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Development of a Novel Formulation with Hypericin To Treat Cutaneous Leishmaniasis Based on Photodynamic Therapy in *In Vitro* and *In Vivo* Studies

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An evaluation of the leishmanicidal activity *in vitro* and *in vivo* of hypericin, an expanded-spectrum photosensitizer found in *Hypericum perforatum*, is presented. Hypericin was evaluated against intracellular amastigotes *in vitro* of *Leishmania (Viannia) panamensis*. A topical formulation containing 0.5% hypericin was developed and assayed *in vivo* in a hamster model of cutaneous leishmaniasis. Results demonstrate that hypericin induces a significant antiamastigote effect *in vitro* against *L. panamensis* by decreasing the number of parasites inside infected cells. The topical formulation of 0.5% hypericin allows healing of *L. panamensis*-induced lesions upon a topical application of 40 mg/day plus visible-light irradiation (5 J/cm², 15 min), twice a week for 3 weeks.

Cutaneous leishmaniasis (CL) is a parasitic disease caused by protozoa of the genus *Leishmania*, which manifests as a chronic infection affecting mainly mononuclear phagocytes of the skin (1). The disease affects the poorest populations in 99 countries in tropical and subtropical regions of the world (2, 3). It is estimated that 14 million people are infected, 350 million are at risk, and there are about 1.5 million new cases per year (3, 4). The parasite is transmitted by the bite of an insect vector belonging to the genus *Lutzomyia* (in America) or *Phlebotomus* (in Europe, Asia, and Africa) in the subfamily Phlebotominae (5).

Only three types of drugs are available to treat CL. They are the pentavalent antimonials (meglumine antimoniate and sodium stibogluconate), pentamidine isethionate, and miltefosine. Miltefosine is the only one available for oral administration (4). These medications are delivered at high doses and for long periods, thus leading to high toxicity. Therefore, their use is contraindicated in pregnant women, in patients with cardiovascular, renal, or hepatic disease, and in children with low body weight (6). Overall, the efficacy of these drugs varies between 55 and 98% depending on the compound and on the *Leishmania* species causing the clinical manifestation (7, 8). However, their elevated toxicity encourages abandonment of treatment and consequently a decrease in the efficacy (9).

More recently, photodynamic therapy (PDT) has emerged as an alternative to treat CL (10). This therapy is based on the application of a photosensitizing agent (PA) that, when excited by light of a certain wavelength, induces the production of reactive oxygen species (ROS) that can destroy the microorganism or the target cell (11). In spite of its potential, only a few PAs have been tested against CL in animal models, and only one in humans. Topical application of 5-aminolevulinic acid (ALA) and light in a murine model of CL caused by *L. major* showed a significant reduction of the parasitic load and the size of lesions (12). Intralesional administration of ALA (one to three PDT sessions) in a patient with CL by *L. major* produced a 10 to 20% cure, although it is probable that clinical outcome resulted from nonspecific destruction of infected macrophages instead of direct killing of parasites (13, 14). On the

other hand, dimethyl and diethyl carbaporphyrin ketals have shown good *in vitro* photodynamic activity against *Leishmania (Leishmania) amazonensis*, *Leishmania (Viannia) panamensis*, and *Leishmania (Leishmania) infantum*. Both compounds were also effective in hamsters infected with *L. (L.) amazonensis*, with a cure efficiency between 80 and 100% (10). Toluidine blue, methylene blue, and phenothiazine have also been shown to have *in vitro* activity against *Leishmania (Viannia) braziliensis* promastigotes (15, 16). When evaluated in hamsters infected with *L. (L.) amazonensis*, methylene blue induced reduction in lesion size but low cure rates (40% to 50%) (16). Meanwhile, aluminum phthalocyanine chloride showed *in vitro* leishmanicidal activity against intracellular amastigotes of *L. (V.) panamensis* and *L. (L.) infantum* (17, 18). To date, there are no clinical studies reported, other than the one with ALA mentioned before.

Hypericin (1,3,4,6,8,13-hexahydroxy-10,11-dimethylphenanthro[1,10,9,8-opqra]perylene-7,14-dione) is a naphthodianthrone, an anthraquinone derivative, which is known to be a non-specific protein kinase inhibitor. This is an expanded-spectrum, naturally occurring photosensitizer that absorbs light in the 510-

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to 540-nm wavelength range (19, 20). Additionally, hypericin is a major component of *Hypericum perforatum*, a plant widely studied in skin and tumor therapies, showing potential as a treatment option for cancer and viral, fungal, and bacterial infections as well as an antidepressant (21, 22). Utility of hypericin has been demonstrated in cancer, photodiagnosis, and wound healing therapy (22–24).

Hypericin is a nontoxic and nonmutagenic compound and is currently approved by the FDA for use in humans with cutaneous lymphoma. More recently, leishmanicidal activity and specific inhibition of spermidine synthase by hypericin in *Leishmania (Leishmania) donovani* promastigotes, which causes visceral leishmaniasis, were reported (25). However, to our knowledge, there are no reports on either the activity of hypericin on intracellular amastigotes (the parasitic stage that has clinical relevance) or its effectiveness for treating CL in animal models. Due to the urgent need to identify new and better alternatives to treat CL, this study aimed to evaluate the potential of hypericin for use in PDT as a therapeutic alternative for CL.

MATERIALS AND METHODS

Chemicals and reagents. Hypericin (CAS 548-04-9) was obtained from Analytik GMBH (Germany). Amphotericin B deoxycholate (AmB) (Sigma-Aldrich, St. Louis, MO, USA) was used as an *in vitro* leishmanicidal control compound. Meglumine antimoniate (MA) (Sanofi-Aventis, Sao Paulo, Brazil) was used as an *in vivo* leishmanicidal drug control. All subsequent dilutions for hypericin, AmB, and MA were freshly made in Roswell Park Memorial Institute (RPMI)-1640 media (Sigma-Aldrich, St. Louis, MO, USA). A water-in-oil emulsion (cream) containing 0.5% hypericin was prepared. Hypericin concentration was selected based on the activity observed *in vitro* and the availability of the active ingredient.

