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(−)-α-Bisabolol, a Promising Oral Compound for the Treatment of Visceral Leishmaniasis

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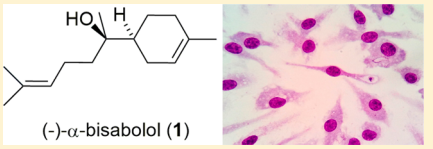
ABSTRACT: The aim of the present study was to assess the in vitro and in vivo activity of (−)-α-bisabolol (**1**) against the etiological agents of visceral leishmaniasis. Bone-marrow-derived macrophages were infected with *Leishmania infantum* or *L. donovani* promastigotes and incubated with (−)-α-bisabolol at different concentrations. Pentamidine isethionate and meglumine antimoniate were used as reference drugs. Inhibitory concentration 50% (IC_{50}) and cytotoxic concentration 50% (CC_{50}) were calculated. Balb/c mice were infected intraperitoneally with stationary-phase promastigotes. They were treated with (−)-α-bisabolol at different doses orally, meglumine antimoniate at 104 mg Sb^V/kg, or a combination of both. (−)-α-Bisabolol proved to be innocuous to mammal cells and active against *L. infantum* and *L. donovani* intracellular amastigotes (IC_{50} 55 and 39 μM, respectively). Compound **1** also proved to be active in an in vivo model of visceral leishmaniasis due to *L. infantum*, as it reduced parasite load in the spleen and liver by 71.60% and 89.22%, respectively, at 200 mg/kg without showing toxicity. (−)-α-Bisabolol (**1**) is a nontoxic compound that was proven to be active against visceral leishmaniasis in an in vivo murine model orally. It was more effective than meglumine antimoniate at reducing spleen parasite load and as effective as this antimonial drug in the liver.



Leishmaniasis is a poverty-associated disease with several clinical forms ranging from localized skin ulcers to a lethal systemic disease, caused by protozoal parasites belonging to the genus *Leishmania* (Trypanosomatida) and transmitted by the bite of female sandflies of the genera *Phlebotomus* and *Lutzomyia* (Diptera) in the Old World and the New World, respectively. It is an endemic disease that occurs in 98 countries across five continents, with 350 million people living at risk. Visceral leishmaniasis, which is fatal if untreated, has been estimated at 300 000 new cases each year, with most of them children. The six most affected countries—Bangladesh, Brazil, India, Ethiopia, Sudan, and South Sudan—represent over 90% of new cases.[1] *Leishmania donovani* is the main causative agent of visceral leishmaniasis and is present in Eastern Africa, the Middle East, and the Indian subcontinent. Among the 20 *Leishmania* species involved in the clinical spectrum of leishmaniasis, *Leishmania infantum* is the only species identified as a causative agent of both cutaneous and visceral diseases. Furthermore, *L. infantum* is the most widely distributed species, being the only one present both in the New World and in the Old World. For visceral leishmaniasis caused by *L. infantum*, the annual incidence has been estimated to be 4500−6800 cases in the American region, 93% of which are occurring in Brazil, and it is also the cause of almost all of the 1200−2000 cases in the Mediterranean Basin and the 5000−10 000 cases in the Middle East.[1] The incidence of cutaneous leishmaniasis due to *L. infantum* is usually underrated; it has recently been identified as a causative agent of mucocutaneous leishmaniasis.[2,3]

The therapeutic requirements for visceral leishmaniasis include an oral, safe, effective, and low-cost treatment. Current drugs used in visceral leishmaniasis present several problems including high toxicity, adverse effects, increasing resistance, high cost, and variability in specificity according to the geographical region.[4] Pentavalent antimonials have been the first-line treatment for decades in spite of their high toxicity, while pentamidine, amphotericin B, and paromomycin are second-line treatment drugs. The use of new drug delivery systems, such as nanoparticles, may improve therapeutic effectiveness and result in a decrease of side effects, as reported for amphotericin B. The last drug to have been added to the therapeutic arsenal for leishmaniasis is miltefosine. Intended initially as an anticancer drug, it turned out to be the first oral treatment against leishmaniasis. However, it is not exempt from problems, as it has been reported to have teratogenicity and to induce resistance.[5−8]

