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Anti-malarial activity and toxicity of *Aspidosperma nitidum* Benth: a plant used in traditional medicine in the Brazilian Amazon

Atividade antimalárica e toxicidade de *Aspidosperma nitidum* Benth: uma planta usada na medicina tradicional na Amazônia brasileira

Actividad antipalúdica y toxicidad de Aspidosperma nitidum Benth: una planta utilizada en la medicina tradicional en la Amazonía brasileña

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Abstract

The objective of this work was to evaluate the antiplasmodial activity and toxicity of the extract and fractions obtained from the bark of *Aspidosperma nitidum*. The ethanol extract obtained from the powdered bark of *plants was acid-base partitioned* and phytochemically analyzed. The antiplasmodial activity, *in vivo* antimalarial activity and *in vitro* cytotoxicity were acessed.

The selectivity index (SI) was calculated. The acute oral toxicity and pathological effects, of the ethanol extract was evaluated in mice. The major constituent of the ethanol extract was suggestive of a β-carboline chromophore. The alkaloid and neutral fractions contained compounds with an aspidospermine core as the major constituent. The ethanol extract (IC_{50} = 3.60 µg/mL), neutral fraction (IC_{50} = 3.34 µg/mL) and alkaloid fraction (IC_{50}= 2.32 µg/mL) showed high activity against *P. falciparum* (W2 strain). The ethanol extract and the alkaloid fraction reduced 80% of the parasitemia of *P. berghei* (ANKA)-infected mice (dose of 500 mg/kg) in the 5th day, which was not sustainable at the 8th day. A similar result was obtained for chloroquine. The ethanol extract (CC_{50} = 410.65 µg/mL; SI = 114.07), neutral fraction (CC_{50} = 452.53 µg/mL; SI = 135.49), and alkaloid fraction (CC_{50} =346.73 µg/mL; SI 149.45) demonstrated low cytotoxicity and high SI. The ethanol extract (5000 mg/kg; gavage) presented low acute oral toxicity, with no clinical or anatomopathological modifications being observed (in comparison to the control group). *In vitro* studies with a chloroquine-resistant clone of *P. falciparum* confirmed the antiplasmodial activity of the *A. nitidum* ethanol extract, and its fractions had low cytotoxicity for HepG2 cells. *In vivo* studies with *P. berghei*–infected mice and acute toxicity studies corroborated these results.

Keywords: Apocynaceae; *Aspidosperma nitidum*; Malaria; Toxicity.

Resumo

O objetivo deste trabalho foi avaliar a atividade antiplasmódica e a toxicidade do extrato e frações obtidas da casca de *Aspidosperma nitidum*. O extrato etanólico obtido dos pós da casca da planta e foi particionado ácido-base e analisado fitoquimicamente. Foram avaliadas a atividade antiplasmódica, atividade antimalárica *in vivo* e citotoxicidade *in vitro*. O índice de seletividade (SI) foi calculado. A toxicidade oral aguda e os efeitos patológicos do extrato etanólico foram avaliados em camundongos. O principal constituinte do extrato etanólico foi sugestivo de um cromóforo β-carbolina. As frações alcalóide e neutra continham compostos com um núcleo de aspidospermina como principal constituinte. O extrato etanólico (IC50 = 3,60 µg / mL), fração neutra (IC50 = 3,34 µg / mL) e a fração alcalóide (IC50 = 2,32 µg / mL) apresentaram alta atividade contra *P. falciparum* (cepa W2). O extrato etanólico e a fração alcalóide reduziram em 80% a parasitemia de camundongos infectados com *P. berghei* (ANKA) (dose de 500 mg / kg) no 5º dia, o que não foi sustentável no 8º dia. Um resultado semelhante foi obtido para a cloroquina. O extrato de etanol (CC50 = 410,65 µg / mL; SI = 114,07), fração neutra (CC50 = 452,53 µg / mL; SI = 135,49) e fração alcalóide (CC50 = 346,73 µg / mL; SI 149,45) demonstrou baixa citotoxicidade e alta SI. O extrato etanólico (5000 mg /

kg; gavagem) apresentou baixa toxicidade oral aguda, não sendo observadas modificações clínicas ou anatomopatológicas (em comparação ao grupo controle). Estudos *in vitro* com um clone de *P. falciparum* resistente à cloroquina confirmaram a atividade antiplasmódica do extrato etanólico de *A. nitidum*, e suas frações apresentaram baixa citotoxicidade para células HepG2. Estudos *in vivo* com camundongos infectados por P. berghei e estudos de toxicidade aguda corroboraram esses resultados.

Palavras-chave: Apocynaceae; *Aspidosperma nitidum*; Malaria; Toxicidade.

