

# Antiplasmodial and Cytotoxic Activity of Raw Plant Extracts as Reported by Knowledgeable Indigenous People of the Amazon Region (Vaupés Medio in Colombia)

## Authors

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## Key words

- antimalarial plants
- indigenous communities
- Amazon region
- *Plasmodium falciparum*

## Abstract

The *in vitro* antiplasmodial activity of 122 raw extracts prepared in ethanol and water from 35 medicinal plants reported by the Cubeo indigenous village of the Amazon region (Vaupés Medio in Colombia) was evaluated for efficacy against 3D7 (sensitive to chloroquine) and FCR-3 (resistant to chloroquine) *Plasmodium falciparum* strains. Five

percent of these extracts presented a significant antiplasmodial activity (<5 µg/mL) and 83% of them were not cytotoxic. These findings highlight the importance of investigating traditional medicinal plants implemented by these ancestral communities of the Amazon region as well as their potential to become a source of new drugs against malaria.

## Introduction

Malaria is known worldwide as a parasitic disease responsible for approximately 700 000 deaths each year. Its high incidence and mortality rate cause large human and economic losses. In 2013, nearly 198 million cases were reported, affecting more than 97 countries [1]. Conventional treatments are now artemisinin combined therapy (ACT; i.e., artemether/lumefantrine), atovaquone + proguanil, quinine (complicated cases), and chloroquine (only in some specific countries). The parasite shows various degrees of resistance, even to the artemisinins, which have become the world's hope to eradicate malaria [2,3]. These facts support the urgency to seek new strategies for the treatment of malaria, focusing on finding new compounds that have better efficacy than current drugs or substances, with new mechanisms of action, but with less or no harmful side effects.

Colombia is the country with the second highest level of biodiversity globally and has a long tradition in the use of plants with curative potential [4, 5]. Moreover, it is widely acknowledged that natural products are a source of new medicines. Several groups of compounds found in plants, like the flavonoids, alkaloids (quinine), naphthoquinones (atovaquone), coumarins, terpenes, and lactones (artemisinins) [6], possess antimalarial properties. Thus, combining the knowledge of tradition-

al plant-derived medicines used in Colombia with current scientific methods is a powerful tool aiding in the urgent search for therapeutic alternatives.

There are four main strategies in use to select plants with probable antimalarial metabolites: 1) At random, which consists of collecting and proving the antiplasmodial activity of all available plants in a certain region without taking into account past experiences or previous knowledge; 2) Ethnobotany, which considers the use of plants for the traditional medicine of a region; 3) Chemotaxonomy, that tests species of plants which are members of a genus or family possessing antimalarial activity as reported in other studies; and 4) Phytochemistry, which looks for and measures antiplasmodial activity in specific structural analogues of compounds or metabolites previously reported as active against the parasite in other studies. The latter two strategies have a greater probability of finding positive results, since their aim is to look for plants or compounds whose activity has already been demonstrated in other studies. Nevertheless, various studies have reported promising results using ethnobotany [7–10]. Thus, the strategy chosen in this work is to implement the knowledge of the ancestral indigenous people of the Colombia Amazon region as a guide for discovering novel antimalarial agents with possibly new modes of action.

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**Table 1** Activity and cytotoxicity of raw extracts from plants reported by the native indigenous people of the region collected through percolation with water.