There is an increasing interest in using natural products against infectious diseases, including leishmaniasis.[9] Many components of natural essential oils like terpenoid derivatives are active against a variety of pathogens. Terpenes are lipophilic compounds that may penetrate the plasma membrane. This interaction with cell membranes might eventually lead to cell death.[10,11]

(−)-α-Bisabolol is a monocyclic sesquiterpene alcohol widely used in fragrances and cosmetics. It is found in chamomile [*Matricaria chamomilla* L. (Asteraceae)] essential oil at up to 50% concentrations, as well as within many other plants from traditional medicine.[12]

(−)-α-Bisabolol has been reported to have anti-inflammatory, anti-irritant, and microbicidal activities.[11,13,14] Associated with its anti-inflammatory activity, the inhibitory properties of **1** were shown against nitric oxide and prostaglandin E-2 production in LPS-stimulated macrophages.[15] During the past

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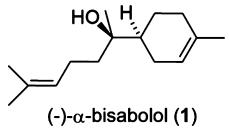

(-)-α-bisabolol (**1**)

decade, several authors have evaluated the cytotoxic properties of **1**, which induces apoptosis in human and rat glioma cells,[16] human pancreatic carcinoma cells,[17] and hepatic carcinoma cells.[18] Other properties have been attributed to this compound such as gastroprotective activity[19] and anesthetic activity.[20] Among its antiparasitic properties, (−)-α-bisabolol has shown insecticidal activity against the white fruit fly,[21] nematocidal activity against *Anisakis simplex* larvae, both in vitro and in vivo,[22] and antimalarial activity in vitro and in vivo.[23] (−)-α-Bisabolol has been reported to show antileishmanial activity against *L. infantum* promastigotes.[24]

(−)-α-Bisabolol (**1**) is nontoxic to animals when administered orally [lethal dose 50% (LD_{50}) 14 g/kg in rats], and it does not exhibit mutagenic activities.[25] Among its properties, (−)-α-bisabolol is effectively absorbed orally and it is widely and uniformly distributed through body tissues and organs.[19] It has very low cytotoxicity on human skin fibroblasts [cytotoxic concentration 50% (CC_{50}) >150 μM].[26]

Visceral leishmaniasis treatment remains a challenging public health need in the regions of the world where it is endemic. The aim of this study was to contribute to the development of new safe and effective oral treatments as either a monotherapy or a combination treatment. Therefore, the activity of the sesquiterpene alcohol (−)-α-bisabolol (**1**) was assessed, both alone and in combination with meglumine antimoniate, against intracellular *L. infantum* and *L. donovani* amastigotes in a primary macrophage in vitro model and in a murine visceral leishmaniasis model.

RESULTS AND DISCUSSION

(−)-α-Bisabolol was effective at the concentrations tested, reducing *L. infantum* infection percentages by 85% and *L. donovani* infection percentages by 89% at 150 μM. The results obtained at 500 and 1000 μM were not considered further due to possible cytotoxicity. A sigmoidal relationship between the variables "**1** concentration" and "percentage of infection reduction" was found for both parasites. The independent variable "infection level" also showed an association with "percentage of infection reduction" ($p < 0.01$) for the *L. infantum* results. A multiple regression model ($R^2 = 0.905$) was obtained including the variable "infection level":

$$\text{Percentage of infection reduction} = 116.38 - \frac{2273.02}{\textbf{1 concentration}} - 0.53 \times \text{infection level}$$

The regression model fitted for *L. donovani* infections was simple, as in vitro infections turned out to be less variable (infection level 32−57%):

$$\text{Percentage of infection reduction} = 102.61 - \frac{2000.58}{\textbf{1 concentration}}$$

These results regarding the influence of the infection level on the efficacy of **1** are consistent with those found by Seifert et al.,[27] who pointed out that the high level of infection in macrophage-amastigote in vitro experiments could result in a decrease of the activity of the test compound. The present study has gone further since a model establishing a relationship between amastigote infection rate and activity decrease has been built. The IC_{50} value for **1** was estimated (for a level of infection of 50%) along with control drugs used, as shown in Table 1. The IC_{50} values for meglumine antimoniate and pentamidine isethionate were calculated using simple linear regression analysis.

