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Scientific Baseline Information for the Potential Use of *Hibiscus surattensis* L against Malaria: Phytochemistry and Biological Studies

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ABSTRACT

Background and aim: *Hibiscus surattensis* L. is a medicinal plant widely used traditionally in Benin to treat malaria. This study was designed to investigate antiplasmodial activity, hemolytic power, acute toxicity, antioxidant activity and phytochemical content of *H. surattensis*.

Methodology: Bioassay-guided isolation approach was adopted and extracts and fractions collected were continuously tested *in vitro* against Chloroquine-sensitive and field isolate strains of *Plasmodium falciparum* by immune-dosage of *Plasmodium* lactate dehydrogenase. Hemolytic effect and acute toxicity of extracts were evaluated respectively on human erythrocytes and according to OECD guideline N°423. 2, 2-diphenyl-1- picrylhydrazyl) radical scavenging, Ferric reducing antioxidant power, superoxyde radical scavenging and hydrogen peroxide radical scavenging methods were used to investigate antioxidant activity.

Results and conclusion: The best antiplasmodial activity was obtained with ethanolic extract of *H. surattensis* L. against field isolate (IC_{50} = 5.03±0.31 µg/mL) and Chloroquine -sensitive (IC_{50} = 7.55±0.59 µg/mL) whereas aqueous extract exhibited moderate activity. Bioassay-guided fractionation of ethanolic extract shows progressive decrease of the antiplasmodial activity. Both extracts exhibited strong antioxidant activity, hemolytic power less than 1%. No mortality of rats was recorded with ethanolic extract at 2000 mg/kg body weight. Flavonoids, anthraquinones, coumarins, and triterpenes are present in both extracts with tannins in the ethanolic extract. In summary, the extracts of *H. surattensis* have interesting antiplasmodial and antioxidant properties probably resulting from a synergetic action of their secondary metabolites, without toxicity effect on rats and human erythrocytes. These findings strengthen the traditional use of *H. surattensis* as antimalarial plants.

Keywords: *Hibiscus surattensis*; antiplasmodial; antioxidant; toxicity; phytoconstituents.

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INTRODUCTION

Malaria is a parasitic disease caused by some species of the genus *Plasmodium* and transmitted to human by the bite of *Anopheles* mosquito [1]. Despite intensive international efforts, malaria infection still remained a public health problem especially in endemic countries. According to the World Health Organization report 2017, malaria cases were estimated to 216 million and 445,000 deaths in worldwide, with 90% of cases and 91% of deaths in WHO Africa region [1]. Bénin is malaria endemic area with 1,268,347 confirmed cases and 1,416 deaths reported in 2015 [2]. *Plasmodium falciparum* is the most virulent species responsible for human malaria in the country and constitute the first cause of death of children under five years [2]. Despite human efforts to control and eradicate malaria, Chloroquine and sulphadoxine/pyrimethamine resistant have been reported in this country and currently artemisinin-based combination therapies (ACTs) are used as first-line treatment of uncomplicated *P falciparum* malaria [3]. Unfortunately, *P falciparum* resistance to ACTs has already been reported in the Grand Mekong sub-region [1] and constitute then a threat for Africa. The appearance of *P. falciparum* resistance to most of antimalarial drugs is a veritable issue in malaria control and can only be overcome by the search for new antimalarial drugs. With the discovery of quinine and artemisinin, the most worldwide used antimalarial drugs medicinal plants represent a potential source for the search of new antimalarial drugs.

According to ethnobotanical surveys, *Hibiscus surattensis* L. (Malvaceae) is one of the most traditionally used medicinal plants to treat malaria in Benin [4,5] and in Uganda [6]. Despite several reports on considerable use of *H. surattensis* in folk medicine, no reports on phytochemical and pharmacological properties of this plant were available. The goal of this study is to assess phytochemicals, acute toxicity, and *in vitro* antiplasmodial and antioxidant activities of *Hibiscus surattensis* L. (Malvaceae).

MATERIAL AND METHODS

Plant material

Hibiscus surattensis was collected from his natural habitat in April 2015 in Tchaada, Municipality of Ifangni/Plateau in southern Benin. The collected plant was further identified and authenticated by the National Herbarium, University of Abomey-Calavi, where voucher specimen was deposited under reference number «YH 311/HNB». The material was dried for two weeks at laboratory temperature (22°C) and ground to a fine powder using an electric grinder.

Preparation of crude extracts and fractions

Crude extracts preparation

Crude ethanolic extract was obtained by maceration of 1 kg of vegetable material overnight in ethanol under mechanical agitation. Filtrate obtain after three washing and filtration with filter paper Whattman N°1 was concentrated using a rotary evaporator (BUCHI Rotavapor RII, Switzerland) at 40-50 °C to obtain the crude ethanolic extract. For aqueous extract preparation, 150 g of vegetable powder were boiled in 1.5 L of water at 100°C for 30 minutes and filtrated using Whattman N°1. The filtrate was concentrated using rotary evaporator coupled with vacuum pump (BUCHI Rotavapor RII, Switzerland; Vacubrand PC101NT, Germany) at 60°C to obtain aqueous extract. All extracts were kept at 4°C until further use for biological analyzes.

