



***In vitro* Assessment of Antiplasmodial Activity and Acute Oral Toxicity of *Dissotis rotundifolia* Extracts and Fractions on *Plasmodium falciparum* Strains**

Rock Djehoue¹, Rafiou Adamou¹, Abdou Madjid O. Amoussa¹,
Adande A. Medjigbodo², Anatole Laleye³, Ambaliou Sanni¹,
Luc S. Djogbenou² and Latifou Lagnika^{1*}

¹Laboratory of Biochemistry and Bioactive Natural Substances, Unit of Biochemistry and Molecular Biology, University of Abomey-Calavi, 04 BP, 0320 Cotonou, Benin.

²Laboratory of Infectious Vector Borne Diseases, Regional Institute of Public Health, University of Abomey-Calavi, 01BP 918, Cotonou, Benin.

³Laboratoire d'histologie, de Cytogénétique et d'Embryologie, Faculté des Sciences de la Santé (FSS), Université d'Abomey-Calavi, Benin.

Authors' contributions

This work was carried out in collaboration among all authors. Authors RD and RA contributed in performing the laboratory work, analysis of data and drafting the manuscript. Authors AMOA and AAM read and contributed to the laboratory training, data analysis and drafting the paper. Authors AL, AS and LSD provided significant editorial assistance. Author LL mentored and supervised the entire work. All authors read and approved the final manuscript.

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ABSTRACT

Aim: *Dissotis rotundifolia* were selected after an ethnopharmacological survey conducted on plants used traditionally for malaria treatment in South Benin, with the aim of discovering new natural active extracts against malaria parasites.

Place and Duration of Study: Laboratory of Biochemistry and Bioactive Natural Substances, University of Abomey-Calavi (Benin)/ Laboratory of Infectious Vector Borne Diseases, Regional

*Corresponding author: E-mail: llagnika@gmail.com;

Institute of Public Health (Benin)/ Laboratoire d'Histologie, de Cytogénétique et d'Embryologie, Faculté des Sciences de la Santé (Benin). The study was conducted from October 2018 to June 2019 in Benin.

Methodology: The antiplasmodial activity of the plant extracts was evaluated using the parasite lactate dehydrogenase (pLDH) immunodetection assay. The extract with the best antiplasmodial activity was used on Wistar rats for acute toxicity.

Results: Ethanolic extract of *Dissotis rotundifolia* showed promising activity (Isolate: $IC_{50} = 22.58 \pm 1.12 \mu\text{g/mL}$; 3D7: $IC_{50} = 6.81 \pm 0.85 \mu\text{g/mL}$) on *Plasmodium falciparum* compared to the aqueous extract (Isolate: $IC_{50} > 100 \mu\text{g/mL}$; 3D7: $IC_{50} > 100 \mu\text{g/mL}$). The aqueous fraction of *D. rotundifolia* exhibit highly potent activity against *P. falciparum* strain (Isolate: $IC_{50} > 100 \mu\text{g/mL}$; 3D7: $IC_{50} = 4.05 \pm 0.72 \mu\text{g/mL}$). Haemolytic effect of active extracts and fractions is less than 5%. Ethanolic extract of *D. rotundifolia* revealed no obvious acute toxicity in rat up to the highest dose administered (2000 mg/kg).

Conclusion: This study justifies traditional uses of *D. rotundifolia* against malaria. A bioguided fractionation of these extracts would identify molecules responsible for their antiplasmodial activity. Moreover, these results could lead to the design of improved traditional medicines in the basis of this plant.

Keywords: Antiplasmodial activity; ethnopharmacology; malaria parasites; *Plasmodium falciparum*.

1. INTRODUCTION

Throughout the history of mankind, malaria has been one of the major causes of human illness and death. More than 435,000 deaths occur every year; the vast majority being children under the age of five [1]. Malaria is a parasitic disease widespread in tropical and subtropical regions of the world. It is endemic particularly in Africa, Asia and South America [1,2].

The emergence and rapid spread of multi-resistant strains of *Plasmodium*, especially *P. falciparum*, becomes a major and serious problem for prophylaxis and treatment in the world. Antimalarial drugs such as chloroquine, sulfadoxine/pyrimethamine and others are no longer effective in most endemic areas, mainly in developing countries. Therefore, WHO has recommended the use of therapeutics based on combinations of artemisinin and derivatives. However, in some countries *P. falciparum* is already resistant to artemisinin combination therapies (ACTs) [3,4].

In Benin, the prevalence of malaria is 36.79% [1]. This parasitic disease is the leading cause of mortality among children under five and of morbidity in the elderly [5]. In recent years, the development of increased resistance of the parasite to the main antimalarial drugs has led Benin to adopt a new malaria control policy focused on three main interventions: (i) use of a polytherapy based on artemisinin for the treatment of simple malaria; (ii) integrated vector control based on the use of long-lasting

insecticide-treated mosquito nets, indoor spraying, the use of larvicides and (iii) intermittent preventive treatment in pregnant women.

