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Biomedical Sciences

Original article

Antileishmanial, immunomodulatory, and pro-regenerative effects of a TC2-SS topical formulation in *Leishmania braziliensis* cutaneous leishmaniasis. In vitro and in vivo studies

Efecto antileishmanial, inmunomodulador y pro-regenerativo de una formulación tópica de TC2-SS en leishmaniasis cutánea por *Leishmania braziliensis.* Estudios *in vitro* e *in vivo*

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Abstract

Leishmania braziliensis cutaneous leishmaniasis (CL) remains a major public health challenge due to the toxicity, variable efficacy, and emerging resistance associated with systemic therapies. However, few topical candidates have been successfully developed despite growing guideline support for local treatment in many patients. This study evaluated the antileishmanial, immunomodulatory, and proregenerative effects of a topical formulation combining triterpene saponins from *Sapindus saponaria* (SS) and a synthetic chroman hydrazone (TC2) using a standardized hamster model of *L. braziliensis* CL. The antiparasitic and immunomodulatory effects of TC2-SS were examined in human monocytederived macrophages infected with *L. braziliensis*, assessing intracellular parasite burden, macrophage morphology, nitric oxide (NO) and reactive oxygen species (ROS) production, and cytokine expression by ELISA and RT-qPCR. In vivo efficacy and early wound-healing responses were evaluated in a dorsalskin hamster model treated topically with 4% TC2SS and compared with intralesional meglumine antimoniate, including epidermal growth factor (EGF) and transforming growth factor β1 (TGFβ1) expression. TC2-SS reduced intracellular infection (EC_{50} 12.83 ± 0.97 µg/mL), decreased parasite burden, and induced macrophage activation-like morphological changes. The formulation preserved infectiondriven NO and ROS and selectively enhanced late NO in infected cells. Cytokine analyses revealed coordinated suppression of IL-1β, TNF-α, IL-4, IL-10, and TGF-β1, preserving MIP-1α. In vivo, TC2-SS achieved cure rates comparable to those of antimonials, with uniform lesion regression, no treatment failures, no systemic toxicity, and an increased ratio of EGF/TGF-β1. Thus, TC2-SS provided dual antileishmanial and host-directed benefits, supporting its advancement as a topical therapeutic candidate for *L. braziliensis* CL.

Keywords: Topical treatment; host-directed therapy; macrophage activation; nitric oxide; cytokines; wound healing; natural products.

Resumen

Leishmania braziliensis, agente causal de la leishmaniasis cutánea (LC), sigue siendo un desafío importante de salud pública debido a la toxicidad, la eficacia variable y la resistencia emergente de las terapias sistémicas. Sin embargo, pese al creciente respaldo de las guías a tratamientos tópicos, son escasos los candidatos desarrollados. Evaluamos aquí los efectos antileishmaniales, inmunomoduladores y pro-regenerativos de una formulación tópica que combina saponinas triterpénicas de *Sapindus saponaria* (SS) y una hidrazona de cromano sintética (TC2), utilizando

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un modelo estandarizado de LC por *L. braziliensis* en hámsters. Los efectos antiparasitarios e inmunomoduladores de TC2SS se analizaron en macrófagos derivados de monocitos humanos infectados, evaluando carga parasitaria, morfología, producción de óxido nítrico (NO) y especies reactivas de oxígeno (ROS), y expresión de citocinas. La eficacia *in vivo* y las respuestas tempranas de cicatrización se valoraron en un modelo de piel dorsal de hámster tratado tópicamente con TC2SS al 4 % y se compararon con las de los antimoniales intralesionales, incluyendo la expresión del factor de crecimiento epidérmico (EGF) y el factor de crecimiento transformante β1 (TGFβ1). La TC2SS redujo la infección intracelular, disminuyó la carga parasitaria e indujo cambios morfológicos compatibles con la activación de macrófagos, preservando la producción de NO y ROS inducida por la infección y aumentando selectivamente el NO tardío. El análisis de citocinas mostró la supresión coordinada de IL1β, TNFα, IL4, IL10 y TGFβ1, así como la preservación de MIP1α. *In vivo*, la TC2SS alcanzó tasas de curación comparables a las de los antimoniales, con regresión uniforme de las lesiones, ausencia de fallos terapéuticos y sin toxicidad sistémica, además de incrementar la relación EGF/TGFβ1. En conjunto, la TC2SS aportó beneficios duales dirigidos al parásito y al huésped, lo que respalda su desarrollo como candidato terapéutico tópico para la LC por *L. braziliensis*.

Palabras clave: tratamiento tópico; terapia dirigida al huésped; activación de macrófagos; óxido nítrico; citocinas; cicatrización de heridas; productos naturales.

Introduction

Cutaneous leishmaniasis (CL) comprises a heterogeneous group of clinical presentations caused by several *Leishmania* species transmitted by phlebotomine sandflies. Although some CL lesions caused by *Leishmania major* may heal spontaneously, others, particularly those caused by *Leishmania* of the *Viannia* subgenus, can progress to chronic, painful, and stigmatizing ulcers, and, in some cases, to destructive mucosal disease (**WHO**, 2010; **Kaye *et al.***, 2020). First-line treatments, including pentavalent antimonials, amphotericin B, and miltefosine, are limited by drawbacks such as toxicity, high cost, prolonged treatment regimens, and emerging drug resistance (**PAHO**, 2022; **Domagalska *et al.***, 2023). These challenges highlight the need for safer, more affordable, and locally applicable alternatives, especially topical therapies capable of reducing parasite burden while minimizing tissue damage and supporting high-quality healing.

In recent years, topical treatments for CL have gained substantial attention as practical, patient-friendly alternatives to systemic therapies. Particularly localized, uncomplicated CL can be effectively managed with topical interventions, reducing the risks associated with systemic drug exposure (**WHO**, 2010; **PAHO**, 2022). Remarkably, both the World Health Organization and the Pan American Health Organization recognize topical therapies as recommended options for specific clinical scenarios; however, the scarcity of well-validated topical products remains a major unmet need, underscoring the urgency for new candidates supported by mechanistic and translational evidence (**WHO**, 2010; **PAHO**, 2022).

The clinical outcome of CL is determined not only by the parasite load but also by complex interactions among infectious species, host genetics, and the balance between inflammatory and regulatory immune responses (**Kaye *et al.***, 2020; **Rőszer**, 2015; **Taslimi *et al.***, 2018; **Akbari *et al.***, 2021). In *Leishmania braziliensis* infection, disease severity is commonly due to excessive inflammatory responses, even when parasites are scarce or undetectable in chronic lesions (**Santos *et al.***, 2018; **Mohammadi *et al.***, 2021). Protective immunity relies on the induction of proinflammatory cytokines, including interferon-γ, transforming growth factor (TGF)-α, and interleukin (IL)-1β, and on microbicidal mechanisms, such as nitric oxide (NO) and reactive oxygen species (ROS) production by classically activated M1 macrophages. Conversely, excessive IL-1β-mediated inflammation, elevated IL-4 and TGF-β1 levels, and M2-like macrophage phenotypes can contribute to parasite persistence, tissue destruction, or impaired healing (**Rőszer**, 2015; **Calegari-Silva *et al.***, 2009). Thus, therapies that can simultaneously reduce parasite viability and modulate macrophage responses toward effective but not pathological inflammation hold promise for improving clinical outcomes. On the other hand, epidermal growth factor (EGF) and

TGF-β1 play key roles in wound healing and fibrosis. An increased EGF/TGF-β1 ratio may favor re-epithelialization and reduce scarring, representing a desirable outcome for CL therapeutics (**Montoya *et al.***, 2018; **Barrientos *et al.***, 2008).

