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Neolignan Licarin A presents effect against *Leishmania (Leishmania) major* associated with immunomodulation *in vitro*

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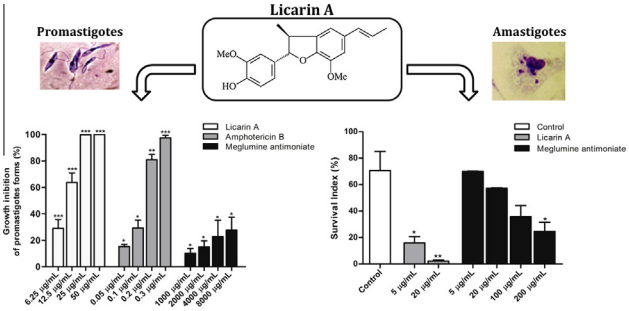
HIGHLIGHTS

- Licarin A inhibits the proliferation of *Leishmania (L.) major* promastigotes *in vitro*.
- This neolignan induces DNA fragmentation on *L. (L.) major* promastigotes.
- Licarin A is more effective against *L. (L.) major* intracellular amastigotes.
- Decrease in IL-6 and IL-10 cytokines levels suggest immunomodulation by licarin A.

GRAPHICAL ABSTRACT



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ABSTRACT

Leishmaniasis' treatment is based mostly on pentavalent antimonials or amphotericin B long-term administration, expensive drugs associated with severe side effects. Considering these aforementioned, the search for alternative effective and safe leishmaniasis treatments is a necessity. This work evaluated a neolignan, licarin A anti-leishmanial activity chemically synthesized by our study group. It was observed that licarin A effectively inhibited *Leishmania (Leishmania) major* promastigotes (IC_{50} of 9.59 ± 0.94 μg/mL) growth, by inducing in these parasites genomic DNA fragmentation in a typical death pattern by apoptosis. Additionally, the neolignan proved to be even more active against intracellular amastigotes of the parasite (EC_{50} of 4.71 ± 0.29 μg/mL), and significantly more effective than meglumine antimoniate (EC_{50} of 216.2 ± 76.7 μg/mL) used as reference drug. The antiamastigote activity is associated with an immunomodulatory activity, since treatment with licarin A of the infected macrophages induced a decrease in the interleukin (IL)-6 and IL-10 production. This study demonstrates for the first time the antileishmanial activity of licarin A and suggests that the compound may be a promising in the development of a new leishmanicidal agent.

1. Introduction

Leishmaniasis, an infectious parasitic diseases complex is caused by protozoans from *Leishmania* genus. These diseases are included in the neglected tropical diseases (NTD) group. NTD are chronic infections that are most common in countries with unfavorable socioeconomic conditions (Feasey et al., 2010; WHO,

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2010). According to World Health Organization (WHO), leishmaniasis is endemic in 88 tropical and subtropical countries, where it is estimated that about 12 million of people are already infected with these parasites, with incidence of 2 million new cases per year, and approximately 350 million of people live in risk areas (WHO, 2010).

Leishmaniasis is characterized by a spectrum of clinical manifestations ranging from simple ulcerative skin lesions to disfiguring mucocutaneous forms, and visceral forms that can be fatal if not treated (Murray et al., 2005; David and Craft, 2009). In the last 60 years the pentavalent antimonials such as meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostan®) have been used as the first-line treatment for all forms of leishmaniasis (Balaña-Fouce et al., 1998; Croft et al., 2006). However, these drugs have several limitations and disadvantages such as painful parenteral administration, high cost, long-term treatment, and serious side effects which include hepato- and cardiotoxicity (Balaña-Fouce et al., 1998; Croft et al., 2006). In refractory cases, second line drugs such as amphotericin B and pentamidine are used. However, these drugs also present high toxicity (Balaña-Fouce et al., 1998; Croft et al., 2006). Thus, the search for new antileishmanial drugs that are more effective against parasite and exhibit low toxicity to the host is a matter of dire necessity.

Neolignans are plants secondary metabolites, usually dimers formed through the oxidative coupling of allyl and propenylphenols, either through intermonomer linkages or crossed-linkages (Barbosa-Filho, 2004). Several biological and pharmacological effects have been reported for this class of secondary metabolites, including antitumoral (Lee et al., 2004; Kang et al., 2012), antimicrobial (León-Díaz et al., 2010), trypanocidal (Cabral et al., 2010; Pelizzaro-Rocha et al., 2011), antileishmanial (Aveniente et al., 2007; Vendrametto et al., 2010) and antimalarial (Zhang et al., 2001) activities among others. Licarin A is a neolignan originally isolated from plants (Barbosa-Filho et al., 1989) but it chemical synthesis is feasible (Chioccara et al., 1993; Nascimento et al., 2000). Licarin A presents several biological activities, among which is the antiparasitic activity against *Trypanosoma cruzi* and *Schistosoma mansoni* (Cabral et al., 2010; Pereira et al., 2011). In the present study, we investigated antileishmanial activity of licarin A. It was observed that the neolignan presents antipromastigote activity associated with fragmentation of the parasites' DNA and antiamastigote activity that correlated well with modulation of cytokine production by macrophages infected with *Leishmania (L.) major*.

