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Comparative Evaluation of Antiplasmodial and Cytotoxic Activities of Alkaloid Extracts from *Coffea arabica* and *Coffea canephora*

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ABSTRACT

The growing resistance of *Plasmodium parasites* to current antimalarial drugs emphasizes the urgent need to discover alternative therapies. Medicinal plants historically used in the treatment of malaria are promising sources for new bioactive compounds, though their effectiveness requires scientific validation. This study aimed to evaluate the antiplasmodial and cytotoxic properties of alkaloid extracts from *Coffea arabica* and *Coffea canephora*. Alkaloids were extracted from powdered dry leaves of both plants using an acidic medium. The alkaloids were identified by Mayer and Wagner reagents. The antiplasmodial activity was assessed using SYBR green fluorescence assays on two *Plasmodium falciparum* isolates and analyzed by flow cytometry. Toxicological evaluations were performed by hemolysis, erythrocyte sensitivity, and cytotoxicity tests on Vero and Raw cells, with measurements taken by spectrophotometry. The results showed that *C. arabica* demonstrated significant activity against both Pf3D7 and PfDd2 strains, with resistance indices of 0.78 and IC50 values of 9.53 ± 1.51 µg/mL and 7.48 ± 0.93 µg/mL, respectively. In contrast, *C. canephora* showed limited activity against Pf3D7 (IC50 > 100 µg/mL) but moderate activity against PfDd2 (IC50 = 85.55 ± 1.17 µg/mL). The toxicity profile for both extracts was promising, as demonstrated by hemolysis results (HC50 > 1000 µg/mL) and cytotoxicity assays (CC50 > 30 µg/mL). This study highlights the potential of these plant extracts as suitable alternatives for malaria treatment.

Keywords: *Coffea arabica*, Alkaloids, *Coffea canephora*, Antiplasmodial activity, Cytotoxicity

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Introduction

Malaria continues to be one of the most fatal parasitic diseases globally, with its greatest impact in tropical and subtropical areas [1]. In 2020, over 200 million cases of malaria were reported in sub-Saharan Africa, a significant increase due to disruptions in malaria control measures during the Covid-19 pandemic [2-4]. In Cameroon, malaria remains a major public health issue, causing high morbidity and mortality, particularly among vulnerable populations [5, 6].

The majority of malaria cases are caused by *Plasmodium falciparum*, responsible for over 95% of infections [7, 8]. Despite efforts to manage the disease through prompt treatment and prevention strategies, the growing resistance of *P. falciparum* to conventional antimalarial drugs has significantly hindered control measures [9, 10]. This has prompted an urgent search for new antimalarial drugs with alternative mechanisms of action.

The search for new treatments often focuses on medicinal plants, which have been used traditionally in various cultures for the treatment of malaria. In Cameroon, there is a longstanding tradition of using plants such as *Coffea* for malaria management, though scientific validation of their effectiveness is still ongoing [11-13]. This study

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aims to assess and compare the antiplasmodial effects of *C. arabica* and *Coffea canephora*, which are commonly used in local medicine.

Materials and Methods

Plant collection

The plant materials used in this research were gathered from different locations in Western Cameroon. *Coffea robusta* was collected from Bantoum, while *C. arabica* was sourced from Bangoua. Identification of the plants was confirmed at the National Herbarium of Cameroon, where they were registered with the numbers 58228/HNC for Coffea and 67456/HNC for *C. canephora*. All extraction processes were carried out in the Laboratory of Pharmacognosy and Pharmaceutical Chemistry at the University of Yaoundé I, while additional analyses were performed at the Phytobiochemistry and Medicinal Plants Laboratory in the Faculty of Sciences at the same university.

Alkaloid extraction process

The alkaloid extraction followed a method adapted from Kémajou *et al*. [14]. A total of 500 grams of dried leaf powder from the plant was weighed and then subjected to extraction with 1800 mL of methanol for 48 hours, using constant stirring via a PRO HPS stirrer from the Lab Plus Series. This procedure was repeated twice. Afterward, the extracts were filtered through Whatman filter paper N°3, concentrated under reduced pressure with a BUCHI R-201 rotary evaporator, and then acidified using 0.5 N hydrochloric acid. The acidified mixture was placed in a separate funnel with 100 mL of chloroform. The resulting aqueous phase was adjusted to a pH of 9-10 by adding 6 mL of a 33% ammonia solution and was then re-extracted with another 100 mL of chloroform. The chloroform phase was collected, dried over anhydrous magnesium sulfate, and filtered to isolate the total alkaloids.