Because a large part of the world's population relies on plants as their primary sources of medicinal agents, it is not surprising to find in many countries a well-established system of traditional medicine. The recognition and validation of traditional medicine is important and could lead to the discovery of new plant-derived drugs, such as quinine isolated from *Cinchona* species and artemisinin isolated from *Artemisia annua* L., since medicinal plants are well known as important sources of bioactive natural metabolites. Many medicinal plants belonging to different botanical families were reported to exhibit antiplasmodial activity against *Plasmodium* strains *in vitro* and *in vivo*. Some of these plants can be considered for the discovery of new antimalarial drugs with various modes of action [6–8].

In Benin, *Dissotis rotundifolia* is one of the most used plants to treat malaria and/or associated symptoms according to ethnobotanical investigations conducted in the country in 2016 [9]. However, to our knowledge, there are no studies on the antiplasmodial activity of extracts and fractions of this plant. This study was therefore designed to investigate the antiplasmodial property and *in vivo* toxicity of extracts, fractions and sub-fractions of whole plant of *Dissotis rotundifolia*.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Materials

The whole plant of *Dissotis rotundifolia* (Sm.) were collected at the morning of 17th, November 2016 in Ifangni, Department of Plateau (Southern Benin). Plant was identified at the National Herbarium of Benin, where voucher specimens were deposited under the reference YH 255/HNB. This plant is not featured in the International Union of Conservation of Nature (IUCN) red list of threatened species and its collect is not prohibited (IUCN red list).

2.2 Preparation of Extracts, Fractions and Sub-fractions

Dissotis rotundifolia whole plant was air-dried for two weeks in laboratory (22°C) and ground into fine powder using an electric grinder (LONGiUE LY-989, China). 1 kg of the plant powder was separately macerated in 10 L of ethanol at room

temperature using ultrasonic sonicator (Vibra Cell 75115, Biobloc Scientific). The aqueous extract were prepared by boiling (100°C) 200 g of powder in 2 L of distilled water for 20 min. Each extraction is repeated three times. The extracts were filtered through Whatman filterpaper CATN_o. 1 001 150 mm, and concentrated using a rotary evaporator (BUCHI Rotavapor RII, Switzerland) at 50-60°C. The dried crude extracts were subsequently subjected to *In vitro* antiplasmodial screening against the sensitive *Pf* 3D7 strains and field isolate. Crude extracts with potent antiplasmodial activity were selected and subjected to successive liquid-liquid solvent partition. Thus, ethanol crude extract (20 g of *D. rotundifolia*) were dissolved in ethanol-water 20/80 (200 mL) and successively extracted with hexane (1 L), dichloromethane (1 L), and ethyl acetate (1 L) to afford hexane (Hex), dichloromethane (DCM), ethylacetate (EtOAc) and aqueous fractions, respectively (Fig. 1). The afforded fractions were concentrated using rotary evaporator. The dried fractions were weighed and stored at 4°C for the biological tests.

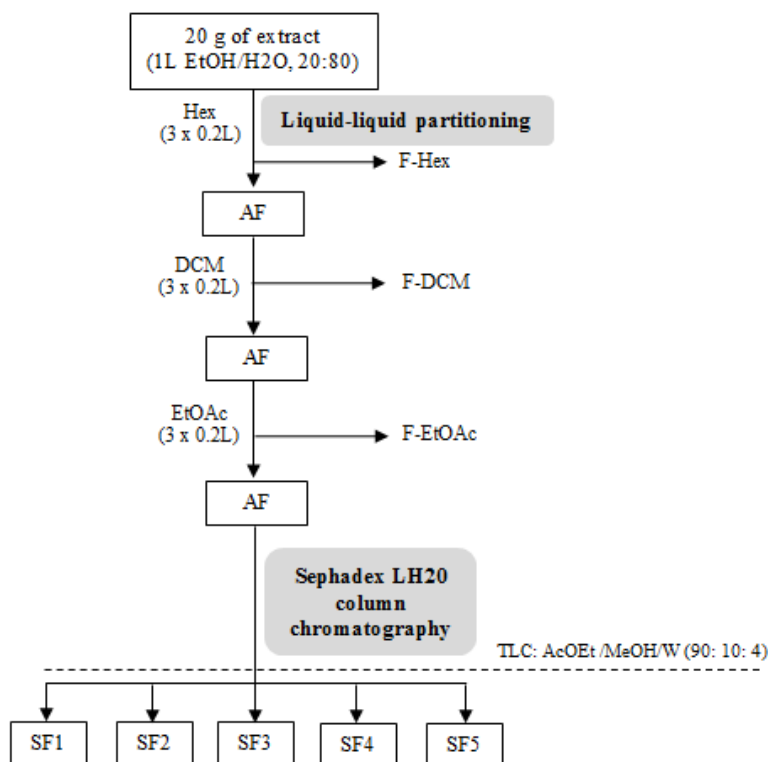


Fig. 1. Diagram of fractionation of *Dissotis rotundifolia* ethanolic extract

F: fraction, Hex: hexane, DCM: dichloromethane, AcOEt: Ethylacetate, AF: aqueous fraction, EtOH: ethanol, Ac/W: acetone/water, SF: sub-fraction, MeOH: Methanol. Column: 200x35 mm; Solid phase: Sephadex LH20; Mobile phase: DCM/EtOH (10:90); Ac/W (50:50)