Parasites. Promastigotes of *L. (V.) panamensis* (MHOM/CO/87/UA140 ePir-GFP) were grown in Novy-MacNeal-Nicolle (NNN) medium at 26°C. Parasites in the stationary phase of growth were used for both *in vitro* and *in vivo* infections (day 5 of culture that correspond to metacyclic or infective promastigotes).

Cell line and culture conditions. U-937 promonocytes (ATCC CRL1593.2) and Detroit 551 fibroblasts (ATCC CCL-110) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured under standard conditions at 37°C and 5% CO₂, with a change of medium every 3 days until use. U-937 cells (suspension) were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). Detroit 551 cells (adherent) were maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma) with similar amounts of FBS and antibiotics.

Human fibrocytes and macrophages were derived from peripheral blood monocytes. Briefly, peripheral mononuclear cells from three healthy donors were isolated from defibrinated blood using density gradient centrifugation (Ficoll-Hypaque 1.077; Sigma-Aldrich), according to the manufacturer's instructions. Differentiation of monocytes to macrophages (huMDM) was achieved as described by Robledo et al. (26), while differentiation of monocytes to fibrocytes (huMDF) was achieved as described by Curnow et al. (27).

Emission properties of 20-W T8 fluorescent light. To validate the use of an efficient and economical light source, the emission properties of a 20-W T8 fluorescent light one fitting was evaluated. Wavelengths and distance required to generate a light exposure of 5 J/cm², a dose suitable for hypericin photoactivation, were determined using an optical spectrum analyzer (AQ6370B; Yokogawa Electric Corporation, Thailand).

***In vitro* cytotoxicity.** The cytotoxic activity of hypericin *in vitro* was determined in huMDM, Detroit-551 cells, and U-937 cells according to the ability of hypericin to induce cell death and was quantified by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich), following the procedure previously described

(28). Briefly, 100 μl of cell suspension containing either 100,000 U-937 cells, 25,000 Detroit-551 cells, or 50,000 huMDM or huMDF in their corresponding culture media supplemented with FBS and antibiotics was dispensed into each well of 96-well cell culture plate. Then, 100 μl of medium with hypericin was added. Six concentrations of hypericin in serial 2-fold dilutions from 10 μM were evaluated. Plates were incubated at 37°C and 5% CO₂ for 2, 4, and 6 h, irradiated at 5 J/cm² (15 min, 15 cm), and incubated again at 37°C and 5% CO₂. After 72 h of incubation, MTT solution was added. The concentration of formazan was determined by spectrophotometric measurement of absorbance (optical density [OD]) at 570 nm on a Varioskan Flash reader (Thermo Scientific). The cytotoxic effect of hypericin in the absence of light irradiation was also evaluated. Cells treated with AmB were used as controls for toxicity (positive controls), while cells incubated in medium alone were used as controls for viability (negative controls). Determinations were done in triplicate in at least two independent experiments. Cytotoxicity was determined according to viability and mortality percentages obtained for each experimental condition (hypericin with or without irradiation, AmB, and culture medium alone). Results were expressed as 50% lethal concentration (LC₅₀), which is the concentration that kills 50% of cells, calculated by Probit analysis (29). Viability (as a percentage) was calculated with the formula $(OD_{\text{treated cells}}/OD_{\text{untreated cells}}) \times 100$, where the OD of untreated cells corresponds to 100% viability. In turn, inhibition cell growth percentage corresponds to $100 - \% \text{ viability}$.

***In vitro* leishmanicidal activity.** The activity of hypericin on intracellular amastigotes of *L. (V.) panamensis* was determined according to the ability of the compound to reduce the infection of macrophages by *Leishmania* parasites following the method described previously by others (10, 30, 31). Briefly, to each well of 24-well cell culture chamber slides containing 300,000 huMDM was added 100 μl containing 9×10^6 promastigotes of *L. (V.) panamensis* (30:1 parasite-to-cell ratio). After 24 h of infection, the RPMI 1640 medium was replaced by fresh culture medium containing hypericin at the corresponding concentration (six concentrations in serial 2-fold dilutions from 10 μM), and plates were incubated at 37°C and 5% CO₂ for 2, 4, and 6 h and then irradiated at 5 J/cm² (15 min, 15 cm). After 72 h, cells were analyzed by flow cytometry. The antileishmanial effect of hypericin in the absence of photoactivation by light irradiation was also evaluated. Infected cells treated with AmB were used as controls for antileishmanial activity (positive control), while infected cells incubated in medium alone were used as controls for infection (negative controls). All determinations for each concentration of hypericin and standard drugs were carried out in triplicate, in at least two experiments. Antileishmanial activity was determined according to the reduction in the number of viable parasites inside infected cells obtained for each experimental condition by the flow cytometer. The percentages of parasites for each concentration tested were calculated by the formula $(\text{MFI of infected and treated cells}/\text{MFI of infected untreated cells}) \times 100$, where the MFI (median fluorescence intensity) of infected untreated cells corresponds to 100% parasites. In turn, the percent inhibition of parasites was calculated as $100 - \% \text{ parasites}$. Antileishmanial activity is expressed as 50% effective concentrations (EC₅₀) measured by the Probit method (29). In addition, the index of selectivity (IS) was calculated by the ratio of cytotoxic activity to leishmanicidal activity (LC₅₀/EC₅₀).

***In vivo* response of topical 0.5% hypericin and toxicity.** The therapeutic response of a 0.5% hypericin topical cream was tested in the hamster (*Mesocricetus auratus*) model for CL (32). Briefly, previously anesthetized (40 mg/kg ketamine and 5 mg/kg xylazine) hamsters were inoculated in the dorsal skin with promastigotes of *L. panamensis* (5×10^8 parasites/100 μl phosphate-buffered saline [PBS]). Three experimental groups, each consisting of four males and four females, were designated as follows: group A, hypericin without light; group B, hypericin with 15 min light; and group C, MA (positive control). Treatment with 0.5% hypericin with or without light (40 mg per dose) or MA (200 μg per dose) was initiated immediately after development of a typical ulcer (4 to 6 weeks postinfection). Hypericin (groups A and B) was applied topically every 3 days for

3 weeks for a total of six doses. After each application, animals of group B were immobilized and directly exposed to visible light (5 J/cm²) for 15 min, similar to the method used for the *in vitro* experiments. In turn, MA (20 µl, 10 mg/ml) was applied intralesionally, also every 3 days for 3 weeks (six doses in total). Animal welfare was supervised daily during the study. Area of the ulcer and body weight were measured every 2 weeks from the beginning of the treatments to the end of the study (three months after completion of treatment). The overall time points of evaluation were pretreatment day (TD0), end of treatment (PTD0), and posttreatment days 30, 60, and 90 (PTD30, PTD60, and PTD90, respectively).