In vitro evaluation of antiplasmodial activity

Preparation of stock solutions for alkaloids, chloroquine, and artemisinin

To prepare the stock solution of alkaloids, 100 mg was dissolved in 1 mL of dimethylsulfoxide (DMSO), creating a concentration of 100 mg/mL. For Chloroquine (98%, Sigma-Aldrich) and Artemisinin (98%, Sigma-Aldrich), stock solutions were made at 1 mM. To achieve intermediate concentrations, either 10 µL of the alkaloids solution (100 mg/mL) or 20 µL of the Chloroquine or Artemisinin (1 mM) solution was added to 190 µL or 180 µL of incomplete RPMI1640 medium, respectively. These mixtures were then serially diluted, creating final concentrations from 8 to 5000 µg/mL for alkaloids and 0.016 to 10 µM for the other compounds.

Culturing plasmodium falciparum parasites

The *Plasmodium falciparum* strains used in this study included a chloroquine-sensitive strain (NF54-E) and a multi-drug-resistant strain (Dd2). The culture of these strains followed the method described by Trager and Jensen [15]. Red blood cells from human group O, Rh-positive, were used at 4% hematocrit in the RPMI 1640 medium. The medium was supplemented with 25 mM HEPES, 0.50% Albumax I, 45 µg/mL of hypoxanthine, and 20 µg/mL gentamicin. The culture was maintained at 37 °C in a humidified incubator with a gas mixture of 92% N2, 5% CO2, and 3% O2. The medium was replaced daily with fresh complete RPMI medium. Giemsa-stained blood smears were prepared to monitor the stages of parasite development and calculate parasitemia.

Testing antiplasmodial activity

The antiplasmodial activity was assessed using the SYBR Green I fluorescence method, as described by Smilkstein *et al*. [16]. A 90 µL volume of parasite culture, containing 2% parasitemia and 1% hematocrit, was added to 10 µL of various concentrations of alkaloids, Artemisinin, or Chloroquine. The plates were incubated for 72 hours at 37 °C in a humidified atmosphere with 92% N2, 5% CO2, and 3% O2. The final concentrations tested ranged from 0.8 to 5000 µg/mL for alkaloids and from 0.0016 to 1 µM for Chloroquine and Artemisinin. All assays were conducted in duplicate for accuracy.

After 72 hours of incubation, 100 µL of SYBR Green I buffer was added to each well. The buffer included 6 µL of a 10,000 × SYBR Green I solution (Invitrogen), 600 µL of red blood cell lysis buffer (25 mM Tris, pH 7.5), 360 µL of EDTA (7.5 mM), and 19.2 µL of a parasite lysis solution containing saponin and 28.8 µL of Triton X-

100 (0.08%; v/v). The samples were then incubated for 1 hour in the dark at 37 °C. Fluorescence was assessed using a Tecan Infinite M200 microplate reader, set to excitation at 485 nm and emission at 538 nm. The resistance index (IR) was calculated as the ratio of IC50 values from the PfDd2 strain to the 3D7 strain, with a resistance index below 1 suggesting that the extract is more effective against the resistant strain [16].

To determine the kinetics of growth inhibition in the multi-resistant PfDd2 strain, different extract concentrations were added to an asynchronous culture of P. falciparum, which included all parasite development stages, followed by incubation. The antiplasmodial effect was measured using SYBR Green-based nuclear staining, and IC50 values were determined. Each total alkaloid extract was incubated for 24, 48, and 72 hours. The IC50 values were then normalized, and the action speed of each extract was classified as either fast or slow based on the comparison with the 72-hour IC50 [17, 18].

The cytotoxicity of the total alkaloid extracts was assessed following protocols from Bowling *et al*. [19] and Al-Daghistani *et al*. [20]. Vero cells (ATCC CRL 1586) from African green monkey kidney and raw cells (ATCC #TIB-71) from murine macrophages were cultured in Dulbecco Eagle medium, supplemented with 10% fetal bovine serum, 0.2% sodium bicarbonate, and 1% penicillin-streptomycin. Cells were seeded into 96-well plates with a density of 10,000 cells per well and incubated at 37°C in 5% CO2 until 90% confluence was reached. After 24 hours, 10 µL of the total alkaloid extracts or control substances were added, with a starting concentration of 500 µg/mL. Podophyllotoxin (20 µM) was used as a positive control. After adding 10 µL of Resazurin solution (0.15 mg/mL in sterile PBS), the plates were incubated for an additional 4 hours, and fluorescence readings were obtained using a Magelan multi-well fluorescence reader (Infinite M200, Tecan) at excitation and emission wavelengths of 530 nm and 590 nm, respectively. The selectivity index (SI) was calculated to evaluate the clinical significance of the extracts by comparing their toxicity to general cells and their selective action against *P. falciparum* [21].

$$Selectivity\ Index\ (SI) = \frac{\text{IC50 of the vero or Raw cell lines}}{\text{IC50 of the Plasmodium cell lines}} \quad (1)$$

Furthermore, the cytotoxicity of the extracts was assessed based on the criteria for crude extract cytotoxicity outlined by the American National Cancer Institute (NCI), which specifies that an extract is considered cytotoxic if its IC50 is less than 30 µg/mL [22].