There are only a few articles focusing on the in vitro activity of essential oils containing (−)-α-bisabolol (**1**) against *Leishmania* spp. promastigotes or axenic amastigotes.[28] Recently, Colares et al. evaluated (−)-α-bisabolol,[29] alone and as an essential oil constituent, against *Leishmania amazonensis* intracellular amastigotes in a peritoneal macrophage model. Those results, in terms of potency (IC_{50} 48 μM), only slightly differed from those obtained in this investigation, and this difference may result from the differences between the in vitro models used. The IC_{50} obtained for (−)-α-bisabolol (**1**) against *L. infantum* intracellular amastigotes was also similar to that found by Morales-Yuste et al. for this compound against *L. infantum* promastigotes (IC_{50} 49 μM).[24] This suggests that (−)-α-bisabolol penetrates the macrophage plasma membrane and the parasitoforous vacuole, reaching the intracellular amastigote. The similarities in these results also suggest that the mechanism of action of **1** is not dependent on the parasite stage.

No cytotoxicity was found for bone-marrow-derived macrophages or L929 fibroblasts when **1** was tested at the concentration range 25−250 μM. At this concentration range, the survival of the cells was close to 100% and there were no differences from the diluent control ($p < 0.01$). At 500 μM, the survival rate was 86.7 ± 11%, and at 1000 μM, the survival rate was 62.3 ± 20%. Therefore, the (−)-α-bisabolol CC_{50} proved to be >1000 μM for normal mammalian cells. These data are consistent with those found by Piochon et al., who found that this compound was innocuous at 150 μM,[26] and with those from Colares et al.,[29] who established its CC_{50} value at 452 μM. Again, these differences might well relate to the in vitro model and procedures performed. The selectivity indexes (SI) could be approximated and were >17 (*L. infantum*) and >25 (*L. donovani*). The CC_{50} values of pentamidine isethionate and

Table 1. Antileishmanial Activity and Cytotoxicity of Compound 1 and Two Positive Controls

	IC_{50} (μM) intracellular amastigote		cytotoxicity CC_{50} (μM)	selectivity index ($CC_{50,L929}/IC_{50,Leishmania}$)	
compound	*L. infantum*[a]	*L. donovani*	L929	*L. infantum*[a]	*L. donovani*
1	56.9 ± 7.9	39.4 ± 7.0	>1000	>17	>25
meglumine antimoniate	144.4 ± 41.0	96.4 ± 7.4	273.1 ± 12.2	2	3
pentamidine isethionate	1.5 ± 0.1	0.88 ± 0.2	6.1 ± 0.2	4	7

[a]The IC_{50} for **1** was calculated for an "infection level" of 50% in the case of *L. infantum*.

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meglumine antimoniate are also reported in Table 1. Although there is no agreement regarding an optimal value for this value,[30] when screening new compounds, it is generally assumed that a promising compound should have an SI of >10.[31] It is important to emphasize that neither SI nor IC_{50} must be seen as an absolute criterion when considering whether in vivo studies should be carried out. In this case, the advantageous properties of (−)-α-bisabolol justified animal studies.

Regarding its mechanism of action, (−)-α-bisabolol (**1**), as for other terpenoidal constituents of essential oils, may affect the parasite plasma membrane. This effect could be either direct, by inhibiting its glycosylation or diminishing its stability, or indirect, by inhibiting the biosynthesis of fatty acids.[11] Also, a disruption in the mitochondrial membrane may lead to parasite death, a mechanism suggested to be involved in (−)-α-bisabolol cytotoxicity effects.[32,33]

Experimental infections in mice were successful, with the parasite loads found in the spleen and liver of treated and untreated animals shown in Figure 1. The use of quantitative

