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

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Publication history: Received on 01 December 2020; revised on 09 December 2020; accepted on 11 December 2020

Article DOI: https://doi.org/10.30574/wjarr.2020.8.3.0464

Abstract

Information collected from nine (09) traditional healers in the Moronou village in the Department of Toumodi revealed that *Anthocleista djalonensis* is regularly used by the population for primary health care in the processing of malaria. Evaluation of the *In vitro* antimalarial activity showed that the aqueous extracts inhibit growth of clinical isolates and chloroquinoresistant strains (K1) with IC₅₀ of 8.29 µg/mL and 10.23 µg/mL while the ethanolic extracts had IC₅₀ 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 antimalarial activity on clinical isolates and on *Plasmodium falciparum* multidrug resistant K1 strain (3 µg/mL <IC₅₀ <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 artemisins. 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 antimalarial 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 have confirmed their antimalarial activities [9, 10]. Accordingly, the present study aimed at investigating the *In vitro* antimalarial activity of *Anthocleista djalonensis*, an important medicinal plant used in the Toumodi region for the treatment of malaria [11]. *Anthocleista djalonensis* is a
woody plant 8-15 m high, growing on river banks. The leaves are 9-35 cm or even 1 m long, opposite, petiolate, with oval or elliptical blades. The flowers are grouped in long corymbbs. 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 100 g of the powder were macerated respectively in 1 L of distilled water and 1 L 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 °C for 48 h to obtain aqueous and hydroethanolic extracts which were stored at 4 °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, 312.51 lgentamycin (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 °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 °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 reference molecules were diluted in a complete medium and 100 μL 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 μL. 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 CO2 and incubated at 37 °C in an incubator for 72 h. After 72 h of incubation, microplates were preserved at -20 °C.

2.4. Determination of IC50

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 °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. IC50 (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 polyterpenes.

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 15 minutes. 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 °C for 5 min and 110 °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 comparisons 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           | FeCl₃            | Black bleu / Dark green        | ++              | +                |
| flavonoids            | RC               | Orange-pink/Purplish           | +               | +                |
| catechic tannins      | SR               | Large flake precipitation      | -               | -                |
| gallic tannins        | FeCl₃            | blue - black                   | +               | +                |
| Quinones              | RBG              | red/purple                     | -               | +                |
| alkaloids             | DR               | orange                         | ++              | +                |
|                       | BR               | Reddish brown                  | +               | +                |
| Saponosides           | FM               | 4 cm of Foam                   | -               | -                |
3.1.3. TLC analysis

Analysis of the chromatograms indicates that the aqueous extract contains only alkaloids and polyphenols. In fact, unlike Polyethylene Glycol, sulfureic 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*

| Compounds        | Aqueous extract | Ethanol extract | Interpretation          |
|------------------|----------------|----------------|-------------------------|
| **Alkaloids**    | Light brown    | Dark brown     | Remarkable presence     |
| **Flavonoids**   | Light brown    | Dark brown     | Yellow                  |
| **Quinones**     | Light brown    | Dark brown     | Pink under UV           |
| **Polyphenols**  | Light brown    | Dark brown     | Remarkable presence     |
| **Terpenes**     | Light brown    | Dark brown     | Purple                  |

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\(_{50}\) 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 IC₅₀ 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 IC₅₀ of clinical quinine isolates is 9.34 ± 5.13 nM with minimum and maximum IC₅₀s 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.

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

|        | 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 IC₅₀ of 42.29 ± 3.76 µg / mL. This extract has an activity half as effective as that of the aqueous extract which has an IC₅₀ 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 IC₅₀ 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 antimalarial activity *In vitro* on *Plasmodium falciparum* with an IC₅₀ = 5 µg / 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 (IC₅₀ = 1.8 ± 1 µg / 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 (IC₅₀ = 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 IC₅₀ 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 IC₅₀ obtained with artesunate, varying from 0.61 nM to 3.62 nM, are below the threshold of 10 nM. These results confirm that artesinisin derivatives remain good antimalarial drugs and that resistance of *P. falciparum* to artesinisin has not yet been encountered in Côte d’Ivoire.

4. Conclusion

Results from this study demonstrate the activity of *Anthochelesta djalonensis* 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 *Anthochelesta djalonensis* 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

Acknowledgments

The authors thank sincerely the director of the health center of Yopougon Wassakara for having permitted this study. We are also grateful to the laboratory technicians for their assistance.
Disclosure of conflict of interest
The authors have none to declare

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