

Evaluation of *in vitro* Antiplasmodial Activity and Chromatographic Analysis for Quinine and Codeine Quantification from the Total Alkaloids Extract of *Mitragyna ciliata*

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ABSTRACT

Background: Malaria is the most common parasitosis in the world and still constitutes a major public health menace, particularly in subsaharan african countries. With the rising resistance of *Plasmodium falciparum* to antimalarial drugs, including artemisinin-based combination, there is a necessary to accelerate the discovery and development of new potential antimalarial drugs. Moreover, Traditional medicinal plants are one of the potential sources of anti-malarial drugs. In Côte d'Ivoire, *Mitragyna ciliata* is commonly used as a medicine in the treatment of malaria in traditional medicine with limited scientific evidence. **Objectives:** The aim of the present work was to evaluate the *in vitro* antiplasmodial activity of the total alkaloids extract of *Mitragyna ciliata* and determine the quantification of codein and quinin, two alkaloids in this extract. **Materials and Methods:** The total alkaloid extract from *Mitragyna ciliata* stem barks was obtained using acid/base extraction method. The *in vitro* antiplasmodial activity of total alkaloids were tested against 4 clinical isolates of *P. falciparum* (ANK 21001, ANK 21002, ANK 21005 and ANK 21006) and revealed using the SYBR Green. Quinin and codein were identify and quantify using chromatographic analysis as HPLC. **Results:** In general, total alkaloids extract of *M. ciliata* exhibited moderate activity against the 4 clinical isolates of *P. falciparum*, with IC₅₀ (inhibitory concentration) ranging between 18 and 37.52 µg/mL whereas dihydroartemisin, used as positive control, showed very active anti-plasmodial activity against these clinical isolates (1.38 nM < IC₅₀ < 1.45 nM). The HPLC profile revealed several peaks suggesting presence of some bioactive compounds including quinin and codein, in respective contents of 21.15 mg/100 g et 1.68 mg/100 g of total alkaloids extract. **Conclusion:** The results obtained constitute a scientific basis to support the traditional use of *M. ciliata* in the treatment of malaria in Côte d'Ivoire.

Key words: Alkaloids, HPLC, Clinical isolates, Antiplasmodial activity, Malaria, *Mitragyna ciliata*.

INTRODUCTION

Malaria is considered as a major global health problem, affecting a large population of the world and that is caused by a protozoa of the genus *Plasmodium*. According to World Health Organization (WHO) latest report, there were an estimated 241 million cases of malaria and 627000 deaths worldwide in 2020.^[1] Most of the cases and the deaths occurred in the WHO African region and affected primarily children and pregnant women.^[1,2] *Plasmodium* are transmitted to people through the bites of infected female Anopheles mosquitoes. Among the species of the parasites that affect humans, *Plasmodium falciparum* is by far the most virulent.^[1,3] Thus, to reduce malaria deaths worldwide, WHO recommended a large-scale vector control (mainly through the massive spreading of dichlorodiphenyltrichloroethane or DDT) and the use of impregnated mosquito nets for preventing infection

and reducing disease transmission.^[4] In addition, for uncomplicated and severe cases of malaria, artemisinin and its derivatives in combinaison with other anti-malarial drugs is the remedy considered in first-line treatment.^[5,6] Unfortunately, the massive and systematic use of insecticides and artemisinin caused, for several years, a parasite resistance against these disease control means, promoting an upsurge in malaria, mainly in developping countries.^[7,8] Given these limitations, there is urgent to find new drugs with novel mechanisms of action against *Plasmodium* parasites. In fact, it is well known that most widely used curative antimalarial drugs are plant products, quinine and artemisinin. The success of quinine and artemisinin isolated from *Artemisia annua* and its derivatives, for the treatment of resistant malaria has focused attention on plants

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as a source of anti-malarial drugs. The benefits of plants containing bioactive anti-malarial compounds, particularly the bitter principles as alkaloids include their use in the preparation of traditional remedies against malaria, fever, and inflammation.^[9] Moreover, Rubiaceae are a family of plants whose high potential in various alkaloids are widely demonstrated.^[10] *Mitragyna ciliata*, one of species from Rubiaceae family, is traditionally used in the treatment of malaria in Côte d'Ivoire.^[11] We report in this study the *in vitro* antiplasmodial activity of total alkaloids from stem barks of *Mitragyna ciliata* to confirm its use as malarial remedy in ivoirian ethnomedicine.

