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Seco-limonoid 11*α*,19*β*-dihydroxy-7-acetoxy-7-deoxoichangin promotes the resolution of *Leishmania panamensis* infection

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ABSTRACT

The high morbidity generated by the infection caused by parasites of the genus *Leishmania*, make of this infection into one of the vector-borne infectious diseases most relevant worldwide, which added to the fact that the drugs used for its treatment are far from be optimal and considering that prophylactic approaches (such as the development of a vaccine) still seems far from being achieved, make of the search for new therapeutic alternatives for safe and effective treatment of this disease one of the most accurate approaches to the control of this disease. In this study we evaluated the antileishmanial and immunomodulatory activity of the compound 11*α*,19*β*-dihydroxy-7-acetoxy-7-deoxoichangin (a *seco*-limonid molecule) through: 1) evaluation of its cytotoxicity over promastigotes and axenic amastigotes of *L.* (V) *panamensis*, 2) determination of its ability to induce the control of *in vitro* infection, using infected murine cells (J774.2) and human dendritic cells (hDCs), 3) quantifying the levels of pro-inflammatory cytokines, *(iv)* evaluating the expression of cell markers associated with hDCs maturation, and *(v)* determinating the production of nitric oxide free radicals (NO). In this regard, this *seco*-limonoid exhibited an antileishmanial activity represented in the reduction of *in vitro* infection in J774.2 cells and hDCs, with a EC_{50} of 7.9 µM (4.48 µg/mL) and 25.5 µM (14.39 µg/mL), respectively, and additionally, we observed an increase on the production of IL-12p70, TNF-*α* and NO, as also, in the number of hDCs HLA-DR-positive in treated infected hDCs. These findings suggest that anti-leishmanial activity of this compound could be associated with the potential "reactivation" of phagocytic cell that is "paralyzed" by the infection, generating an immune phenotype associated with protection.

Keywords: Leishmaniasis; Treatment; Immunomodulation; *Seco*-Limonoid

1. INTRODUCTION

Leishmaniasis is a parasitic disease of global public relevance, which is transmitted by the bite of sandfly species belonging to the genus *Lutzomyia* or *Phlebotomus,* infected with promastigotes of the genus *Leishmania.* According to reports getting for the World Health Organization (WHO), the transmission of the pathogens responsible for this disease occurs endemically in at least 98 countries, primarily affecting developing countries located in tropical and subtropical regions around the world [1,2]. Annually, at least 1.5 million of new cases of the cutaneous form of the disease (CL) and approximately 500,000 new cases of the visceral leishmaniasis (VL) are reported around the world, being the VL form the clinical presentation responsible for the deaths associated with the pathogen and the CL form the responsible of its high levels of morbidity [3].

Chemotherapy against leishmaniasis is based on the administration of pentavalent antimonial salts (as first-choice drugs) or the use of formulations of pentamidine®, paromomycin®, miltefosine® or amphotericin B®, as second-choice drugs. However, the mechanism of action of these drugs has not been well clarified yet, and this added to the fact that none of them was originally designed for the treatment of this disease and limitations associated with their administration routes [4,5], high costs and duration of the treatment, the emergence of parasites resistant to these drugs [3,6] and the high toxicity of some formulations, make necessary and urgent the develop of safer and more effective therapeutic alterna-

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tive to the better control this disease [1,7]. Among the elucidated to date in relation to the mechanism of action postulated for the drugs mentioned above, it have been found that the formulations of pentavalent antimony salts act as pro-drugs, needing for that, their reduction to trivalent form to perform their action, which is proposed to be directed against the trypanothione reductase and zinc-finger protein (proteins that are related with the protection of the parasites from oxidative damage and toxic heavy metals, and with the process of replication and repair of DNA, respectively) [8,9]. On the other hand, the mechanism of action of pentamidine (second drug of choice for the treatment of this disease, used when patients cannot tolerate the antimonial drugs, or in cases of resistance to these drugs) is associated with the accumulation and altering of the function of the kinetoplast, as well as the collapse of the membrane potential of the microorganism [10,11], while the mechanism of action of amphotericin B (a macrolide used for treatment of mucocutaneous and visceral leishmaniasis) is associated with its capability to bind to the ergosterol molecule (the major sterol of the *Leishmania* parasite membrane), leading to the formation channels that would alter the permeability of the cell membrane [12].

Recently, the study of compounds derived from plant species has become in a major tool for the developed of novel therapeutic agents against several infectious diseases, such as leishmaniasis [13-15], and in this regard, several research groups have screened a large number of natural compounds, with the aim of find potential chemical entities (prototypes) that can be used for the development of more effective and safer alternative therapies against these pathogens [16-18].

In previous studies, our research group demonstrated that the compound 11α,19β-dihydroxy-7-acetoxy-7-deoxoichangin (a *seco*-limonoid) exhibits an antileishmanial specific activity against the intracellular form of the parasite (corresponding to the most relevant stage for studying and designing possible therapeutic targets against this pathogen) [19,20]. This compound was isolated from the vegetal species *Raputia heptaphylla*, a species belonging to the family Rutaceae, and whose geographical distribution is concentrated in tropical areas of Central and South America (mainly reports its location in Costa Rica, Panama, Colombia, and forested areas of Brazil and Peru). Species belonging to the genus *Raputia* have been characterized by the present of abundant secondary metabolites (primarily alkaloids and limonoid), being an example of this, the studies carried out in *Raputia prateermisa* (other species of the genus *Raputia*), whose leaves have been used in decoction for treatment of cutaneous leishmaniasis and chagas disease in selvatic areas of Brazil, and whose phytochemical study showed that limonoids compounds are an important part of its composition to the secondary metabolites level [21], being them possibly responsible for some of the biological activities attributed to these plants.

In this study, we sought to confirm the antileishmanial activity of this *seco*-limonoid using for *in vitro* assays, murine macrophages and human dendritic cells (hDCs) infected with this protozoan, as also, we wanted to characterize its possible immunomodulatory effect on antigen-presenting cells (APCs), which are a critical population for the resolution of natural infections by parasites of the genus *Leishmania*, observing that the compound presents an antileishmanial activity associated with an immunomodulatory effect, that it is evident by the differential induction on the increase in the production of soluble pro-inflammatory mediators, such as IL-12p70 and TNF-α, and a significant increase in the expression of MHC class II molecules as also in the levels of nitric oxide (NO) on infected cells exposed to the *seco*-limonoid compared with infected cells without treatment.