Resumen

El objetivo de este trabajo fue evaluar la actividad antiplasmodial y la toxicidad del extracto y fracciones obtenidas de la corteza de *Aspidosperma nitidum*. El extracto de etanol obtenido de la corteza en polvo de plantas se repartió ácido-base y se analizó fitoquímicamente. Se redujeron la actividad antiplasmodial, la actividad antipalúdica in vivo y la citotoxicidad in vitro. Se calculó el índice de selectividad (SI). Se evaluó la toxicidad oral aguda y los efectos patológicos del extracto de etanol en ratones. El componente principal del extracto de etanol sugirió un cromóforo de β-carbolina. Las fracciones alcaloide y neutra contenían compuestos con un núcleo de aspidospermina como constituyente principal. El extracto de etanol (CI50 = 3.60 µg / mL), la fracción neutra (CI50 = 3.34 µg / mL) y la fracción alcaloide (CI50 = 2.32 µg / mL) mostraron alta actividad contra *P. falciparum* (cepa W2). El extracto de etanol y la fracción alcaloide redujeron el 80% de la parasitemia de ratones infectados con *P. berghei* (ANKA) (dosis de 500 mg / kg) en el quinto día, lo que no fue sostenible en el octavo día. Se obtuvo un resultado similar para la cloroquina. El extracto de etanol (CC50 = 410.65 µg / mL; SI = 114.07), fracción neutra (CC50 = 452.53 µg / mL; SI = 135.49) y fracción alcaloide (CC50 = 346.73 µg / mL; SI 149.45) demostraron citotoxicidad baja y alta SI. El extracto de etanol (5000 mg / kg; sonda) presentó baja toxicidad oral aguda, sin observarse modificaciones clínicas ni anatomopatológicas (en comparación con el grupo control). Los estudios in vitro con un clon de *P. falciparum* resistente a la cloroquina confirmaron la actividad antiplasmodial del extracto etanólico de A. nitidum, y sus fracciones tenían baja citotoxicidad para las células HepG2. Los estudios in vivo con ratones infectados por *P. berghei* y los estudios de toxicidad aguda corroboraron estos resultados.

Palabras clave: Apocynaceae; *Aspidosperma nitidum*; Malaria; Toxicidad.

1. Introduction

Malaria is one of the world's leading causes of mortality, with approximately 405 000 deaths annually. The highest number of cases (94%) occurred in Africa. Every year, 292 000 African children die of malaria. However, there was an impressive 48% decrease in global mortality between 2000 and 2015 (WHO, 2019).

Parasite resistance to antimalarials remains an ever-present obstacle to the elimination of malaria (Wicht, et al., 2020). The extensive use of anti-malarial drugs imposes selective pressure on the parasites. *Plasmodium falciparum* resistance has been extensively described (White, 2004).

Because of this growing drug resistance, new therapeutic alternatives are necessary. Traditional medicines can contribute to the search. In this regard, some plant species have been proven to be active against the chloroquine-resistant *P. falciparum* clone W2, such as, plants of the genus *Aspidosperma*, Apocynaceae (Dolabela, et al., 2012).

Indeed, the ethanol extract obtained from *A. olivaceum* bark has also been shown to be active against W2 clones (Chierrito, et al., 2014).Moreover, alkaloid extracts and alkaloid-rich fractions from *A. olivaceum*, *A. ramiflorum*, *A. spruceanum*, and *A. parvifolium* have all shown activity against both chloroquine-resistant and chloroquine-sensitive clones of *P. falciparum* (IC_{50} < 10 μg/mL). The antiplasmodial activities of these extracts and fractions are related to the alkaloids they contain (Dolabela, et al., 2012).

Likewise, the hydroethanolic extract from *A. excelsum* bark exhibits antiplasmodial activity (24 h, IC_{50} = 5.2 μg/mL) against the W2 clone. However, the alkaloid fraction obtained from this extract showed lower activity (24 h, IC_{50} = 37.2 μg/mL; Gomes, 2011).

The present study evaluated the antimalarial activity of the ethanol extract and fractions from the bark of *Aspidosperma nitidum*. We also describe the results of the phytochemical study, investigations into HepG2 cytotoxicity, and acute oral toxicity in Swiss mice.

2. Material and Methods

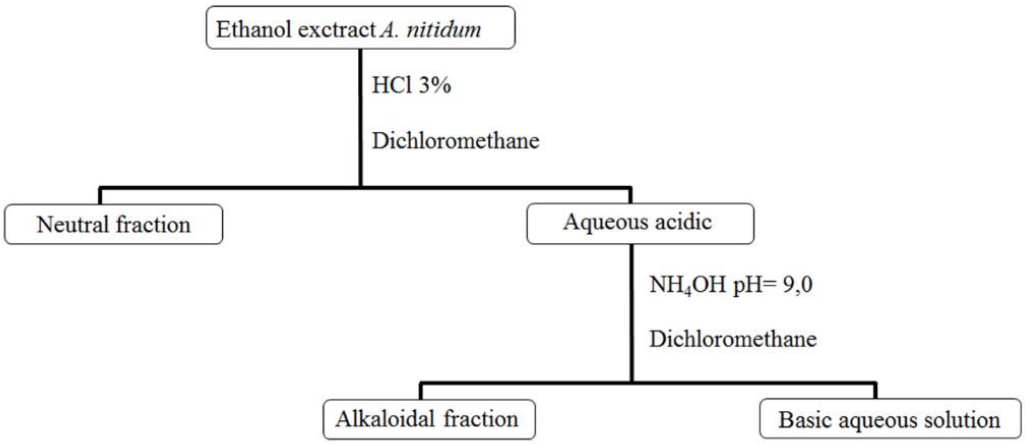
This work used the experimental scientific model of comparison with positive and negative controls of biological activities *in vivo* and *in vitro* (Pereira, et al., 2018).