MATERIALS AND METHODS

Plant Collection

The stem bark of *Mitragyna ciliata* was collected in swampy areas of Toukouzou, Department of Grand-Lahou, in south of Côte d'Ivoire, during February 2020. The plant was identified by comparison with authentic specimens deposited in National Floristic Center of the University, University Félix Houphouët-Boigny of Abidjan (n°8114). The collected plant material was washed and dried under room temperature. Stem barks were cut up and stored in tigh-seal dark containers until needed.

Total alkaloids extraction

The dried stem barks of *M. ciliata* were ground in fine powder with a mechanical gringer (IKAMAG-RCT). The powder of plant (50 g) was macerated with 1.5 L of methanol for 24 hr, at room temperature, using magnetic stirrer (Heidolph Lab-Mix 35). The methanolic solution obtained was filtered with Whatmann no 1 filter paper and concentrated to dryness at 40°C using a rotary evaporator (Buchi) under reduced pressure. The dried extract was then dissolved in 10 mL of methanol/water (v/v) and the pH of solution was acidified with HCl. Then, the acidified solution was washed with dichloromethane (3 × 5 mL). The aqueous phase, rich in alkaloids, was separated to organic phase by decantation. The acidic aqueous phase was subjected to basification with sodium carbonate (Na₂CO₃) until reaching a pH of 10 and dichloromethane (3 × 5 mL) was added, to extract the alkaloids which were retained in the organic phase. The different organic phases were combined and water residues are removed with anhydrous magnesium sulfate. Finally, the resulting solution was evaporated under reduced pressure in the rotary evaporator at 40°C, to obtain the total alkaloids extract.

In vitro antiplasmodial activity

Collection of samples (clinical isolates)

Sampling for this study was carried out with patients diagnosed positive for uncomplicated malaria caused by *Plasmodium falciparum*, at Community Health and Urban Training of Anonkoua-Kouté-Abobo (Abidjan, Côte d'Ivoire). However, informed consent was obtained from all patients in this study prior to clinical isolates of *P. falciparum* collection. Thereby, blood samples of each confirmed case of mono infection of *Plasmodium falciparum* were collected in vacuum tubes containing EDTA and sent to Malariology Laboratory of Pasteur Institute (Abidjan, Côte d'Ivoire).

In vitro Cultivation of *Plasmodium falciparum*

Fresh blood plasma were removed and the blood pellets resuspended and washed thrice in RPMI 1640 medium (Rose Park Mary Medium Insitute, Gibco, USA) by centrifugation at 3000 rpm for 5 min. Then, these blood pellets were diluted with uninfected human type O positive red blood cells to reach a parasitemia under 0.3 % and 1.3 % of hematocrit. Finally, four clinical isolates of *P. falciparum* named ANK 21001, ANK 21002, ANK 21005 and ANK 21006, were obtained

for the *in vitro* antiplasmodial test. The antiplasmodial activity of total alkaloids extract from stem barks of *M. ciliata* was screened against clinical isolates of *P. falciparum* obtained from continuous cultures. The parasites were cultured in human O Rh⁺ red blood cells according to the method of Trager and Jensen,^[12] using RPMI 1640 complete medium culture prepared with 450 mL of RPMI 1640, 5 mL of L-glutamyl (2 mM, Eurobio), 12.5 mL of HEPES buffer (25 µM, Eurobio) and 10 mL of stock solution containing 500 mL of RPMI 1640, 25 g of albumax II, 10 g of D-glucose (20 g/L, Wagtech) and 625 µL of gentamycin (40 mg/mL, Eurobio). The solution was filtered in sterile condition and stored at 4°C.

