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Leishmanicidal effect of LLD-3 (**1**), a nor-triterpene isolated from *Lophanthera lactescens*

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ABSTRACT

Leishmanicidal activity of 6α, 7α, 15β, 16β, 24-pentacetoxy-22α-carbometoxy-21β,22β-epoxy-18β–hydroxy-27,30-bisnor-3,4-secofriedela-1,20 (29)-dien-3,4 *R*-olide (LLD-3 (**1**)) isolated from *Lophanthera lactescens* Ducke, a member of the Malpighiaceae, was demonstrated against intramacrophage amastigote forms (IC_{50} of 0.41 μg/mL). The *in vitro* leishmanicidal effect of Glucantime, the first choice drug for leishmaniasis treatment, was increased by LLD-3 (**1**) association. The leishmanicidal effect of LLD-3 (**1**) was not due to stimulation of nitric oxide production by macrophages. LLD-3 (**1**) was also not cytotoxic for mouse peritoneal macrophages or B cells as assessed by the XTT and Trypan blue exclusion assays. LLD-3 (**1**) was unable to affect proliferation of naïve or activated B and T cells, as well as the B cells immunoglobulin synthesis. Cellularity of different tissues, liver and kidney functions were not altered in mice treated with LLD-3 (**1**), as well as the histology pattern of different organs. Our results add LLD-3 (**1**) as a potential drug candidate for treatment of leishmaniasis.

1. Introduction

Leishmaniasis is a neglected tropical disease prevalent worldwide, affecting over 12 million people in 88 countries, with an annual incidence of 2 million cases that is increasing with both urbanization and HIV co-infection (Rocha et al., 2005). The protozoan parasite *Leishmania* is responsible for several pathologies. These being comprised of cutaneous, mucocutaneous, and visceral leishmaniases; the latter form, may lead to death, if untreated. These protozoa have a digenetic life cycle. The motile flagellated promastigotes are transmitted by an insect vector to the vertebrate host, and these forms are phagocytosed by macrophages and transform into non-motile, replicative amastigotes inside the phagolysosome. These forms maintain the infection state while in the mammalian host. The first choice treatment for all forms of leishmaniasis still relies on pentavalent antimonials, which have been in clinical use since the beginning of the last century. Amphotericin B and pentamidine constitute alternative drugs for antimonial unresponsive cases. However, all of these compounds have serious side-effects such as high toxicity, development of parasite resistance, requirement for parenteral administration and high cost (Croft et al., 2005). Miltefosine, recently approved for the treatment of visceral leishmaniasis, has the advantage of being effective orally, but its use is restricted due to teratogenicity, high cost, and severe gastrointestinal side-effects (Mishra et al., 2007; Chappuis et al., 2007). Moreover, there are no vaccines in routine use. All of these problems and the few drugs that have emerged over the last years have stimulated the search for new compounds for leishmaniasis treatment, thereby renewing interest in plants as a source of new compounds (Berman, 2003; Croft et al., 2005).

Plants have been used mostly by people from leishmaniasis endemic areas. Their usage relies on phytotherapy, using topical and oral preparations for treatment of cutaneous and visceral forms of this disease, respectively. Based on this knowledge, different plants and isolated compounds have been evaluated for anti-*Leishmania* properties (Kayser et al., 2003; Anthony et al., 2005; Rocha et al., 2005). With the aim to search for leishmanicidal activity, we studied *Lophanthera lactescens* Ducke, a member of the Malpighiaceae found in the Amazon region of Brazil. It has been described that an infusion from leaves and bark of this plant has been used for malaria treatment by Amazonian Indians (Abreu et al., 1990). Phytochemical studies analyzing the wood of *L. lactescens* have described the presence of four steroids (stigmasta-4,22-dien-3-one; ergost-4en-3-one; stigmasterol; ergost-5-en-3-ol) and two triterpenes (β-amyrenone and 6α, 7α, 15β, 16β, 24-pentacetoxy-22α-carbometoxy-21β,22β-epoxy-18β–hydroxy-27,30-bisnor-3,4-secofriedela-1,20 (29)-dien-3,4*R*-olide [the last one named LLD-3 (**1**)]) (Abreu et al., 1990). Preliminary studies in rats have indicated that LLD-3 (**1**) (Fig. 1), a nor-triterpene that comprises 3% of the

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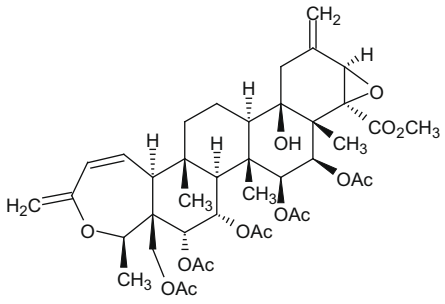

Fig. 1. LLD-3 (**1**) chemical structure.

wood dry weight, has analgesic effects and decreases the threshold for pentylenetetrazole-induced seizure (Abreu, 1985).

