mg of each substance in 10 mL of distilled water to obtain a concentration of 1 mg/mL. Extract stock solutions were autoclaved at 121 ^{o}C for 15 min to sterilize them. As for reference molecules a 0.22 µm Millipore filter was used for filtration. Aliquot of extracts and refence molecules were diluted in a complete medium and 100 of each aliquot was a twofold serial dilutions (100 µL) were performed in a 96 well microplate and concentrations ranged from 100 to 1.56 µg/mL for crude extracts and from 1600 to 3.125 nM for chloroquine. *Plasmodium falciparum* multidrug resistant K1 strains and *Plasmodium falciparum* chloroquine sensitive NF54 strains were synchronized by 10% D sorbitol (w/v) treatment at the ring stage prior to test. Then a volume of 100 µL of the inoculum (parasitized erythrocytes) was added to each well to reach a final volume of 200 lL. Infected erythrocytes non-treated with drugs were used as negative control whereas infected erythrocytes treated with chloroquine (CQ) were used as positive control. All experiments were run in duplicate. Microplates were confined in a candle jar saturated with CO_2 and incubated at 37 ^{o}C in an incubator for 72 h. After 72 h of incubation, microplates were preserved at - 20 ^{o}C.

2.4. Determination of IC_{50}

After thawing of the 96 well microplates 100 µL of each well containing a volume of 200 µL was transferred in a new 96 well microplate and 100 µL of SYBR Green I lysis buffer (5 µL of SYBR Green was mixed to 25 mL of lysis buffer) was added to each well using a multi-channel pipette and incubated in a dark room at 37 ^{o}C for 1 h. Fluorescence was measured with a spectro-fluorimeter BIOTEK microplate reader (BIOTEK, FLX 800) with excitation and emission wavelength bands centered at 485 and 530 nm, respectively. IC_{50} (concentration of a tested substance inhibiting 50% of parasites growth) was determined through analysis of dose–response curves using the software IVART (*In vitro* Analysis and Reporting Tool) of WWARN [16].

2.5. Phytochemical Screening

Detection of major chemical groups was carried out according to the analytical techniques described by Tona et al. (1998) [17], Longanga et al. (2000) [18] improved by Békro et al. (2007) [19]. Phytochemical groups sought are essentially sterols, polyterpenes, alkaloids, tannins, polyphenols, flavonoids, quinones and saponins.Sterols and polyterpens.

2.6. Sterols and polyterpenes

Extracts (0.1 g) were dissolved in 1 mL of hot acetic anhydride in a capsule. The resulted solutions were poured and added with 0.5 mL H2SO4. A violet coloration that turned in blue, and then in green revealed the presence of sterols and triterpenes.

2.7. Polyphenols

A drop of alcoholic solution of 2% ferric chloride was added to 2 mL of extracts. A blue-blackish to green darkish coloration indicated a positive reaction.

2.8. Flavonoids

In a tube containing 3 mL of extract, a few drops of 10% NaOH were added. Appearance of yellow-orange color indicated the presence of flavonoids.

2.9. Catechic tannins

Two milliliters of water and few drops of 1% ferric chloride were added to 1 mL of extract. The appearance of a blue, blue-black or black coloration indicated the presence of gallic tannins, the green or dark green coloration showed the presence of catechic tannins.

2.10. Gallic tannins

Previous solution was filtered and saturated with sodium acetate. Addition of 3 drops of 2% FeCl3 causes appearance of an intense blue-black color denoting gallic tannins presence.

2.11. Quinonic substances

An aliquot (0.1 g) of extract was dissolved in 5 mL of diluted HCl (1/5) and heated in a boiling water bath for 30 minutes, and then extracted with 20 mL of CHCl3 after cooling. To the organic phase was added 0.5 mL of 50% NH4OH diluted solution. The positivity of the reaction was indicated by a red to violet color.

2.12. Alkaloids

Two drops of Bouchard's reagent (reagent of iodine-iodide) were added to 1 mL of each extract. A red-brown precipitate indicated a positive reaction.

2.13. Saponins (foam index)

Samples (0.1 g of dry extract) were dissolved in 10 mL of distilled water. The samples were shaken vigorously up and down for 30-45 seconds and then left for 15minutes. The height of the foam was measured. Persistent foam for more than 1 cm high indicated the presence of saponins.

2.14. Characterization on thin layer chromatography (TLC)

Analyses were carried out in the normal phase, with silica plates (Silicagel 60F254, 0.25 mm thick) deposited on aluminium sheets (stationary phase). On the prepared plates, 10 µL of each extract was deposited. Then, the plates were introduced into glass vats previously saturated on the mobile phase (ethyl acetate+methanol+water 100:12:8). After development, the TLC plates were dried, observed under UV lamp and sprayed with reagents including 5% methanolic potassium hydroxide, Godin and Dragendorff reagents. Plates were dried at 60 ^{o}C for 5 min and 110 ^{o}C for 10 min to reveal the spots resulting from the separation [20]. Each substance was identified by its fluorescence under UV light, by its frontal ratio (Rf) in a specific solvent system and by its colour after revelation with a specific reagent.

