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Original Article

Antileishmanial activity of a dillapiole derivative obtained from *Piper aduncum* L. (Piperaceae)

Atividade antileishmania de um derivado do dilapiol obtido a partir de *Piper aduncum* L. (Piperaceae)

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Abstract

Cutaneous leishmaniasis (CL) is considered a public health problem. Current treatments have disadvantages because they are invasive and have serious side effects, and thus there is a need for research into new, more effective pharmacological alternatives. Plants are promising sources of bioactive substances, and new analogues can be obtained through chemical reactions. The present study aimed to evaluate the antileishmanial effects of the analog dillapiole n-butyl ether (DBE) extracted from *Piper aduncum* leaves. The cytotoxic potential of DBE was evaluated at concentrations of 15.62 to 500 µM in peritoneal macrophages for 48 h, and in RAW 264.7 macrophages for 72 h using a dose-response method. The antileishmanial activity in *L. amazonensis* promastigotes used concentrations of 0.2 to 4.5 µM for 24, 48 and 72 h and the quantification of the cellular infection rate used a concentration of 4.5 µM of DBE against the amastigote forms internalized in macrophages for 24 h and 48 h. Nitric oxide was quantified from macrophages previously treated with DBE for 24 h and 48 h. The dosage of reactive oxygen species used a concentration of 4.5 µM of DBE incubated together with dichlorofluorescein acetate for 1, 3, 6, and 24 h. For the molecular modeling of DBE, the *Leishmania* protein, available in the "Protein Data Bank" database, was used. The studied molecule was not toxic to cells and presented a CC_{50} of 413 µM in peritoneal macrophages and 373.5 µM in RAW 264.7. The analogue inhibited promastigote forms of *L. amazonensis* with an IC_{50} of 1.6 µM for 72 h. DBE presented an infection rate of 17% and 12%, dillapiole of 24% and 14% and Pentacarinat® of 10% and 9% over 48 h. DBE demonstrated a binding energy of -7.8 for the U53 enzyme. It is concluded that the analogue showed promising antileishmanial activity for future *in vivo* tests.

Keywords: analog, dillapiole *n*-butyl ether, *Leishmania*.

Resumo

A leishmaniose cutânea (LC) é considerada um problema de saúde pública. Os tratamentos atuais apresentam desvantagens por serem invasivos e possuem efeitos colaterais graves, levando a pesquisa de novas alternativas farmacológicas mais eficazes. As plantas são fontes promissoras de bioativos, e por meio de reações químicas pode-se obter novos análogos. O presente estudo objetivou avaliar os efeitos antileishmania do análogo éter *n*-butil do dilapiol (EBD) extraído das folhas de *Piper aduncum*. Foi avaliado o potencial citotóxico do EBD nas concentrações de 15,62 a 500 µM em macrófagos peritoneais por 48 h, e em macrófagos RAW 264.7 por 72 h utilizando o método dose-resposta. A atividade antileishmania em promastigotas de *L. amazonensis* usou-se concentrações de 0,2 a 4,5 µM durante 24, 48 e 72 h e a quantificação da taxa de infecção celular usou-se uma concentração de 4,5 µM do EBD contra as formas amastigotas interiorizado em macrófagos por 24 h e 48 h. O óxido nítrico foi quantificado a partir de macrófagos tratados previamente com o EBD durante 24 h e 48 h. A dosagem das espécies reativas de oxigênio utilizou-se a concentração de 4,5 µM do EBD incubado junto ao acetato de diclorofluoresceína por 1, 3, 6, e 24 h. Para a modelagem molecular do EBD, usou-se a proteína de origem de *Leishmania*, disponível no banco de dados "Protein Data Bank". A molécula do estudo não foi tóxica para as células, apresentou uma CC_{50} de 413 µM em macrófagos peritoneais e de 373,5 µM em RAW 264.7. O análogo inibiu as formas promastigotas de *L. amazonensis* com uma CI_{50} de 1,6 µM por 72 horas. O EBD apresentou uma taxa de infecção de 17% e 12%, dilapiol de 24% e 14% e Pentacarinat® de 10% e 9% durante 48 h. O EBD demonstrou uma ligação de energia de -7,8 pelo sítio de ação da enzima U53. Conclui-se que o análogo mostrou atividade antileishmania promissora para futuros testes *in vivo*.

Palavras-chave: análogo, éter *n*-butil dilapiol, *Leishmania*.

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1. Introduction

Leishmaniases are anthropozoonoses that constitute a set of clinical manifestations and, due to epidemiological, socio-economic diversity and the low interest of the pharmaceutical industry, are considered neglected diseases and a public health problem (BRASIL, 2017). Cutaneous leishmaniasis (CL) is an infection caused by *Leishmania* parasites and, in view of the different species, it can present several clinical forms, such as cutaneous, mucocutaneous, diffuse cutaneous and disseminated forms, which in many cases leads to tissue destruction in such a way as to leave the individual deformed or even cause death (WHO, 2010).

CL occurs in 88 countries, which together cover the five continents (Americas, Europe, Africa, Asia and Oceania), and annually 0.7 to 1.3 million cases are reported (WHO, 2017). In Brazil, the incidence of CL has increased significantly, with an annual record of 21,161 cases in practically in all states of the Federation (BRASIL, 2017); of these, 1,732 were recorded in the state of Amazonas, and the capital city Manaus had 733 cases in 2017 (SINAN, 2017).

Currently, the treatment that is used in the pharmacotherapy of patients with CL is based on pentavalent antimonials (Sb^v), and the first-choice drug for most cases is sold in Brazil as Glucantime® (N-methylglucamine antimonate), the Pentacarinat® and Amphotericin B® are used in exceptional cases (BRASIL, 2017). These pharmacotherapies have disadvantages because they are hepatoxic, nephrotoxic and cardiotoxic (BRASIL, 2013; Demicheli and Frézard, 2005), in addition to having a parenteral route of administration, limited efficacy and a high cost (Maciel Santos et al., 2013).