Our group has recently reported that an *Tabernamontanae australis* stem extract and an indolic alkaloid (coronaridine) purified from this extract had potent anti-leishmania (Delorenzi et al., 2001, 2002; Soares et al., 2007) and anti-HIV-1 activities (Silva et al., 2004). Coronaridine and its congener, 18-methoxycoronaridine, are ibogaine-derived alkaloids that have been shown to decrease self-administration of drugs (e.g., morphine, methamphetamine, nicotine) and attenuate opioid withdrawal in rats (Glick et al., 2006; Taraschenko et al., 2007). Opioids modulate the host immune response directly interacting with cell surface receptors and indirectly through the neuroendocrine circuits (Singh and Singal, 2007). Since preliminary studies have indicated that components from the extract of *L. lactescens* have morphinomimetic effects (Abreu, 1985), we decided to test whether LLD-3 (**1**) would have anti-leishmanial activity like coronaridine. Additionally, the toxicity of LLD-3 (**1**) was tested in macrophages, B and T lymphocytes as well as *in vivo,* analyzing different tissues in treated mice. Our results demonstrated that LLD-3 (**1**) inhibited growth of *Leishmania amazonensis* amastigotes in infected macrophages, presenting no cytotoxicity for the host cells. These findings characterize LLD-3 (**1**) as a potential drug for treatment of leishmaniasis.

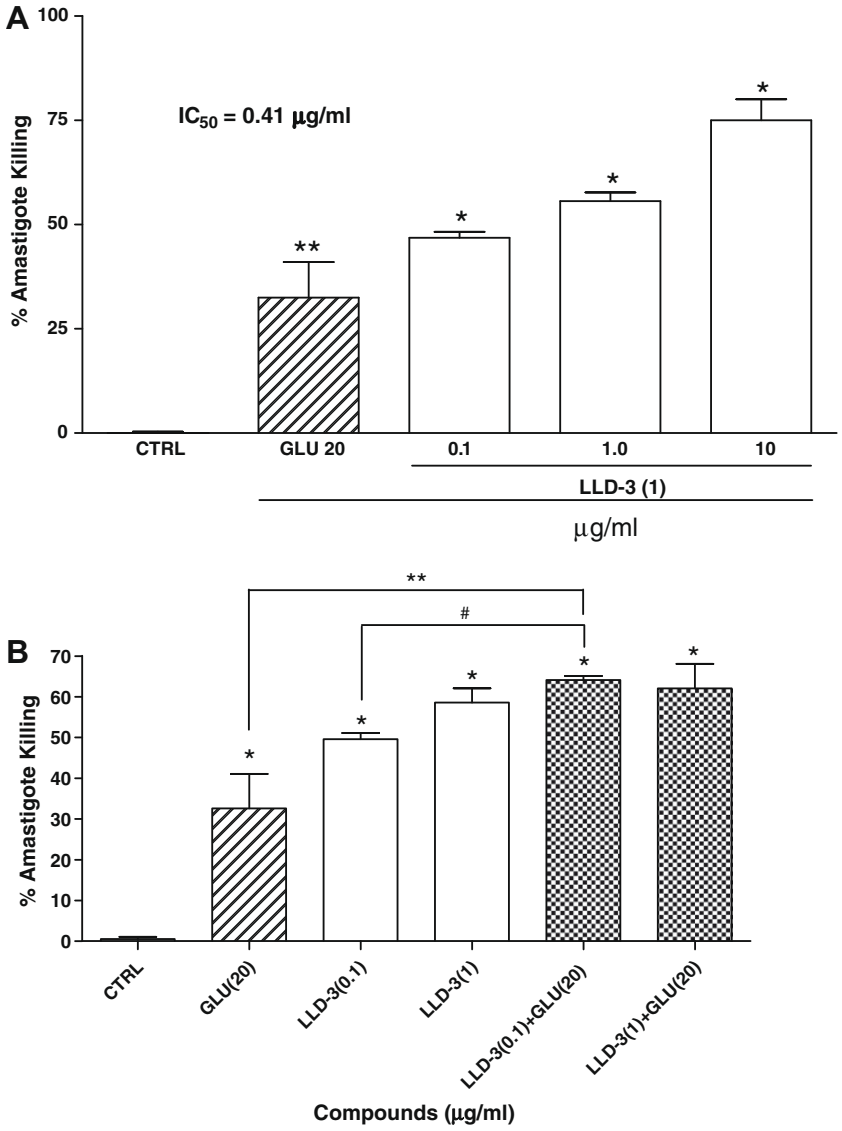

Fig. 2. Leishmanicidal activity of LLD-3 (**1**) on amastigote survival. *L. amazonensis*-infected mouse peritoneal macrophages were treated 24 h after infection with different concentrations of LLD-3 (**1**) (A) or with LLD-3 (**1**) associated or not with Glucantime (Glu) (B). Amastigote mortality rate was assessed 24 h after treatment. Results from three experiments performed in triplicates, are shown as percentage of amastigote killing + SEM in relation to untreated control (CTRL). (A) LLD-3 (**1**) treatment in relation to control, $^{*}p < 0.0001$; Glucantime treatment versus control, $^{**}p = 0.0008$. (B) Control versus all treatments $^{*}p = 0.0004$; $^{**}p < 0.05$; $\#p < 0.01$.