Natural products remain a valuable source of antileishmanial compounds, particularly those with dual leishmanicidal and immunomodulatory properties (**Peer *et al.***, 2024; **Sakyi *et al.***, 2021). In a previous study, we demonstrated that the following triterpenic saponins: (i) hederagenin-3-O-(3,4-O-diacetyl-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinofuranoside); ii) hederagenin-3-O-(3,4-O-diacetyl-α-L-arabinopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinofuranoside), and iii) hederagenin-3-O-(4-O-acetyl-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinofuranoside) from Sapindus saponaria (SS) (**Correa *et al.***, 2014), combined with the synthetic chroman-4-ylidene-benzohydrazide (TC2) (**Vargas *et al.,*** 2017) at a 1:1 ratio, exerted potent in vitro and in vivo antileishmanial activity (**Upegui *et al.***, 2020; **Upegui *et al.***, 2019; **Rodríguez-Hernández *et al.***, 2017), inducing mitochondrial dysfunction and impairing reinfection processes. Additionally, a topical formulation containing a 4% TC2-SS mixture (1:1 ratio) achieved high cure rates in dogs and human CL patients, with minimal systemic absorption, supporting its safety as a topical therapy (**Piragauta *et al.***, 2022).

Despite these encouraging findings, the immunomodulatory properties of the TC2-SS combination, particularly its effects on cytokine networks, NO, and ROS production, macrophage polarization, and growth factor expression, remain poorly characterized. In particular, it is not known whether TC2-SS can reshape the local immune environment toward a balanced M1/M2 phenotype while simultaneously modulating EGF and TGF-β1 levels in CL lesions. Elucidating these host-directed effects is crucial, as CL resolution depends not only on parasite elimination but also on coordinated tissue repair.

To address these gaps, we evaluated the dual antiparasitic and host-directed actions of TC2-SS using complementary in vitro and in vivo approaches. First, we examined the effects of TC2-SS on human monocyte-derived macrophages (huMDMs) infected with *L. braziliensis*, focusing on intracellular parasite burden, morphological activation, NO and ROS production, and the expression of key cytokines associated with M1/M2 polarization (**Bernaerts & Vandooren**, 2025; **Mei *et al.***, 2023; **McWorther *et al.*,** 2015). Second, we analyzed the impact of the 4% TC2-SS topical formulation on EGF and TGF-β1 expression in skin lesions of golden hamsters with dorsal CL as parallel clinical outcomes. Together, these experiments provided a comprehensive assessment of TC2-SS as a topical candidate that integrates direct leishmanicidal activity with immunomodulatory and pro-regenerative effects, thereby laying the groundwork for its further translational development and eventual evaluation in controlled clinical trials.

Materials and methods

Human monocyte-derived macrophages (huMDMs)

Peripheral blood mononuclear cells were isolated from buffy coats of healthy donors recruited through the Hospital Alma Mater blood bank. Mononuclear cells were separated using Ficoll-Hypaque (Sigma-Aldrich) density-gradient centrifugation. The peripheral blood mononuclear-cell layer was washed twice with phosphate-buffered solution (PBS) and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were seeded at 1×10^6 cells/well in 24-well plates containing sterile round coverslips and incubated for 4 h at 37 °C, 5% CO_2 to facilitate monocyte adherence. Non-adherent cells were removed, and adherent monocytes were differentiated into macrophages by culturing them in RPMI supplemented with 10% autologous serum for 24 h (**Bernaerts & Vandooren**, 2025).

Parasites and culture conditions

Leishmania braziliensis promastigotes (MHOM/CO/88/UA301) were maintained at 26 °C in modified Novy–MacNeal–Nicolle medium overlaid with PBS, as previously described (**da Silva *et al.***, 2015). Promastigotes in the stationary phase (day 5–6) were used for infections.

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Preparation and characterization of the triterpene saponin mixture (SS)

The refined mixture of triterpene saponins was obtained from pericarps of *S. saponaria* via ethanol extraction, solvent evaporation, and fractionation on an XAD-7 resin column. Fractions were eluted with water and ethanol to yield a crude saponin fraction, which was further purified using Sephadex LH-20 chromatography. Purity and composition were confirmed by analytical one-dimensional/two-dimensional nuclear magnetic resonance (1D/2D NMR), following the methods of **Correa *et al.*** (2017) (**Figure S1A,** https://raccefyn.co/index.php/raccefyn/article/view/4172/5393).

Synthesis and purification of TC2

The chroman hydra zone TC2 was synthesized as previously described (**Vargas *et al.***, 2017) by reacting chromane-4-one with benzoic acid hydrazide in ethanol under acidic conditions. Crystals were filtered, dried, recrystallized, and characterized by NMR (purity ≥ 99%) (**Figure S1B,** https://raccefyn.co/index.php/raccefyn/article/view/4172/5393).

Preparation of the TC2-SS mixture

Stock solutions of TC2 ($C_{16}H_{14}N_2O_2$; molecular weight 266.1055; CAS No. 949087-80-3) and SS (50 mg/mL in DMSO) were combined in a 1:1 (w/w) ratio immediately before use. This ratio was selected based on previous structure-activity relationship studies, which demonstrated synergistic antileishmanial activity (**Upegui *et al.***, 2020; **Upegui *et al.***, 2019).

Infection of huMDMs and in vitro leishmanicidal assay

Macrophages (2 x 10^5 cells/well) were infected with stationary-phase *L. braziliensis* promastigotes at a parasite-to-cell ratio of 10:1 in FBS-free RPMI 1640 medium at 34 °C for 3 h. Non-internalized parasites were removed by washing, and cultures were incubated for 24 h before treatment.

Infected macrophages were exposed to four 1:4 serial dilutions of TC2-SS (starting concentration: 50 µg/mL) for 48 h. Untreated infected cells served as negative controls. Amphotericin B (AMB) was included as an internal control for assay validation but not for comparing the potency of TC2-SS with that of amphotericin and was tested at four two-fold serial dilutions (5, 1.25, 0.3125, and 0.078 µg/mL). These concentrations were used to calculate the half-maximal effective concentration (EC_{50}) of amphotericin B.

Cells were fixed in methanol, stained with Giemsa, and examined under light microscopy (100×). For each treatment, 100 macrophages were counted, and the parasite load was calculated using Equation 1:

$$\text{Parasite load} = \text{mean amastigotes per infected macrophage} \quad (1)$$

The EC_{50} for TC2-SS and AMB were estimated by non-linear regression using Prism 8 (GraphPad Prism 8 software, USA), following standard dose-response analysis procedures (**Motulsky**, 2018).