2. MATERIALS AND METHODS

2.1. Cells

RPMI 1640 (Gibco BRL-Life Technologies Inc., Grand Island, NY, USA) medium was used for culturing the phagocytic cell line J774.2 (a murine macrophage cell line derived from the murine J774A.1 cells, which are monocyte/macrophage cells, obtained from reticulum cell sarcoma of ascites in BALC/c mouse, which are susceptible to the infection with *Leishmania* parasites), human dendritic cells (hDCs) derived from peripheral blood monocytes (obtained from healthy volunteers) and the promastigotes of *Leishmania* (Viannia) *panamensis* (*L.* (V) *panamensis*), which were either untransfected (MHOM/88CO/UA140) or transfected (MHOM/88/CO/UA140irGFP) with a plasmid (ir) encoding green fluorescent protein (GFP) (kindly donated by Dr. Sara Robledo from the Program for the Study and Control of Tropical Diseases; PECET-Programa de Estudio y Control de Enfermedades Tropicales, of the Universidad de Antioquia, Colombia [22]). The RPMI medium was supplemented with 2 mM L-glutamine (Gibco BRL-Life Technologies Inc.), 1% non-essential amino acids, 1000 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 ug/ml amphotericin B (Sigma Chemical Co., St. Louis, MO, USA), 24 mM sodium bicarbonate (Sigma Chemical Co.), and 25 mM HEPES (Gibco BRL-Life Technologies Inc.). The culture media were enriched with 10% fetal bovine serum (FBS) (Microgen, Bogotá, Colombia) for the J774.2 cells and parasites or with AB negative human plasma that was filtered and inactivated (Lonza Walkersville, MD, USA) for the culture of hDCs.

The mammalian cells were incubated at 36˚C - 37˚C in

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an atmosphere of 5% CO_2 and 90% humidity, whereas the promastigotes of *L.* (V) *panamensis* were grown at 25˚C - 26˚C.

The axenic amastigotes were produced from the culture of promastigotes of *L.* (V) *panamensis* in RPMI medium enriched with 20% FBS at pH 5.4, and differentiation was achieved by the gradual exposure of the cells to increases in temperature (29˚C, 32˚C, and 34˚C).

It should be note that depending on the assay, the monocytes used to differentiate hDCs were derived from 2 different sources of peripheral blood taken, either of them, from healthy donors. For the *in vitro* infection assays, the monocytes were obtained by leukopheresis and density gradient centrifugation of a single donor sample (that was screened for HIV-1, hepatitis B, hepatitis C, HTLV-1 and HTLV-2) (Lonza Walkersville, MD, USA), whereas to evaluate the compound-induced immunomodulation, the monocytes were obtained from heparinized blood samples from healthy volunteers without and with a medical history of active cutaneous presentation of the disease prior the treatment of the disease (six (6) and seven (7) individuals, respectively), and whose samples were taken after the explanation and subsequent signing of the informed consent (approval by the Ethics Committee, act 04 of June 2009 at the Faculty of Sciences of the Universidad Nacional de Colombia).

Briefly, for the monocytes derived from blood of volunteers without and with a medical history of leishmaniasis, the samples were centrifuged at 2500 rpm for 15 minutes, and the leuko-platelet layer (buffy coat) was collected and diluted in RPMI-1640 (Gibco BRL-Life Technologies Inc.). Subsequently, the cell suspension was used to prepare the density gradient by adding 1 volume of the ficoll-hypaque reagent (Invitrogen, USA) to 2 volumes of diluted blood sample, and the gradients were centrifuged for 30 minutes at 3000 RPM at room temperature, producing a layer of peripheral blood mononuclear cells (PBMCs), which was collected and washed by centrifugation at 2000 RPM for 10 minutes and at 2000 RPM for 7 minutes. Then, the PBMCs were cultured for 2 h at 37˚C in sterile Petri dishes (TPP, Techno Plastic Products, Switzerland) using the previously described culture medium. Ended the incubation time, the non-adherent cells were removed, and the adherent cells were incubated in RPMI-1640 medium supplemented with recombinant human GM-CSF at 1ng/mL (BD Biosciences, San Diego, USA) and human recombinant IL-4 at 1 ng/mL (BD Biosciences) for 5 - 7 days. The differentiation process of the hDCs, was monitored by evaluation of the cell morphology using an inverted microscope and the purity of hDCs was evaluated using flow cytometry. Finally, the yield and the cell viability were determined using trypan blue dye staining, and the cells were counted in a Neubawer chamber [23].

2.2. Compound

The plant material from the bark of *Raputia heptaphylla* used in this study was collected in Albán (Cundinamarca) in Colombia (a specimen of this species rests in the National Herbarium of Colombia, Institute of Natural Sciences, Universidad Nacional de Colombia, under the code COL. 511102). Briefly, to obtain the crude ethanolic extract, the dried plant material was subjected to extraction by percolation in 96% ethanol at room temperature, and the organic solvent was removed by distillation under reduced pressure in a rotavapor. Next, the metabolites were extracted from the ethanolic extract, and the purification of the *seco*-limonoid was performed using conventional chromatographic methods [18]. To elucidate the structure of this *seco*-limonoid compound (**Figure 1(a)**), spectroscopic methods were used (including ultraviolet, infrared (IR), nuclear magnetic resonance (NMR) of hydrogen and carbon, circular dichroism, and nuclear Overhauser enhancement spectroscopy (NOESY) experiments), as also data reported in the literature of related *seco*-limonoid.

Furthermore, the extracts, fractions, and the compounds isolated from the plant material as well, the culture medium used were subjected to evaluation for the presence of endotoxins using the LAL test and the reagent Pyrogent™ Plus Gel Clot LAL assay (Lonza Walkersville, MD, USA). This procedure was performed to ensure that any functional regulation of the hDCs infected with *L.* (V) *panamensis* and treated with the compound was due to the treatment and not to the presence of contaminating lipopolysaccharide (LPS) in the material. For this purpose, the experimental procedures were made according to the instructions recommended by Lonza.

2.3. Assays to Evaluate the Effects of the Cytotoxic Activity on Mammalian Phagocytic-Cell Targets of Infection with *L.* (V) *panamensis*

Using the resazurin test for cell metabolism detection, the toxicity of the *seco*-limonoid was evaluated according to its ability to damage murine macrophage cells (J774.2) and hDCs [24]. Briefly, 2×10^4 J774.2 cells or 3×10^4 hDCs were exposed to different concentrations of the compound [sequential 1:4 dilutions were used, using as maximum concentration 354.5 µM (200 µg/mL) and a minimum of 22 µM (12.5 ug/mL)] for 72 h at 36˚C - 37˚C in an atmosphere containing 5% CO_2 and 90% humidity. The controls included cells exposed to the reagents that were used to solubilize the *seco*-limonoid [dimethylsulfoxide (DMSO), ethanol, and chloroform] and cells cultured in the absence of the compound. After the 72 h of incubation period, the resazurin solution was

