Acta Tropica 237 (2023) 106744

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Thermosensitive system formed by poloxamers containing carvacrol: An effective carrier system against *Leishmania amazonensis*

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ARTICLE INFO

Keywords:
Polymer
Hydrogel
Drug delivery system
Cytotoxicity
Leishmaniasis
Monoterpene

ABSTRACT

The drugs used in the treatment of leishmaniasis show problems concerning side effects and toxicity. As a result, the search for new actives is necessary, and natural products like carvacrol - *5-isopropyl-2-methylphenol*, become a relevant alternative. To enable the use of carvacrol as an antileishmanial agent, thermosensitive hydrogels were developed from poloxamer triblock copolymers 407 (P407) and 188 (P188). Carvacrol-free and carvacrol-containing hydrogels were obtained from P407 alone and from the mixture of P407 and P188. The hydrogels were subjected to *Differential scanning calorimetry, Small-angle X-ray scattering, Scanning electron microscopy,* and *Rheology analysis*. The activity of hydrogels and carvacrol isolated against promastigotes and intracellular amastigotes of *Leishmania amazonensis* and their cytotoxicity in mammalian cells was determined. The sol-gel transition temperature for the binary hydrogel containing carvacrol (HG407/188CA) was 37.04 ± 1.35 °C. HG407/188CA presented lamellar structure at temperatures of 25 °C and 37 °C. HG407/188CA and carvacrol presented IC_{50} against *Leishmania amazonensis* promastigotes of 18.68 ± 1.43 μg/mL and 23.83 ± 3.32 μg/mL, respectively, and IC_{50} against *Leishmania amazonensis* amastigotes of 35.08 ± 0.75 μg/mL and 29.32 ± 0.21 μg/mL, respectively. HG407/188CA reduced the toxicity of carvacrol in all mammalian cells evaluated, raising the CC_{50} in murine peritoneal macrophages from 40.23 ± 0.21 μg/mL to 332.6 ± 4.89 μg/mL, obtaining a Selectivity Index (SI) of 9.5 against 1.37 of the isolated carvacrol. HG407/188CA provided higher selectivity of carvacrol for the parasite. Thus, the binary hydrogel obtained may enable the use of carvacrol as a potential antileishmanial agent.

1. Introduction

Leishmaniasis is a group of infectious diseases caused by protozoa of the genus *Leishmania*, family Trypanosomatidae, transmitted to humans through the bite of female infected sandflies (Freitas-Junior et al., 2012). According to the World Health Organization, the leishmaniasis are endemic in 98 countries around world and more than 1 billion people are exposed to the risk of contracting the infection, with an approximate record of 700,000 to 1 million new cases per year of the different clinical forms (WHO, 2021). In Brazil, *Leishmania amazonensis* and *Leishmania braziliensis* are an epidemiologically relevant species that cause Tegumentary leishmaniasis. *Leishmania amazonensis* stands out for causing a broad spectrum of clinical manifestations, ranging from cutaneous to visceral leishmaniasis (Christensen et al., 2018; Mendonça et al., 2016; Silva et al., 2021). Leishmaniasis is reported as endemic and is considered a public health challenge (Rokni et al., 2021). Furthermore, the

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https://doi.org/10.1016/j.actatropica.2022.106744
Received 4 May 2022; Received in revised form 15 July 2022; Accepted 3 November 2022
Available online 11 November 2022

drugs used in the treatment of leishmaniasis are usually associated with adverse effects and toxicity, resulting in the discontinuity of treatments and the increase of resistant forms of the parasite (Pham et al., 2014, 2013). According to Caridha et al. (2019), there is no new drug or procedure that is effective and widely applicable against cutaneous leishmaniasis, as well as limited advances in the research and development of these new drugs. Thus, new drug candidates with leishmanicidal action are being investigated and, among them, natural products and/or their isolated components of natural or synthetic origin can be highlighted (Lima et al., 2015; Ribeiro et al., 2014; Singh et al., 2014). Natural products are sustainable, economical, available, with a low probability of causing adverse effects and have immunomodulatory effects compared to common antileishmanial compounds (Rokni et al., 2021).

A successful development drug against the *Leishmania* parasite must either kill the parasite in the macrophage or activate the macrophage to kill it (Caridha et al., 2019). Carvacrol (*5-isopropyl-2-methylphenol*), a phenolic monoterpene, is the major component (up to 80%) of oregano essential oil (*Origanum vulgare*) (Tenci et al., 2017), stands out for its leishmanicidal action against different species of *Leishmania* where the IC_{50} values found against promastigote and amastigote forms vary between 7.35 μg/mL and 25.4 μg/mL (de Melo et al., 2013; Essid et al., 2015; Silva et al., 2017; Youssef et al., 2019).

Carvacrol has antileishmanial activity, but it has a low rate of selectivity for *Leishmania* observed in the study by Monzote et al. (2014) who found an IC_{50} 13.6 μg/mL against *Leishmania amazonensis* amastigotes and CC_{50} 32.3 μg/mL in a murine macrophage, obtaining a selectivity index (SI) equal to two. However, in addition to its cytotoxicity in mammalian cells, carvacrol, as well as essential oils and their isolated components, has limitations regarding its direct use (high volatility and low aqueous solubility), making the incorporation of carvacrol into a delivery system relevant (Lucia et al., 2017; Sajomsang et al., 2012).

Thermosensitive systems (micelles and/or hydrogels) formed by amphiphilic copolymers of the triblock type have been studied for solubilization, stabilization, and transport systems of bioactive substances and drugs (Jindal and Mehta, 2015). Poloxamers have been gaining prominence due to the results achieved in several nanometric formulations, such as increased drug solubility, protection of substances against degradation, improved efficiency, slow and prolonged release, reduction of adverse effects, and directing the drug to the desired target (Kupetz et al., 2013; Mendonça et al., 2016; Nascimento et al., 2016).

Considering the use of poloxamers in thermosensitive systems to treat cutaneous leishmaniasis, Lage et al. (2016) observed that hydrogels with poloxamer 407 containing 8-hydroxyquinoline are highly efficient in treating mice chronically infected with *Leishmania amazonensis* and can be considered an alternative to treating cutaneous leishmaniasis alone or in combination with other drugs. Furthermore, Brugués et al. (2015) developed a polymeric micellar system formulated with poloxamer 407 for controlled transdermal release of paromomycin in the treatment of cutaneous leishmaniasis, the system reduces cytotoxicity in Vero cells presented by paromomycin and is more effective against *Leishmania* when compared to drug-free. However, these studies use a single polymer and synthetic drugs.

In recent years, thermosensitive hydrogels made up of binary systems have started to attract interest due to their advantages over methods formed with a single type of copolymer. The limiting aspects of a mono polymeric system, such as low drug load, larger micelle size, and low system stability, can be compensated by mixing different polymers to generate integrated micellar systems (Attia et al., 2011; Kulthe et al., 2011). Binary systems formed by poloxamers 407 and 188 reduce the cytotoxicity of incorporated drugs and prolong their effect (Akkari et al., 2016b; Querobino et al., 2019; Santos et al., 2015). However, the application of these binary systems in antileishmanial activity has not yet been investigated.

