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Research Article

ASSESSMENT OF *IN VITRO* ANTIPLASMODIAL ACTIVITY AND ANALYSIS OF THE CHEMICAL EFFECTS ON ERYTHROCYTES OF BARK EXTRACTS OF *MARGARITARIA DISCOIDEA* (EUPHORBIACEAE) FROM CÔTE D'IVOIRE

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A B S T R A C T

Background: Resistance of malaria parasites to existing antimalarial drugs complicates treatment. It is therefore necessary to find new effective and affordable drugs. From this perspective, medicinal plants are used as active ingredients against various pathologies and constitute an important source of new molecules for the pharmaceutical industry. Thus, medicinal plants contribute a lot to the modern pharmacopoeia and constitute a credible alternative in health.

Material and Methods: In this study, the *in vitro* antiplasmodial activities of aqueous and ethanolic extracts of the bark of *Margaritaria discoidea* (B. Dismar) are assessed. These extracts were tested on both clinical isolates of *Plasmodium falciparum* and reference sensitive strains NF54 and resistant K1 by the fluorimetric method with SYBR green. A hemolysis test was used to analyze the effect of the extracts on erythrocytes.

Results: The results of the *in vitro* antiplasmodial tests are as follows: the ethanolic extract had an IC_{50} of 2.4 nM on the chloroquine-resistant (CQ-R) isolate, 14.76 ± 2.78 nM on the chloroquine-sensitive isolates (CQ -S), and 6.8 nM on the K1 strains; the aqueous extract had an IC_{50} of 12.08 nM on the CQ-R isolates, 12.73 ± 1.54 nM on the CQ-S isolates and 13.28 nM on the K1 strain. Both extracts had a hemolysis rate of less than 5%.

Conclusion: We can deduce that the ethanolic extract of B. Dismar shows good activity on both CQ-R isolates and CQ-S isolates and does not have a hemolytic effect.

INTRODUCTION

Malaria remains a serious worldwide health problem due to the emergence and spread of parasite resistance to well-established antimalarial drugs and mosquito vectors resistant to insecticides. [1, 2] In sub-Saharan African populations, malaria is one of the diseases causing most morbidity and mortality. It is estimated that each year malaria causes nearly 405 000 deaths.[3, 4] mostly of African children aged below 5 years. In Côte d'Ivoire, everyone is at risk of malaria. It is the leading cause of morbidity and mortality in children under 5, 43% of the reasons for consultation in health centers, 40% of the causes of absenteeism in school and work. The Ivorian populations spend around 25% of their income on the prevention and treatment of this disease.[5] So, new antimalarial drugs that are effective and accessible to the population are essential to fight this scourge. In order to decrease the risk of chemo-resistance to most of the antimalarial drugs, the World Health Organization has recommended artemisinin-based combination therapies (ACTs) for the management of uncomplicated *P. falciparum* malaria cases. Unfortunately, ACT treatment failures have been reported in some countries.[6,7] In addition, these drugs are expensive, limiting their use in a population with average annual income around $100. Thus the use of traditional and less expensive preparations is common [8]. Historically, many drugs effective against parasitic diseases stem from traditional medicine, such as quinine and artemisinin.[9,10] Today, 30% of drugs on the pharmaceutical market come from nature [11, 12, 13] and medicinal plants constitute a popular source of potential antimalarial agents. The millennial use of these plants by African populations is generally confirmed by the results of scientific research. This is why WHO is proposing a simplified protocol for the production of herbalgalenic preparation, effective and safe dosage formulations to meet local need. In Côte d'Ivoire, various scientific studies carried out on medicinal plants have made it possible to isolate active ingredients with antimalarial, antibacterial, antidiabetic, antioxidant, anti-inflammatory, antihypertensive activity. [14, 15, 16, 17] From this perspective we have shown interest in *Margaritaria discoidea*, a plant commonly used as a purgative and anthelmintic in

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West and Central Africa. [18, 9] Bark powder is applied to swelling and inflammation as well as toothache in Malawi and Sierra Leone. [20] In addition, studies in Ghana have shown that extracts of *Margaritaria discoidea* are a curative remedy. [21] Ethnopharmacological surveys carried out in Côte d'Ivoire and Guinea have shown that this plant is used for the treatment of various diseases such as diabetes, helminthiasis, wounds, diarrhea, malaria, gastric disorders, erectile dysfunction. [22, 23] However, very little scientific data has been reported on antimalarial activity and toxicity.