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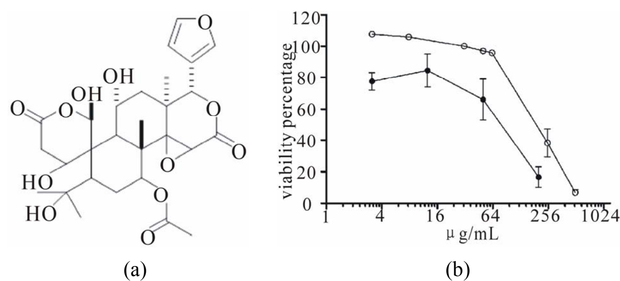

Figure 1. (a) correspond to the *seco*-limonoid compound structure (11*α*,19*β*-dihydroxy-7-acetoxy-7-deoxoichangin-C28H36 O12, 565.2285) and (b) shows the cytotoxic activity of the *seco*-limonoid on monocyte/macrophage cells derived from mice-J774.2 cells (open circles) and hDCs (filled circles). The graph depicts the average percentage viability ± SEM of the cells exposed to different concentrations of the compounds.

added at a final concentration per well of 44 μM (Sigma-Aldrich), and after 4 h of incubation at 36˚C - 37˚C, the plates were read using a spectrofluorometer at λ_{exc} 535 nm and λ_{emi} 590 nm (Tecan, Genios, Salzburg, Austria). The lethal concentration 50% (LC_{50}) of the *seco*-limonoid which corresponds to the concentration at which 50% of the cells die, was determined using the statistical software package GraphPad Prism version 5.00 Demo (GraphPad Software, USA). Each assay was performed in duplicate in at least three independent experiments.

2.4. Antileishmanial Activity Evaluation

2.4.1. Antileishmanial Activity against Promastigotes of *L.* (V) *panamensis*

The *in vitro* antileishmanial effect of *seco*-limonoid on the extracellular flagellar form of the parasite was evaluated by culturing promastigotes of *L.* (V) *panamensis* (expressing GFP) in the presence of different concentrations of the compound, using for this experiment, *seco*-limonoid concentrations lower than the LC_{50} (that had previously been determined in cytotoxicity assays over cells of mammals). Briefly, 2 × 10^5 promastigotes were incubated for 72 h at 25˚C - 26˚C in the presence of 106.3 μM (60 μg/mL), 26.6 μM (15 μg/mL), 6.6 μM (3.75 μg/mL), 1.1 μM (0.63 μg/mL), 0.55 μM (0.31 μg/mL) or 0.28 μM (0.16 ug/mL) of *seco*-limonoid. As a positive control for the direct cytotoxicity of the compound against the extracellular form of the parasite, the promastigotes were exposed to different concentrations of pentamidine isethionate (Pentacarinat®, Sanofi-Aventis, France) at 1:4 dilutions starting at a maximum concentration of 5 μg/mL to a minimum concentration of 1 × 10^{-5} μg/mL, using the same experimental conditions as those used in the evaluation tests of the activity of the compound. Next, the viability of the parasites was determined by flow cytometry (FacsCantoII) (BD Biosciences) and resazurin metabolic assay (Sigma Chemical Co.), where the reduction in the fluorescence emission from the promastigotes and the decrease in their metabolic activity, was associated with non-viable parasites.

2.4.2. Antileishmanial Activity against the Intracellular Form of the Parasite

The antileishmanial effect on intracellular amastigotes was determined by *in vitro* APC infection assays using J774.2 cells and hDCs. 2 × 10^5 cells were infected with promastigotes of *L.* (V) *panamensis* (constitutively expressing GFP) at ratios between 1:40 and 1:50 (cell per parasites) for 6 h, time after which the non-internalized parasites were removed by washing with RPMI-1640 medium, and once the infection was established, the cells were exposed to various concentrations of *seco*-limonoid [consecutive 1:4 dilutions were used starting from a maximum concentration of 53.2 μM (30 μg/mL) down to a minimum concentration of 0.81 μM (0.47 μg/mL)] for 48 h at 36˚C in an atmosphere of 5% CO_2. Similarly, infected cells treated with 2000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 40 μg/mL, and 20 μg/mL of sodium stibogluconate® (Ryan Laboratories, Colombia) were used as positive controls for the resolution of the *in vitro* infection. Completed the incubation times, the supernatants were collected and cryopreserved at −20˚C until they were required for quantification of the cytokines associated with inflammatory processes. The J774.2 cells were transferred to flow cytometry tubes to quantify the percentage of infected cells by flow cytometry (FacsCanto II, BD, San José, CA, USA), and thereby, the fluorescence emitted by the fluorescent parasites was captured in the green channel using a 530/30 nm filter, and was used to distinguish the infected cells from the uninfected population. Because of the complexity of hDCs, clear differences among the infected cells population and uninfected cells were not easily observed using flow cytometry, due mainly at the characteristic autofluorescence of this kind of cells, factor for which is limited the analysis using this technique; therefore, light microscopy with slides containing Giemsa-stained cell preparations were used to evaluate infection on hDCs. For this assay, the protocol described above was used to evaluate the antileishmanial activity against the intracellular form of the pathogen; however, the hDCS were placed onto the chamber slides, containing 16 wells (Nunc, Rochester, NY, USA) instead of 96-well flat-bottom plates. Free parasites, uninfected cells, infected cells in the absence of the compound, and infected cells exposed to the conventional treatment (pentavalent antimonials) were used as controls for each of the assays. In these assays, reductions in the percentage of infected cells and/or in the emitted fluorescence by the internalized parasites were the parameters that were indicative of the parasiticidal activity of the compounds. Antileishmanial activity against the promastigotes and intracellular

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amastigotes was evaluated in at least three independent experiments.

Using these assays, the effective concentration 50 (EC_{50}), which corresponds to the concentration that reduces the parasite burden by 50% in experimentally infected phagocytes, was determined, and the division of the values obtained from the cytotoxic antileishmanial activity on the intracellular pathogens [Lethal Concentration 50 (LC_{50})/Effective Concentration 50 (EC_{50})] corresponds to the selectivity index (SI), a parameter related to the selectivity and safety of the tested molecule. For the compounds with potential leishmanicidal activity, arbitrary SI values have been reported, considering a value ≥ 2 to be significant.

2.4.3. Antileishmanial Activity against Axenic Amastigotes

The evaluation of antileishmanial activity against the axenic forms of the parasite was performed by seeding 2 - 5 × 10^5 axenic amastigotes per well in 96-well flat-bottom plates (TPP, Switzerland) which were exposed to different concentrations of the compound for 72 h at 36˚C, 5% CO_2 and 90% humidity (using the concentrations mentioned previously for the evaluation of activity against intracellular amastigotes). Pentamidine isethionate® and sodium stibogluconate® were used as treatment controls, and cells that were not exposed to treatment or compounds were included in the assays, as negative controls. After the incubation period, the viability of the cells was determined using the indirect resazurin metabolic assay (Sigma-Aldrich, St. Louis, USA), following the protocol described in a previous section.