Plant name	Numbers of voucher specimen	Part of the plant (extraction)*	Presence of alkaloids (Dragendorff)**	Cytotoxicity on HepG2***	Activity 3D7****	Activity FCR-3	SI
<i>Picrolemma sprucei</i>	194 114	SB	–	1.9 ± 0.8	8.0E-02 ± 1.0E-03	4.0E-02 ± 1.0E-03	50.9
<i>Arrabidaea cf. verrucosa</i>	194 126	SB	–	9.9 ± 0.5	11.1 ± 0.2	NA	
<i>Hiraea apaporiensis</i>	194 135	SB	–	NT	17.3 ± 1.6	18.6 ± 0.4	
<i>Psidium acutangulum</i> DC.	94 128	SB	–	NT	18.2 ± 0.6	23.2 ± 4.3	
<i>Anaueria brasiliensis</i>	194 122	SB	–	NT	18.4 ± 2.7	15.8 ± 0.0	
<i>Guarea guidonia</i>	194 112	SB	–	NT	29.7 ± 6.1	NA	
<i>Aspidosperma excelsum</i>	194 115	SB (juvenile)	++	NT	NA	NA	
		SB (adult)		NT	32.4 ± 3.1	83.5 ± 1.9	
		SB (adult) + RB from Abuta		NT	75.3 ± 11.5	35.8 ± 3.7	
<i>Plinia cf. duplipilosa</i> McVaugh	194 129	SB		109.2 ± 10.4	33.2 ± 14.5	36.9 ± 2.8	2.9
<i>Abuta grandifolia</i>	194 130	SB	+	NT	39.7 ± 4.9	50.1 ± 1.2	
		RB	+	NT	42.6 ± 5.3	62.4 ± 4.1	
<i>Potalia resinifera</i> Mart.	194 119	L	–	NT	55.5 ± 55.1	NA	
<i>Rudgea cornifolia</i>	194 116	SB		74.2 ± 4.3	89.7 ± 15.2	3.3 ± 0.3	21.8
<i>Inga umbellifera</i>	194 110	SB	–	NT	92.1 ± 7.3	74.9 ± 15.5	
<i>Citrus x limón</i>	194 125	RB	+	NT	NA	NA	
<i>Matisia cf. glandifera</i>	194 125	SB	–	NT	NA	NA	
<i>Cissampelos</i> sp.	194 132	SB	+	88.1 ± 4.3	NA	NA	
<i>Abarema laeta</i>	194 124	SB	–	83.0 ± 7.8	NA	NA	
<i>Callichlamys latifolia</i>	194 120	SB	+	NT	NA	NA	
<i>Guarea guidonia</i>	194 134	SB	–	NT	NA	NA	
<i>Jacaranda copaia</i>	194 112	SB	–	NT	NA	NA	
<i>Anaueria brasiliensis</i>	194 113	SB	–	101.3 ± 2.0	NA	NA	
<i>Clathrotropis macrocarpa</i>	194 122	SB	–	NT	NA	NA	
<i>Tovomita cf. spruceana</i>	194 109	SB	–	NT	NA	NA	
<i>Pleonotoma jasminifolia</i>	194 127	SB	+	176.9 ± 15.7	NA	NA	
<i>Pleonotoma variabilis</i>	194 107	RB	+++	187.7 ± 0.4	NA	NA	
<i>Aspidosperma excelsum</i>	194 108	SB		NT	NA	NA	
<i>Senna spinescens</i>	194 115	SB	–	NT	NA	NA	
<i>Rudgea woronovii</i>	194 118	SB	–	NT	NA	NA	
<i>Mangifera indica</i>	129 691	SB	–	NT	NA	NA	
<i>Pleurisanthes flava</i>	194 216	SB	–	NT	NA	NA	
<i>Machaerium</i> sp.	194 123	SB	–	NT	NA	NA	
<i>Abuta rufescens</i> Ubl.	194 121	SB	–	NT	NA	NA	
Fabaceae (Pagapuniuore)	194 133	SB		NT	NA	NA	
<i>Dolioscarpus dentatus</i>	194 111	SB		NT	NA	NA	
<i>Viola decorticans</i>	194 117	SB		NT	NA	NA	
<i>Clathrotropis macrocarpa</i>	194 109	SB	–	NT	NA	NA	
**** <i>Cinchona pubescens</i> (control of raw extract)		SB	++	80.2 ± 12.2	0.2 ± 4.0E-03	0.3 ± 2.0E-02	
Chloroquine (control of antiplasmodial assays)				50.1 ± 4.2	1.0E-02 ± 2.6E-03	1.0E-01 ± 5.2E-03	486.0

Values are means ± SD in µg/mL. \* Parts of the plant used: SB: stem bark; RB: root bark; L: leaf. \*\* Presence of alkaloids: through Dragendorff reagent: – absent; + present in a small amount; +++: present in an abundant amount. \*\*\* Cytotoxicity: average TC<sub>50</sub> of two independent tests with. NT: not toxic at the highest concentration used (200 µg/mL).

