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Antiplasmodial activity of the andiroba (*Carapa guianensis* Aubl., Meliaceae) oil and its limonoid-rich fraction

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ABSTRACT

Ethnopharmacological relevance: From seeds of *Carapa guianensis* the Amazon native people extracts the andiroba oil, which is traditionally used as febrifuge, anti-malarial, insecticidal and repellant. The non-saponifiable fraction separated from the oil is rich in limonoids, which assigns its pharmacological effects.

Materials and methods: The andiroba oil and its limonoid-rich fraction were submitted to *in vitro* antiplasmodial bioassay using W_2 and Dd_2 strains of *Plasmodium falciparum*. The acute toxicity of andiroba oil was evaluated. The limonoid-rich fraction was subjected to fractionation and identified its major constituents.

Results: Andiroba oil and its limonoid-rich fraction inhibited the growth of W_2 clone in 100%, between 24 and 72 h, at concentrations of 8.2 μg/mL and 3.1 μg/mL, respectively. Under the same conditions, the parasitaemia of Dd_2 clone provoked by the andiroba oil showed inhibition of 31% (IC_{50} > 82 μg/mL) with a time-dependent relationship of 24 h and inhibition of 88% (IC_{50} 8.4 μg/mL) after 72 h, while for the limonoid-rich fraction the inhibition of Dd_2 clone was 56% (IC_{50} 2.8 μg/mL) at 24 h and 82% (IC_{50} 0.4 μg/mL) after 72 h. Andiroba oil in acute toxicity test with a fixed dose (LD_{50} > 2000 mg/kg) was not toxic The limonoids identified in the oil were gedunin, 6α-acetoxygedunin, 7-deacetoxy-7-oxogedunin, 7-deacetylgedunin, 1,2-dihydro-3β-hydroxy-7-deacetoxy-7-oxogedunin and andirobin. Gedunin and derivatives has been reputed as anti-malarials.

Conclusion: The results support the traditional use of andiroba oil as antiplasmodial, which additionally proved not to be toxic in bioassays conducted with mice.

1. Introduction

About 40% of the world's population lives in countries where the malaria is endemic and more than 300 million people are suffering from the disease every year (WHO, 2008). Most cases of malaria have been reported for the sub-Saharan Africa and it is responsible for the almost 1 million deaths each year. Outside Africa malaria still claims for more than 100 thousand lives each year. In Latin America, reaching 99%, the largest number of cases is verified in the Brazilian Amazon, with an incidence of 400 to 700 thousand cases a year. According to the Brazilian Ministry of Health, just in the Amazonas, Rondônia and Pará states, was registered about 350 thousand cases of malaria in 2007 (Ministério da Saúde, 2008).

The etiological agents of malaria are protozoans that belong to four parasite species pathogenic to humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. *Plasmodium falciparum* is the parasite responsible for most cases of malaria (80% worldwide) and for the most severe forms of the disease, frequently fatal.

The resistance of *P. falciparum* to the available drugs has become the most important threat to the effective control of the disease. Naturally occurring populations are known to vary considerably in their relative sensibility to mefloquine. Chloroquine-resistant strains of *P. falciparum* occur in all endemic areas except in Panama, Mexico, Hispaniola and parts of China, as well as multidrug-resistant strains exist in Southeast Asia, South America, and in the sub-Saharan Africa (Griffth et al., 2007). The chloroquine-resistant parasites accumulate low levels of the drug compared to drug-sensitive parasites. It is understood that the resistance of parasites is due to exclusion of the drug from its site

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of accumulation, possibly the food vacuole (Yayon et al., 1984). The boundary between Thailand and Cambodia, with respect to malaria, has become an area of concern with the emergence of numerous cases of resistance to the drug artemisinin. For this reason, some warning signs have been sent to the entire world by the WHO as a result of human migration in the region that is greatly facilitated and this differential resistance can spread throughout the African continent (World Health Organization (WHO), 2007). The resistance to artemisinin was observed by the change and selective inhibition of protein-type ATPase in the genome of *P. falciparum* (Eckstein-Ludwig et al., 2003; Mugittu et al., 2006).