2. Results and discussion

The leishmanicidal activity of LLD-3 (**1**) was evaluated in *L. amazonensis*-infected mouse macrophages. Our results demonstrated that LLD-3 (**1**) inhibits the parasite survival in a dose-dependent manner with 75%, 55% and 46% inhibition of *Leishmania* growth at 10, 1 and 0.1 μg/mL, respectively (Fig. 2A). The calculated IC_{50} value of LLD-3 (**1**) was 0.41 μg/mL. This inhibition was mainly due to a decrease in the percentage of infected macrophages. A 50% decrease in the percentage of infected macrophages was obtained after treatment with 10 μg/mL of LLD-3 (**1**) in relation to untreated controls, although parasite numbers in macrophages treated or not with LLD-3 (**1**) were not significantly affected (data not shown). LLD-3 (**1**) is a potent compound, considering that an important leishmanicidal activity was observed in *L. amazonensis*-infected macrophages after a treatment of only 24 h. Actually, in this assay model, LLD-3 (**1**) was even more active than Glucantime, the first choice drug for leishmaniasis treatment. Glucantime used as a control inhibited 32% of amastigote growth. Interestingly, a potentiation in the Glucantime killing effect was observed with LLD-3 (**1**) association (Fig. 2B). The association of both compounds almost doubled the *Leishmania* mortality rate when compared with Glucantime alone ($p < 0.05$). In relation to LLD-3 (**1**), this association significantly increased parasite killing only at the lower LLD-3 (**1**) concentration assayed ($p < 0.01$). The mechanism of action of LLD-3 (**1**) is unknown and that of Glucantime is still poorly understood, although, for this last one, some targets have been suggested (Pathak and Yi, 2001; Walker and Saravia, 2004). This potentiation effect should be further analyzed, since combination therapy for leishmaniasis has been suggested to increase treatment efficacy, prevent the development of resistance and shortening the duration of treatment (Chappuis et al., 2007).

Nitric oxide (NO) production is considered as the most effective mechanism involved in *Leishmania* killing (Green et al., 1990). In

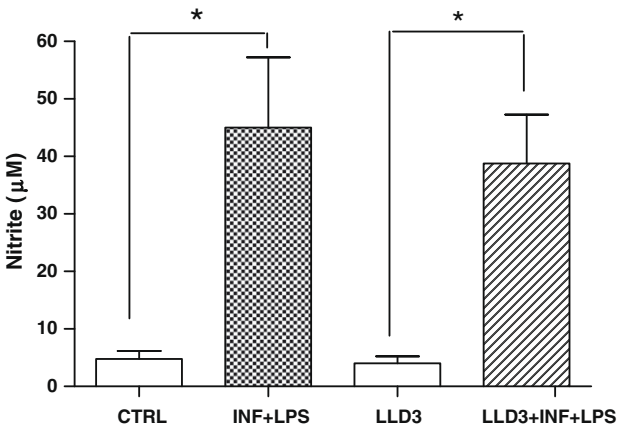

Fig. 3. Effect of LLD-3 (**1**) on nitric oxide production by macrophages. Non-activated (open bars) and IFN-γ plus LPS-activated peritoneal macrophages (filled bars) were treated with LLD-3 (**1**). Supernatants were harvested 24 h after treatment, and nitrite concentration was estimated by the Griess method. LLD-3 (**1**) was tested at 10 μg/mL. Data represent means + SEM of four different experiments performed in duplicate. (*$p < 0.05$).

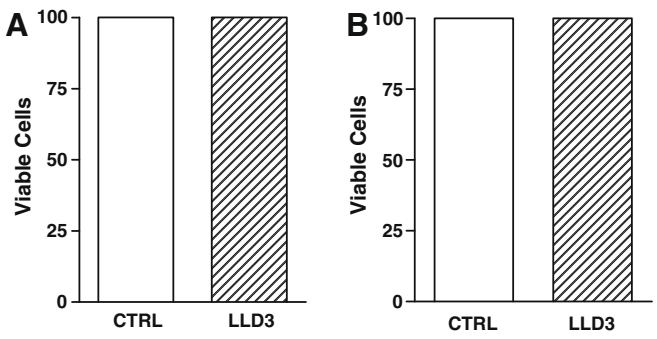

Fig. 4. LLD-3 **(1)** cytotoxicity for macrophages. Adhered macrophages were treated (LLD-3 **(1)**) or untreated (CTRL) with the 10 μg/mL of LLD-3 **(1)** and cytotoxicity was measured by XTT (A) and Trypan blue dye exclusion (B) methods. The results of two independent experiments performed in duplicate are expressed as percentage of viable cells + SEM.

