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Dissection of phospholipases A_2 reveals multifaceted peptides targeting cancer cells, *Leishmania* and bacteria

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ABSTRACT

Cationic peptides bio-inspired by natural toxins have been recognized as an efficient strategy for the treatment of different health problems. Due to the specific interaction with substrates from biological membranes, snake venom phospholipases (PLA_2s) represent valuable scaffolds for the research and development of short peptides targeting parasites, bacteria, and cancer cells. Considering this, we evaluated the *in vitro* therapeutic potential of three biomimetic peptides (pCergo, pBmTxJ and pBmje) based on three different amino acid sequences from Asp49 PLA_2s. First, short amino acid sequences (12–17 in length) derived from these membranolytic toxins were selected using a combination of bioinformatics tools, including AntiCP, AMPA, PepDraw, ToxinPred, and HemoPI. The peptide, from each polypeptide sequence, with the greatest average antimicrobial index, no toxicity, and no hemolysis predicted was synthesized, purified, and characterized. According to *in vitro* assays performed, pBmje showed moderate cytotoxicity specifically against MCF–7 (breast cancer cells) with an EC_{50} of 464.85 μM, whereas pBmTxJ showed an antimicrobial effect against *Staphylococcus aureus* (ATCC 25923) with an MIC of 37.5 μM, and pCergo against *E. coli* (ATCC 25922) with an MIC of 75 μM. In addition, pCergo showed antileishmanial activity with an EC_{50} of 93.69 μM and 110.40 μM against promastigotes of *Leishmania braziliensis* and *L. amazonensis,* respectively. Altogether, these results confirmed the versatility of PLA_2-derived synthetic peptides, highlighting the relevance of the use of these membrane-interacting toxins as specific archetypes for drug design focused on public health problems.

1. Introduction

Bacterial infections, cancer, and leishmaniasis represent important medical challenges, which produce a significant impact on global public health due to the high infection and mortality rate worldwide [1,2]. Despite the different clinical manifestations, these diseases lack an adequate and efficient treatment, mainly limited by the parasites, cells, or bacteria resistance to the clinically available drugs and also to the high toxicity characterized by multiple side effects [1,3–5]. As a result, new specific and innovative approaches are imperative. In this pharmaceutical scenario, peptide therapy has been developed as a possible solution and strategy for new biomedical advances [1,6].

Antimicrobial peptides (AMPs) are small molecules, in contrast with their high versatility and heterogeneity, in terms of both their physicochemical and biological properties, which has allowed the *in vitro* and *in vivo* evaluation on several human diseases [1,7,8]. The mode of action described for most cationic peptides involves the membrane permeabilization, which responds to an initial electrostatic interaction with the membrane component targets. From this recognition and key event, multiple intracellular processes responsible for antimicrobial and anticancer effects are stimulated [7,9–11]. In line with this, most cellular pathogenic agents have the peculiarity of having membranes predominantly formed by anionic phospholipids and different structural components, such as the variable content of sterols, in relation to mammalian or health cells [8,12–15]. In general, the membrane patterns in combination with the characteristic of the peptides, including

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hydrophobicity, amphiphilicity and hydrophobic moment, play complementary roles for selective properties and disruptive activity typically determined by AMP-membrane interactions [16,17].

Proteins that bind or metabolize phospholipids represent outstanding sources for the search and design of possible candidate compounds, for instance, the phospholipases A_2 (PLA_2s). These lipid-modifying toxins are highly expressed in the proteome of many snake species. In addition, many studies have evidenced the medical and scientific interest, as a consequence of its particular features and properties [10,18,19]. A diversity of PLA_2s has been reported in different venoms by proteomic and structural studies [18,20–24]. According to the ability to coordinate calcium and hydrolytic effect, they are mainly classified in two isoforms: Asp49 PLA_2s and Lys49 PLA_2s, which are catalytically active and inactive, respectively [12]. Despite their enzymatic differences, these proteins are able to remodel membrane elements specifically through their C-terminal region, which is typically recognized by its high density of cationic and hydrophobic amino acids [1]. This property turns these small fragments into interesting molecular models for obtaining possible new functional peptides with activity against different pathogens and tumor cells [9].