Bioassay-guided fractionation of crude ethanolic extract.

The crude ethanolic extract (most active against *P. falciparum* strains used) was subjected to successive fractionations including liquid-liquid partition and column chromatography. The liquid partition was performed using three solvent of varying polarity. The extract (5 g) was dissolved in 250 mL of ethanol/distilled water (20:80, v/v) and extracted successively with hexane, dichloromethane, and ethyl acetate using a separating funnel. Extraction procedure was repeated tree times with each solvent. The hexane (F-Hex), dichloromethane (F-DCM), ethyl acetate (F-AcOEt) and the last remaining aqueous (ARF) fractions were concentrated with rotary evaporator and tested against *P. falciparum* strains using pLDH assay. The ARF was sub-fractionated using Sephadex LH-20 column chromatography (30 x 200 mm) which was eluted successively with Dichloromethane-Ethanol (20-80) and water-acetone (1-1. The sub-fractions obtained were assembled into 4 groups after TLC (Ethyl acetate/Formic acid/Acetic acid/Water: 100:11:11:26).

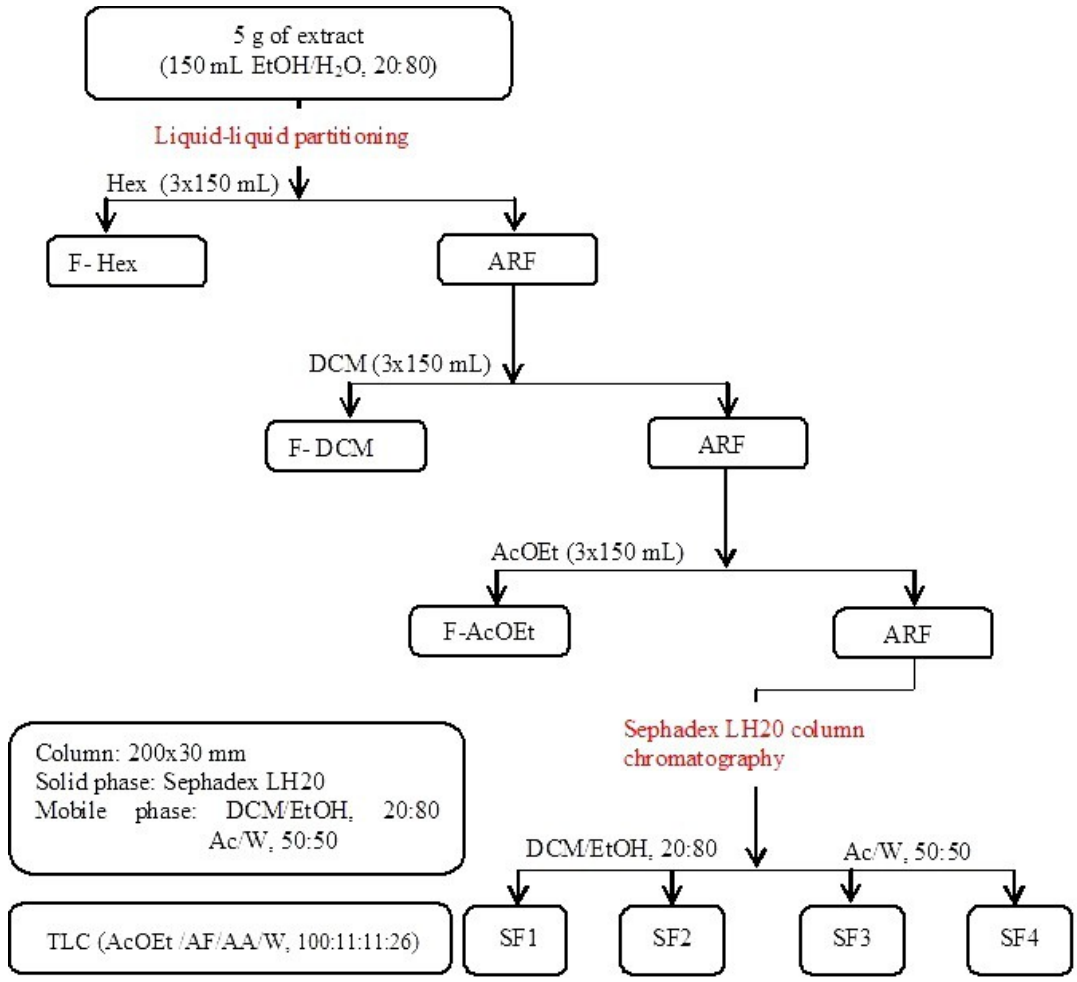

Figure 1: Diagram of fractionation of *H. surattensis* ethanolic extract. Hex = hexane, DCM = dichloromethane, AcOEt = Ethyl acetate, ARF = aqueous residual fraction, EtOH = ethanol, Ac/W = acetone/water, SF = sub-fraction, AF = formic acid, AA = Acetic acid.