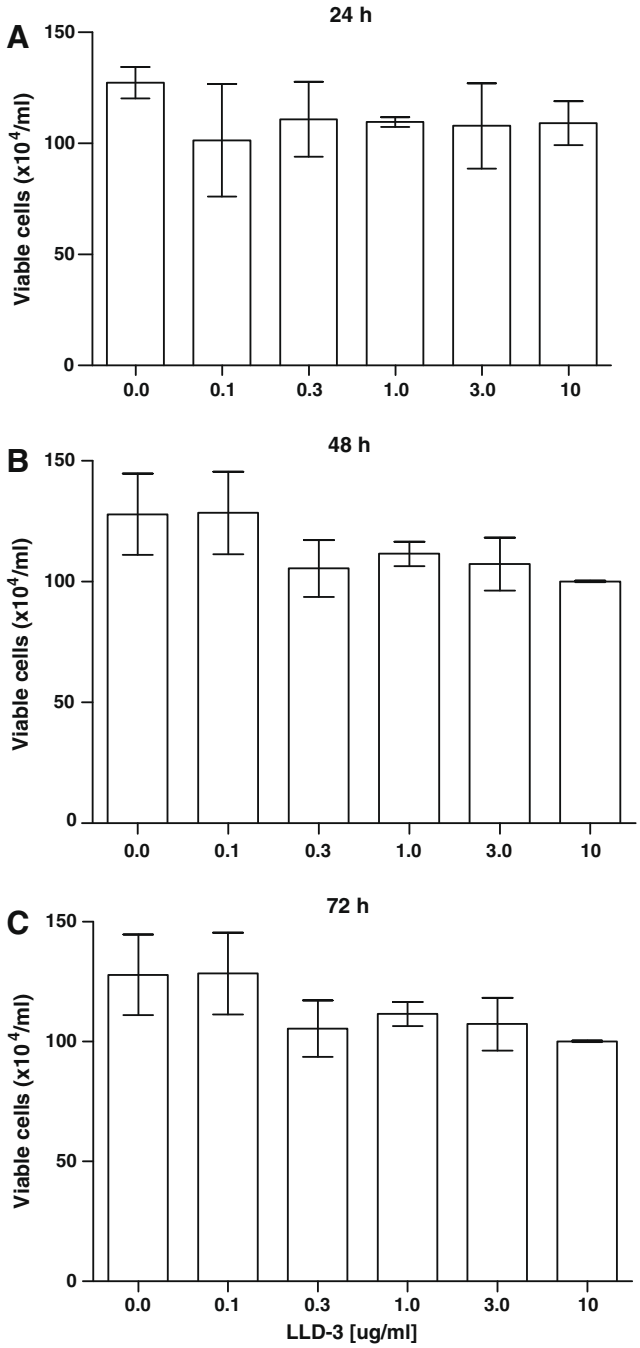

Fig. 5. LLD-3 (**1**) effect on spleen B cells viability. Spleen B cells were cultured in the presence of 10 μg/ml of LPS and different concentrations of LLD-3 (**1**). Cell viability was determined by Trypan blue exclusion assays at 24, 48 and 72 h after treatment. Results from three independent experiments are shown as mean + SEM.

general, a drug may act directly against the parasite or indirectly by activating macrophage killing mechanisms such as NO production. The anti-leishmanial effect of LLD-3 (**1**) could then be due to its ability to activate cell killing mechanisms in the host macrophages. Measurement of NO production showed that even at a higher dose of LLD-3 (**1**) (10 μg/mL) tested (Fig. 3), macrophages produced the same amount of nitrite (4.7 μM) as untreated cells (4.0 μM). Similarly, the same levels of nitrite were produced by IFN-γ plus LPS-activated macrophages, treated or not with LLD-3 (**1**) (45 μM versus 39 μM of nitrite). Also, LLD-3 (**1**) was unable to change nitrite production induced by macrophage activation with IFN-γ plus LPS. These results strongly suggest that the *Leishmania* killing mediated by LLD-3 (**1**) was independent of NO production.

Modulation of the immune response by morphine, a classical opiate, is reported to occur through suppression as well as stimulation effects (Singh and Singal, 2007). On leishmaniasis, morphine presented a dose-dependent biphasic modulation outcome: leishmanicidal activity in lower doses and exacerbation of parasite growth by higher doses, both *in vitro* and *in vivo* (Singal et al., 2002–2003; Singal and Singh, 2005; Singh and Singal, 2007). Also, morphine has been reported to modulate nitric oxide (NO) production in a bell-shaped dose–response curve. The dual effect of morphine occurs with NO up-regulation in lower doses and the opposite effect in higher doses (Singh and Singal, 2007). Although LLD-3 (**1**) shared morphine-mimetic effects (Abreu, 1985), its leishmanicidal activity in the tested concentrations seems to be different from morphine, as the anti-leishmanial activity and the NO production seems not to be biphasically modulated.