\*\*\*\* Activity: average IC<sub>50</sub> of two independent tests with. NA: not active at the highest concentration used (100 µg/mL). SI: TC<sub>50</sub>/IC<sub>50</sub> of FCR-3. \*\*\*\*\* *Cinchona pubescens* was also used as a positive control bark (processed and donated by the laboratory IPHYM in France). It has a minimum total alkaloid content of 6.5% by weight of the dried bark, of which 30–60% is quinine.

The population of the Department of Vaupés consists of 85% indigenous people representing 19 ethnic groups, with the Cubeo, Tucano, Desano, and Guanano being the most numerous [11]. The traditional healers of these communities commonly use medicinal plants to treat their diseases, including malaria. The aim of this study was to evaluate *in vitro* antiplasmodial and cytotoxic activity of the aqueous and ethanolic extracts prepared from plants that have been reported as antimalarial by the traditional healers of the region of Middle Vaupés.

## Results and Discussion



During two field trips that took place in November 2011 and December 2012, 76 samples were collected from 35 plant species known by the indigenous people of the Vaupés Medio for their medicinal use. From those samples, 122 extracts were prepared. Of those, 76 were extracted using percolation with ethanol and 46 were aqueous extractions.

**Table 2** Activity and cytotoxicity of raw extracts from plants reported by the native indigenous people of the region prepared through percolation with ethanol.