Data processing

The data collected were organized into an Excel spreadsheet for analysis and calculation. Fluorescence readings were used to calculate the inhibition percentages with the help of Microsoft Excel 2016. The IC50 values were determined by plotting concentration-response curves, where the logarithm of the concentration was plotted against the percentage of inhibition. GraphPad Prism 9 software was utilized for this analysis.

Results and Discussion

The extraction yields for each plant species are summarized in **Table 1**.

Table 1. Extraction yields

Botanical name	Mass of powder (g)	Mass of total alkaloids (g)	Extraction yield (%)
C. arabica	500	0.23	0.05
C. canephora	500	0.38	0.08

The data shows that *C. canephora* yields more alkaloids (0.08%) compared to *C. arabica* (0.05%), even though both were extracted from 500 grams of plant powder. This difference can be attributed to the varying alkaloid content between the two species. Caffeine, a major alkaloid in both plants, is more abundant in *C. canephora* [23], which likely explains the higher yield [24]. Additionally, environmental factors, such as climate and cultivation conditions, can impact the chemical composition and, consequently, the extraction yield [25-27].

The results of the in vitro antiplasmodial activity tests and resistance index are shown in **Table 2**.

Table 2. Antiplasmodial activity and resistance index

Botanical name and control	IC50 (µg/mL) - PfDd2	IC50 (µg/mL) - Pf3D7	Resistance index
AT (*C. arabica*)	7.48 ± 0.93	9.53 ± 1.51	0.78
AT (*C. canephora*)	85.55 ± 1.17	> 100	ND
Artemisinin	26.63 ± 0.00	43.86 ± 0.00	0.6
Chloroquine	517.4 ± 0.03	40.36 ± 0.00	517.4 ± 0.03

Pf: plasmodium falciparum; IC50: inhibitory concentration 50; AT: total alkaloids; and ND: Not determined.

Activity was classified as: high (IC50 < 5 µg/mL), active (5 ≤ IC50 < 50 µg/mL), moderate (50 ≤ IC50 < 100 µg/mL), or inactive (IC50 > 100 µg/mL) [28].

The total alkaloid extract from *C. arabica* exhibited significant activity against both the chloroquine-sensitive Pf3D7 strain and the multi-drug-resistant PfDd2 strain, with IC50 values of 9.53 ± 1.51 µg/mL and 7.48 ± 0.93 µg/mL, respectively. On the other hand, the alkaloid extract from *C. canephora* displayed weak activity (IC50 >100 µg/mL) against Pf3D7 and moderate activity (IC50 = 85.55 ± 1.17 µg/mL) against PfDd2 **(Table 2)**. These findings differ from those of Lacroix *et al*. [29], who worked with ethyl acetate extracts of *C. arabica* leaves. The discrepancy could be due to the solvent used for extraction, as ethyl acetate is more effective at extracting compounds like mono- and diglycosidic flavonoids but may extract alkaloids less efficiently [30]. These results suggest that the alkaloids play a significant role in the antiplasmodial activity of Coffea species.

Moreover, while *C. canephora* yielded more alkaloids **(Table 1)**, its lower antiplasmodial activity suggests differences in the alkaloid composition between *C. arabica* and *C. canephora*. The resistance index (0.78) indicates that the alkaloid extract from *C. arabica* may have a preferential action against the multi-resistant PfDd2 strain, possibly interacting with resistance genes not present in the chloroquine-sensitive Pf3D7 strain.

Regarding the kinetics of growth inhibition in vitro of the multi-resistant strain (PfDd2), the IC50 values were assessed for both the control substances (chloroquine and artemisinin) and the total alkaloid extracts from *C. arabica* and *C. canephora*. These measurements were taken after 24, 48, and 72 hours of incubation **(Figure 1; Table 3)**.