Preparation of stock solution

The stock solution of total alkaloids extract of *M. ciliata* was prepared at 1 mg/mL in dimethyl sulfoxide (DMSO). Then, 10 mg of this extract was dissolved in 1 mL of DMSO and the volume was made up to 10 mL with distilled water. The solution was vortexed in order to completely dissolve the extract and sterilized in an autoclave at 121°C for 15 min. Dihydroartemisin stock solution, used as standard drug, was prepared at 1 mM in DMSO.

Preparation of Inoculum

A volume of 12 mL of inoculum was prepared with 240 µL of parasitized erythrocytes suspension (hematocrit at 2 % and parasitemia under 0.3 %) and 11.76 mL of RPMI complete medium culture.

In vitro anti-plasmodial assay

The stock solutions were subsequently diluted with RPMI complete culture medium at 7 concentrations of two-fold dilutions into a 96-well microtiter plate. Then, drug concentrations ranged from 50 µg/mL to 0.78 µg/mL for total alkaloids extract and from 200 nM to 0.78 nM for dihydroartemisinin. The volume of each drug in the wells was 100 µL. A volume of 100 µL of inoculum (parasitized erythrocytes suspension) was added to each well to reach a final volume of 200 µL. Therefore, wells with parasitized erythrocytes and without plant extract served as negative controls whereas wells containing cultures with dihydroartemisinin served as positive controls. The antiplasmodial assay was carried out with duplicate, plates were confined in a candle jar saturated with CO₂ and incubated at 37°C in an incubator for 72 hr. After 72 hr of incubation, the plates were removed from the incubator and frozen at -20°C and evaluation of parasitemia was assessed using the SYBR Green method previously described.^[13]

Evaluation of parasitemia

After thawing of the 96-well plates, 100 µL of the cell suspension of each well was dispensed in a new 96-well plates containing 100 µL of SYBR Green I fluorescent lysis buffer. The lysis buffer was prepared by dissolving 0.93 g of Tris-Base in 200 mL of distilled water, under magnetic stirring. The pH of the solution was adjusted to 7 by adding a volume of HCl. The solution was completed with 100 mL of distilled water. Then, 0.93 g of EDTA, 40 mg of saponin and 400 µL of Triton X were added. Finally, SYBR Green I fluorescent lysis buffer consists to mix 5 µL of SYBR Green I (Invitrogen, Waltham, Massachusetts, USA) and 25 mL of lysis buffer. The solution obtained was filtered with 0.22 µm membrane filter and stored at room temperature. The plate was incubated for 1 hr at room temperature, in the darkness. The SYBR Green fluorescence was measured with a microplate reader (FLX 800, BIOTEK) at the excitation and emission wavelengths of 485 nm and 528 nm, respectively.

Determination of IC₅₀ and test interpretation

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.^[14,15] The antiplasmodial activity of extracts was classified by Bero *et al.* and Jansen *et al.*^[16,17] as follows: high (IC₅₀ < 5 µg/mL), promising (5 < IC₅₀ < 15 µg/mL), moderate (15 < IC₅₀ < 50 µg/mL) and inactive (IC₅₀ > 50 µg/mL).

HPLC analysis of alkaloids

Preparation of standard and sample solutions

For HPLC analysis of alkaloids, quinine sulfate and dihydroartemisinin are used as standard. The stock standard solutions were prepared by dissolving 125 mg of quinine sulfate and 100 mg of dihydroartemisinin, separately, in 50 mL of mixture containing 450 mL of methanol, 150 mL of acetonitrile and 400 mL of ammonium acetate. Furthermore, 1 g of total alkaloids extract of *M. ciliata* were homogenized in 50 mL of hexane. A volume of 5 mL of filtrate obtained were added to 50 mL of the solvents mixture. Each solution was filtered through a 0.45 mm nylon membrane filter before injection.