2.5. Immunomodulatory Activity Assessment

2.5.1. Determination of Nitric Oxide (NO)

J774.2 cells or hDCs (4 × 10^5 cells) were infected at a ratio of between 1:40 and 1:50 with promastigotes of *L.* (V) *panamensis* (not transfected parasites) for 6 h at 36˚C - 37˚C in an atmosphere with 5% CO_2 and 90% humidity. Once the experimental infection was established, the non-internalized parasites were removed by gentle washes using a sterile saline solution, followed by treatment of the cells with the concentrations of the *seco*-limonoid mentioned previously (see protocols for the evaluation of antileishmanial activity). Sodium stibogluconate® was used as control drug (as described previously). Similarly, a sample of cells was treated with 10 µg/mL phorbol myristateacetate for 24 h as a positive control for the induction of NO, as also, a sample of cells not exposed to any type of treatment (with and without infection) was used to determine the basal levels of NO production for each population. The NO production was monitored after 48 h of treatment with the *seco*-limonoid or the control drug, and latter, the cells was transferred to flow cytometry tubes and staining with 4 µM DAF-FM diacetate solution (Invitrogen, USA) for 1 h at 36˚C - 37˚C. Subsequently, the cells were washed, with the aim to remove the unincorporated probe and the readings were performed in a BD FacsCanto II flow cytometer using the blue laser (λ_{exc} 488 nm) as the excitation source and the 530/30 nm filter for detection.

2.5.2. Quantification of Cytokines in Supernatants Cultures

Briefly, 15 µL of a mixture comprising capture beads for each of the cytokines to be evaluated (IL-6, IL-10, MCP-1, IFN-γ, TNFα, and IL-12p70 for the J774.2 cells or IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70 for the hDC) was mixed with 15 µL of the PE detection reagent and 50 µL of the culture supernatants. The mixtures were incubated at ambient temperature for 2 - 3 h protected from the light, and after this incubation period, the samples were washed to remove the unbound PE detection antibody. On the other hand, using the same conditions mentioned before, standards for each cytokine were run simultaneously. The readings were performed using the FacsCanto II flow cytometer (BD Biosciences, San Diego, CA, USA) and the BD FACSDiva (BD Biosciences) acquisition software. The data were analyzed using the FCAP Array™ (Soft Flow) software.

2.5.3. Evaluation of Cell Surface Marker Expression

For the phenotypic analysis of the hDCs, the surface marker expression was evaluated using the following antibodies: clone L243, (APC-conjugated anti-HLA-DR), clone DCN46 (FITC-conjugated anti-CD209), clone MφP9 (PerCP-conjugated anti-CD14), clone HB15e (PE-conjugated anti-CD83), and clone L307.4 (PE-conjugated anti-CD80). The acquisition and analysis were performed using a FacsCanto II (BD, Biosciences) flow cytometer and the acquisition software FACSDiva (BD Biosciences).

2.6. Statistical Analyses

The results from each experimental protocol were analyzed according to the parameters for each technique and were validated by comparison with the established controls for each assay. Therefore, the data obtained allowed the determination of the lethal concentration 50 (LC_{50}) and the effective concentration 50 (EC_{50}) as expected according to the technique used. Using nonparametric tests, it was possible to evaluate differences between the experimental groups, according to the parameters of the technique employed. For these analyses, the statistical software package GraphPad Prism version 5.00 Demo (GraphPad Software, USA) was used.

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3. RESULTS

3.1. Cytotoxic Activity on Mammalian Cells Targeted by *L.* (V) *panamensis* Infection

The cytotoxic activity of the *seco*-limonoid on cells of the murine monocyte/macrophage lineage (the murine macrophage cell line J774.2) and hDCs was determined using the resazurin metabolic assay, which indirectly demonstrates the viability of a cell population through evaluating of the metabolism of the non-fluorescent indicator (resazurin) to a fluorescent molecule (resorufin) [25]. In these assays, we observed a decreased susceptibility of the murine cells to the toxicity induced by the *seco*-limonoid, determining an LC_{50} of 368.5 µM (207.9 µg/mL) for the J744.2 cells versus 116.8 µM (65.89 µg/mL) for the hDCs. The cytotoxic effect induced by the greatest concentration of the compound observed in both cell lines was statistically significant (P *value* < 0.0001).

The cell viability results in these cell populations exposed to varying concentrations of the *seco*-limonoid are shown in **Figure 1(b)**, where it's shows that to achieve similar death levels in the hDCs (filled circles) as in the J774.2 cells (empty circles) a lower concentration of the compound is required. Moreover, pentavalent antimonyals (sodium stibogluconate) did not induce toxicity, as demonstrated by the resazurin metabolic assay, which could not determine an LC_{50} for the two cell populations evaluated ($LC_{50} > 2000$ µg/mL).

3.2. Antileishmanial Activity

With the aim of evidencing whether previously demonstrated activity of *seco*-limonoid was conserved in the different stages of the pathogen, tests were developed on the extracellular form (promastigotes), as well as the intracellular and axenic amastigotes forms. The parasites used in the present study belong to the genus *Leishmania*, subgenus Viannia, specie *L. panamensis*, which is one of the most important epidemiological species in South America, being the primarily responsible for the cases of cutaneous leishmaniasis in Colombia. The data obtained show that the activity of the *seco*-limonoid compound observed here appears to be directed specifically against the intracellular form of the pathogen (intracellular amastigotes) determining an EC_{50} of 7.9 µM (4.48 µg/mL) and 25.5 µM (14.39 µg/mL) using the *in vitro* infection model of J774.2 cells and hDCs, respectively. **Figure 2** shows the representative results of the *in vitro* infection assays using infected murine cells (panel 2a) or hDCs (panels 2b and 2c). In the panel a of the **Figure 2**, the broken line of the histogram, and panel b, are the infected cells without treatment, while, the solid lines in panel 2a and the panel c correspond to cells exposed to 30 µg/mL of the *seco*-limonoid.

Here, we show that the *in vitro* infection is controlled when cells were treated with compound, as demonstrated, by the decrease in the percentage of fluorescent cells (62.5% in untreated infected cells versus 10.32% in infected cells treated with *seco*-limonoid at 30 µg/mL) as also, was observed in the slides stained with Giemsa solution a decrease in uninfected cells in relation to the total cell population (**Figures 2(d)** and **(e)**). Similarly, **Figure 3(a)** shows the antileishmanial effect on axenic amastigotes of *L.* (V) *panamensis*, with an EC_{50} of 86.3 µM (48.7 µg/mL), demonstrating a concentration dependent response, which was not observed on the flagellar form of the pathogen (statistically significant differences were found when comparing the response obtained in both stages with 60 µg/ml of the compound, being most susceptible to the effect of the compound the axenic amastigotes form that promastigotes with a P *value* of 0.0024) (**Figure 3(b)**). **Figure 3(c)** shows the results obtained with pentamidine drug on the flagellar extracellular form of the pathogen (promastigotes) determining an EC_{50} of 0.6 µg/ml **Table 1** shows a summary of the cytotoxicity results and the efficacy observed in the tests described.