2.1 Plant material and phytochemical studies

Stem-bark samples were collected from *A. nitidum* growing beside Highway PA-150 State (S 02° 09' 5 0.3" and W 048° 47' 56.9") in the Brazilian Amazon. The dried specimen was deposited in the Museu Paraense Emilio Goeldi, Belém, Pará, Brazil (voucher specimen: MG-206619).

The bark was washed, dried in an air-vented kiln, and triturated in a knife mill. The resulting powder (500 g) was macerated with ethanol (yield 10.33 g; 2.06%). The solution was concentrated in a rotary evaporator to yield the ethanol extract. Part of the extract (2.0 g) was solubilized with hydrochloric acid (3% v/v) and subsequently dichloromethane partition. The dichloromethane solution was concentrated in a rotary evaporator (neutral fraction = 0.475 g). The aqueous acid solution was alkalized to pH 9 with ammonium hydroxide to yield the dichloromethane fraction. This solution was concentrated in the rotary evaporator, yielding the alkaloid fraction (0.395 g; Figure 1; Henriques, et al., 2010).

Figure 1. Acid–base fractionation of the crude extract from the stem bark of *Aspidosperma nitidum*



Source: Authors

The ethanol extract and fractions were analyzed with high-performance liquid chromatography coupled with a diode array detector (Waters e2695 and Waters 2998). The analysis used column RP 18 (particles of 5 μm, 45 × 250 mm) and, as the mobile phase, a mixture of water acidified with 0.01% trifluoroacetic acid (eluent A) and acetonitrile acidified with 0.01% trifluoroacetic acid (eluent B). A linear gradient was created using 70-30% eluent B for 15 minutes, 60-40% eluent B for 20 minutes and 50-100% eluent B for 25 minutes. The

oven temperature was maintained at 26°C, flow was kept at 1 mL per minute, and wavelengths from 220 to 400 nm were scanned in a methodology adapted from Coutinho, et al. (2013).

2.2 Cytotoxicity assay

Cell viability was determined by the MTT (3- (4.5-dimethyltrazol-2-yl)-2,5-diphenyltetrazolium bromide) method according to Mosman (1983). HepG2 A16 cells (4×10^5 cells per 0.1 mL) were grown in RPMI medium (Roswell Park Memorial Institute 1640 medium; Sigma Aldrich, USA) supplemented with 5% fetal calf serum and kept under a 5% CO_2 atmosphere (37°C).

After 24 hours, samples of extract or fraction were added at different concentrations (1, 10, 100, or 1000 µg/mL), followed by 24 hours of further incubation. Then, MTT (2.0 mg/mL) was added, followed by incubation for 4 hours. Dimethyl sulfoxide (99.7 %,100 µL) was added to each well, and plaques were mixed to solubilize formazan crystals. The optical density was determined at 570 nm (Stat Fax 2100 microplate reader, Awareness Technology, USA).

Cell viability was expressed as the percentage of the absorbance in the untreated control cells. The cytotoxic concentration (CC_{50}) was determined by linear regression.

2.3 *In vitro* anti-plasmodial activity

Plasmodium falciparum (Indochina - W2) cultivated according to Trager & Jensen (1976) was synchronized with 5% sorbitol (Lambros & Vanderberg, 1979). The activity of the *A. nitidum* extract and fractions was evaluated against chloroquine (CQ)-resistant *P. falciparum* blood parasites by *in vitro* testing with monoclonal anti-histidine-rich protein (HRPII) antibody (Noedl, et al., 2002). This test used the trophozoite stage (parasitemia, 2%; hematocrit, 1%) and different concentrations of test samples (50.00, 25.00, 12.50, 6.25, 3.125 and 1.56 µg/mL). Normal noninfected red blood cells (RBCs) were used as a negative control. Parasitized, untreated RBCs were the positive control. Chloroquine was used as the standard anti-malarial drug.

Serial dilutions of test samples and chloroquine (20 µL per well; 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/mL), plus 180 µL per well of parasite culture, were distributed in standard 96-well plates. All plates were incubated in a CO_2 chamber (37°C) for 48 hours. Subsequently, plates were freeze-thawed twice to obtain complete hemolysis of the RBCs. After incubation,

plates were processed immediately or frozen at –20°C until further processing (Noedl, et al., 2002).

One hundred microliters of the antibody MPFM-55A (1.0 µg/mL in PBS) was added to each well, followed by overnight incubation at 4°C. The supernatant was discarded. Blocking solution (BSA 2% in PBS) was added (200 µL per well), and plaques were incubated at room temperature for 2 hours. The plates were washed three times with 0.5% PBS/Tween solution (200 µL per well). Thereafter, 100 µL of the hemolyzed culture was added to samples and controls (background; RBCs parasitized and frozen at time 0 h). Plaques were incubated for 1 hour at room temperature in a humidity chamber. Subsequently, plaques were washed three times with 0.5% PBS/Tween (200 µL per well; Noedl, et al., 2002).

MPFG-55P antibody conjugated with HRP (0.05 µg/mL in 2% BSA + 1% Tween in PBS) was added (100 µL per well) and incubated at room temperature for 1 hour in a humidity chamber. Subsequently, plaques were washed three times with 0.5% PBS/Tween solution (200 µL per well; Noedl, et al., 2002).

