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Thermoresponsive Leishmanicidal Emulgel Based on *Copaifera reticulata* Ducke: in Vitro Studies with the Amastigote Forms

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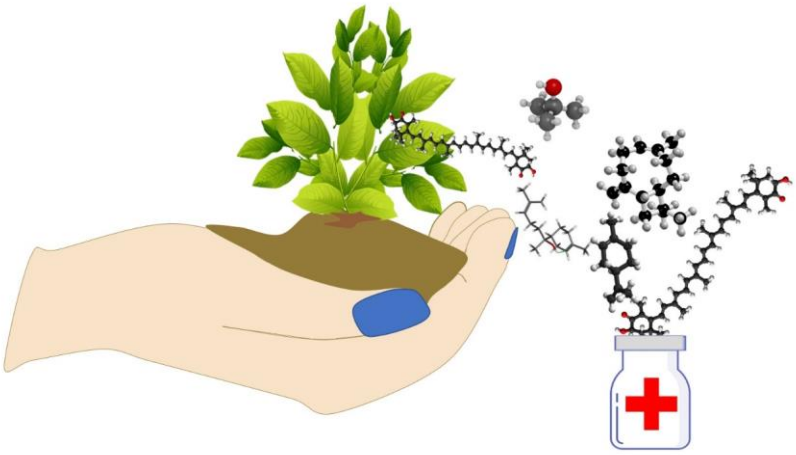
HIGHLIGHTS

- The studies showed the in vitro application of a recently reported drug.
- Thermoresponsive stimulus gel containing high levels of copaiba oil.
- Formulation that increases the leishmanicidal potential of the drug.
- A viable treatment alternative for leishmaniasis patients.

Abstract: Leishmaniasis is a neglected disease that causes disfiguring skin damage. In a previous publication, we characterized a thermoresponsive medicine developed for the dermal release of the *Copaifera reticulata* Ducke. The medicine showed chemical and physical stability for two years, proven by validation studies. Furthermore, the topical medicine undergoes gelation when body-stimulated, which increases the time spent on the injured skin. The present study aims to complement the characterization of the developed medicine by showing the *in vitro* results against amastigote forms of *Leishmania amazonensis and Leishmania infantum*. The results showed a notable antiproliferative capacity, especially against *L. infantum*, due to the excellent selectivity of the copaiba oil by the protozoan. Furthermore, the drugs were more effective than pure copaiba oil, with lower IC_{50} values for the incorporated oils, since the IC_{50} of the incorporated oil and the free oil decreased more than fourfold (from 14.8 to 3.4 µg/mL for phytotherapeutic gel containing 8 % w/w copaiba oil resin - named PHY-8). These results, together with those recently published for these formulations, guarantee the medicine's potential as a leishmanicidal agent and make it a potential candidate for future *in vivo* studies.

Keywords: Gel; thermoreversible system; cutaneous leishmaniasis; copaiba oil-resin; amastigotes forms.

GRAPHICAL ABSTRACT



INTRODUCTION

Tegumentary leishmaniasis is a disfiguring infectious disease that affects people and causes ulcers on the skin and in the mucous membranes of upper airways. This disease is caused by an intracellular protozoan of the *Leishmania* genus. Parasites have two main forms during their life cycle: amastigotes (occurring in the vertebrate host) and promastigotes (occurring in the invertebrate host) forms[1]. Currently, leishmaniasis is treated with N-methylglucamine and amphotericin B [2]. However, the cost of these drugs is high (N-methylglucamine R$ 599.14; 50 ampoules; a daily dose of 1-3 ampoules per day and Amphotericin B R$ 1.122; 50 mg - 25 ampoules, dosage of 1- 5 mg/kg/day), which makes it challenging to access medicines without public contribution. Furthermore, these drugs have the inconvenience of requiring hospitalization for their administration due to intrinsic toxicity. These facts encourage the development of new, more tolerable, safer therapies for leishmaniasis.

Recognition of the healing, antibiotic [3], and leishmanicidal potential of copaiba oil have led us to develop medicine with thermo-responsive properties (emulsion-filled gels or emulgel) [4]. The high content of copaiba oil has guaranteed effects against the promastigote forms of the protozoa *Leishmania amazonensis and Leishmania infantum*. However, this form exists in the invertebrate host, which serves as a vector. Therefore, this study aims to demonstrate the benefits of using thermoresponsive medicine for the amastigotes forms, which infect humans. The medicinal previously published by us are composed of F127 copolymer 18% w/w, Carbopol C934P 0.25 %w/w, and copaiba oil in concentrations between 8-12% w/w[4,5].