In order to verify the safety of LLD-3 (**1**) for macrophages, Trypan blue and XTT tests were carried out. In the XTT method, cells with damaged mitochondria were unable to metabolize XTT to a water-soluble formazan dye (Roehm et al., 1991). Our results showed that LLD-3 (**1**) was not toxic, as the mitochondrial activity or the membrane integrity of peritoneal macrophages was unaffected by any of the LLD-3 (**1**) concentrations (0.1–10 μg/mL) tested (Fig. 4). We also assayed LLD-3 (**1**) cytotoxic effect on spleen B cells from naïve mice cultured with 10 μg/mL of LPS and different concentrations of LLD-3 (**1**), at different time intervals (Fig. 5). LLD-3 (**1**) was not toxic for spleen B cells at any concentration or time interval (Fig. 5). A toxicity of 35% was observed at 100 μg/mL of LLD-3 (**1**) in all time points assayed (data not shown). Membrane integrity of red blood cells was also not affected by LLD-3 (**1**) treatment. Hemolysis induced by 0.1 and 1 μg/mL of LLD-3 (**1**) was, respectively, 2.4% and 3.1%, and 2.4% of hemoglobin was released by red blood cells incubated in PBS control (data not shown).

Immunological response of mice to LLD-3 (**1**) was tested *in vitro* analyzing T and B lymphocyte functions. Spleen B cells were stimulated with or without LPS (10 μg/mL) in the presence of 10 μg/mL LLD-3 (**1**) and proliferation measured after 48 h of culture. Our results demonstrated that LLD-3 (**1**) was unable to induce B cell proliferation as well as, incapable to significantly affect B cell proliferation induced by LPS (Fig. 6). It also neither induced proliferation of spleen T cells nor significantly changed T cell proliferation induced by Concanavalin A or Phytohaemagglutinin (Fig. 7). Immunoglobulin synthesis was also not affected by this compound. Its effect on immunoglobulin secretion was tested assaying IgM production using either naïve B cells and or LPS-activated B cells (Fig. 7A and B). Although a slight increase in IgM production

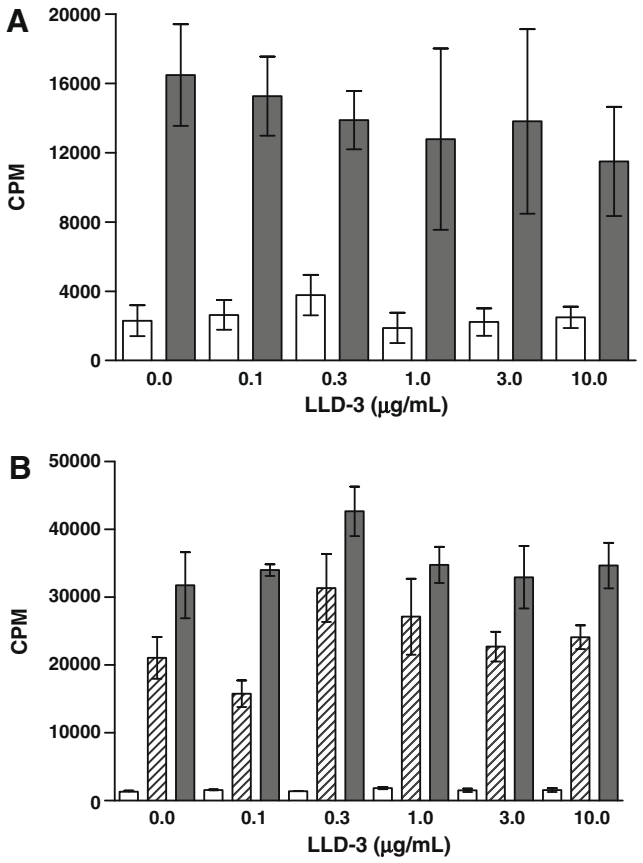

Fig. 6. LLD-3 (**1**) effect on spleen cells proliferation. (A) Spleen B cells were cultured in the absence (open bars) or in the presence of 10 μg/ml of LPS (black bars) and different concentrations of LLD-3 (**1**). (B) Spleen T cells were cultured in the absence (open bars) or in the presence of 40 μg/ml of Concanavalin A (hatched bars) or 40 μg/ml of phytohaemagglutinin (black bars) and different concentrations of LLD-3 (**1**). Both cells proliferation were determined by ^{3}H-timidine incorporation. Results from three independent experiments are shown as mean CPM + SEM.