Chromatographic conditions

The analysis of quinine and artemisinin in total alkaloids extract of *M. ciliata* was carried out using a Waters® HPLC chain, USA. Samples were separated on a 250 mm × 4.6 mm, 5 µm particle, C₁₈ column. The mobile phase consisted of a mixture of 450 mL of methanol, 150 mL of acetonitrile and 400 mL of ammonium acetate, at a flow rate of 1 mL/min, with an injection volume of 10 µL. The detection of two standard alkaloids were carried out at UV-Visible at the wavelength of 347 nm. The peaks of quinine and artemisinin in total alkaloids extract of plant were identified by comparing their retention times of the peaks with that of the standard. Quinine and artemisinin contents were determined from the peak areas and according to the equation of the calibration of standard.

RESULTS

Antiplasmodial activity of the total alkaloids extract

The antiplasmodial activity of total alkaloids extract from stem barks of *M. ciliata* was evaluated against four clinical isolates of *P. falciparum* and summarized in Table 1. The IC₅₀ values obtained were 35.88 µg/mL, 37.52 µg/mL, 31.49 µg/mL and 18.65 µg/mL, respectively for clinical isolates ANK 21001, ANK 21002, ANK 21005 and ANK 21006. Based on previous data, total alkaloids extract of *M. ciliata* showed moderate antiplasmodial activity. Therefore, artemisinin indicated a very high activity against the four clinical isolates with IC₅₀ ranging from 1.38 nM to 1.45 nM.

Quantification of quinine and codeine using HPLC

HPLC chromatogram of total alkaloids extract from stem barks of *M. ciliata* was illustrated in Figure 1. The HPLC profile revealed several peaks suggesting the presence of some bioactive compounds. From the HPLC-chromatogram, the peaks of quinine and codeine appeared at retention times of 5.70 min and 6.48 min, respectively. Table 2 showed the amount of two alkaloids detected in the total alkaloids extract. Quinine content indicated 21.15 mg/100 g of extract while codeine was detected in least quantity (1.68 mg/100 g of extract).

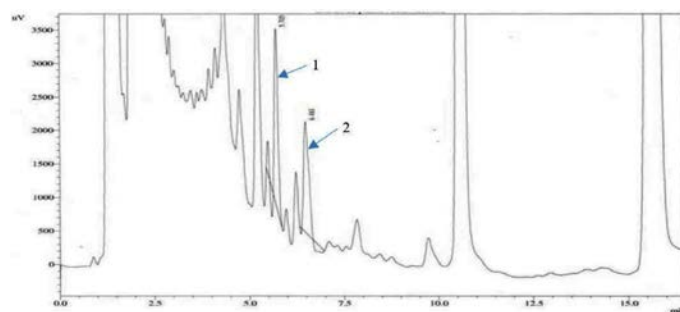
DISCUSSION

Several species of the Rubiaceae family are well known for their uses in a traditional medicine as antimalarial plants.^[18] In the present study, one

Table 1: Antiplasmodial activity of total alkaloids extract of stem barks of *M. ciliata*.

	Antiplasmodial activity (IC ₅₀)			
	Clinical isolates			
	ANK 21001	ANK 21002	ANK 21005	ANK 21006
Total alkaloids	35,88	37,52	31,49	18,65
Dihydroartemisinin	1.45	1.42	1.38	1.40

IC₅₀ values of total alkaloids extract of *M. ciliata* and dihydroartemisinin (standard) were expressed, respectively, in µg/mL and nM.



1. Quinine; 2- Codeine

Figure 1: HPLC profile of total alkaloids extract from stem barks of *M. ciliata*.

Table 2: Determination of quinine and codeine contents in total alkaloids extract of *M. ciliata* using HPLC.