In this context, this study aims at developing thermosensitive hydrogels, carrier systems based on triblock copolymers, with the poloxamers 407 (HLB 22) and 188 (HLB 29), for the incorporation of carvacrol, providing its solubility in aqueous media and reducing its cytotoxicity, thus enabling its use as a drug candidate with antileishmanial action in formulations for topical or subcutaneous use. For this reason, physicochemical characterizations were carried out to evaluate the influence of carvacrol on the structural organization of the hydrogels, as well as in vitro carvacrol release from hydrogels, in vitro cytotoxicity, and leishmanicidal activity assays of free and incorporated carvacrol were carried out.

2. Materials and methods

2.1. Chemical and reagents

Poloxamer 407 (Pluronic® F-127, MW 12,600 Da, Poly(ethylene glycol)$_{100}$-Poly(propylene glycol)$_{70}$-Poly(ethylene glycol)$_{100}$), Poloxamer 188 (Pluronic® F-68, MW 8400 Da, Poly(ethylene glycol)$_{76}$- Poly (propylene glycol)$_{29}$-Poly(ethylene glycol)$_{76}$), Carvacrol (*5-isopropyl-2-methylphenol*, $C_{10}H_{14}O$, MW 150.22 g/mol, ≥ 98%) and Amphotericin B (250 μg/mL) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Salts and other reagents were analytical grades.

2.2. Parasites cultures

Culture of *Leishmania amazonensis* (strain MHOM/BR/77/LTB 0016) promastigotes was maintained at 24 °C in Schneider`s medium (Sigma-Aldrich, St. Louis, MO, USA; pH 6.7) supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum (FBS, Gibco by Thermo Fischer Scientific, Carlsbad, CA, USA), ampicillin 500 mg mL^{-1}, 1% and gentamicin 40 mg mL^{-1}, 0.1% (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Animals

Swiss mice were obtained from the Institute of Science and Technology in Biomodels (ICTB-FIOCRUZ). Mice were housed at 5 mice per cage and maintained under standard environmental conditions. All of the animal procedures used in this study were performed according to the recommendations of the Ethics Committee for Animal Use of the Institute Oswald Cruz (CEUA-FIOCRUZ) (licence number L-02/2022). Experimental procedures were conformed to the Guide for the Care and Use of Laboratory Animals (National Institute of Health, publication number 85–23, 1996).

2.4. Cell cultures

Culture of the mouse fibroblast cell line (L929, ATCC CCL-1) and murine macrophages was maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco by Thermo Fischer Scientific, Carlsbad, CA, USA) and 1% streptomycin/penicillin (5000 units + 5 mg mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA), and kept in a humid atmosphere at 37 °C and 5% CO_2.

Murine peritoneal macrophages were obtained from adult, male Swiss mice. Peritoneal lavage was performed with 10 mL of RPMI medium (Sigma-Aldrich Corp, St Louis, MO, USA) supplemented with 10% fetal bovine serum, 1% pyruvate, and 1% glutamine, 1% penicillin, and streptomycin (Gibco). The macrophage concentration was obtained by counting in the Neubauer Chamber. For cytotoxicity experiments, 2×10^6 cells/mL were plated in 96-well plates. To evaluate the leishmanicidal activity in intracellular amastigotes, 1×10^6 cells/mL were plated in chambers mounted on glass slides (16 chambers, Lab-Tek®, Nunc Co.). After cell adherence for 1 hour at 37 °C, 5% CO_2 atmosphere, the experiments were carried out (Andrade-Neto et al., 2021).

2.5. Systems preparation

The thermosensitive hydrogels were obtained by the cold method described by Schmolka (1972). Concentrations were selected through previous studies with poloxamers based on the Santos et al., al.(2015) work, using as a criterion the formation of gel close to body temperature (37 °C). To obtain the formulations, the copolymers were added slowly, under constant stirring, to a pH 7.4 buffer solution (Phosphate Buffer Saline - PBS) placed in an ice bath, keeping the temperature at 5 °C. The dispersions were kept under refrigeration for a minimum period of 12 h until obtaining a translucent solution. Hydrogels were prepared with the triblock poloxamer copolymer 407 (P407) and from the mixture of P407 with poloxamer 188 (P188), keeping the total polymer concentration at 15% (w/v), for P407 (HG407) 15% (w/v) and for the binary mixture P407 14% (w/v) and P188 1% (w/v) (HG407/188). Hydrogels without carvacrol (HG407IN and HG407/188IN) and hydrogels containing carvacrol at 3000 μg/mL (HG407CA and HG407/188CA) were obtained.

2.6. Micellar hydrodynamic diameter

The micellar hydrodynamic diameter was determined to evaluate the drug-polymers interaction. For this purpose, polymeric solutions (micelles) were obtained, according to Akkari et al. (2016b) adapted, using P407 at a concentration of 5% (w/v) (MI407) and a mixture of P407 with P188, with concentrations (w/v) of 4.7% and 0.3%, respectively (MI407/188), maintaining a total of 5% (w/v) polymer. Micelles without carvacrol (MI407IN and MI407/188IN) and micelles containing carvacrol at a concentration of 1000 μg/mL (MI407CA and MI407/188CA) were obtained, considering the proportions of polymers and carvacrol in the hydrogel. The determination of the average diameter and the Polydispersity Index (PDI) of the polymeric micelles was performed by Dynamic Light Scattering (DLS) using the Zetasizer Nano ZS equipment (Malvern Instruments®, UK). The samples were inserted in an appropriate cuvette for measurements at a fixed angle of 173° at 37 °C to simulate the micelle's behavior at body temperatures. The measurements were performed with at least 12 readings and in triplicate for each sample. The measurement parameters were the indexes of refraction of the material and the dispersing medium.

2.7. Differential scanning calorimetry (DSC) analysis

The micellization temperature (T_m) of the hydrogels was obtained using the DSC 200 F3 Netzsch equipment. Approximately 20 mg of the inert and carvacrol hydrogels were placed in an aluminum sample holder, which was subjected to three successive heat-cooling cycles from 0 °C to 50 °C, with a rate of 5 °C/min in heating and 10 °C/min in cooling. The measurements were performed in triplicate ((Mello et al., 2016), adapted).

2.8. Rheological behavior assays

The rheological analysis of the hydrogels was performed using the TA Instruments Discovery HR-1 rheometer. An oscillatory test of the systems was performed to determine the evolution of the storage (G`) and loss (G``) modules as a function of temperature using 40 mm diameter plate/plate geometry and 200 μm distance between the plates. The temperature scanning test was performed to determine the phase transition temperature (Tsol-gel) with a fixed frequency of 1 Hz and temperature variation between 10 °C and 50 °C. Each sample was carefully applied to the lower plate of the rheometer, ensuring minimum shear and allowing a resting time (relaxation of the tension induced before analysis) of 1 min before each determination. The data obtained were analyzed using the Origin Pro-software v.8 (Akkari et al., 2016b).

