Comparative Antiplasmodial and Cytotoxic Activities of *Coffea arabica* and *Coffea canephora alkaloids extracts*

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ABSTRACT

The increase of Plasmodial parasite resistance to available antimalarial drugs underscores the emergency to research alternatives to new drugs development. Medicinal plants traditionally used against malaria are potential source of new molecules; however, their efficiency must be scientifically validated. The present study is therefore aimed at evaluating the antiplasmodial potential and the cytotoxicity effect of alkaloids from *Coffea Arabica* and *Coffea canephora*. Alkaloids were extracted in acidic medium from powder of the dry leaves of each plant. Characterization of alkaloids was carried out using Mayer and Wagner reagents. Antiplasmodial tests based on the fluorescence of SYBR green were carried out on two isolates of *Plasmodium falciparum*. The revelation was made by flow cytometry. Toxicological evaluation of the extracts had been carried out through hemolysis test, erythrocyte sensitivity as well as cytotoxicity on Vero cells and Raw cells using spectrophotometry. Evaluation of antiplasmodial activity showed that *C. arabica* was active on isolates Pf3D7 and PfDd2 with resistance index of 0.78 and IC_{50} values of 9.53 ± 1.51 µg/mL and 7.48 ± 0.93 µg/mL respectively; On the other hand, *C. canephora* showed a weak activity on the strain Pf3D7 (IC_{50} >100µg/mL) and a moderate activity on the strain PfDd2 (IC_{50} = 85.55 ± 1.17 µg/ mL). The toxicological profile was favorable for both extracts concerning hemolysis test (HC_{50} >1000 µg/mL) and for cytotoxicity test (CC_{50} >30 µg/mL). The present study revealed that these extracts could constitute glimmers of hope by offering an alternative to the management of malaria.

Key Words: *Coffea arabica, Coffea canephora, Alkaloids, Antiplasmodial activity, Cytotoxicity*

eIJPPR 2022; 12(1):54-59

HOW TO CITE THIS ARTICLE: Zibi RDN, Tala VRS, Mbopi PY, Bayaga NH, Tcheuffa GMN, Ngoupayo J. Comparative Antiplasmodial and Cytotoxic Activities of *Coffea arabica* and *Coffea canephora alkaloids extracts*. Int J Pharm Phytopharmacol Res. 2022;12(1):54-9. https://doi.org/10.51847/md2J5bMnQF

INTRODUCTION

Malaria is one of the deadliest parasitic diseases in the world [1]. It is one of the most important health problems in tropical and subtropical regions [2]. In sub-Saharan Africa, malaria infected more than 200 million people in 2020 indicating its exponential increase attributed to the interruption of malaria-control services during the wake of Covid-19 pandemics [3, 4]. In Cameroon, malaria is still an important public health threat; it remains the first cause of morbidity and mortality among the most vulnerable groups [5, 6].

Plasmodium falciparum is the main parasite responsible for over 95% of malaria cases [7, 8]. The control strategy of early and effective management of malaria has not been satisfactory. Unfortunately, the increasing spread of drug-resistant *P. falciparum* strains has worsened the situation [9, 10]. The rise in resistance of *Plasmodium falciparum* to conventional antimalarial drugs in humans [9] has

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Received: 10 December 2021; **Revised:** 17 February 2022; **Accepted:** 22 February 2022

therefore led to research of new drugs with new modes of action.

Research of new cures from medicinal plant species used in the treatment of malaria depends on precise and specific ethnopharmacological information obtained from local healers [2]. In Cameroon, research about the evaluation of antiplasmodial activity [11] of medicinal plants such as Coffea used for the treatment of malaria in traditional medicine is not new [12, 13]. The present study is aimed at comparing the antiplasmodial activity of *Coffea arabica* and *Coffee canephora.*

MATERIALS AND METHODS

Plant collection

Plants were harvested in the Western region of Cameroon more specifically in Bantoum for *Coffea robusta* and Bangoua for *Coffea arabica*. Botanical identification numbers are given at the National Herbarium of Cameroon based on comparison with documented samples by botanists for *Coffea arabica* and *Coffea canephora* 58228/HNC and 67456/ HNC, respectively. Extractions were made in the Laboratory of Pharmacognosy and Pharmaceutical Chemistry of the Faculty of Medicine and Biomedical Sciences (FMSB) of the University of Yaoundé I. Various tests were carried out in the Laboratory of Phytobiochemistry and Study of medicinal Plants of the Faculty of Sciences, University of Yaoundé I.