Antiplasmodial assay

All crude extracts, fractions and sub-fractions were evaluated *in vitro* for their antiplasmodial activity against field isolate and chloroquine-sensitive (3D7) strains of *Plasmodium falciparum* obtained graciously from «Laboratoire des Maladies Infectieuses à Transmission Vectorielles (LMITV), Institut Régional de Santé Publique /Université d'Abomey-Calavi, Benin and Institut Pluridisciplinaire Hubert Curien, UMR 7178-CNRS/Unistra, France». Parasites strains were maintained on culture in fresh O^+ human erythrocytes in complete medium (RPMI 1640 with 0.8% AlbuMAX II, 25 mM HERPES, 1mM L-glutamine, 0.4mM Hypoxanthine and 0.05 mg/ml Gentamicin) as previously described [7]. The cultures were maintained in a standard gas mixture (1% O_2, 3% CO_2, 96% N_2) at 37°C. Parasitaemia was maintained daily between 1% and 5% using smears colored with Giemsa. *Plasmodium falciparum* sensitivity was carried out in 96-well plates. Crude extracts and fractions were preliminary tested at single concentration of 100 µg/mL and only active fractions were submitted to dose-response assay (concentration ranging from 100 to 0.78 µg/ml). In triplicate, 100 µL of parasite suspension (1% parasitaemia, hematocrit, 4%) were mixed with 100µL of extracts or fractions previously dissolved in complete medium and the plate was incubated for 96 h at 37°C in *P falciparum* culture conditions. Parasites viability was measured by immune-dosage of *Plasmodium* lactate dehydrogenase (*p*LDH) using ELISA malaria antibody test kit (ApDia, Belgium) according to manufacturer protocol. The concentrations of *p*LDH were determinated at 450 nm using microplate reader (Rayto R 6500, China) against a positive control (parasite suspension) and negative control (red blood cells). The positive and negative controls of the kits were also plotted to access efficacy of test following the recommendations of the manufacturer. The percentage of parasite growth inhibition (PI) was extrapolated using the following formula:

$$PI = 100-[(OD_S - OD_{nc})/ OD_{pc}]*100$$

Where ODs = optical density generated by sample, OD_{nc} = optical density generated by negative control, OD_{pc} = optical density generated by positive control.

The PI was plotted as a function of extract or fraction concentration and the IC_{50} was estimated by nonlinear regression analysis using Graphpad prism version 8.0.2 (GraphPad Prism software Inc., San Diego CA). Chloroquine diphosphate and Artesunate were used as antimalarial drug.

Hemolytic assay

Hemolytic activity was evaluated as described by Sarr et al., 2011 [8] with slight modification. Briefly, 100 µL of extracts or fractions in complete medium (200 µg/mL - 1.56 µg/mL) were mixed with 100 µL of non-infected erythrocytes (4% hematocrit) in 96-well plates. The mixture was incubated at 37°C under circular agitation. After 1 hour, the plates was centrifuged at 2000 rpm for 5 min and 150 µL of supernatant was collected in others 96-well plates. 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 the blank was constituted with erythrocytes suspension. Hemolysis percentage was expressed according to following formula:

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

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

Acute oral toxicity

Experimental Animals

Experiments were performed on nulliparous and non-pregnant females Albinos Wistar rats, aged 10 to 12 weeks and with body weight ranged from 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.

Acute oral toxicity assay

The acute oral toxicity of crude ethanolic extracts of *H. surattensis* was carried out using OECD 423 guidelines [9]. A total of 6 rats divided in 2 groups of 3 animals each were ranged in different cage during experiments. The animals were deprived of food for over-night before administration of extracts. The control group (group 1) received distilled water (10 ml/kg body weight). A single dose of 2000 mg/kg body weight of *H. surattensis* crude ethanolic extract firstly dissolved in distilled water has been administrated to the rats of group 2. After administration, the rats 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 and blood samples were collected into tubes with EDTA and tubes without EDTA respectively for hematological and biochemical analyzes.

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).

Antioxidant evaluation

DPPH radical scavenging activity assay

The free radicals scavenging activity of extracts was evaluated on DPPH according to the method previously described [10]. Briefly, 1.5 mL of a freshly prepared methanolic solution of DPPH (0.04%) were mixed with 0.75 mL of methanolic solution of extracts in varying concentrations (300 µg/mL to 2.34 µg/mL). Mixture were shaken and incubated for 20 minutes in the dark at room temperature. Absorbencies were 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:

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

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

The percentage of inhibition was plotted as a function of extracts concentration and curve fitting was obtained by nonlinear regression analysis using Graphpad prism version 8.0.2 (GraphPad Prism software Inc., San Diego CA). The IC_{50} value was extrapolated as the concentration that induced 50% inhibition of DPPH.

Potassium ferricyanide Reducing antioxidant power (FRAP) assay

The ferric reducing capacity of extracts was evaluated following the potassium ferricyamide-ferric chloride method [10]. 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 to reduce ferricyanide into ferrocyanide, 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 iron (III) reducing activity assay was performed in triplicate and expressed in mmol of Ascorbic Acid Equivalent (AAE)/g of extract.

Superoxide radical scavenging assay

This test consists to measure the capacity of extract to quench superoxide radical generated by alkaline DMSO [11]. In microplate 96 wells, the reaction mixture was constituted by 50 µL of extract (100 µg/mL in DMSO) and 170 µL of Alkaline DMSO (1mL DMSO, 100 µL NaOH 5mM). 30 µL 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 calculated 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.