2.9. Small-angle X-ray scattering (SAXS)

The SAXS measurements were carried out at temperatures of 25 °C and 37 °C. The data were collected at a measurement station at the National Laboratory of Synchrotron Light (LNLS, Campinas, SP, Brazil) using an incident beam energy of 8.3 keV (lambda = 1.488 Å) with the distance between sample and detector 1007 mm (MarCCD detector with a diameter of 165 mm) and measuring range (brand measuring range) from 0:13 to 3:34 nm^{-1}. The measured scattered intensity was displayed as a function of the scattering vector modulus $q = 4\pi\sin(\theta)/\lambda$, where 2θ is the scattering angle and λ is the radiation wavelength. The typical q range was from 0.075 to 0.23 $\AA-1$ (Akkari et al., 2016b).

2.10. Scanning electron microscopy (SEM)

Scanning electron microscopy was used to visualize the material surface and morphological parameters. The samples were deposited on a metallic substrate and dried for about 1 hour in an oven at a fixed temperature of 37 °C. After drying, the samples were covered with a gold film of approximately 10 nm, following the procedure adapted to prepare the samples for the morphological analysis by Querobino et al. (2019). The images were obtained using the Scanning Electron Microscope JEOL JSM-6510 LV.

2.11. In vitro release assays

The release test was performed in triplicate, using a cellulose acetate membrane (Jindal and Mehta, 2015). First, 0.1 mL of the formulations were transferred to the dialysis tube, which was introduced in a double-walled beaker containing 9.9 mL of release medium (phosphate buffer pH 7.4 and DMSO in the ratio 1:1, selected from previous solubility tests) maintained at 37 °C. Then, at time intervals of 0.5; 1; 2; 4; 6; 8; 10, and 24 h, 2 mL of the release medium outside the dialysis tube was removed, and the same volume was replaced to maintain sink conditions. The amount of carvacrol released in each time interval was determined by UV/visible spectrophotometry at a wavelength of 274 nm. The carvacrol triplicate calibration curve was previously determined.

2.12. Cytotoxicity evaluation in fibroblasts

Cytotoxicity assays were performed on L929 fibroblasts. Cells were plated in 96-well culture plates (2×10^4 cells/well) and maintained for 24 h at 37 °C and 5% CO_2 for cell adherence. Then cells were treated with carvacrol, inert- and containing carvacrol hydrogels in concentrations of 6.25, 12.5, 25, 50, and 100 μg/mL, for 24 h under the same conditions as before. Cellular activity/viability was obtained by the colorimetric method using Methyl-thiazole-tetrazolium - MTT (Sigma-Aldrich, USA). After the treatment, the cells were washed twice with PBS pH 7.4, 200 μL of MTT (0.5 mg/mL) was added to each well, and the cells were incubated at 37 °C for 3 h. After removing the MTT, 200 μL of DMSO were added for formazan solubilization. After 10 min, the optical density (OD) was read in an automated plate reader at a wavelength of 570 nm (Silva et al., 2017). The untreated cells were used as a control and considered 100% cell viability. The tests were performed in triplicate and then normalized according to the following equation Eq. (1):

$$\%\,\text{Cell Viability} = (\text{absorbance of the treated cell}/\text{absorbance of negative control}) \times 100 \quad (1)$$

CC_{50} was obtained by non-linear regression analysis of cell viability data in the GraphPad Prism 5.0 program.

2.13. Cytotoxicity evaluation in peritoneal murine macrophages

To evaluate the activity of the compounds in the host cell, toxicity tests were performed on murine peritoneal macrophages that were not infected with *Leishmania*. The cells were grown (or incubated) for 24 and 72 h in the absence or presence of carvacrol and inert and containing carvacrol hydrogels in concentrations ranging from 3.75 to 960 μg/mL. Amphotericin B (0.289 - 18.5 μg/mL) was used as a positive control. After the incubation time, resazurin (50 μM) was added to each well. The cell viability was assessed by fluorimetry, using Spectra Max GEMINI XPS equipment (Molecular Devices, Silicon Valley, USA), with excitation at 560 nm and emission at 590 nm.

2.14. Antileishmanial activity against promastigotes

Leishmania amazonensis promastigotes (strain MHOM/BR/77/LTB 0016) in the exponential growth phase were used for the experiments. The promastigotes (2×10^6 parasites/mL) were incubated with different concentrations of carvacrol, inert hydrogels, hydrogels containing carvacrol (6.25 – 100 μg/mL), and Amphotericin B (0.03125 – 0.5 μg/mL) (positive control) in Schneider medium at 24 °C for 24 h. As a negative control, the promastigotes were kept without any treatment. After the incubation time, 20 μL of resazurin solution (3 mM) were added to each well, and the plate was incubated at 24 °C for 2 h. The fluorescence measurements were obtained in a microplate reader at wavelengths of 560 nm excitation and 590 nm emission and used to calculate the promastigote viability (%). In addition, IC_{50} values were obtained from viability data by non-linear regression of the sigmoidal growth inhibition curve in the GraphPad Prism 5.0 program (Andrade-Neto et al., 2021).

2.15. Antileishmanial activity against amastigotes

Murine peritoneal macrophages were infected with *Leishmania amazonensis* (strain MHOM/BR/77/LTB 0016) promastigotes forms at a 3:1 (parasite: macrophage) ratio in RPMI medium (Sigma-Aldrich Corp, St Louis, MO, USA) supplemented with 10% serum fetal bovine, 1% pyruvate, and 1% glutamine. After 24 h of interaction at 34 °C in a 5% CO_2-air mixture, the chambers were washed with RPMI 1640 medium to remove non-interiorized parasites. Afterward, the infected macrophages were treated with carvacrol and inert and containing carvacrol hydrogels in concentrations ranging from 7.5 to 240 μg/mL for 24 and 72 h at 37 °C and 5% CO_2. Amphotericin B (0.04375 - 1 μg/mL) was used as a positive control. The infection rate was determined after the slides were stained by counting infected cells under an optical microscope. The infection rate was calculated according to the following equation Eq. (2): (Andrade-Neto et al., 2021).

$$\text{Infection Rate} = \%\,\text{infected macrophages} \times (\text{number of amastigotes/total macrophages}) \quad (2)$$

2.16. Statistical analysis

Analysis of variance (ANOVA) followed by the Bonferroni post-test was used for multiple comparisons. The calculations were performed using the GraphPad Prism statistical software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistically significant differences were defined as $p < 0.05$. The data were presented as means ± S.D. of three independent experiments performed in triplicate.

3. Results and discussion

3.1. Micellar hydrodynamic diameter

Initially, micelles using a total of 5% of polymer were assessed by DLS for evaluating drug-polymer interaction. The analyzes were performed at 37 °C. The measurement at this temperature is important to simulate the behavior of the micelles when in contact with the physiological temperature after the dissolution of the hydrogels.

In general, the micelles were homogeneous with low Polydispersity Index (PDI) values, 0.113, 0.124, 0.131, and 0.100 to MI407IN, MI407CA, MI407/188IN, and MI407/188CA, respectively. The hydrodynamic diameter increased with the incorporation of P188 as well as with carvacrol, from 17.4 ± 0.13* nm to 18.26 ± 0.17* nm, respectively, to MI407IN, MI407CA and from 18.7 ± 0.15* nm to 19.86 ± 0.24* nm, respectively, to MI407/188IN and MI407/188CA. These values were significantly different considering $^*p < 0.05$. It is possible to suggest that carvacrol (hydrophobic) was inserted into the micellar nucleus, connecting the polymers' PPO units, increasing the micelles' hydrodynamic diameter. The results corroborate with data from the literature of Akkari et al. (2016b), which show higher hydrodynamic diameter values for binary systems consisting of P188 and P407 and lower for P407. Since P188 is more hydrophilic (HLB 29) than P407 (HLB 22), P188 forms micelles with larger diameters and is less homogeneous due to greater hydration of the PEO (hydrophilic) units. PPO (hydrophobic) units increase the system's viscosity and form a homogeneous spherical micellar system (Akkari et al., 2016b).