The effectiveness of each treatment was assessed by comparing the lesion sizes prior to and after treatments. Treatment outcome at the end of study was recorded as cure (healing of 100% of the area and complete disappearance of the lesion), clinical improvement (reduction in the size of the lesion of >30% of the area), failure (increase in the size of the lesion), or relapse (reactivation of lesion after initial cure). To compare the effectiveness among groups of treatments, an arbitrary score was assigned to each treatment: 3 = cure, 2 = clinical improvement, 1 = relapse, and 0 = failure.

The toxicity of 0.5% hypericin cream or MA was evaluated according to hepatic and renal functions of hamsters in treated and untreated animals as described previously (32). At TD0 and day 8 of treatment (TD8), blood was drawn from the heart, and serum was separated by centrifugation at 5,000 × g for 2 to 3 min. The serum was stored at -80°C until use. Hepatic and renal functions were assessed by measuring the levels of alanine amino transferase (ALT), blood urea nitrogen (BUN), and creatinine using commercially available kits (Biosystems, Spain). The hepatic and renal functions were also evaluated in healthy (uninfected and untreated) hamsters. Toxicity of treatments was determined by comparing serum levels of ALT, BUN, and creatinine and postmortem histological changes in liver and kidney. Severity of histological changes was also graded as severe, moderate, or mild.

Quantification of parasite load. Quantification of parasites present in the lesions was based on amplification of a single copy gene of *Leishmania* using quantitative real-time PCR (qPCR) and a standard curve as described previously (33). At days 0 and 15 of treatment of hamsters, scrapings of the lesions were taken for subsequent RNA extraction and quantification of growth factors associated with healing of wounds (see below). At the end of the study, hamsters were humanely sacrificed, and after necropsy, liver and kidney biopsy specimens were taken for histopathological studies. A skin biopsy specimen (from the site where the injury occurred) was also taken to determine parasite load by qPCR.

Initially, a 600-bp fragment of DNA of *L. (V.) panamensis* was amplified by conventional PCR (T1000 thermocycler; Bio-Rad) and purified (using a QIAquick gel extraction kit according to the manufacturer's instructions), and the product was ligated to an InsTAclone pTZ57R/T vector. Then, DH5α cells (Invitrogen, USA) were transformed with the construct. The construct was purified, and quantitative reverse transcription-PCR (qRT-PCR) was set to amplify a 120-bp fragment within the 600-bp sequence initially cloned. The number of copies per plasmid was determined based on the size of the cloned fragment and the size of the insert. A standard curve ranging from 1 to 1 million parasites in log increases from 10 was established. The QuantiFast SYBR green qRT-PCR kit (Qiagen Inc., USA) was used for qPCR. The PCR amplifications were performed in a SmartCycler II (Cepheid, Sunnyvale, CA, USA) using a final volume of 25 µl containing 100 ng of DNA, 12.5 µl of the reaction mix, 100 nmol/liter of each primer, and nuclease-free water. The amplification efficiency of each was measured using the PCR program LinReg. Tissue samples were weighted and lysed with 500 µl of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, and 0.5% SDS [pH 8.0] and 0.1 mg/ml proteinase K) by incubation in a water bath for 4 h at 56°C. Then, DNA was extracted with 1 ml of phenol-chloroform-isoamyl alcohol (25:24:1). After centrifugation for 10 min at 1,700 rpm, the aqueous layer was carefully removed, washed with 90% ethanol, centrifuged 2 min at 1,700 rpm, and dried at room temperature. The pellet was resuspended

in 300 µl of autoclaved nuclease-free water. DNA was quantified using NanoDrop 1000 (Thermo Scientific, New Hampshire, USA) at 260 nm of absorbance and stored at -20°C until further use.

Dermal absorption. *In vitro* skin penetration/absorption of hypericin was evaluated following OECD (Organisation for Economic Co-operation and Development) test guideline 428 (34). Hamster skin was cut in 2-by 2-cm² pieces and kept in Dulbecco's phosphate-buffered saline (DPBS). The receptor chamber of a Franz-type diffusion cell having a permeation area of 0.95 cm² was filled with 10 ml of DPBS, and the skin piece was appropriately clamped between the donor and the receptor chamber. Then, 40 mg of 0.5% hypericin cream was applied to the skin. Diffusion cells were warmed at 32°C and 5% CO₂ and continuously stirred with magnetic bars. To quantify the hypericin present in the receptor chamber, the receptor solution was collected at 30 min and 1, 2, 4, and 8 h. At the end of the experiment, skin samples were washed twice with DPBS and collected. Samples of the receptor solution and washing were lyophilized and stored in the dark at -20°C until use. Lyophilized samples were resuspended in ethanol (1-ml vial), and 200 µl was used for quantification by spectrofluorimetry on a Varioskan Flash reader (Thermo Scientific) using a standard curve (0.0004 to 0.2 mg/ml hypericin in ethanol). Each sample was measured in duplicate.