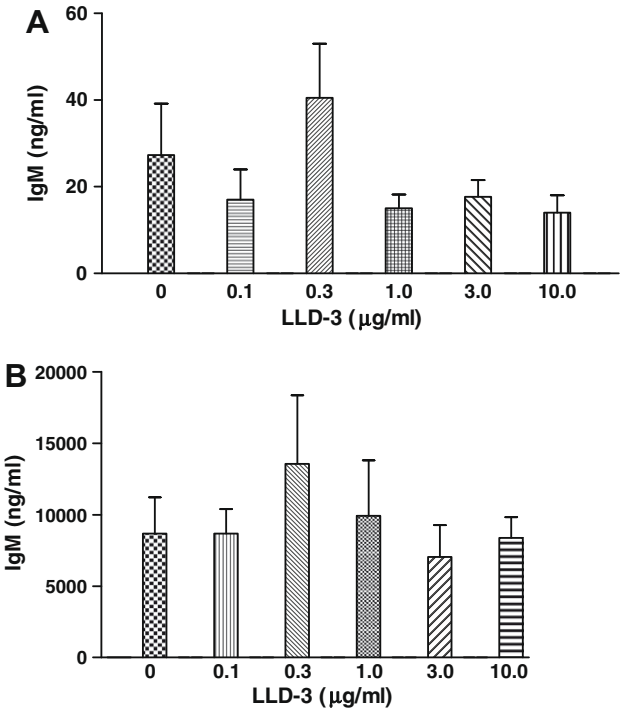

Fig. 7. LLD-3 (**1**) activity on IgM secretion by spleen B cells. Spleen B cells were cultured in the absence (A) or in the presence of 10 μg/ml of LPS (B) and the different concentrations of LLD-3 (**1**). IgM secretion was determined by ELISA using specific antiserum. Results from three experiments are shown as IgM concentration (ng/ml) + SEM.

Table 1
LLD-3 (**1**) Toxicity for mice tissue and organs.

Dose (μg/kg)	Blood ($\times 10^4$/μl)	Spleen ($\times 10^6$/ml)	Peritoneal Cells ($\times 10^5$/ml)	Bone marrow ($\times 10^6$/ml)	Thymus ($\times 10^6$/ml)
0.0	$19.16^a \pm 3.060$	$2.06^a \pm 0.261$	$0.98^a \pm 0.320$	$2.44^a \pm 0.830$	$6.84^a \pm 1.322$
50.0	$19.28^a \pm 3.961$	$2.06^a \pm 0.378$	$0.56^a \pm 0.270$	$3.64^a \pm 0.620$	$6.86^a \pm 1.422$
500.0	$15.48^a \pm 3.261$	$1.63^a \pm 0.403$	$0.86^a \pm 0.230$	$3.32^a \pm 0.642$	$7.22^a \pm 1.381$

Groups of five mice were IP injected twice a week with LLD-3 (**1**) (50 or 500 μg/Kg/animal) or the LLD-3 (**1**) vehicle (80% DMSO–H_2O) during 45 days. Tissues and organs were processed as described in the Section 4. Results are expressed by the group average ± standard deviation for five animals. Data were analyzed by ANOVA and (a) means no significant differences ($p > 0.05$).

was observed with naïve and LPS-activated B cells treated with 0.3 μg/mL of LLD-3 (**1**), it was not statistically significant, suggesting that it had no effect on immunoglobulin production. All these results reinforce parasite selectiveness of LLD-3 (**1**). Moreover, no alterations in blood levels of different enzymes, of urea as well as in the cell numbers recovered from several organs and tissues were observed in LLD-3 (**1**) treated mice.

LLD-3 (**1**) toxic properties were assayed *in vivo* treating three groups of mice twice a week by either intraperitoneal injection (50 or 500 μg/Kg/animal) or using a LLD-3 (**1**) vehicle for 45 days (Table 1). Cellularity in peripheral blood, thymus, bone marrow, peritoneal cavity and spleen was not different among the group inoculated with the LLD-3 (**1**) vehicle and the groups inoculated with either doses of LLD-3 (**1**) (Table 1). The LLD-3 (**1**)-treated animals were also tested for hepatic and kidney toxicity. No alterations in the blood levels of creatinine, urea, glutamic–oxalacetic and glutamic–pyruvic transaminases were observed (data not shown). Histopathological alterations were also not observed in livers and kidneys of LLD-3 (**1**) treated animals (data not shown).

Our results suggest that the anti-*Leishmania* activity of LLD-3 (**1**) could be due to a direct effect on the parasite. Actually, terpenes have been shown to affect several targets on *Leishmania* parasites, such as adenosine phosphoribosyl transferase, cysteine proteases and microtubules (Kapoor et al., 1999; Havens et al., 2000; Tiuman et al., 2005; Gray et al., 2006). Further studies need to be done to determine its parasite target.

The search for new classes of anti-leishmanial drugs has become imperative considering the increasing reports on host toxicity and parasite resistance to the few drugs currently in use for leishmaniasis therapy (Ouellette et al., 2008). In conclusion, this study showed the anti-amastigote activity of LLD-3 (**1**), adding a new potential drug candidate for treatment of this important disease.