3.2. Rheological assays

Rheological studies can be used to examine differences in the viscosity and elasticity properties of materials and determine the sol-gel transition temperature ($T_{sol\text{-}gel}$), an important parameter to estimate the influence of components on the structural organization of hydrogels (Vigato et al., 2019). The obtained hydrogels showed temperature-dependent rheological behavior where $T_{sol\text{-}gel}$ is defined by the intersection between the G' (storage module) and G" (loss module) curves (Zhang et al., 2013). As seen in Fig. 1, below 20 °C, the samples showed only G'' and above that temperature, they started to show G' however, lower than G" which only becomes higher than G" when $T_{sol\text{-}gel}$ is achieved. Above the $T_{sol\text{-}gel}$ the hydrogels were presented as non-Newtonian fluids, viscoelastic, with G' > G". This behavior is characteristic of its thermosensitive property that allows perfecting formulations, being in a liquid state at room temperature, facilitating administration and in a gel state close to or above the physiological temperature, promoting the prolonged release of pharmacological agents (Akkari et al., 2016b).

According to the results obtained from $T_{sol\text{-}gel}$, it is possible to observe that the presence of P188 increased the $T_{sol\text{-}gel}$ of hydrogels, from 32.23 °C ± 0.67 to above 40 °C for inert hydrogels HG407 and HG407/188, respectively, and from 24.02 °C ± 0.34 to 37.04 °C ± 1.35 for hydrogels containing carvacrol HG407CA and HG407/188CA, respectively, this can be explained because the presence of P188 decreases the system viscosity by increasing hydration of PEO units. However, the incorporation of carvacrol decreased the $T_{sol\text{-}gel}$ of hydrogels. These results suggest that the incorporation of carvacrol possibly promoted dehydration of the polymer PPO units increasing the viscosity of the systems and therefore decreasing the $T_{sol\text{-}gel}$. Sharma et al. (2008) evaluated the influence of drugs and pharmaceutical additives on the gel-forming behavior of P407. They observed that incorporating hydrophobic substances can reduce the $T_{sol\text{-}gel}$ or the polymer concentration required to form a gel.

The binary hydrogel HG407/188CA showed a $T_{sol\text{-}gel}$ closer to the physiological temperature than the hydrogel HG407CA and is, therefore, more suitable for use in an antileishmanial formulation for cutaneous or subcutaneous use.

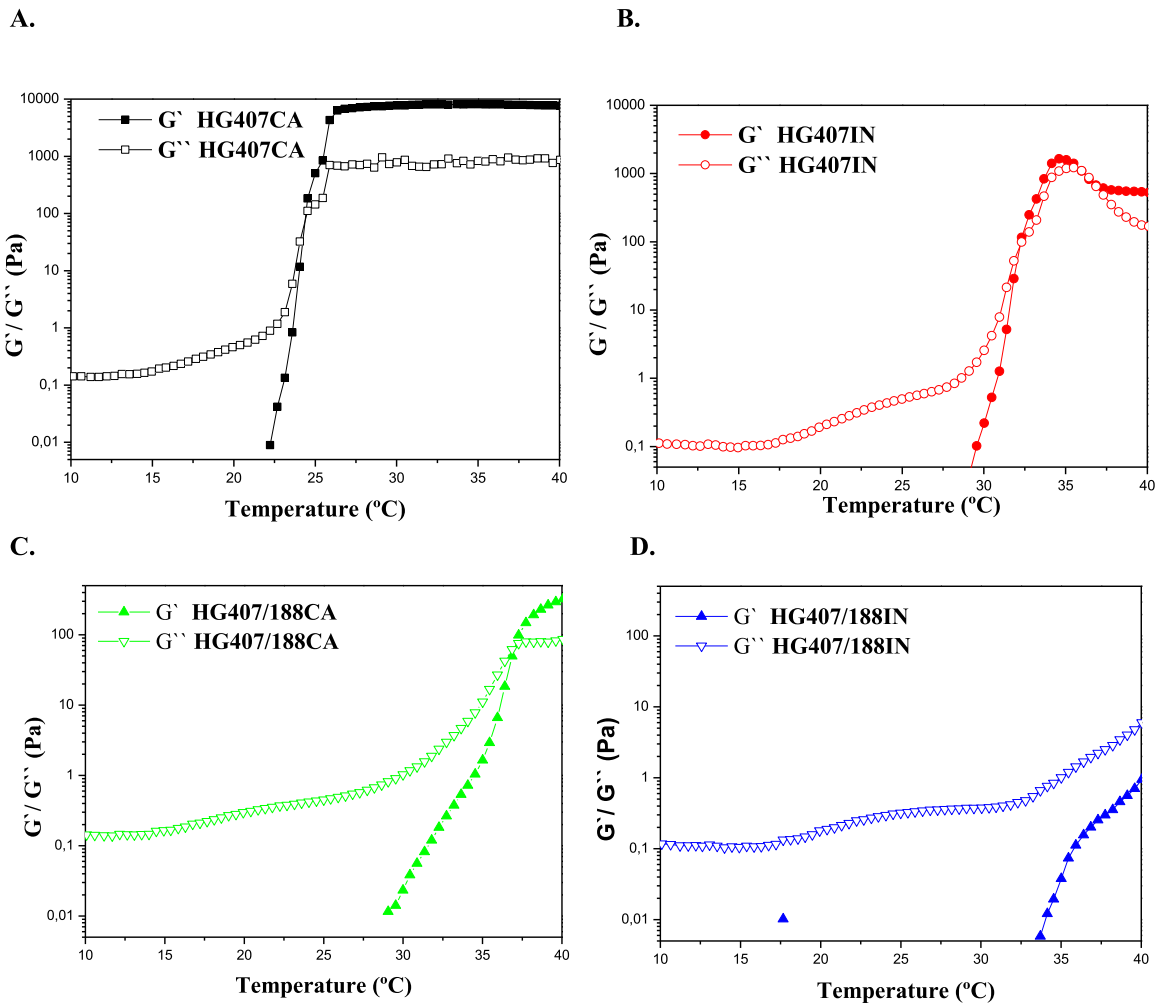

Fig. 1. Oscillatory temperature sweep of hydrogels obtained in PBS: **A.** P407 15% (w/v) containing 3000 μg/mL of carvacrol (HG407CA); **B.** Inert P407 15% (w/v) (HG407IN); **C.** P407 14% (w/v) and P188 1% (w/v) containing 3000 μg/mL of carvacrol (HG407/188CA); and **D.** Inert P407 14% (w/v) and P188 1% (w/v) (HG407/188IN).