Hydrogen peroxide radical scavenging assay

The ability of different extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al., 1989 [12] with slight modification. A solution of hydrogen peroxide (100 mM) was prepared in phosphate buffer (0.1mM, pH 7.4). Hydrogen peroxide concentration (control) was determined spectrophotometrically at 295 nm. Extracts (0.5 mL, 100 µg/mL) in distilled water were added to hydrogen peroxide solution (0.6 mL, 100mM). The absorbance of the mixture was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of extracts and Gallic acid (standard) was calculated using the following formula:

$$\text{Scavenged } (H_2O_2)\ \% = (Ac - As)/Ac \times 100$$

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

Phytochemical screening

The both crude extracts were screened for the presence of selected phytoconstituents including Alkaloids, flavonoids, triterpenes, tannins, coumarins, anthraquinone, saponins and anthocyanins by time layer chromatography (TLC) and colorimetric test in glass tube using respectively a standard protocol [13].

Estimation of Total Phenolic content

Total phenolic content (TPC) was estimated by colorimetric method using Folin-ciocalteu reagent [10]. Briefly, 200 µL of sample (100 µg/mL) were added to 1 mL of Folin-ciocalteu reagent (10%). After 4 min, 800 mL 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 essays were performed in triplicate and the results were expressed as mg of Gallic acid Equivalents (GAE) per gram of extract.

Estimation of total flavonoid content

Total flavonoid content was measured by the aluminum chloride colorimetric assay (10). 250 µL of extract were mixed with 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.

Statistical analysis

The results were expressed as means ± standard error of mean (S.E.M.). Student T test was used to compare data and difference was considered statistically significant when the $P< 0.05$. The graphical representations of the data were performed using the Graph Pad Prism 8.0.2 version (GraphPad Prism software Inc., San Diego CA) and Microsoft Excel 2010 software.

RESULTS

Antiplasmodial activity

The antiplasmodial activity of *H. suraattensis* was assessed according to the bioassay-guided fractionation process using *p*LDH assay against field isolates and chloroquine sensitive (3D7) strains of *P. falciparum*. At the single dose of 100 µg / mL, both crude extracts showed parasites growth inhibition upper than 60 % against both *Plasmodium* strains. However, crude ethanolic extract exhibited the highest inhibition on both strains (PI > 70 %) with a significant difference ($P<0.05$) when compared to aqueous extract (Figure 2A). Moreover, the field isolates strain is more sensitive than 3D7 strain to both crude extracts with a statistically significant difference (Figure 2A). Both extracts presented dose dependent inhibition against both *Plasmodium* strains. However, the crude ethanolic extract exhibited the best dose-response-inhibition with respective IC50 values of 5.03±0.31 µg/mL against field isolates and 7.54±0.59 µg/mL against 3D7 strain while aqueous extract showed moderate dose-response-inhibition with IC50 values of 47.81±5.53 µg/mL and 23.75±2.83 µg/mL respectively on both strains (Figure 2 D and E). Among the fractions obtained from the solvent-solvent partition of crude ethanolic, the aqueous residual fraction (ARF-HS) inhibited 3D7 strain viability at more than 70% (IC_{50} of 2.86±0.46 µg/mL, Figure 2D) and field isolate less than 50% at single dose of 100µg/mL. The fractions hexane (HEX-HS), dichloromethane (DCM-HS) and ethyl acetate (AcOEt-HS) inhibited both strain less than 50% at the single dose (Figure 2B). The ARF fraction was sub-fractionated by column chromatography and the sub-fractions were also tested against both strain of *P. falciparum*. The sub-fractions 1 (Sf1) showed moderate activity with IC_{50} of 36.72±4.76 µg/mL against 3D7 (Fig. 2C and D). A poor activity was observed with all sub-fractions against field isolate with an inhibition less than 50% (Fig. 2C). Artesunate exhibited IC_{50} values of 2.57±0.38 µg/mL on field isolates. Chloroquine showed IC_{50} values of 1.87±0.32

µg/mL and 19.85±2.71 µg/mL respectively on 3D7 and field isolates strains.