2. Materials and methods

2.1. Parasites

Promastigote forms of *L.* (*L.*)*major* (MHOM/IL/1980/FN) used in this work were generously provided by Dr. Maria Norma Melo (Laboratório de Biologia de *Leishmania* – Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil). The culture was maintained *in vitro* at 25 ± 1 °C in test tubes containing biphasic culture medium, consisting of a solid medium agar-blood "Novy-MacNeal and Nicolle" (NNN) and Schneider medium (Sigma–Aldrich™, St. Louis, USA) supplemented with 20% fetal bovine serum (FBS) (Cultilab, São Paulo, Brazil), streptomycin (100 μg/ml) and penicillin (100 U.I/mL) (Cultilab, São Paulo, Brazil) (Jacob et al., 2013). The parasites were maintained *in vivo* in Balb/c mice infected in the left hind paw.

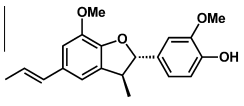

Fig. 1. Chemical structure of neolignan licarin A.

2.2. Licarin A and commercial drugs

Licarin A (Fig. 1) was synthesized by our study group according to our patent application PI 1101946-8, dealing with "Biocatalyst oxidative process using coconut water from *Cocos nucifera* as medium reaction and peroxidase source". The licarin A was subjected to nuclear magnetic resonance (NMR) of ^{1}H e ^{13}C for identification, and was identified as the enantiomer (−)-licarin A. Yield: 60%; m.p. 108 °C (CHCl3). αD25 = −20° (CHCl3; c 1,0). 1H-NMR (d, 200 MHz, CDCl3): 6,96 (1H, s, H6′), 6,88 (2H, sl, H2′eH5′), 6,77 (1H, sl, H6), 6,75 (1H, sl, H2), 6,36 (1H, d, Ha, J = 15,7), 6,09 (1H, dq, Hb, J = 15,7 e 6,6 Hz), 5,64 (1H, s, OH), 5,08 (1H, d, Ha′, J = 9,5 Hz,), 3,88 (3H, s, OCH3), 3,86 (3H, s, OCH3), 3,43 (1H, dq, Hb′, J = 9,5 e 6,8 Hz), 1,86 (3H, dd, Hl, J = 6,6 e 1,7 Hz), 1,36 (3H, d, Hl′, J = 6,8 Hz). 13C- NMR (d, 50,3 MHz, CDCl3): 146,60 (C4),146,48 (C5′), 145,70 (C5), 144,08 (C4′), 133,20 (C3), 132,13 (C1′), 132,00 (C1), 130,87 (Ca), 123,44 (Cb), 119,93 (C2′), 114,00 (C3′), 113,23 (C2), 109,10 (C6), 108,83 (C6′), 93,72 (Ca′), 55,89 (OMe), 45,60 (Cb′), 18,39 (Cl), 17,50 (Cl′). The data obtained was in concordance with the literature (Barbosa-Filho et al., 1989; Pereira et al., 2011)

The neolignan obtained was diluted in DMSO (Sigma–Aldrich™, St Louis, USA) to prepare a 20 mg/mL stock solution. For each experiment, working solutions (0.1% DMSO) were prepared by diluting the stock solution in culture medium. We used as reference drugs the pentavalent antimony (Sbv) meglumine antimoniate (Glucantime® – Aventis Pharma™, SP, Brazil) and amphotericin B (Cristália, SP, Brazil).

2.3. Animals

Female Balb/c mice (8–10 weeks) obtained from Keizo Asami Immunopathology Laboratory (Universidade Federal de Pernambuco, Recife, Brazil) and female Swiss mice (*Mus musculus*) (8–10 weeks) obtained from Professor Thomas George Animal House (Centro de Biotecnologia, Universidade Federal da Paraíba, João Pessoa, Brazil) were used. All animals were kept in an environment with temperature controlled at 21 ± 2 °C, light and dark cycles of 12/12 h and had free access to food and water. All experimental protocols were previously approved by the Ethics Committee for Animal Research (CEPA) of Universidade Federal da Paraíba (case number 0410/10).