Most studies have explored the potential of PLA_2-based peptides from the Lys49 isoforms. For instance, the peptides pEM–2, p–Appk, and the fragment derived from BPB–BthTX–I presented anticancer potential [12,25,26], while the peptides pC–CoaTxII and pEM–2 exhibited antimicrobial activity against drug resistant microorganisms [27,28]. Furthermore, pEM–2 has shown multiple activities which implies that a single peptide could be the basis for the development of a wide-range drug that could bring several health benefits [12,27]. Conversely, Asp49 PLA_2s are still largely unexplored as sources of multifunctional peptides, representing an opportunity for the discovery of new chemical entities for future therapeutic modalities. Given this background and the pharmacological value of PLA_2s, we evaluated three peptides derived from the C-terminal of three catalytically active PLA_2s as inhibitory agents of Gram-positive and Gram-negative microorganisms, cancer cell lines, and species of *Leishmania*, which are clinically relevant agents of leishmaniasis in America.

2. Material and methods

2.1. Bioinformatics study: Physicochemical properties, structural analysis, and toxicity predictions

Three Asp49 PLA_2s were selected from the Uniprot database (https://www.uniprot.org/): entries Q6EER5 (PA2B_CERGO), P0C8M1 (PA2B1_BOTMO), and P86803 (PA2B1_BOTMA). So far, studies evaluating the effect of these Asp49 PLA_2s on cancer cells and various pathogens such as bacteria and parasites have not been conducted. AMPA web application (http://tcoffee.crg.cat/apps/ampa/do) was used to detect antimicrobial regions in these sequences, and BLAST to assess homologies. Each toxin region, with the highest *in silico* antimicrobial potential determined by AMPA, was also analyzed with ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) to evaluate the toxicity probability towards mammalian cells, HemopI (https://webs.iiitd.edu.in/raghava/hemopi/) to predict the hemolytic activity, and AntiCP (https://webs.iiitd.edu.in/raghava/anticp/submit_prot.php) to verify the possible activity against cancer cells. Based on this previous analysis, three regions of 10–20 amino acids, one from each PLA_2, were selected to carry out the experimental stage. The size of the peptide obeys exactly to the length of the fragment with the greatest potential identified by AMPA. Their sizes transform them into attractive molecules, especially due to their high cost-effective production, easy modification, tunable functionalization and clinical success of peptides of similar lengths [29]. Table 1 shows the principal characteristics of these potential antimicrobial fragments. The PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/) tool was applied to determine the charge, hydrophobicity, isoelectric point (pI), and peptide mass. Amidations at the C-terminal ends were considered for these determinations. Since, the main secondary structure predicted for AMPs is the alpha-helix [30], we used I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) and NetWheels (http://lbqp.unb.br/NetWheels/) to understand structural aspects of peptides.

2.2. Peptide synthesis, purification and characterization

2.2.1. Synthesis strategy

The automatic synthesizer CEM Liberty Blue^{TM1} was applied for automatic production of individual peptides using the Fmoc (9-fluorenylmethyloxycarbonyl) strategy. We used 0.192 g of Rink Amide Novabiochem resin (0.52 granulation) as polymeric support and N, N'-dimethylformamide as a solvent for the synthesis. Fmoc protector groups were removed with piperidine and, once the peptide completion was achieved, an acid cocktail including 95% trifluoroacetic acid (TFA), 2.5% triisopropyl silane (TIPS) and 2.5% water was used to remove the protecting groups from the side chains and to uncouple the synthetic product from the resin. Crude peptide was washed with cold ethyl ether and lyophilized at –80 °C at a pressure of 0.09 mT for 24 h.

2.2.2. Characterization and purification of synthetic peptides

Reversed-phase liquid chromatography (RP-HPLC) was performed with a four-pump chromatograph (Waters) coupled to a C18 analytical column (250 × 4.6 mm) to verify the purity of each peptide. An amount of 0.5 mg of crude peptide was dissolved in 1% acetic acid solution and 50 μL were injected into the system. Peptides were eluted using a linear gradient system from 95% mobile phase A (99.9% water, 0.1% formic acid) to 100% mobile phase B (99.9% acetonitrile, 0.1% formic acid) at a flow rate of 1 mL/min for 60 min. A UV–VIS detector monitored the fractionation at 220 nm. The molecular identity was confirmed by matrix-assisted laser ionization/desorption time-of-flight mass spectrometry (MALDI-TOF MS).

Crude peptides that did not present purity percentages ≥ 90% were purified by chromatography using a FLASH equipment (BUCHI) coupled to a C18 column (7 × 2 cm, 40 μm, 4 g). Then, 10 mg of peptide were

Table 1
Origin and structural characteristics of Asp49 PLA_2-derived peptides. Primary structure of selected peptides, precursor Asp49 PLA_2, and specimens are detailed.