The spread of resistance of the mosquito vector to currently available insecticides and the limited success of potential anti-malarial vaccines contribute to the urgent necessity of finding new chemotherapeutic agents for the treatment of malaria, in particular, against *P. falciparum*, the strain responsible of the most severe forms of malaria (Corbett et al., 2004). There is a consensus that new drugs to treat malaria are urgently needed. These drugs need to have schizontocidal and gametocytocidal activities for the blood and tissues, as well as they must be inexpensive, safe, long-acting and effective in preventing relapses. Treatment is aimed to the interruption of blood schizogony, responsible for the pathogenesis and clinical manifestations of infection (Fundação Nacional da Saúde (FUNASA), 2001).

The search for novel compounds effective against *Plasmodium* strains resistant to widely used synthetic drugs has led to increased interest in new and existing information about malaria remedies from natural sources (Phillipson and Wright, 1991; Angerhofer et al., 1992; Wells et al., 2009). Natural products comprising alkaloids, lignans, triterpenes, coumarins, flavonoids and limonoids isolated from medicinal plants and used worldwide were examined *in vitro* for antimalarial activity against *P. falciparum* (Khalid et al., 1986; Leaman et al., 1995; Batista et al., 2009; Nogueira and Lopes, 2011). Some Meliaceae species in Tropical America, as *Cedrela odorata* L., *Carapa guianensis* Aubl., and *Swietenia mahagoni* (L.) Jacq., have been used in traditional medicine for the treatment of fevers. (Ayensu, 1981). MacKinnon et al. (1997) examined extracts from 22 Meliaceae species, by characterizing their *in vitro* antiplasmodial activities against the clones chloroquine-sensitive (D_6) and chloroquine-resistant (W_2) of *P. falciparum*.

The Meliaceae family is rich in limonoids, highly oxygenated tetranortriterpenoid compounds that are reported to possess a wide range of biological activities, such as insecticidal, antifeedant and growth-regulator on insects, antibacterial, antifungal, antimalarial, and antiviral (Roy and Saraf, 2006). Gedunin, nimbin, nimbolide and many other limonoids isolated from *Azadirachta indica*, *Carapa odorata*, *Guarea multiflora*, *Khaya grandiflora* and *K. grandifoliola* have been identified for their *in vitro* antimalarial activities on *P. falciparum* (Bickii et al., 2000; Kayser et al., 2003; Saxena et al., 2003). Finally, new antiplasmodial limonoids known as ceramicines were reported from *Chisocheton ceramicus* C. DC. (Mohamad et al., 2009).

The species *Carapa guianensis* Aubl., known as andiroba, is another rich source of limonoids. It is an oilseed crop that grows wild throughout South America. From its seeds the native people of the Amazon extracts the andiroba oil, traditionally used as febrifuge, anti-bacterial, anti-parasitic and anti-inflammatory, as well as insecticidal, pain-relieving, and repellant (Tropical Plant Database, 2012, www.rain-tree.com/andiroba.htm). The andiroba oil is a source of fatty acids, such as oleic, palmitic, stearic and linoleic acids. The non-saponifiable part of the oil, between 2 and 5%, basically consists of a rich fraction containing limonoids. The compounds methyl angolensate, 7-deaceto xy-7-oxogedunin, deacetylgedunin, 6α-acetoxygedunin, gedunin, andirobin, 17β-hydroxyazadiradione, 1,2-dihydro-3β-hydroxy-7-deacetoxy-7-oxogedunin and xyloccensin were previously isolated from *C. guianensis* by various chromatographic techniques (Ambrozin et al., 2006; Silva et al., 2009).

In view of the importance of Meliaceae in the search for compounds that may act as anti-malarial drugs, the aim of this paper is to report the *in vitro* evaluation of the andiroba oil and its limonoid-rich fraction against chloroquine-resistant *P. falciparum* strains, as well as the fractionation and identification of the main compounds existing in the limonoid-rich fraction. In addition the acute toxicity of andiroba oil was also determinate.

2. Materials and methods

2.1. Andiroba oil (AO)

It was obtained by traditional process, as it is done by native peoples of the upper Tocantins River, Brazil. The seeds (500 g) of andiroba were collected in the campus of University of Pará, in March 2008, boiled in a pot of water, stored for some two weeks until start to get rotten (the time to occur the hydrolysis process), and then subjected to pressing and filtering to separate the oil (60 g). This specimen was identified with an authentic voucher (MG 144540) of *Carapa guianensis* Aubl., deposited in the herbarium of Museu Emílio Goeldi, Belém, PA, Brazil.