In this respect, an antileishmanial effect was demonstrated for the amastigote forms of the parasite (being mostly susceptible the intracellular form that the axenic); finding that the concentration of compound required to control 50% of the population for the intracellular stage of the parasite was lower (4.48 and 14.39 µg/mL for the infected J774.2 and hDCs that were treated with the compound, respectively) than for the axenic stage (48.7 µg/mL), which allows us to suggest that the compound, in addition to exhibiting antiparasitic activity capable of controlling the pathogen, may be acting on some metabolic pathways of APCs, leading to the complete resolution of the infection, possibly by the recovery of the antimicrobial activity that was silenced by the pathogen. The data indicate that this compound needs to be metabolized to carry out its activity or its antileishmanial activity is associated with an event caused by the immunomodulation of APCs by this molecule.

3.3. Immunomodulatory Activity

3.3.1. Nitric Oxide (NO) Production

Given the importance of the free nitrogen radicals in the control of intracellular infectious agents (such as *Leishmania* spp.) in this study, we determined the levels of NO in infected murine J774.2 macrophages and hDCs treated with the *seco*-limonoid by flow cytometry using the indicator DAF-FM diacetate (4-amino-5-methylamino- 2',7'-difluorofluorescein diacetate) as a probe. In both *in vitro* infection models (J774.2 and hDCs) the *seco*-limonoid induced an increase in NO production in infected and treated cells (with significant differences for

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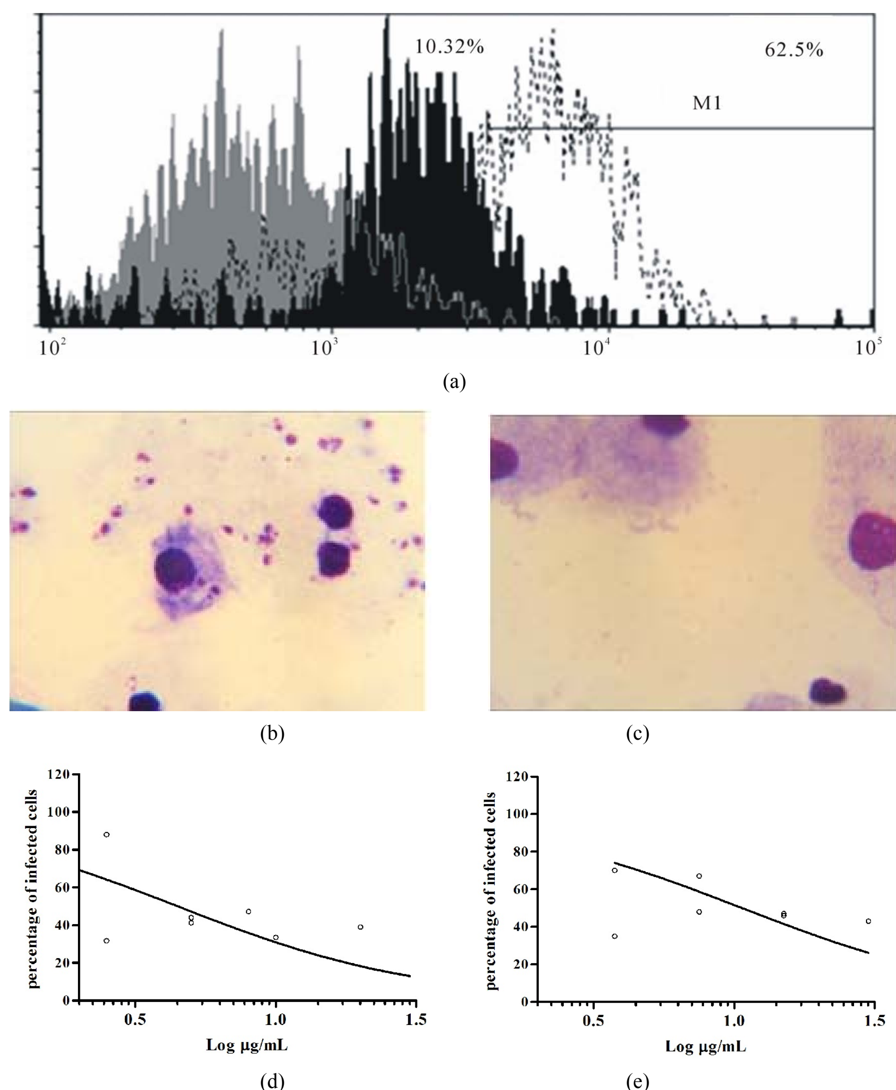

Figure 2. Cells infected with *L*. (V) *panamensis* and treated with *seco*-limonoid. (a) correspond to a histogram (MFI detected by 530/30 filter) of uninfected murine macrophages (continuous gray line), macrophages infected with *L*. (V) *panamensis* transfected with GFP without exposure to treatment after 48 h of infection *in vitro* (broken line) and macrophages infected and treated with 30 µg/ml of the *seco*-limonid (continuous black line); (b) and (c) correspond to hDCs infected with promastigotes of *L*. (V) *panamensis* without exposure to any treatment and exposed to 30 µg/ml of the *seco*-limonid after 72 h of infection *in vitro*, respectively, showing the internalized parasites observed by light microscopy on Giemsa-stained slides; (d) and (e) correspond to the graphs of concentration versus response in both *in vitro* models of infection treated with compound.

Table 1. Cytotoxic and antileishmanial activity on murine macrophages J774.2 cells and human dendritic cells.

Compound	*L*. (V) *panamensis* promastigotes	*L*. (V) *panamensis* axenic amastigotes	Murine Macrophages (cell line J774.2)			Human Dendritic Cells (hDCs)		
	EC_{50} (µg/mL)	EC_{50} (µg/mL)	LC_{50} (µg/mL)	EC_{50} (µg/mL)	SI	LC_{50} (µg/mL)	EC_{50} (µg/mL)	SI
Sodium stibogluconate	>1000	3018	>2000	40.17	>50	>2000	210	>10
seco-limonoid	>60	48.7	207.9	4.483	46	65.89	14.39	4.6

the case of hDCs treated) in a concentration-dependent manner when compared to the levels of NO in the untreated infected cells (**Figure 4**). Therefore, despite the variability in the response, in the murine cells and the