2.15. Statistical analyses

Graphics were performed using Graphpad prism 5 software (Microsoft, San Diego California, USA). All values were expressed as mean ± Standard of deviation. Data analysis were performed using one way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparisms test using Graphpad instat® software. Values were statistically significant at $p<0.05$.

2.16. Ethical consideration

Informed consent was obtained from all patients enrolled in this study.

3. Results and discussion

3.1. Results

This study evaluated the anti-plasmodic activity of aqueous and ethanolic extracts from the leaves of *Anthocleista djalonensis* and qualitatively analyzed the chemical compound of these extracts.

3.1.1. Preparation of extracts

Decoction of water is the extraction method used by traditional healers to treat malaria. This technique was used to extract the bioactive compounds contained in *Anthocleista djalonensis*. The masses of the aqueous decoctate and that of the hydro-ethanolic organic extract are given in Table 1. The hydro-ethanolic and aqueous extracts gave yields of 8.8% and 7.5% respectively.

Table 1 Extraction report

Solvent	Mass obtained	Yield (%)	Appearance
Water	7,5g	7,5%	Light brown
Ethanol	8,8g	8,8%	Dark brown

3.1.2. Phytochemical screening

Phytochemical screening has shown that the solvents used solubilized several secondary metabolites. Alkaloids, polyphenols, polyterpenes, flavonoids and catechetic tannins were found to be present in both extracts with a predominance of polyphenols and alkaloids in the aqueous extract. Catechetical tannins and saponosides were absent in both extracts. The results of the phytochemical screening are reported in Table 2.

Table 2 Phytochemical screening of *Anthocleista djalonensis* leaves

Chemical compound	Reaction/reagent	Coloration	Aqueous extract	Ethanolic extract
Sterols/polyterpenes	LR	Blue turns to green	+	+
Polyphenols	$FeCl_3$	Black bleu / Dark green	++	+
flavonoids	RC	Orange-pink/Purplish	+	+
catechic tannins	SR	Large flake precipitation	-	-
gallic tannins	$FeCl_3$	blue - black	+	+
Quinones	RBG	red/purple	-	+
alkaloids	DR	orange	++	+
	BR	Reddish brown	+	+
Saponosides	FM	4 cm of Foam	-	-

LR = Liebermann reagent; FeCl3 = Ferric chloride; RC = Reaction to Cyanidine; SR = Stiasny reagent; RBG = Borntraëger's reagent; DR = Dragendorff's reagent; RB = Burchard's reagent; FM = Foam Test; (++) = Positive reaction (abundant presence); (+) = Positive reaction (weak presence); (-) = Negative reaction (absence)

3.1.3. TLC analysis

Analysis of the chromatograms indicates that the aqueous extract contains only alkaloids and polyphenols. In fact, unlike Polyethylene Glycol, sulfuric vanillic acid and potassium hydroxide which gave a negative reaction, Dragendorff's reagent and iron trichloride showed a positive reaction by the respective appearance of orange color. On the other hand, the ethanolic extract contains in addition to these two compounds, quinones, flavonoids and terpenes in small quantities. Details of these results are shown in Table 3.

Table 3 Description of the chromatograms of the aqueous and ethanolic extracts of the leaves of *Anthocleista djalonensis*

Alkaloids			
	Color before reaction	color after reaction	Interpretation
Aqueous extract	Light brown	Dark orange	Remarquable presence
Ethanolic extract	Dark brown	orange	Presence
Flavonoïdes			
	Color before reaction	color after reaction	Interpretation
Aqueous extract	Light brown	Light brown	Absence
Ethanolic extract	Dark brown	yellow	Presence
Quinones			
	Couleur before reaction	color after reaction	Interpretation
Aqueous extract	Light brown		Absence
Ethanolic extract	Dark brown	Pink under UV	Presence
Polyphenols			
	Color before reaction	color after reaction	Interpretation
Aqueous extract	Light brown	brown	Remarquable presence
Ethanolic extract	Dark brown	brown	Presence
Terpenes			
	Color before reaction	color after reaction	Interpretation
Extrait aqueux	Light brown	Light brown	Absence
Extrait éthanolique	Dark brown	Purple	Presence