2.2. Limonoid-rich fraction (LRF)

After being extracted, the andiroba oil (10 g) was submitted to fractionation in a silica gel chromatographic column (Merck 60, 0.040-0.063 mm), using for elution a gradient mixture of hexane and ethyl acetate and yielding a limonoid-rich fraction (0.4 g), according to their similarity in TLC plates (Merck 60G). After pretreatment in C18 SPE cartridge it was subjected to HPLC reversed-phase (Shimadzu, LC-8A and UV detector) using gradient mixture of acetonitrile and ultra-pure water as mobile phase and keeping the flow at 1.0 mL/min and detection at 217 nm, yielding six pure compounds (1–6). The detection and identification of the limonoids were performed by TLC and HPLC-DAD with co-injection of authentic samples, as well as by the ^{1}H and ^{13}C NMR chemical shifts. The TLC plates were revealed with cerium sulfate/sulfuric acid reagent, after heating to 100 °C. The HPLC-DAD conditions were mentioned above.

2.3. Antiplasmodial assay

The clones of *Plasmodium falciparum* – Dd_2 (resistant to chloroquine, mefloquine and pirimetamin) and W_2 (resistant to chloroquine and sensitive to mefloquine) were kept in continuous cultures of human erythrocytes, suspended in RPMI 1640 and supplemented with 10% human serum, according the method described by Trager and Jensen (1976). The antiplasmodial activity of the andiroba oil and its limonoid-rich fraction were performed in 96-well tissue culture plates as previously described (Rieckmann, 1980; Carvalho et al., 1991; Mitaine-Offer et al., 2002). Twofold serial dilutions of limonoid-rich fraction dissolved in sterile methanol, and andiroba oil dissolved in DMSO solution, were placed in micro titer plates and diluted with the culture medium (RPMI 1640 plus 10% human serum). A suspension of parasitized erythrocytes (0.5–1% parasitaemia, 2.5% hematocrit) containing mainly trophozoites was added to the wells to reach a final volume of 100 μL. For andiroba oil were used the concentrations 820, 82, 8.2, 0.82 and 0.082 μg/mL, while for limonoid-rich fraction the concentrations were 100, 50, 25, 12.5, 6.25 and 3.125 μg/mL. Chloroquine (clone Dd_2) and quinine (clone W_2)

were used as positive control and infected erythrocytes were included as negative controls. The plates were incubated at 37 °C and after 24 and 48 h the culture medium was replaced by fresh medium with or without test samples. Samples were taken 24 h, 48 h and 72 h later, smeared, Giemsa stained and microscopically examined to determine the percentage of parasitaemia by counting 2000 erythrocytes. All material used in this essay was endotoxin free. Results were expressed as the mean IC_{50} of three independent experiments for each sample. The Student *t* test was used to compare the inhibition of the two different *P. falciparum* strains.

2.4. Animals

Female Swiss albino mice (20–30 g) were obtained from colonies maintained in the Instituto Evandro Chagas (Belém, Brazil) and kept under environmentally controlled conditions (24 °C; 12–12 h dark/light cicle) for seven days. Food was withheld overnight prior to experiments while water was still provided *ad libitum*. The experimental procedures and use of animals was approved by Animal Experimentation Ethics Committee of Federal University of Pará.

2.5. Acute toxicity

The test of acute toxicity was performed according the National Institute of Environmental Health Sciences (NIEHS 2001). Mice were divided into three groups (10 animals by group). The groups received by oral gavages (single dose): 100 μL of ultra pure water; 100 μL of water and tween 20 (1%); and 100 μL of a solution containing water (490 μL), andiroba oil (500 μL) and tween 20 (10 μL), corresponding to a dose of 2.0 g/kg. The animals were observed for the first three hours and every 24 h for 14 days. Body weight and clinical evaluation of the animals were measured daily. Hematological and biochemical parameters were made on the 14th day.

3. Results

3.1. Limonoid-rich fraction

The extraction performed in the seeds (500 g) of *C. guianensis* yielded 12% of andiroba oil (60 g). The fractionation of andiroba oil (10 g) on silica chromatographic column provided a yield of 3.5% for the limonoid-rich fraction (350 mg). The limonoids occurring in the limonoid-rich fraction (compounds 1–6) were identified by co-injection of authentic standards using HPLC-DAD and TLC, as well as by the ^{1}H and ^{13}C NMR chemical shifts (Ambrozin et al., 2006; Silva et al., 2009). The following limonoids were isolated and identified in the limonoid-rich fraction: gedunin (1) (24 mg), 6α-acetoxygedunin (2) (44 mg), 7-deacetoxy-7-oxogedunin (3) (21 mg), 7-deacetylgedunin (4) (22 mg), 1,2-dihydro-3β-hydroxy-7-deacetoxy-7-oxogedunin (5) (25 mg) and andirobin (6) (24 mg) (Fig. 1).