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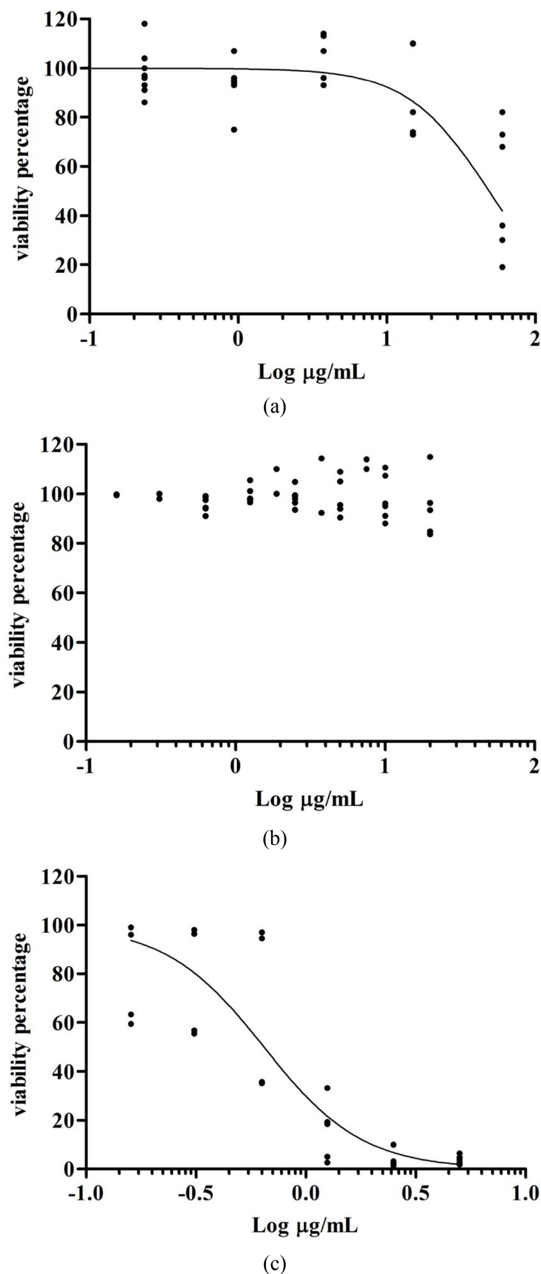

Figure 3. (a) shows the cytotoxic activity of seco-limonoid on axenic amastigotes (the graph shows the average percentage viability ± SD); while (b) and (c) correspond to cytotoxic activity of the seco-limonoid and Pentacarinat® against the extracellular flagellar form of the parasite (promastigotes). The graphs show the average percentage mortality ± SD.

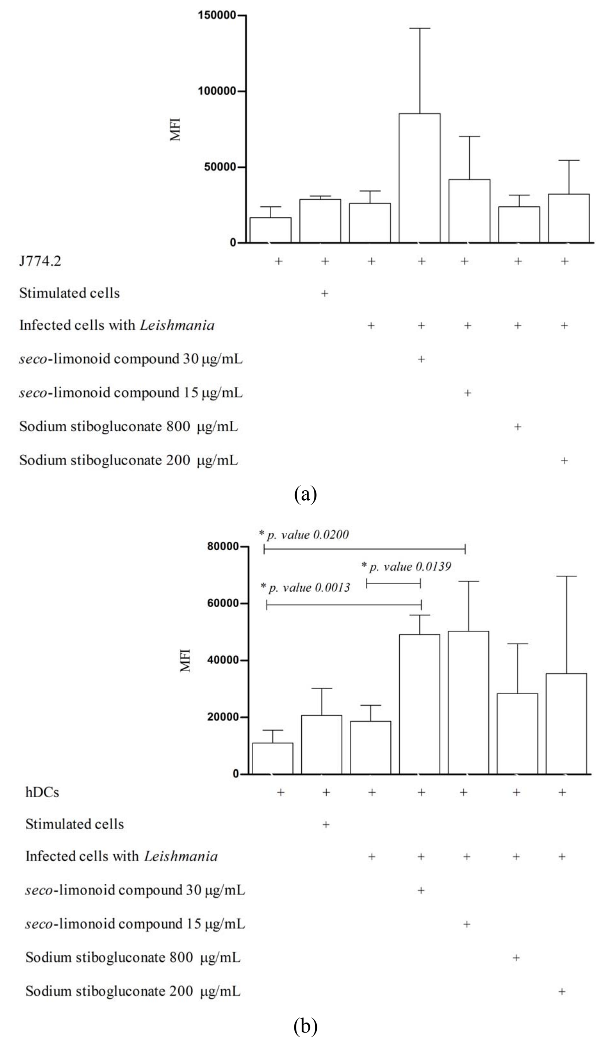

Figure 4. The diagrams correspond to nitric oxide (NO) production by murine macrophages (J774.2) (a) and human dendritic cells (hDCs) (b) The graph depicts the mean of the mean fluorescence intensity (MFI) and its SEM.

hDCs, there is a marked tendency toward NO production at the highest concentration of *seco*-limonoid, which reinforces the concept of its potential immunomodulatory activity. It should be noted that the compound used in this study, as well the culture media used here for the maintenance of the cell populations described before, did not yield positive results in the determination of endotoxin assay (commercial kit which present a sensibility range of 0.06 EU/mL)

3.3.2. Quantification of Cytokines by Flow Cytometry

To evaluate the possible immunomodulatory effect of the *seco*-limonoid, we quantified cytokines associated with inflammatory processes using the cytometric bead array (CBA) method for flow cytometry. Thereby, IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 were assayed in the supernatants of the J774.2 cells, and IL-8, IL-1β, IL-6, IL-10, TNFα, and IL-12p70 were assayed in the supernatants of the hDCs from patients with active disease (cutaneous presentation) and control volunteers with no history of the disease. In this regard, we determined that the *seco*-limonoid modulates cytokine secretion selectively in cells infected with *L.* (V) *panamensis* in the two *in vitro* infection models used (see **Figures 5** and **6**)