2.4. Antipromastigote activity of licarin A

The sensitivity of *L. (L.) major* promastigotes to licarin A, amphotericin B and meglumine antimoniate was evaluated as previously described (Amorim et al., 2013). Briefly, logarithmic growth phase of *L. (L.)* major promastigotes (1×10^6 cells/mL) were incubated in Schneider medium supplemented with 20% FBS, streptomycin (100 μg/mL) and penicillin (100 U.I./mL) in the presence and absence of different concentrations of the substances evaluated. After 72 h of growth at 25 °C, samples were quantified in Neubauer chamber under optical microscope. Each experiment was performed in duplicate and was repeated at least as three independent experiments. The 50% inhibitory concentration (IC_{50}) was calculated by Probit analysis (SPSS for Windows 8.0).

2.5. DNA fragmentation analysis

For DNA fragmentation analysis, logarithmic growth-phase of *L. (L.) major* promastigotes were treated with different licarin A concentrations for six hours at 25 ± 1 °C. In parallel the promastigotes

were treated with H_2O_2, used as positive control for inducing DNA fragmentation in promastigotes of *Leishmania* sp. (Das et al., 2001). These parasites were subjected to DNA extraction using chloroform/phenol method (Sambrook et al., 1989). The DNA samples were analyzed in agarose gels (2%) stained with ethidium bromide, and the electrophoresis was performed for 1.5 h at 60 mV. The result was visualized using a UV transilluminator (ImageMaster®).

2.6. Peritoneal macrophages isolation

Peritoneal macrophages from Swiss and Balb/c mice were isolated as described by Oliveira et al. (2005). Briefly, mice were elicited with 1 mL of thioglycolate (3%) injected intraperitoneally. After 5 days, animals were euthanized by cervical dislocation and the peritoneal cavity was washed with 10 mL of PBS (phosphate buffer saline pH: 7.4) supplemented with FBS (3%) to obtain the macrophages. The cells were centrifuged for 10 min at 111*g* and suspension was adjusted with RPMI 1640 medium (Cultilab, SP, Brazil) supplemented with 10% FBS.

2.7. Cytotoxicity assay and selectivity index determination

Licarin A cytotoxicity was evaluated against Swiss mice peritoneal macrophages using the colorimetric assay of 3-(4,5-dimethylthiazol-2-yl) -2,5-difeniltetrazolium bromide (MTT) (Mosman, 1983) and dye exclusion assay trypan blue vital (Freshney, 1994) as performed by Monte Neto et al. (2008). Briefly, for MTT analysis, murine peritoneal macrophages (4×10^5 cells/200 μL) were incubated in 96-well plates in the presence or absence of licarin A at 37 °C in a 5% CO_2 atmosphere for 24 h. Then, MTT (5 mg/mL) was added in each well and after 4 h, 10% sodium dodecyl sulfate (SDS) was added. The results were analyzed on spectrophotometer at 540 nm. For trypan blue viability dye assay, murine peritoneal macrophages (1×10^6 cells/mL) were incubated in polypropylene tubes in the neolignana presence or absence. After 24 h at 37 °C in 5% CO_2 atmosphere, cells were quantified in Neaubauer chamber with 0.4% trypan blue. Viability of the cells exposed to licarin A was determined by comparing the culture with control (no drug). Each experiment was performed in duplicate, with at least three independent experiments. The concentration that caused a 50% reduction in cell viability (CC_{50}) was calculated by Probit analysis (SPSS for Windows 8.0). The selectivity index (SI) was calculated by dividing the CC_{50} for peritoneal macrophages by IC_{50} for *L. (L.) major* promastigote forms (Amorim et al., 2013).

2.8. Licarin A activity against intracellular amastigotes

Macrophages infected with *L. (L.) major* treatment was performed as described by Amorim et al. (2013). Briefly, peritoneal macrophages from Balb/c mice (5×10^5 cells/mL) were incubated in 24-well plates for 2 h at 37 °C and 5% CO_2, and then infected with stationary growth-phase of *L. major* promastigotes (5×10^6 cells/mL) in the ratio of 10 promastigotes: 1 macrophage. After 3 h of infection, each well was washed three times with prewarmed RPMI 1640 medium to remove non-ingested promastigotes and fresh medium was added. The infected macrophages were treated with different concentrations of licarin A or meglumine antimoniate, used as a positive control, for 24 and 72 h. About 300 macrophages were analyzed in each stained coverslip under light microscopy. Each experiment was performed in duplicate and repeated at least three times. The survival index was determined by multiplying the number of infected macrophages and the number of amastigotes per infected macrophage (Vendrametto et al., 2010). These values were used to determine the 50% effective concentration (EC_{50}) of licarin A by Probit analysis (SPSS for Windows 8.0).