	Molecules	Retention time (min)	Area	Height	Content (mg/100 g)
1	Quinine	5.705	16793	2646	21.15
2	Codeine	6.481	13712	1657	1.68

member of this family, *Mitragyna ciliata*, was investigated with the view to providing a scientific justification for the traditional use in treatment of malaria. Thereby, total alkaloids extracted from stem barks of *M. ciliata*, obtaining by acid-base extraction, were evaluated for antiplasmodial activity using the SYBR Green assay. SYBR Green is an asymmetrical cyanine dye, which binds to any double-stranded DNA, including the DNA intrinsically present in whole blood samples, preferring G and C base pairs.^[19] The DNA bases emit a fluorescence which is proportional to the level of DNA in the medium which is itself proportional to the number of parasites. Antiplasmodial activities of plant extracts/fractions were based on the IC₅₀ values obtained. As previously mentioned, for *in vitro* studies, the antiplasmodial activity of an extract was considered high if IC₅₀ < 5 µg/mL, promising if 5 µg/mL < IC₅₀ < 15 µg/mL, moderate if 15 µg/mL < IC₅₀ < 50 µg/mL and inactive if IC₅₀ > 50 µg/mL. Based on this classification, results from this study indicate that total alkaloids extract of *M. ciliata*, with IC₅₀ values ranging from 18.65 µg/mL to 37.52 µg/mL, exerted moderate activity against clinical isolates of *P. falciparum*. Therefore, the four clinical isolates of *P. falciparum* were sensitive, both to total alkaloids extract and dihydroartemisinin, used as positive control. Certain plants from Rubiaceae family have previously been described as having significant antiplasmodial activity. This was the case for ethanolic extract of stem barks of *Nauclea latifolia* which inhibited FcB1 strain of *Plasmodium falciparum* growth, with an IC₅₀ value of 8.9 µg/mL,^[20] while the petroleum ether extract of leaves of *Morinda lucida* exhibited an antiplasmodial activity with IC₅₀ values less than 5 µg/mL against chloroquine-resistant *P. falciparum* (K1)

strain.^[21] The alkaloids contained in chloroform extracts, purified from the hydromethanol extract of *M. inermis*, another species of the genus *Mitragyna*, induced a significant decrease of *P. falciparum* proliferation ($IC_{50} = 4.36-4.82 \mu\text{g/mL}$).^[22] In addition, Rubiaceae are also known for their high potential in alkaloids.^[10] Alkaloids are considered as an important class of phytoconstituents exhibiting diverse biological activities, particularly antimalarial activity. They constitute an important class of structurally diversified compounds that are having the nitrogen atom in the heterocyclic ring and are derived from amino acids.^[23] In this study, quinine and codeine contents, two alkaloids of stem barks of *M. ciliata* were investigated using HPLC. Quinine, isolated from the bark of *Cinchona* species (Rubiaceae), and its derivatives were widely used as antimalarial drugs, even today. The basis for the antimalarial activity of quinine is unclear, but three mechanisms have been proposed such as intercalation with parasite DNA, interrupting replication and transcription, interaction with erythrocyte fatty acids, promoting hemolysis and preventing schizont maturation, and alkanization of parasite digestive vacuoles, interfering with hemoglobin degradation.^[24-27] Codeine is an opioid analgesic used to treat moderate to severe pain when the use of an opioid is indicated. Like morphine, codeine binds to receptors in the brain (opioid receptors) that are important for transmitting the sensation of pain throughout the body and brain.^[28,29] Thus, the antiplasmodial activity of alkaloids from *M. ciliata* may be explained by the presence of these two alkaloids. Previous studies reported by Adjetei et al.^[30] and Dongmo et al.^[31] were revealed also the antiplasmodial and anti-inflammatory activities of leaves and stem barks of *M. ciliata*, respectively. Over the years, malaria parasites have developed resistance to several commonly used antimalarial drugs.^[32] Diminished sensitivity of *P. falciparum* to quinine has been widely documented.^[33-36] In order limit parasite resistance to antimalarial drugs, promising antimalarial alkaloids from the Rubiaceae family have been isolated. Naucleorine was identified from the stems of *Nauclea orientalis* by He et al.^[37] and showed high antiplasmodial activities against the *P. falciparum* D6 and W strains with IC_{50} values of $6.9 \mu\text{M}$ and $6.0 \mu\text{M}$, respectively. In another study, gardenine, obtained from the investigation of crude extract of the aerial parts of *Canthium multiflorum*, exhibited an antiplasmodial activity against the K1 strain of *P. falciparum*, with an IC_{50} value of $32.12 \mu\text{M}$,^[38] while from *Nauclea officinalis*, naucleofficine A, an indole alkaloid was isolated and exhibited moderate antimalarial activity against FCC1-HN with an IC_{50} value of $9.7 \mu\text{M}$.^[39]