3.1.4. In vitro assessment of antiplasmodial activity

The mean IC_{50} for clinical isolates in aqueous extract was 21.87 ± 8.70 µg / mL with minimum and maximum IC_{50s} of 8.29 µg / mL and 31.71 µg / mL, respectively. This extract has an IC_{50} of 28.36 µg / mL on strain NF54 and 10.23 µg / mL on K1. The aqueous extract exerts activity on all isolates and on the reference strains (Figure 1). The mean IC_{50} of the ethanolic extract on clinical isolates was 42.29 ± 3.76 µg / mL with respective minimum and maximum IC_{50} of 37.65 µg / mL and 47.91 µg / mL. This extract has an IC_{50} of 48.30 µg / mL on strain NF54 and of 46.07 µg / mL on K1. Chloroquine has an IC_{50} of 12.8 nM on strain NF54 and 114 nM on K1. This molecule faces resistance (IC_{50}> 100 nM) with isolate W6622. The mean IC_{50} of clinical artesunate isolates is 1.98 ± 1.29 nM with minimum and maximum IC50s of 0.61 nM and 3.62 nM, respectively. The isolates and strain NF54 are susceptible to it. The mean IC_{50} of clinical quinine isolates is 9.34 ± 5.13 nM with minimum and maximum IC_{50s} of 3.06 nM and 16.39 nM, respectively. The data from the *In vitro* sensitivity tests of the extracts and of the reference molecules on the clinical isolates and the strains NF54 and K1 are given in Table IV

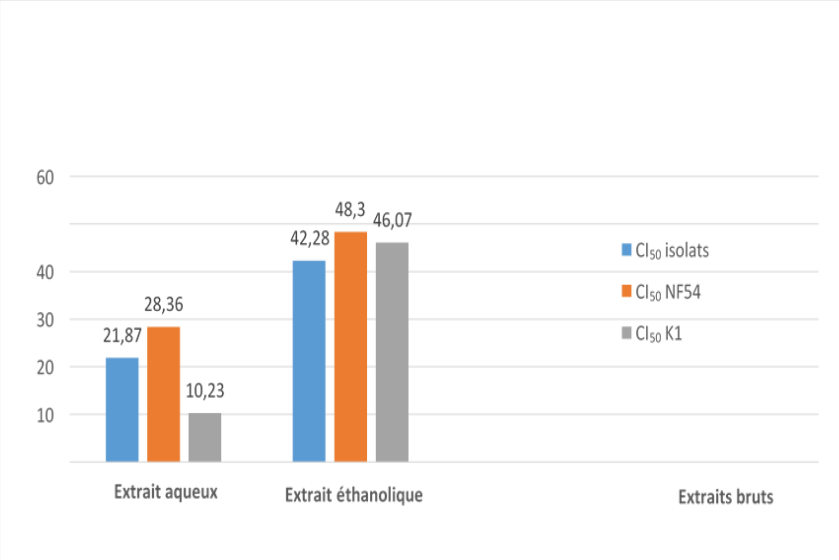

Figure 1 IC_{50} of aqueous and ethanolic extracts on clinical isolates and reference strains NF54 and K1

Table 4 Inhibitory concentration 50% of extracts (µg / mL) and pure molecules (nM) on clinical isolates and strains NF54 and K1

			IC_{50} drug				
			Aqueous extract	**Ethanolic extract**	**Chloroquine**	**Quinine**	**Artesunate**
Isolates	CQ-S	W6708	26,46	47,91	5,92	16,3	3,62
		W6743	31,71	37,65	16,22	11,94	2,89
		W7177	21,04	40,53	12,04	6,07	0,83
	CQ-R	W6622	8,29	43,06	106,39	3,06	0,61
Strain	NF54		28,36	48,3	12,8	1,19	3,29
	K1		10,23	46,07	114	nd	nd

nd : not determined

3.2. Discussion

The 70% hydro-ethanolic solvent has a better extraction yield than the aqueous extract and contains more secondary metabolites. Chemical analysis showed that the aqueous extract consists predominantly of alkaloids and polyphenols, in abundant quantities, while the organic extract contains in addition to the two compounds, polyterpenes, flavonoids and anthraquinones. These results are similar to those obtained in Côte d'Ivoire by Kabran et al (2012) [21] and in Nigeria by Nduche et al (2015) [22] which showed that the ethanolic extract of *A. djalonensis* contains a variety chemical compounds including polyphenols, flavonoids and alkaloids. The abundance of alkaloids and polyphenols in the aqueous extract indicates that these compounds have a high affinity for water. The two extracts exhibit antiplasmodial activity on the four clinical isolates and on the two reference strains. An *In vivo* study carried out in Nigeria also showed that the ethanolic extract of the leaves of *Anthocleista djalonensis* had good activity on strains of Plasmodium berghei, comparable to the activity of 4-aminoquinolines [23]. Another study evaluated the antiplasmodial and antipyretic activity of the roots of *A. djalonensis* on *Plasmodium berghei* and concluded that this plant has a prophylactic action and acts not only as a schizonticide but also as a usable antifolinic like pyrimethamine [24]. An earlier study demonstrated the efficacy of extracts from the leaves of this plant by comparing their antiparasitic activity to that of standard antibiotics [25].