2.9. Cytokines and nitric oxide production

The supernatant obtained from the antiamastigote assays were stored at −20 °C for TNF-α, IL-6, IL-10 and nitric oxide (NO) production analysis. Cytokines determination was performed by sandwich ELISA assay according to manufacturer's instructions (eBioscience™, USA). Supernatants were also evaluated for nitrite production by the Griess reaction as a measure of NO production (Green et al., 1982). The cytokines limit detection varied from 7 to 1000 pg/mL for TNF- α and IL-6 and from 31 to 4000 pg/mL for IL-10.

2.10. Statistical analysis

The data obtained were presented as mean ± standard error of the mean (S.E.M.). Student's *t*-test was used to evaluate the individual data significance, and one-way analysis of variance (ANOVA) for comparison between groups. For statistical analysis GraphPadPrism software version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used. Only values of $P \leqslant 0.05$ were considered as significant.

3. Results

3.1. Licarin A presents antipromastigote activity associated with DNA fragmentation

In order to determine licarin A effect against *L. (L.) major* promastigote forms, parasite growth inhibition in the presence of the neolignan was analyzed (Fig. 2). Licarin A inhibited *L. (L.) major* promastigotes growth at all concentrations analyzed, resulting in IC_{50} of 9.59 ± 0.94 μg/mL (29.38 ± 2.88 μM). In parallel, the reference drugs meglumine antimony (Sb^v) and amphotericin B were also analyzed. It was found that the Sb^v inhibited the promastigotes growth only at high concentrations (IC_{50} > 8.0 mg/mL). On the contrary, amphotericin B inhibited *L. (L.) major* promastigotes growth as little as from 0.05 μg/mL concentration, resulting in an IC_{50} of 0.13 ± 0.008 μg/mL.

In order to investigate the mechanisms associated with the antipromastigote activity of licarin A, we analyzed if this neolignan could be inducing cell death by apoptosis. Licarin A was found to induce *L. (L.) major* promastigotes genomic DNA fragmentation after 6 h of exposure to 4 and 8 times of its IC_{50} concentration

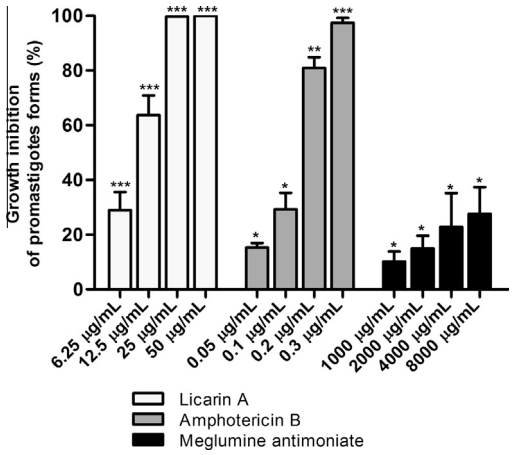

Fig. 2. *L. (L.) major* promastigotes growth inhibition in the presence of different licarin A, amphotericin B and meglumine antimoniate concentrations. The graph represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. $^*P \leqslant 0{,}05$; $^{**}P \leqslant 0{,}01$; $^{***}P \leqslant 0{,}001$.

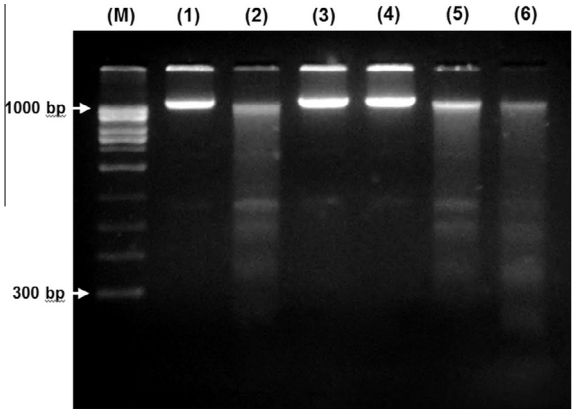

Fig. 3. *L. (L.) major* promastigotes genomic DNA treated with different licarin A concentrations or H_2O_2 4 mM. The agarose gel (2%) was stained with ethidium bromide (0.25 μg/ml) and the analysis was performed under UV light. (M) molecular marker 1 kb DNA (1) control; (2) H_2O_2 4 mM (positive control); Licarin A at concentrations of IC_{50} (3), 2× IC_{50} (4), 4× IC_{50} (5) e 8× IC_{50} (6).