In the search for new alternative treatments for CL, researchers have sought to better understand the chemical diversity of molecules found in natural sources, in order to develop new technologies for obtaining new drugs (Pinto et al., 2002). The technological evolution in bioanalytical chemistry within computational chemistry have allowed us to identify and modify biological chemical structures in new derivatives that can be applied in the development of new drugs. In fact, technological evolution has been significant in the development of new drugs that precede computational chemistry, structural molecular biology that can be applied, and that allow structural modifications of one or more molecules, and thus obtain new more active derivatives (Agarwal and Fishwick, 2010).

Studies with analogs obtained from *Piper aduncum* L. (Piperaceae) Vianna and Akisue (1997), such as the promising 2',6'-dihydroxy-4'-methoxychalcone isolate, demonstrated activity against the intra- and extracellular forms of parasites of the genus *Leishmania* (Torres-Santos et al., 1999). Farah et al. (2010) demonstrated antileishmanial activity of dillapiole and isodillapiole against *L. chagasi* Cunha and Chagas (2010), Parisi-Filho et al. (2012) demonstrated antileishmanial activity against promastigote forms of *L.* (*L.*) *amazonensis* Lainson and Shaw (1972), *L.* (*V.*) *braziliensis* Vianna (1911) using dillapiole and its analogs (dihydrodillapiole and isodillapiole). Given the search for new prototypes with greater safety, greater efficacy and potency, the present study evaluated the *in vitro* and *in silico* antileishmanial activity of dillapiole n-butyl ether obtained from *P. aduncum* in order to, in the future, develop a drug for the treatment of cutaneous leishmaniasis *in vivo*.

2. Materials and Methods

2.1. Ethical information

This project is part of the umbrella project of the Laboratory of Leishmaniasis and Chagas Disease, submitted to and approved by the Ethics Committee on the Use of Animals (CEUA/CEP/ INPA) under Nº 014/2015.

2.2. Acquisition of the plant material

The plant material of the species *P. aduncum* L. was collected at highway markers 26, 27, 43 and 58 of the Manaus-Itacoatiara highway (AM-010) in 2017 (exsiccate voucher # 10.480/2013, collector: E. S. Silva). Leaves were taken from *P. aduncum* L. plants cultivated at Embrapa Amazônia Occidental (Manaus, Amazonas state, Brazil) (2°53'36.2" S, 59°58'22.8" W). About 40 kg of the plant material was transported to Fazenda Litiara, located in Itacoatiara, where the plant material was spread on a tarpaulin for drying during two days in a covered area. After drying, the material was weighed and an amount of 25 kg of dried plant was obtained.

2.3. Preparation of DBE

The preparation of the DBE analog was performed by Dr. Ana Cristina da Silva Pinto. Dillapiole was obtained via fractional distillation and the DBE was prepared from this compound using some reactions already cited in the literature (Tomar et al., 1979a, b). The DBE was purified in flash chromatography column and identified using spectroscopic methods (1^H, 13^C NMR).

2.4. Obtaining peritoneal macrophages for cytotoxic assay

To obtain the peritoneal macrophages, ten anesthetized BALB/c mice (*Mus musculus*) were used and RPMI medium without sterile fetal bovine serum was injected intraperitoneally. Subsequently, the medium was recovered via aspiration. The abdominal lavage was centrifuged, resuspended in 1 mL of the medium, the cells were adjusted in a Neubauer chamber to a concentration of $5x10^5$ macrophages/mL. Then, these were plated in a 96-well plate in triplicate and exposed to concentrations of 15.62 to 500 µM of DBE, and afterwards incubated in an oven at 37 ºC with 5% CO_2 for 48 h.

2.5. Obtaining Macrophages of the RAW 264.7 cell line for cytotoxic assay

The macrophages of the RAW 264.7 cell line (from cryopreservation at -80 ºC) were supplemented with 10% fetal bovine serum (Gibson by life technologies), and 50 U/mL of penicillin and 50 µg/mL of streptomycin were added. The cells were adjusted to a concentration of 10^6 macrophages/mL. These were plated in triplicate and exposed to concentrations of 15.62 to 500 µM of DBE, then incubated in an oven at 37 ºC with 5% CO_2 for 72 h.

For cell viability, the oxide-reduction method was applied. As controls, Pentacarinat® (positive), RPMI medium supplemented with cells and DMSO at 0.2% plus cells (negatives), After 24 h of incubation, the cells were treated. After the treatment periods, 10 µL of 0.4% resazurin was added for 2 h (Nakayama et al., 1997). The fluorescence reading was performed in a spectrophotometer at 560 nm.

2.6. Acquisition and maintenance of the parasites

The species of *L.* (*L.*) *amazonensis* (MHOM/BR/2009/IM5584) was used for the test. The specimens were maintained and cryopreserved in liquid nitrogen at the Laboratory of Leishmaniasis and Chagas Disease at INPA, then cultivated in Schneider 1640 medium, supplemented with 10% inactivated fetal bovine serum (IFBS) and incubated in an oven at 25 °C.

2.7. Determination of antileishmanial activity using the 50% inhibitory concentration (IC_{50})

The antileishmanial activity of the DBE analog was evaluated by the inhibition of growth and mortality of promastigote forms of *L.* (*L.*) *amazonensis* in the periods of 24, 48 and 72 h, at 25 °C. In the bioassay, Schneider culture medium supplemented with 10% IFBS was used. The promastigote forms were centrifuged at 4,400 rpm/15 minutes, then diluted and adjusted in a Neubauer chamber to the concentration of $2x10^6$ parasites/mL. The test was in triplicate using concentrations of 1.31 to 21.1 µM of DBE, for 24, 48 and 72 h as screening. Later, another test was performed with concentrations of 0.2812 to 4.5 µM using the same durations. The negative control was only the parasite and the medium, and DMSO to evaluate its influence as a diluent in the samples. For the positive control, Pentacarinat® was used at the same concentrations. The bioassays were analyzed using the dose-response test (Alamar Blue®), according to the protocol adapted from Nakayama et al. (1997) with the reading performed in a spectrophotometer.