***In vitro* wound healing (scratch) assay.** The effect of hypericin on wound closure was investigated with a CytoSelect 24-well wound healing assay kit (Cell Biolabs, Inc., San Diego, USA) using a method similar to that used by others (35). Detroit 551 fibroblasts (250,000/ml) in DMEM containing 10% FBS and antibiotics were seeded into 24-wells tissue culture plate containing proprietary treated inserts in the plate wells with their "wound field" aligned in the same direction and incubated for 24 h, allowing the cells to adhere and reach the 80% confluence. After the inserts were removed from the wells, the medium was carefully aspirated, and wells were washed with test medium (DMEM containing 10% FBS and antibiotics) to remove dead cells and debris. Finally, the cells were treated with three concentrations of hypericin (1.5, 0.75, and 0.375 µM) for 2 h, irradiated with 5 J/cm² (15 min), and incubated for an additional 36 h. Cells treated with EDO5 (EDO Laboratories, Cali, Colombia) were used as positive controls for wound healing. Migration into the wound field was determined every 12 h by using manual fixing with cell stain solution according to the manufacturer's instructions. Representative images focused on the center of the wound field were obtained. Microscopic imaging of wound closure was analyzed using NIS-Elements imaging software (Nikon). Two sets of experiments in duplicate were performed. The influence of test compounds on wound closure was assessed by comparison to untreated controls. The density of cells in wells without treatment was used as 0% wound closure. The effect on wound closure was followed every 12 h. Migration of cells into the wound was compared to that in untreated control in DMEM with 10% FBS and EDO5. The level of cellular filling within the wound area in response to substances was compared to the wound-filling response in the untreated cells.

Production of growth factors associated with wound healing. The effect of hypericin to induce expression of growth factors associated with wound healing, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor beta (TGF-β), was evaluated by qRT-PCR in huMDF treated with hypericin at 3.0 µM with and without photoactivation (*in vitro* studies). Briefly, huMDF were incubated in the presence of 3.0 µM hypericin. Six hours later, cells were irradiated with 5 J/cm². After 24, 48, and 72 h, RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control gene.

Expression of the same growth factors was determined in samples from skin scraping of hamsters treated with 0.5% hypericin cream with or without exposure to light. In this case, gamma actin was used as a housekeeping control gene, while RNA was extracted using TRIzol as described above. RNA (1 µg) was transcribed to cDNA using a Maxima first-strand cDNA synthesis kit for qRT-PCR (Fermentas, Thermo Scientific, USA),

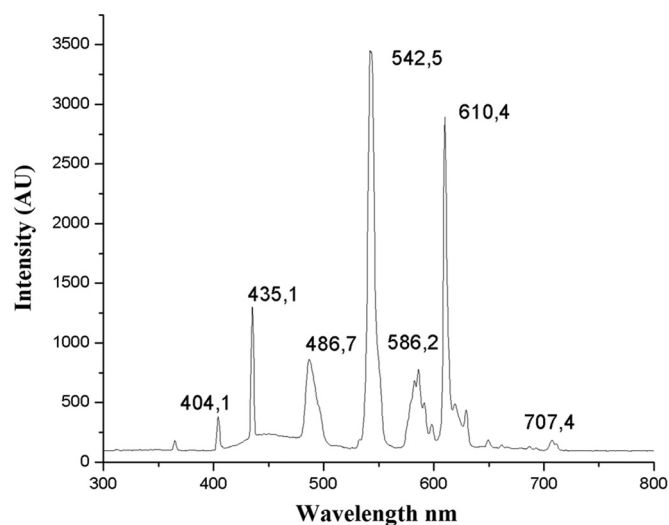


FIG 1 Emission spectrum of a T8 MY-20W fluorescent lamp. The spectrum shows emission bands between 400 and 700 nm. The most intense is at 542.5 nm.

according to the manufacturer's instructions. Then, cDNA (1 μ l) was amplified using primers and TaqMan probes in a Smart Cycler II (Cepheid, Sunnyvale, CA, USA) with the following amplifier conditions: 95°C for 600 s and 40 cycles of 95°C for 15 s and 60°C for 60 s. The efficiency was assessed with the LinReg PCR program. Expression level of growth factors was calculated using the $\Delta\Delta C_T$ method, comparing expression early during treatment (TD15) with expression before treatment (TD0). Additionally, the level of expression was compared in terms of treatment outcome observed at the end of the study (cure, improvement, or failure) versus expression level before treatment (TD0). The number of times that an expression level increased is called the increment factor (IF).

Statistical analysis. *In vitro* experiments were performed at least in triplicate in two independent assays. Eight hamsters per group were used for all *in vivo* experiments. Data presented are means and standard deviations. The statistical significance of differences between experimental groups was determined as described in the figure legends using analysis of variance (ANOVA) followed by Tukey's test with GraphPad Prism 6 software. A *P* value below 0.05 was considered statistically significant.

Ethics. The Ethics Committee for Animal Research of the University of Antioquia approved all procedures involving the uses and care of animals (act 63, 2010). The Bioethical Committee of the University of Antioquia approved the utilization of blood from healthy donors (act 10-5-331, 2010).

RESULTS

Calibration lamp. In order to explore the utilization of a low-cost, highly accessible illumination source, this work utilizes a conventional fluorescence lamp. Therefore, the emission spectrum of a T8 fluorescent lamp fitting MY-20W was determined. We found that this fluorescent lamp has an emission spectrum between 400 and 700 nm (Fig. 1). The most intense emission band peaks at 542.5 nm, a wavelength that is within the photoactivation range of hypericin. In addition, we established that 5 J/cm² of radiation exposure is obtained when the lamp is used at a 15-cm distance for 15 min.

Hypericin exhibits selective cytotoxicity against cell lines and primary culture cells. The effect of hypericin on cell viability was assessed in human macrophages (U937 and huMDM) and fibroblasts (Detroit 551 and huMDF), which are cell types that interact directly with *Leishmania* parasites during infection and

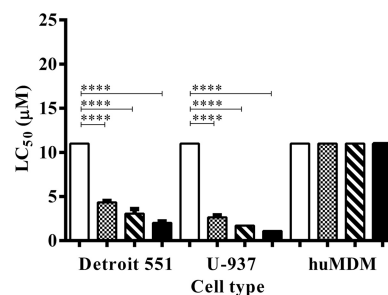


FIG 2 *In vitro* cytotoxicity of hypericin. Bars represent means and standard deviations (SD) obtained with Detroit 551 cells, U-937 cells, and huMDM after 72 h of incubation with hypericin without irradiation (white bars) or with irradiation after 2 h (cross-hatched bars), 4 h (hatched bars), and 6 h (black bars). ****, *P* < 0.0001.

wound resolution. We observe that hypericin is more toxic for cell lines (U937 and Detroit 551) than primary cell cultures (huMDM and huMDF) (Fig. 2). Moreover, cytotoxicity of hypericin increases after photoactivation at 5 J/cm². The increases in cytotoxicity correlate with time of light exposure. Thus, the LC₅₀ was 2.8 ± 0.11 μ M, 1.7 ± 0.15 μ M, and 1.1 ± 0.01 μ M after 2, 4, and 6 h of treatment of U937 cells, respectively. In Detroit 551 cells, the LC₅₀ is 4.34 ± 0.15 μ M, 3.06 ± 0.5 μ M and 2.02 ± 0.16 μ M after 2, 4, and 6 h of light exposure. In contrast, the LC₅₀ in huMDM and huMDF was >10 μ M (maximum concentration evaluated) regardless of the time of light exposure. In all nonirradiated cells, the LC₅₀ was >10 μ M, implying that hypericin is not cytotoxic at the maximum concentration evaluated and time of exposure.