These studies show that extracts of leaves of *A. djalonensis* have antiplasmodial potential and are a probable source of new bioactive compounds for the development of new antimalarials. However, the ethanolic extract, although having solubilized a greater number of secondary metabolites, has a very moderate activity tending towards the threshold of inactivity with an average IC_{50} of 42.29 ± 3.76 µg / mL. This extract has an activity half as effective as that of the aqueous extract which has an IC_{50} of 21.87 ± 8.70 µg / mL. These results revealed that the aqueous extract has better antiplasmodial potential than the organic extract. The aqueous extract has an inhibitory effect on the *In vitro* growth of all clinical isolates, including the chloroquine resistant (CQ-R) isolate and K1 strain. This antiplasmodial activity could be explained by the abundant presence of alkaloids and polyphenols in this extract. A study conducted in Mali found that a decoction of *Argemone mexicana* L had an antimalarial efficacy comparable to that of artesunate-amodiaquine. Three alkaloids (allocryptopine, protopine and berberine) isolated from this plant were responsible for this activity against *P. falciparum* [26]. In Gabon, the antiplasmodial effect of the methanolic extract of *Monodora myristica* has been attributed to the alkaloids and polyterpenes present in this extract with an IC_{50} of 6.1 µg / mL [27].

An alkaloid is a natural organic compound, most often of plant origin, heterocyclic with nitrogen as a heteroatom, with a more or less basic complex molecular structure and endowed with pronounced physiological properties even at low doses. Alkaloids isolated from plants have antiparasitic properties and show antiplasmodial activity *In vitro* on *Plasmodium falciparum* with an IC_{50} = 5µg / mL and on certain resistant strains of *Plasmodium falciparum* [28]. Quinine, a natural antimalarial drug, effective against strains resistant to chloroquine, is an alkaloid. In 2012, a study showed that the alkaloidic extracts of *Pyrostria major* (*Rubiaceae*) and *Gonioma malagasy* (*Apocynaceae*), two plants from the Malagasy pharmacopoeia, showed quite remarkable antiplasmodial activity against the chloroquine-resistant strain FcB1 [29].

Polyphenols are a family of molecules with nearly 8,000 natural compounds. They have in common a benzene ring carrying at least one hydroxyl group. Phenolic compounds, natural antioxidants, show activity against a spectrum of parasites and have interesting antiplasmodic activities [30]. Two studies showed that the *In vitro* efficacy (IC_{50} = 1.8 ± 1µg / mL) of crude aqueous extracts from the stems and leaves of *Chrozophora senegalensis* (*Euphorbiaceae*) tested on chloroquine-resistant strains of *P. falciparum* was due to a polyphenol. This is very active on the trophozoite and young schizont stages while showing good prophylactic activity [31, 32]. A case of CQ-R was obtained with the W6622 isolate (IC_{50} = 106.39 µg / mL) thus confirming the presence of chloroquine resistance in our country and helping to explain the disqualification of chloroquine as a first-line antimalarial in Côte d 'Ivoire [33].

The minimum IC_{50} of 3.06 nM and maximum of 16.39 nM obtained with quinine are far from its resistance threshold equal to 800 nM. These results confirm the good efficacy of this antimalarial, which remains one of the antimalarial drugs of last resort in the event of drug resistance or severe malaria. Since 1992, no quinino-resistant isolate has yet been detected in Côte d'Ivoire [34]. The IC_{50s} obtained with artesunate, varying from 0.61 nM to 3.62 nM, are below the threshold of 10 nM. These results confirm that artemisinin derivatives remain good antimalarial drugs and that resistance of *P. falciparum* to artemisinin has not yet been encountered in Côte d'Ivoire

4. Conclusion

Results from this study demonstrate the activity of *Anthocleista djolonensis* extracts *against P. falciparum*, the causative agent of malaria. To a reasonable extent, they also partly support the traditional uses of this plant in ethno-medicine to treat malaria. However, full validation of this use will depend on the results of detailed toxicological studies of the active extracts. The findings reported here have great scientific significance as they highlight for the first time the antiplasmodial activity of *Anthocleista djolonensis* in Côte d'Ivoire. Aqueous extracts with good antiplasmodial potential and selectivity against reference strains will be further fractionated following activity-guided approach, and the isolated hit compounds polished and progressed towards novel antimalarial drugs development.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have none to declare

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