2.8. Determination of the selectivity index

To determine the selectivity of the concentrations with antileishmanial activity against the macrophages, the selectivity index (SI) was determined using the following equation (Nakamura et al., 2006):

SI = CC_{50} in macrophages (cytotoxic concentration 50%)/ IC_{50} against promastigotes (inhibitory concentration 50%).

2.9. Quantification of the infectivity rate in peritoneal macrophages infected with L. (L.) amazonensis

The macrophages were adjusted to a concentration of $5x10^5$ cells/mL, and sterile glass slips were placed on the 24-well plates. Then, the cells and supplemented RPMI medium were added, and incubated in an oven at 37 °C with 5% CO_2 for 24 h. The promastigote forms of *L.* (*L.*) *amazonensis* were added to plates containing macrophages at a ratio of 5:1 (promastigotes/macrophage) and incubated with DBE, Pentacarinat® and dillapiole at a concentration of 4.5 µM. The negative control was 0.2% DMSO. This was then placed in an oven at 37 °C with 5% CO_2 for a period of 24 h and 48 h.

At the end of the assay, the coverslips were stained using a Panoptic kit and observed under an optical microscope (1000x). Infectivity was determined by counting 100 macrophages/well, in triplicate. The number of infected, uninfected, and infected and treated macrophages was quantified. The mean and standard deviation were calculated.

2.10. Quantification of nitric oxide

The production of nitric oxide was measured by the dosage of its degradation products, based on the Griess reaction. In this method, nitrite first reacts with sulfanilamide in an acidic medium to form an intermediate compound, i.e., diazonium salts. This compound then reacts with N-(-naphthyl)ethylenediamine (NED) to form a more stable azo compound of a purple coloration and can thus be quantified spectrophotometrically at 560 nm (Green et al., 1982).

For the determination of NO• production, 50 µL of cell culture supernatant (peritoneal macrophages) was obtained at a concentration of 10^6 macrophages/mL. This was treated with the DBE analog at a concentration of 4.5 µM with and without cellular infection, in triplicate. As a positive control Pentacarinat® was used and, as a negative control, the parasite plus RPMI medium and DMSO at 0.2%, and a group stimulated by lipopolysaccharide (LPS) at 2 µg/mL for 24 and 48 h. For the preparation of this reagent, stock solutions of N-(1-naphthyl)ethylenediamine chloride (0.1%) dissolved in H_2O and 1% sulfanilamide dissolved in H_3PO_4 (5%) were used. After the incubation period of 10 min, the samples were read in a microplate reader (DTX 800, Beckman) at 560 nm.

To perform the MTT assay, three more plates were prepared with the same procedures mentioned previously. After reading the plate with the biological supernatant, the test plate was incubated with 5 mg/mL of MTT at 37 ºC, with 5% CO_2 to evaluate cell viability through the formation of formazan salts as per the protocol of Sieuwerts et al. (1995).

2.11. Evaluation of reactive oxygen species (ROS) production

Peritoneal macrophages with and without infection with *L. amazonensis* and treated infected macrophages were used at a concentration of $6x10^4$ cells/mL. DBE was used at a concentration of 4.5 µM, the positive control was Pentacarinat® and the negative controls were the infected cell plus medium and 0.2% DMSO. AAPH (2,2-azobis (2-amidinopropane) dihydrochloride) at a concentration of 600 µM was used as a stimulator. The test was performed in triplicate and observed at 1, 3, 6 and 24 h. The supernatant from this assay was discarded and then H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) (Invitrogen, Eugene, USA) was added at a final concentration of 20 M per sample. The analyses were performed with excitation at 485 nm and emission at 530 nm in a spectrophotometer (Fonseca-Silva et al., 2011).

2.12. Molecular spatial modeling

The molecular modeling procedure was performed with the crystallographic structure, using proteins of

parasite origin (*Leishmania*), available in the database "Protein Data Bank" (PDB) [code: ID PDB 5 g1z, 5ebk], with a resolution of 2.7 Å (Orlando and Malkowski, 2016). The three-dimensional (3D) structures of the ligands of dillapiole and dillapiole n-butyl ether were obtained from the 2D structure, with subsequent conversion to 3D, using Chem 3D 15.0 software.

Geometric optimization of all the ligands was performed using the semi-empirical method PM7 (Stewart, 2013), available in the software MOPAC2016 (Stewart Computational Chemistry, 2016). The preparation of the receptor and ligands was achieved through the Autodock tools software (Morris et al., 2009), and the grid box was defined in relation to the active site occupied by the ligand (U53 and RDS). The anchoring procedure was performed using the Autodock Vina software (Trott and Olson, 2010). The results were processed through the Discovery Studio software (Accelrys Software Inc, 2016). The analysis was carried out with the collaboration of Dr. Rodrigo Otávio Silva De Souza.

2.13. Statistical analysis

The IC_{50} and CC_{50} values were obtained through a nonlinear regression by inhibition analysis, and the statistical differences between concentrations and controls were analyzed using an ANOVA (two-way) for multiple comparisons, followed by Bonferroni's test with a 95% confidence interval. The analyses were performed in the GraphPad Prism 6® program.

3. Results

3.1. Evaluation of the cytotoxic activity of dillapiole n-butyl ether (DBE) in macrophages

In this study, cell viability was analyzed in relation to the action of the substances over time. It was observed that DBE obtained a 50% cytotoxic concentration (CC_{50}) of 413.3 ± 3.1 µM, Pentacarinat® of 15.6 ± 0.2 µM demonstrating that DBE was less toxic (26.5 times) than Pentacarinat®. In relation to dillapiole, it was not possible to obtain the CC_{50}, since it was not toxic to peritoneal cells at the concentrations used for 48 h.