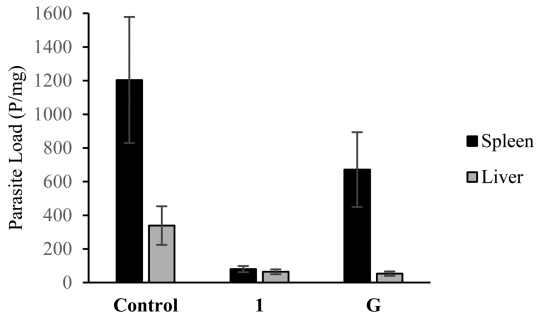

Figure 1. Average parasite loads for the spleen and liver of infected mice (parasites/mg). Control: mice treated with olive oil; **1**: mice treated with 200 mg/kg (−)-α-bisabolol; G: mice treated with meglumine antimoniate 104 mg Sb^V/kg.

real-time PCR (qPCR) for the evaluation of antileishmanial compounds has been reported earlier, as several authors have suggested this to overcome the major drawbacks of classical methods (organ culture microtitration or amastigote counting in imprint smears), such as low sensitivity and nonuniform dispersion of the parasites in the imprints.[34] Recently, this technique was compared to Leishman Donovan units (LDU), reporting a higher sensitivity, accuracy, and reproducibility of qPCR and highlighting its validity in the evaluation of antileishmanial compounds.[35] Parasite loads in the spleen of control mice were quadruplicated when compared with those detected in the liver (1204 and 339 parasites/mg, respectively). In terms of reduction of infection percentages (Figure 2), statistically significant differences could be found among the groups of mice. Data at 1000 mg/kg are not shown. Treatment with (−)-α-bisabolol (Figure 2) proved to be effective in the spleens of infected mice at the doses used in comparison to the control group (50 mg/kg, p 0.05; and 200 mg/kg, p 0.003). However, treatment with meglumine antimoniate or with **1** in combination with meglumine antimoniate was not effective in comparison to the control groups ($p > 0.05$).

In the livers of infected mice, treatment with **1** (Figure 2) proved to be effective at the doses used (50 mg/kg, p 0.045; and 200 mg/kg, p 0.038). The effectiveness of meglumine antimoniate in the liver was very high, reducing the parasite load (p 0.014). In addition, the combination **1**/meglumine

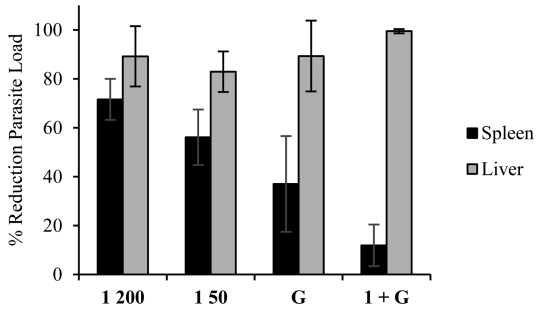

Figure 2. Parasite load reduction (%) in the liver and spleen of infected mice. **1** 200: mice treated with 200 mg/kg (−)-α-bisabolol; **1** 50: mice treated with 50 mg/kg (−)-α-bisabolol; G: mice treated with meglumine antimoniate 104 mg Sb^V/kg; **1** + G: mice treated with 200 mg/kg (−)-α-bisabolol + meglumine antimoniate 104 mg Sb^V/kg.

antimoniate was effective in comparison to the control group ($p < 0.01$) and even more effective than the positive control drug (p 0.027).

Treatment with (−)-α-bisabolol (**1**) at 50 and 200 mg/kg proved innocuous and did not negatively affect biochemical or enzymatic plasma values in comparison to the control or to healthy groups ($p < 0.05$). Neither did this cause hepatomegaly or splenomegaly related to treatment. Analysis of histological sections did not reveal any changes at the tissue level. These results were also obtained for meglumine antimoniate and the combination (−)-α-bisabolol/meglumine antimoniate. Although toxicity results for 1000 mg/kg were not statistically different from the control or healthy groups, these results were not considered further due to possible alterations on AST plasma values.