Hypericin exhibits *in vitro* antileishmanial activity against intracellular amastigotes of *L. (V.) panamensis*. The antileishmanial activity of hypericin was assessed in huMDM infected with fluorescent *L. (V.) panamensis*. The effect of hypericin was determined by monitoring reduction of intracellular infection (parasite load). Hypericin exhibited a high leishmanicidal activity with time-dependent reduction of intracellular amastigotes. Moreover, antileishmanial activity increases upon light exposure. Thus, EC₅₀ was 2.5 ± 0.14 μ M in infected huMDM treated with hypericin without light and 1.2 ± 0.007 μ M in infected cells treated with hypericin and light. However, no statistically differences were observed (*P* < 0.05). As expected, AmB (positive control) is highly active against *Leishmania* parasites (EC₅₀, 0.61 ± 0.005 μ M). There were statistically significant differences between hypericin (with and without light) and AmB (*P* < 0.001). The IS was >8.3 and >4.0 in irradiated and nonirradiated experiments, respectively. In AmB, the IS was >45. These results suggest that the biological activity of hypericin was selective, being more active against *Leishmania* parasites than huMDM.

Response of 0.5% hypericin in an animal model of disease. Treatment of *L. (V.) panamensis* at 40 mg/day twice per week for 3 weeks with 0.5% hypericin with light exposure of 5 J/cm² (group A) resulted in cure of 6/8 hamsters (with 100% reduction of lesion size) and clinical improvement in 2/8 hamsters with approximately 80% of reduction in their lesion sizes. Parasite load in the animals healed was <100 parasites/mg of tissue, while in the two animals with clinical improvement it was 1,355 and 2,273 parasites/mg of tissue. On the other hand, treatment with hypericin without irradiation (group B), at the same dose, produced cure in 3/8 hamsters, clinical improvement with more than an 80% re-

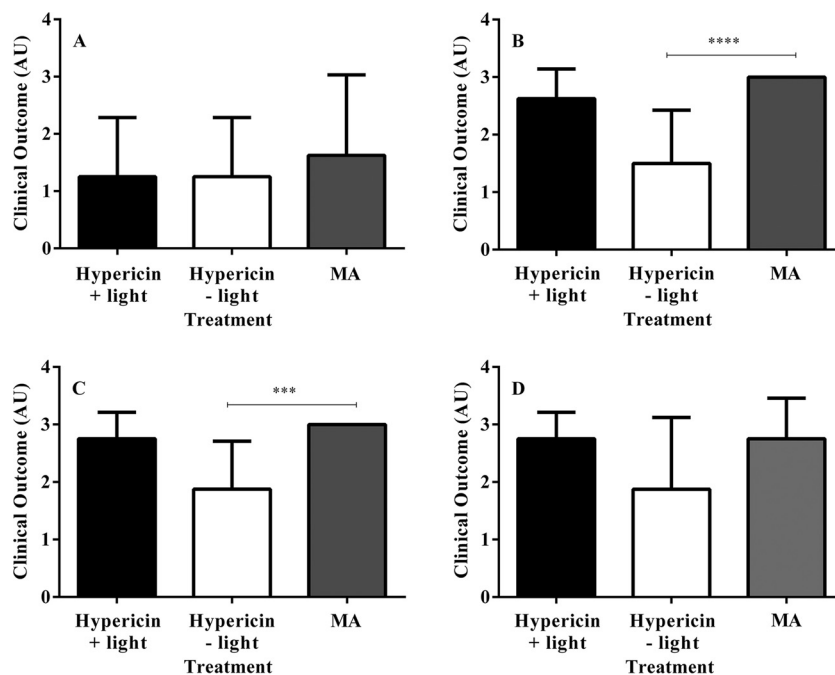


FIG 3 Response of hypericin in treatment of CL *in vivo*. Treatment outcome was evaluated at the end of treatment (A) and on days 30 (B), 60 (C), and 90 (D) posttreatment. Bars represent means \pm SD. ***, $P = 0.003$; ****, $P = 0.0012$.

duction in their lesion size in 3/8 hamsters, and failure in 2/8 hamsters (reduction $< 30\%$). In this group, parasite load was < 50 parasites/mg of tissue in cured animals. In contrast, parasite loads in animals that showed clinical improvement and failure were 7,000 and 27,000 parasites/mg of tissue, respectively. Finally, treatment with intralesion injection of MA 200 $\mu\text{g}/\text{day}$ twice a week for 3 weeks (group C) cured 7/8 hamsters, and 1/8 hamster experienced relapse, 2 months after treatment. The amount of parasites in hamsters cured was < 30 parasites/mg of tissue. The hamster that relapsed had 890 parasites/mg of tissue. Differences were not statistically significant ($P > 0.05$).

Differences in treatment effectiveness were based on results obtained in each of the different stages of evaluation (PTD0, PTD30, PTD60, and PTD90), using the arbitrary scale described in Materials and Methods. The mean value of treatment outcomes obtained at each time point for each treatment group is summarized in Fig. 3. Effectiveness of topical treatment with hypericin and light was similar to that observed with intralesion injection of MA at any time point during follow up. Differences were not statistically significant ($P > 0.05$). On the other hand, the effectiveness of topical hypericin without light was lower than that of MA at PTD30 and PTD90. These differences were statistically significant ($P = 0.003$ at PTD 60 and $P = 0.0012$ at PTD90).