In RAW 264.7 macrophages, DBE obtained a CC_{50} of 373.5 ± 41.7 µM, Pentacarinat® of 14.4 ± 7.7 µM; DBE showed a toxic safety that was 25.9 times higher than the standard drug. It was not possible to obtain the CC_{50} of dillapiole due to the substance not killing 50% of the cells.

The BALB/c Peritoneal macrophages exposed to LPS at 2 µg/mL for 24 and 48 hours remained viable. These results were obtained after the measurement of nitric oxide using the MTT reduction assay. Pentacarinat® presented a toxic profile within 48 h.

3.2. Morphological evaluation of the viability of peritoneal macrophages exposed to dillapiole n-butyl ether

Micrographs of the macrophages without infection exposed to the substances (DBE, Pentacarinat® and dillapiole) showed delineated nucleus, cytoplasm and membranes of preserved cells, indicating that there was no change in cell morphology at the concentration used (4.5 µM) for 24 and 48 h (Figure 1).

3.3. Evaluation of DBE activity against promastigote forms of Leishmania (L.) amazonensis

The results obtained for activity of DBE against *L.* (*L.*) *amazonensis* at different concentrations are shown in Figure 2. It was observed that in the highest concentration (4.5 µM) of DBE presented greater activity, reduced promastigote forms and presented statistical differences, with P<0.0001 in relation to the negative control for 24, 48 and 72 h, Dillapiole did not inhibit the multiplication of parasites in the concentration used, thus, we did not obtain the IC_{50}.

Table 1 shows the IC_{50} values and the confidence intervals of the substances obtained through the exposure of

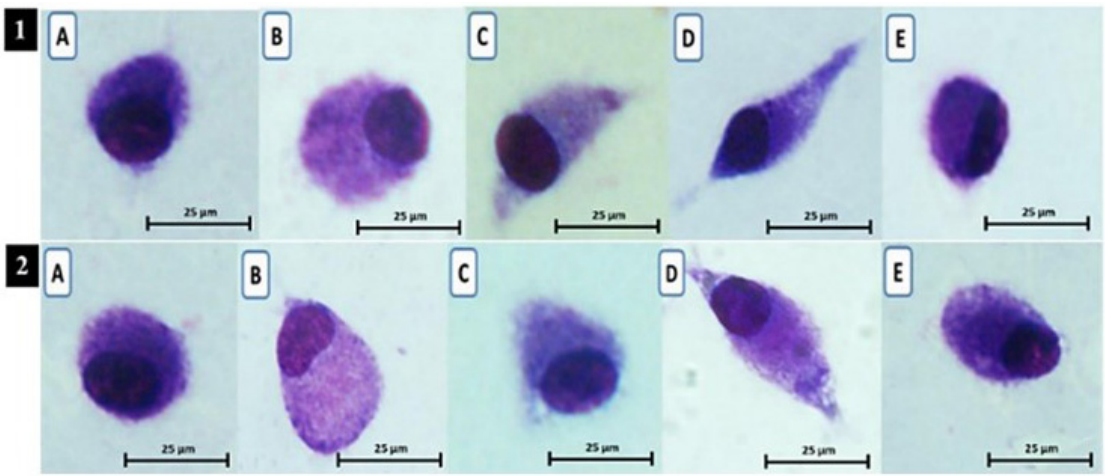

Figure 1. Morphology of peritoneal macrophages from BALB/c mice, when exposed to the effects of substances at a concentration of 4.5 µM; (A) Dillapiole; (B) DBE; (C) Pentacarinat®, (D) DMSO, (E) Macrophages and medium for 24 h (1) and 48 h (2).

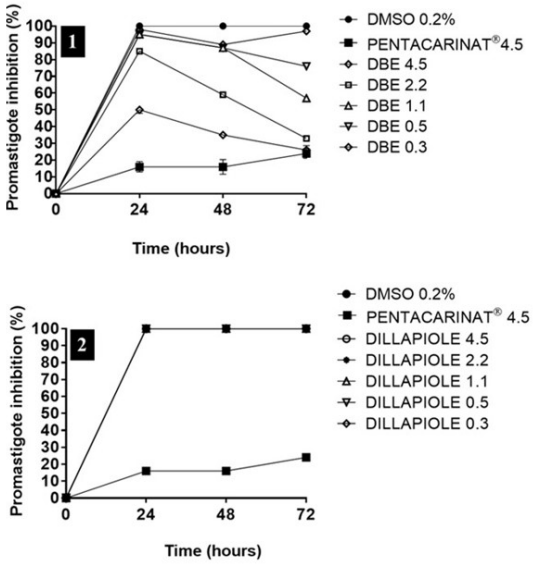

Figure 2. Assessment of the activity of DBE (1) and dillapiole (2) against promastigote forms of *Leishmania* (*L.*) *amazonensis* – quantification of viable forms of the parasites at 24, 48 and 72 h. The results were obtained through analysis using an ANOVA (two-way) and Bonferroni's test with a 95% confidence interval using the GraphPad Prism 6® program. Mean IC_{50} values are presented in µM.

Table 1. IC_{50} values of dillapiole n-butyl ether (DBE) and Pentacarinat® in *Leishmania* (*L.*) *amazonensis* promastigotes at 24, 48 and 72 h (mean ± standard deviation of three replicates). Means of the IC_{50} are presented in µM.

Test samples	24 h	48 h	72 h
DBE	10.0 (7.2 ± 14)[a]	5.6 (2.2 ± 8.9)[a]	1.6 (0.8 ± 3.3)[a]
PENTACARINAT®	5.4 (3.6 ± 8.0)[b]	1.8 (1.0 ± 2.6)[b]	2.2 (0.0 ± 4.5)[b]

promastigotes with the substances over time. DBE showed a similar efficacy to the standard drug at 72 h. The IC_{50} for dillapiole could not be obtained because it did not inhibit 50% of the promastigote forms.

