



Assessment of Antiplasmodial and Antioxidant Activities, Total Phenolics and Flavonoids Content, and Toxicological Profile of *Cola millenii* K. shum (Malvaceae)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors LL and LSD conceived the idea and designed the study. Authors GHT and AAM carried out experiments. Authors GHT, AMOA and RA analyzed data. Authors LL, GHT and LSD drafted the manuscript. All authors approved the final manuscript.

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ABSTRACT

Aims: This study was designed to assess antiplasmodial and antioxidant activities in relation to phytochemical contents and toxicological profile of crude extracts of *Cola millenii* leaves.

Place and Duration of Study: The study was carried out from April 2015 to November 2018 at University of Abomey-Calavi, Laboratory of Biochemistry and Bioactives Natural Products and Laboratory of Infectious Vector-borne Diseases.

Methodology: Phytochemical screening of aqueous and ethanolic crude extracts was performed using standard methods. Estimation of total phenolics contains (TPC) and total flavonoids contains

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(TFC) was done using colorimetric methods. Antioxidant activity was assessed *in vitro* by 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical-Scavenging, Reducing power, superoxide radical scavenging and Hydrogen peroxide scavenging assay. Antiplasmodial activity was investigated *in vitro* using both field isolate and laboratory 3D7 strains of *P. falciparum* using *Plasmodium* lactate dehydrogenase (pLDH) assay. Hemoglobin release was measured spectrophotometrically to assess hemolytic power. Acute oral toxicity of extracts was evaluated on rats according to OECD 423 guideline.

Results: Flavonoids, anthraquinones, coumarins, and triterpenes were recorded in both extracts while tannins were recorded only in ethanolic extract. High TPC and TFC were recorded with ethanolic extract with significant difference ($P < 0.01$) when compared to aqueous extract. Ethanolic extract exhibited the highest parasite growth inhibition against both field isolate ($41.62 \pm 1.78\%$) and 3D7 ($45.89 \pm 1.66\%$) strains at $100 \mu\text{g/mL}$. Both extracts exhibited strong antioxidant activity according to different methods used. Toxicological profile revealed no hemolytic effect on human red blood cells as well as acute toxicity signs in rats.

Conclusion: This study demonstrated strong antioxidant and moderate antiplasmodial activities of *C. millenii* extracts without toxicity effect on rats and human erythrocytes. It would play an important role in malaria and oxidative damage control.

Keywords: *Cola millenii*; phytoconstituents; antioxidant activity; antiplasmodial activity; toxicity.

ABBREVIATIONS

TPC	:	Total phenolic content,
TFC	:	Total flavonoid content,
TLC	:	Time layer chromatography,
GAE	:	Gallic acid Equivalents,
QE	:	Quercetin Equivalent,
DPPH	:	2, 2-diphenyl-1 picrylhydrazyl,
AAE	:	Ascorbic Acid Equivalent,
DMSO	:	Dimethylsulfoxide,
NBT	:	nitro blue tetrazolium,
NaOH	:	Sodium hydroxide,
IRSP	:	Institut régional de santé publique,
UAC	:	Université d'Abomey-Calavi,
HEPES	:	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid,
RPMI	:	Roswell Park Memorial Institute medium,
AlbuMAX	:	Lipid rich bovine serum albumin,
pLDH	:	Plasmodium lactate dehydrogenase,
SDS	:	Sodium Dodecyl Sulfate,
OECD	:	Organization for Economic Cooperation and Development,
EDTA	:	Ethylene diamine tetraacetic,
HCT	:	haematocrit, Hgb: haemoglobin,
CQ	:	Chloroquine, Art: Artesunate,
MCHC	:	Mean corpuscular haemoglobin concentration,
RBC	:	Red blood count, MCV: Mean corpuscular volume differential,
MCH	:	Mean corpuscular haemoglobin,
PLT	:	Platelet count,
WBC	:	White blood cells count,
Crea	:	Creatinine,
AST	:	Aspartate aminotransferase,
ALT	:	Alanine aminotransferase,
Glu	:	Glucose,
Et-ex	:	Ethanolic extract,
Aq-ex	:	Aqueous extract.

1. INTRODUCTION

Native to West Africa, *Cola millenii* K. Schum (*Malvaceae*) is a semi-deciduous wet dense forest and swampy forest species encountered from Ivory Coast to Nigeria [1]. In Benin, it is encountered in the Guinean and Soudano-Guinean zones [1]. Commonly called *Alovi aton* (Fon, goun: Benin), *Kakara agba* (Nago: Benin) and *Monkey kola* (English), the plant has several medicinal uses in Benin republic. The decoction of leaves is used in folk medicine to treat malaria [2–6], jaundice, abscess, rashes, varicella [1,7]. The use in treatment of ringworm, scabies, gonorrhoea, dysentery and ophthalmia have been reported in Nigeria [8].