(−)-α-Bisabolol was also effective in a murine model of visceral leishmaniasis due to *L. infantum*, reducing the spleen and liver parasite loads by 71.6% and 89.2% respectively, at 200 mg/kg. This sesquiterpene alcohol appears to be at least as safe as the current standard leishmaniasis treatment. Regarding its antileishmanial activity, this natural product was more effective than meglumine antimoniate at reducing the spleen parasite load and as effective as this antimonial drug at decreasing parasite load in the liver. However, combining (−)-α-bisabolol (**1**) and meglumine antimoniate led to disappointing results, with the data showing high efficacy in the liver but with no activity in the spleen in comparison with control mice. This lack of activity cannot be explained satisfactorily, but it may be pointed out that meglumine antimoniate has been reported to cause oxidative stress in the organs of mice such as the spleen, and this could interfere with the activity of (−)-α-bisabolol.[36]

Therefore, the antileishmanial activity and cytotoxicity profile of (−)-α-bisabolol (**1**) has been assessed in a macrophage−amastigote model and in an in vivo model for the first time. There is no previous report that has evaluated this sesquiterpene alcohol against any trypanosomatid parasite in vivo. The possibility of administering (−)-α-bisabolol orally is also an important advantage since current therapeutic options, except for miltefosine, allow only parenteral administration. All these factors, along with its low cost, make (−)-α-bisabolol a promising compound for the treatment of leishmaniasis. Although this substance has been tested only on visceral leishmaniasis, in the future its evaluation on a cutaneous leishmaniasis model could be carried out.

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EXPERIMENTAL SECTION

Chemicals. (−)-α-Bisabolol (**1**) (purity >95%, gas chromatography) was purchased from Sigma-Aldrich (ref 14462). For in vitro experiments it was diluted in dimethyl sulfoxide (DMSO) and properly added to every well to obtain the intended concentration and 0.5% DMSO in the medium. For oral administration in in vivo experiments **1** was diluted with olive oil to a maximum dose volume of 200 μL. The reference drugs, pentamidine isethionate (Pentacarinat) and meglumine antimoniate (Glucantime), were purchased from Sanofi-Aventis (Paris, France). For in vitro experiments they were added to each well up to the intended concentration. For intraperitoneal administration in in vivo experiments, meglumine antimoniate was diluted with physiological saline solution to a maximum volume of 500 μL.

Parasites. For in vitro and in vivo experiments two autochthonous isolates of *Leishmania infantum*, MHOM/ES/2007/DP532JFJ, obtained from a local human visceral leishmaniasis case, and MCAN/ES/2007/DP534, obtained from a local canine leishmaniasis case, were used. These strains were properly characterized through isoenzyme electrophoresis and identified as *Leishmania infantum* MON-1. Both strains were isolated and characterized by the authors. A *Leishmania donovani* strain, MHOM/IN/00/LEM138, was used for in vitro experiments. This strain was kindly donated by Prof. M. Gállego (University of Barcelona) and had been previously identified through isoenzyme electrophoresis as *Leishmania donovani* MON-2. The parasites were grown as promastigotes in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 200 U/mL penicillin at 24 °C.

Animals. ICR (CD-1) mice, approximately 30 g in weight, were used as the source of bone-marrow-derived macrophages (BMDM). Female 4−6-week-old Balb/c mice were used for in vivo experiments. Animals were handled in accordance with Guidelines for Animal Experimentation recommended by the Federation for Laboratory Animals Science Associations (FELASA). They were properly housed under 12 h light cycles and provided with water and food ad libitum. Animals were purchased from Harlan Ibérica (Barcelona, Spain). All experiments were performed in accordance with the recommendations and guidelines of FELASA and were approved by the Ethics Committee of Animal Experimentation (CEEA) of the University of Granada (CEEA 455-2013), which evaluated and approved each protocol carried out in the research, namely, *Leishmania* parasites intraperitoneal inoculation (E.2.24), drug intraperitoneal administration (E.1.17c), (−)-α-bisabolol (**1**) oral administration (E.1.17e), and euthanasia (E.1.19.133).