Extraction of total alkaloids

Extraction was performed on the samples according to a slightly modified method described by Kemajou *et al*. (2012) [14]. Five hundred grams (500 g) of leave powder from each plant species weighed on an ARCHMED brand electronic scale (sensitivity of 0.01g and a range of 510 g) were macerated with 1800 mL of methanol for 48 hours with constant stirring using a PRO HPS stirrer -7 Lab Plus Series. This operation was repeated twice. The crude extracts that had been obtained after filtration through Whatman paper N°3 and concentration under reduced pressure using a BUCHI R-201 rotary evaporator were acidified with 0.5N hydrochloric acid and then placed in a separatory funnel with 100 mL of chloroform. The collected aqueous phase was alkalinized by 6 mL of a 33% ammonia solution to reach a pH between 9 and 10, then placed again in a separatory funnel with 100 mL of chloroform. The chloroformic phase was then collected, dried using anhydrous magnesium sulfate, and then filtered to obtain total alkaloids.

In vitro antiplasmodial activity test

Preparation of stock solutions of total alkaloids, chloroquine, and artemisinin

A stock solution of the different total alkaloids (100 mg/mL) was prepared by dissolving 100 mg of total alkaloids in 1mL of dimethylsulfoxide (DMSO). Chloroquine (98 %. Sigma-Aldrich) and Artemisinin (98%, Sigma-Aldrich) were prepared at a concentration of 1 mM. Preparation of intermediate concentrations was carried out by adding either 10 µL of total alkaloids (100 mg/mL) or 20 µL of chloroquine or artemisinin (1mM) in 190 µL or 180 µL of incomplete RPMI1640 medium respectively contained in a 96-well microplate followed by a geometric dilution of order 5 leading to concentrations ranging from 8 to 5000 µg/ mL for total alkaloids and from 0.016 to 10 µM for Artemisinin and Chloroquine.

Cultivation of the malaria parasite

Two strains of *plasmodium falciparum*, one chloroquine-sensitive (NF54-E) and another multi-resistant (Dd2) were cultured according to the method described by Trager and Jensen in 1976 [15]. The strains were cultured in human red blood cells of group O Rhesus positive at 4% hematocrit in RPMI 1640 medium (500 mL) supplemented with 25 mM HEPES, 0.50% Albumax I, 45 µg/ mL of hypoxanthine, and 20 µg/ mL gentamicin (0.5mL) and incubated at 37ºC in a humidified incubator containing 92% N_2, 5% CO_2 and 3% O_2. The medium was replaced daily by a complete RPMI medium to allow parasite growth in culture. Subsequently, blood smears were made, stained with Giemsa 10%, and then observed under optical microscope objective 100 with immersion oil to follow all the stages of the cell cycle and evaluate the parasitemia.

Antiplasmodial activity test

The antiplasmodial activity test was evaluated according to the method described by Smilkstein *et al*. (2004) [16], based on the fluorescence of SYBR Green I. Ninety microliter s (90 µL) of parasite suspension synchronized in stage at rings of 2% parasitemia and 1% hematocrit were incubated with 10 µL of different concentrations of each pre-diluted total alkaloid, Artemisinin or Chloroquine solution. Plates were incubated 72 hours at 37° C. in a humidified incubator consisting of 92% N_2, 5% CO_2, and 3% O_2. Final concentrations in the test plates ranged from 0.8 to 500 µg/ mL (DMSO < 1%) for each extract and from 0.0016 to 1µM (DMSO 0.1%) for Artemisinin and Chloroquine in a final volume of 100 µL. The experiments were carried out in duplicates.