3. Concluding remarks

The compound 6α, 7α, 15β, 16β, 24-pentacetoxy-22α-carbometoxy-21β,22β-epoxy-18β−hydroxy-27,30-bisnor-3,4-secofriedela-1,20 (29)-dien-3,4 *R*-olide (LLD-3 (**1**)) isolated from *L. lactescens* has a leishmanicidal effect on intramacrophage amastigote forms (IC_{50} of 0.41 μg/mL). Its leishmanicidal effect was not due to stimulation of nitric oxide production by macrophages. It was not cytotoxic for mouse cells (peritoneal macrophages or B cells), and was unable to affect proliferation of naïve or activated B and T cells, as well as B cell immunoglobulin synthesis. Moreover, cytotoxicity tested *in vivo* by cellularity of different tissues, liver and kidney functions, as well as the histology pattern of different organs were unaltered in mice treated with LLD-3 (**1**). Likewise, the Glucantime leishmanicidal effect potentiated by LLD-3 (**1**) merits further analysis as combination therapy has been suggested as a strategy to increase treatment efficacy. Taken together, our results indicate that LLD-3 (**1**) has promising anti-leishmanial activity and should be considered as a potential drug candidate for treatment of leishmaniasis.

4. General experimental procedures

4.1. LLD-3 (*1*). isolation

L. lactescens stems collected at Instituto de Floresta (voucher number 1419), Universidade Federal Rural Rio de Janeiro, Rio de Janeiro, Brazil, were dried for 6 months at room temperature and the wood was ground after bark removal. LLD-3 (**1**) was obtained as described by Abreu et al. (1990). Briefly, the wood powder, was extracted with benzene and the residue obtained treated with MeOH. LLD-3 (**1**) was obtained in the MeOH insoluble fraction and was crystallized using MeOH. LLD-3 (**1**) was used in all assays diluted in DMSO–(4:1 v/v) (DMSO, Sigma). Identification and purity of LLD-3 (**1**) was checked by chromatography (TLC) using an standard, as well by infrared spectroscopy and $^{13}C/^{1}H$ NMR spectroscopy, as reported by Abreu et al. (1990).

4.2. Parasites

L. amazonensis, (WHOM/BR/75/Josefa) promastigotes were cultured at 26 °C in Schneider Insect Medium (Sigma) supplemented with 10% fetal calf serum (FCS – Cripion) and 40 μg/mL of gentamycin (Schering–Plough, Rio de Janeiro, Brazil).

4.3. Anti-amastigote activity

Mouse peritoneal macrophages were stimulated with 3% thioglycolate during 4 days, harvested in RPMI, plated on 13 mm^2 coverslips inside 24-well plates and allowed to adhere for 2 h at 37 °C in 5% CO_2. Non-adherent cells were removed by washing, and macrophages were incubated overnight in RPMI supplemented with 10% FCS, at 37 °C, 5% CO_2. Adhered macrophages were infected with *L. amazonensis* promastigotes (stationary growth phase) at a 10:1 parasite/macrophage ratio and incubated for 1 h at 35 °C, 5% CO_2. Free parasites were washed out with PBS, pH 7.2 and cultures were maintained for 24 h at 35 °C in 5% CO_2 in RPMI supplemented with 10% FCS. LLD-3 (**1**) was added to the cultures and, after 24 h incubation as above, the cells were washed with PBS, fixed in MeOH, and stained with Giemsa. The number of amastigotes and the percentage of infected macrophages were determined by counting at least 200 cells in triplicate cultures. Endocytic indices were obtained by multiplying the percentage of infected macrophages by the mean number of amastigotes per infected macrophage. Experiments were made in accordance with ethical guidelines for care and handle of laboratory animals.

4.4. Nitric oxide production

Thioglycolate-stimulated mouse peritoneal macrophages obtained as above (5×10^5 cells/well in 24-well plates) were incubated with 10 μg/mL of LLD-3 (**1**) concomitant or not with 10% IFN-γ (4-days culture supernatant of L1210 cell line transfected with IFN-γ gene) and 100 ng/mL of LPS (*E. coli* O111:B4). After 24 h at 37 °C in 5% CO_2, nitrite concentrations in culture supernatants were determined by the Griess method (Green et al., 1990).

The reaction was read at 540 nm, and the concentration of nitrite was determined with reference to a standard curve using sodium nitrite. Results are expressed as micromolar concentrations of nitrite.

4.5. Macrophage cytotoxicity assays

Thioglycolate-stimulated mouse peritoneal macrophages (5×10^6 cells/mL) obtained as above were incubated for 24 h at 37 °C, 5% CO_2 with different concentrations of LLD-3 (**1**). Macrophages were then washed with PBS, incubated with 3% Trypan blue solution and scored for viable cells in an inverted microscope. Additionally, LLD-3 (**1**) cytotoxicity to mouse macrophages was determined by the reduction of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxinilide inner salt (XTT, Sigma) assay, according to Roehm et al. (1991).