The aim of this study was to determine the antiplasmodial activity of aqueous and ethanolic extracts of the barks of *M. discoidea* on clinical isolates and NF54 and K1 strains and to evaluate their effect on erythrocytes in order to develop a natural compound with time to effectively fight against malaria.

MATERIAL AND METHODS

Collection and preparation of plant extract

The fresh bark of *Margaritaria discoidea* (Figue 1) were harvested in the Moronou village at the Department of Toumodi (Central Côte d'Ivoire) between July and September 2014. After identification at the National Floristic Center and a sample deposit at the herbarium, plant samples were air dried in shade at room temperature and ground into powder. Two times 100g of the powder were macerated respectively in 1L of distilled water and 1L of 70% ethanol hydroalcoholic solvent using a blender. Macerates were filtered twice on hydrophilic cotton and once on Whatmann filter paper. Filtrates were evaporated through rotary vacuum evaporator and dried in an oven at 45 ^{o}C for 48 h to obtain aqueous and hydroethanolic extracts which were stored at 4 ^{o}C for further use. [24, 25]

The activity of crude extracts or pure compounds tested *in vitro* on *P. falciparum* is expressed in IC_{50} (inhibitory concentration of 50%), in µg/mL for crude extracts and in nM for pure compounds. The antiplasmodial activity of the extracts and of the pure compounds was determined according to the standards in Table 1. [26, 27]

Figure 1 Leafy twig of *Margaritaria discoidea* from Toumodi (Central, Côte d'Ivoire).

Table 1 Classification of the antiplasmodial activity of plant extracts and pure compounds

Extract	IC_{50} (µg/mL ou nM)	Classification
Crude extracts (µg/mL)	>50	Inactive
	15< IC_{50}< 50	Moderate effect
	5< IC_{50}< 15	Promising effect
	< 5	Strong effect
Pure compounds (nM)	> 50	Inactive
	11< IC_{50}<50	Low activity compound
	2< IC_{50}<11	Active Compound
	< 1	Very active compound (lead compound)

Phytochemical Screening

Detection of major chemical groups was carried out according to the analytical techniques described by Tona *et al*. (1998)[28], Otshudi *et al*. (2000).[29] Phytochemical groups sought are essentially sterols, polyterpenes, alkaloids, tannins, polyphenols, flavonoids, quinones and saponins. Sterols and polyterpens.

Sterols and polyterpenes

Extracts (0.1 g) were dissolved in 1 mL of hot acetic anhydride in a capsule. The resulted solutions were poured and added with 0.5 mL H2SO4. A violet coloration that turned in blue, and then in green revealed the presence of sterols and triterpenes.

Polyphenols

A drop of alcoholic solution of 2% ferric chloride was added to 2 mL of extracts. A blue-blackish to green darkish coloration indicated a positive reaction.

Flavonoids

In a tube containing 3 mL of extract, a few drops of 10% NaOH were added. Appearance of yellow-orange color indicated the presence of flavonoids.

Catechic tannins

Two milliliters of water and few drops of 1% ferricchloride were added to 1 mL of extract. The appearance of a blue, blue-black or black coloration indicated the presence of gallic tannins, the green or dark green coloration showed the presence of catechic tannins.

Gallic tannins

Previous solution was filtered and saturated with sodium acetate. Addition of 3 drops of 2% FeCl3 causes appearance of an intense blue-black color denoting gallic tannins presence.