Morphological assessment of macrophage activation

Morphological activation of huMDMs was assessed according to established criteria, including increased cell spreading, cytoplasmic expansion, and the appearance of membrane protrusions such as filopodia and lamellipodia (**Mei *et al.***, 2023; **Atri *et al.***, 2018; **McWorther *et al.***, 2015). A macrophage was considered activated when it displayed at least well-defined cytoplasmic projections exceeding 2 µm in length, clearly distinguishable from membrane ruffling, together with an increase in cell area relative to resting macrophages. For each experimental condition, 100 macrophages were randomly selected and analysed from calibrated micrographs. Cell area (µm²) was quantified using ImageJ software (National Institutes of Health, USA), and all values were expressed as mean ± standard deviation (SD).

Quantification of NO and ROS by flow cytometry

Infected and uninfected huMDMs were treated with TC2-SS at its EC_{50} for 24 h and 60 h. Phorbol 12-myristate 13-acetate (PMA, 1 µg/mL) was used as a positive inducer of oxidative responses, and untreated cells served as negative controls (**Lund *et al.***, 2016).

NO production was measured using 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate (30 µM) (**Li *et al.***, 2021), while ROS production was assessed using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA, 10 µM) (**Eruslanov *&* Kusmartsev**, 2010). After treatment, cells were incubated with DAF-FM diacetate for 1 h or H_2DCF-DA for 30 min at 37 °C in the dark, then detached, washed with PBS, and analyzed by flow cytometry (Cytomics FC-500 MPL; excitation wavelength: 488 nm, emission 525 nm). A total of 10,000 events were collected per sample.

The threshold for NO and ROS cells was established using unstained or untreated control cells, and positive cells were defined as those exhibiting fluorescence intensity above this threshold. Nitric oxide production was quantified using DAF-FM fluorescence, whereas intracellular ROS levels were determined using DCF fluorescence and expressed as mean fluorescence intensity (MFI) in viable cells (**Li *et al.***, 2021).

Cytokine production by L. braziliensis-infected huMDMs after treatment with TC2-SS using ELISA

Cytokine production was quantified in infected and uninfected huMDMs treated with TC2-SS using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA). The panel included M1-associated cytokines, MIP-1**α**, TNF-α, and IL-1β, and M2-associated cytokines (IL-4, IL-10, and TGF-β1) (**Barik *et al.***, 2025; **Kaye *et al.***, 2020; **Santos *et al.***, 2018). This panel also corresponds to the consensus minimal cytokine sets commonly recommended for macrophage-polarization studies.

Twenty-four hours post-infection, the medium in each well was replaced with RPMI 1640 medium supplemented with 10% autologous serum, and TC2-SS was added at the corresponding EC_{50} at a 1:1 weight/weight ratio. The plates were then incubated at 34 °C with 5% CO_2, and cytokine levels in the cell supernatant were measured 60 hours post-treatment (**Na & Engwerda**, 2024; **Berdusky *et al.***, 2000). Infected and uninfected huMDMs treated with 20 µg/mL phytohemagglutinin were used as positive controls for proinflammatory cytokine production. Cells cultured in RPMI 1640 medium were used to measure basal cytokine production. Cytokine concentrations were quantified in picograms per milliliter (pg/mL), and color intensity was measured at 540 nm using a Multiskan Flash Multimode reader (Thermo Scientific), with results recorded as optical density.

Cytokine gene expression by RT-qPCR

Cytokine gene expression was assessed in huMDMs. After 60 h of treatment with TC2-SS, infected and uninfected cells were washed with pre-warmed PBS (Mg^{2+}/Ca^{2+}-free) and lysed with 500 µL of TRIzol reagent according to the manufacturer's instructions. Samples were stored at −80 °C in DNase- and RNase-free tubes until RNA extraction (**Montoya *et al.***, 2018). For RNA extraction, 200 µL of chloroform was added to TRIzol-treated samples, followed by vigorous shaking (15 s) and a 5-minute incubation at room temperature. Samples were centrifuged at 14,000 rpm for 20 min at 4 °C, producing three phases. The aqueous phase containing RNA was transferred to a new tube, mixed with 500 µL isopropanol, and centrifuged. The RNA pellet was washed twice with 70% ethanol, air-dried, and resuspended in 20 µL RNase-free water. RNA concentration and purity were determined with a Nanodrop 1000 spectrophotometer.

For RT-qPCR, a 10-µL master mix was prepared for each sample using the AccuPower GreenStar kit, which contains M-MLV reverse transcriptase, dNTPs, SYBR Green, and stabilizing agents. Each reaction mixture included 2 µL of RNA, 0.5 µL of each primer, and nuclease-free water. RNA was reverse-transcribed into cDNA and amplified using a CFX96™ Real-Time PCR System (Bio-Rad). The RT-qPCR cycling conditions were fully integrated into the protocol as follows: reverse transcription at 50 °C for 30 min, an

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initial denaturation at 95 °C for 5 min followed by 39 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 20 s, and extension at 72 °C for 20 s, with a final extension at 72 °C for 5 min. A melting curve analysis was performed at the end of the run to confirm the specificity of the amplification product. Primer sequences, amplicon sizes, and annealing temperatures for MIP-1α, IL-4, TGF-β1, IL-1β, and GAPDH (housekeeping gene) are listed in supplementary **Table 1** (ST1). Relative gene expression was calculated using the $^{\Delta\Delta}$Ct method ($2^{-\Delta\Delta Ct}$).

Efficacy of the topical formulation confirmed in a hamster model of cutaneous leishmaniasis induced in dorsal skin

Six-week-old male and female golden hamsters (*Mesocricetus auratus*) weighing 120-170 g (average 150 g) were obtained and housed under specific pathogen-free conditions in ventilated racks equipped with a controlled ventilation system providing 10 to 15 air changes per hour at the animal facility of the Universidad de Antioquia, Medellín, Colombia. Environmental conditions were maintained at a temperature of 22 ± 2 °C and relative humidity of 55–60%, under a 12 h light/12 h dark cycle. Animals were provided free access to autoclaved drinking water and a standard rodent diet. To enhance welfare and reduce stress, environmental enrichment materials such as paper bedding, hay, hydrophobic cotton, and cardboard tubes were included. All animal procedures, including handling, clinical monitoring, sample collection, and euthanasia, were performed in accordance with the Centers for Disease Control and Prevention guidelines for animal and human health diagnostics and the ARRIVE guidelines (**Percie du Sert *et al.***, 2020a; **Percie du Sert *et al.***, 2020b).