The chromogen *o*-phenylenediamine (OPD) was added to each well (100 µL). Plaques were incubated in the dark for an additional 10 minutes, and 50 µL of 1 M sulfuric acid was added. Spectrophotometric analysis was performed using an ELISA plaque reader (ELISA Stat Fax, mod. 2100) at 492 nm (Noedl, et al., 2002).

The following antiplasmodial activity classification criteria were adopted in accordance with the IC_{50} range: IC_{50} < 10.0 µg/mL, active; IC_{50} between 10 and 50 µg/mL, moderately active; and IC_{50} > 50 µg/mL, inactive (Dolabela, et al., 2008).

The selectivity index (SI) for the antiplasmodial activity was calculated based on the ratio between CC_{50} and IC_{50} for the *in vitro* activity against *P. falciparum*. Compounds with SI > 10 were considered nontoxic (Dolabela, et al., 2012).

2.4 *In vivo* antimalarial activity

The parasites (*P. berghei* ANKA) were maintained through weekly blood passages in mice intraperitoneally (i.p.), using 3.8% sodium citrate as an anticoagulant. The traditional suppressive test of Peters (1965, 1967), as modified by Carvalho, et al. (1991), was used. Briefly, adult Swiss albino mice (*Mus musculus*; 18–22 g; n= 24; 4 animals per group obtained from the Animal Facility of the Evandro Chagas Institute-IEC; Belém, Pará State, Brazil) were inoculated (i.p.) with 10^5 *P. berghei*–infected RBCs. Mice were divided into groups of four per cage and orally treated for three consecutive days with daily doses of the extracts. Two control

groups were used in each experiment, one treated with chloroquine (30 mg/kg, orally) and the other kept untreated. After 5 and 8 days of parasite inoculation, blood smears were prepared from the tail tip of all mice, fixed with methanol, stained with Giemsa, and then microscopically examined (×1000).

Parasitemia was determined from blood smears using coded and counted randomized double-blind trials, counting 10 000 erythrocytes in cases of 0% parasitemia, 5000 erythrocytes for up to 5% parasitemia, 2000 erythrocytes for 5-10% parasitemia, 1000 erythrocytes for 11-20% parasitemia, and 300 erythrocytes for parasitemia higher than 20%. Overall mortality was monitored daily in all groups for a period of 4 weeks following inoculation. The extract and alkaloid fraction were tested in three independent experiments at daily doses of 500, 250 and 125 mg/kg body weight. Samples were considered partially active when there was a reduction of parasitemia greater than 30% (Carvalho, et al., 1991).

2.5 Acute oral toxicity

Male (n = 8) and female (n = 8, nulliparous and non-pregnant) albino Swiss mice weighing 20 ± 2 g were obtained from the Animal Facility of the Evandro Chagas Institute (IEC; Belém, Pará State, Brazil). Mice were kept in the vivarium of the Faculdade de Farmácia of the Universidade Federal do Pará (UFPA) under controlled temperature and humidity, light/dark cycle of 12 hours each, with unlimited pelleted food and filtered tap water. Animals were weighed daily, and food and water intake were recorded. At the end of the experiment, animals were anesthetized and euthanized, blood samples were drawn, and target organs were removed for anatomopathological and histopathological evaluation (brain, cerebellum, stomach, intestine, mesentery, heart, lungs, liver, spleen, pancreas and kidney; OECD, 2001).

Acute oral toxicity was measured using the Fixed Procedure Test, according to NCCLS guidelines from OECD (2001). Animals were treated with a single dose (5000 mg/kg; gavage) and evaluated with the Hippocratic test after 14 test days.

Signs of toxicity were recorded, as well as its decline and duration. Changes in the conscious state and disposition, activity, coordination, motor system, reflexes, and central nervous system and peripheral nervous system activity of the mice were evaluated (Malone & Robichaud, 1983).

On the 14th day after exposure, animals were euthanized and their brain, cerebellum, stomach, intestine, mesentery, heart, lungs, liver, spleen, pancreas and kidney were removed for anatomopathological and histopathological studies. The histological sections of excised

organs were fixed in buffered formalin (10% formaldehyde solution) and cut for histopathological processing after 24 hours. The process involved samples being dehydrated with a series of increasing alcohol concentrations (70–100%), cleared in xylene, impregnated and embedded in paraffin according to standard methods. In a microtome, tissue fragments were sectioned at a thickness of 3.0 mm and subsequently underwent hematoxylin-eosin and Masson's trichrome staining and were examined under an optical microscope (Bacha & Wood, 1990).

All procedures were carried out in accordance with the ethical principles of animal experimentation, according to the standards of the Brazilian Society of Sciences in laboratory animals and of Brazilian's National Council of Animal Experimentation Control (CONCEA). The experimental protocol was approved by the Ethics in Animal Research Committee of the Universidade Federal do Pará (report CEPAE no. BIO063-12).

2.6 Statistical analysis

Linear regression in dose-response curves was used for the cytotoxicity and antiplasmodial assays, the data regression on sigmoidal dose-response curves. Student's t-test for non-paired data was used to evaluate acute oral toxicity, with a significance level set at $p < 0.05$.