The presences of alkaloids, flavonoids, saponins, tannins, phenols, glycosides, terpenes and steroids have been revealed in the plant [9–11]. Previous studies have revealed that *C. millenii* have different biological properties including antimicrobial [9,11], sedative, anticonvulsive [12], hepato-protective and cardio-protective [10].

Despite several reports on considerable use of *Cola millenii* in traditional medical system to treat malaria in Benin, until now, no studies reporting its antiplasmodial and antioxidant properties as well as toxicological profile. The present study was designed to assess *in vitro* antiplasmodial and antioxidant potentials and to determine total phenolics and flavonoids content, acute oral toxicity and hemolytic power of this plant.

2. MATERIALS AND METHODS

2.1 Plant Material and Extracts Preparation

Leaves of *Cola millenii* were collected in April 2015 in Daagbe, Municipality of Ifangni/Plateau in southern Benin. The plant was further identified at the National Herbarium (Herbier National du Bénin, Université d'Abomey Calavi, Bénin) where the voucher specimen was deposited under reference number «YH 354/HNB».

2.2 Preparation of Crude Extracts

Leaves were dried for two weeks at laboratory temperature (22°C) and ground into a fine powder. Crude ethanolic extract was prepared by maceration of 1 Kg of powder in ethanol (70%) overnight under mechanical agitation at room temperature. Filtrate obtain after three

washing and filtration with filter paper Whatman N°1, was concentrated using a rotary evaporator coupled with vacuum pump (BUCHI Rotavapor R11, Switzerland) at 40-50°C to obtain the crude ethanolic extract. The aqueous extract was obtained by boiling of 150 g of vegetable powder in 1.5 L of water at 100°C for 30 minutes. After cooling, the filtrate obtained was concentrated using rotary evaporator coupled with vacuum pump (BUCHI Rotavapor R11, Switzerland; Vacubrand PC101NT, Germany) at 60°C. All extracts were kept at 4°C for biological analyzes.

2.3 Qualitative Phytochemical Screening

The phytochemical screening of both crude extracts was carried out using thin layer chromatography (TLC) and colorimetric test in glass tube according to respective standard protocol [13,14] to detect the absence or presence of certain phytoconstituents including Alkaloids, flavonoids, triterpenes, tannins, coumarins, anthraquinone, saponins, essential oil, lignans and anthocyanins.

2.4 Estimation of Total Phenolic Content

The estimation of total phenolic content (TPC) was done by colorimetric method using Folin-ciocalteu reagent [15]. A volume of 200 µL of sample (100 µg/mL) was mixed to 1 mL of Folin-ciocalteu reagent (10%). After 4 min, 800 µL of saturated sodium carbonate (75 g/L) were added. The mixture was incubated for 2 hours at room temperature and the absorbance was measured at 765 nm using a UV/vis spectrophotometer (VWR UV-1600PC, China). Standard regression curve ($y = 0.0428x - 0.052$; $R^2 = 0.9937$) for estimation of TPC was plotted with gallic acid. The assays were performed in triplicate and the results were expressed as mg of Gallic acid Equivalents (GAE) per gram of extract.

2.5 Estimation of Total Flavonoid Content

Total flavonoid contents was measured by spectrophotometer according to aluminum chloride colorimetric assay [16]. The reaction mixture was constituted with 250 µL of each sample (100 µg/mL), 750 µL of ethanol (96%), 50 µL of potassium acetate (1M), 50 µL of aluminum chloride (10%) and 1400 µL of distilled water. After 30 minutes of incubation at room temperature, the absorbance was measured at 450 nm. Quercetin was used to plot standard calibration curve ($y = 0.0162x - 0.0347$; $R^2 =$

0.9997) and total flavonoid content was expressed as mg of Quercetin Equivalent (QE)/ g of extract.

2.6 Antiplasmodial Activity

The crude ethanolic and aqueous extracts of *C. millenii* were tested *in vitro* against a field isolate and chloroquine-sensitive (3D7) *Plasmodium falciparum* parasites supplied respectively by the Laboratory of Infectious Vector Borne Diseases, IRSP/UAC, Benin and Institut Pluridisciplinaire Hubert Curien, UMR 7178-CNRS/Unistra, France. Parasites were maintained in continuous culture in fresh O⁺ human erythrocytes maintained in malaria complete medium (RPMI 1640 with 0.8% AlbuMAX II, 25 mM HEPES, 1mM L-glutamine, 0.4mM Hypoxanthine and 0.05 mg/mL Gentamicin) according to the modified method of Trager and Jensen [17]. The cultures were maintained in a standard gas mixture (3% O₂, 5% CO₂, 92% N₂) at 37°C. Parasitaemia was monitored and daily adjusted between 1% and 5% by microscopy (OPTIKA MICROSCOPES DM-25, Italy) using thin smears colored with Giemsa. *Plasmodium falciparum* sensitivity to extracts was carried out in 96-well plates. A volume of 100 µL of parasite suspension (1% parasitaemia, 4% hematocrit) was mixed with 100µL of each extract (100 µg/mL) previously dissolved in malaria complete medium and the plate was incubated for 96 hours in *P. falciparum* culture conditions as describe above. All assays were performed in triplicate. Parasites viability was assessed by immune-dosage of *Plasmodium* lactate dehydrogenase (pLDH) using ELISA malaria antibody test kit (ApDia, Belgium) according to manufacturer protocol. The concentrations of pLDH in the tests well were measured at 450 nm with microplate reader (Rayto R 6500, China) against a positive control (parasite suspension only) and negative control (red blood cells only). The positive and negative controls of the kits were also plotted to access efficacy of test following the recommendations of the manufacturer. Chloroquine diphosphate and artesunate were used as antimalarial drug. The percentage of parasite growth inhibition (PI) was extrapolated using the following formula:

