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Antioxidant activity and ultrastructural alterations in *Leishmania amazonensis* promastigotes induced by limonoid-rich fractions from andiroba oil

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ABSTRACT

In the Amazon region, andiroba (*Carapa guianensis*) oil is among the most used products by the local population due to its medicinal properties. Previously, our group described the leishmanicidal activity of three limonoid-rich fractions from andiroba oil. This work aimed to evaluate the antioxidant activity and ultrastructural changes in *Leishmania amazonensis* treated with these limonoid-rich fractions from andiroba oil: 6α,11β-diacetoxygedunin; 11β-hydroxygedunin; and a fraction with both 6α,11β-diacetoxygedunin +11β-hydroxygedunin. The antioxidant activity was evaluated by the DPPH method and ultrastructural changes were evaluated by transmission electron microscopy. The fraction with both 6α,11β-diacetoxygedunin + 11β-hydroxygedunin displayed the best antioxidant activity (IC_{50} 330.35 ± 8.60 μg mL^{-1}; p=0.0022) compared to the 6α,11β-diacetoxygedunin fraction. The three fractions induced structural changes to mitochondria and kinetoplasts of *L. amazonensis*, in addition to lipid bodies, vacuolization, and vesicles in the flagellar pocket, indicating that the limonoid-rich fractions from andiroba oil can induce structural damage to *Leishmania*.

KEYWORDS: leishmaniasis, crabwood, DPPH, transmission electron microscopy, *Carapa guianensis*

Atividade antioxidante e alterações ultraestruturais em *Leishmania amazonensis* induzidas por frações ricas em limonóides do óleo de andiroba

RESUMO

Na região Amazônica, o óleo de andiroba (*Carapa guianensis*) é um dos produtos mais utilizados pela população local devido às suas propriedades medicinais. Anteriormente, nosso grupo descreveu a atividade leishmanicida de três frações do óleo de andiroba ricas em limonóides. Este trabalho objetivou avaliar a atividade antioxidante e alterações ultraestruturais em *Leishmania amazonensis* tratadas com essas frações ricas em limonóides do óleo de andiroba: 6α,11β-diacetoxigedunina; 11β-hidroxigedunina; e uma fração com ambas 6α,11β-diacetoxigedunina + 11β-hidroxigedunina. A atividade antioxidante foi avaliada pelo método DPPH, e alterações ultraestruturais foram avaliadas por microscopia eletrônica de transmissão. A fração com ambas 6α,11β-diacetoxigedunina + 11β-hidroxigedunina exibiu a melhor atividade antioxidante (IC_{50} = 330,35 ± 8,60 μg mL^{-1}; p = 0.0022) comparado com a fração 6α,11β-diacetoxigedunina. As três frações induziram alterações estruturais nas mitocôndrias e cinetoplasto de *L. amazonensis*, além de corpúsculos lipídicos, vacuolizações e vesículas na bolsa flagelar, indicando que as frações ricas em limonóides do óleo de andiroba podem induzir dano estrutural a *Leishmania*.

PALAVRAS-CHAVE: leishmaniose, andiroba, DPPH, microscopia eletrônica de transmissão, *Carapa guianensis*

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In the Amazon region of Brazil, andiroba (*Carapa* sp.) (Meliaceae) stands out in popular medicine due to the properties of the oil extracted from the seeds (Costa and Marenco 2007). Andiroba oil is well known in traditional communities and used in alternative medicine in northern and northeastern Brazil to treat arthritis, cold, fever, flu, cough, and malaria, to relieve arthritis pain, to treat ear infections, wounds, bruises, insect bites in people and animals, and as an insect repellant (Henriques and Penido 2014). *Carapa* was described as one of the most used genera to treat and prevent leishmaniasis in Amazonian communities (Odonne et al. 2017).

Leishmaniases comprise a group of infectious-parasitic illnesses caused by several protozoa of the genus *Leishmania* that occur in different regions of the world, mainly in tropical and subtropical regions. The disease is endemic in the Amazon region, and cutaneous leishmaniasis is the predominant form of the disease (PAHO 2023).

There are only two studies on the antileishmanial activity of andiroba oil. One study tested an andiroba oil nanoemulsion against *Leishmania amazonensis* Lainson & Shaw, 1972, and *Leishmania infantum* Nicolle, 1908, however they did not describe the chemical compounds involved in the antileishmanial activity (Dhorm Pimentel de Moraes et al. 2018). In the other study, our research group did not observe antileishmanial activity of whole oil from andiroba, *Carapa guianensis* Aublet (IC_{50} > 500 µg mL^{-1}), in contrast with three of its fractions rich in diverse limonoids, which showed promising antileishmanial activity against promastigote and intracellular amastigote forms of *L. amazonensis* (Oliveira et al. 2018). The antileishmanial activity of limonoids was also observed with orthoacetates from *Pseudocedrela kotschyi* (Schweinf.) Harms. roots (Hay et al. 2007) and trigilgianin from *Trichilia gilgiana* Harms. stem bark (Kowa et al. 2020) against *Leishmania donovani* Laveran & Mesnil, 1903, *in vitro*. Although these studies report promising antileishmanial activity of limonoid-rich fractions, there are no reports on the effect of limonoids on ultrastructures of *Leishmania* parasites.