Plant Name	Part of the plant used*	Presence of alkaloids (Dragendorff)**	Cytotoxicity***	Activity 3D7	Activity FCR-3	SI
<i>Picrolemma sprucei</i>	SB	+	2.3 ± 0.1	3.0E-02 ± 2.0E-04	3.0E-02 ± 7.0E-07	83.2
<i>Rudgea cornifolia</i>	SB	+	39.2 ± 2.8	2.1 ± 5.0E-02	2.54 ± 5.0E-02	15.4
<i>Aspidosperma excelsum</i>	SB (juvenile)	+	104.0 ± 5.5	3.8 ± 0.7	6.7 ± 0.7	15.4
	SB (adult)	+	74.7 ± 13.1	NA	NA	
<i>Citrus x limón</i>	RB	+++	37.5 ± 1.7	9.3 ± 0.1	7.1 ± 0.1	5.2
<i>Psidium acutangulum</i> DC.	SB	-	95.2 ± 3.9	10.6 ± 1.0	12.9 ± 1.0	7.3
<i>Matisia</i> cf. <i>glandifera</i>	SB	-	142.8 ± 5.4	11.9 ± 3.5	NA	
<i>Anaueria brasiliensis</i>	SB	-	102.0 ± 14.6	14.5 ± 2.0	10.5 ± 2.0	9.6
<i>Abuta rufescens</i> Ubl.	SB	+	69.7 ± 4.7	16.8 ± 2.5	19.0 ± 2.1	3.6
<i>Senna spinescens</i>	SB	-	NT	26.2 ± 3.2	84.2 ± 3.6	
<i>Plinia</i> cf. <i>duplipilosa</i> McVaugh	SB	-	115.8 ± 3.9	27.4 ± 2.7	NA	
<i>Tovomita</i> cf. <i>spruceana</i>	SB	-	32.5 ± 3.7	31.6 ± 3.3	29.4 ± 3.2	1.1
<i>Hiraea apaporiensis</i>	SB	-	116.9 ± 8.3	43.1 ± 4.4	41.7 ± 4.3	2.8
<i>Abuta grandifolia</i>	SB	+	NT	43.9 ± 7.2	35.2 ± 7.1	
	RB	+	NT	80.8 ± 10.41	NA	
<i>Clathrotropis macrocarpa</i>	SB	+	56.0 ± 0.7	44.4 ± 2.8	71.9 ± 2.8	0.7
<i>Virola decorticans</i>	SB	-	89.2 ± 0.8	46.6 ± 4.5	NA	
<i>Doliocarpus dentatus</i>	SB	-	19.1 ± 2.6	51.6 ± 2.3	NA	
<i>Anaueria brasiliensis</i>	SB	-	95.6 ± 5.3	57.2 ± 1.0	NA	
<i>Pleonotoma variabilis</i>	RB	++	141.0 ± 29.6	62.4 ± 15.1	NA	
<i>Clathrotropis macrocarpa</i>	SB	+	80.0 ± 2.3	64.0 ± 1.2	NA	
<i>Abarema laeta</i>	SB	-	22.5 ± 4.6	64.1 ± 3.6	72.9 ± 3.6	0.3
<i>Pleurisanthes flava</i>	SB	-	42.8 ± 13.6	64.2 ± 5.5	51.5 ± 5.5	0.8
<i>Guarea guidonia</i>	SB	+	86.0 ± 5.6	67.19 ±	64.0 ± 4.9	1.3
<i>Jacaranda copaia</i>	SB	-	198.9 ± 3.5	87.97 ± 1.8	NA	
<i>Matisia</i> cf. <i>glandifera</i>	SB + L	-	NT	90.12 ± 14.5	NA	
<i>Inga umbellifera</i>	SB	-	NT	NA	NA	
<i>Cissampelos</i> sp.	SB	+	NT	NA	NA	
<i>Callichlamys latifolia</i>	SB	++	NT	NA	NA	
<i>Guarea guidonia</i>	SB	+	138.1 ± 13.0	NA	31.6 ± 2.4	4.3
<i>Pleonotoma jasminifolia</i>	RB	-	124.4 ± 8.1	NA	NA	
<i>Arrabidaea</i> cf. <i>verrucosa</i>	SB	-	23.5 ± 6.5	NA	NA	
<i>Rudgea woronovii</i>	SB	+	NT	NA	NA	
<i>Mangifera indica</i>	SB	-	NT	NA	NA	
<i>Potalia resinifera</i> Mart.	L	-	NT	NA	NA	
<i>Machaerium</i> sp.	SB	-	154.3 ± 3.3	NA	NA	
Fabaceae (Pagapuniuore)	SB	-	95.0 ± 4.4	NA	NA	
	RB	-	156.0 ± 22.8	NA	NA	
Chloroquine			50.1 ± 4.2	1.0E-02 ± 2.6E-03	1.0E-01 ± 5.2E-03	486.0

\* Parts of the plant used: SB: stem bark; RB: root bark; L: leaf. \*\* Presence of alkaloids: through Dragendorff reagent: - absent; + present in a small amount; +++: present in an abundant amount. \*\*\* Cytotoxicity: average TC<sub>50</sub> of two independent tests with. NT: not toxic to the highest concentration used (200 µg/mL). Activity: average IC<sub>50</sub> of two independent tests with. NA: not active to the highest concentration used (100 µg/mL). SI: TC<sub>50</sub>/IC<sub>50</sub> of FCR-3. Data are given in µg/mL

From the 122 extracts prepared, 6 were highly active (IC<sub>50</sub> < 5 µg/mL) in the 3D7 strain and 5 in the FCR-3 strain of *Plasmodium falciparum* (5% and 4%, respectively). Seven percent presented promising activity (< 15 µg/mL), 22% average and low activity, and about 70% did not show antiplasmodial activity (IC<sub>50</sub> > 50 µg/mL). Eighty-three percent of the extracts were not cytotoxic (101 extracts) and only 4% (5 extracts) were highly toxic (TC<sub>50</sub> < 10 µg/mL). Nevertheless, the selectivity index (SI: TC<sub>50</sub>/IC<sub>50</sub>) was above 15%, which meant that the extracts prepared from these plants were not antiplasmodial because of their cytotoxicity.