$$PI = 100 - [(ODs - ODnc)/ODpc] * 100$$

Where,

ODs = optical density generated by sample,
ODnc = optical density generated by negative

control and ODpc = optical density generated by positive control.

2.7 Antioxidant Assay

2.7.1 DPPH radical scavenging activity assay

The DPPH radical (2,2-diphenyl-1 picrylhydrazyl) scavenging activity of extracts was evaluated spectrophotometrically according to the method previously described by Amoussa et al. [15] with slight modification. The reaction mixture was constituted with 1.5 mL of a freshly prepared methanolic solution of DPPH (0.04%) and 0.75 mL of methanolic solution of extracts in varying concentrations (300 µg/mL to 2.34 µg/mL). The mixture was shaken and incubated for 20 minutes in the dark at room temperature. DPPH radical reduction was measured at 517 nm against methanolic DPPH solution as a blank. All tests were realized in triplicate. The DPPH radical scavenging percentage was determinate according to the formula:

$$\text{Inhibition (\%)} = [(Ab - As)/Ab] \times 100$$

Where,

Ab is absorbance of the blank and As is absorbance of the test sample.

Sample concentration providing 50% inhibition of DPPH (IC₅₀) was determinate from graph plotting inhibition percentage against extracts concentration.

2.7.2 Reducing power assay

The capacity of extracts to reduce iron (III) to iron (II) was evaluated following the potassium ferricyanide-ferric chloride method [16]. Briefly, 2 mL of each extract (100 µg/mL) were mixed with 2 mL of phosphate buffer (0.2 M, pH 6.6) and 2 mL of potassium ferricyanide (10 mg/mL). After 20 min of incubation at 50°C, the reaction was stopped with 2 mL of trichloroacetic acid (100 mg/L). The mixture was then centrifuged at 3000 rpm for 10 min. 2 mL of the upper solution were mixed with 2 mL of distilled water and 0.4 mL of fresh ferric chloride (0.1%: w/v). Absorbencies were measured at 700 nm after 10 min of reaction. Ascorbic acid was used to produce the calibration curve ($y=0.0069x+0.015$; R²=0.9625). The assays were performed in triplicate and the iron (III) reducing activity was expressed in mmol of Ascorbic Acid Equivalent (AAE)/g of extract.

2.7.3 Superoxide radical scavenging activity

This test consists to measure the capacity of extract to quench superoxide radical generated by alkaline DMSO [18]. Superoxide radical scavenging activity of extract is proportional to nitro blue tetrazolium (NBT) reduction by superoxide into formazan dye at room temperature and can be measured around 630 nm. In microplate 96 wells, the reaction mixture was constituted by 50 μ L of extract (100 μ g/mL in DMSO) and 170 μ L of Alkaline DMSO (1mL DMSO, 100 μ L NaOH 5mM). 30 μ L of NBT (1 mg/mL in DMSO) were added and incubated for 5min at room temperature. The absorbance was measured at 630 nm using microplate Reader (Rayto R 6500, China). Quercetin was used as reference. The percentage of superoxide quenching (PI) of extracts was determinate using the following formula:

$$PI = [(A_0 - A_1) / A_0] * 100$$

Where,

A_0 is absorbance of the blank constituted by Alkaline DMSO with NBT and A_1 is absorbance of the tested sample.

2.7.4 Hydrogen peroxide radical scavenging activity

The ability of extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al. [19] with slight modification. A volume of 500 μ L of extract (100 μ g/mL) dissolved in distilled water were mixed with 600 μ L of hydrogen peroxide solution (100mM) previously prepared in phosphate buffer (0.1mM, pH 7.4). The absorbance of the mixture was determined spectrophotometrically 10 minutes later against a blank (phosphate buffer) and control (hydrogen peroxide solution) at 295 nm. The percentage of hydrogen peroxide scavenging of extracts and Gallic acid (standard) was calculated using the following formula:

$$\text{Scavenged (H}_2\text{O}_2\text{) \%} = (Ac - As) / Ac \times 100$$

Where,

Ac is absorbance of control and As is absorbance of sample or standard. All tests were performed in triplicate.