There are a few reports of andiroba oil antioxidant activity (Milhomem-Paixão et al. 2016, Araujo-Lima et al. 2018), but none associated to limonoids. Yet the antioxidant activity of limonoids is already known. For example, the antioxidant activity of limonin occurs through activation of intracellular antioxidative pathways (Yang et al. 2020).

In this study, we evaluated the antioxidant activity and the ultrastructural changes that the treatment with the abovementioned limonoid-rich fractions from *C. guianensis* induce in promastigote forms of *L. amazonensis*.

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Andiroba seed oil was acquired from extractive producers in the Rosário microregion, Maranhão state, Brazil. Botanical identification was carried out at the Botany Laboratory and Herbarium of Universidade Estadual do Maranhão (voucher deposit # 4896). Details about plant acquisition, fractionation and chemical characterization are described elsewhere (Oliveira et al. 2018). We used the three fractions that previously exhibited leishmanicidal activity: fraction with 6α,11β-diacetoxygedunin (LF3); fraction with 11β-hydroxygedunin (LF5); and fraction with both 6α,11β-diacetoxygedunin and 11β-hydroxygedunin (LF4).

The antioxidant activity was evaluated by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA) free radical scavenging method as described by Wang et al. 2022. Concentrations of the fractions in methanol ranging from 1,000 to 7.8125 µg mL^{-1}, and 6-hydroxy-2,5,7,8-tetramethychroman-2- carboxylic (Trolox) standard curve ranging from 12.5 to 0.097 µg mL^{-1}, were obtained by two-folding dilutions in 96-well microplates in triplicate. Trolox is a well know antioxidant used as a standard in several commercial kits and antioxidant activity studies (Xiao et al. 2020; Rumpf et al. 2023). The inhibitory concentration of 50% (IC_{50}) of parasites was calculated by fitting a regression curve of the log concentration on the normalized response using GraphPad Prism 7.00 (GraphPad Software Inc., USA).

Ultrastructural analyses were carried out with *L. amazonensis* (MHOM/BR/76/MA-76) promastigote forms kept in Schneider's Insect medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}) (Sigma-Aldrich, USA) at 26 ºC. The parasites were treated for 72 hours with the IC_{50} of each fraction, 10.53 µg mL^{-1} (6α,11β-diacetoxygedunin), 56.90 µg mL^{-1} (11β-hydroxygedunin fraction), and 25.30 µg mL^{-1} (6α,11β-diacetoxygedunin + 11β-hydroxygedunin). Ultrastructures of the parasites were evaluated by transmission electron microscope following Almeida-Souza et al. 2021, by treating 10^6 parasites mL^{-1} with the limonoid-rich fractions (in duplicate) and comparing the alterations in plasma membrane, nuclei, flagella and all the visualized organelles with those of untreated parasites.

In the radical scavenging assay, the 6α,11β-diacetoxygedunin + 11β-hydroxygedunin fraction showed the best, though moderate, antioxidant activity ($p = 0.0022$) when compared to the 6α,11β-diacetoxygedunin fraction (Table 1). This result is similar to the IC_{50} obtained for andiroba oils from Belém, Pará state (northeastern Amazon) (Milhomem-Paixão et al. 2016). The 11β-hydroxygedunin fraction did not exhibit antioxidant activity at the concentrations evaluated.

Untreated parasites exhibited normal morphology (Figure 1a). The three fractions induced alteration in nuclei of the promastigotes with variations in chromatin condensation (Figure 1e,g,h). Promastigotes treated with the 6α,11β-diacetoxygedunin fraction (Figure 1b-e) showed an increase in kinetoplast and mitochondria volume, alterations in mitochondrial cristae, electron-dense material in the flagellar pocket, lipid bodies, increase of the endoplasmic reticulum and irregular membranous-shape structures near the flagellar pocket. Parasites treated with the 6α,11β-diacetoxygedunin + 11β-hydroxygedunin fraction (Figure 1f-g) displayed vacuoles and large lipid corpuscles, increase and fragmentation of the endoplasmic reticulum, and electron-dense material in the flagellar pocket. Parasites treated with the 11β-hydroxygedunin fraction exhibited electron-dense lipid bodies, large-volume vacuoles with electron-dense material close to the nucleus and to the flagellar pocket, change in mitochondrial morphology with circular membranes inside, and kinetoplast kDNA barely visible (Figure 1h).

To the best of our knowledge, this is the first report of ultrastructural alterations induced by limonoid-rich andiroba oil fractions in *Leishmania* parasites. *Leishmania infantum* and *L. amazonensis* promastigotes treated with an andiroba oil nanoemulsion and analyzed by electron microscopy scanning showed alterations like oval cell shape and retracted flagella as early as one hour after treatment (Dhorm Pimentel de Moraes et al. 2018).