The extracts that were prepared with water, in the same manner as the traditional healers, did not present cytotoxicity, except for those prepared from *Picrolemma sprucei* Hook. fil. (Simaroubaceae) and *Arrabidaea verrucosa* (Standl.) A. Gentry (Bignoniaceae) plants. However, the cytotoxicity value of these extracts

was minor compared to the ethanolic extracts (● Tables 1 and 2). Overall, the ethanol extracts yielded the best results for antiplasmodial activity when compared with the aqueous extracts. In most cases, the native experts used the stem bark to prepare malaria treatments from plants. In this study, the raw extracts prepared from the stem bark of *P. sprucei*, *Rudgea cornifolia* (Kunth) Standl. (Rubiaceae), and *Aspidosperma excelsum* Benth. (Apocynaceae) presented the best results for antiplasmodial activity. More specifically, both the ethanolic and aqueous extracts from the stem bark of *P. sprucei* showed good antiplasmodial activities with an SI > 50. Even ethanolic extracts from the leaves of this plant showed promising activities. Similarly, this species has been reported as antimalarial by Silva et al. and de Andrade-Neto et al., who isolated two compounds from the stems and roots of this plant. The compounds extracted were the quassinoid neosergeolide with an IC<sub>50</sub> = 1.0E-03 ± 2.5E-03 µg/mL, and isobrucein

**Table 3** Antiplasmodial activity of promising extracts from the part of the plant used by the native indigenous people of Vaupes Medio.

Plant Name	Part of the plant (extraction)*	Used by traditional healers of the region of Middle Vaupés	Type of extract**	Presence of alkaloids (Dragendorff)	Cytotoxicity***	Activity 3D7	Activity FCR-3	SI	Referenced activity
<i>Picrolemma sprucei</i>	SB	Yes	E	+	2.3 ± 0.1	3.0E-02 ± 1.0E-03	3.0E-02 ± 8.0E-03	83.3	Yes
	L	No	A	-	1.9 ± 0.8	8.0E-02 ± 1.0E-03	4.0E-03 ± 1.0E-03	51.0	
<i>Rudaea cornifolia</i>	L	No	E	N.D.	8.8 ± 3.4	0.5 ± 0.1	5.7 ± 1.0E-02	1.6	
	SB	Yes	E	+	39.2 ± 2.8	2.12 ± 5.0E-02	2.54 ± 0.5	15.4	NO
<i>Cissampelos</i> sp.	SB + RB from Abuta	Yes	E	+	161.3 ± 15.3	9.78 ± 1.7	13.11 ± 0.8	12.3	
	RB	No	E		31.3 ± 3.9	7.86 ± 0.1	7.33 ± 0.5	4.3	Yes
<i>Citrus x limón</i>	RB	Yes	E	+++	37.5 ± 1.7	9.28 ± 0.1	7.13 ± 1.2	5.3	Yes
<i>Psidium acutangulum</i> DC.	SB	Yes	E	-	95.2 ± 3.9	10.62 ± 1.02	12.94 ± 1.9	7.4	
<i>Arrabidaea cf. verrucosa</i>	SB	Yes	A	-	9.9 ± 0.2	11.1 ± 0.2	NA		
<i>Jacaranda copaia</i>	L	No	E	N.D.	84.4 ± 3.8	11.23 ± 1.9	5.57 ± 0.6	15.2	Yes
Fabaceae (Pagapuniuore)	L	No	E	N.D.	187.4 ± 27.9	11.53 ± 2.5	NA		
<i>Matisia</i> cf. <i>glanidifera</i>	SB	Yes	E	-	142.8 ± 5.4	11.9 ± 3.5	NA		
<i>Anaeria brasiliensis</i>	SB	Yes	E	-	102.0 ± 14.6	14.5 ± 3.5	10.5	9.6	
Chloroquine					50.1 ± 4.2	1.0E-02 ± 2.6E-03	1.0E-01 ± 5.2E-03	486.0	

\* Parts of the plant used: SB: stem bark; RB: root bark; L: leaf. \*\* Type of extracts: E: ethanolic; A: aqueous. \*\*\* Cytotoxicity: average  $TC_{50}$  of two independent tests with; NT: not toxic to the highest concentration used (200 µg/mL). Activity: average  $IC_{50}$  of two independent tests with; NA: not active to the highest concentration used (100 µg/mL). SI:  $TC_{50}/IC_{50}$  of FCR-3. Data are given in µg/mL.