2.8 Toxicological Study

2.8.1 Hemolytic power

Hemolytic power assay was performed to assess cytotoxicity effects of extracts on human red

blood cells. The test was evaluated following the method used by Sarr et al. [20] with slight modification. Briefly, 100 μ L of extracts previously dissolved in RPMI 1640 (ranging from 200 μ g/mL to 1.56 μ g/mL by two fold dilution) were mixed with 100 μ L of red blood cells (4% hematocrit) in 96-well plate. The mixture was incubated at 37°C under circular agitation. After 1 hour, the plate was centrifuged at 2000 rpm for 5 min and 150 μ L of supernatant was transferred into new 96-well plate. Haemoglobin content in the supernatants was determined by absorbance measurements at 450 nm in microplate reader. 100% hemolysis (positive control) was obtained with 5% Sodium Dodecyl Sulfate (SDS) and non-infected erythrocytes suspension was used as the blank. Hemolysis percentage was expressed according to the formula:

$$\text{Hemolysis \%} = [(As - Ab) / Ac] * 100$$

Where,

As is absorbance of the sample, Ab is absorbance of the blank and Ac is absorbance of positive control.

2.8.2 Acute oral toxicity assay

Experiments were performed on nulliparous and non-pregnant females Wistar rats, aged 10 to 12 weeks and weighing 180 to 200 g. Animals were kept in animal house and maintained under laboratory conditions (22 – 25°C; 12 h light/dark cycle). They were fed with standard laboratory diets and water available ad libitum.

The acute toxicity of *C. millenii* crude ethanolic extracts was carried out using the median lethal dose according to Organization for Economic Cooperation and Development (OECD) guidelines 423 [21]. A total of 6 rats divided in 2 groups of 3 animals each were ranged in different cage where the test group was orally crammed with a single dose 2000 mg/kg body weight of extract and the control group has received distilled water (10 ml/kg body weight). The animals were deprived of food for over-night before administration of extracts. The animals were observed in detail during the first eight hours and daily further for a period of 14 days. The monitoring was based on general toxicity signs, behavior changes, body weight evolution (days 0, 7 and 14) and mortality. The animals were anesthetized with ether on 15th day after an over-night food privation and blood samples

were collected into tubes with EDTA and tubes without EDTA respectively for hematological and biochemical analyzes.

2.8.3 Hematological and biochemical parameters

Hematological analyzes were carried out using an automated hematology analyzer (Sysmex XP-300, Japan). Parameters as hematocrit (HCT), haemoglobin (Hgb), Mean corpuscular haemoglobin concentration (MCHC), red blood count (RBC), leukocytes formula (lymphocytes), mean corpuscular volume differential (MCV), mean corpuscular haemoglobin (MCH), platelet count (PLT), white blood cells count (WBC) were examined.

Biochemical parameters including blood glucose, blood urea, creatinine (Crea), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were analyzed using clinical chemistry analyzer (CHEM-7 ERBA DIAGNOSTICS MANNHEIM GmbH, Germany).

2.9 Statistical Analysis

The results were expressed as means \pm standard error of mean (S.E.M.). Student T test was used to compare data and difference was considered statistically significant when the $p < 0.05$. Graph Pad Prism 8.0.2 version (GraphPad Prism software Inc., San Diego CA) was used to carry out the statistical analysis. Nonlinear regression analysis was used to determine IC_{50} value for the DPPH antioxidant test. The graphical representations of the data were performed using the Graph Pad Prism and Microsoft Excel 2010 software.

3. RESULTS

3.1 Phytochemical Constituents

Phytochemical screening of crude ethanolic (Et-ex) and aqueous (Aq-ex) extracts of *C. millenii* leaves showed presence of coumarins, flavonoids, anthraquinones, triterpenes in both extracts and tannins in ethanolic extract only (Table 1). The phytoconstituents such as saponins, alkaloids, anthocyanins, essential oil and lignans are absent in both extracts.

Total phenolics and flavonoids contents were estimated respectively on the basis of standard regression of gallic acid and quercetin (Fig. 1). The highest Total phenolics content (TPC) was quantified in ethanolic extract (9.12 ± 0.17 mg GAE/g of extract) with significant difference ($P < 0.01$) when compared to aqueous extract (6.91 ± 0.37 mg GAE/g of extract). Total flavonoids content (TFC) was revealed as highest also in ethanolic extract (32.93 ± 2.45 mg QE/g of extract) when compared to aqueous extract (13.28 ± 1.17 mg QE/g of extract) with significant difference ($P < 0.01$).

3.2 Antiplasmodial Activity

The antiplasmodial activity of crude ethanolic and aqueous extracts of *C. millenii* leaves was tested against field isolate and chloroquine sensitive (3D7) *Plasmodium falciparum* at the single dose of 100 μ g/mL and the results are showed in Fig. 2. The crude ethanolic extract exhibited the highest parasite growth inhibition against field isolate ($41.62 \pm 1.78\%$) and 3D7 ($45.89 \pm 1.66\%$) parasites with significant difference ($P < 0.01$) when compared respectively to aqueous extract ($32.68 \pm 2.54\%$, $31.35 \pm 1.19\%$). The chloroquine used as control inhibited both strains at more than 70 % at the same concentration.