3.2. Antiplasmodial assay

Andiroba oil and its limonoid-rich fraction inhibited the growth of W_2 clone in 100%, between 24 and 72 h, at concentrations of 8.2 μg/mL and 3.1 μg/mL, respectively. Table 1 compares these results with those of quinine, the drug used as standard. Under the same conditions, the parasitaemia of Dd_2 clone provoked by the andiroba oil showed inhibition of 31% ($IC_{50} > 82$ μg/mL) with a time-dependent relationship of 24 h, followed by inhibition of 71% (IC_{50} 9.4 μg/mL) at 48 h and inhibition of 88% (IC_{50} 8.4 μg/mL) after 72 h. For the limonoid-rich fraction the inhibition of Dd_2 clone was 56% (IC_{50} 2.8 μg/mL) at 24 h, 64% (IC_{50} 2.4 μg/mL) at 48 h and 82% (IC_{50} 0.4 μg/mL) after 72 h. Table 2 compares these results with those of chloroquine, the drug used as standard.

For Dd_2 clone, in both experiments with andiroba oil and limonoid-rich fraction, the final response at 72 h (IC_{50} 8.4 μg/mL and IC_{50} 0.4 μg/mL) was more positive than the initial response of 24 h (IC_{50} > 82 μg/mL and IC_{50} 2.8 μg/mL). A concentration-

Table 1
Inhibition percentages of the andiroba oil and limonoid-rich fraction against the W_2 clone.

Samples	Concentration (μg/mL)	W_2 strain (% inhibition)		
		24 h	48 h	72 h
AO	8.2	100	100	100
LRF	3.1	100	100	100
Quinine	0.016	71	73	75

AO = andiroba oil; LRF = limonoids-rich fraction.

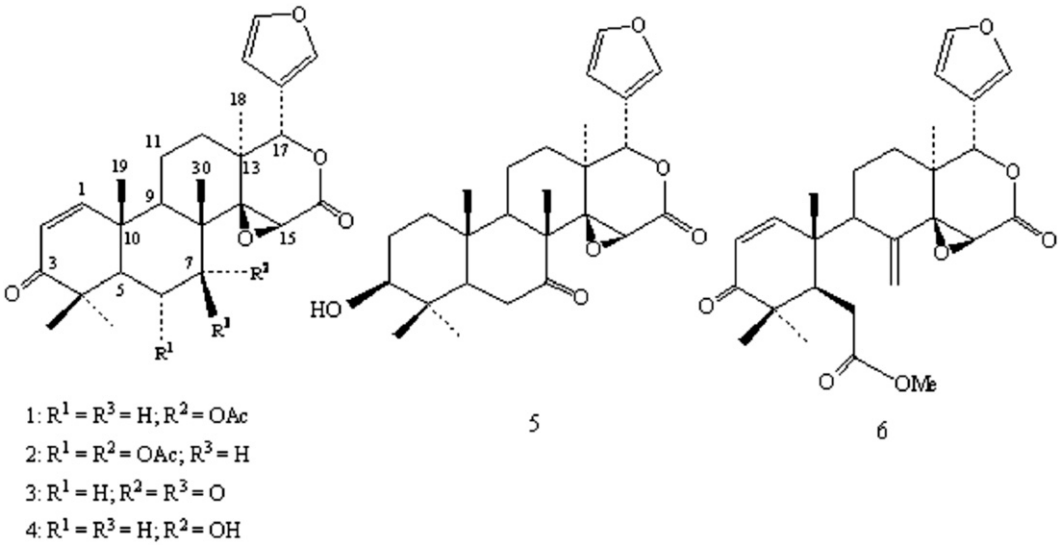

Fig. 1. Limonoids isolated from andiroba oil.

Table 2
Inhibition percentages and IC_{50} values of the andiroba oil and limonoid-rich fraction against the Dd_2 clone.