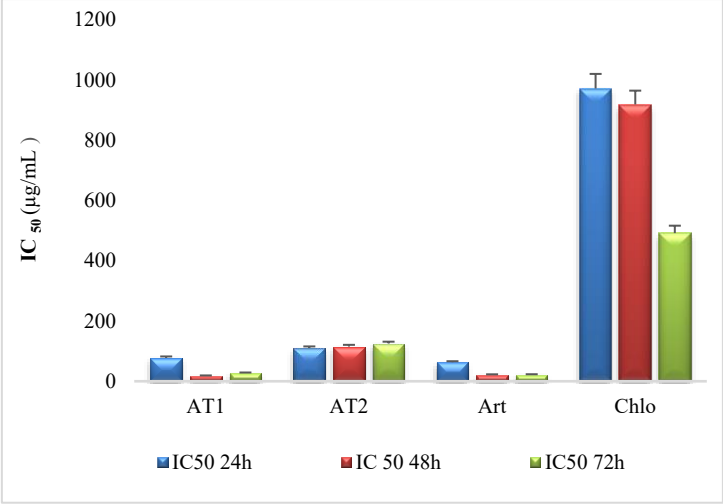

Figure 1. IC50 inhibition kinetics (unsynchronized Dd2 culture); AT1: total alkaloids of *C. arabica*, AT2: total alkaloids of *C. canephora*, Art: artemisinin, and Chlo: chloroquine

Table 3. Kinetics data and action speed

IC50 (µg/mL) on *P. falciparum* Dd2 strain	24 hours	48 hours	72 hours	R	Conclusion
AT (*C. arabica*)	78.33 ± 0.00	18.35 ± 0.00	27.56 ± 0.00	2.89	Fast action
AT (*C. canephora*)	> 100	> 100	> 100	ND	Slow action
Artemisinin	63.28 ± 0.00	21.91 ± 0.00	22.16 ± 0.00	2.86	Fast action
Chloroquine	970.9 ± 0.01	918.0 ± 0.02	491.4 ± 0.00	1.97	Fast action

R: ratio of IC50 at 24 hours to IC50 at 72 hours; ND: not determined

The IC50 values after 24 hours and 72 hours for the different treatments were as follows: for AT1, 78.33 ± 0.00 (24 hours) and 27.56 ± 0.00 (72 hours); for artemisinin, 63.28 ± 0.00 (24 hours) and 22.16 ± 0.00 (72 hours); and chloroquine, 970.9 ± 0.01 (24 hours) and 491.4 ± 0.00 (72 hours). The calculated ratios show that IC50 values at 24 hours were 2.89, 2.86, and 1.97 times higher than at 72 hours for AT1, artemisinin, and chloroquine, respectively **(Table 3)**. These results indicate that the total alkaloid extract from *C. arabica* and the standards have rapid action, while the *C. canephora* extract (AT2) showed higher IC50 values at all time points, indicating slower activity. This suggests that the alkaloid composition in *C. arabica* is more effective across all parasite developmental stages (rings, trophozoites, schizonts), supporting the hypothesis that the alkaloid profiles of these two species differ.

The biological effectiveness of an extract is generally considered minimal in the case of high cytotoxicity when the selectivity index (SI) is below 10 [31]. In this study, low cytotoxicity was defined by a selectivity index of less than 10, and high cytotoxicity was indicated by an SI greater than 10, based on the criteria set by Waiganjo *et al*. [22]. As shown in **Table 4**, the total alkaloid extracts from both *C. arabica* and *C. canephora* exhibited a selectivity index of ≥ 10 against Vero and Raw cell lines, with IC50 values greater than 30 µg/mL, suggesting that these extracts do not display significant cytotoxicity [31].

Table 4. Selectivity index (SI) of total alkaloid extracts evaluated against vero, raw cells, and Dd2 strain of *P. falciparum*

Extract	IC50 (µg/mL) on Dd2 Strain	IC50 (µg/mL) on Cells	SI (on Cells)
	Vero	Raw	Vero
AT1	7.48 ± 0.93	>500	ND
AT2	85.55 ± 1.17	>500	115.05 ± 8.13

AT1: total alkaloids of *C. Arabica*; AT2: total alkaloids of *C. canephora*; ND: not determined; SI: selectivity index

Conclusion

This study provides the first evidence of the promising antimalarial and antiplasmodial activities of total alkaloid extracts from *C. arabica* and *C. canephora*, with an impressive selectivity index. The data revealed that *C. arabica* exhibited superior activity while maintaining a low level of cytotoxicity. Given the limited current knowledge of its alkaloid composition, further investigation into *C. arabica*'s phytochemicals are warranted. These findings support the use of these Coffea species in traditional medicine for malaria treatment and suggest their potential for use in developing enhanced traditional medicines (ETMs).

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Conflict of Interest: None

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Ethics Statement: None

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