CONCLUSION

The results of this study indicate that the total alkaloids extract from stem barks of *Mitragyna ciliata* possesses a moderate in vitro antiplasmodial activity against clinical isolates of *P. falciparum*. Our study justifies and confirms the use of this plant in ivoirien folk medicine for malaria treatment. Therefore, further work could be carried out in order to identify and isolate another alkaloid compounds besides quinine and codeine, evaluate their antiplasmodial activity including bioassay-guided fractionation and toxicity testing to find new anti-malarial drug candidates.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

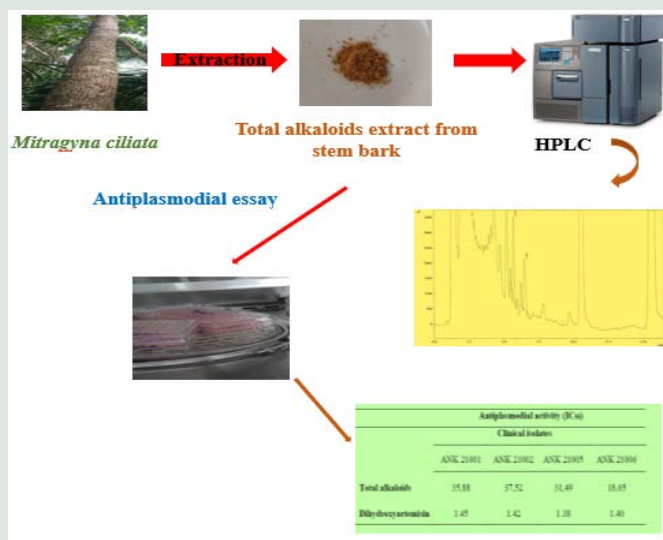
HPLC: High performance liquid chromatography; **IC_{50} :** Half-maximal inhibitory concentration; **DMSO:** Dimethyl sulfoxide; **CO_2 :** Carbon dioxide; **Na_2CO_3 :** Sodium carbonate; **ANK:** Anonkoua-Kouté; **HCl:** Chloride acid; **RPMI:** Rose Park Mary Medium Institute; **rpm:** Rounds per minute; **%:** Percentage, **pH:** Potential of hydrogen; **HEPES:** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; **EDTA:** Ethylene diamine tetra acetic acid; **min:** Minute.

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GRAPHICAL ABSTRACT



SUMMARY

Alkaloids are known for their more pharmacological actions such as antiplasmodial activity. *Mitragyna ciliata* is frequently used by old folks, in Cote d'Ivoire, to treat and reduce clinical symptoms of malaria. In this study, total alkaloids from stem bark of *M. ciliata* was extracted. *In vitro* antiplasmodial activity of this extract was evaluated against clinical isolates *Plasmodium falciparum*. Total alkaloids extract from *M. ciliata* inhibited, moderately, malaria parasites, with IC₅₀ values ranging from 18.65 µg/mL to 37.52 µg/mL. In addition, quantification of quinine and codeine, two alkaloids with antimalarial and anti-inflammatory actions, respectively, was determined, using HPLC. Total alkaloids from stem barks of *M. ciliata* possess promising antimalarial potential and could be a good candidate for new drug.

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