The aqueous residual phase was selected for bioassay-guided fractionation. It was subjected to medium pressure liquid chromatography (MPLC) over a column packed with Sephadex LH20 (25-100 μm ; Fluka) and eluted with solvent gradient composed of DCM/EtOH (10: 90) (Fig. 1). A total of 13 eluates of 250 mL each were collected and combined on the basis of thin layer chromatography behaviour, to get 5 sub-fractions which were then tested for *In vitro* antiplasmodial activities.

2.3 *In vitro* Cultivation of *Plasmodium falciparum*

The *Plasmodium falciparum* Chloroquine (CQ)-sensitive strain 3D7 obtained graciously from "Institut Pluridisciplinaire Hubert Curien, UMR 7178- CNRS/University of Strasbourg" and field isolates obtained from "Laboratoire des Maladies Infectieuses à Transmission Vectorielle/Institut Régional de Santé Publique (Ouidah/Benin)" were used for *In vitro* blood stage culture to test the antiplasmodial efficacy of plant extracts, fractions and sub-fractions. The continuous culture of *P. falciparum* was maintained according to the method described by Trager and Jensen [10] with minor modifications, in fresh O⁺ human erythrocytes suspended at 5% haematocrit in RPMI 1640 (Lonza) supplemented of 0.1 M L-glutamin, 1 M HEPES, 8% albumax II, 0.004 M hypoxanthine, and 40 $\mu\text{g}/\text{ml}$ gentamicin (Sigma-Aldrich, New Delhi, India). Conditions of incubation used in all the experiments were as follows 37°C, 92% nitrogen, 5% carbon dioxide, 3% oxygen (Air Liquid, France Industry). Infected erythrocytes were transferred to fresh complete medium to propagate the culture, daily.

2.4 Dilution of Crude Extracts, Fractions, Sub-fractions and Positive Control

Sample solutions were prepared at 1 mg/mL in dimethyl sulfoxide (DMSO) 1%. Chloroquine diphosphate (Sigma-Aldrich, Saint Quentin Fallavier, France) and artesunate stock were used as standard drugs and were prepared in malaria culture medium (MCM) at 1 mg/mL. All stocks solutions were then diluted in 96-well, round-bottom, (Corning, USA) with fresh RPMI 1640 culture medium to achieve the required concentrations for testing. In all cases, the final solution contained 0.5% DMSO, which was found to be non-toxic to the parasite. Extracts, fractions and sub-fractions were tested at concentrations ranging from 100 to 0.78 $\mu\text{g}/\text{mL}$.

2.5 *In vitro* Antiplasmodial Assay

The antiplasmodial activity of crude extracts, fractions and sub-fractions was tested on *P. falciparum* field isolates and 3D7 lab strain. Samples testing were performed in triplicate with parasites cultures mostly at ring stages at 1% parasitaemia (haematocrit, 5%). Parasite culture (100 μL) was mixed with each drug (100 μL) in 96-well plate and the latter was incubated at 37°C in candle jar for 96 h in culture conditions. For plants extracts, fractions and sub-fractions screening, parasites viability was assessed by immune-dosage of *Plasmodium* lactate dehydrogenase (*pLDH*) using ELISA malaria antibody test kit (ApDia, Belgium) according to manufacturer protocol [11]. The absorbance was read with a microplate spectrophotometer (Rayto R 6500, China) at 450 nm. The positive control is parasites suspension and negative control is the healthy red blood cells. The percentage of parasite growth inhibition (PI) was determinate using the following formula:

$$\text{PI} = 100 - \left[\frac{(\text{ODs} - \text{OD}_{\text{nc}})}{\text{OD}_{\text{pc}}} \right] * 100$$

Where,

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

2.6 Toxicity Assays

2.6.1 Haemolytic activity

The samples Haemolytic effect was performed to determine whether the observed *In vitro* antiplasmodial activity is not related to haemolysis of red blood cells. It was done according to a modified method described by Kumar and Ravikumar [12]. Extracts and fractions were serially diluted with 1% DMSO (Dimethyl Sulfoxide) in MCM (Malaria Culture Medium) at concentrations ranging from 200 to 1.56 $\mu\text{g}/\text{mL}$ in 96-well culture plates and each concentration was incubated with the same volume (100 μL) of healthy red blood cells (5% haematocrit) at 37°C for 40 min under circular agitation. Erythrocytes were sedimented in the microtitre plate wells by centrifugation (3000 rpm for 10 min, Heraeus Megafuge 16 R, Germany). The supernatants were diluted 1/4 in the plates with distilled ultra-pure water. A haemolytic agent, 5% Sodium Dodecyl Sulfate (SDS), was

used as positive control. Negative control contained erythrocytes diluted (v/v) with the sterile MCM. Haemoglobin content in the supernatants was determined by absorbance measurements at 450 nm in a microtiter plate spectrophotometer (Rayto R 6500, China). Hemolysis percentage was expressed according to the formula:

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

Where,

As, Ab and Ac were respectively absorbances of sample, blank and positive control.