Under anesthesia with ketamine (40 mg/kg) and xylazine (5 mg/kg), hamsters were intradermally inoculated in the dorsal skin with 1×10^8 stationary-phase promastigotes of *L. braziliensis* (day-6 culture) suspended in 100 µL PBS, following previously described

Table 1. Nitric oxide (NO) and reactive oxygen species (ROS) production in huMDMs infected with *Leishmania braziliensis* and treated with TC2-SS

Condition	Time (h)	NO^+ cells (%)	ROS^+ cells (%)
Untreated-non-infected	24	1.7 ± 0.3	2.4 ± 0.5
	60	0.1 ± 0.0	1.2 ± 0.3
Untreated-infected	24	15.4 ± 2.1$^{\#}$	13.5 ± 1.8$^{\#}$
	60	2.2 ± 0.1$^{\#}$	10.0 ± 1.5$^{\#}$
TC2-SS-non-infected	24	1.4 ± 0.2	4.5 ± 0.7
	60	0.6 ± 0.2	3.4 ± 0.6
TC2-SS-Infected	24	13.4 ± 1.9$^{\#}$	14.8 ± 2.0$^{\#}$
	60	12.2 ± 2.9*$^{\#}$	12.5 ± 1.7$^{\#}$
PHA-non-infected	24	8.2 ± 1.6	12.0 ± 1.5
	60	12.4 ± 1.1	10.0 ± 1.2
PHA-infected	24	8.2 ± 1.3	16.0 ± 2.1
	60	26.8 ± 3.5$^{\#}$	14.0 ± 1.8
PMA-non-infected	24	9.1 ± 1.7	30.4 ± 3.2
	60	12.5 ± 2.0	28.5 ± 2.7
PMA-infected	24	12.8 ± 1.4	30.8 ± 3.5
	60	25.0 ± 3.8$^{\#}$	34.5 ± 4.1

Values are expressed as mean ± standard deviation (SD) from three independent experiments performed in duplicate. *Significant difference compared to untreated infected cells ($p < 0.05$). $^{\#}p < 0.05$ vs. non-infected cells. PHA: phytohemagglutinin; PMA: phorbol 12-myristate 13-acetate

protocols (**Robledo *et al.***, 2022; **Robledo *et al.***, 2012). Once ulcerative lesions larger than 25 mm² had developed (4-6 weeks post-infection), animals were randomly assigned to the following treatment groups (n = 6 per group): Group 1 (TC2-SS): topically treated for 30 days with 40 mg/day of a 4% TC2-SS cream (2% TC2 and 2% SS incorporated in lanolin: petrolatum at 2:1 w/w ratio, with 1% glycerine added). This formulation was previously evaluated in an observational study in dogs with cutaneous leishmaniasis, where it demonstrated high therapeutic efficacy and an excellent safety profile (**Piragauta *et al.***, 2022); Group 2 (MA): treated with intralesional meglumine antimoniate (200 mg/mL), administered three times per week for four weeks.

A vehicle-only control group was not included because the excipients used (lanolin, petrolatum, glycerin) are well-characterized inert agents with moisturizing and barrier-repair properties, and they lack intrinsic leishmanicidal activity (**Lodén**, 2003). Their role in this study was limited to improving formulation consistency and skin tolerability.

Animals were monitored daily for changes in behaviour or mortality, and body weight was recorded biweekly. Lesion size was measured using an electronic digital caliper at baseline (TD0) and every 30 days thereafter, up to 90 days post-treatment (PTD90). At PTD90, animals were euthanized via intraperitoneal administration of an overdose of ketamine (600 mg/kg) and xylazine (30 mg/kg). Therapeutic response was classified by comparing lesion area at PTD90 with that at baseline (TD0) as follows: Cure (complete re-epithelialization); improvement (≥20% reduction in lesion area); failure (increase in lesion size during treatment), and relapse (reappearance or enlargement of a lesion after initial cure). At TD0 and day 7 of treatment (TD7), lesion scrapings were collected, preserved in RNAlater, and stored at −20 °C for subsequent RNA extraction and quantification of EGF and TGF-β1 gene expression.

Growth factor expression in hamster lesions

Expression of wound-healing–associated growth factors was evaluated in skin samples following previously described procedures (**Eruslanov & Kusmartsev**, 2010) [12]. Total RNA was isolated using TRIzol reagent (Invitrogen) and quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific).

For cDNA synthesis, 100 ng of RNA were treated with1 µL of DNase I (Fermentas, VWR International) in the presence of 1 µL of DNase buffer and 8 µL of nuclease-free water, and incubated in a PTC-100™ thermocycler (MJ Research Inc.) at 37 °C for 30 min, 4 °C for 5 min, and at 65 °C for 10 min. First-strand cDNA was subsequently synthesized using the Maxima First Strand cDNA Synthesis Kit, according to the manufacturer's instructions. Each reaction mixture consisted of 4 µL of master mix, 2 µL of reverse transcriptase enzyme mix, 2 µL of DNase-treated RNA, and 12 µL of nuclease-free water. The reaction was incubated at 25 °C for 10 min, 50 °C for 15 min, and 85 °C for 5 min.

Primer sequences and FAM-labeled hydrolysis probes for EGF and TGF-β1 are shown in ST2. PCR amplification was performed using 1 µL of cDNA under the following cycling conditions: an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 60 s, using a Smart Cycler II system (Cepheid, USA). Amplification efficiency was evaluated using LinReg software. Gene expression was quantified by the ΔΔCt method, with expression levels at TD7 compared to those at TD0 and stratified by clinical outcome (cure, improvement, relapse, failure).

Ethical statement

All procedures involving huMDMs were conducted according to the Declaration of Helsinki and approved by the Institutional Ethics Committee (Act 16-05-727). Written informed consent was obtained from all blood donors. Animal experiments were conducted following national and institutional guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Ethics Committee (Act 140, 2021).

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Statistical analysis

Data from four independent donors (n = 4 per group) were analysed using two-tailed Welch's t-tests for pairwise comparisons (infected *vs*. non-infected and treatment *vs*. NT within each infection condition). A one-way ANOVA with Tukey's post hoc test was used for multigroup comparisons. Statistical calculations were performed using Prism 8 (GraphPad Prism 8 software, USA). Results are presented as mean ± SD, and $p < 0.05$ was considered statistically significant. Quantitative data correspond to the mean values of two independent experiments performed in triplicate.

Results

TC2-SS reduces intracellular L. braziliensis infection in human macrophages

Treatment of infected macrophages with TC2-SS produced a significant reduction in infection parameters. After 48 h of exposure to TC2-SS, the proportion of infected macrophages decreased from 49% in non-treated cells to 15.5% (3.2-fold reduction, $p<0.001$) (**Figure 1A**). Similarly, the control group AMB showed a comparable trend, with a lower percentage of infected cells (22.2%) ($p<0.05$). Also, parasite load decreased from approximately seven amastigotes per cell in non-treated macrophages to three amastigotes per cell in TC2-SS-treated macrophages ($p<0.05$). In contrast, infected macrophages exposed to AMB contained an average of four amastigotes per cell (**Figure 1B**).

On the other hand, treatment of infected huMDMs with the TC2-SS mixture resulted in an EC_{50} of (12.83 ± 0.97) µg/mL. AMB, used solely as a reference standard to validate assay performance, exhibited high antileishmanial activity with an EC_{50} of (0.34 ± 0.07) µg/mL ($p<0.05$).

TC2-SS induces morphological activation of huMDMs

Treated macrophages displayed marked morphological changes consistent with activation. After 60 h, 61% of TC2-SS–treated cells exhibited ≥2 cytoplasmic extensions compared with 41% in untreated infected controls ($p<0.001$) (**Figure 2A**). Quantification of cell spread showed a modest increase in mean cell area (TC2-SS: 42 µm² vs. control: 35 µm²). However, this difference was not statistically significant. Giemsa-stained micrographs revealed more pronounced cytoplasmic extension and vacuolization in treated cells (**Figure 2**), which is consistent with activation patterns previously reported for macrophages undergoing enhanced phagocytic and secretory activity.