MATERIAL AND METHODS

Materials

F127 Pluronic® was acquired from Sigma-Aldrich (Missouri, USA), Carbopol C934P® (Cb) was obtained from Lubrizol Advanced Materials (Sao Paulo, SP, Brazil), and Triethanolamine (TEA) was purchased from Synth (Sao Paulo, SP, Brazil). The copaiba oil resin *(Copaifera reticulata* Ducke) was obtained from the *Copaíba da Amazônia* company, according to the georeferencing described previously [4]. All experiments were carried out using ultra-pure water.

All the processes follow the environmental legislation in force through the National Biodiversity Authorization and Information System (SISBIO nº 72922-1) and the National System for Genetic Heritage Management (SISGEN nº AE28797), as well as duly authorized by the same association.

Preparation of medicine

First, Cb (0.25 %, w/w) was dispersed in purified water under vigorous stirring (ca 6 h). Then, the solution received F127 copolymer (18%, w/w), and the system was maintained at 5 ± 2ºC for 24 h. Afterwards, the mixture was mechanically stirred at 100 rpm (Quimis stirrer, model Q235-2, Sao Paulo, SP, Brazil). After complete homogenization of dispersion (30 min), the pH was adjusted to 7 using TEA. Next, the copaiba oil (8, 10, and 12%, w/w) was slowly added to the preparation, which remained in constant agitation for 30 min. The formulations obtained were stored at 5 ± 2°C for at least 24 h before analysis. The phytotherapeutic gel (generically named PHY) in each oil concentration was named PHY-8, PHY-10, and PHY-12. Additionally, gel without the addition of copaiba oil was obtained and named standard gel (Std-gel).

Cell cultures

Promastigote forms of *Leishmania amazonensis* (IFLA/BR/1967/PH8 transfected with pIR1SAT-LUC(a)DsRed2(b) B5947) and *Leishmania infantum* (MHOM/MA/67/ITMAP-263 transfected with plasmid pSP72αHYGαLuc1.2) were cultivated in Warren medium (brain and heart infusion, hemin and folic acid; pH 7.2) supplemented with 10% inactivated fetal bovine serum (FBS) and incubated at 25ºC.

J774A.1 macrophages were maintained in RPMI 1640 medium (Gibco; NY, USA), pH 7.2, supplemented with *L*-glutamine and 10% FBS, incubated at 37°C in a 5% CO_2 atmosphere.

Leishmanicidal activity of the phytotherapeutic medicine

For activity against amastigote forms of *Leishmania*, $5x10^5$ macrophages/mL were added with $5x10^6$ promastigotes/mL (ratio 1:10), in a stationary growth phase, in 96-well white plates. The plates were incubated at 34°C, with 5% CO_2 for 24 h, for internalization and differentiation of the parasites into amastigote forms. The cells were then washed to remove the non-internalized parasites, treated with increasing copaiba oil (or PHY-concentrations medicinal), and incubated for 48 h. After the incubation period, the Pierce Firefly Luc One-Step Glow Assay kit (ThermoFicher, Rockford, USA) was added according to the manufacturer's instructions. The luminescence was quantified in a SpectraMax L luminometer (Molecular Devices) with λ_{em} = 570 nm and an integration time of 1s. The percentage of inhibition was calculated based on the luminescence of the control (untreated). The values were plotted and the IC_{50} inhibitory concentrations were calculated by non-linear regression [6]. Results were expressed as the mean ± standard deviation of at least three independent experiments.

The selective index (SI) was calculated using the cytotoxicity that we have recently published for these drugs, making CC_{50} J774A.1 cells/IC_{50} protozoa [4].

Statistical Analysis

The averages were compared using the free software R version 3.6.0, with the RStudio interface version 1.1.463 [7], with *t.test* algorithm. The significance level used for the rejection of the null hypothesis was 5% ($p<0.05$) [8].

RESULTS AND DISCUSSION

The developed medicines have high copaiba oil contents, gelation at approximately 12°C, and behaviors typical of semisolid systems. The rheological studies exhibited pseudoplastic behavior, with thixotropy and viscoelasticity at body temperature. Additionally, the emulgels showed higher skin permeation capacity than pure copaiba oil, indicating that the polymers act as human skin permeation promoters. A complete study of these phytotherapeutic medicines has recently been published [4,5]. This paper complements such studies and brings the *in vitro* results of the formulations (and the pure oil) against the intracellular amastigotes forms. This parasite form infects humans and is the main target of therapies in patients affected by these diseases. The antiproliferative activities of medicinal-loaded Copaiba oil-resin against the *L. amazonensis* and *L. infantum* amastigotes are shown in Table 1.