Table 1. Qualitative phytoconstituents of crude extracts of *C. millenii*

Chemical components	<i>C. millenii</i>	
	Aqueous	Ethanol
Alkaloids	-	-
Flavonoids	+	+
Saponins	-	-
Tannins	-	+
Anthraquinones	+	+
Anthocyanins	-	-
Coumarins	+	+
Triterpenes	+	+
Lignans	-	-
Essential oil	-	-

- Absence, + Presence

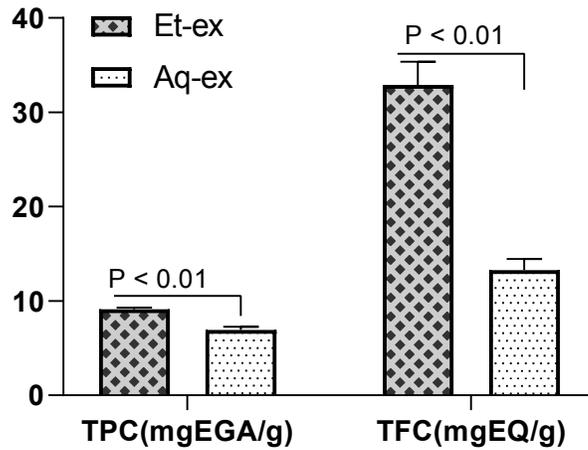


Fig. 1. Total phenolic (TPC) and flavonoids (TFC) contents of *C. millenii* extracts
 Et-ex ethanolic extract, Aq-ex aqueous extract, EGA Equivalent of Gallic acid, EQ Equivalent of Quercetin
 $P < 0.05$ indicating statistically significant difference. Data represents means for three experiments \pm SD ($n = 3$)

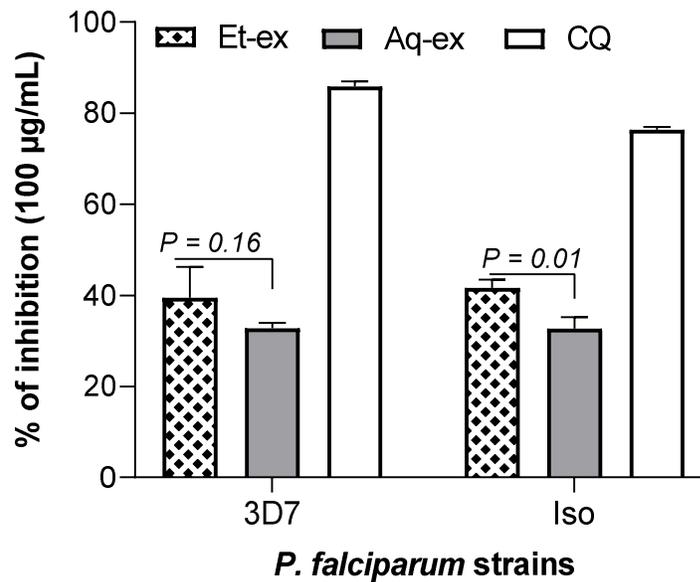


Fig. 2. Antiplasmodial activity of *C. millenii* against chloroquine sensitive (3D7) and field isolates (Iso) *P. falciparum*

Et-ex ethanolic extract, Aq-ex aqueous extract, CQ chloroquine diphosphate $P < 0.05$ indicating statistically significant difference. Data represents means for three experiments \pm SD ($n = 3$)

3.3 Antioxidant Activity

The antioxidant potential of *C. millenii* leaves extract was assessed *in vitro* by determining DPPH radical scavenging activity, iron III reducing power, superoxide anion and hydrogen

peroxide scavenging capacity. The Fig. 3 summarizes the antioxidant activity of *C. millenii*. Both extracts show dose-dependent DPPH radical scavenging activity. The best IC_{50} (half maximal inhibitory concentration) value was observed with ethanolic extract (70.05 ± 3.66

$\mu\text{g/mL}$) with significant difference ($P < 0.01$) when compared to aqueous extract ($137 \pm 5.94 \mu\text{g/mL}$) (Fig. 3A). The ferric reducing antioxidant capacity of both extract was respectively $1448.45 \pm 157.02 \mu\text{M AAE/g}$ of extract and $1344.21 \pm 25.14 \mu\text{M AAE/g}$ of extract ($P = 0.32$) at the single dose of $100 \mu\text{g/mL}$, indicating strong reducing capacity of extracts when compared to Ascorbic acid ($1004.24 \pm 62.03 \mu\text{M AAE/g}$) with significant difference ($P \leq 0.01$) (Fig. 3B). Both extracts also exhibited strong superoxide anion scavenging capacity with an inhibition percentage more than 90 per cent when tested at $100 \mu\text{g/mL}$ of extract (Fig. 3C). Comparing to quercetin (83.58%), both extracts showed significant difference ($P = 0.001$). At the same concentration, hydrogen peroxide inhibition percentage of both extracts where respectively 55.58 ± 1.51 for ethanolic extract and 38.50 ± 2.85

for aqueous extract. These results indicate significant difference between hydrogen peroxide quenching capacities of both extracts ($P = 0.001$) on the one hand and between both extracts compared to gallic acid ($P < 0.001$) on the other hand (Fig. 3D).