3.4. Evaluation of the selectivity index (SI)

During 48 h, the SI of 76.5 was observed for DBE, and Pentacarinat® was 8.7 when using a concentration of 4.5 µM. DBE was 8.8 times less toxic than the standard drug. At 72 h, DBE showed an SI of 233.4 and Pentacarinat® of 6.5 when using RAW 264.7 macrophages.

3.5. Evaluation of cellular morphological aspect and the Leishmania (L.) amazonensis infection rate in peritoneal macrophages

Figure 3 shows the substances DBE, Pentacarinat® and dillapiole at a concentration of 4.5 µM in contact with infected peritoneal macrophages over time. It was observed that the analog DBE showed inhibition of the amastigote forms and presented several vacuoles and a greater amount of uninfected cells when compared to the negative control

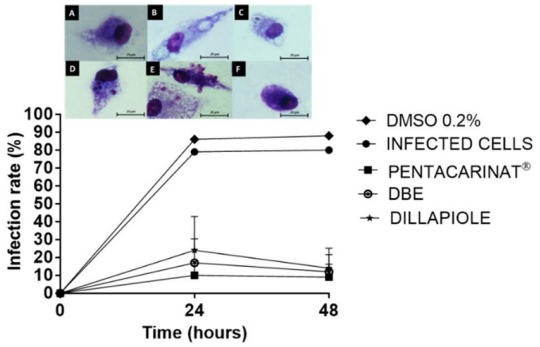

Figure 3. Morphological aspects of cells exposed to the substances used at a concentration of 4.5 µM against amastigote forms of *Leishmania* (*L.*) *amazonensis* in peritoneal macrophages for 48 h. (A) Dillapiole, (B) DBE, (C) Pentacarinat®, (D) Infected macrophages, (E) DMSO, (F) Uninfected macrophages. M.O. 1000x and assessment of the infection rate of DBE, dillapiole and Pentacarinat® for 24 and 48 h. The results were obtained through analysis using an ANOVA (two-way) and Bonferroni's test with a 95% confidence interval, using the GraphPad Prism 6® program. Previously, the percentages were calculated using the percentages of the means - % NC (mean percentage of the negative control value).

group (parasite plus macrophages) and DMSO at 0.2%. Therefore, it demonstrated promising antileishmanial activity against intracellular forms during 48 h.

In the infection of the macrophages by *L. amazonensis*, it was observed that the negative control (DMSO 0.2%) presented a percentage of cellular infection that was close to 100%. From these data, the infection rate was determined by treatment with DBE, Pentacarinat® and dillapiole at a concentration of 4.5 µM for 24 and 48 h. A mean of the groups was obtained, and it was observed that DBE showed promise in significantly inhibiting the proliferation of amastigote forms ($P<0.0001$) over time, and this was similar to Pentacarinat® for 48 h (Figure 3).

3.6. Standardization and measurement of nitric oxide production in peritoneal macrophages

It was observed that, at the concentration of 10^6 cells/mL, basal peritoneal macrophages produced low levels of nitric oxide (NO). However, when macrophages were stimulated by LPS at concentrations 10^5, 10^6, 10^7 they showed high levels of NO production in relation to time and cell concentrations over 24, 48 and 72 h. Through the data obtained, it was possible to select a more appropriate cell concentration for the study, namely 10^6 cells/mL.

Through the nitrite quantification test, it was observed that DBE did not stimulate NO production in cells of the basal group. This was also the case for the other substances used in the experiment when compared with the basal control group (without treatment). It was observed that the group infected with *L.* (*L.*) *amazonensis* and treated did not significantly produce nitric oxide in macrophages for 24 and 48 h when compared to the infected group without treatment. The group stimulated with LPS and treated with the substances inhibited nitric oxide production at all times when compared to the control group stimulated with LPS and without treatment (Figure 4).

3.7. Quantification of the production of cellular reactive oxygen species (ROS)

Figure 5 shows that the substances (DBE, Pentacarinat® and dillapiole), at the concentration of 4.5 µM, did not show significant activation of ROS. When the basal control group was compared with the treated basal group, it was observed that there was no stimulation of ROS at any of the times. In the other groups treated with the substances, biological normality was observed in the quantification of ROS at all the times. In relation to the stimulated group, it was observed that the treatments did not significantly reduce or increase ROS concentrations at 1 h and 24 h.

3.8. Enzyme inhibition simulations using molecular docking

The results obtained through molecular docking demonstrated a concordance with the experimental results regarding the *in vitro* antileishmanial activity using the analog dillapiole n-butyl ether (DBE). In the *in silico* study, two enzymes were tested: trypanothione reductase (ID PDB 5ebk) and N-myristoyl transferase (ID PDB 5g1z). DBE showed greater affinity for the active site of the enzyme N-myristoyl transferase with a binding energy of -7.8; a value very close to that of the standard inhibitor U53 and higher than the binding energy of dillapiole (Figure 6). It was observed that DBE and dillapiole did not present a

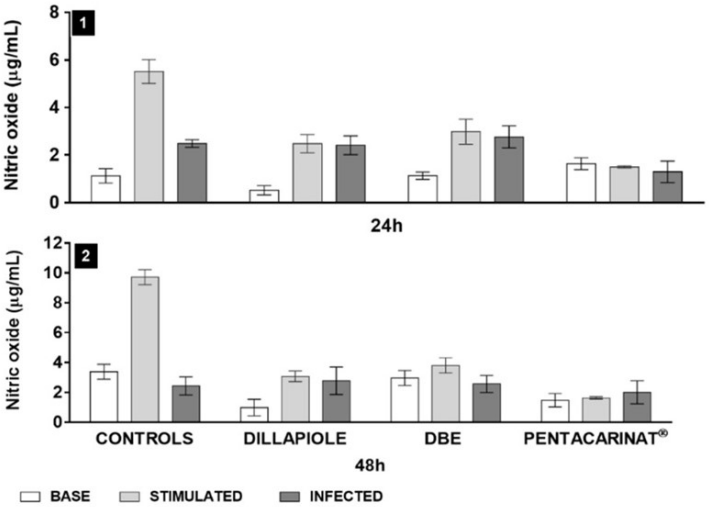

Figure 4. Estimation of nitric oxide production in peritoneal macrophages of BALB/c mice infected with *Leishmania* (*L.*) *amazonensis* for 24 (1) and 48 (2) h and exposed to substances (DBE, Pentacarinat® and dillapiole) at the concentration of 4.5 µM. The results were obtained through analysis using an ANOVA (two-way) and Bonferroni's test with a 95% confidence interval using the GraphPad Prism 6® program. The mean and standard deviation were obtained through tests in triplicate.