Only a few animals in each treatment group experienced weight loss ($< 10\%$). Thus, weight loss could not be associated with toxic effects of treatment with hypericin. Levels of serum ALT for liver dysfunction and BUN and creatinine for renal dysfunction, measured 8 days after treatment with hypericin and light and hypericin without light as well as in uninfected untreated hamsters, demonstrated normal levels of the serum enzymes. No histological alterations attributable to treatment were observed in animals treated with hypericin and either irradiated or nonirradiated. In contrast, hamsters treated with MA exhibited the follow-

ing changes in the liver: cloudiness, vacuolar and fat degeneration, karyomegaly, binucleation, and pigmentation. These occurred to a moderate to severe degree. MA treatment also induced changes in kidneys, including vacuolar and fat degeneration and binucleation to a mild to moderate degree.

Dermal absorption of hypericin. The skin absorption assay determines the amount of hypericin that is absorbed through the skin as well as the amount that is retained in the skin. In this assay, 40 mg of 0.5% hypericin cream were in contact with the surface of a skin sample. Only 0.011 mg was detected in the washing solution, and no hypericin was detected in the receiving solution at any time tested. These results suggest that the remaining 0.189 mg of hypericin was accumulated and retained in the skin.

Hypericin promotes *in vitro* wound healing. To investigate whether hypericin promotes wound closure, we used the *in vitro* wound-healing scratch assay in Detroit 551 fibroblasts, which mimics cell migration during wound healing *in vivo*. Specifically, this model assessed cellular wound fill, the “net effect” of all cellular events contributing to the *in vitro* wound healing process. The assay has proved to be a valuable tool to obtain first insights into how preparations can positively influence wound closure (24, 36). The scratch wound assay revealed that hypericin significantly increased the rate of fibroblast-mediated wound closure compared to untreated cells (Fig. 4).

After the wound field was created, Detroit 551 fibroblasts were exposed for 36 h to hypericin (1.5, 0.75, and 0.375 μM) with or without irradiation. Cell cultures treated with hypericin and light experienced only $11.7 \pm 3.2\%$, $23.9 \pm 6.0\%$, and $19.1 \pm 4.3\%$ (for 1.5, 0.75, and 0.375 μM hypericin, respectively) wound closure after 12 h in comparison to time zero (Fig. 5). Cells cultures treated with hypericin without light showed $24.8 \pm 4.5\%$, $42.2 \pm 5.2\%$, and $29.4 \pm 3.8\%$ of wound closure upon incubation with

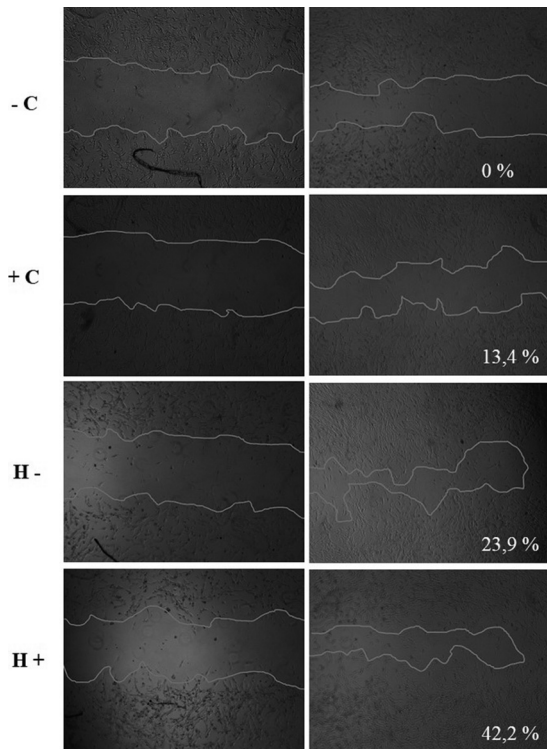


FIG 4 Light microscope images of wound closure *in vitro*, obtained by using confluent monolayers of Detroit 551 fibroblasts. Microphotographs show one representative experiment of cell migration into the created wound area in response to treatment. The wound area at 0 h (left) and after 12 h (right) for an untreated control (–C) (set to 0%), a positive control (+C), 0.75 μM hypericin plus light (H+), and hypericin without light (H–) is shown. Percent wound closure was normalized to the untreated control.

1.5, 0.75, and 0.375 μM hypericin, respectively (Fig. 5). The positive control EDO5 caused $13.4 \pm 3.5\%$ wound closure.

Wound closure after 24 h in comparison to time zero in cells treated with 1.5, 0.75, or 0.375 μM was $18.0 \pm 3.7\%$, $22.5 \pm 5.2\%$, and $18.6 \pm 3.2\%$ in hypericin without light and $15.9 \pm 3.3\%$, $17.9 \pm 4.3\%$, and $4.5 \pm 2.3\%$ in hypericin with light, respectively. The positive control EDO5 caused $5.0 \pm 2.5\%$ wound closure (Fig. 5). Significant differences were observed between any concentration of hypericin with light versus negative controls and hypericin without light versus negative controls ($P < 0.0001$). Statistical differences were also observed between hypericin with light and hypericin without light (0.075 μM and 0.375 μM) versus positive controls ($P < 0.0001$). Thus, hypericin, whether photoactivated or not, exerted significant effects. Interestingly, the level of wound closure by hypericin without light was about twice the value of hypericin with light. The differences were significant ($P < 0.0001$).

Hypericin induces production of growth factors associated with wound healing. To measure the expression of multiple angiogenic and antiapoptotic genes after hypericin priming, a qRT-PCR analysis was performed. Expression of factors associated with wound-healing growth was increased during treatment. However, the level of expression of each factor varied according to treatment and growth factor profile. Generally, levels of expression of growth factors in animals treated with hypericin and light were higher than those in animals treated with hypericin without light

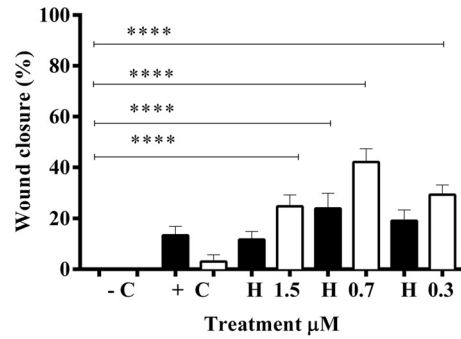


FIG 5 Wound closure effect of hypericin. The effects of hypericin without light (white bars) and hypericin with light (black bars) at different concentrations on the wound closure of Detroit fibroblasts (250,000/well) are expressed as percent wound closure after 12 h. EDO5 was the positive control. Bars represent means and SD from two independent experiments. ****, $P < 0.0001$.