(Fig. 3). The result was similar to that observed on treatment of the parasites with hydrogen peroxide (H_2O_2) at 4 mM, used as positive control of cell death by apoptosis.

3.2. Licarin A cytotoxicity and selectivity index

Licarin A cytotoxicity on murine macrophages analysis was investigated (Fig. 4) by trypan blue exclusion assay (Fig. 4a) and MTT reduction method (Fig. 4b). Licarin A cytotoxicity tests revealed that there was no observed change in cell viability at up to 400 μg/mL concentration by the MTT reduction method (Fig. 4b). A significant reduction in cell viability was only observed at 800 μg/mL concentration, resulting in a CC_{50} of 729.80 μg/mL. These values were used to calculate the selectivity index, thus obtaining a value of 76.10. Licarin A cytotoxicity assessed by trypan blue exclusion assay gave 308.96 μg/mL as CC_{50} (Fig. 4a), which resulted in a selectivity index of 32.21.

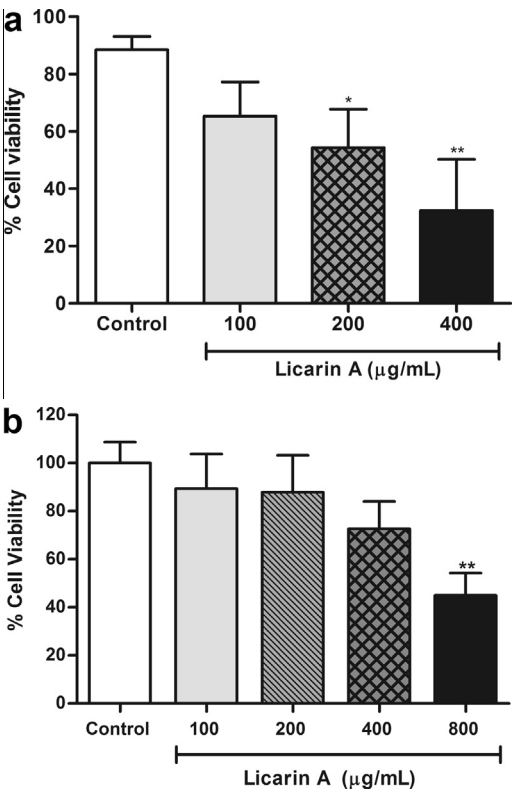

Fig. 4. Licarin A cytotoxicity on Swiss mice peritoneal macrophages. Cell viability was assessed by trypan blue exclusion assay (a) and by MTT reduction method (b). The graph represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$.

3.3. Licarin A antiamastigote activity on macrophage infected with L. (L.) major

To evaluate the antiamastigote activity, murine macrophages infected *in vitro* with *L. (L.) major* were exposed to licarin A (Fig. 5). The treatment for 24 h with the neolignan induced a survival index decrease of macrophages infected with *L. (L.) major* for both concentrations analyzed (Fig. 5a). After 72 h of exposure we observed an even greater reduction in the survival index, resulting in an EC_{50} of 4.71 ± 0.29 μg/mL (Fig. 5b). In parallel, the reference drug meglumine antimoniate (Sb^v) was only able to induce a significant reduction in the survival index at 200 μg/mL, after both periods of treatment evaluated. Thus, the EC_{50} of Sb^v was calculated to be 216.2 ± 76.7 μg/mL after 72 h of treatment.

3.4. Cytokines and nitric oxide production

To evaluate possible immunomodulatory activity of licarin A on macrophages infected with *L. (L.) major*, the levels of TNF-α, IL-6, IL-10 and NO were measured in the supernatant of infected macrophages (Fig. 6). We observed that treatment with the neolignan do