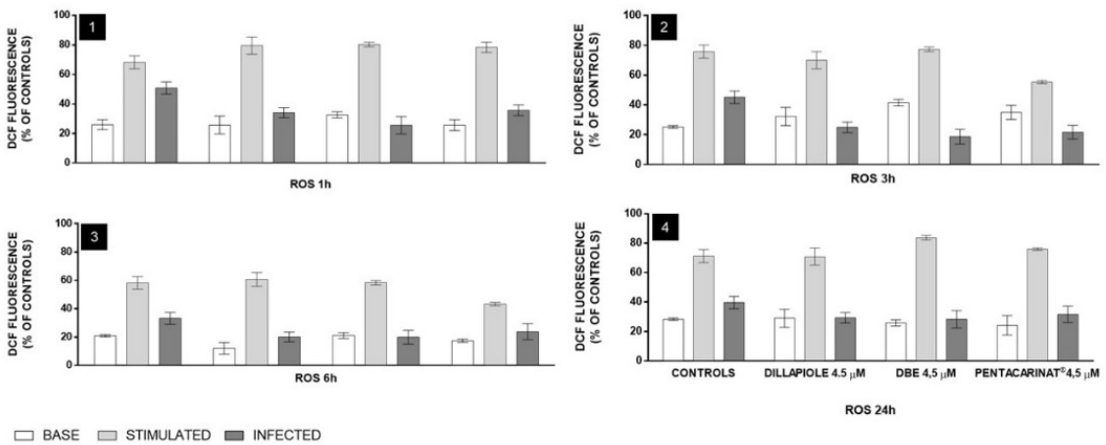

Figure 5. Quantification of reactive oxygen species in macrophages in the basal form without infection, stimulated by LPS and infected with *Leishmania* (*L.*) *amazonensis* during the first 24 h, and exposed to substances (DBE, Pentacarinat® and dillapiole) at the concentration of 4.5 µM. Results shown in graphs 1 (1 h); 2 (3 h); 3 (6 h) and 4 (24 h). Data were obtained by mean ± SD (standard deviation of the mean) using the GraphPad Prism 6® program. The test was performed in triplicate.

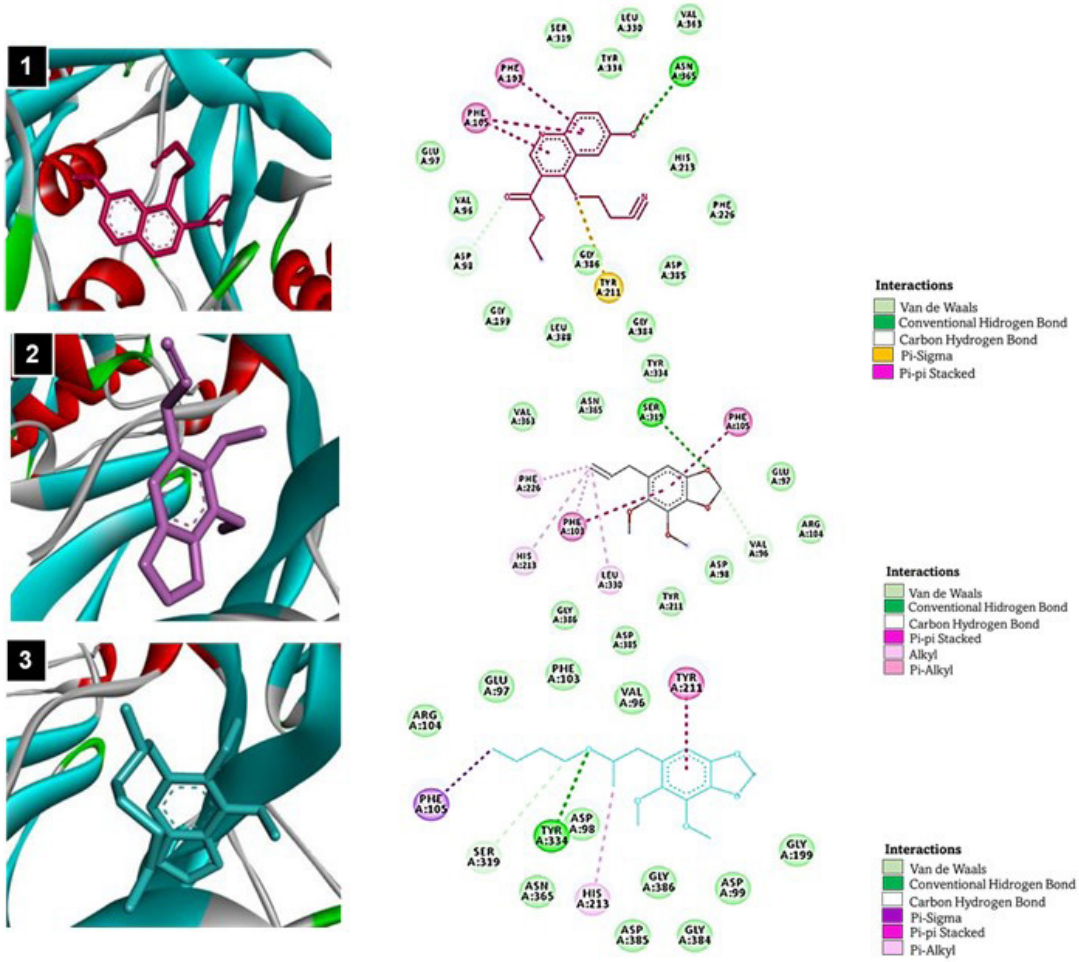

Figure 6. N-myristoyltransferase: standard inhibitor U53 with binding energy of -8.2 (1). Interactions and docking of dillapiole at the site of action of the enzyme myristoyltransferase ID PDB 5g1z with binding energy of -6.8 (2). Interactions and docking of dillapiole n-butyl ether at the site of action of the enzyme myristoyltransferase ID PDB 5g1z with binding energy of -7.8 (3).