4.6. Spleen B cells cytotoxicity

Murine spleen cell suspension was treated with a mixture of anti-Thy-1, anti-CD4 and anti-CD8 antibodies and Low Tox rabbit complement (Cedarlane Labs., Canada) as previously described (Brunswick et al., 1988). Enriched B cells suspension was then applied to a 1.086, 1.081, 1.074 and 1.062 g/mL Percoll (Pharmacia, Sweden) gradient as previously described (Rabin et al., 1985). High-density (resting) B cells recovered at the 1.086–1.081 interface (2.5×10^6 cells/mL) were incubated in RPMI (Gibco-BRL, NY, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), 2 mM L-glutamine (Sigma), 50 mM 2-mercaptoethanol (Sigma), 50 μg/mL gentamycin and 10 μg/mL of LPS (*E. coli* 0111:B4, Difco Lab. Inc.) with different concentrations of LLD-3 (**1**). Cell viability was determined by the Trypan blue exclusion assay at 24, 48 and 72 h of incubation.

4.7. Hemolytic assay

Hemolytic activity was tested mixing (v/v) 25% human red blood cell suspension with: distilled H_2O (100% hemolytic activity); 0.01 M Phosphate Buffered Saline (PBS) pH 7.2 and 0.1 and 1 mg/mL LLD-3 (**1**). Experiments done in triplicates were read at 541 nm after incubation at 37 °C for 24 h. The degree of hemolysis was calculated taking lysed red blood cells absorbance as 100%.

4.8. Proliferation assay

Splenic B cells obtained as above, and nylon wool purified splenic T cells (Hathcock, 1994) were cultivated with different concentrations of LLD-3 (**1**), on 96-well flat bottom plates in the presence or absence of different activators (B cell/LPS *E. coli* 0111:B4 [10 μg/mL; T cells/Concanavalin A [40 μg/mL, [Sigma] and phytohaemagglutinin HA16 [40 μg/mL [Murex Diagn.]) in a final volume of 0.2 mL in supplemented RPMI medium. Cell proliferation was measured after 48 h incubation by tritiated thymidine incorporation. Cultures were pulsed with 1 μCi of thymidine (specific activity of 5 Ci/mmol, Amersham Pharmacia Biotech) during the last 18 h of culture and thymidine incorporation determined by liquid scintillation spectroscopy. The results are expressed as the arithmetic mean of counts per minute (cpm) of triplicate cultures.

4.9. Measurement of Immunoglobulin (Ig) levels in culture supernatant

B cell (5×10^4 cells/well) obtained as above were cultured for 7 days with or without 10 μg/mL of LPS and different concentrations of LLD-3 (**1**). Afterwards culture supernatants were harvested and IgM levels were measured by sandwich ELISA (Snapper and Paul, 1987). Briefly, polystyrene plates were coated with polyclonal goat anti-mouse IgM (Sigma), followed by the addition of culture supernatants and standard solutions with known concentrations of IgM. After incubation, binding of alkaline phosphatase-labeled goat anti-mouse IgM antibody (Sigma) was detected by addition of the chromogenic substrate *p*-nitrophenylphosphate (Sigma). Absorbance of the product was measured at 405 nm in a Microplate Reader (BIO-RAD Labs, CA, USA). Absorbance at 405 nm values were converted to Ig concentrations by extrapolation from standard curves determined in each assay by using purified myeloma proteins of know concentrations (ICN Biomedical Inc., CA, USA).

4.10. In vivo toxicity assays

Female BALB/c mice ($n = 15$) were randomly allotted and equally divided into three groups and treated as follow: (1) inoculated with 80% DMSO (LLD-3 (**1**) vehicle); (2) treated with 50 μg of LLD-3 (**1**)/Kg/animal; (3) treated with 500 μg of LLD-3 (**1**)/Kg/animal. The stock solution of LLD-3 (**1**) and DMSO–H_2O (4:1, v/v) were diluted in PBS, pH 7.2, and animals were injected intraperitonealy twice a week during 45 days. Animals were maintained with food and water *ad libitum* during the whole experiment. Three days after the last dose animals were sacrificed and the following items were observed and measured: **(A) Liver and kidney functions:** were evaluated assaying glutamic–oxalacetic transaminase activity, glutamic–pyruvic transaminase activity, creatinine and urea in animals' pool of sera as recommended by manufacturer (Diagnostic Kits from BIOCLIN/Belo Horizonte, MG, Brazil). **(B) Histopathology:** microscopical analysis of liver and kidney was done after formalin (10%, pH 7.8) fixation. Organ thin sections were stained by haematoxilin–eosin (HE) and analyzed by optical microscopy. **(C) Cell counts:** cell numbers were determined in peripheral blood, thymus, bone marrow, peritoneal cavity and spleen by counting in a hemocytometer. Peritoneal cells were obtained after injection and recovery of 5 mLof RMPI. Organ cell suspensions were obtained by maceration of the whole organ in 10 mLof Türk solution.

5. Statistical analysis

Data were analyzed by Student's *t-test* when comparing two groups or one-way ANOVA for more than two groups followed by Tukey's multiple comparisons post-test, using the GraphPad Program. *P* values of less than 0.05 were considered significant.

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