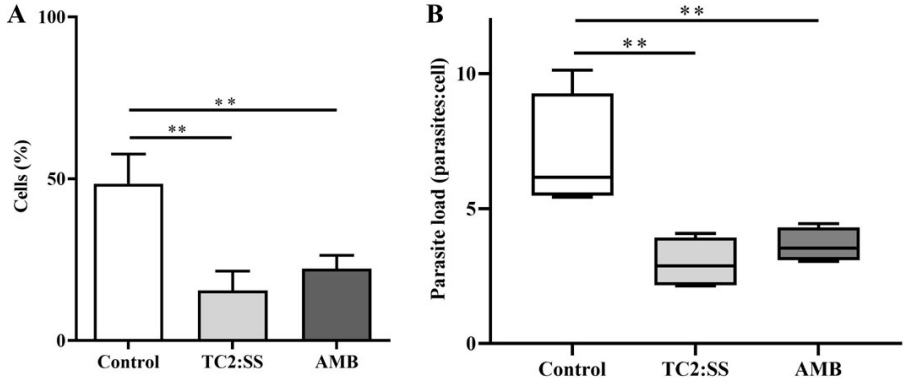

Figure 1. Leishmanicidal effect of TC2-SS in *Leishmania braziliensis*-infected huMDMs. **(A)** Percentage of infected huMDMs and **(B)** parasite load (mean number of amastigotes per infected cell) after 60 hours of treatment. Human monocyte-derived macrophages (huMDMs) were either left untreated (RPMI + 10% FBS), treated with TC2-SS, or treated with amphotericin B (AMB) as a reference drug. Bars and box plots represent mean ± SD from at least three independent experiments performed in duplicate. **$p < 0.005$ for treated groups vs. untreated infected control.

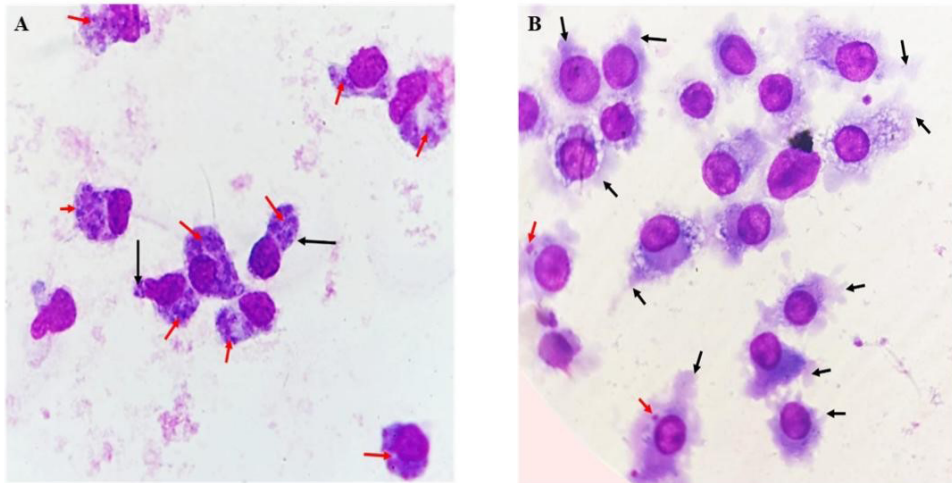

Figure 2. Morphological appearance of *Leishmania braziliensis*-infected huMDMs exposed to TC2-SS. Representative Giemsa-stained images of huMDMs at 60 hours post-treatment under two experimental conditions: **(A)** untreated infected cells and **(B)** infected cells treated with TC2-SS. Black arrows indicate cytoplasmic extensions (pseudopodia), and red arrows point to intracellular amastigotes. Images illustrate the increased cytoplasmic spreading and membrane projections consistent with macrophage activation in TC2-SS–treated cells.

TC2-SS preserves infection-driven NO and ROS production

NO and ROS production demonstrated a distinct infection-driven pattern, with infected macrophages exhibiting higher levels than non-infected cells under all conditions, including stimulation with TC2-SS, PMA, and PHA. TC2-SS maintained this response, resulting in NO and ROS levels similar to those observed in untreated infected macrophages at 24 hours. At 60 hours, TC2-SS significantly increased NO production in infected macrophages compared to untreated infected controls, while ROS levels remained unchanged. TC2-SS did not induce NO or ROS production in non-infected macrophages, indicating a lack of non-specific oxidative activation. As anticipated, PMA and PHA elicited strong and moderate activation responses, respectively (**Table 1**).

MFI analysis revealed a time-dependent, infection-associated increase in intracellular NO levels. At 24 hours, differences between infected and non-infected macrophages were inconsistent and not statistically significant across most conditions. At 60 hours, infected macrophages exhibited higher MFI values than non-infected cells across all treatments, with significant differences observed in both untreated and PMA-stimulated conditions. Infected macrophages treated with TC2-SS also demonstrated increased NO levels compared to their 24-hour counterparts, indicating a time-dependent increase in intracellular NO. These findings indicate that infection promotes intracellular NO accumulation, which is further sustained or enhanced over time, particularly in the presence of TC2-SS treatment.

MFI values for ROS could not be determined due to technical limitations. However, the proportion of ROS-positive cells followed a consistent infection-driven pattern, supporting increased oxidative activity in infected macrophages.

Cytokine production by ELISA

The protein-level cytokine response in huMDMs was markedly modulated by infection with *L. braziliensis* and by treatment with TC2-SS or PHA (**Table 2**). Cytokine production in human monocyte-derived macrophages (huMDMs) was significantly altered by *L. braziliensis* infection and by TC2-SS treatment (**Table 2**). Infection alone resulted in a proinflammatory and partially regulatory cytokine profile, as indicated by elevated TNF-α, IL-4, and MIP-1α levels.

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Table 2. Cytokine levels produced by huMDM at 60 hours post-treatment and infection with *Leishmania braziliensis*

Cytokine (pg/mL)	Treatment	Non-infected	Infected	p-value (infected vs. non-infected)	p-value (treatment vs. non-treatment in infected cells)
IL-1β	NT	10.5 ± 1.1	12.0 ± 1.4	ns	na
	TC2-SS	12.0 ± 1.2	1.5 ± 0.2	***	***
	PHA	22.0 ± 3.0	19.5 ± 2.8	ns	***
TNF-α	NT	10.1 ± 1.0	13.8 ± 1.5	**	***
	TC2-SS	13.1 ± 1.3	2.0 ± 0.3	***	***
	PHA	18.1 ± 2.5	15.2 ± 2.0	ns	ns
IL-4	NT	13.1 ± 2.8	28.3 ± 8.3	*	na
	TC2-SS	12.1 ± 2.0	10.8 ± 0.8	ns	*
	PHA	21.9 ± 7.4	15.7 ± 2.4	ns	ns
MIP-1α	NT	341.8 ± 48.3	801.5 ± 116.3	*	na
	TC2-SS	767.9 ± 3.3	947.6 ± 129.0	ns	ns
	PHA	731.4 ± 12.2	905.4 ± 21.8	***	ns
TGF-β1	NT	704.8 ± 110.0	829.8 ± 143.2	ns	na
	TC2-SS	292.7 ± 85.6	509.7 ± 49.7	*	*
	PHA	526.6 ± 105.2	735.7 ± 41. 3	*	ns
IL-10	NT	2.3 ± 0.6	1.5 ± 0.4	ns	na
	TC2-SS	1.7 ± 0.2	0.5 ± 0.1	***	*
	PHA	2.0 ± 0.2	1.4 ± 0.6	ns	ns