3.3. Differential scanning calorimetry (DSC)

The formation of micelles occurs at a particular concentration, the so-called critical micellar concentration (CMC) of polymer in an aqueous medium at a fixed temperature, the block copolymers (monomers) aggregate forming micelles (mycelization), in addition to CMC, this process also occurs at a given temperature, the critical micellar temperature (T_{mc}) with fixed concentration (Nie et al., 2011). Therefore, the DSC technique was performed to determine the T_{mc} of the hydrogels.

The addition of P188 for the inert system reduced the T_{mc} from 15.9 °C to 14.9 °C and the ΔH value from −1.128 to −0.5921, respectively, to HG407IN and HG407/188IN. The addition of a more hydrophilic polymer (P188) induces changes in the micellar arrangement of the less hydrophilic copolymer (P407), such as increased hydration of the micellar crown, favoring the formation of micelles, which justifies the lower T_{mc} for the binary system (Akkari et al., 2016b).

The incorporation of carvacrol increased the T_{mc} of the systems, respectively, from 15.9 °C to 19.2 °C, for HG407IN and HG407CA, and from 14.9 °C to 21.8 °C, for HG407/188IN and HG407/188CA. In addition, increased the value of ΔH from −1128 to 0.1995 for the P407 system and from −0.5921 to 0.1563 for the binary system. Santos et al. (2015) also observed an increase in the enthalpy variation with the incorporation of tramadol into the P407/P188 binary system, probably because it promotes dehydration of the PPO units of the micellar nucleus.

3.4. Small-angle x-ray scattering (SAXS)

The SAXS technique was used to characterize the supramolecular structure of the obtained hydrogels. The experiment was carried out at temperatures of 25 °C and 37 °C, and the structures formed were determined through the positions of the Bragg diffraction peaks obtained, considering the relationships between the peaks of 1: 2: 3: 4 … and 1: $\sqrt{3}$: 2: $\sqrt{7}$: 3 …, for lamellar and hexagonal structures, respectively. The parameters of lamellar periodicity (d) and distance between the centers of adjacent cylinders (a), the lamellar and hexagonal structures, respectively, were obtained from the position of the first and most intense diffraction peak (q_{max}) from the Eq. (3) and Eq. (4) (Alexandridis et al., 1998).

$$q_{\max} = 2\pi/d\,(lamellar) \tag{3}$$

$$q_{max} = 4\pi \Big/ a\sqrt{3}(hexagonal) \tag{4}$$

The hydrogels showed different structures with carvacrol, with an increase in temperature, and with the incorporation of P188 (Table 1). The inert P407 hydrogel presented a lamellar phase structure at 25 °C, and with the incorporation of carvacrol, a more ordered structure was formed, a hexagonal phase system (Fig. 2A). As observed in the rheology behavior experiments, the incorporation of carvacrol increased the viscosity of the system by reducing the phase transition temperature from 32 °C (HG407IN) to 24 °C (HG407CA), this higher organization can also

Table 1
Structural parameters obtained from the SAXS curves at 25 °C and 37 °C of the inert hydrogels of P407 15% (w/v) (HG407IN) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188IN); hydrogels containing 3000 μg/mL of carvacrol of P407 15% (w/v) (HG407CA) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188CA), obtained in PBS.

Formulations	q^1_{max} (nm)	q^2_{max} (nm)	q^3_{max} (nm)	q^4_{max} (nm)	d/a (nm)	Structure
25 °C						
HG407IN	0.33	0.65	–	–	19.04	LAMELLAR
HG407CA	0.32	0.53	0.62	0.82	22.67	HEXAGONAL
HG407/188IN	0.34	0.64	–	–	19.63	LAMELLAR
HG407/188C	0.30	0.60	–	–	20.94	LAMELLAR
37 °C						
HG407IN	0.33	0.57	0.66	0.87	21.98	HEXAGONAL
HG407CA	0.32	0.54	0.62	0.82	22.67	HEXAGONAL
HG407/188IN	0.32	0.62	–	–	19.63	LAMELLAR
HG407/188C	0.30	0.58	–	–	20.94	LAMELLAR

be observed in SAXS experiments with the increase in temperature, at 37 °C the hydrogel HG407IN changed from the lamellar phase to the hexagonal phase. However, the HG407CA remained in the hexagonal phase presenting similar values of q_{max} for all peaks (Fig. 2C and Table 1).

The inert binary hydrogels (HG407/188IN) and containing carvacrol (HG407/188CA) presented a lamellar phase for both evaluated temperatures (Figs. 2B and 2D). As can be seen in rheology, the incorporation of P188 reduced the viscosity of the hydrogel by raising the phase transition temperature from 32 °C to values above 40 °C for the inert hydrogel and from 24 °C to 37 °C for the hydrogel containing carvacrol. Although there was no phase change of the binary hydrogels as observed for the HG407 hydrogel, the addition of carvacrol shifted the peaks to lower angles, with lower values of $q_{máx}$ for HG407/188CA compared to HG407/188IN (Table 1), increasing the value of *d*, indicating the greater distance between the lamellae and thus the structuring.