After 72 hours of incubation, 100 µL of SYBR Green I buffer [6 µL of 10,000 × SYBR Green I (Invitrogen) + 600 µL of red blood cell lysis buffer {Tris (25 mM; pH 7.5)} + 360 µL of EDTA (7.5 mM) + 19.2 µL of parasite lysis solution {saponin and 28.8 µL of Triton X-100 (0.08%; v/v)}] were added to each well and incubated 1 hour in the dark at 37 °C. Fluorescence was measured using an Infinite

M200 (Tecan) Microplate reader with excitation and emission wavelength of 485 and 538 nm, respectively.
Resistance index was determined through IC_{50} ratio expressed as **IR = CI_{50} extracted on PfDd2 / CI_{50} of the same extract on 3D7**. A resistance index below **1** indicates that the inhibitor (plant extract) acts preferentially on the resistance strain.

Kinetics of growth inhibition of multi-resistant strain (PfDd2) in vitro
Evaluation of the kinetics of the growth inhibition of the resistant strain in vitro was carried out using the method described by Le Manach *et al*. (2013) [17]. On an asynchronous culture (containing all stages of development) of *P. falciparum*, different concentrations of the extracts were added and the mixture was incubated. The antiplasmodial activity was evaluated using a method based on nuclear staining with SYBR Green and expressed as an IC_{50} value. For each extract of the total alkaloids, three incubation times were used, namely 24 h, 48 h, and 72 h. Subsequently, the ratio of normalized IC_{50} compared to 72 hours IC_{50} was used to classify the action time effect (fast or slow) of the different extracts.

Cytotoxicity assay
Cytotoxicity assessment of the total alkaloid extracts was evaluated according to the method described by Bowling *et al*. (2012) and Al-Daghistani *et al*. (2021) [18-20]. Vero cell line (ATCC CRL 1586) from normal African green monkey kidney and Raw (ATCC #TIB-71) from murine macrophages were maintained in modified complete Dulbecco Eagle medium supplemented with 10% fetal bovine serum, bicarbonate of sodium 0.2% (w/v) with a penicillin-streptomycin combination 1% (v/v). A cell with a density of 10,000 cells per well in a 100 µL suspension was seeded on 96-well plates and incubated at 37°C for 24 hours at 5% CO_2 to reach 90% confluence. Ninety microliters (10 µL) of total alkaloid extracts or control were added after 24 hours with an initial 500 µg/ml concentration. Podophyllotoxin as Positive control (20 µM) was added. Thereafter, 10 µL of Resazurin solution (0.15 mg/mL in sterile PBS), was added to all wells and incubated for an additional 4 hours at the same condition. Fluorescence was subsequently read via a Magelan multi-well plate fluorescence reader, Infinite M200 (Tecan) at the excitation and emission wavelengths of 530 and 590 nm respectively [18]. Selectivity Index (SI) was used as a parameter of the clinical significance of test samples by comparing general toxins and selective inhibitory effect of *P. falciparum* using the following equation [21]:

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

In addition, the cytotoxicity activity of our extracts was evaluated according to the cytotoxicity criteria for crude extracts as established by the American National Cancer Institute (NCI) stipulating that IC_{50} of the extract < 30 µg/ mL [22].

Data processing
Data collected were transferred to an Excel table for interpretation and calculation. Fluorescence values obtained were used to calculate the inhibition percentages using Microsoft Excel 2016 software. Inhibitory concentrations 50 (IC_{50}) was then determined using the concentration-response curves plotted to the logarithm of the concentration as a function of the percentage inhibition. The software used was Graph-pad software Prism 9.

RESULTS AND DISCUSSION

Determination of yields led to obtaining values for each extract as is shown in **Table 1**.