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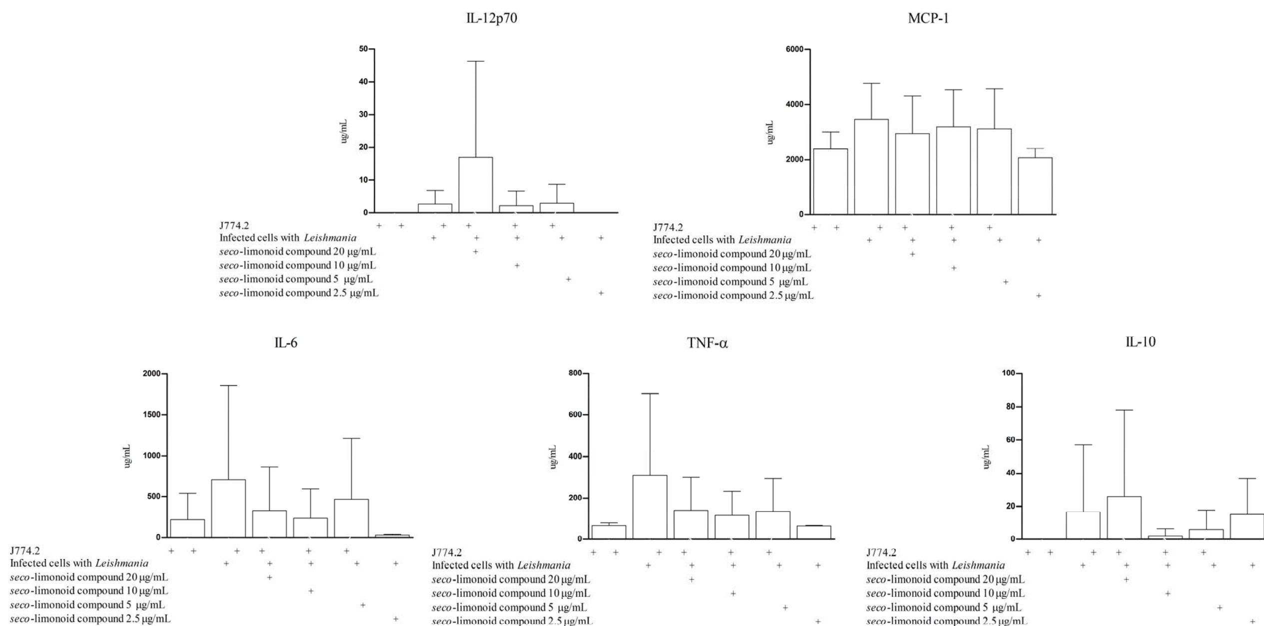

Figure 5. Correspond to levels of cytokines present in the supernatans of murine cells J774.2.

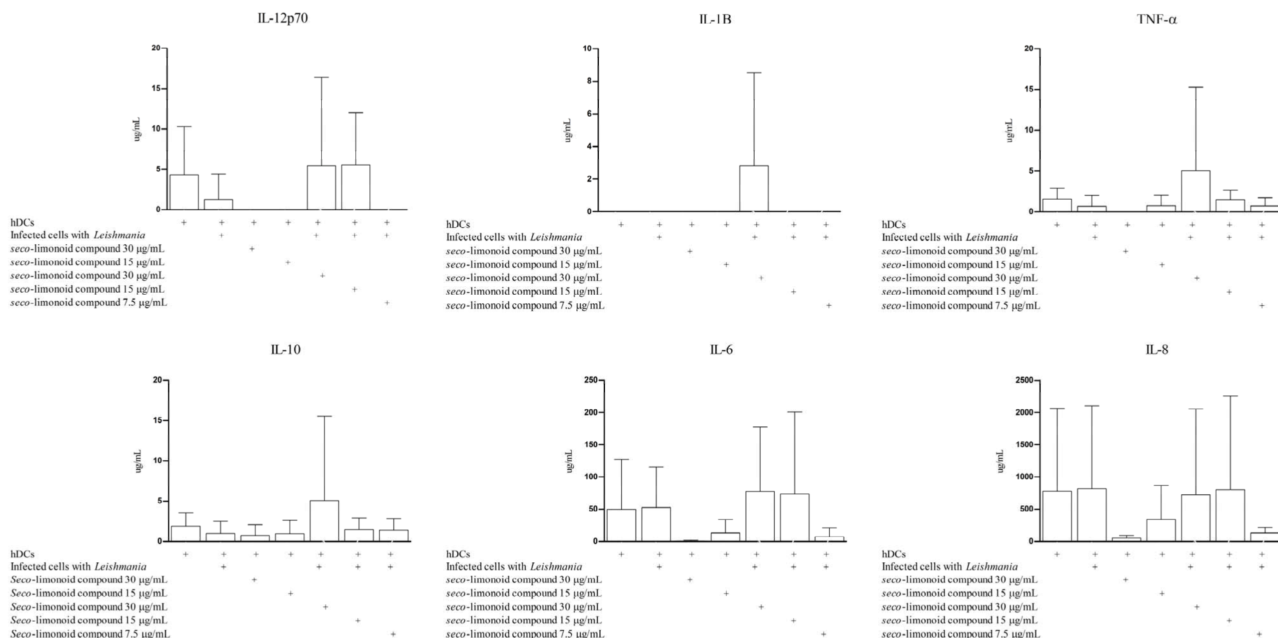

Figure 6. Correspond to cytokines levels in the supernatans of hDCs.

preferentially to the pro-inflammatory phenotype. Notably, despite the trend observed, significant differences between the two *in vitro* models when compared to the untreated infected cells were not observed.

Furthermore, the evaluation on the production of soluble mediators involved in inflammatory processes in cells derived from active leishmaniasis patients and the control volunteers demonstrated that the hDCs derived from patients with active disease produced more of these cytokines compared with the control group of unexposed patients; however, because of the variability of the results, significant differences were not observed.

3.3.3. Evaluation of Cell Surface Markers by Flow Cytometry

To explore the possible immunomodulatory effect of the *seco*-limonoid on hDCs (critical cell population for the development of adequate immune response for the resolution of this disease) the expression of cell surface markers associated with the maturation of the hDCs was

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assessed. The cells were labeled with commercial monoclonal antibodies in the presence of HLA-DR, DC-SIGN, and the co-stimulatory molecule human CD83, allowing, in this way the evaluation of the basic phenotypic characteristics of the hDCs population of infected that was treated with the compound. The results demonstrate that in infected cells treated with 15 µg/mL of the *seco*-limonoid, the expression of HLA-DR was enhanced (significant differences between the untreated infected cells and the infected cells treated with the compound in the two experimental groups, *P value* < 0.05) (**Figure 7**).

Similar results were obtained when evaluating the expression of the DC-SIGN molecule and CD83 (data not shown); however, no significant differences were observed for these molecules, when comparing the untreated infected cells with the treated cells. It is noteworthy that the number of positive events in the hDCs derived from patients with active disease was less than the number of positive events in the hDCs derived from the control volunteers for the three molecules tested; however, despite this marked tendency, significant differences between the two experimental groups of individuals were not observed.

4. DISCUSSION

The current first-choice treatment for leishmaniasis is based on the administration of pentavalent antimonial salts, which is still used despite their associated [7,11] adverse effects, as well as, the appearance of drug-resistant parasite [6,26] and the high cost associated with patient care. Also, despite the existence of second-line drugs (as amphotericin B, hexadecilphosphocholine, pentamidine isethionate, and paromomycin sulfate), these also have presented some drawbacks (such as adverse effects induced in the treated individuals and the high costs involved in the access to certain formulations). Considering this, it acquires even more relevance the urgent need for the development of new therapeutic agents for the treatment of this disease [5]. For many years, the study of compounds derived from natural products has been an important source of information for the design and/or discovery new therapeutic alternatives for the control of diverse diseases, such as leishmaniasis disease [13,17]. Therefore, various research studies have focused on the search for new, safer, and more effective bioactive molecules against leishmaniasis.