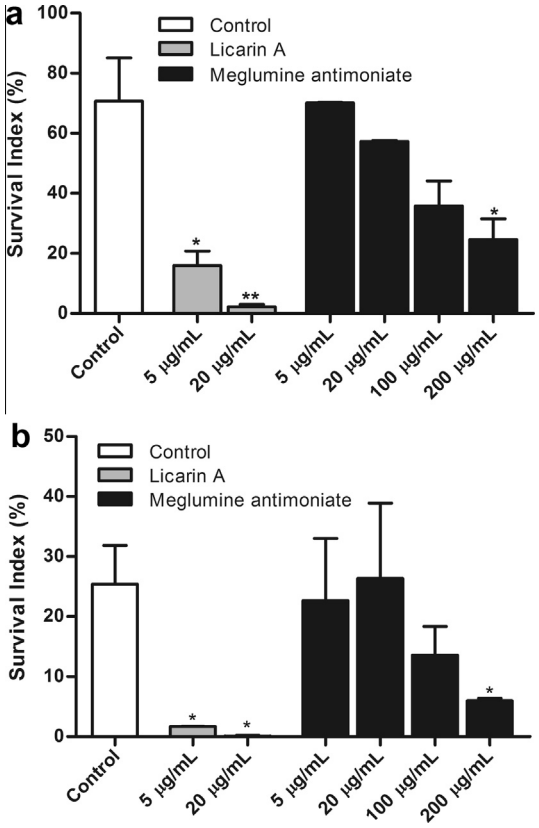

Fig. 5. Survival index of macrophages infected with *L. (L.) major*. The macrophages were infected with *L. (L.) major* and treated with licarin A or meglumine antimoniate for 24 h (a) and 72 h (b). The graph represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$.

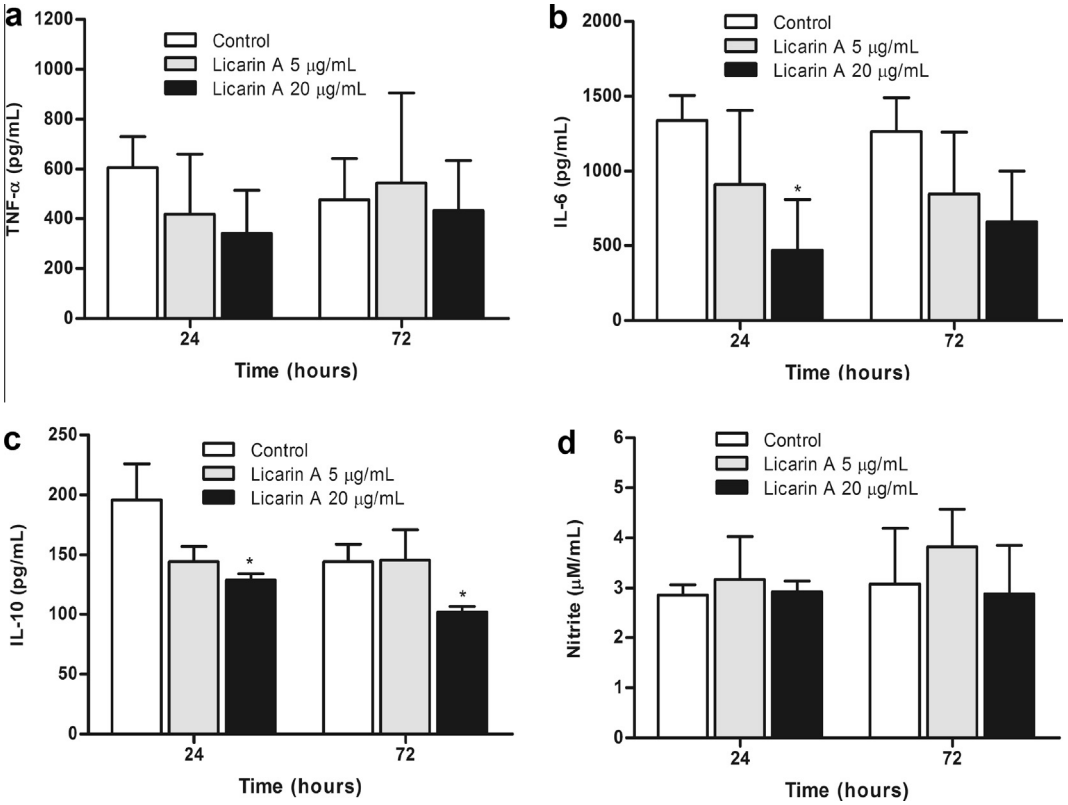

Fig. 6. Licarin A effect in TNF-α (a), IL-6 (b), IL-10 (c) and NO (d) production. The cytokines and NO concentrations were measured in the supernatants culture of macrophages infected with *L.* (*L.*) *major* and treated with licarin A for 24 h or 72 h. The graph represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. $^{*}P \leqslant 0.05$.

not alter TNF-α production (Fig. 6a). However, IL-6 production (Fig. 6b) and IL-10 (Fig. 6c) were reduced after treatment with 20 μg/mL of licarin A. NO levels were not affected after different treatments with licarin A (Fig. 6d).

4. Discussion

Drugs commonly used in the treatment of leishmaniasis, such as pentavalent antimonials and amphotericin B, are parenterally administered drugs which have high toxicity (Balaña-Fouce et al., 1998 Croft et al., 2006), requiring in many cases hospitalization of the patient, which makes this treatment very expensive. Thus, researches aimed at obtaining alternative treatments for these diseases are warranted.