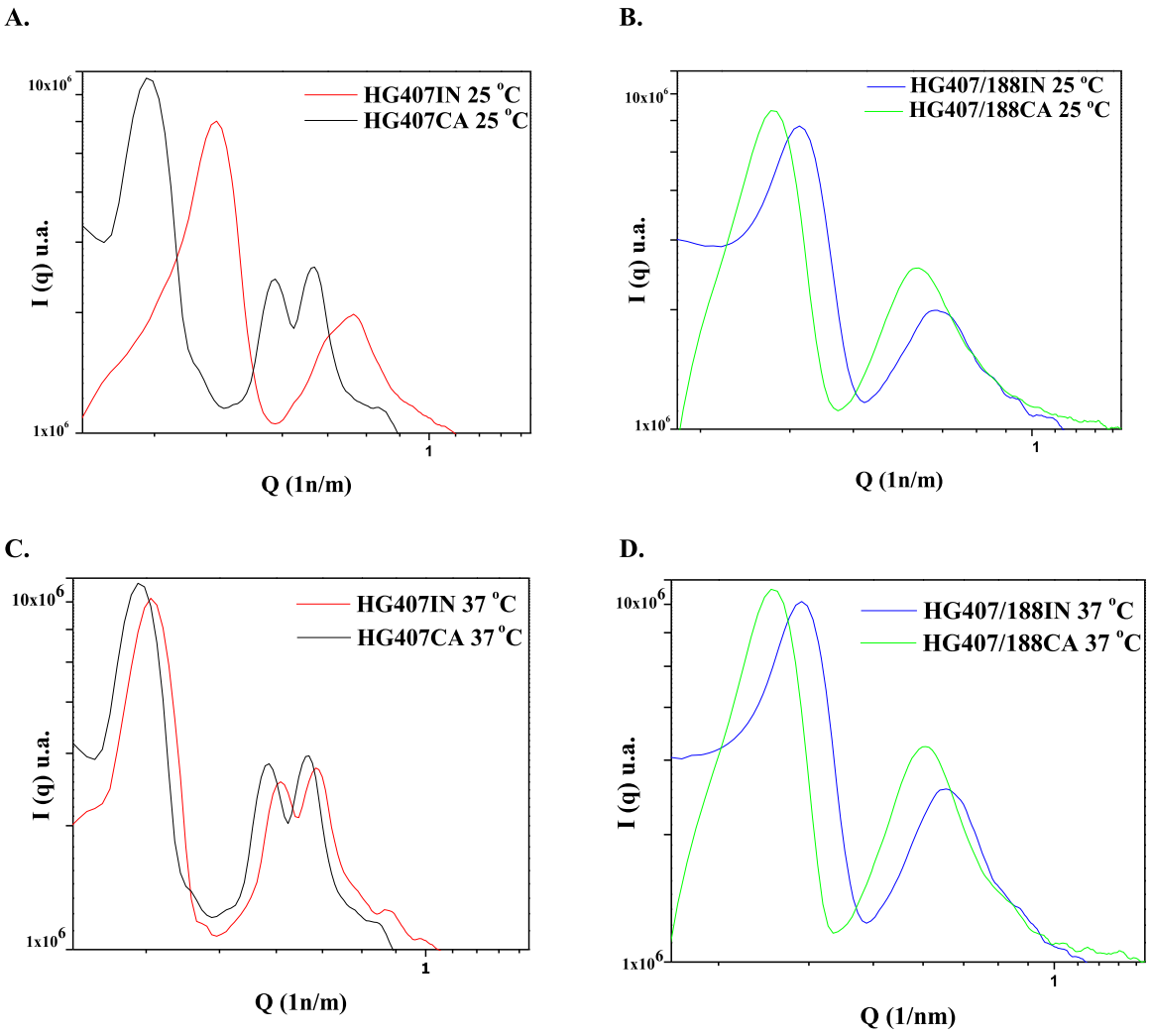

Fig. 2. SAXS curves at the temperatures of 25 °C (**A** and **B**) and 37 °C (**C** and **D**) of the inert hydrogels of P407 15% (w/v) (HG407IN); and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188IN); hydrogels containing 3000 μg/mL of carvacrol of P407 15% (w/v) (HG407CA) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188CA), obtained in PBS.

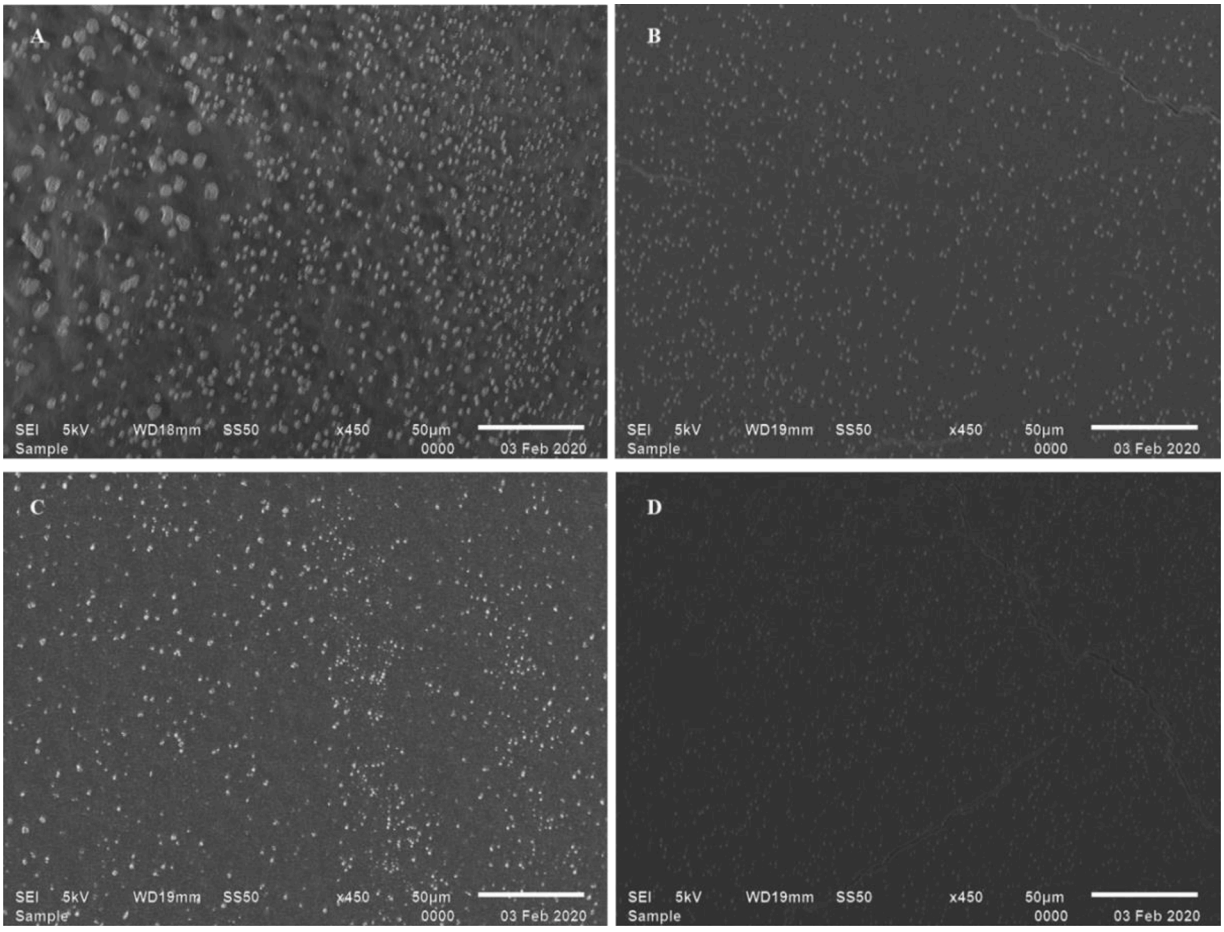

Fig. 3. SEM images of hydrogels: **A.** P407 14% (w/v) and P188 1% (w/v) containing 3000 µg/mL of carvacrol (HG407/188CA); **B.** Inert P40714% (w/v) and P188 1% (w/v) (HG407/188IN); **C.** P407 15% (w/v) containing 3000 µg/mL of carvacrol (HG407CA); and **D.** Inert P407 15% (w/v) (HG407IN), obtained in PBS.

3.5. Scanning electron microscopy (SEM)

Scanning electron microscopy was used to verify the morphology of the hydrogels. In systems formed by poloxamers, it is possible to observe structure in layers or sheets (Akkari et al., 2016a). The hydrogels had similar homogeneous nanometric structures with uniform distribution (Fig. 3). The incorporation of carvacrol provided a higher organization of the structures observed, with the HG407CA being the system with the most significant organization corroborating the results obtained in the SAXS technique. According to the results obtained and following the literature data, where layered morphologies (Akkari et al., 2016b), regular, homogeneous, and uniform morphology (Akbar et al., 2018), and absence of morphological differences after addition of drug (Akkari et al., 2016a), it can be inferred that hydrogels formed by poloxamers do not undergo morphological changes with the addition of other poloxamers, active and/or inclusion complexes.