(Table 1). For instance, treatment with hypericin plus light largely increased the expression of EGF, PDGF, and FGF but did not increase the level of TGF- β much. In contrast, treatment with hypericin without light largely increased only the expression of PDGF, while the increase of FGF, TGF- β , and EGF levels was low (< 2.0). These results suggest that photoactivation of hypericin affects both the expression levels and profiles of the growth factors studied.

The large increase in the expression of EGF but small increase in the expression of TGF- β by treatment with hypericin plus light agrees with a higher cure rate observed in this treatment group (6/8 hamsters). Animals treated with hypericin plus light that were cured showed the greatest increase in expression of PDGF, FGF, and EGF, and the increase in TGF- β expression was low (Table 1). Animals with improvement expressed a little more TGF- β , while there were no substantial changes in expression of the other factors. Animals that were treated with hypericin without light and cured showed increased expression of PDGF and moderate increases in the expression of FGF, TGF β , and EGF. In animals showing improvement, there was a small decrease in expression of EGF and TGF- β , while changes in expression of FGF and PDGF

TABLE 1 Expression levels of growth factor genes after treatment with hypericin *in vivo*

Treatment ^a	Comparison ^b	No. ^c	Change in expression level of growth factor ^d			
			EGF	FGF	PDGF	TGF- β
A	TD15 vs TD0	8	7.41	5.25	6.02	0.83
B	TD15 vs TD0	8	0.18	1.94	7.44	1.44
A	C vs TD0	6	3.37	5.25	6.02	0.83
	CI vs TD0	2	0.14	0	0	1.1
B	C vs TD0	3	0.18	1.94	7.44	1.43
	CI vs TD0	3	0.28	0	0	0.64
	F vs TD0	2	4.52	0	0.03	7.53

^a A, hypericin with light; B, hypericin without light.

^b Comparison between time points or treatment outcomes. C, cure; CI, clinical improvement; F, failure.

^c Number of hamsters used for data comparison.

^d Number of times that expression increased.

were not observed. Finally, in hamsters with failure, the highest increase was observed in levels of TGF β and EGF. No changes were observed in expression of PDGF and FGF (Table 1).

The production *in vitro* of growth factors was not as significant as that *in vivo*. After 24 h of treatment with 3.0 mM hypericin with or without light, huMDF exhibited an increase in EGF expression. The increment was higher in irradiated cells than nonirradiated cells (IF = 4.4 and 2.9 hypericin without light and hypericin with light, respectively). The increase in expression of FGF was similar in irradiated and nonirradiated hypericin-treated cells (IF, 2.7 and 2.6 for irradiated and nonirradiated huMDF, respectively). Expression of TGF β and PDGF increased only in huMDF treated with hypericin with light (IF, 7.6 and 1.9, respectively). After 48 h of treatment with 3.0 μ M hypericin with light, only the expression of EGF increased (1.1 times). No increase was detected in the expression of these growth factors in huMDF after 72 h of exposure to 3.0 μ M hypericin with or without light.

DISCUSSION

Cutaneous leishmaniasis affects populations with limited economical resources and availability of proper health centers. Indeed, one of the limitations for implementing the extensive use of hypericin in PDT for leishmaniasis is the need for expensive light sources (LEDs and lasers) for photoactivation, which, in the case of hypericin, occurs at around 540 nm and 5 J/cm² (37, 38). The T8 fluorescent lamp one fitting MY-20W has an emission spectrum covering the photoactivation range of hypericin. These findings will facilitate the use of hypericin in different therapies at a lower cost.

Results of assays for the *in vitro* toxicity of hypericin suggest that hypericin is selective, being more toxic to tumor than to normal cells. The selective cytotoxicity observed here is in agreement with a previous study, which demonstrated that hypericin is preferably internalized by tumor cells, where it accumulates into the membranes of mitochondria, the Golgi apparatus, and the endoplasmic reticulum and then induces apoptosis (39). This accumulation is apparently due to alterations in the expression of protein complexes like E cadherin (40). Although Detroit 551 cells are normal cells, they express high levels of cadherin-like proteins (41). It is possible that during the immortalization process, the expression of these proteins is altered. The increased susceptibility of Detroit 551 cells to hypericin may also be due to the fact that these cells have a finite useful life span from the tissue of origin. This could make the cells more susceptible to oxidative stress caused by hypericin after this is activated by light exposure.

Leishmanicidal activity of both irradiated and nonirradiated hypericin is probably due to the specific inhibition of spermidine synthase, which may result in decreased trypanothione but increased glutathione levels inside parasites. This leads to a redox imbalance with ROS production and necrosis, as demonstrated in *L. donovani* promastigotes (25). Leishmanicidal activity could also be enhanced due to the production of ROS and proinflammatory cytokines (TNF- α , IL-6, and IL-1 β) produced by huMDM upon activation by PDT (42). These are involved in elimination of *Leishmania* parasites during activation of acquired immune response. However, the effect of hypericin on the activation of inflammatory response remains to be determined.

The *in vivo* evaluation of the therapeutic response confirmed the leishmanicidal activity of hypericin observed *in vitro*. Photoactivation of hypericin was also confirmed. Thus, a topical cream

formulation containing 0.5% hypericin combined with visible light irradiation allowed wound healing in most animals. Although some hamsters treated with topical 0.5% hypericin cream in the absence of irradiation were also cured, the proportion of cure was higher with irradiation. Differences, however, were not statistically significant ($P > 0.05$). Hypericin also exhibited no effects on the body weight gain or tissues of hamsters, suggesting no toxicity *in vivo*.