B with an  $IC_{50} = 1.0E-03 \pm 2.0E-03$  mg/mL in the *P. falciparum* K1 strain [12, 13].

This work reports for the first time that *R. cornifolia* has promising antiplasmodial activity, with an SI > 15. Interestingly, another plant belonging to the Rubiaceae family, *Canthium multiflorum* (Schumach. & Thonn.) Hiern, is used in Burkina Faso for the treatment of malaria. 19 $\alpha$ -Hydroxy-3-oxo-ursa-1,12-dien-28-oico acid was isolated from ethanolic extracts derived from *C. multiflorum* roots. This acid presented antiplasmodial activity with an  $IC_{50}$  value of 26 µg/mL in the *P. falciparum* 3D7 strain. Moreover, it did not induce changes in the shape of the erythrocyte membrane [14]. Other plants from this family with known *in vitro* antiplasmodial activity or “*in vivo*” efficacy are *Rennellia elliptica* Korth. [15], *Crossopteryx febrifuga* (Afzel. ex G.Don) Benth. [16], and *Nauclea pobeguunii* (Hua ex Pobég.) Merr. [17].

Notably, as in this study, *A. excelsum* was among some Peruvian plants screened for their antiplasmodial activity by Kvist et al. in 2006. They reported that ethanolic extracts from the *Aspidosperma* plant had  $IC_{50}$  values between 10 µg/mL and 50 µg/mL in the *P. falciparum* 3D7 strain [18]. Similarly, *A. excelsum* extracts from this study demonstrated  $IC_{50}$  values below 10 µg/mL (see **Table 2**). *In vitro* antiplasmodial activity from this plant has also been reported from the preparation used in traditional medicine, i.e., a decoction of the leaves in water for 2 h [19]. Isolated compounds from other species of the genus *Aspidosperma* with antiplasmodial activity are *A. vargasii* and *A. desmanthum* [12].

In relation to the *Cissampelos* species that showed very promising antiplasmodial activity in this work (see **Table 3**), Fischer et al., in 2004, evaluated the fractions rich in alkaloids from ethanolic extracts of the leaves of *Cissampelos andromorpha* DC. (Menispermaceae) and *Cissampelos ovalifolia* DC. They found  $IC_{50}$  values below 3.3 µg/µL in the *P. falciparum* K1 strain [20].

In this research, the extracts of the plant *Citrus x limon* (L.) Osbeck presented promising antiplasmodial activities with  $IC_{50}$  values below 9.2 µg/mL (see **Table 3**). Similarly, Kvist et al., in 2006, reported an  $IC_{50}$  value below 10 µg/mL from the ethanolic extract of this plant [18]. On the other hand, Valadeau et al., in 2009, evaluated the *in vitro* antiplasmodial activity of the ethanolic extract of the plant *Jacaranda copaia* (Aubl.) D.Don (Bignoniaceae), and found an  $IC_{50}$  of  $8.1 \pm 1.5$  µg/mL in the chloroquine resistant *P. falciparum* FCR3 strain [21]. In this study, extracts from this plant demonstrated  $IC_{50}$ s of 11.2 and 5.6 µg/mL in the 3D7 and FCR3 strains, respectively (see **Table 3**).

In conclusion, this study supports the use of ethnobotany from the Vaupés Medio region to aid in the acquisition of raw plant extracts with high antiplasmodial *in vitro* activity ( $3.0E-02 \pm 1.0E-03$  µg/mL). Importantly, the findings reported here further validate the traditional use of native medicinal plants by these Amazonian ancestral communities. Until now, the antimalarial activity of plant species such as *R. cornifolia* had not been reported. These findings warrant further research focused on finding the metabolites responsible for such activity as well as the mechanisms behind the antiplasmodial effects.