Data represent the mean cytokine concentrations ± standard deviation (SD) measured in culture supernatants of human monocyte-derived macrophages (huMDM) at 60 hours post-infection with *L. braziliensis* and treatment with TC2-SS or PHA, as measured by ELISA. NT: Non-treated; na: Not applicable; ns: Non-significant: * $p < 0.05$: ** $p < 0.01$; *** $p < 0.001$

TC2-SS exerted a pronounced and selective immunomodulatory effect in infected macrophages, significantly decreasing major proinflammatory cytokines (IL-1β, TNF-α) and regulatory cytokines (IL-4, IL-10, TGF-β1), while maintaining or increasing MIP-1α production. This effect was predominantly observed in infected cells, with minimal influence on cytokine levels in non-infected macrophages.

In contrast, phytohemagglutinin (PHA) elicited a broader and less selective cytokine response, increasing proinflammatory mediators without consistent modulation of regulatory cytokines. Briefly, TC2-SS supports a regulated immunological profile by suppressing excessive inflammatory and regulatory pathways, while sustaining chemokine-mediated antimicrobial recruitment.

Gene expression profile of cytokines in huMDMs by RT-qPCR

At 60 hours post-treatment, cytokine gene expression in huMDMs demonstrated modulation dependent on both treatment and infection status (**Table 3**). Infection alone did not significantly alter baseline transcriptional levels for most cytokines under untreated conditions.

TC2-SS induced a pronounced and selective immunomodulatory profile, characterized by substantial suppression of proinflammatory cytokines (IL-1β, TNF-α) and regulatory or M2-associated cytokines (IL-4, IL-10, TGF-β1), particularly in infected macrophages. In contrast, MIP-1α expression was maintained and significantly increased in infected cells, indicating preservation of chemokine-mediated antimicrobial responses. These effects were predominantly observed in infected macrophages, with minimal modulation detected in non-infected cells.

PHA treatment elicited a broader and less selective transcriptional response, increasing proinflammatory cytokine expression without consistent modulation of regulatory pathways.

Overall, TC2-SS promotes a controlled transcriptional profile that restricts excessive inflammation and regulatory signaling while preserving essential antimicrobial mechanisms.

Therapeutic effectiveness of topical 4% TC2-SS formulation in cutaneous leishmaniasis in hamsters

The topical administration of the 4% TC2-SS formulation resulted in a pronounced therapeutic effect in hamsters infected with *L. braziliensis* (**Figure 3**). Lesion size decreased progressively, leading to complete clinical cure in four out of six animals by PTD90. The remaining animals exhibited substantial improvement. TC2-SS induced a uniform healing response, with no observed lesion progression or relapse.

MA achieved a comparable cure rate, with four out of six animals cured. However, MA treatment was associated with greater inter-animal variability, including partial responses and one case of lesion progression in a non-responder.

Although cure rates were similar, TC2-SS demonstrated a more consistent therapeutic profile, with sustained lesion reduction observed in all treated animals. Body weight remained stable in both groups, and no macroscopic alterations were detected at necropsy, indicating the absence of systemic toxicity. The progression of lesions in individual animals is presented in **SFigure 2,** https://raccefyn.co/index.php/raccefyn/article/view/4172/5393.

Modulation of EGF and TGF-β1 mRNA expression

Figure 4 demonstrates that TC2-SS treatment significantly increased EGF mRNA expression in infected animals (6.53-fold) and concurrently reduced TGF-β1 expression

Table 3. Cytokine gene expression at 60 hours in huMDMs infected or not with *Leishmania braziliensis* and treated with TC2-SS

Cytokine (pg/mL)	Treatment	Non-infected	Infected	p-value (infected *vs.* non-infected)	p-value (treatment *vs.* non-treatment in infected cells)
IL-1β	NT	1.0 ± 0	1.0 ± 0	ns	na
	TC2-SS	0.8 ± 0.2	0.25 ± 0.07	***	***
	PHA	3.3 ± 2.5	11.9 ± 6.3	*	*
TNF-α	NT	1.0 ± 0	1.0	ns	na
	TC2-SS	0.67 ± 0.2	0.45 ± 0.1	*	***
	PHA	1.4 ± 1.2	1.2 ± 0.5	ns	*
IL-4	NT	1.0 ± 0	1.0 ± 0	ns	na
	TC2-SS	1.0 ± 0.1	1.4 ± 0.3	*	*
	PHA	1.2 ± 0.6	1.1 ± 0.5	ns	ns
MIP-1α	NT	1.0	1.0	ns	na
	TC2-SS	0.67 ± 0.1	0.40 ± 0.05	***	***
	PHA	1.0 ± 0.2	1.2 ± 0.8	ns	ns
TGF-β1	NT	1.0 ± 0	1.0 ± 0	ns	na
	TC2-SS	1.2 ± 0.2	0.3 ± 0.01	***	***
	PHA	4.0 ± 2.0	8.0 ± 3.0	**	**
IL-10	NT	1.0 ± 0	1.0 ± 0	ns	na
	TC2-SS	0.8 ± 0.02	0.4 ± 0.01	***	***
	PHA	1.5 ± 0.4	1.7 ± 0.5	ns	ns

Data represent the relative gene expression levels of cytokines in human monocyte-derived macrophages (huMDM) measured by RT-qPCR ± standard deviation (SD) at 60 hours post-infection with *L. braziliensis* and treatment with TC2-SS or PHA, as measured by ELISA. NT: Non-treated; na: Not applicable; ns: Non-significant: * $p < 0.05$: ** $p < 0.01$; *** $p < 0.001$

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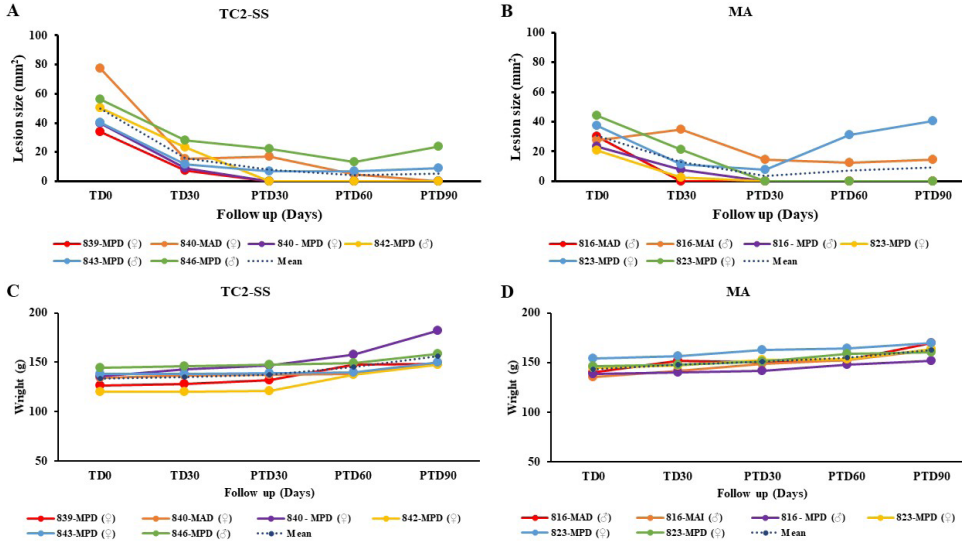