3.4 Toxicological Profile

3.4.1 Hemolytic power of extracts

The hemolytic effect of crude ethanolic and aqueous extracts of *C. millenii* leaves is illustrated by Fig. 4. The results show very low cytotoxic effect of both extracts on human red blood cells with a hemolysis percentage less than 1%. This indicates non cytotoxic effect of both extract against human red blood cells.

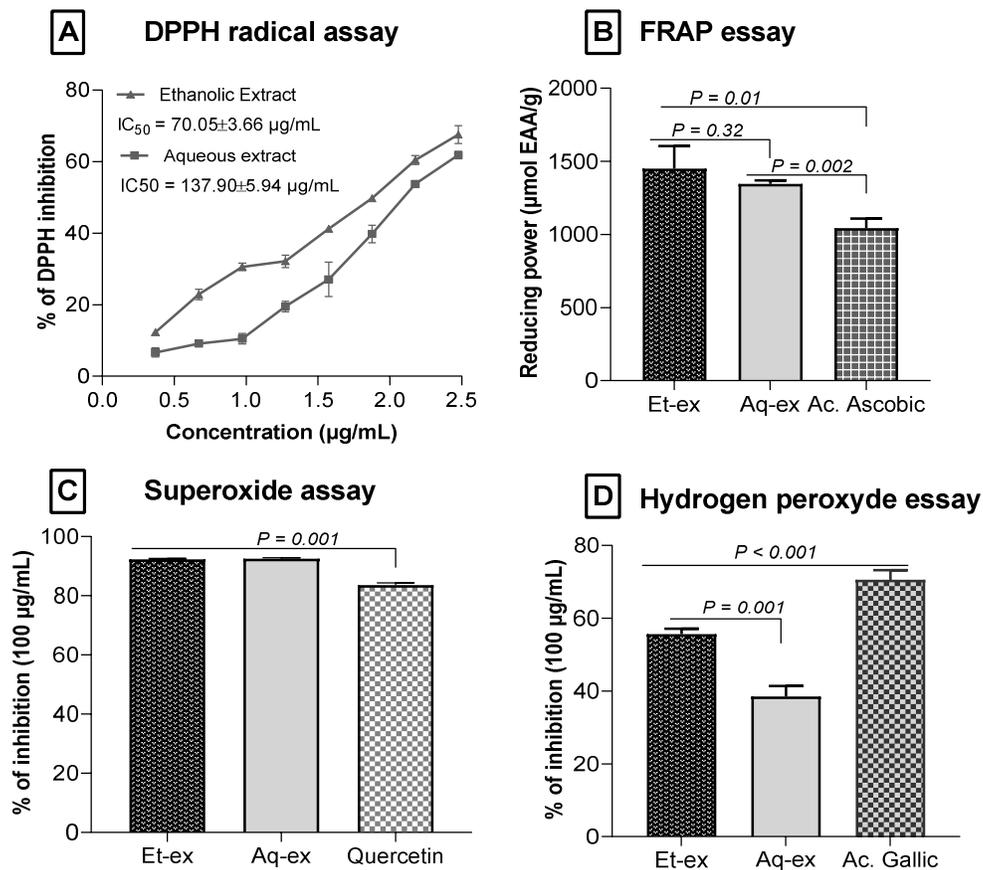


Fig. 3. Antioxidant activity of *C. millenii* crude ethanolic (Et-ex) and aqueous (Aq-ex) extracts
In vitro free radicals scavenging activity of both extracts obtained against DPPH radical (A), iron III (B), superoxide anion radical (C) and hydrogen peroxide (D). Each value represents mean for three experiments \pm SD (standard deviation). $P < 0.05$ indicating statistically significant difference

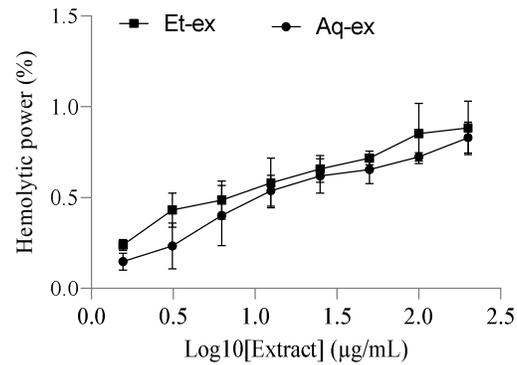


Fig. 4. Hemolytic effect of crude ethanolic and aqueous extracts of *C. millenii*
Et-ex = ethanolic extract, Aq-ex = aqueous extract. Data represent means for three experiments \pm SD (standard deviation)