## Materials and Methods

Approval from the indigenous communities through “Asociación de Autoridades Tradicionales del Vaupés Medio” (AATIVAM) was obtained for the sharing of ethnobotany knowledge and for specimen collection. Likewise, permission was obtained for the development of the research by “Corporación para el Desarrollo del

Norte y el Oriente Amazónico” (CDA) and “Ministerio del Interior de Colombia”. Each traditional healer involved in the study signed the informed consent, which was approved by the ethics committee from the Facultad de Medicina of the Universidad de Antioquia by the Minute # 11M790 ADV on April 20th, 2009.

### Plant material

The collection of plant materials was carried out in November 2011 and December 2012 in collaboration with the knowledgeable indigenous people in 10 indigenous communities in middle Vaupés (south-west of Colombia 0°48'11.09" N, 70°57'16.20" W; 180 m above sea level) [22]. Approximately 2 kg of plant material from 35 plants used by traditional healers to cure malaria were collected. A specimen of every plant collected is in the herbarium of Universidad de Antioquia (HUA), catalogue Colombian flora. The plant material was dried at room temperature for 10 days and it was ground to a 5-mm particle size.

### Obtaining raw extracts

- ▶ **Ethanolic:** Approximately 5.0 g of plant material were weighed. Next, it was percolated (3 days/3 times), using analytical grade ethanol as the solvent (approximately 250 mL), to a ratio of 2 g of plant material for every 100 mL of solvent. Subsequently, the extract was filtered with Whatman paper and it was concentrated using a rotary evaporator until the solvent was completely evaporated.
- ▶ **Aqueous:** Approximately 5.0 grams of plant material were added to an erlenmeyer, then deionized water was added (250 mL). The sample was then subjected to a process of heating by lamp for 60 min, starting from room temperature (~8 °C) up to 60 °C. Later, the hot extract was filtered and then concentrated by freeze-drying.

### Evaluation of *in vitro* antiplasmodial activity

To evaluate *in vitro* antiplasmodial activity of raw extracts (aqueous and ethanolic), the methodologies described by Bravo et al., in 1999, as well as the ones described by Desjardins et al., in 1979, were used [23,24]. Briefly, a basic solution of 10 mg/mL from each extract was prepared in DMSO. To obtain a final concentration of 0.5 mg/mL, 50  $\mu$ L of the 10 mg/mL solution were added to 950  $\mu$ L of complete RPMI-1640 medium, without hypoxanthine.

The tests to assess *in vitro* antiplasmodial activity were carried out in 96 Falcon® flat well plates. Seven concentrations of every compound were evaluated in a range between 100  $\mu$ g/mL to 1.56  $\mu$ g/mL. When the extract was very active (< 1.56  $\mu$ g/mL), another seven concentrations were prepared from the lower concentration. Each assay was done twice. The DMSO concentration was 1% in the first dilution, which was not toxic for the parasite. Chloroquine diphosphate salt solid ( $\geq$  98%; C6628 SIGMA) was used as the control treatment. It was evaluated in serial dilutions in a range between 1.04  $\mu$ g/mL and 0.0024  $\mu$ g/mL (2.0  $\mu$ M and 0.0046  $\mu$ M). Furthermore, bark from *Cinchona pubescens* Vahl (Rubiaceae; also known as red or officinal Quine), which was processed and donated by the laboratory IPHYM in France, was used as a positive control. The dried bark from this plant has a minimum total alkaloid content of 6.5% by weight, of which 30–60% is quinine. Plain medium was used as a growth control.

Antiplasmodial activity was evaluated in the strain 3D7 (chloroquine sensitive) and FCR-3 (chloroquine resistant) of *P. falciparum*. A suspension of red blood cells with parasites was prepared,

along with a hematocrit of 2% and a parasitemia of 1%. The final concentration of  $^3$ H-hypoxanthine per well was 0.8  $\mu$ Ci/mL.