Quinonic substances

An aliquot (0.1 g) of extract was dissolved in 5 mL of diluted HCl (1/5) and heated in a boiling water bath for 30 minutes, and then extracted with 20 mL of CHCl3 after cooling. To the organic phase was added 0.5 mL of 50% NH4OH diluted solution. The positivity of the reaction was indicated by a red to violet color.

Alkaloids

Two drops of Bouchard's reagent (reagent of iodine-iodide) were added to 1 mL of each extract. A red-brown precipitate indicated a positive reaction.

Saponins (foam index)

Samples (0.1 g of dry extract) were dissolved in 10 mL of distilled water. The samples were shaken vigorously up and down for 30-45 seconds and then left for 15minutes. The height of the foam was measured. Persistent foam for more than 1 cm high indicated the presence of saponins.

Malaria parasites

Informed consent was obtained from all patients in this study prior to clinical isolates collection. Four fresh clinical isolates of *Plasmodium falciparum* such as W6622, W6708, W6743 and W7177 were obtained from symptomatic patients at the Urban Health Unit (FSU-COM) of Wassakara in the district of Yopougon (Abidjan). Moreover, *Plasmodium falciparum* multidrug resistant K1 strain and *Plasmodium falciparum* chloroquine sensitive NF54 strains obtained from Medicine for Malaria Venture (MMV) were used for this study. The parasites were cultivated and maintained continuously in a human type O positive erythrocytes according to the method described by Trager and Jensen [30].

Determination of antiplasmodial activity

Determination of antiplasmodial activity Antiplasmodial activity of test samples (extracts and fractions)was evaluated, against chloroquine-sensitive NF54 and chloroquine-resistant K1 strains of *P. falciparum*, using the fluorescence-based SYBR® Green I assay measurement in 96-well microplates [31].The parasites were cultured in a complete medium constituted by RPMI 1640 medium with L-glutamine and 25mM HEPES buffer supplemented with 25mg Gentamycin, 5% $NaHCO_3$, 10 mg hypoxanthine, 10 mL sterile human serum and 2.5 mg Albumax II. Cultures were fed with 5% CO_2 and incubated at 37°C. Every day, infected red blood cells were transferred into fresh complete medium to propagate the culture[32]. The haematocrit was kept at 1.5-2%. Symptomatic blood samples of patients collected in EDTA collecting tubes were centrifuged at 3000 rpm for 5 min, then blood serum and buffy coat were removed and blood pellet washed thrice in RPMI 1640 medium (Gibco USA) and diluted with uninfected human type O positive red blood cells to reach a parasitemia of 0.24% at 1.5% hematocrit. Thawing of *Plasmodium falciparum* K1 strain was performed according to the method described by Witkowski *et al*. (2013)[33]. After withdrawing the cryovial from the nitrogen liquid, it was left thawing inside the Biosafety hood Class II (STERILGUARD) and transferred in a Falcon tube (15 mL) and then centrifuged at 3000 rpm for 5 min. The supernatant was removed, an equal volume of NaCl (3.5%) was added dropwise to blood pellet and slowly stirred. The tube was left resting for 1 min, then 12 mL of RPMI 1640 washing medium preheated at 37 oC was added and centrifuged at 3000 rpm for 5 min and the supernatant was removed. Then 50 µL of the blood pellet was suspended in 8 mL of complete medium in a culture flask cells (25 mL, Nunc WVR) and a volume of 110 µL of uninfected human type O positive red blood cells were added at 2% hematocrit. Daily, the infected blood pellets were transferred into fresh complete medium to propagate the culture. The stock solution of both crude extracts and Chloroquine were dissolved separately, 10 mg of each substance in 10 mL of distilled water to obtain a concentration of 1 mg/mL. Extract stock solutions were autoclaved at 121 °C for 15 min to sterilize them. As for reference molecules a 0.22 µm Millipore filter was used for filtration. Quinine and chloroquine tested in the study were obtained from Sanofi-Adventis (France). Stock solutions were prepared in 70% methanol andtwo-fold serial dilutions of the stock solutions were prepared in RPMI 1640 medium. The final concentrations of quinine and chloroquine ranged from 12.5 to 1,600 nM and 100 to 1.56 µg/mL for crude extracts. *Plasmodium falciparum* multidrug resistant K1 strains and *Plasmodium falciparum* chloroquine sensitive NF54 strains were synchronized by 10% D sorbitol (w/v) treatment at the ring stage prior to test. Then a volume of 100 µL of the inoculum (parasitized erythrocytes) was added to each well to reach a final volume of 200 L. Infected erythrocytes non-treated with drugs were used as negative control whereas infected erythrocytes treated with chloroquine (CQ) were used as positive control. All experiments were run in duplicate. Microplates were confined in a candle jar saturated with CO_2 and incubated at 37 °C in an incubator for 72 h. After 72 h of incubation, microplates were preserved at - 20 °C.