2.6.2 Acute toxicity assay

The assay was carried out on females Albino Wistar aged 8-12 weeks. The rats were nulliparous and non-pregnant with body weight ranged from 180±20 g and come from "Laboratoire d'Histologie, de Cytogénétique et d'Embryologie, UFR Biologie Humaine" of Faculty of Health Sciences of Cotonou. The animals are acclimated to laboratory temperature (22°C) and have free access to water and food. A total of 6 six animals (3 tests and 3 controls) were used in this study.

The ethanolic extract of *D. rotundifolia* were tested (gavage) in Wistar rats using the procedures described by Organization for Economic Co-operation and Development guidelines N°423 [13]. The automatic haematologic analyzer (Sysmex, XP-300, Japan) were used for haematological parameters. Blood collected into non heparinized tubes were analyzed to evaluate biochemical parameters using clinical chemistry analyzer (CHEM-7 Erba Diagnostics Mannheim GmbH, Germany).

2.7 Statistical Analysis

For antiplasmodial activities the IC₅₀ (concentration at which the parasite growth was inhibited by 50%) was determined by analysis of dose-response curves using non-line ar regression with GraphPad Prism version 8.04 (San Diego, California, USA). All results included IC₅₀ are reported as mean ± standard deviation (SD) of experiments. The graphical representation of the data was performed using GraphPad Prism. The difference was considered statistically significant when $p < 0.05$.

3. RESULTS

3.1 Bioassay-guided Fractionation of *D. rotundifolia*

Fig. 2 illustrates the results of bioguided fractionation of ethanolic extract of *D. rotundifolia*. These results show that at 100 µg/mL, the most active samples showed an inhibition greater than 70% on both strains of *P. falciparum* (Fig. 2).

The results of the sensitivity test show that the ethanol extract (3D7: IC₅₀ = 6.81 ± 0.85 µg/mL, isolate: IC₅₀ = 22.58 ± 1.12 µg/mL) is more active than aqueous extract (3D7: IC₅₀ > 100 µg/mL, Isolate: IC₅₀ > 100 µg/mL) on both strains and, on the other hand, more active on the 3D7 laboratory strain (IC₅₀ = 6.81 ± 0.85 µg/mL) than on the field isolate (IC₅₀ = 22.58 ± 1.12 µg/mL) (Table 1).

Subsequent solvent-solvent partitioning of potent ethanolic crude extract (20 g) yielded four fractions, namely hexane (1.42 g), dichloromethane (0.721 g), ethyl acetate (15.57 g) and aqueous (3.408 g). Following the solvent-solvent partitioning procedure the next step was to identify the most potent fraction, as observed by inhibitory concentration 50 on *in vitro* *P. falciparum* asexual stages viability (IC₅₀ < 5 µg/mL). Only the aqueous fraction (IC₅₀ = 4.05 ± 0.72 µg/mL) showed potent activity on 3D7 strain of *Plasmodium*. The EtOAc fraction (IC₅₀ = 22.44 ± 2.10 µg/mL) showed moderate activity (Table 2). None of the three other fractions (IC₅₀ > 100 µg/mL) are active on the field isolate. The potency order was aqueous > ethyl acetate > DCM/Hex.

Based on these results, the aqueous fraction phase was selected for further fractionation, whereas the Hex, DCM and EtOAc fractions were not considered for further study due to their low activity on *Plasmodium* strains.

The aqueous fraction, passed on a Sephadex LH-20 gel chromatography column, made it possible to obtain 5 sub-fractions. The results of the sub-fractions sensitivity test showed that sub-fraction SF5 (3D7: IC₅₀ = 4.11 ± 0.79 µg/mL, fieldisolate: IC₅₀ = 3.24 ± 0.22 µg/mL) showed better activity on *Plasmodium* strains compared to other sub-fractions (3D7: IC₅₀ > 100 µg/mL, fieldisolate: IC₅₀ > 100 µg/mL) which are inactive on the both strains (Table 3).