good binding energy interaction (-5.2 and -4.8) respectively, when compared with the binding energy of the standard inhibitor RDS (-6.0) of the enzyme trypanedione reductase, in addition to not presenting hydrogen bonding.

4. Discussion

One study shows a high viability of macrophages when exposed to dillapiole and its analogue isodillapiole starting from 50 µg/mL for 72 h (Farah et al., 2010). These data corroborate our findings when the peritoneal macrophages of BALB/c mice were exposed for 48 h at serial concentrations of 15.62 to 500 µM of DBE and dillapiole, thus reinforcing the non-specificity for macrophages. On the other hand, several lines of immortalized macrophages are often used for studies, since they present responses that are very similar to primary cells (Knapp, 2009). In macrophages of the murine lineage RAW 264.7, due to their ability to exhibit a phenotype of rapid and incessant growth, the cytotoxicity of the substances was evaluated for 72 h (Moffat et al., 2014). Furthermore, cells of various origins can exhibit different responses when exposed to the same natural compounds (Marques-Santos et al., 2023). In our findings, in this strain, we observed a higher toxicity of the standard drug than for DBE when using the same concentrations and the period.

Studies with secondary cells conducted by Parisi-Filho et al. (2012) demonstrated the cytotoxic effects of dillapiole starting from 25 µM in 3T3 fibroblasts during 24 h of treatment. This cell is important for the *Leishmania* parasite during the chronic phase of infection (Vargas-Inchaustegui et al., 2010). It was observed that dillapiole and DBE were non-toxic in RAW 264.7 macrophages for 72 h at a concentration of 4.5 µM. The study by Sousa et al. (2008) demonstrated that *Piper aduncum* oil had a high margin of safety of 240 mg/kg and 120 mg/kg, with minimal toxic effects on biochemical and hematological parameters *in vivo*.

Study of knowledge of Amazonian biodiversity must be strengthened or implemented to protect sources of natural bioactives that can be studied to develop future medicines (Vieira et al., 2008). The review by Rocha et al. (2005) described 101 plants from several countries that had leishmanicidal activity and 239 isolated natural substances with activity in different species of *Leishmania*. Farah et al. (2010) demonstrated the antileishmanial activity of the isodillapiole derivative isolated from *P. aduncum*, using a concentration of 50 µg/mL in promastigotes of *L. chagasi* for 72 h, which inhibited 96% of the viable parasites. In our study, we used a 4.5 µM concentration of DBE to inhibit viable promastigote forms of *L. amazonensis* for 24, 48 and 72 h, thus demonstrating that it is an antileishmanial molecule. The study by Parisi-Filho et al. (2012) demonstrated the effects of dihydrodillapiole and isodillapiole analogs on promastigotes of *L. amazonensis* and *L. braziliensis*, which presented an IC_{50} of 99.9 ± 10.4 and 122.9 ± 13.9 µM and 90.5 ± 8.6 and 109.8 ± 9.5 µM, respectively. Dillapiole presented an IC_{50} of 69.3 ± 4.9 µM for *L. amazonensis* and 59.4 ± 4.0 for *L. braziliensis*. These results diverge from our findings since DBE showed IC_{50} values of 10.0 (7.2-14), 5.6 (2.2-8.9), 1.6 (0.8-3.3) at 24, 48 and 72 h, respectively. It was shown to be more potent than dillapiole against promastigote forms of *L. amazonensis* in all periods and similar to the standard drug (Pentacarinat®).

The study by Nakamura et al. (2006) demonstrated that fractions of *P. regnellii* Pessini et al. (2003) showed antileishmanial activity when calculating the selectivity index (SI) using a mathematical ratio (CC_{50}/IC_{50}), which is the method used in this study. In this way, it was possible to evaluate the cytotoxic sensitivity of cells when exposed to a new molecule under study and the efficacy of the substance on the microorganism. For a prototype to be considered promising and to be a candidate for a future drug, it must present an SI that is equal to or greater than 10 (Chiaradia et al., 2008). DBE proved to be a promising prototype since it presented a cellular SI of 76.5 in relation to Pentacarinat® with 8.7. These results differ from the study by Parisi-Filho et al. (2012) who demonstrated that dillapiole has a greater effect on promastigote forms than its analogs, in addition to obtaining a CC_{50} of 22 µM in 3T3 fibroblasts for 24 h and an SI of 0.3 was obtained, demonstrating that it is not selective for the *Leishmania* parasite or toxic to the cell.

In our study, DBE at 4.5 µM inhibited viable intracellular forms of the *L. amazonensis* parasite, exhibiting an infection rate of 17% and 12% for 24 and 48 h, respectively. These results indicate that the molecule in our study was promising because, when compared with the values of Pentacarinat® of 10% and 9% and dillapiole with 24% and 14%, similar results were shown. The negative control presented 88% infection. Thus, the substances used in the tests did not present a statistically significant difference between them. The study by Martins (2011) demonstrated activity of the derivative LASSBIO 1108 with 75% inhibition of the amastigote forms of *L. major* for 24 h, with an IC_{50} of 6.6 µM and an infection rate of 23%, and in *L. amazonensis* a parasitic inhibition of 76%, with an IC_{50} of 11.2 µM presenting an infection rate of 24% for 48 h.