Licarin A is a neolignan isolated from several plant species (Barbosa-Filho et al., 1989; Barros et al., 2009) but its chemical synthesis is also viable (Chioccara et al., 1993; Nascimento et al., 2000). Given the poor yield of licarin A isolation from plants (Barbosa-Filho et al., 1989), the possibility of chemical synthesis is a viable solution for the production of this neolignan in satisfactory quantity. In this context, licarin A used in the present study was synthesized by our study group through a methodology using *Cocos nucifera* coconut water as reaction medium and source of peroxidase according to our patent application PI 1101946-8 (manuscript in preparation). This synthesis demonstrates the economic viability, not only due to the use of an abundant natural source of peroxidase, but also to because it presents good yield and have stereoselectivity, producing mainly the (−)-licarin A enantiomer.

In the searching for new molecules with anti-leishmanial activity, this study demonstrated that licarin A presents *in vitro* activity against *L. (L.) major*. The results show that licarin A has a dose-dependent effect on the promastigotes growth of *L. (L.) major*. The IC_{50} value presented by the neolignan demonstrated that it is more active than the reference drug, meglumine antimoniate (Sb^{v}). However, available information from literature show that Sb^{v} has lower toxic action against *Leishmania* promastigotes forms compared with the amastigotes forms (Ephros et al., 1999; Vermeersch et al., 2009). It was observed that amphotericin B was more effective than licarin A against *L. (L.) major* promastigotes. Although this antifungal is used as second-line drug in leishmaniasis treatment, it has been reported to be highly toxic with serious associated side effects, such as nephrotoxicity (Balaña-Fouce et al., 1998).

Previous studies have shown that licarin A has several important pharmacological properties, among them the antimicrobial activity against multidrug resistant *Mycobacteria* (León-Díaz et al., 2010), cytotoxic and antiproliferative effect on human cancer cell lines (Lee et al., 2004; Park et al., 2004), schistosomicidal effect (Pereira et al., 2011) and trypanocidal activity (Cabral et al., 2010; Pereira et al., 2011). Considering licarin A antiparasitic activity, we noted that the trypanocidal activity observed against *T. cruzi* trypomastigotes for the enantiomer (−)-licarin A (IC_{50}/24 h 23 μM) (Pereira et al., 2011) was similar to the anti-leishmanial effect investigated in this study on *L. (L.) major* promastigotes (IC_{50}/72 h 29.38 ± 2.88 μM). However, comparing our antipromastigote results with those of Cabral et al. (2010) on *T. cruzi* epimastigotes (IC_{50}/96 h 462.7 μM) and trypomastigotes (IC_{50}/24 h 960 μM), these data seem to suggest that licarin A presents a greater *in vitro* anti-leishmanial than trypanocidal activity. Nevertheless, Cabral et al. (2010) did not report in their work the type of licarin A enantiomer used for tests against *T. cruzi* parasites.

The induction of programmed cell death (apoptosis) in parasites, such as if *Leishmania* species is associated with mechanism

actions of some anti-leishmanial drugs such as trivalent antimony (SbIII) (Sudhandiran and Shaha, 2003), amphotericin B (Lee et al., 2002) and miltefosine (Paris et al., 2004). In this context, genomic DNA fragmentation is one of the typical characteristics of cells undergoing apoptosis that occurs both in the metazoans (Stewart, 1994) as well as in the unicellular organisms (Das et al., 2001; Ardestani et al., 2012), in response to an inductor stimulus. The results obtained here demonstrate that treatment of *L. (L.) major* promastigotes with licarin A induced genomic DNA fragmentation ladder similar to that seen after treatment with hydrogen peroxide (4 mM), which is typical of apoptotic cells (Das et al., 2001).

One of the limitations of drugs used in leishmaniasis treatment is the problem of high toxicity (Balaña-Fouce et al., 1998; Croft et al., 2006). In the course of search for new effective and save drugs, some studies have demonstrated a strong correlation between the results of *in vitro* cytotoxicity evaluation using mammalian cells and in vivo acute toxicity in animals and humans (Evans et al., 2001). Thus, licarin A cytotoxicity assay demonstrated that the neolignan is more toxic to *L. (L.) major* parasite than to macrophages, the host cells of the amastigotes form. The trypan blue exclusion test, which evaluates cell membrane integrity, seems to be more sensitive in the cytotoxicity analysis than the MTT reduction colorimetric assay, as previously observed (Monte Neto et al., 2008). While MTT reduction assay measures the succinate dehydrogenase mitochondrial enzyme activity (Mosmann, 1983), the trypan blue exclusion test evaluates possible damage of licarin A to the plasma membrane integrity which may results in cell death (Freshney, 1994). Thus, the observed difference in the results of the *in vitro* cytotoxicity assay can partly be explained due to the difference in the methodological principles used. Additionally, other factor that may influence the differences in the cytotoxicity results is that in trypan blue tests the cells were in suspension and, therefore, presented a greater surface area of contact with the drug in comparison to MTT assay, in which cells adhered to plates. Nevertheless, considering the more sensitive of the assay, licarin A was approximately 32 times more toxic to *L. major* promastigotes than to the murine macrophages, demonstrating a high selectivity index. The low licarin A cytotoxicity against murine macrophages has also been reported by Cabral et al. (2010).