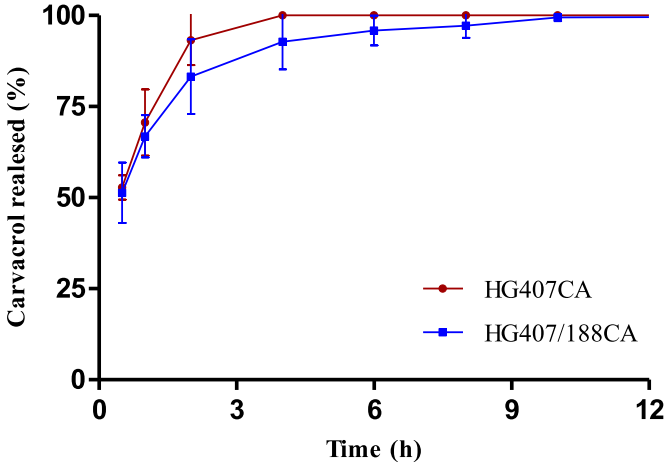

Fig. 4. Release curves of hydrogels containing 3000 µg/mL of carvacrol from P407 15% (w/v) (HG407CA) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188CA), obtained in PBS.

3.6. In vitro release assays

Fig. 4 shows the release curves of hydrogels containing carvacrol. The difference was not statistically significant considering $p < 0,05$. However, the addition of P188 promoted a slower release of carvacrol from the hydrogel HG407/188CA compared to HG407CA, as P188 provided an increase in the hydrophilic crown of the micelles, avoiding rapid dissolution and prolonging the release time. Carvacrol was quickly released from the hydrogel HG407CA, 50% in the first hour and 100% in up to 4 h, however, the binary hydrogel, although it also released about 50% of the carvacrol in the first hour, prolonged the time of total release to up 10 h. Carvacrol was 100% released in both hydrogels. The result obtained was similar to that of Querobino et al. (2019) who obtained 100% release of voriconazole within 4 h for the P407 system, and up to 75% release within 8 h for the P407/188 system.

The mathematical models of zero order, first order, and pseudo-first-order (Higuchi) were applied. The correlation coefficients (R^2) of HG407CA and HG407/188CA were 0.8886 and 0.856, respectively, for the zero order model and 0.8288 and 0.8018 for the first order model,

Table 2
Cytotoxic concentration for 50% murine peritoneal macrophages from carvacrol; Amphotericin B; inert hydrogels of P407 15% (w/v) (HG407IN) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188IN); hydrogels containing 3000 μg/mL of carvacrol of P407 15% (w/v) (HG407CA) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188CA), obtained in PBS. Data are expressed as mean ± SD.

Formulations	CC_{50} μg/mL	
	24 h	72 h
HG407IN	579.1 ± 3.17*	514 ± 4.24*
HG407CA	203.2 ± 0.23*	119.3 ± 0.17*
HG407/188IN	884 ± 15.67ns	880.6 ± 4.03ns
HG407/188CA	374.4 ± 2.76*	332.6 ± 4.89*
CARVACROL	75.91 ± 2.80*	40.23 ± 0.21*
AMPHOTERICIN B	>18.5	>18.5

ns, no significant statistical difference.
* $p < 0.05$, significant statistical difference.

respectively. The model with the highest correlation coefficient was Higuchi's for HG407/188CA ($R^2 = 0.9192$) and HG407CA ($R^2 = 0.957$), indicating that the release follows Fick's law associated with the diffusion of carvacrol through hydrogels. Higuchi's model was also obtained by Akkari et al. (2016b) in the release of ropivacaine from P407 (25% and 30% w/v) and P407/P188 (25/5% and 20/10% w/v) hydrogels. Literature data indicate that poloxamers, alone or in mixtures, depending on concentration and types, may be released characterized by an anomalous release mechanism with transport controlled by Fickian diffusion and swelling due to the slow relaxation of the polymeric matrix in contact with the release medium (Querobino et al., 2019) and as well as an anomaly or non-Fickian release, suggesting a coupled erosion-diffusion mechanism, where the drug can be released by diffusion from the micellar nucleus or by dissociation of the micelle followed by the separation of the drug from the monomer (Jindal and Mehta, 2015).

3.7. Cytotoxicity evaluation

Although *Leishmania* spp. mandatory infect macrophages, fibroblasts also show a high degree of injury during the development of cutaneous and/or mucosal lesions in cutaneous leishmaniasis (Holanda et al., 2020), so cytotoxicity evaluation in L929 fibroblasts was performed. Carvacrol showed cytotoxic concentration (CC_{50}) of 42.93 ± 3.25 μg/mL. The hydrogels HG407/188CA and HG407CA reduced the cytotoxicity of carvacrol, providing viabilities greater than 80% and 60%, respectively, for concentrations of 56.25 and 450 μg/mL. The inert hydrogels HG407IN and HG407/188IN also did not show cytotoxicity, providing high percentages of viability, around 90%.

3.8. Cytotoxicity in murine macrophages

In investigating cytotoxicity in murine macrophages, the experiments were carried out in two treatment times (24 h and 72 h) to determine the SI after trials against *Leishmania amazonensis* amastigotes. As observed in L929 fibroblasts, hydrogels had CC_{50} higher than CC_{50} for carvacrol (Table 2). In the experiment conducted within 24 h, carvacrol did not show high cytotoxicity, presenting CC_{50} of 75.91 ± 2.80 μg/mL. However, in 72 h, carvacrol showed CC_{50} of 40.23 ± 1.03 μg/mL, being considered cytotoxic. The results were significantly different considering $p < 0.05$ for all samples at other times, except for comparing 24 and 72 h of the inert hydrogel HG407/188IN. Both inert hydrogels and hydrogels containing carvacrol were not cytotoxic to murine macrophages at both times evaluated (Table 2).

Hydrogels containing carvacrol significantly reduced ($p < 0.05$) the toxicity of carvacrol. For the 24 h time, hydrogels provided 100% cell viability at a 120 μg/mL concentration, unlike carvacrol, which showed high cytotoxicity with just over 25% viable cells. At 72 h, carvacrol was highly cytotoxic, causing 100% cell death at a concentration of 120 μg/mL, while binary hydrogel HG407/188CA, provided 100% cell viability (Supplementary data 1).

3.9. In vitro antileishmanial activity

In assessing activity against promastigote forms of *Leishmania amazonensis*, carvacrol, HG407/188CA and HG407CA showed an IC_{50} of 18.68 ± 1.43 μg/mL, 23.83 ± 3.32 μg/mL, and 23.82 ± 2.31 μg/mL, respectively. The result obtained for carvacrol was below the values found by Galvão et al. (2020) and Silva et al. (2017) who obtained IC_{50} of 28.1 ± 0.3 μg/mL and 25.4 ± 2.4 μg/mL, respectively, against *Leishmania amazonensis* promastigotes. HG407/188CA and HG407CA presented IC_{50} similar to that obtained by Galvão et al. (2020), who carried carvacrol in nanostructured lipid carriers obtaining IC_{50} of 28.2 ± 3.25 μg/mL. The viability of *Leishmania amazonensis* promastigotes exposed to different concentrations of carvacrol and hydrogels is shown in Fig. 5. Carvacrol showed significantly different results for HG407/188IN and HG407IN considering $p < 0.05$. The inert hydrogels,