In this study it was possible to quantify $NO^{\bullet}$ in BALB/c peritoneal macrophages, and thus present the findings. $NO^{\bullet}$ can be used in a beneficial or harmful way, and this depends on the amount which is produced (Preiser et al., 2011). It was observed that the substances tested did not have the ability to induce nitric oxide synthase (iNOS) to produce nitric oxide in relation to the groups of basal and LPS-stimulated macrophages. This possible mechanism of iNOS inhibition can be explained by the inhibitory effect of a particular compound in $NO^{\bullet}$ production and/or by $NO^{\bullet}$ radical sequestration (Miranda, 2012). The Study by Ikeda et al. (2008) demonstrated the inhibition of nitric oxide by ursolic acid in the attenuation of nitric oxide synthase induced by the NF-kB pathway in RAW 264.7 murine macrophages. Miranda (2012) showed a gradual increase in nitric oxide production in basal peritoneal macrophages at 1, 6 and 24 h of incubation with a significant difference. In this study, a small production of nitric oxide was observed at 24 h and 48 h, but without any significant difference per group. The substances (DBE, Pentacarinat® and dillapiole) at the concentration of 4.5 µM also did not stimulate the production of nitric oxide in infected macrophages over time. This inhibition can be explained by the fact that the production of nitric oxide depends on the formation of arginine, which is involved in the body's defense (Hallemeesch et al., 2002) and inhibits $NO^{\bullet}$ production in BALB/c peritoneal macrophages when they are infected by *L. amazonensis* (Fortéa et al., 2009). These are similar results to those found in this study since, the promastigote and amastigote forms of *L. amazonensis* are sensitive to the action of $NO^{\bullet}$, but have an active dose-dependent cytotoxicity, and promastigotes are more sensitive than amastigotes (Genestra et al., 2008).

In the study by Mukbel et al. (2007), after 24 h of infection of macrophages by promastigotes of *L. major* and *L. amazonensis* and activated by LPS, a lower significant parasitic load was observed in macrophages infected by *L. major* Yakimov and Schockov 1914 and not in macrophages infected by *L. amazonensis*. On the other hand, there are studies that demonstrate that when nitric oxide was stimulated by LPS in BALB/c peritoneal macrophages and infected with the parasite of *L. infantum* Nicolle 1908 there was an increase in $NO^{\bullet}$ and a reduction in the number of amastigotes. However, when infected with *L. amazonensis,* there was no reduction in parasitic load (Sousa et al., 2008). In this study, it was observed that the basal control group and the infected group did not induce the production of $NO^{\bullet}$, as also occurred in the infected groups treated with the study substances.

In our study, it was observed that the substances (DBE, Pentacarinat® and dillapiole) did not stimulate ROS production in the periods tested, because macrophages infected by *L. amazonensis* and treated with the substances did not activate ROS production in any of the periods. The induction mechanism in the activation of the NADPH oxidase enzyme to produce ROS through an oxidative burst can act beneficially in the defense against infections by bacteria, fungi and parasites and destroy these microorganisms (Ferreira and Matsubara, 1997). ROS have an important role in the inhibition of *L. amazonensis* (Fonseca-Silva et al., 2013). The study by Ribeiro et al. (2013) demonstrated that the derivative LQB-118 induced ROS production in *L. amazonensis* promastigotes in the first

hours, which suggests that this event is one of the triggers of parasite death. However, in our study, we suggest that the mechanism of death of the parasite is directly related to exposure to DBE.

In the search for a possible mechanism of the antileishmanial action of the DBE analog, a screening was performed with two enzymes (trypanothione reductase and N-myristoyltransferase) found in the *Leishmania* parasite, which have specific functions for the survival and/or parasitic infectivity. Through *in silico* analysis, it was possible to discover the degree of energy affinity of DBE with the active site of the enzyme U53. In *L. major*, the deletion of genes was used to demonstrate that the single copy NMT (N-myristoyltransferase) gene is essential for the viability of *Leishmania* promastigotes, and responsible for parasite virulence (Price et al., 2013). Wright et al. (2015) demonstrated that the N- myristoylation protein known as HASPB can be detected by western blot at both stages of the *Leishmania* parasite's life. Thus, these data predict a strongly pleiotropic effect of NMT inhibition on parasite cell function and an evident target of leishmanicidal drugs. Ogungbe and Ng (2013) performed a molecular docking screening with 209 molecules of antiparasitic alkaloids. Among the indole alkaloids, those that presented a stronger docking were the flinderoles A, B and C. These bonds showed selectivity for the active site of N-myristoyltransferase of *L. major*, as well as for pteridine reductase one and t-methionyl-tRNA synthetase.

Based on the molecular docking analyses, in this study, it was observed that the DBE analog presented six bonds with amino acid residues similar to the standard ligand: ASP 98, ASP 385, GLY 199, GLY 384, GLY 386, VAL 96 and GLU 97, and dillapiole with GLY 386, ASP 386, TYR 334, GLU 97 and VAL 363. Since standard NMT has thirteen amino acid residue bonds with Van der Waals bonds, DBE has eleven and dillapiole has nine. Thus, we suggest that DBE has a higher specificity for NMT than its precursor.

5. Conclusion

DBE presented a non-toxic profile in peritoneal macrophages for 48 h and in macrophages of the RAW 264.7 lineage for 72 h at the concentrations used and it did not alter cellular morphological characteristics. In addition to inhibiting the promastigote and amastigote forms of *L. amazonensis* when tested at the concentration of 4.5 µM, it was similar to Pentacarinat® and superior to dillapiole, with a reliable selectivity index. Regarding the production of nitric oxide in basal and infected peritoneal macrophages, DBE did not induce NO and did not stimulate NADPH oxidase to produce reactive oxygen species. It was demonstrated that the inhibition of *L. amazonensis* did not occur by these mechanisms, but by the direct effect of DBE. In addition, it showed a higher binding energy affinity to the active site of the enzyme U53.

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