Figure 3. Therapeutic response to topical 4% TC2-SS and intralesional meglumine antimoniate (MA) in hamsters with *Leishmania braziliensis* cutaneous leishmaniasis. **(A)** Lesion size (mm²) over time in hamsters treated topically with 4% TC2-SS. Four of six animals achieved complete clinical cure by post-treatment day 90 (PTD90), and the remaining two showed marked partial healing (57.2% and 78.2% reduction in lesion area). **(B)** Lesion evolution in MA-treated animals: four of six hamsters were cured, one showed partial improvement (47.8% reduction), and one exhibited lesion worsening (+7.5%). **(C–D)** Body weight curves for animals treated with TC2-SS and MA, respectively, showing no significant weight loss or overt systemic toxicity during follow-up. Data indicate that topical TC2-SS is therapeutically comparable to systemic antimonials, with more homogeneous lesion regression and no treatment failures. TD0: baseline (before treatment); TD30: end of treatment; PTD30, PTD60, PTD90: 30, 60, and 90 days after treatment completion, respectively. Representative ulcer photographs illustrating individual healing trajectories are shown in Supplementary **Figures S1A** (TC2-SS) and **S1B** (MA) (www.raccefyn.co/index.php/raccefyn/article/view/4172/5393).

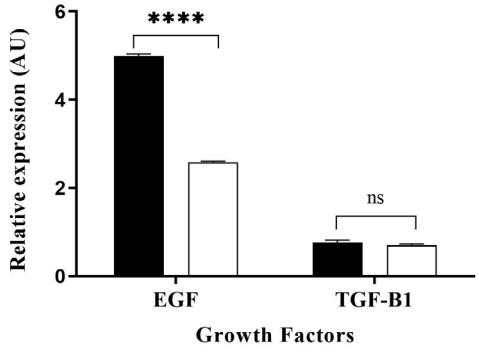

Figure 4. Modulation of EGF and TGF-β1 mRNA expression in skin ulcers of hamsters with *Leishmania braziliensis* cutaneous leishmaniasis treated with topical 4% TC2-SS or meglumine antimoniate. Relative mRNA expression of EGF and TGF-β1 was quantified in ulcer tissue obtained from hamsters (n = 6 per group) after 7 days of treatment with topical 4% TC2-SS (black bars) or meglumine antimoniate (MA) (white bars). Gene expression levels were calculated using the ΔΔCt method with GAPDH as the endogenous reference gene and normalized to the non-treated infected control group. Bars represent mean ± SD. TC2-SS induced a pronounced upregulation of EGF and a concomitant reduction in TGF-β1, resulting in a markedly higher EGF/TGF-β1 ratio than in MA. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. ****$p < 0.0001$; ns: non-significant

compared to untreated controls. In comparison, MA-treated animals exhibited a smaller increase in EGF expression and a less substantial reduction in TGF-β1 levels. These results suggest that TC2-SS induces a gene expression profile conducive to tissue repair and diminished pro-fibrotic signaling.

Discussion

Cutaneous leishmaniasis caused by *L. braziliensis* results from a complex interplay between parasite persistence, macrophage activation, and a dysregulated inflammatory response. Unlike other *Leishmania* species, pathology in *L. braziliensis* infection often arises despite low parasite numbers, mainly driven by exaggerated inflammation that contributes to chronic ulcers, delayed healing, and mucosal complications (**Mohammadi *et al.***, 2021; **Santos *et al.***, 2018). These features underscore the need for therapeutic approaches that simultaneously reduce parasite burden and restore a balanced local immune environment. In this context, we evaluated the combined chroman hydrazone–saponin formulation TC2-SS as a topical candidate integrating direct parasiticidal activity with immunomodulatory and pro-regenerative effects.

Our in vitro findings demonstrate that TC2-SS induces significant intracellular antileishmanial activity in primary human macrophages, reducing both the proportion of infected cells and intracellular parasite burden. The associated morphological changes, particularly increased cytoplasmic projections, indicate activation of macrophage effector functions (**Fan *et al.***, 2025; **Mei *et al.***, 2023; **Atri *et al.***, 2018). Importantly, TC2-SS preserved the infection-driven production of nitric oxide and reactive oxygen species, and enhanced NO at later time points without inducing oxidative activation in uninfected cells. Because *L. braziliensis* can suppress macrophage oxidative pathways to facilitate intracellular persistence (**Muxel *et al.***, 2018; **Calegari-Silva *et al.***, 2009), this selective amplification of NO in infected macrophages aligns with the observed decline in parasite load. It supports a model in which TC2-SS augments host microbicidal mechanisms while exerting direct parasiticidal effects.

The cytokine profile elicited by TC2-SS reinforces this interpretation. ELISA results showed that TC2-SS induced a highly regulated cytokine signature in huMDMs, characterized by selective suppression of key inflammatory and regulatory mediators exclusively in infected macrophages. IL-1β, TNF-α, and IL-4 were markedly reduced in infected cells, while cytokine levels in uninfected macrophages remained near baseline, indicating infection-restricted immunomodulation rather than generalized activation. In contrast, PHA broadly increased cytokine production across conditions. MIP-1α was the only cytokine consistently enhanced by TC2-SS in both infected and non-infected cells, suggesting preservation of chemokine-driven antimicrobial recruitment despite the suppression of excessive inflammatory signals. TC2-SS also produced the strongest down-modulation of the regulatory and pro-fibrotic cytokines TGF-β1 and IL-10, limiting M2-associated and wound-healing–delaying pathways. This integrated cytokine pattern, characterized by reduced IL-1β and TNF-α, preserved MIP-1α, and decreased IL-4, IL-10, and TGF-β1, defines a balanced, microbicidal M1-leaning phenotype that supports parasite control without inducing harmful inflammation (**Na & Engwerda**, 2024; **Santos *et al.***, 2018; **Rözer**, 2015). Such a controlled activation profile is particularly relevant in *L. braziliensis* infection, where therapeutic failure frequently stems from either insufficient microbicidal responses or destructive inflammatory pathology (**Akbari *et al.***, 2021; **Kaye *et al.***, 2020).