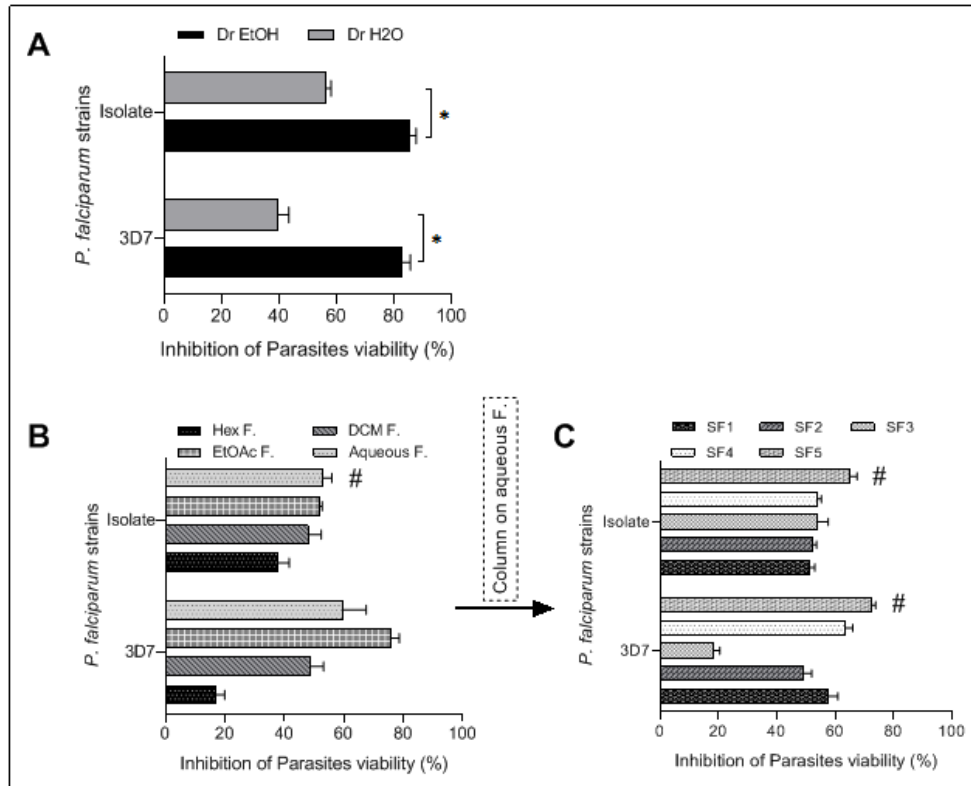


Fig. 2. Bioassay-guided fractionation of ethanolic extract of *D. rotundifolia*. Inhibition of *in vitro* viability of asexual stage of *P. falciparum* (3D7 and field isolate strains) by crude extracts (A), fractions (B) and sub-fractions (C) of *D. rotundifolia*

Data are the mean \pm SEM of three assays. Positive drug control for inhibition of viability of asexual stage of *P. falciparum* were Chloroquine and artesunate. DrEtOH: ethanolic extract of *D. rotundifolia*, Ec H₂O: aqueous extract of *D. rotundifolia*, Hex F: hexane fraction, DCM F: dichloromethane fraction, EtOAc F: ethylacetate fraction, Aqueous F: aqueous fraction, SF: sub-fraction, Inhibition of parasites viability of ethanolic extract is significantly different than that of aqueous extract ($P < 0.05$, Student's t-test), # higher inhibition of parasites viability

Table 1. Test of sensitivity of *P. falciparum* to extracts

Extracts	<i>In vitro</i> antiplasmodial activity (IC ₅₀ in μ g/mL)	
	3D7	Isolate
EtOH	6.81 \pm 0.85	22.58 \pm 1.12
H ₂ O	> 100	> 100
Chloroquine	5.30 \pm 1.02	19.64 \pm 2.66
Artesunate	0.16 \pm 0.03	2.60 \pm 0.38

Each reading is mean ($n = 3$) \pm SD (standard deviation, EtOH: ethanol, H₂O: aqueous

Table 2. Test of sensitivity of *P. falciparum* to fractions at 100 μ g / ml

Fractions	<i>In vitro</i> antiplasmodial activity (IC ₅₀ in μ g/mL)	
	3D7	Isolate
Hex	> 100	> 100
DCM	> 100	> 100
EtOAc	22.44 \pm 2.10	> 100
Aqueous	4.05 \pm 0.72	8.26 \pm 1.07

Each reading is mean ($n = 3$) \pm SD (standard deviation), Hex: Hexane; DCM: Dichloromethane; EtOAc: Ethylacetate

Table 3. IC₅₀ of sub-fractions

Sub-fractions	<i>In vitro</i> antiplasmodial activity (IC ₅₀ in µg/mL)	
	3D7	Isolate
SF1	> 100	> 100
SF2	> 100	> 100
SF3	> 100	> 100
SF4	> 100	> 100
SF5	4.11 ± 0.79	3.24 ± 0.22

Each reading is mean ($n = 3$) ± SD (standard deviation)

3.2 Toxicity Test

significantly ($p > 0.05$) different from the control group.