Cell Cultures and Culture Media. L929 fibroblasts, properly identified (ECACC: 85011425), were purchased from the Cell Bank of University of Granada. They were grown in RPMI-1640 medium supplemented with 10% FBS and 200 U/mL penicillin at 37 °C in 5% CO_2. These were used both for obtaining L929 Cell Conditioned Medium (LCCM) and for cytotoxicity assays. BMDM were obtained as undifferentiated bone marrow cells from bone marrows of healthy ICR (CD-1) mice. They were differentiated into macrophages, according to Zamboni and Rabinovitch,[37] by culturing in RPMI-1640 supplemented with 20% FBS, 30% LCCM, and 200 U/L penicillin at 37 °C 5% CO_2. For in vitro experiments, both compound efficacy tests and cytotoxicity assays, macrophages were cultured in RPMI-1640 supplemented with 20% FBS, 5% LCCM, and 200 U/L penicillin at 37 °C, 5% CO_2.

Cytotoxicity Assay. A Trypan Blue assay was employed to determine the toxicity of **1** for BMDM and L929 fibroblasts. Cells were distributed in 24-well plates (4×10^5 BMDM/well or 5×10^4 fibroblasts/well) and incubated with (−)-α-bisabolol (**1**) at increasing concentrations (25, 50, 100, 150, 250, 500, and 1000 μM), pentamidine isethionate (0.1, 0.2, 0.5, 1, 5, 10, and 20 μM), or meglumine antimoniate (50, 100, 150 200, and 500 μM). Control groups incubated with diluent (DMSO) or physiological saline solution were also included. After 72 h, cells were collected and incubated with Trypan Blue (0.4%) in culture medium (1:1). After a few minutes, alive and dead cells were counted in a hemocytometer, and a survival ratio was established for every concentration by comparing them with control groups. Thus, the cytotoxic concentration 50% (CC_{50}), the concentration that inhibits cell growth by 50%, was approximated for (−)-α-bisabolol (**1**) and for the reference drugs. The selectivity index was then calculated using the following formula: $SI = CC_{50} \times IC_{50}^{-1}$.

In Vitro Assays for Intracellular Amastigotes. BMDM were used as an infection model according to Zauli-Nascimento et al.[38] They were counted and distributed (4×10^6 macrophages/well) in 24-well cell culture plates (GreinerBioOne) with round coverslips. Macrophages were left a day to allow them to stick to the coverslips. Then, they were infected with stationary-phase *L. infantum* or *L. donovani* promatigotes in a 10:1 parasite/macrophage ratio. Once infected, macrophages were incubated with the test compound at increasing concentrations (25, 50, 100, 150, 250, 500, and 1000 μM) for 48 h. Experiments were performed at least in triplicate, and wells untreated or treated with diluent (DMSO 0.5%) were included as control. Wells treated with pentamidine isethionate and meglumine antimoniate were included as reference drug controls as well.

Evaluation of in Vitro Results. The coverslips with macrophages were washed, fixed with methanol, and stained with Giemsa. The percentage of infection was quantified by optical microscopy. Macrophages were considered infected with at least one amastigote inside.

A linear regression analysis was performed in order to establish a dose−response relationship and to estimate IC_{50} values. This analysis included two parameters as independent variables: "**1** concentration" and "infection level" calculated from nontreated wells. A dependent variable, "percentage of infection reduction", was calculated as the difference between the average percentage of infection of control wells and the percentage of infection of treated wells, divided by the average percentage of infection of control wells.

Experimental Infection. Mice were infected intraperitoneally with 10^7 stationary-phase promastigotes, freshly isolated from the spleen of infected Balb/c mice (less than three culture passages), in 200 μL of 0.9% saline solution. Then, the mice were divided into groups of seven. This experimental infection model, which has been reported before,[39] was chosen over a variety of infection models including the following variables: infection dose (10^7 or 10^8 parasites), parasite stage (amastigote or stationary-phase promastigote), sacrifice times and inoculation route (intraperitoneal or intravenous), and considering parasite load through qPCR, parasite isolation from target organs, and parasite load through imprint smears of liver and spleen. This approach provides a stable infection in the liver and spleen with high parasite loads (up to 2500 parasites per milligram for at least two months after infection) and reaches the bone marrow (data not shown).