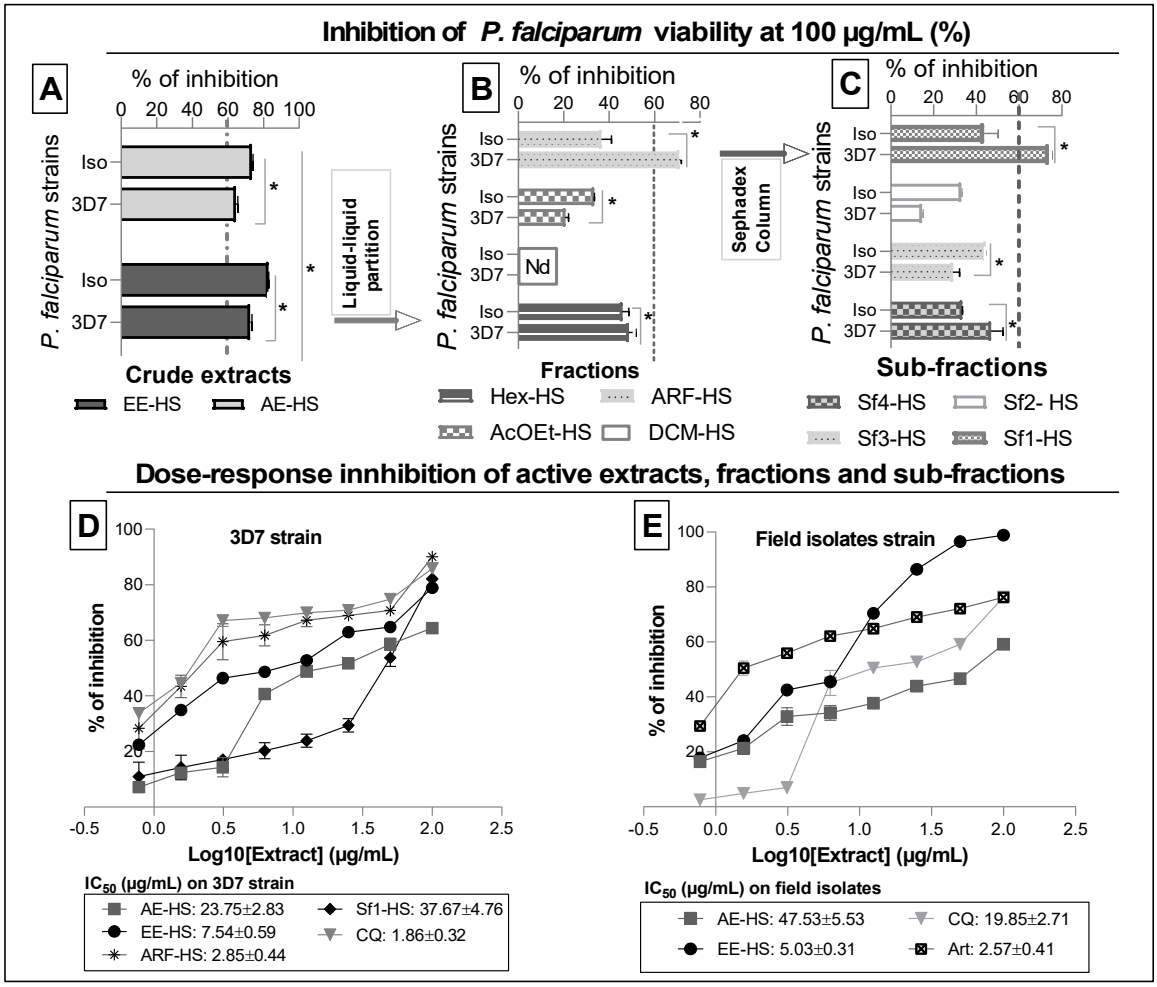

Figure 2: Biossay-guided fractionation of crude extracts of *H. surattensis* against chloroquine sensitive (3D7) and field isolate strains of *P. falciparum*. *In vitro* inhibition of parasites viability by crude extracts (A), by fractions (B) and by sub-fractions (C) at 100 µg/mL. Dose-response inhibition of extracts, fractions and sub-fractions against 3D7 strain (D) and field isolate (E). Data represent means for three experiments ± SD. Artesunate (Art) and chloroquine diphosphate (CQ) are positive drugs control. * indicating statistically significant difference (P<0.05). EE-HS Ethanolic extract, AE-HS Aqueous extract, Hex-HS fraction hexane, DCM-HS fraction dichloromethane, AcOEt-HS fraction ethyl acetate, ARF-HS aqueous residual fraction, Sf sub-fraction, Nd not determined (total hemolysis)

Hemolytic power

The figure 3 illustrates the hemolytic power of *H. surattensis*. Both crude extracts, AcOEt-HS and ARF-HS showed very low hemolytic effect (<2%) on human red blood cells while HEX-HS exert hemolytic effect less than 10%. Meanwhile, DCM-HS showed total hemolysis at 100 µg/mL.

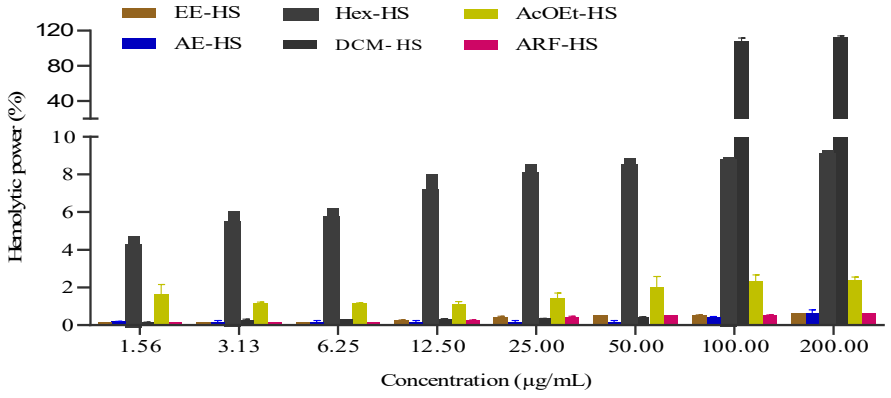

Figure 3: Hemolytic activity of extracts and fractions of *H. surattensis*. EE-HS = Ethanolic extract, AE-HS = Aqueous extract, Hex-HS = fraction hexane, DCM-HS = fraction dichloromethane, AcOEt-HS = fraction ethyl acetate, ARF-HS = aqueous residual fraction. Data represent means for three experiments ± SD.