3. Results and Discussion

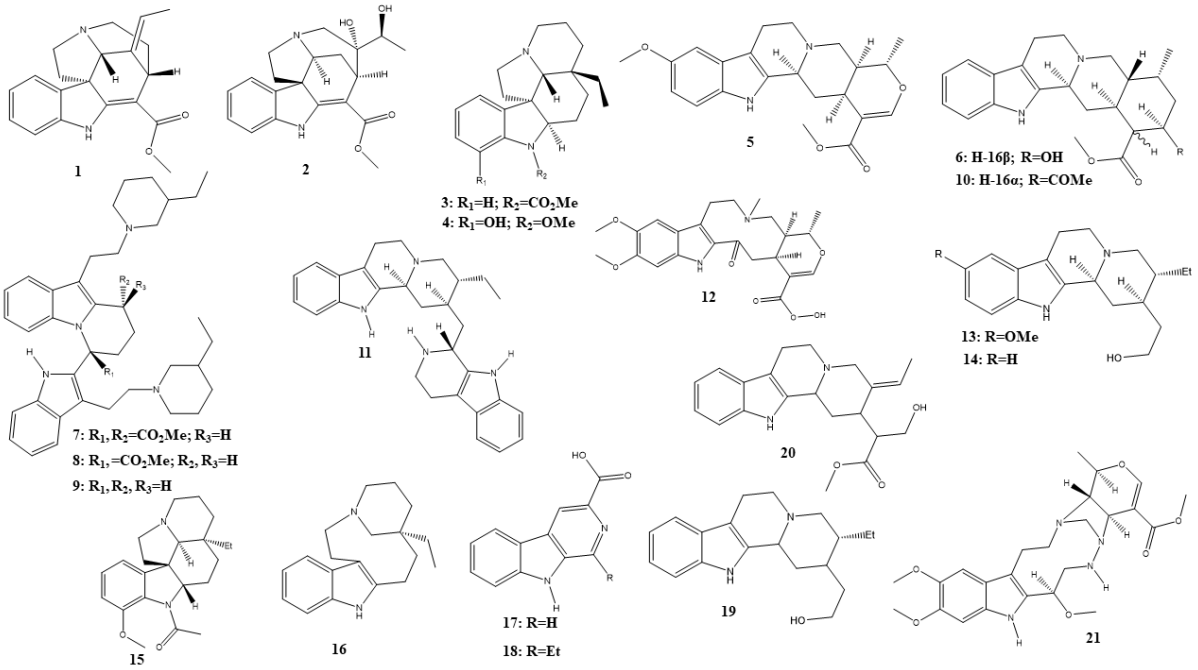
3.1 Phytochemical analysis

The following alkaloids have been isolated from *A. excelsum*: 11-methoxytubotaiwin (Figure 2-1), compactinervine (Figure 2-2), N-acetyl aspidospermidine (Figure 2-3), O-desmethyl-aspidospermidine (Figure 2-4), aricine (Figure 2-5), yohimbine (Figure 2-6), tetrahydrosecamine (Figure 2-7), 16-desmethoxy-carboxyl-tetrahydrosecamina (Figure 2-8), didesmethoxy-carboxyl-tetrahydrosecamine (Figure 2-9), O-acetyl yohimbine (Figure 2-10), ocrylfuanine (Figure 2-11; Verpoorte, et al., 1983), and excelsinine (Figure 2-12; Marques, 1988).

Phytochemical studies of *A. nitidum* have isolated 10-methoxy-dihydro-corynantheol (Figure 2-13), corynantheol (Figure 2-14; Arndt, et al., 1967), aspidospermine (Figure 2-15), quebrachamine (Figure 2-16), yohimbine (Figure 2-6; Marques, et al., 1996), carboxylic

harman acid (Figure 2-17), 3-carboxylic ethylharman (Figure 2-18; Pereira, et al., 2007), dihydrocorynantheol (Figure 2-19), dehydrositsiriquine (Figure 2-20; Nascimento, et al., 2006) and braznitidumine (Figure 2-21; Nascimento & Silveira, 2006).

Figure 2. Chemical structure of compounds occurring in *Aspidosperma nitidum*.



11-methoxytubotaiwin (1), compactinervine (2), N-acetyl aspidospermidine (3), O-desmethyl aspidospermidine (4), aricine (5), yohimbine (6), tetrahydrosecamine (7), 16-desmethoxy-carboxyl-tetrahydrosecamina (8), didesmethoxy-carboxyl tetrahydrosecamine (9), O-acetyl yohimbine (10), ocrylfuanine (11), excelsinine (12), 10-methoxy-dihydro-corynantheol (13), corynantheol (14), aspidospermine (15), quebrachamine (16), carboxylic harman acid (17), 3-carboxylic ethylharman (18), dihydrocorynantheol (19), dehydrositsiriquine (20), braznitidumine (21). Source: Authors.