3.2.1 Haemolytic activity

Ethanollic extract and active fractions of *D. rotundifolia* were evaluated for their haemolytic activity. Haemolytic effect observed with this extract and actives fractions was always below 1% of haemolysis (Fig. 3).

3.3.2 Effect of ethanolic extract on the concentration levels of haematological parameters

The hematological parameters profile is presented in Table 4. The reported results indicated that the oral administration of ethanolic extract of *D. rotundifolia* at the oral dose of 2000 mg/kgbw in acute toxicity, did not affect concentration levels of evaluated hematological parameters and no significant difference between the treated animals compared to untreated was deduced ($p > 0.05$). They remained all in normal limits and presented no sign of a particular pathologic state.

3.3 Acute Oral Toxicity

3.3.1 Effect of ethanolic extracts on mean body weight of rats

The oral acute toxicity evaluation showed that animals have tolerated the ethanolic extract of *D. rotundifolia* at 2000 mg/kg body weight. Throughout the 14 days of study period, the eating, drinking habit and behaviour of all the animals used were normal. No mortality and no visible symptoms of acute toxicity were observed. These results indicated that the medium lethal dose (LD₅₀) of ethanolic extract is greater than 2000 mg/kg body weight. Fig. 4 shows a slight increase of rats' body weight during the study period but this gain is not

3.3.3 Effect of ethanolic extract on the concentration levels of biochemical parameters

The biochemical parameters (Table 5) measured in the treated rats also do not differ significantly ($p > 0.05$).

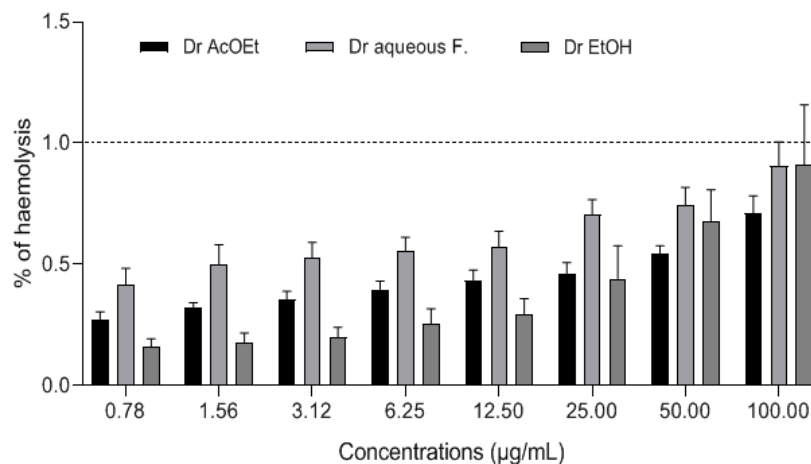


Fig. 3. Haemolytic activity of EtOH extract of *D. rotundifolia* and its active fractions

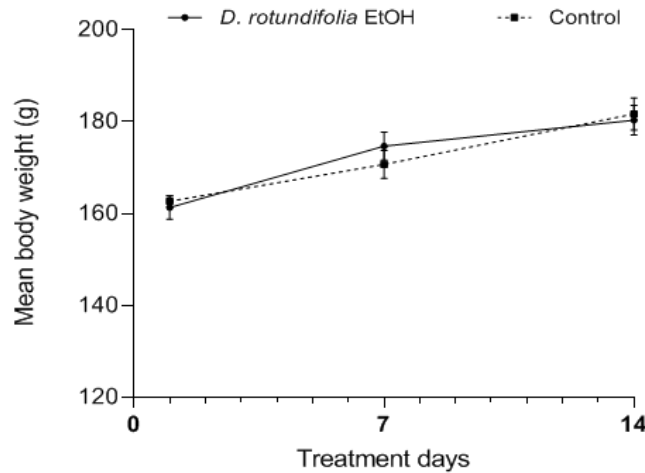


Fig. 4. Effect of ethanolic extract (2000 mg/kg) on Mean body weight of rats for 14 days

Table 4. Effect of ethanolic extract of *D. rotundifolia* on haematological parameters

Parameters	Control	Experimental
HGB (g/dl)	15.67 ± 0.80	14.63 ± 0.96
HCT (%)	47.6 ± 3.36	43.96 ± 2.80
MCV (fl.)	59.1 ± 0.95	59.8 ± 2.19
MCH (Pg)	19.67 ± 0.32	19.7 ± 1.01
MCHC (g/dL)	32.93 ± 0.76	33.26 ± 0.38
RBC (x 10 ⁶ / μL)	7.45 ± 0.59	7.38 ± 0.32
WBC (x10 ³ / μL)	14.3 ± 0.85	15.17 ± 1.07
LYM (%)	77.33 ± 5.02	79.87 ± 7.29
PLT (x 10 ⁶ / μL)	1229.67 ± 32.72	1128 ± 8.89
MPV (fL)	7.57 ± 0.12	7.7 ± 0.36