Acute oral toxicity

The acute toxicity test conducted with single dose of 2000 mg/kg of ethanolic extract of *H. surattensis* caused no deaths in rats. Rigorous observation of the behavior of the treated animals revealed no clinical toxicity signs throughout the study period. There was no significance change in the body weight in the treated rats vs control (*P>0.05*). The body weights of test animals of both *H. surattensis* crude ethanolic extract treated and control groups were increased progressively throughout the study period (Figure 4).

Analysis of serum creatinine and urea level revealed no significant change in acute toxicity test group compared to control group. No significant changes were observed in the biochemical markers of liver function analyzed, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST). There was no significant difference between test group and control group for serum glucose (Table 1). Hematological analysis revealed significant (*P*<0.05) decrease in white blood cells count in acute toxicity test group compared to control group (Table 1). Others hematological parameters were not significantly different from the control group (Table 1).

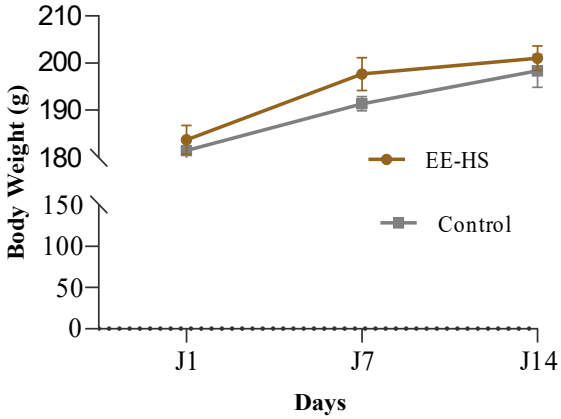

Figure 4: Effect of *H. surattensis* crude ethanolic extract on body weight of rat wistar. EE-HS = Ethanolic extract. *P<0.05* indicates statistically significant difference. Data represent means for three experiments ± SD (n = 3).

Table 1: Effect of ethanolic extract of *H. surattensis* on biochemical parameters and hematological parameters.

Biochemical parameter	Control	EE-HS	*P value*
GLU (g/L)	1.00±0.30	1.15±0.62	0.67
CREAT (mg/L)	6.40±0.98	6.33±0.89	0.95
UREE (g/L)	0.53±0.15	0.42±0.07	0.19
ASAT (IU/L)	116.67±8.85	155.57±34.13	0.12
ALAT (IU/L)	43.02±5.40	60.70±12.79	0.17
Hematological parameter			
WBC ($x10^3$/µL)	14.30±2.05	9.43±0.25	0.01
Red blood count ($x10^6$/µL)	7.98±0.24	7.60±0.62	0.70
Haemoglobin (g/dL)	15.67±0.40	14.53±1.07	0.40
Hematocrit (%)	47.60±1.49	43.03±3.25	0.35
MCV (fL)	59.80±1.31	56.63±1.10	0.23
MCH (pg)	19.70±0.12	19.13±0.21	0.50
MCHC (g/dL)	32.93±0.58	33.77±0.35	0.27
Platelet ($x10^3$/µL)	1229.67±126.06	1075.00±76.62	0.12
Lymphocytes ($x10^3$/µL)	9.17±2.12	9.23±1.21	0.92

P<0.05 indicate statistically significant difference. Each value represents mean for three experiments ± SD (n = 3).

Antioxidant activity

In vitro free radicals scavenging activities of ethanolic (EE-HS) and aqueous (AE-HS) extracts of *H. surattensis* were presented in figure 5. DPPH radical inhibition (Fig.5A) generated by both crude extracts is dose dependent. However, EE-HS exhibited the strongest activity (IC_{50} = 58.12±5.43 µg/mL) with significant different (*P*<0.05) when compared to AE-HS (IC_{50} of 108.50±16.73 µg/mL). The highest reducing power (Fig.5B) was also obtained with EE-HS (1366.15±49.38 µmol EAA/g) compared to aqueous extract (1179.61±25.14 µmol EAA/g) and ascorbic acid (1042.44±66.52 µmol EAA/g) with significant difference (*P*<0.05). Both extracts showed superoxide radical quenching more than 90% at 100 µg/mL without significant difference (*P>0.05*) (Fig. 5C). EE-HS presents the highest hydrogen peroxide radical scavenging activity with an inhibition of 52.56±1.72 % when aqueous extract exert an inhibition of 40.62±1.58%. Otherwise, significant difference (*P<0.05*) was observed with gallic acid (70.53±3.67%) in the same condition (Fig. 5D).