Table 1. Extraction yield

Botanical name	Mass of Powder (g)	Mass of total alkaloids (g)	Extraction yield (%)
Coffea arabica	500	0.23	0.05
Coffea canephora	500	0.38	0.08

This result shows that from the same initial mass of plant powder, *Coffea canephora* is richer in alkaloids with a yield of 0.08% than *Coffea arabica* contents 0.05%.
The difference between these two yields could be explained by each plant's contents in alkaloids. Indeed, caffeine is the major alkaloid of these two species [23], and caffeine content is higher in *C. canephora* [24]. In addition, some parameters like climate and culture conditions can modify the chemical composition [25] and influence extraction yield [26, 27].
In vitro antiplasmodial test of total alkaloids of the two species and the resistance, the index is presented in **Table 2**.

Table 2. Antiplasmodial activity of total alkaloids and resistance index

Botanical name and control	IC_{50} (µg/ mL)		Resistance index
	PfDd2	Pf3D7	
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
Chloroquin	517.4 ±0.03	40.36 ± 0.00	517.4±0.03

Pf: *Plasmodium falciparum;* **IC_{50}**: Inhibitory Concentration 50; **AT:** Total Alkaloids; **ND:** Not determined

The results were interpreted as follows: high activity (IC_{50} < 5 µg/ mL), active (5 ≤ IC_{50} < 50 µg/ mL), moderate activity (50 ≤ IC_{50} < 100 µg/ mL), inactive (IC_{50} > 100 µg/ mL) [28].

Total alkaloids extract from dried leaves of *Coffea arabica* is active both on the chloroquine-sensitive Pf3D7 strain and on multi-resistant PfDd2 strain with $IC_{50\ values}$ of 9.53 ± 1.51 µg/ mL and 7.48 ± 0.93 µg/ mL respectively, while total alkaloid extract from dried leaves of *Coffea canephora* showed weak activity (IC_{50} >100 µg/ mL) on the Pf3D7 strain and moderate activity (IC_{50} = 85.55 ± 1.17 µg/ mL) on the PfDd2 strain **(Table 2)**. These results are different from those of Lacroix *et al*. (2011) [29] who worked on ethyl acetate extract of leaves of *Coffea arabica*. This difference may be due to the nature of the extract and the solvent used. Indeed, ethyl acetate will weakly extract alkaloids and more other compounds such as mono and diglycosidic flavonoids [30]. These results show that alkaloids could therefore be responsible for the antiplasmodial activity of the Coffea genus. Moreover, the antiplasmodial activity of total alkaloids of *C. arabica* being greater than that of *C. canephora* while alkaloid extraction yield being higher in *C. canephora* **(Table 1)** would suggest some differences in the alkaloid composition of the leaves of these two species. Furthermore, antiplasmodial activity **(Table 2)** may show that extracts could act preferentially on the multi-resistant PfDd2 strain with a resistance index of 0.78 (IR<1) probably because it acted on the resistance genes carried on the genomes of multi-resistant isolates (PfDd2) but absent in chloroquine-sensitive isolates (3D7).

Concerning kinetics of *in vitro* growth inhibition of multi-resistant strains (PfDd2), IC_{50} values were determined for the two standards (chloroquine and artemisinin) as well as for the two total alkaloid extracts after 24, 48, and 72 hours of totals culture time incubation **(Figure 1 and Table 3)**.

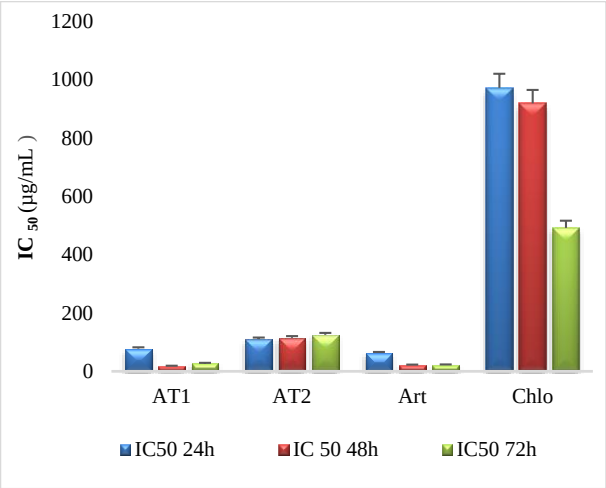

Figure 1. $IC_{50\ inhibition\ kinetics}$ (unsynchronized Dd2 culture)