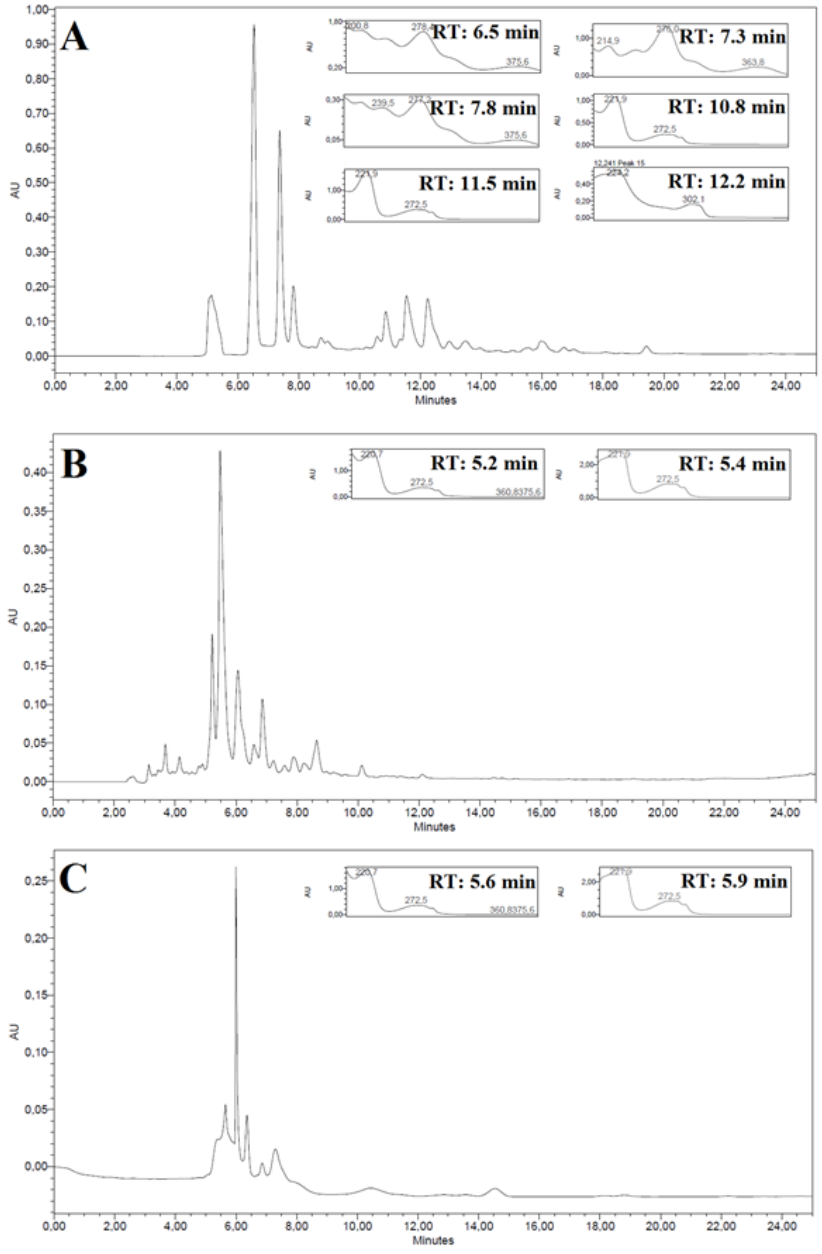
The chromatogram of the ethanol extract of *A. nitidum* showed peaks of substances of high, medium and low polarity. The major peaks at 6.5 minutes (λ_{max} 200.8; 278.4 and 375.6 nm), 7.3 minutes (λ_{max} 214.9; 276.0 and 363.8 nm) and 7.8 minutes (λ_{max} 239.5; 277.2 and 375.6 nm) were likely harman β-carboline alkaloids (Pena et al., 1986). The peaks with retention times of 10.8 minutes (λ_{max} 221.8 and 272.5 nm), 11.5 minutes (λ_{max} 221.9 and 272.5 nm) and 12.2 minutes (λ_{max} 224.2 and 302.1 nm) were likely indole alkaloid chromophores (Figure 3 A; Rapaport, et al., 1960).

The chromatogram of the alkaloid fraction showed major peaks at 5.2 and 5.4 minutes (λ_{max} 220.7 and 272.5 nm). The neutral fraction showed major peaks with retention times of 5.6 and 5.9 minutes for the same chromophores (λ_{max} 220.7 and 272.5 nm) suggestive of indolic alkaloid with an aspidospermine core, which is the aspidospermine described for this species (Figure 3 B and C; Pereira, et al., 2007).

Acid–basic partitioning was not efficient for the extraction of the major alkaloid of the extract, as peaks corresponding to chromophores of β-carboline alkaloids were not found in the alkaloid and neutral fractions.

Harman alkaloids, when in contact with hydrochloric acid, tend to become their phenol-harmol pairs. Phenol-harmol alkaloids can be precipitated and retained in aqueous alkaline solution (Perkin & Robinson, 1919). Thus, only indole alkaloids with an aspidospermine core are seen in the chromatograms of alkaloid and neutral fractions.

Figure 3. Chromatograms profiles.