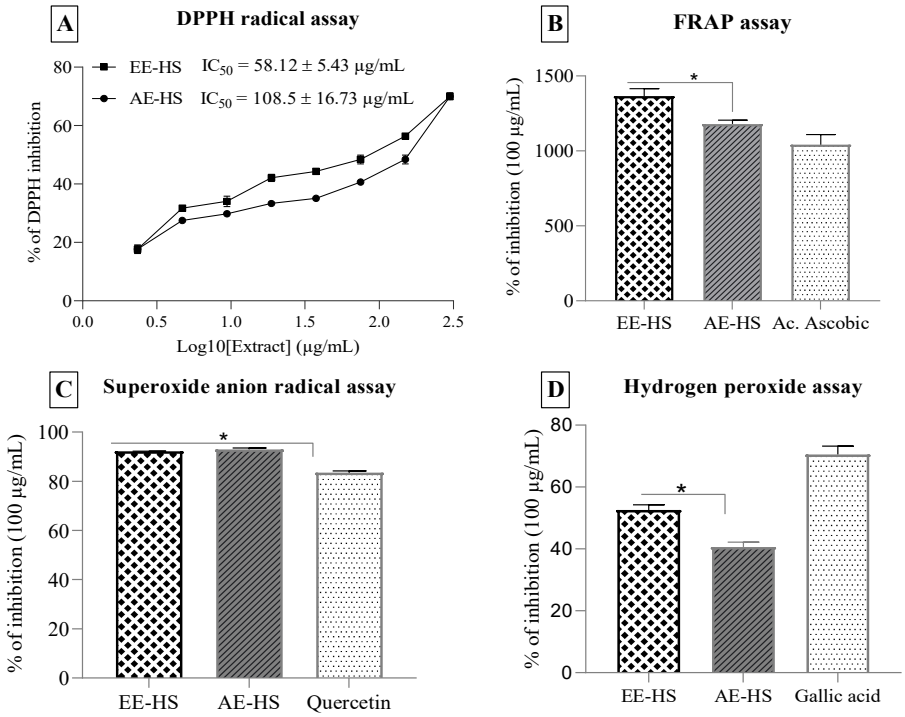

Figure 5: Antioxidant activity of *H. surattensis* crude ethanolic (EE-HS) and aqueous (AE-HS) extracts. *In vitro* free radicals scavenging capacity of both extracts obtained against DPPH radical (A), iron III (B), superoxide anion radical (C) and hydrogen peroxide (D). Each value represents mean for three experiments ± SD. * indicating statistically significant difference (*P<0.05*).

Phytochemical constituents

Preliminary phytochemical analysis of crude extracts of *H. surattensis* revealed the presence of several secondary metabolites such as anthraquinones, flavonoids, coumarins, tannins and triterpenes (Table 2). The presence of saponins, alkaloids, anthocyanins, essential oil and lignans haven't been noticed in both extracts likewise tannins in the aqueous extract.

The estimation of total phenolics content (TPC) and total flavonoids content (TFC) are summarized in Figure 6. The highest TPC was recorded in the ethanolic extract (12.35±0.03 mg EGA/g of extract) with a significant difference (*P<0.05*) when compared to TPC of aqueous extract (10.54±0.11 mg EGA/g of extract). The high amount of TFC was observed with the aqueous extract (20.48±1.17 mg EQ/g of extract) but without significant difference (*P>0.05*) when compared to ethanolic extract (18.53±0.64 mg EQ/g of extract).

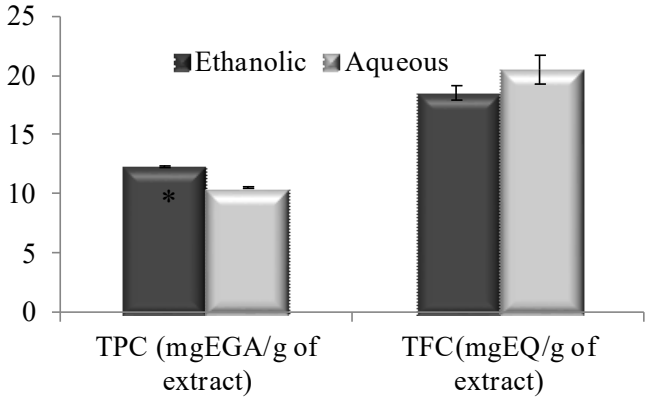

Figure 6: Total phenolic (TPC) and flavonoids (TFC) contents of *H. surattensis* extracts. EGA = Equivalent of Gallic acid, EQ = Equivalent of Quercetin. * indicating statistically significant difference (P<0.05). Data represents means for three experiments ± SD (n = 3).

Table 2: Secondary metabolites present in *H. surattensis* extracts

Chemicals components	*Hibiscus surattensis*	
	Aqueous extract	Ethanolic Extract
Alkaloids	-	-
Flavonoids	+	+
Saponins	-	-
Tannins	-	+
Anthraquinones	+	+
Anthocyanins	-	-
Coumarins	+	+
Triterpenes	+	+
Lignans	-	-
Essential oil	-	-