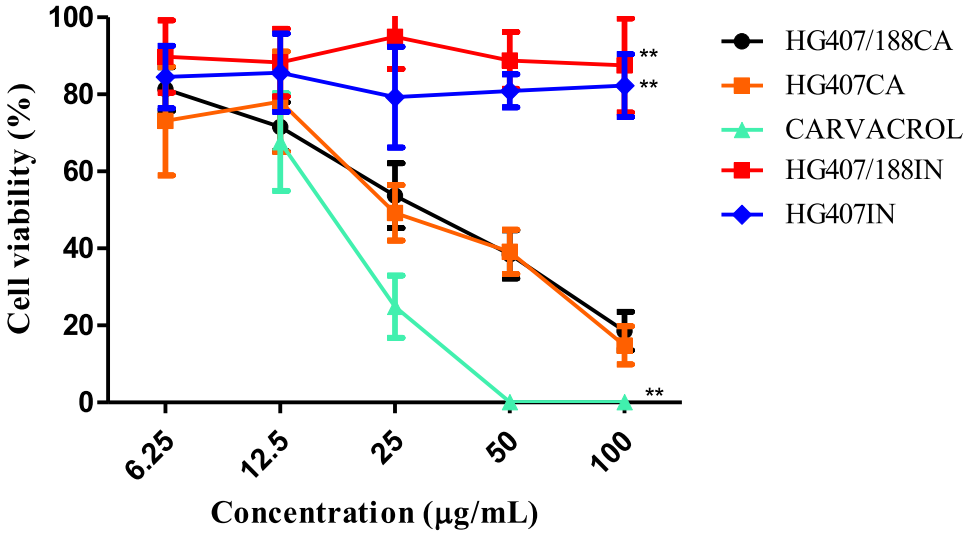

Fig. 5. Viability of *Leishmania amazonensis* promastigotes treated with different concentrations of carvacrol; inert hydrogels of P407 15% (w/v) (HG407IN) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188IN); hydrogels containing 3000 μg/mL of carvacrol of P407 15% (w/v) (HG407CA) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188CA), obtained in PBS. **$p < 0.05$, significant statistical difference.

Table 3
Inhibitory concentration for 50% of *Leishmania amazonensis* (IC_{50}) and SI (CC_{50}/IC_{50}) of Amphotericin B; carvacrol; inert hydrogels of P407 15% (w/v) (HG407IN) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188IN); hydrogels containing 3000 μg/mL of carvacrol of P407 15% (w/v) (HG407CA) and binary with P407 14% (w/v) and P188 1% (w/v) (HG407/188CA), obtained in PBS. Data are expressed as mean ± SD.

Formulations	24 h		72 h	
	IC_{50} μg/mL	SI	IC_{50} μg/mL	SI
HG407IN	>240	—	>240	—
HG407CA	57.07 ± 1.94*	3.5*	42.41 ± 2.03*	3*
HG407/188IN	>240	—	>240	—
HG407/188CA	53.91 ± 0.93*	6.9*	35.08 ± 0.75*	9.5*
CARVACROL	38.05 ± 0.61*	2.0*	29.32 ± 0.21*	1.37*
AMPHOTERICIN B	0.36 ± 0.01*	—	0.13 ± 0.029*	—

* $p < 0.05$, significant statistical difference.

HG407/188IN and HG407IN, showed no activity, different from Mendonça et al. (2016) who obtained an IC_{50} of 22.1 μM against *Leishmania amazonensis* promastigotes for the inert system of P407 18% (w/v).

3.10. Treatment of infected macrophages

In assessing the antiamastigote activity of carvacrol and inert hydrogels containing carvacrol, *Leishmania amazonensis* amastigotes were treated at 24 h and 72 h, according to a cytotoxicity experiment on murine macrophages. IC_{50} and IS values determined are shown in Table 3. The infection rate was above 80%.

The inert hydrogels, HG407IN and HG407/188IN, did not show antiamastigote activity in the investigated concentrations for both times 24 and 72 h. Amphotericin B, carvacrol, and hydrogels containing carvacrol (HG407CA and HG407/188CA) showed higher activity against *Leishmania amazonensis* amastigotes in 72 h (Supplementary data 2). This result was expected considering that the compounds need to transpose the macrophage to reach the parasite. All samples showed significantly different results considering $p < 0.05$.

Considering that the SI is determined by the ratio between CC_{50} and IC_{50} of substances, the polymeric micellar systems have made it possible to reduce cytotoxicity (increase in the CC_{50} value) and consequently increase the SI. The binary hydrogel containing carvacrol (HG407/188CA) provided SI of 9.5 against amastigotes of *Leishmania amazonensis*, significantly different ($p < 0.05$) from carvacrol and HG407CA, which showed SI of 1.37 and 3, respectively, the binary system allows carvacrol to be a new drug against cutaneous leishmaniasis. The results obtained corroborate with the data in the literature, even for different species of *Leishmania* spp. and concentrations of polymers, the systems obtained with poloxamers elevate the SI of the carried compounds, making them viable for use against leishmaniasis (Duarte et al., 2016b, 2016a; Lage et al., 2016; Mendonça et al., 2016). Furthermore, the obtained system (HG407/188CA) was more effective than the system developed by Galvão et al. (2020) that carried carvacrol in nanostructured lipid carriers obtaining CC_{50} of 49.05 ± 2.04 μg/mL in murine peritoneal macrophages and SI of 2.5 against amastigotes of *Leishmania amazonensis*, against 332.6 ± 4.89 μg/mL and 9.5 for HG407/188CA, respectively. Therefore HG407/188CA is a promising system for the treatment of cutaneous leishmaniasis. However, future in vivo investigations of these system on development of new pharmaceutical formulations for the treatment of leishmaniasis should be performed.

4. Conclusion

Based on the results of the present study, it can be concluded that the copolymers used were suitable for the development of hydrogels containing carvacrol, being suitable for topical or subcutaneous formulations. The binary hydrogel containing carvacrol has demonstrated antipromastigote and antiamastigote activity against *Leishmania amazonensis* and reduced the cytotoxicity of carvacrol in fibroblasts and macrophages, obtaining a good Selectivity Index, therefore enabling the use of carvacrol as a promising antileishmanial agent. The results finding point out the HG407/188CA as a strategy for future investigations on development of new pharmaceutical formulations for the treatment of leishmaniasis.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Amanda Mendonça Barros Costa: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Audrey Rouse Soares Tavares Silva:** Investigation. **Adriana de Jesus Santos:** Investigation. **Juliana Gouveia Galvão:** Investigation. **Valter Viana Andrade-Neto:** Methodology, Investigation, Formal analysis. **Eduardo Caio Torres-Santos:** Methodology, Investigation, Formal analysis. **Marcelo Massayoshi Ueki:** Methodology, Investigation. **Luis Eduardo Almeida:** Methodology, Investigation. **Victor Hugo Vitorino Sarmento:** Methodology, Investigation. **Silvio Santana Dolabella:** Investigation. **Ricardo Scher:** Methodology, Resources, Supervision, Writing – review & editing. **Ana Amélia Moreira Lira:** Writing – review & editing. **Rogéria de Souza Nunes:** Supervision, Conceptualization, Project administration, Funding acquisition, Visualization, Resources.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

We are grateful to the Brazilian Synchrotron Light Laboratory for SAXS facilities and to the Nanotechnology Multi-User Center at the Federal University of Sergipe for SEM. We also acknowledge the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2022.106744.

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