Treatment. Each treated group received a daily oral dose of **1** diluted in olive oil for a maximum volume of 200 μL. Doses administered were 50, 200, and 1000 mg/kg (−)-α-bisabolol. The treatment started 28 days after the infection day and lasted for 14 days. As a reference treatment, mice treated intraperitoneally with 104 mg Sb^V/kg meglumine antimoniate daily for 14 days were included. Mice treated only with diluent (olive oil) orally for 14 days were included as a control. A combination group was also included, and this was treated with (−)-α-bisabolol 200 mg/kg orally and meglumine antimoniate 104 mg Sb^V/kg intraperitoneally.

Parasite Evaluation. Mice were sacrificed by cervical dislocation, and samples of the spleen and liver were taken. Both organs were divided into three parts for microscopy, culture, and qPCR. Liver and spleen imprints were made, fixed in pure methanol, and then Giemsa-stained in order to detect amastigotes. Cultures were made with macerates of 20 mg of each organ, using a combination of EMTM solid phase made with rabbit blood and RPMI supplemented with 20% FBS and 5% human urine as the liquid phase. The cultures were kept for 3 months before being rejected as negative. In vitro subinoculations were performed weekly.

The organ parts were processed in a room exclusively designed for DNA extraction. DNA was obtained from the different weighted samples using the REAL DNA SSS Extraction Kit (RBME01). Each

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DNA extract was rehydrated in a final volume of 20 μL of sterile water. To make sure there was no contamination at this stage, extraction controls were carried out. These consisted of tubes of sterile water to which the whole extraction process was applied simultaneously with the biological samples. One control was used for every group of seven biological samples. The extracted DNA was kept at −20 °C until its amplification by PCR.

As a totally independent procedure from the DNA extraction from biological samples taken from mice, DNA was also extracted from *L. infantum* promastigotes (MCAN/ES/2007/DP534) taken from cultures. The parasites were washed and counted with a hemocytometer and adjusted to a final concentration of 1000 parasites/μL to be used as a positive control in the PCR. Various negative controls were used: (i) tube of PCR reagents without DNA, (ii) extraction controls, (iii) DNA from an uninfected mouse.

Evaluation of parasite load in the liver and spleen was performed by using a qPCR specific for *L. infantum* derived from the PCR-ELISA described by Martín-Sánchez et al.[40] Each sample was analyzed in triplicate, adding 1, 1, and 2 μL of DNA in a final reaction volume of 25 μL. The parasite load was obtained interpolating the threshold cycle values obtained for each biological sample in a previously constructed calibration curve. The final parasite density data were expressed in terms of number of parasites/milligram of liver or spleen and were calculated from the values returned in the three replicates taking an adjusted average. Once the parasite load was quantified, percentage of reduction in parasite load is calculated by comparing the parasite load of the treated mice to the parasite load of the untreated mice.

This technique has been calibrated to be very sensitive (capable of detecting 0.09 parasite) and reliable at quantifying *L. infantum* DNA, having an efficiency of 95.8% (R^2 = 97.2%) (data not shown).

Toxicity Evaluation. For in vivo toxicity evaluation, three doses were tested: 50, 200, and 1000 mg/kg. A noninfected healthy group of mice was included as a reference group. Mice were weighed weekly in order to observe weight loss associated with product administration. They were also investigated to look for signs such as stress, pain, cutaneous signs, or diarrhea. After the sacrifice, the spleen and liver were weighed in order to look for spleno- or hepatomegaly. Blood samples were taken in order to carry out biochemical functional tests: urea, creatinine, alkaline phosphatase, and transaminase plasma level tests were carried out by using commercially available kits (Spinreact, Spain). In addition, structural integrity research was performed: histological slides were obtained from the kidney, spleen, and liver, stained with hematoxylin−eosin, and fixed in paraffin. These preparations were investigated under light microscopy in order to find tissue damage.

Statistical Analysis. A mathematical model was built using linear regression analysis in order to estimate the reduction of *Leishmania* infection from product concentration and infection level. Thus, IC_{50} was calculated as the concentration of **1** that reduces the macrophage infection rate by 50%. In order to evaluate the in vivo effectiveness results, a nonparametric Mann−Whitney's test was performed, while an ANOVA test was used for analyzing toxicity data. The statistical software package SPSS 20.0 was used.

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Notes

The authors declare no competing financial interest.

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