+ = Presence, - = absence

DISCUSSION

In the last decades, medicinal plants screening has gradually increased in order to discover the most effective herbs and or news compounds able to treat various diseases including malaria. The preliminary screening is generally performed on plant extracts, a complex mixture of different compounds. Bioassay-guided isolation is a classical commonly used approach in the malaria field for separation of active compounds before structural characterization [14]. In this study, i*n vitro* antiplasmodial screening of *H. surattensis* was assessed using bioassay-guided approach. According to the classification of antiplasmodial activity in the literature [14], the ethanolic extract showed very promising activity on both strains of *P falciparum* while the aqueous extract exhibited moderate activity on both strains. The difference observed in activity of the both crude extracts could be due to the variability between the phytoconstituents. Moreover, the results show that the field isolates strain is sensitive to EE–HS as to artesunate, but less sensitive to chloroquine diphosphate and AE-HS. This indicates that the EE-HS would be effective against resistant strains of *P. falciparum. P falciparum* resistant strain to Chloroquine and sulphadoxine/pyrimethamine have already been reported in Benin [15], origin of field isolates collection. However, the results obtained from bioassay-guided fractionation of the ethanolic extract (Table 1) indicate a gradual decrease of its activity. The strong antiplasmodial activity obtained with this extract could, therefore, be attributed to a synergistic effect of the compounds present in this crude extract. Various alkaloids (cinchonine, quinidine, and cinchonidine) presents in the crude extract of cinchona bark have shown synergetic effect improving quinine activity against resistant *P falciparum in vitro*. Flavonoids from *Artemisia annua* are also reported to potentiate artemisinin activity against *P. falciparum* [16]. The qualitative phytochemical screening of both extracts revealed the presence of several phytoconstituents such as flavonoids, triterpenes, coumarins, and anthraquinones. The presence of tannins in ethanolic extract only can be explained by the difference of polarity between both solvents. It is reported that some condensed tannins are insoluble in water [17]. However, these secondary metabolites are known for their outstanding activity against *P falciparum* either single or in synergy [14,16]. The best antiplasmodial activity observed with ethanolic extract in comparison to aqueous extract could be a synergetic or a potentiation effect brought by the presence of tannins in this extract. The presence of this metabolite could also explain the significant difference observed between the TPC of both extracts. Tannins belonging to phenolic compounds [16]. It is also reported, the correlation between TPC of plant extract and its antiplasmodial activity [16].

The pathophysiology of malaria involves oxidative stress arising mainly from excessive production of reactive oxygen and nitrogen species by the host immune response and energy metabolism of the *plasmodium* [18]. In fact, the *Plasmodium*-infected red blood cell and uninfected are constantly exposed to oxidative stress leading to lesions of erythrocyte membranes and early aging of cells and thus contributing to serious complications such as anemia, increased sequestration of red blood cells, brain damage and failure of several organs in patients [19]. Thus, it's important to have antimalarial drug which also have antioxidant activity to minimize oxidative damage in malaria patients. Therefore, free radical scavenging capacity of the extracts was evaluated. The results obtained in this study (Figure 5) show that both extracts of *H. surattensis* mainly ethanolic extract possess strong antioxidant capacity attributable to polyphenolic compounds [20] identified in these extracts and their concentration [21]. The significant difference between the antioxidant activities of extracts could be explained by the variation observed in the phytoconstituents. However, these results indicate that the compounds present in *H. surattensis* extracts have several antioxidant properties and can interact as scavengers, electron or hydrogen donors, or as reducing agents [22]. These antioxidant properties can be useful in the management of oxidative damage caused by malaria infection, mainly the protection of red blood cells against oxidative stress. They can also be useful to eliminate the parasite by blocking the oxidation of heme.

Generally, phytotherapeutic products from medicinal plants are often regarded as safe just because they are a natural source but unfortunately, no scientific data that prove the

safety of these products [23]. The acute toxicity conducted for this purpose revealed that the LD_{50} of this extract is greater than 2000 mg / kg body weight, as indicated in the OECD Guidelines. The absence of changes in behavior, of an overt sign of distress, of death, and the normal increase of body weight in the test animals during the experimentation period is good evidence for negligible toxicity [23]. Moreover, the absence of statistically significant change in serum biomarkers indicates that the ethanolic extract of *H. surattensis* does not induce probable alteration of liver and renal functions [24]. Hematological parameters provide useful information on the physiological and pathological status of animals and human. The results indicate no statistically significant changes in hematological parameters between control and rats treated with *H. suratensis* extract. Furthermore, both ethanolic and aqueous extracts of *H. surattensis* do not have hemolytic effect on healthy human red blood cells. Red blood cells membrane is a delicate structure that can be easily altered by plant extract [25] resulting in erythrocyte rupture. These results indicate the probable nontoxic nature of *H. suratensis* ethanolic extract and support the usage of this plant in folk medicine by indigenous populations. However, chronic and sub-chronic toxicity assessment will provide more safety of its use.

CONCLUSIONS

The results of the present study show that the *H. surattensis* ethanolic extract possess the high antiplasmodial activity against chloroquine-sensitive (*3D7*) and field isolate strains of P. falciparum as well as a lack of toxicity. Antiplasmodial bioassay guided isolation indicate the possible synergetic effects attributable to secondary metabolites contained in this extract. Both ethanolic and aqueous extracts exhibited strong antioxidant capacity able to modulate oxidative damage induced by malaria infection. The present study strengthens the importance of herbal medicine in primary health care mainly the use of *H. suratensis* in folk medicine for malaria management.

CONFLICTS OF INTEREST

The authors declared that there is no conflict of interest.

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