A: ethanol extract of *A. nitidum;* B: alkaloid fraction; C: fraction of neutral compounds. Source: Authors

3.2 Antiplasmodial activity and cytotoxicity

The ethanol extract of *A. nitidum* was shown to be active against the W2 clone of *P. falciparum* (IC_{50} = 3.6 µg/mL; Table 1). Another study evaluated the antiplasmodial activity of the ethanol extract of *A. nitidum* using the [^{3}H]-hypoxanthine uptake method and obtained a CI_{50} of 21.93 µg/mL (moderate activity). In that study, the fractioning of the extract led to the isolation of the following alkaloids: harman carboxylic acid, 3α,20β-10methoxy-18,19-dihydrocorynantheol and braznitidumine (Pereira, 2005). The difference between the results of that previous study and the present study can be related to differences in methodologies used in the evaluation of antiplasmodial activity. Moreover, the fact that plants were collected in different locations for each study must be taken into account. Pereira (2005) collected plants from Southeast Brazil. The present study used plants collected from the Brazilian Amazon (Pará State). Although both samples possessed alkaloids with the same chromophore, there may be differences in their concentrations. Differences in climate, rainfall, altitude, ultraviolet radiation exposure, and collection location, among other factors, can significantly alter the composition and concentration of secondary metabolites (Gobbo-Neto & Lopes, 2007).

The neutral fraction showed high activity against the W2 *P. falciparum* clone (IC_{50} = 3.34 µg/mL; Table 1). The chromatogram of the neutral fraction showed major peaks at 5.6 and 5.9 minutes. The UV spectrum was suggestive of the indole alkaloid. Similar spectra were found in the alkaloid fraction, although there were differences in retention times. These different retention times suggest that the chromophores have different substituents, which would explain the differences in biological activity. Nevertheless, it was expected that the neutral fraction would not contain alkaloids. This was not observed, indicating incomplete separation during the extraction.

The alkaloid fraction showed high activity against *P. falciparum* (IC_{50} = 2.32 µg/mL; Table 1). In another study, the alkaloid fraction of the hydroethanolic extract of *A. excelsum* was moderately active against clone W2 (Gomes, 2011). These different responses may be due to different major alkaloids. In the study conducted by Gomes (2011), most of the alkaloid fraction and alkaloid extract was yohimbine. In the present study, the major alkaloid was also of indole origin but with a different maximum ultraviolet absorption, probably reflecting an alkaloid with an aspidospermine core. Aspidospermine has already been shown to be active against chloroquine-resistant *P. falciparum* strains (IC_{50} = 5.6 µg/mL; Mirtaine-Offer, et al., 2002).

The ethanol extract and its neutral fraction have similar antiplasmodial activities (Table 1). Fractionation can result in alkaloids with high activity. The antiplasmodial activity of alkaloids has been widely described, and in many cases, the IC_{50} lies on the order of nanograms or micrograms. Examples of indole alkaloids active against *P. falciparum* are ellipticine (IC_{50} = 0.073 µg/mL), aspidoscarpine (IC_{50} = 0.007 µg/mL), aspidospermine (IC_{50} = 5.6 µg/mL), 10-methoxy-aspidospermidine (IC_{50} = 3.2 µg/mL), N-formyl-aspidospermidine (IC_{50} = 5.6 µg/mL), palosine (IC_{50} = 12.7 µg/mL), demethyl-aspidospermine (IC_{50} = 15.1 µg/mL), demethoxy-aspidospermine (IC_{50} = 7.4 µg/mL) and uleine (IC_{50} = 2.8 µg/mL; Dolabela 2007). The greater activity in the fraction of alkaloids is due to higher concentrations of alkaloids in this fraction, as can be observed in Figure 3.

The ethanol extract of *A. nitidum* and its fractions showed low cytotoxicity for HepG2 cells (Table 1). When the 50% cytotoxic concentration corresponds to the 50% inhibitory concentration, the compound is highly selective (SI > 114; Table 1).

One factor contributing to the toxicity or the antiplasmodial activity of alkaloids is the presence of the tetrahydrofuran ring. The ring decreases the antimalarial activity and contributes to cytotoxicity. The presence of aspidospermane core-derived alkaloids increases antiplasmodial activity and decreases toxicity (Mirtaine-Offer, et al., 2002). Thus, low cytotoxicity in *A. nitidum* may be related to the absence of alkaloids with the tetrahydrofuran ring. However, the presence of aspidospermane derivatives explains the high antiplasmodial activity displayed.

Table 1. Antiplasmodial activity and cytotoxicity of the ethanol extract and fractions of *Aspidosperma nitidum.*

Sample	Antiplasmodial activity IC_{50}±SD (µg/mL)	Cytotoxicity CC_{50}±SD (µg/mL)	SI
EEAN	3.60 ± 0.37	410.65 ± 9.84	114.07
FAAN	2.32 ± 0.19	346.73 ± 14.17	149.45
FNAN	3.34 ± 0.31	452.53 ± 21.68	135.49
Chloroquine	0.08 ± 0.02	247.23 ± 4.7	3169.61

EEAN: ethanol extract of *Aspidosperma nitidum*; FAAN: alkaloid fraction of *A. nitidum*; FNAN: neutral fraction of *A. nitidum*; Values presented as the mean ± standard deviation; IC_{50}: inhibitory concentration to *P. falciparum* W2 stain; CC_{50}: cytotoxicity in the HepG2 cell line; SI: selectivity index. Source: Authors.