To evaluate the drug effect on the amastigotes, which is the stage present in vertebrate hosts, macrophages were infected with *L. (L.) major* and subsequently treated with the neolignan. Licarin A showed a significant antiamastigote activity, being able to reduce the survival index of intracellular amastigotes at non toxic concentrations for the host cell. On analyzing the EC_{50} value of licarin A, it was noted that the concentration required was approximately 45 times lower than the concentration required to reduce by 50% the survival index of the intracellular amastigotes when compared to meglumine antimoniate (Sb^v), after 72 h of treatment. These results demonstrate that the neolignan is more effective in the treatment of the macrophages infected with *L. (L.) major* than the Sb^v ($P < 0.025$), one of the first-line drug in leishmaniasis treatment (Balaña-Fouce et al., 1998; Croft et al., 2006).

The comparative analysis of licarin A anti-leishmanial activity shows that *L. (L.) major* intracellular amastigotes were more sensitive (EC_{50}/72 h 4.71 ± 0.29 μg/mL) to the neolignan than the promastigote forms (IC_{50}/72 h 9.59 ± 0.94 μg/mL) ($P < 0.0039$). Thus, the antiamastigote activity observed can be because of the drug direct action on the parasite as well as an indirect action, through induction of the macrophages activation to contain the infection. To analyze this possible immunomodulatory effect, production of TNF-α, IL-6 and IL-10 and nitric oxide by the infected macrophages *L. (L.) major* exposed to the neolignan was evaluated.

It is reported in literature that resistance to infection with *L. (L.) major* in inbred mice is related to a Th1-type immune response, which secretes cytokines such as IFN-γ and TNF-α resulting in macrophage activation and ultimately parasites death (Sacks and Noben-Trauth, 2002; Vila-del Sol et al., 2007). On the other hand, a Th2 response development, with cytokines production such as IL-4, IL-10 and TGF-β, contributes to *L. (L.) major* infected mice susceptibility due to a down regulation of Th1 cell differentiation and macrophage activation suppression (Sacks and Noben-Trauth, 2002). Among the oxygen metabolites toxic to *Leishmania* produced by activated macrophages are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and especially nitric oxide (NO) (Liew et al., 1990; Vila-Del Sol et al., 2007).

In the present work it was observed that licarin A antiamastigote activity is associated with a decrease in IL-6 and IL-10 production. Previous studies demonstrated that IL-6 participates in the differentiation of $CD4^+$cells into Th2 cells by inducing the IL-4 production, and inhibiting the signaling pathway of IFN-γ, preventing a Th1 response formation (Rincón et al., 1997; Diehl et al., 2000). This cytokine also inhibits, *in vitro*, anti-leishmanial activity induced by TNF-α and IFN-γ on macrophages infected with *L. (L.) amazonensis* (Hatzigeorgiou et al., 1993), and have a suppressive effect, *in vivo*, in the containment of *L. (L.) donovani* liver infection (Murray, 2008). IL-10 is an anti-inflammatory cytokine produced by various cells types such as macrophages, B lymphocytes, and various subsets of T lymphocytes (Couper et al., 2008), which inhibits macrophage activation, and consequently, the leishmanicidal activity of these cells (Vouldoukis et al., 1997; Trinchieri, 2001). Therefore, infection containment observed in macrophages infected with *L. (L.) major* after treatment with licarin A may be associated with this immunomodulatory activity, since decreased levels of IL-6 and IL-10 may be allowing the activation of macrophages by TNF-α produced by macrophages, although the TNF-α levels were not changed significantly after treatment with this neolignan. Furthermore, the antiamastigote activity observed was independent of NO production.

This work reveals that licarin A presents anti-leishmanial activity against *L. (L.) major* promastigote forms, associated with parasites DNA fragmentation, suggesting a programmed cell death mechanism, and on intracellular amastigotes, modulating cytokine production by host macrophages. Considering the feasibility coupled with the high yield of the licarin A synthesis, this compound is thus considered promising in the development of a new leishmanicidal agent.

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