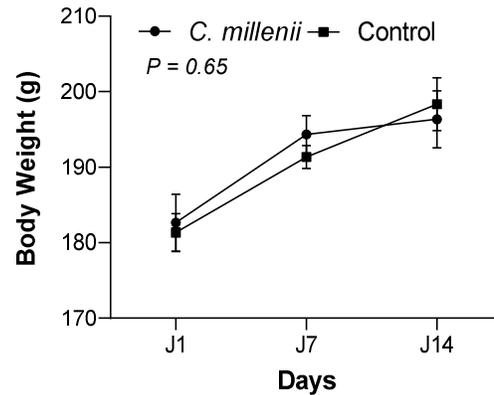


Fig. 5. Effect of *C. millenii* crude ethanolic extract on body weight of rat wistar
Data represent means for three experiments \pm SD (standard deviation). $P < 0.05$ indicates statistically significant difference

Table 2. Effect of ethanolic extract on biochemical and hematological parameters

Biochemical parameter	Control	Ethanolic extract	<i>P</i> value
GLU (g/L)	1.00 \pm 0.30	1.10 \pm 0.22	0.77
CREAT (mg/L)	6.40 \pm 0.98	7.29 \pm 1.53	0.53
UREE (g/L)	0.53 \pm 0.15	0.68 \pm 0.05	0.23
ASAT (IU/L)	116.67 \pm 8.85	138.33 \pm 30.14	0.31
ALAT (IU/L)	43.02 \pm 5.40	48.70 \pm 3.73	0.14
Hematological parameter	Control	Ethanolic extract	<i>p</i> -value
WBC ($\times 10^3/\mu\text{L}$)	14.30 \pm 2.05	11.37 \pm 0.22	0.22
Red blood count ($\times 10^6/\mu\text{L}$)	7.98 \pm 0.24	7.35 \pm 0.24	0.21
Haemoglobin (g/dL)	15.67 \pm 0.40	15.03 \pm 0.96	0.12
Hematocrit (%)	47.60 \pm 1.49	43.60 \pm 1.49	0.14
MCV (fL)	59.80 \pm 1.31	59.33 \pm 1.31	0.78
MCH (pg)	19.70 \pm 0.12	20.43 \pm 0.12	0.30
MCHC (g/dL)	32.93 \pm 0.58	34.47 \pm 0.58	0.17
Platelet ($\times 10^3/\mu\text{L}$)	1229.67 \pm 126.06	1166.33 \pm 74.78	0.40
Lymphocytes ($\times 10^3/\mu\text{L}$)	9.17 \pm 2.12	10.48 \pm 0.38	0.11

3.4.2 Acute oral toxicity

The result of acute oral toxicity test of *C. millenii* leaves ethanolic extract showed no noticeable toxicity signs in rats during the experiment period and no death recorded. This indicates that the LD₅₀ (Lethal Dose 50) is greater than 2000 mg/kg body weight. The body weight of treated animals in comparison to control showed progressive increasing without significant change ($P = 0.65$) between both groups (Fig. 5). Hematological parameters analyzed as well as biochemical markers revealed no significant difference between treated and control groups (Table 2).

4. DISCUSSION

Medicinal plants are still the first recourse for the primary health care of populations living in mainly in developing countries. Unfortunately, this practice is supported by broadly accepted beliefs and traditions that could not guarantee the efficacy of herbal medicines. The present study investigates the pharmacological properties of *C. millenii* through its antiplasmodial and antioxidant potential, its toxicological profile as well as its phytochemical constituents. The initial qualitative screening, firstly conduct to ensure the presence of phytoconstituents biologically actives, confirm the presence of flavonoids, coumarins, anthraquinones, triterpenes in aqueous extract and tannins in ethanolic extract. This variability could be justified by the difference of polarity of extraction solvents. It is also reported that some condensed tannins are insoluble in water [22]. The presence of tannins and terpenes have also been observed in *C. millenii* leaves ethanolic extract by [9,23]. Contrary, the present study reported the presence of flavonoids and anthraquinones when these authors revealed the presence of alkaloids and saponins [9,23]. Akinnibosun and Adewumi report the presence of flavonoids in pulp of *C. millenii* [23]. The phytochemicals difference observed in the present study compared to others here reported could be explained by the phenology which is in direct relationship with biomolecules synthesis in plant [24]. The difference observed in TPC and TFC of both extracts could also be explained by this variability of phytoconstituents. However, our results show the presence of important bioactive secondary metabolites belong to phenolic compounds with diverse chemical structures which could facilitate their various biological properties mainly antioxidant [25] and antiplasmodial [26–28].