Samples	Concentration (μg/mL)	Dd_2 strain					
		% Inhibition			IC_{50} (μg/mL)		
		24 h	48 h	72 h	24 h	48 h	72 h
AO	8.2	31	71	88	>82	9.4	8.4
LRF	3.1	56	64	82	2.8	2.4	0.4
Chloroquine	0.031	10	35	60	>1	0.1	0.01

AO=andiroba oil; LRF=limonoids-rich fraction.

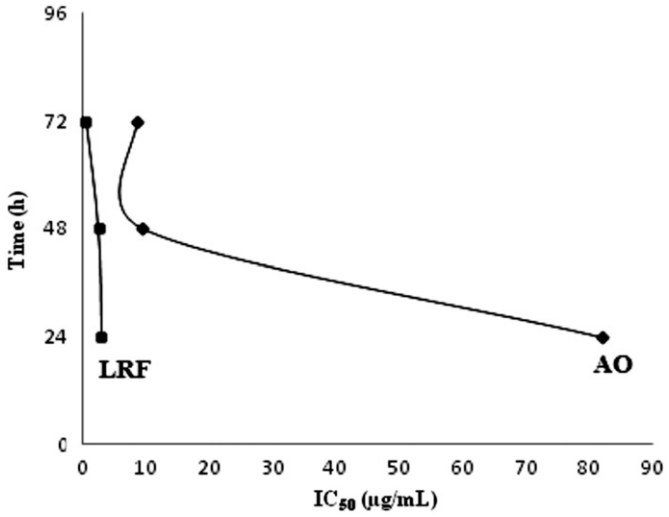

Fig. 2. Concentration-response curve for the andiroba oil (AO) and limonoid-rich fraction (LRF) against the Dd_2 clone.

response curve for andiroba oil and limonoid-rich fraction, at the different time-points, was constructed to substantiate the determination of IC_{50} values (see Fig. 2).

3.3. Acute toxicity

The acute toxicity indicated that andiroba oil by oral route of a single dose of 2.0 g/kg did not produce any sign of toxic effect or death in mice during 14 days of observation. In addition, no alteration in their hematological and biochemical parameters was detected, as can be seen in Table 3.

4. Discussion

The results for the andiroba oil and its limonoid-rich fraction are in agreement with those previously reported to Meliaceae species that occur in Africa and Asia, in which the antiplasmodial effects of crude extracts of *Azadirachta indica*, *Carapa odorata*, *Guarea multiflora*, *Khaya grandiflora* and *K. grandifoliola* showed IC_{50} values varying from 1.0 to 10 μg/mL (Khalid et al., 1986; Phillipson and Wright, 1991; Bickii et al., 2000), at same range of activity observed for the W_2 clone in our experiments. The inhibition of parasite growth was 100% for the W_2 clone (resistant to chloroquine and sensitive to mefloquine), using andiroba oil and its limonoid-rich fraction at concentrations of 8.2 μg/mL and 3.1 μg/mL, respectively, in comparison to quinine (standard drug, 0.016 μg/mL). It is likely that the parasite inhibition still be 100% if the concentrations of andiroba oil and limonoid-rich fraction

Table 3
Effects of andiroba oil on hematological and biochemical parameters by oral route in mice.

	Group 1*	Group 2*	Group 3*
Hematological parameters			
Erythrocytes ($\times 10^6$ μL)$^{-1}$	8.73 ± 0.2	8.35 ± 0.1	8.38 ± 0.3
Hemoglobin (g/dL)	13.7 ± 0.2	13.6 ± 0.21	14.1 ± 0.1
Hematocrit (%)	42.6 ± 0.4	42.2 ± 0.7	42.6 ± 0.4
MCV (fL)	50.6 ± 0.8	49.6 ± 0.9	51.9 ± 1.9
MCH (pg)	17.1 ± 0.7	17.1 ± 0.4	17.5 ± 0.5
MCHC (g/dL)	31.4 ± 0.3	30.8 ± 0.2	31.2 ± 0.2
Leukocytes ($\times 10^3$ μL)$^{-1}$	7.74 ± 0.2	7.66 ± 0.2	8.04 ± 0.1
Biochemical parameters			
Glicose (mg/dL)	88.2 ± 4.0	87.2 ± 3.68	83.1 ± 2.79
AST (IU/L)	64.4 ± 2.5	64.5 ± 1.8	69.2 ± 2.6
ALT (IU/L)	49.8 ± 2.3	49.6 ± 1.9	51.8 ± 1.7
Creatinine (mg/dL)	0.56 ± 0.02	0.62 ± 0.03	0.58 ± 0.02

Group 1: water (100 μL, control); Group 2: water (100 μL)+tween (1%); Group 3: water (490 μL)+tween (10 μL)+andiroba oil (500 μL). The values are expressed as mean ± S.E.M (n=10 animals/group).