AT1: Total alkaloids of *C. arabica*; **AT2**: Total alkaloids of *C. canephora*; **Art**: Artemisinin; **Chlo**: Chloroquine

Table 3. Data on inhibition kinetics and speed of action

IC_{50} (µg/ mL) on the *P. falciparum* Dd2 strain					
	24 hours	**48 hours**	**72H**	**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
Chloroquin	970.9± 0.01	918.0 ± 0.02	491.4 ± 0.00	1.97	Fast action

R: Ratio CI_{50} 24h / CI_{50} 72h; **ND:** not determined

The 24-hour and 72-hour dosage of AT1, Artemisinin and Chloroquine gave IC_{50} values of 78.33 ± 0.00; 63.28 ± 0.00 and 970.9 ± 0.01 (24 hours) and 27.56 ± 0.00; 22.16 ± 0.00 and 491.4 ± 0.00 (72 hours) respectively. Calculated ratios show that IC_{50}s were 2.89; 2.86 and 1.97 times higher at the 24-hour time point compared to those generated at the 72 hour time point **(Table 3)**. These three days assessment data are a good indicator that the AT1 extract and the standards state with rapid action. AT2 extract values on the other hand were high at the three points (24, 48, and 72 hours) describing slow action. These results show that unlike total alkaloid extract of *C. canephora* (AT2), total alkaloid extract of *C. arabica* (AT1) could act at all evolutionary stages of the parasites (rings, trophozoites, schizonts) explaining the alkaloid difference composition hypothesis of the two species.

Biological efficiency is generally not due to *in vitro cytotoxicity* when the selectivity index (SI) is higher or equal to 10 [31]. In this study, *in vitro* cytotoxicity is said to be low when the Selectivity index is less than 10 and high when the Selectivity index is higher than 10 according to Waiganjo *et al*. (2020) [21]. Results are summarized and presented in **Table 4** in which total alkaloid extracts of *C. arabica* and *C. canephora* have high selectivity index (SI ≥ 10) compared to Vero and Raw cells, and their IC_{50s} were > 30 µg/ mL **(Table 4)**. This result indicates that the total alkaloids of the two species of the *Coffea* are non-cytotoxic [31].

Table 4. Selectivity index (SI) of total alkaloid extracts assayed against Vero, Raw cells, and the Dd2 resistant strain of *P. falciparum*

Extract	**IC_{50} (µg/ mL) on Dd2 stump**	**IC_{50} (µg/ mL) on the cells**		**IF on the cells**	
		Vero	Raw	Vero	Raw
AT1	7.48 ±0.93	>500	>500	ND	ND

AT2	85.55 ± 1.17	>500	115.05±8.13	ND	1.34

AT1: Total alkaloids of *C. arabica;* **AT2:** Total alkaloids of *C. canephora*; **ND**: not determined
SI: Selectivity Index

CONCLUSION

For the first time, a promising antiplasmodial and antimalarial activity presenting an interesting selectivity index has been demonstrated about total alkaloids of two species of Coffea (*C. arabica* and *C. canephora*). Results revealed that *C. arabica* showed a better activity with very low cytotoxicity. This plant is particularly interesting for further investigation as its phytochemical composition is poorly known, precisely concerning alkaloids. All these results justify their uses in traditional medicine for the treatment of malaria and can be a substrate for Improved Traditional Medicines (MTA).

Acknowledgments: None

Conflict of interest: None

Financial support: None

Ethics statement: None

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