The culture with the treatments was incubated at 37 °C for 48 h in an atmosphere of 5% CO<sub>2</sub> and 5% O<sub>2</sub>, and balanced with N<sub>2</sub>. The plates were then frozen at -20 °C causing hemolysis of erythrocytes, and the next day they were defrosted. Nucleic acids were deposited in a filter of fiberglass with the help of a semiautomatic collector and they were read in a beta counter. The reading was expressed in cpm. Any reading below 2500 cpm from the control wells (wells with parasites + medium) was a means to reject the experiment.

These data were analyzed using the GraphPad Prism 5.1 program to find the IC<sub>50</sub> (concentration that inhibits radiolabel incorporation by 50%) through a variable slope model [log (inhibitor) vs. response-variable slope]. It is also called a four-parameter dose-response curve or four-parameter logistic curve. In this model, data were also normalized and assumed a maximal (Top = 100%) response and the maximally inhibited (Bottom = 0%) response. The coefficient of variation was also calculated (%CV) to estimate the grade of dispersion of the obtained IC<sub>50</sub> from the two independent tests. When %CV was superior to 20% [25], a third test was carried out.

In this work, extracts were classified in terms of their activity (measured with IC<sub>50</sub> average) as: highly active, those which presented an IC<sub>50</sub> average < 5.0  $\mu$ g/mL; promising: 6.0–15.0  $\mu$ g/mL; middle activity: 16.0–30.0  $\mu$ g/mL; low activity: 31.0–50.0  $\mu$ g/mL; and not active: > 51.0  $\mu$ g/mL.

### Evaluation of cytotoxicity

The potential toxic effect on human hepatocytes was modeled by measuring the cytotoxicity of ethanolic and aqueous extracts. This was determined through the measurement of *in vitro* viability and proliferation at the HepG2 cellular level. This is important because the hepatocytes constitute the first host cell for *Plasmodium* sp. in humans and are the only cells invaded by the parasite with the ability to split. More specifically, to determine *in vitro* viability and proliferation, the MTT reduction method was used, as described by Mosmann in 1983. This method reveals cellular damage at the mitochondrial level. MTT, which has a yellow color, is reduced by the metabolically active cells, partly by the action of an enzyme dehydrogenase, generating cellular reducing equivalents such as NADH and NADPH. The final result is the formation of formazan crystals, a violet compound that is solubilized and quantified by spectrophotometry [26].

For every test, a stock solution of each compound at a concentration of 20 mg/mL in 96% DMSO was prepared. Seven serial dilutions from the stock solution were prepared with DMEM F-12 (10% of FBS). The highest concentration of DMSO was 2%, which had no toxic effect on the HepG2 cells under the conditions of this test. In the Neubauer chamber, HepG-2 cells were placed on a flat grid - 2 × 10<sup>5</sup> cells/mL in 100  $\mu$ L with half DMEM-F-12 supplemented with 10% FBS. The cells were then incubated for 24 h at 37 °C in 5% CO<sub>2</sub> in air to allow for the formation of a monolayer. The cells were washed with 0.85% saline solution and then 100  $\mu$ L from each treatment were added to the test plate. Plain medium was used as a growth control.

Each concentration of the samples and controls were evaluated in triplicate in two tests. Thereafter, plates were incubated for 48 h at 37 °C, the culture media was removed and the cells were washed with 0.85% saline solution, 30  $\mu$ L of MTT prepared in culture media (Sigma-Aldrich) were added to a final concentration of 2 mg/mL, and the cells were incubated again for 4 h. After the

incubation, 130  $\mu$ L of 96% DMSO were added, they were mixed softly in a vortex and incubated during 20 min at room temperature to allow the MTT crystals to be dissolved. Then, the absorbance of every well was read at 570 nm [26]. Finally, the optical densities obtained were analyzed with the GraphPad Prism 5.1 program to find the toxic concentration 50% (TC<sub>50</sub>) and the coefficient of variation was calculated (%CV) to estimate the extent of dispersion of the TC<sub>50</sub> obtained from the two independent tests.

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## Conflict of Interest

The authors have no conflicts of interest.

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