Healing in nonirradiated hamsters might result from inhibition of spermidine synthase as described previously for *L. donovani* promastigotes (25). It is also plausible that any degree of exposure to ambient visible light during animal manipulation during treatment administration could have induced hypericin excitation and direct activation of ROS-mediated microbicide response. Another explanation of the leishmanicidal effect observed in the absence of light could be related to the ability of hypericin to inhibit some enzymes, such as glutathione reductase, which may induce a decrease in pH, which in turn would indirectly favor subsequent oxidative response and parasite clearance (43).

Hypericin was not absorbed but retained in the skin. These results are consistent with previous studies of penetration and distribution of hypericin in mice, where after application on the skin, hypericin penetrated after 4 h and was retained mainly in the stratum corneum (44). A previous study showed that the passage of hypericin into the circulatory system is minimal, with undetectable levels in plasma or serum (45). Retention of hypericin in the skin guarantees a treatment that targets the affected area, thus avoiding possible systemic toxic effects, as happens with other drugs used for the treatment of CL or even with other photosensitizers used to treat other diseases (46, 47).

The cutaneous wound healing process is a well-orchestrated molecular and biological event consisting of cell proliferation, angiogenesis, extracellular deposition, and remodeling (48, 49). In addition, the most important clinical endpoint in wound management is wound closure, or 100% epithelialization. The level of wound closure was higher in hypericin without light than hypericin with light treatment. These findings agree with a mechanism in which the induction of cell proliferation mediated by hypericin is not enhanced by photoactivation, as reported by others (43). Furthermore, in the absence of photoactivation, hypericin has better potential in the formation of granular tissue vascularization and re-epithelialization associated with wound healing (24).

Photoactivation of hypericin affects not only the expression levels of the factors associated with wound healing but also the expression profile of these factors. It has been demonstrated that expression of growth factors may be mediated by ROS, which could explain the wound healing process induced by PDT. ROS activate EGF signaling pathways, which accelerates re-epithelialization of wounds by the migration and proliferation of keratinocytes (50, 51). Similarly, activation of PDGF is also mediated by ROS through the STAT3 signaling pathway (52). However, further studies are necessary to understand the delicate balance between ROS release and the production of growth factors, because exacerbated ROS levels can affect the migration and proliferation of fibroblasts and impair wound healing (53).

It could be hypothesized that that besides killing parasites, the combination of hypericin and light promotes or accelerates the expression of EGF, with subsequent ulcer healing. On the contrary, in animals treated with hypericin without light, the relative increased expression of TGF β may favor the persistence of para-

sites and therefore the presence or even exacerbation of the ulcer. It has been demonstrated in other *in vivo* models that TGF- β promotes the proliferation of fibroblasts under normal conditions; however, overexpression of this factor delays the wound healing process (54).

The increases in the expression levels of growth factors only after 24 h after light irradiation suggest that photoactivation consumes hypericin, and therefore, the capability of hypericin to stimulate production of growth factors may be lost. The dynamics of the production of growth factors both *in vitro* and *in vivo* confirm the healing potential of hypericin previously reported by others (24). Increased expression of TGF- β was detected only *in vitro* after treatment of huMDF with hypericin and light, not in hamsters with CL. This difference could be due to the fact that although TGF- β is a growth factor associated with wound healing and tissue repair, in CL it is associated with persistence of parasites and therefore persistence of ulcers (55). This differential effect could also be explained because hormones exert an antagonistic effect on signaling pathways responsible for the expression of TGF- β , directly affecting the regeneration of tissues (56). On the other hand, tissue repair is a dynamic process in which cells, extracellular matrix proteins, and growth factors are involved (49). Induction of FGF expression could allow wound healing and it induces angiogenesis and contributes to the proliferation and migration of keratinocytes to the injured site, thereby facilitating wound closure (57). Meanwhile, EGF stimulates mitosis and migration of keratinocytes and fibroblasts, thus contributing to the formation of granulation tissue and deposition of extracellular matrix (58). To date, no studies to establish the usefulness of growth factors in the process of tissue repair in the LC are known. However, results shown here suggest that when the parasite load decreases and expression of the growth factors EGF and FGF are induced, the ulcer healing process is accelerated. These findings are consistent with those reported by other investigators, where hypericin has been used to treat burns and found to enhance the production of growth factors such as TGF- β and vascular endothelial growth factor (EVGF) (24).

In conclusion, here we demonstrate that hypericin exhibits antileishmanial activity against intracellular amastigotes of *L. (V.) panamensis*. The leishmanicidal activity was further confirmed in golden hamsters infected with *L. (V.) panamensis* which developed CL. Topical treatment with a cream containing 0.5% hypericin and exposure to light (5 J/cm²) induced cure in 6/8 of hamsters treated (75%), while the remaining 2 hamsters had 80% improvement of their lesions. Moreover, cured animals had 99.6% inhibition of parasite burden, while 75% of parasite load was inhibited in hamsters that showed improvement. The leishmanicidal effect is mediated by photoactivation of hypericin, as evidenced by the increases in the leishmanicidal activity relative to treatment in the absence of light. In addition, hypericin promotes fibroblast migration and production of growth factors associated with wound healing. Both events are necessary for the regeneration of tissue that has been damaged as a result of CL. Moreover, no cytotoxic effect was observed on human macrophages and fibroblasts and there was no impairment of liver and kidney functions of hamsters topically treated with hypericin.

Our results suggest that once hypericin is in contact with the skin, it exerts a wound-healing effect by increasing expression of growth factors. Exposure to visible light and photoactivation improve its leishmanicidal activity. However, only after elimination

of parasite stimuli can tissue repair processes occur. The results shown here suggest that topical administration of hypericin (0.5% cream) is as effective as intralesion injection of MA and safer for use in animals. Because hypericin can be photoactivated by inexpensive light sources, photodynamic therapy with hypericin could become an alternative for treating CL, particularly in regions where there has been a decrease in the effectiveness of the therapy of pentavalent antimony. In addition, the healing properties of hypericin could help improve the appearance of scars on people affected by CL. This study expands the scope for designing and strengthening chemotherapy strategies for better management of this stigmatizing disease. Given that hypericin is FDA approved for use in humans, it is feasible to proceed with clinical trials to demonstrate the efficacy and safety of photodynamic therapy using hypericin for the treatment of CL.

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