The major ultrastructural changes in our treated parasites were observed in kinetoplast/mitochondria. The kinetoplast is a structure with DNA (kDNA) in the mitochondria formed by a unique network of circular interlocked minicircles and maxicircles, and the morphological traits and segregation pattern of the kinetoplast are associated with the cell cycle stage (Minocha et al. 2011). Alterations in kDNA, such as the decrease in chromatin condensation induced by the treatments, can be related to the cell death process. Mitochondrial morphological changes, such as the swelling and cristae disorganization, are associated with a phenotype similar to apoptosis observed in mammalian cells *in vitro* (Basmaciyan and Casanova 2019).

Table 1. Antioxidant activity of limonoid-rich fractions of andiroba (*Carapa guianensis*) oil. Fractions are identified by their predominant limonoid compounds. Trolox = positive control. Values are the mean ± standard deviation of three repetitions.

Fraction	DPPH IC_{50} (μg mL^{-1})
6α,11β-diacetoxygedunin	619.51 ± 11.41[a]
11β-hydroxygedunin	>1000[b]
6α,11β-diacetoxygedunin + 11β-hydroxygedunin	330.35 ± 8.60[c]
Trolox	6.15 ± 0.57[d]

IC_{50} = inhibitory concentration of 50% of the parasites when compared to an untreated group. Different letters in the column indicate statistical difference between groups according to a Mann-Whitney test.

The other alterations are related to the flagellar pocket, an invagination of the cell membrane around the proximal end of the flagellum. In *Leishmania* parasites, endo/exocytosis occurs exclusively through the flagellar pocket (Halliday et al. 2021). The electron dense material observed in the flagellar pocket, and the irregular shaped vacuole with membranous structures inside or near the flagellar pocket, indicate an improvement in exocytosis by the promastigotes. The increase in vacuolization, with some vacuoles presenting material inside, as well as the increase in lipid bodies, is a signal of exacerbated production/recycling of proteins by the cells to survive. The increase in exocytic activity, that is also associated with drug resistance (Perea et al. 2016), demonstrates the need of the parasite cell to remove intracellular material as an attempt to survive drug action (Almeida-Souza et al. 2020).

The set of ultrastructural alterations, some related to accidental cell death, such as the increase in the exocytic activity, the increase in lipid bodies and vacuolization, and some related to the regulation of cell death, like the mitochondria/kinetoplast alterations, indicate anti-*Leishmania* effects of the limonoid-rich fractions that may act by

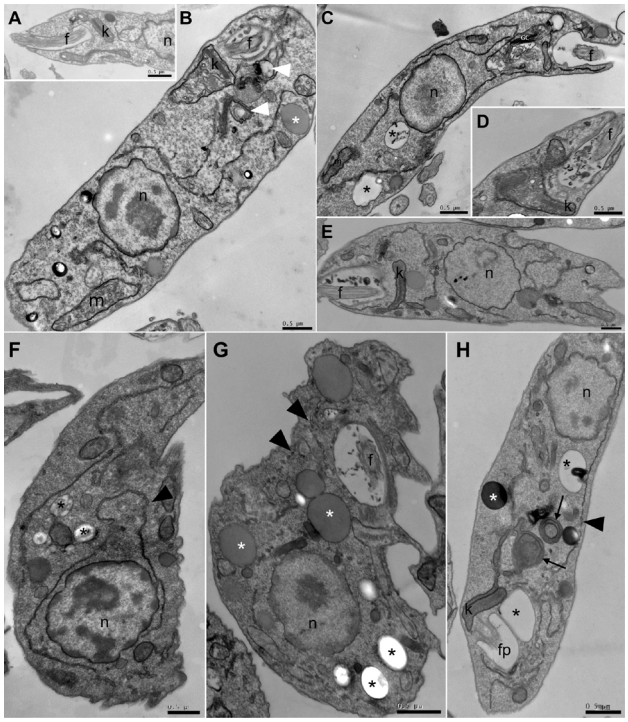

Figure 1. Ultrastructural alterations in *Leishmania amazonensis* treated with limonoid-rich fractions of oil from andiroba, *Carapa guianensis*. Promastigotes remained untreated (A); or were treated for 72 hours with fractions containing 6α,11β-diacetoxygedunin (B–E); 6α,11β-diacetoxygedunin + 11β-hydroxygedunin (F–G); and 11β-hydroxygedunin (H). White asterisks = lipid bodies; black asterisks = vacuoles; white arrowhead = irregularly shaped vacuole with membranous structures inside near the flagellar pocket; black arrowheads = increase and fragmentation of endoplasmic reticulum; arrows = change in mitochondrial morphology with circular membranes inside; f = flagella; k = kinetoplast; m = mitochondria; n = nucleus; fp = flagellar pocket. Scale bar = 0.5 μm.

different mechanisms. Further studies should isolate the limonoid compounds in the tested fractions and elucidate the mechanisms involved in the tendency to leishmanicidal and antioxidant activity observed in our study.

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DATA AVAILABILITY

The data that support the findings of this study are available, upon reasonable request, from the corresponding author, Kátia da Silva Calabrese.