The antiplasmodial activity of aqueous and ethanolic crude extracts of *C. millenii* leaves was tested against field isolates and chloroquine sensitive 3D7 strains of *P. falciparum* using pLDH assay. On the basis of appreciation grid of natural substances [29,30], both extracts are moderately active against the strains of *P. falciparum* used. However, the highest activity observed with the ethanolic extract in comparison to aqueous extract could be explained by variability of their secondary metabolites or by the high amount of phenolic contents observed in ethanolic extract. It has been reported the correlation between phenolic contents and antiplasmodial activity [31]. Although this is the first report on antiplasmodial activity of *C. millenii*, there are reports on related species in the literature. In 2006, Ménan et al. [32] have reported similar antiplasmodial activity with *C. caricaefolia* extracts. In other study conducted in Nigeria, promising antimalarial activity have been reported with ethanolic extract of *C. nitida* [33].

Oxidative stress has been implicated in several diseases including diabetes, rheumatoid arthritis, cardiovascular diseases, atherosclerosis, neurodegenerative diseases (Parkinson, Alzheimer and Huntington), cancer and aging [34]. Moreover, it has been reported to be implicated in serious complications of malaria such as anemia, increased sequestration of red blood cells, brain damage and failure of several organs in patients [35,36]. Antioxidants, mainly from natural sources, are thus important for minimizing or reversing the oxidative damage that may occur during malaria. It also reported that antioxidants can block the polymerization of free heme to hemozoin, thus making the host environment toxic to the survival of the parasite [37]. Therefore, free radical scavenging capacity of both extracts of *C. millenii* was evaluated *in vitro* using four different antioxidant methods. The results obtained (Fig. 3) show that both extracts of *C. millenii* mainly ethanolic extract possess very good antioxidant activity and can act as electron or hydrogen donors, scavengers, or as reducing agents [38]. However, the difference observed in the antioxidant potential of both extracts could be attributed to the variability of the phytochemicals constituents mainly phenolic compounds and their concentrations [25,39]. Moreover, the quantification of TPC and TFC revealed important amount of these phenolics groups in both extracts. Numerous studies correlated antioxidant potential of medicinal plants extracts to their qualitative and

quantitative richness in flavonoids and polyphenolics [26,40–42]. These extracts mainly ethanolic could be useful in the management of either malaria as well as erythrocytes lysis induced by the infection or oxidative damages of the body.

The cytotoxic effect of aqueous and ethanolic extracts of *C. millenii* leaves on human red blood cells (Fig. 4) show no hemolytic effect induced by both extracts. These results suggest that the extracts have not affected the osmotic fragility of erythrocytes and can confirm the direct effect of extracts on *Plasmodium* parasites. It is reported that red blood cells membrane is a delicate structure that can be easily altered by plant extract causing in erythrocyte rupture [43]. The acute oral toxicity profile assessed on ethanolic extract indicates a lethal dose (LD₅₀) upper than 2000 mg/kg body weight, according to the OECD Guidelines. These findings agree with those observed by Oyemitan et al. [12]. Ubon et al. [10] reported intraperitoneal LD₅₀ upper than 2000 mg/kg body weight with ethanolic extract of *C. millenii* seed. The absence of statistically significant difference between the body weight of test and control groups during the experimentation period, of death, of changes in behavior, and of an overt sign of distress indicate absence of eventual acute toxicity. It was reported that alteration of body weights gain of mice after exposure to toxic substances would reflect the toxicity [44]. The effects of *C. millenii* ethanolic extract on liver (ALT and AST) and kidneys (creatinine and urea) enzymes as well as serum glucose revealed no statistically significant difference indicating no alteration of liver and renal functions of albinos rats treat with this extract [45,46]. Ethanolic extract of *C. millenii* seed is reported to possess Hepato-protective property [10]. Moreover, *C. millenii* ethanolic extract not induce statistically significant change in hematological parameters of group treated and control group (Table 2). This indicates normal physiological condition of treated animals. Contrary to the present results, Ubon et al. [10] reported significant decrease of Hb, PCV, and MCV with ethanolic extract of *C. millenii* seed. This difference could be justified by the presence of saponin in seed extract. Saponins are known to induce erythrocytes lysis or to suppress red blood cells synthesis [47].

5. CONCLUSION

The present study clearly demonstrated that the leaf extracts of *C. millenii* possess a significant

antioxidant activity attributable to their qualitative and quantitative richness in phenolic compounds. These extracts were found to be moderately active against chloroquine-sensitive (3D7) and field isolates *P. falciparum*, and safe of hemolytic effect as well as acute toxicity signs on rats. The present study claims the potential use of *C. millenii* in management of malaria as well as oxidative damage in human body and strengthens the phytosanitary capacity of this plant in traditional medical system. Further investigations should be done on the use of this plant.

ETHICAL APPROVAL

The experimental protocol of this study was reviewed and approved under N° UAC/FAST/ED-SVT/10203707 by the scientific committee of research protocols of Doctoral School of Life and Earth Sciences of University of Abomey-Calavi, Bénin République.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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