In this study, we confirmed the antileishmanial activity

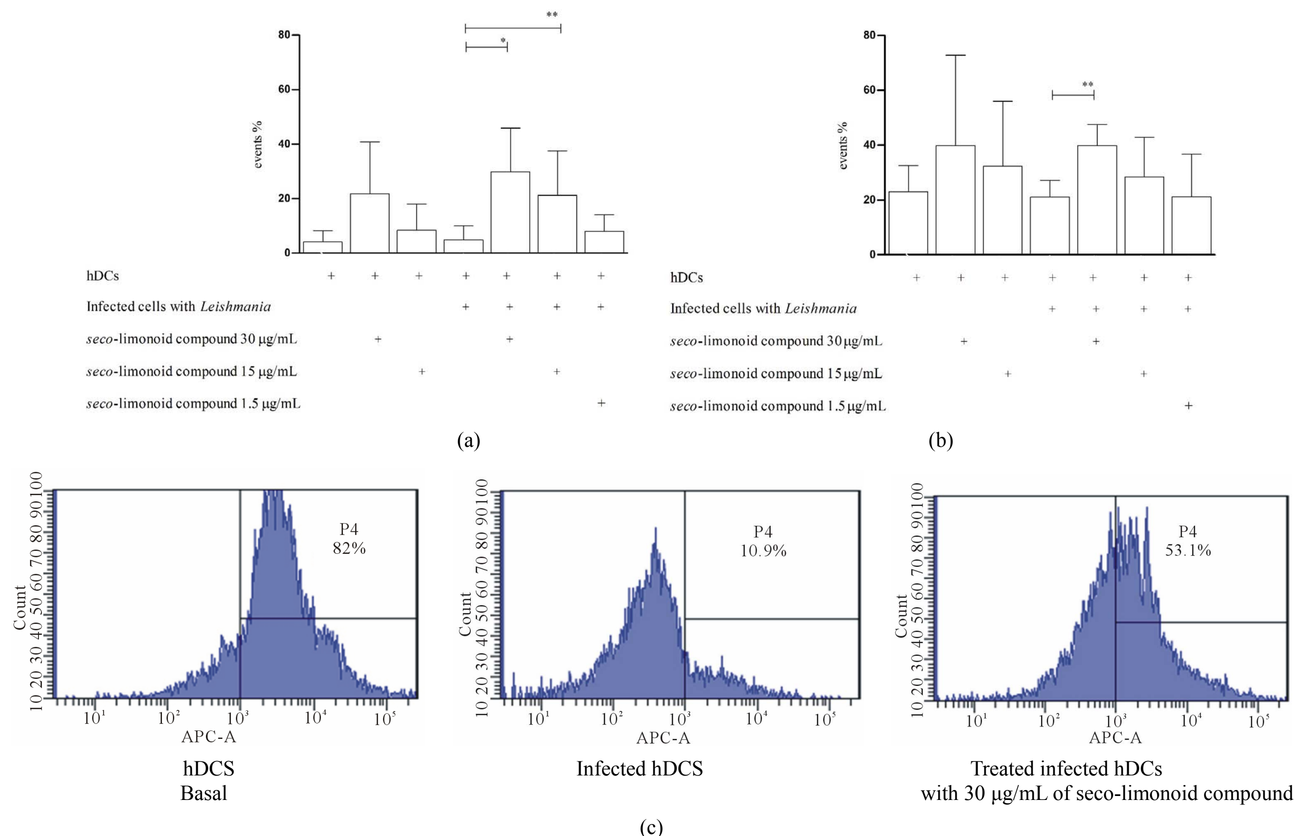

Figure 7. (a) and (b) shows the expression of the HLA-DR molecule in hDCs derived from individuals with and without clinical history of *Leishmania* infection, respectively. **correspond to significant differences finding when comparing the response found it with the basal response with a *P value* < 0.05, *significant differences with a *P value* < 0.01. (c) corresponds to histograms representing the HLA-DR expression in hDCs.

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of a *seco*-limonoid (11α,19β-dihydroxy-7-acetoxy-7-deoxoichangin), which was isolated from the bark of *Raputia heptaphylla* (Rutaceae family), which was previously described by Coy and colleagues [18]. We suggest here a possible mechanism for the antiparasitic activity of this compound that is related to the immunomodulatory effect on APCs.

In this regard and taking into account the information currently available regarding the parasite responsible for leishmaniasis and the information regarding its vertebrate host [20,27-29] suggest that immunomodulation is an attractive and promising tool [7,30,31] for developing new therapies for the adequate control of intracellular pathogens, such as *Leishmania spp.* [32]. Here, we observed that the *seco*-limonoid exhibits a specific activity against intracellular amastigotes of *Leishmania* and that this activity is possibly mediated or promoted by the immunomodulatory effect that this compound induces only in infected cells (depending on their interaction with pathogen). Thereby, this *seco*-limonoid has been shown to be a bioactive molecule exhibiting an interesting specific projection (because it would only modulate the microbicidal activity of infected cells) that can be used for the design of a leishmanicidal therapy.

The immunomodulatory potential of the *seco*-limonoid 11α,19β-dihydroxy-7-acetoxy-7-deoxoichangin is supported by the production of nitric oxide and the expression of HLA-DR, which is down-regulated following infection and exhibits a tendency to recover after exposure to this *seco*-limonoid. It is also important to highlight the importance of developing studies to confirm this activity *in vivo* (results using the hamster model *Mesocricetus auratus* have demonstrated the resolution of experimental cutaneous lesions of leishmaniasis).

Notably, for many years, numerous molecules exhibiting microbicidal activity has been reported; however, these molecules have not been developed into drug prototypes. One of the reasons for this delay is the difficulty in establishing the mechanism of action, which reinforces the importance of this study with respect to the immunomodulatory potential of the *seco*-limonoid in infected cells.

Furthermore, this study enhances the importance of considering the highly complex life cycle of the pathogen responsible for leishmaniasis [33]. When developing *in vitro* screens to study bioactive molecules for the control of this microorganism, there is a necessity for developing assays to evaluate possible antiparasitic activities against parasites of the genus *Leishmania* at the different life cycle stages of this pathogen. It is particularly important to develop antiparasitic therapies against the form of the pathogen responsible for the disease in the vertebrate host during the clinical manifestations of the disease [34,35], and is in this way, that in the present study we confirmed the efficacy of a *seco*-limonoid (triterpene) [18] which exhibits activity against only the intracellular form of the parasite, leading to a decrease and subsequent resolution of the infection in treated infected cells. We demonstrated that this activity is related to an immunomodulatory mechanism of action in which the compound induces an apparent "reactivation" of the "paralyzed" APC caused by the survival mechanisms developed by the pathogen.

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