Table 1. Antiproliferative activity of emulgels compositions and copaiba resin oil against amastigotes forms of *Leishmania amazonensis* and *L. infantum*.

Medicine	J774A.1	*L. amazonensis*		*L. infantum*	
	CC_{50}^{**} (µg/mL)[a]	IC_{50}^{*} (µg/mL)	SI	IC_{50}^{*} (µg/mL)	SI
Copaiba oil resin	26.34 ± 1.68 [a]	20.20 ± 1.70	1.30	14.80 ± 1.48	1.78
PHY-8	249.92 ± 34.01 [a] (19.99 ± 2.72) [a]	145.50 ± 9.32 (11.64 ± 0.75)	1.72	41.88 ± 2.43 (3.35 ± 0.19)	5.97
PHY-10	217.63 ± 16.49 [a] (21.76 ± 1.65) [a]	132.70 ± 9.63 (13.27 ± 0.96)	1.64	34.99 ± 1.84 (3.50 ± 0.18)	6.22
PHY-12	195.05 ± 21.80 [a] (23.41 ± 2.62) [a]	128.50 ± 10.33 (15.42 ± 1.24)	1.52	32.50 ± 0.59 (3.90 ± 0.07)	6.00
Std-gel	>1000 [a]	>250	ND	>250	>250

a: data published by Campanholi and coauthors (2022) [4] and used to determine the selectivity index. The results were expressed as three independent experiments' mean ± standard deviation. Results expressed concerning total PHY-8, PHY-10, and PHY-12, and in parentheses present the copaíba oil resin relative concentration in IC_{50}. *IC_{50} is the inhibitory concentration for 50% of the protozoa, and **CC_{50} = cytotoxic concentration for 50% of the cell. SI= selectivity index (CC_{50}/IC_{50} *L. amazonensis or L. infantum*). ND = not determined.

Copaiba oil resin showed activity against amastigote forms of *L. infantum* and *L. amazonensis*, with IC_{50} between 15 and 20 µg/mL. The emulgels were more effective than copaiba oil for both protozoa ($p<0.05$). For example, the copaiba oil resin showed a mean IC_{50} value of 14.80 µg/mL for *L. infantum*, while PHY-8 showed a mean IC_{50} value of 3.35 µg/mL, considering the 8% w/w copaiba oil content in the respective medicine. The antiproliferative activity of emulgels (PHY-8, PHY-10, and PHY-12) against *L. amazonensis* was similar ($p>0.05$), while for *L. infantum* it was statistically different ($p<0.05$).

The cytotoxicities of copaiba oil resin and emulgels on J774A.1 macrophage cells (Table 1) were previously published [4]. These values were used by the determination of selective index (SI) (ratio: CC_{50} J774A.1 cells/IC_{50} protozoa). A value greater than 1.0 is considered more selective for the parasite. The selectivity index values between the copaiba oil and the emulgels (PHY-8, PHY-10, and PHY-12) were statistically different ($p<0.05$) for both protozoa. The results were noteworthy for *L. infantum* treated with emulgels, in which the selectivity was four times higher than copaiba oil, therefore justifying the lower IC_{50} values obtained. The selectivity index values reported for copaiba range from 1-125, due to variations in oil composition [9,10].

Overall, the results obtained are in agreement with the literature [9–12]. However, compared to those previously published, the medicine developed here is superior in thermoresponsive performance, chemical and physical stability, in addition to the dermal release performance verified in human skin[4]. Thus, like amphotericin B, whose activity was previously reported (IC_{50} value of 0.231 µg/mL [10]), the PHY-emulgel products were adequate and constituted a consistent proposition for future clinical trials.

Author Contributions:

Katieli da Silva Souza Campanholi: Conceptualization, Formal analysis, Writing - original draft. Rodolfo Bento Balbinot: Methodology, Investigation. Danielle Lazarin-Bidóia: Investigation, Formal analysis. Flávia Amanda Pedroso de Morais: Methodology, Investigation. Tânia Ueda Nakamura: Resources, Supervision, Project administration, Funding acquisition, Investigation, Formal analysis. Celso Vataru Nakamura: Resources, Supervision, Project administration, Funding acquisition, Investigation, Formal analysis. Wilker Caetano: Resources, Supervision, Project administration, Funding acquisition, Investigation, Formal analysis.

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Data Availability Statement: Research data are only available upon request for corresponding author

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