* No significant difference between the parameters by analysis of variance, ANOVA.

are smaller. The same observation could be made to the inhibition of the Dd_2 clone (resistant to chloroquine and mefloquine). Despite the low concentration of chloroquine (0.031 μg/mL, standard drug) its inhibition was only 60%, while the andiroba oil and its limonoid-rich fraction showed higher inhibition values, 88% (IC_{50} 8.4 μg/mL) and 82% (IC_{50} 0.4 μg/mL), respectively, after 72 h. This latter value is comparable to that of other limonoids from Meliaceae, as ceramicine B (IC_{50} 0.23 μg/mL) active against the 3D7 clone and isolated from *Chisocheton ceramicus* occurring in Malaysia (Mohamad et al., 2009) and as trichirubine A (IC_{50} 0.3 μg/mL) active against the FcB1 clone and identified in *Trichilia rubescens* existing in Uganda, Africa (Krief et al., 2003). Moreover, among the limonoids, gedunin (1) has been regarded as one of the most active compounds tested in *in vitro* antimalarial assays, whose IC_{50} values (0.7 to 1.25 μg/mL) inhibit the W2 and KI clones, resistant to chloroquine (Khalid et al., 1989; MacKinnon et al., 1997; Bickii et al., 2000). The limonoid derivatives 7-deacetyl-7-oxogedunin (3) and 7-deacetylgedunin (4), previously isolated from the roots of *Pseudocedrela kotschyi* and occurring in Mali, have been reported to display moderate antimalarial activity (IC_{50}=1.36 and 1.77 μg/mL, respectively) (Hay et al., 2007), which should also contribute to the antiplasmodial activity observed in andiroba oil and limonoid-rich fraction.

Some criteria have been used in establishing the *in vivo* antiplasmodial activity of crude extract and compounds. Basco et al. (1994) adopted the following criteria: IC_{50} <10 μg/mL, good activity; IC_{50} of 10–50 μg/mL, moderate activity; IC_{50} of 50–100 μg/mL, low activity; and IC_{50} >100 μg/mL, inactive. Batista et al. (2009) have used combined criteria: IC_{50} <1 μM, excellent/potent activity; IC_{50} of 1–20 μM, good activity; IC_{50} of 20–100 μM, moderate activity; IC_{50} of 100–200 μM, low activity; and IC_{50} >200 μM, inactive. In view of our results and the criteria presently adopted, we can consider the andiroba oil and its limonoid-rich fraction in the range from good to excellent antiplasmodial activity.

According to the experiments, it can be suggested that acute toxicity of andiroba oil in mice is practically null by oral route. At the dose used is possible to estimate a LD_{50} >2.0 g/kg. In a previous study the dose of 5.0 mg/kg was administered to rats and not produced toxicity or deaths of the animals (Costa-Silva et al., 2008). Therefore, in toxicity bioassays using mice and rats could be accepted doses greater than 2.0 g/kg and 5.0 g/kg, respectively. At the same time, this result precludes the possibility of which clones W_2 and Dd_2 were eliminated by the toxicity of

oil. This fact highlights the significant antiplasmodial action of andiroba oil.

The different responses of the clones of *P. falciparum* to the *in vitro* antimalarial assays can be explained by their genetics, since the chloroquine-resistant clones (as W2 and Dd2 strains) may have their genes modified by the action of the drug and thus altering their sensitivity to chloroquine (Viana et al., 2006).

5. Conclusion

The andiroba oil and its limonoid-rich fraction, both derived from *Carapa guianensis*, a Meliaceae species with occurrence in the Brazilian Amazon, showed a significant antiplasmodial activity against the clones W_2 and Dd_2 of *Plasmodium falciparum*. For gedunin (1), 6α-acetoxygedunin (2), 7-deacetoxy-7-oxogedunin (3), 7-deacetylgedunin (4), 1,2-dihydro-3β-hydroxy-7-deacetoxy-7-oxogedunin (5) and andirobin (6), the limonoids that were identified from the non-saponifiable part of the andiroba oil, we are attributing this anti-plasmodial activity.

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