Hematological values of experimental and control rats in the treatment. HCT: hematocrit; RBC: red blood cells; HGB: hemoglobin concentration; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin levels; WBC: white blood cells; LYM: lymphocytes, PLT: platelets MPV: mean platelet volume. Values are mean ± SEM (n=3 per group), differences were considered significant when p-values were less than 0.05 (p < 0.05)

Table 5. Effect of ethanolic extract of *E D. rotundifolia* on biochemical parameters

Parameters	Control	Experimental
GLU	1.07 ± 0.18	1.06 ± 0.12
CREA	6.40 ± 0.98	7.63 ± 0.54
Urée	0.6 ± 0.04	0.64 ± 0.049
AST	116.67 ± 8.85	159.13 ± 12.45
ALT	43.02 ± 5.40	59.43 ± 3.37
AST/ALT	2.71 ± 0.57	2.68 ± 0.35

GLU, Glucose; CREA, Creatinine; ALT, alanine amino transferase; AST, aspartate transaminase. Values are mean ± SEM (n=3 per group), differences were considered significant when p-values were less than 0.05 (p < 0.05)

4. DISCUSSION

Over the years, plant medicine research has gradually increased as a means of finding promising herbs and novel chemical compounds

to fight diseases including malaria [14]. Traditional medicine is one of the most patronized means of treatment in Benin [15,16]. Traditional Medicine Practitioners in Benin use plants such as *D. rotundifolia* to manage malaria.

Due to the emergence and spread of malaria parasite with resistance to antimalarial drugs, discovery and development of alternative options are urgently needed. Extracts of plants are inherently a complex mixture of different compounds. For the purpose of drug discovery these mixtures of compounds need to be separated and the active compounds isolated and subsequently identified. In this study, the bioassay-guided procedure was used to identify sub-fraction from *D. rotundifolia* primarily active *in vitro* against erythrocytes stage of *P. falciparum* parasites.

D. rotundifolia is one of the medicinal plants used to treat malaria in Benin [9]. The aqueous and ethanolic extract of whole plant of *D. rotundifolia* demonstrated varying degrees of antiplasmodial activity against 3D7 strains and field isolated strains of *P. falciparum* (Table 1). Several strategies are available for the discovery of new anti-malarial drugs. The first important steps in the search for new antimalarial compounds are the *in vitro* screening for inhibitory activity and the identification of traditionally used plant extracts or fractions with IC_{50} values below 10 μg / mL against *P. falciparum*. Similar approaches have led to the discovery of potent plant natural products, such as quinine [17] and artemisinin [18,19].

In this study, extract with inhibitory concentration 50 (IC_{50}) less than 15 $\mu\text{g}/\text{mL}$ were selected for liquid liquid fractionation to locate active fractions containing at least one phytochemical group as major constituent. Crude extract fractionation led to fractions with varied activities against *P. falciparum* parasites. According to WHO guidelines, antiplasmodial activity was classified as follows: highly active at $IC_{50} < 5 \mu\text{g}/\text{mL}$, promising at 5-15 $\mu\text{g}/\text{mL}$, low at 15-50 $\mu\text{g}/\text{mL}$ and inactive at $IC_{50} > 50 \mu\text{g}/\text{mL}$ [20].

D. rotundifolia ethanolic extract showed antiplasmodial at IC_{50} value of $6.81 \pm 0.85 \mu\text{g}/\text{mL}$ (Table 1) against clinical isolates of *P. falciparum* and this might be due to the presence of secondary metabolites. The findings of this report, as well as all previous data, highlight the ability of *D. rotundifolia* to produce secondary metabolites with potent antiplasmodial activity. Furthermore, recent reports on phytochemical studies of *D. rotundifolia* mainly showed the presence of phenols, flavonoids, tannins, saponins, alkaloids and coumarins [21,22]. Many compounds belonging to these classes of phytochemicals have been found to be highly