In RT-qPCR results, TC2-SS induced a selective, infection-restricted cytokine profile in huMDMs, supporting a controlled, microbicidal M1-leaning response. In infected macrophages, the formulation produced a profound suppression of IL-1β, limiting the excessive proinflammatory signaling that contributes to tissue damage in *L. braziliensis* ulcers (**Mohammadi *et al.***, 2021; **Santos *et al.***, 2018), while leaving cytokine levels in non-infected cells unchanged. TC2-SS also strongly inhibited M2-associated and regulatory mediators, including IL-4, TGF-β1, and IL-10, counteracting parasite-promoting

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pathways and preventing the emergence of regulatory macrophage states known to support intracellular survival (**Na & Engwerda**, 2024; **Rözer**, 2015). In contrast, MIP-1α was preserved and mildly enhanced, indicating maintenance of chemokine-driven antimicrobial recruitment, consistent with its role in sustaining early microbicidal responses (**Bhavsar *et al.*,** 2015). Together, this coordinated signature shows that TC2-SS dampens harmful hyperinflammation while suppressing parasite-permissive regulatory circuits, generating a balanced activation state that supports parasite control and aligns with the improved microbicidal activity and wound-healing outcomes observed in vitro and in vivo (**Kaye *et al.***, 2020; **Barrientos *et al.***, 2008).

ELISA and RT-qPCR showed strong overall concordance in the immunomodulatory profile induced by TC2-SS. In infected macrophages, both assays demonstrated pronounced suppression of IL-1β, TNF-α, IL-4, TGF-β1, and IL-10, confirming that TC2-SS downregulates these immunological mediators at transcriptional and protein levels. MIP-1α was the only cytokine consistently increased across both datasets, reinforcing the preservation of chemokine-driven antimicrobial recruitment, consistent with its role in the early innate immune response by promoting the recruitment and activation of monocytes/macrophages and amplifying local inflammatory signalling through CCR1- and CCR5-dependent pathway activation (**Bhavsar *et al.***, 2015). Minor discrepancies, such as high fold changes detected by RT-qPCR, are expected because transcriptional regulation precedes protein accumulation and clearance (**Atri *et al.*,** 2018; **Rözer**, 2015). Overall, the agreement between ELISA and RT-qPCR supports a coherent, infection-restricted, M1-leaning activation pattern induced by TC2-SS, aligned with the regulated pro-microbicidal responses described in *L. braziliensis* infection (**Kaye *et al.***, 2020; **Santos *et al.***, 2018).

In the hamster model, topical TC2-SS achieved a cure rate comparable to systemic MA yet produced more homogeneous lesion regression, with no treatment failures. The in vivo efficacy of TC2-SS indicates that topical TC2-SS is safe, well-tolerated, and therapeutically comparable to systemic antimonials, while offering the advantage of uniform lesion regression without treatment failures and a non-invasive, locally acting formulation that avoids the systemic exposure and toxicity associated with injectable antimonials. Notably, this study employed a dorsal-skin infection model, which provides a more physiologically stringent and human-relevant environment than ear or footpad infections typically used in murine leishmaniasis research. Hamster dorsal skin provides a clinically relevant model because it closely resembles human skin in its overall architecture, vascularization, collagen density, and immunological complexity of human skin (**Militzer *et al.***, 1990), generating deep, persistent, and destructive ulcers that closely resemble the clinical lesions caused by *L. braziliensis*. Therapeutic effects observed in this model, therefore, reflect meaningful challenges related to parasite persistence, intense Th1-driven inflammation, and impaired epithelial repair. In this context, the consistent lesion contraction and absence of non-responders in TC2-SS–treated animals represent a strong signal of therapeutic potential.

TC2-SS promotes a transcriptional environment favoring epidermal repair and re-epithelialization driven by EGF while simultaneously maintaining lower levels of TGF-β1, a cytokine associated with fibrosis, collagen deposition, and delayed wound healing (**Legrand & Martino**, 2022; **Sanjabi *et al.*,** 2017; **Barrientos *et al.***, 2008). The greater EGF predominance in the TC2-SS group suggests an accelerated regenerative response compared with the MA group, consistent with the more uniform macroscopic lesion resolution observed in treated animals.

Further mechanistic insight was provided by the marked shift in the EGF/TGF-β1 balance observed in ulcers after only seven days of treatment. TC2-SS induced a pronounced predominance of EGF over TGF-β1, creating a transcriptional environment favourable for re-epithelialization, keratinocyte proliferation, and reduced fibrosis. Because persistent TGF-β1 signalling contributes to collagen deposition, delayed closure, and chronicity in L. *braziliensis* ulcers, the strong EGF dominance in TC2-SS–treated skin aligns with the macroscopically cleaner borders, healthier granulation tissue, and more uniform epithelial

closure observed in treated animals. This regenerative phenotype distinguishes TC2-SS from pentavalent antimonials, which focus on parasite clearance without directly enhancing wound repair pathways.

Together, these findings support a dual mechanism of action in which TC2-SS induces direct intracellular leishmanicidal effects, selectively immunomodulates, and early activates regenerative pathways. TC2-SS addresses two central challenges in the treatment of *L. braziliensis* cutaneous leishmaniasis, parasite persistence and poor-quality healing, by simultaneously reducing parasite burden, promoting regulated M1-like macrophage activation, limiting pathological inflammation, and enhancing epithelial repair. The favourable therapeutic response observed in the hamster dorsal-skin model, plus the absence of systemic toxicity, suggest that TC2-SS could be a promising, safe, and non-invasive topical alternative worthy of further preclinical development and evaluation in clinical settings.

Conclusions

Our study demonstrates that the TC2-SS combination exerts a dual therapeutic effect against *L. braziliensis* CL with potent intracellular antileishmanial activity, significantly reducing infection rates and parasite burden and preserving and selectively enhancing nitric oxide production driven by infection. Concomitantly, the formulation induced a regulated, M1-skewed activation profile characterized by maintenance of chemokine (MIP-1α)-mediated antimicrobial functions, reduction of excessive IL-1β and TNF-α, and down-modulation of IL-4, IL-10, and TGF-β1, thereby supporting parasite control without exacerbating inflammatory damage.

In vivo, topical 4% TC2-SS produced a therapeutic outcome comparable to systemic meglumine antimoniate in a hamster dorsal-skin model of *L. braziliensis* infection, but with more homogeneous lesion regression and no treatment failures. The absence of weight loss or macroscopic organ alterations supports an excellent local safety profile. The notable increase in the EGF/TGF-β1 ratio in treated ulcers also indicates that TC2-SS promotes a regenerative environment favourable to re-epithelialization and fibrosis reduction. These findings suggest that TC2-SS could be a promising topical candidate that combines direct parasiticidal activity, host-directed immunomodulation, and early activation of wound-healing pathways.

Supplementary information

See the supplementary information in https://raccefyn.co/index.php/raccefyn/article/view/4172/5393

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Author contributions

All authors contributed substantially to the development of this work. YAU, FE, and SMR: Conceptualization and definition of the intellectual content. EQ, YAU, and SMR: Study design. EQ, JA, VO, JM, WQ, and SMR: Experimental studies and data acquisition. EQ, JA, and SMR: Statistical analysis. EQ, JA, and SMR: Manuscript preparation. EQ, JM, and SMR: Manuscript editing. All authors participated in the literature search, data analysis, and critical review of the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest**.**

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