Determination of IC_{50}

After thawing of the 96 well microplates 100 µL of each well containing a volume of 200 µL was transferred in a new 96 well microplate and 100 µL of SYBR Green I lysis buffer (5 µL of SYBR Green was mixed to 25 mL of lysis buffer) was added to each well using a multi-channel pipette and incubated in a dark room at 37 °C for 1 h. Fluorescence was measured with a spectro-fluorimeter BIOTEK microplate reader (BIOTEK, FLX 800) with excitation and emission wavelength bands centered at 485 and 530 nm, respectively. IC_{50} (concentration of a tested substance inhibiting 50% of parasites growth) was determined through analysis of dose-response curves using the software IVART (*In vitro* Analysis and Reporting Tool) of WWARN [34, 35].

Assessment of chemical injury to erythrocytes

Hemolysis tests were performed to assess the effects of the extracts on red blood cells during antiplasmodial testing, because, if the extracts cause hemolysis, the parasite cannot grow properly and therefore the antiplasmodial effect measured would be biased. The extracts were tested at two concentrations: 100 µg / mL and 200 µg / mL. Indeed, a product is determined to have hemolytic power if its hemolysis rate is greater than 5%. [27, 36]

The aim of this test is to verify whether the antiplasmodial activity observed *in vitro* is not linked to a simple hemolysis of the red blood cells by the extracts. In this test, we used human red blood cells at 10% hematocrit in PBS. The hemolytic activity was carried out according to the method described by Robert *et al*. (2010)[37].We collected 5 mL of human blood in EDTA tubes (50 mM) and stored in an ice bath. The blood was then washed with PBS buffer at pH 7.4 and then centrifuged at 2200 rpm at 4 ° C. for 15 min. After removing the supernatant, the operation was repeated twice in a row on the pellet obtained. Then the optical density was adjusted to 1.5 at 550nm. The red blood cells are then diluted to 10% (v / v) in PBS at room temperature. To verify that these are still whole, 10 µL of PBS are placed in an Eppendorffmicrotube and 10 µL of Triton X-100 (20% in water, v / v) in a second microtube. Then 190 µL of red blood cells are added to the two Eppendorff tubes. The tube containing the Triton should not be pelletized after three minutes of centrifugation at 2200 rpm, while the tube containing the PBS should show a red pellet and clear supernatant. A stock solution of the samples to be tested is prepared in the appropriate solvent at the concentrations 100

µg / mL and 200 µg / mL, ensuring that the volume of the solvent is not greater than 1% in the final solution. To perform the hemolysis test, 10 µL of stock solution are placed in an Eppendorffmicrotube and mixed with 190 µL of 10% red blood cells. Control solutions are also prepared. The negative control comprising 10 µL of PBS + 190 µL of 10% red blood cells and the positive control comprising 10 µL of 20% Triton X-100 + 190 µL of 10% red blood cells. Each sample is tested in triplicate and incubated for one hour at room temperature with slow stirring. Then, the tubes are centrifuged for 5 minutes at 2200 rpm and 150 µL of supernatant are placed in a 96-well plate. The absorbance is read at 550 nm with a plate reader (Multiskan FC, Thermo Scientific).