3.3 Acute oral toxicity and *in vivo* anti-malarial activity

Doses of 125, 250 and 500 mg/kg of the extract and fractions were used to assess anti-malarial activity. The ethanol extract and the alkaloid fraction were active in all doses tested at the 5th day of analysis. The anti-malarial activities for both samples were similar to the chloroquine standard (30 mg/kg). The extract and its fraction did not interfere with parasitemia on the 8th day. These results suggest that the anti-malarial response is not sustained (Table 2).

Table 2. Reduction of the parasitemia of *Plasmodium berghei*-infected mice treated orally.

Sample	**Dose (mg/Kg)**	**5° day**			**8°day**	
		Parasitaemia Media±SD	**Reduction of parasitaemia Media±SD %**	***p***	**Parasitaemia Media±SD**	**Reduction of parasitaemia %**
EEAN	500	0.12±0.05	86.11±5.55	0.18*	4.16±4.61	0
	250	0.65±0.26	75.48±11.79	0.27*	5.85±0.49	0
	125	0.2±0.14	77.77±15.71	0.76*	3.45±0.21	0
FAAN	500	0.17±0.09	80.55±10.64	0.99*	4.40±1.84	0
	250	0.52±0.45	66.66±11.11	0.17*	7.47±1.33	0
	125	0.35±0.12	61.1±12.42	0.13*	4.40±0.56	0
Chloroquine	30	0.17±0.05	80.54±5.55	-	0.53±0.11	84.31±3.39
Negative control	-	0.9±0.25	-	-	3.4±1.08	-

EEAN: ethanol extract of *Aspidosperma nitidum*. FAAE = alkaloid fraction of *A. nitidum*. Average parasitemia of animals (4± mice/group). The reduction in parasitemia was calculated from the average parasitemia of the negative control group. *Statistically no significant difference (p value >0.05, Teste Student's t test) compared to the corresponding activity of chloroquine. Source: Authors.

To minimize the number of animals in the experiment, the neutral fraction was not tested. This fraction presented a profile alike the alkaloid fraction. Similar to the *in vitro* test, fractioning of the extract did not increase anti-malarial activity. The *in vivo* study showed no significant differences in parasitemia ($p > 0.05$, Student's t test) between doses (Table 2), but the *in vitro* study showed a direct concentration-response relation. In another study, mice infected with *P. berghei* and treated with ethanol extract of *A. nitidum* (doses= 250 and 125 mg/kg) showed a similar reduction in parasitemia (Coutinho, et al., 2013).

In the present study and Coutinho et al. (2013), the greatest reduction in parasitemia was observed at the 5th day (Table 2), and the presence of alkaloids was probably responsible for *A. nitidum* activity. Notwithstanding, the alkaloid Manzamine F caused a rapid and unsustained reduction in parasitemia of mice infected with *P. berghei* (Ang, et al., 2000).

Likewise, only the fixed dose of 5000 mg/kg of the ethanol extract was used for the oral acute toxicity test. The animals were divided into two groups (test and control), again minimizing the number of animals used (Brito, 1994).

During the first 4 hours of testing, no signs of toxicity were observed, except that the mice were resistant to handling during the first 15 minutes. There were no animal deaths during the 14 days of the experiment. Based on this, the ethanol extract was classified as toxicity V (2000 mg/kg < LD_{50} < 5000 mg/kg; OECD, 2001).

There were no significant changes in weight, and the consumption of water and feed was similar between groups (Table 3). Moreover, there were no anatomo-histopathological alterations in the organs analyzed (brain, cerebellum, stomach, intestine, mesentery, heart, lungs, liver, spleen, pancreas, and kidney) compared to the control group.

Table 3. Ponderal evolution, food intake and water consumption of female mice treated with EEAN and observed for 14 days.

Group	Change in weight (g)	Food intake (g/day)	Water consumption (mL/day)
Treated with EEAN 5000 mg/Kg	7.34±1.45*	26.7±3.38 *	47.83±5.29*
Control	5.51±1.67	21.14±3.05	46.69±14.02

Legend: EEAN= ethanol extract of *Aspidosperma nitidum*. *Statistically no significant difference (p value >0.05, Student's t test) compared to the control. Source: Authors.

This result confirms the low toxicity of the ethanol extract. The *in vitro* and *in vivo* results suggested low toxicity of the ethanol extract from *A. nitidum*. The acute oral toxicity of

the hydroethanolic extract of *A. excelsum* showed low toxicity in mice (5000 mg/kg). Additionally, changes in weight, feed intake and water were not observed (Gomes, 2011).

4. Conclusion

The ethanol extract and fractions proved highly active against resistant strains of *P. falciparum*. The fractioning of the extract did not significantly increase its antiplasmodial activity. In the *in vivo* anti-malarial activity test, both the extract and the alkaloid fraction reduced parasitemia with values of reduction equal to the control. The cytotoxicity assay showed low cytotoxicity. The correlation of these results to the results of the *in vitro* antiplasmodial tests indicates a high selectivity index for all samples tested, implying that the effect is pharmacological and not toxicological. The acute oral and fixed-dose toxicity tests confirmed the low toxicity of the extract. Thus, *A. nitidum* has high antiplasmodial potential and is worth studying for the development of new drugs for the treatment of this disease.

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