potent against several sensitive and resistant strains of *P. falciparum* [23–26]. The presence of phytochemical and bioactive compounds in plants is responsible for the medicinal values and is indicative of their many possible therapeutic uses [27]. The purpose of this bio-assay-directed phytochemical investigations was to identify the active(s) sub-fraction(s) of the plant. According to the fact that the antiplasmodial activity resided preferentially in the ethanolic extract of the plant rather than in traditional preparations, such as decoctions [28]. Ethanol extracts were generally more active *in vitro* than water extracts probably due to active lipophilic constituents, which do not extract into the water extract. This justify why the ethanolic extract showed the most antiplasmodial activity than the aqueous extract [28]. The traditional method of preparation often involves boiling in water for quite some time and it is not uncommon to make a concoction of more than one plant part, which in the aqueous phase would possibly enhance the extraction of lipophilic constituents and act in synergy against the malaria parasites. The results from the investigation of ethanolic extract show that aqueous fraction of *D. rotundifolia* is the most active on the both *Plasmodium* strains (Table 2). This suggests the localization of the active molecules with both preventive and curative effects. Indeed, the aqueous fraction is more active on the 3D7 strain ($IC_{50} = 4.05 \pm 0.72 \mu\text{g}/\text{mL}$) than on the field isolates ($IC_{50} = 8.26 \pm 1.07 \mu\text{g}/\text{mL}$). This activity discrepancy might be explained by the specific features of parasites relating to drug susceptibility. Therefore, there might be a relationship between *in vitro* adaptation to culture of *P. falciparum* and drug-resistant characteristic of the strain. There is also the possibility of the emergence of a drug-resistant sub-population or of changes in the metabolic pathways during the course of *in vitro* routine culture maintenance [29]. Among the sub-fractions obtained from aqueous fraction, the SF5 sub-fraction showed a very potent activity ($IC_{50} < 5 \mu\text{g}/\text{mL}$) on the two strains of *Plasmodium* (Table 3). The molecules responsible for antiplasmodial activity therefore reside in this sub-fraction. To our knowledge, the antiplasmodial activities of extracts, fractions, sub-fractions and antiplasmodial isolated compounds from *D. rotundifolia* species have not been reported. However, this study is reporting for the first time the antiplasmodial activity of this plant. Results of the antiplasmodial testing of aqueous fraction of *D. rotundifolia* and its sub-fractions indicated that they exhibited a dose dependent inhibitory effect on the development

of trophozoites to schizonts when tested at a concentration from 100 to 0.78 µg/ml on the both strains (Fig. 2).

The ethanolic extract and its active fractions showed a haemolytic capacity less than 1% (Fig. 3). According to [30], a substance is considered toxic when its haemolytic capacity is upper to 5%. This study indicating the weak cytotoxicity potential of the extract and fractions.

Changes in body weight and food consumption have been used as an indicator of adverse effects of drugs and chemicals [31]. Since no significant changes were observed in the general behavior, food consumption and body weight in the treated groups as compared to the control group. The study of haematological parameters in rats after administration of the ethanolic extract of *D. rotundifolia* shows a non-significant decrease of RBC, HCT and HGB. This decrease could be due to the direct hemolytic effect of certain compounds such as alkaloids and triterpenes [32] contained in the extract.

In case of tissue injury, ALT and AST are released at proportional levels in the blood [33]. The AST/ALT ratio can be used to determine whether the liver or other tissue is damaged although its application is of limited value [34]. In the case of this study, the values of the AST/ALT ratio of the extract tested did not show a significant difference ($p > 0.05$) compared to the control and there was no significant increase of the ALT, which gives the impression that hepatocytes have been preserved from any lesion. The non-significant increase in ALT could be due to vitamin B6 deficiency resulting from phytochemical effects of the extract [35]. In addition, analysis of urea and creatinine revealed that the administration of the extract did not result in any significant ($p > 0.05$) change. Serum urea and creatinine are considered the major markers of nephrotoxicity, although serum urea is often considered to be a more reliable predictor of renal function than serum creatinine [35]. Consequently, the results showed that a single oral maximum dose of 2000 mg/kg ethanolic extract of *D. rotundifolia* caused no adverse or observable (death) effects in the treated rats. Likewise, no significant disturbances have been detected with respect to hematological and biochemical parameters.

These results provide validity to the traditional medicinal usage of *D. rotundifolia* as antimalarial plant. Taking account of the high activity on asexual stage of the ethanolic extract, whole

plant of *D. rotundifolia* may be considered as potential raw materials for the preparation of ameliorated antimalarial herbal medicines to be used by the local people.

5. CONCLUSION

The ethanolic extract of *D. rotundifolia* exhibited good antimalarial properties as they targeted the asexual parasites. Aqueous fraction from ethanolic extract of this plant exhibited very high activity against both 3D7 and field isolates strains. Both activities as well as the safety studies further confirm the ethnopharmacological usefulness of *D. rotundifolia* as antimalarial in Benin. The current study has clearly shown that a bioassay-guided strategy led to the separation of one sub-fraction (SF5) which also exhibited very high activity against both 3D7 and field isolates strains of *Plasmodium*. Thus, the antiplasmodial compounds leave to this sub-fraction of aqueous fraction of *D. rotundifolia*. They could be selected as good candidates for further investigations in the field of new antimalarial drug discovery. Further investigation will be conducted either to isolate the active components and elucidate the *in vivo* antiplasmodial activity or evaluate the efficacy of this plant on gametocytes and then on malaria transmission and develop a standardized Improved Traditional Medicine.

ETHICAL APPROVAL

The scientific committee of research protocols of Doctoral School of Life and Earth Sciences of University of Abomey-Calavi, Benin (UAC/FAST/ED-SVT) reviewed and approved the experimental protocol of this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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