The following formula is used to calculate the percentage of hemolysis:

[(A tested sample- A negative control) / (A positive control- A negative control)]x100

A: absorbance at 550 nm
A substance is said to be hemolytic when it has a rate of hemolysis

Statistical analysis

Graphics were performed using Graphpad prism 5 software (Microsoft, San Diego California, USA). All values were expressed as mean ± Standard of deviation. Data analysis were performed using one way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparisms test using Graphpadinstat® software. Values were statistically significant at $p<0.05$.

Ethical approval

The patient's selections were carried out in accordance with the provisions of the Helsinki Declaration of 1964, revised in 2013 and the relevant regulatory provisions. All the study participants were wellinformed about the purpose, nature, and outcomes of the study prior to the process of getting informed consent forms. A consent form was signed by them before any inclusion in the study.

RESULTS

Phytochemical screening

The results indicate that the aqueous and ethanolic extracts contain abundant polyphenols and alkaloids. In addition, these two extracts contain trace amounts of polyphenols, flavonoids and quinones. On the other hand, no extract contains saponosides and tannins (Table 2).

Antiplasmodial tests

The ethanolic extract had an IC_{50} of 2.4 nM on the chloroquine-resistant (CQ-R) isolate and an average IC_{50} of 14.76 ± 2.78 nM on the chloroquine-sensitive (CQ-S) isolates. This same extract has an IC_{50} of 6.8 nM on the chloroquine-resistant reference strains K1 and 15.61 nM on sensitive NF54. In contrast, the aqueous extract had an IC_{50} of 12.08 nM on the CQ-R isolate, 12.73 ± 1.54 nM on the CQ-S isolates and 13.28 nM on the K1 strain (Table 3). Quinine, a molecule used as a second-line treatment in the event of failure of first-line treatment, had an IC_{50} of 3.3 nM on the CQ-R isolate, 2.4 nM on the K1 strain and 11.43 ± 5 , 13 nM on the CQ-S.

Hemolysis test

A substance with hemolytic power is defined as a substance with a percentage of hemolysis greater than 5%. The data in Table 4 show a hemolytic activity of less than 1% for the aqueous and ethanolic extracts at concentrations of 100 and 200 µg / mL.

DISCUSSION

Drug resistance is a serious problem in the strategy to fight against malaria and several studies mentioned decreases in susceptibility of *Plasmodium falciparum* isolates to antimalarials in endemic areas. [38] New active ingredients are needed to diversify the therapeutic management and treat severe forms of malaria and fight against multidrug-resistant parasites. The use of herbal remedies in the treatment of malaria and other diseases is a common tradition in many developing countries, especially in Africa. Therefore, natural substances of therapeutic value are an alternative for the populations in low-income countries for the treatment of malaria. The aqueous and ethanolic extracts of the bark of *Margaritariadiscoidea* have been the subject of a phytochemical study aimed at determining the main chemical groups of which they are composed, in order to establish a relationship between their chemical composition and their activity on the growth of plasmodia. The phytochemical screening was performed in a tube and mainly targeted alkaloids, sterols / polyterpenes, quinone substances, polyphenols, flavonoids, tannins and saponins because of their pharmacological properties. Thus, ethanolic and aqueous extracts have been shown to be rich in polyphenols and alkaloids. The other compounds, except tannins and saponins, are present in varying but low concentrations.

Table 2 Phytochemical screening of crude extracts

Plante	Extract	Sterols/polyterpenes	polyphenols	flavonoids	tannins	Quinones	Alkaloids	Saponins
B. Dismar	Ethanolic	(+++)	(+)	(+)	(-)	(+)	(+++)	(-)
	Aqueux	(+++)	(+)	(+)	(-)	(+)	(+++)	(-)

+++: abundance, +: trace; -: absence: B. dismar: *Bark of Margaritaria discoidea*

Tableau 3 *In vitro* antimalarial effects of plant extracts and reference compounds.

Plantes	Extrait	CI50(µg/mL)					
		Isolatscliniques (CQ-R/CQ-S)				Souchesde référence	
		Is.W6622	Is.W6708	Is.W6743	Is.W7177	NF54	K1
E. Dismar	Aq	12,08	11,36	14,41	12,43	12,73	13,28
	EthOH	2,4	14,01	17,85	12,43	15,61	6,8
Chloroquine (nM)		**106,39**	5,92	16,22	12,04	12,4	130
Quinine (nM)		3,06	16,30	11,94	6,07	1,19	2,4

Aq: Extraitaqueux; **EthOH**: Extraitethanolique; **MeOH**:**E. Dismar**: Ecorcede *Margaritaria discoidea* ; **Is**: Isolat **CI_{50}**= Concentration inhibiting 50% of parasites; **CQ-R**=chloroquine-resistant; **CQ-S**=chloroquine-sensitive

However, it should be noted that the ethanolic extracts concentrate these metabolites at relatively lower levels than in the aqueous extracts. This could be explained by the fact that water having a high polarity with respect to ethanol concentrates better the compounds highlighted.[39] The aqueous and ethanolic extracts showed varying degrees of activity against clinical isolates and reference strains K1 and NF54. The antiplasmodial activities of the extracts were assessed according to the classification of Jansen *et al*. (2012). [27] Thus, the aqueous extract was highly active (IC_{50}<5 µg / mL) on the chloroquine resistant (CQ-R) isolate and had a promising effect on *P. falciparum* K1 (5 <IC_{50}<15 µg / mL). In contrast, this extract and the ethanolic extract had a moderate effect on both CQ-R and CQ-S as well as on the reference strains. The phytochemical screening analysis showed that the aqueous extract, in addition to being rich ingallic tannins, phenols and alkaloids also contains flavonoids and polyterpenes. These compounds are believed to be responsible for the strong effect on CQ- and *P. falciparum* K1 isolates as reported by other authors in similar studies. [14, 28, 40, 41] Likewise, according to several pharmacological studies, terpenoids have shown remarkable activity against protozoa and malaria. [42, 43] Consequently, the antiplasmodial activity exhibited by the two plant extracts could be due to these phytocompounds which would have acted alone or in combination to produce the observed antiplasmodial effect. In addition, the promising activity could be due to inhibition of parasite growth through inhibition of parasite fatty acid biosynthesis. [44, 45]

Table 4 Hemolytic activity of the extracts expressed as a percentage of hemolysis

Extract	Hemolytic activity (%)	
	100 µg/mL	200 µg/mL
E. Dismar (Aq)	0,86	0,
E. Dismar (EthOH)	0,55	0,78

E = Bark; Aq = aqueous extract; Et OH = ethanolic extract

Hemolysis tests were performed to verify the absence of false positives in *in vitro* anti-malarial tests. Hemolysis of red blood cells is characterized by lysis of the membrane of red blood cells leading to the release of hemoglobin into the surrounding plasma. The osmotic fragility of red blood cells is conventionally used as an *in vitro* test to assess the effects of chemicals on the cell membrane. [37, 46] Because if the sample causes hemolysis, the parasite cannot develop properly and therefore the measured antimalarial effect would not reflect the action of the sample on *Plasmodium*. The extracts showing a very low hemolytic power for the two extracts (<1 µg / mL). The results showed hemolytic activity of less than 1% in most cases. The highest hemolytic activity was that of the 200 µg/ mL concentration of *Harungana madagascariensis* (Madhar), however it hardly exceeds 1.02%. It can therefore be concluded that the results obtained during antiplasmodial activity are not influenced by this weak hemolytic action.

The results of the hemolytic test confirmed the safety of the extracts tested. We can deduce that our extracts do not contain constituents causing lysis of red blood cells. They are therefore non-toxic for the integrity of red blood cells. Furthermore, the phytochemical screening which indicated an absence of saponosides, confirms the non-hemolytic nature of the extracts.

Conflicts of interest

The authors declare they have no relevant conflicts of interest.

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