	Peptides		
	pCergo	**pBmTxJ**	**pBmje**
Peptide sequence	NLRTYKKRYMFY-**NH_2**	YNKKYMKHLKPCKKA-**NH_2**	YNKKYRYHLKSCKKADK-**NH_2**
PLA_2s precursor specimen	*Bothrops godmani*	*Bothrops moojeni*	*Bothrops marajoensis*
Specimen characteristics	Venomous snake found in southern Mexico and Guatemala. Viperidae family.	Venomous snake endemic to South America (Argentina, Brazil, Paraguay). Viperidae family.	Venomous snake endemic to Brazil. Viperidae family.
Asp49 PLA_2 length	122	121	121
Peptide region within Asp49 PLA_2	99–110	103–117	103–119
Uniprot code	Q6EER5 (PA2B_CERGO)	P0C8M1 (PA2B1_BOTMO)	P86803 (PA2B1_BOTMA)
Reference	[31]	[20]	[22]

diluted in 1 mL of 1% acetic acid solution and filtered using a 0.45 μm membrane. The same mobile phases described above were used. The sample was injected with a flow rate of 2.5 mL/min for 30 min following the linear gradient starting from 95% mobile phase A to 100% mobile phase B. Purified peptides were lyophilized at –80 °C and 0.09 mbar for 5 days and reevaluated by RP-HPLC and MALDI-TOF MS following the same protocols.

2.3. Evaluation of effects on red blood cells

A 4% red blood cells suspension was prepared using 2 mL of human type O Rh + blood from a healthy volunteer. At the same time, different dilutions were prepared with pCergo, pBmTxJ, and pBmje, thus obtaining the 12.5, 25, 37.5, 50, 75, 100, and 125 μM concentrations. Subsequently, 200 μL of the red cells suspension were incubated with 200 μL of the diluted peptide in 1.5 mL tubes, and then incubated at 37 °C for 2 h. The tubes were then centrifuged and the supernatants were transferred to a 96-well plate. Finally, the plate was analyzed in a microplate reader (Glomax DS, Promega) at 550 nm. Triton X-100 (2% v/v) was used as a positive control and phosphate-buffered saline (PBS) as negative control, as described by Proaño [32].

2.4. Effects against cancer cells

2.4.1. Cell culture

The cell lines MCF–7 (breast adenocarcinoma ATCC®-CRL3435), Caco–2 (colon adenocarcinoma ATCC®-HTB37), and Hep G2 (hepatocyte carcinoma ATCC®-HB8065) were provided by the cell culture laboratory of the National Center for Aquaculture and Marine Research (CENAIM) and kept at 37 °C with 5% CO_2. MCF–7 cell line was cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 μM MEM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.01 mg/mL of bovine insulin. Meanwhile, Caco–2 cells were cultured in EMEM medium with 10% FBS, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Finally, Hep G2 cells were cultured in EMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate.

2.4.2. MTT assay

The cytotoxic effect of the three peptides was evaluated on the three cancer cell lines mentioned above by the MTT colorimetric assay ((3-(4,5-dimethylthiazol-2-yl) −2,5-diphenyltetrazolium bromide)). The cells were seeded ($1.0x10^4$ cells/well) in 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h. Subsequently, the medium was removed and the adherent cells were incubated with 90 μL of complete medium and 10 μL of pure peptide (50 μM and 250 μM). Cells were also cultured without peptide as a negative control. As a positive control, doxorubicin (50 μM) was used instead of the peptide. The plates were incubated at 37 °C and 5% CO_2 for 48 h. After the treatment with peptides, the cells were incubated with MTT (5 mg/mL in 9 mL of medium without phenol red) for 2 h, and then the formazan crystals were dissolved with 200 μL of dimethyl sulfoxide (DMSO). Finally, the plate was analyzed in a Varioskan LUX detector at 620 nm. The data obtained were treated with the GraphPad Prism 8 statistics package, taking into consideration 100% cell viability as the average of the six negative control repetitions. The results were expressed in function of the mean ± standard deviation (SD). The synthetic peptides that showed activity were evaluated at the concentrations of 75, 100, 200, 300, 400, and 500 μM, following the methodology previously described for the MTT assay [33]. Probit analysis was performed by calculating the data obtained at 620 nm.

2.5. Antibacterial effects: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) assays

Six different concentrations of each synthetic peptide were prepared by serial dilution with PBS (4.68, 9.4, 18.75, 35.5, 75 and 150 μM) for the assay against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). The microorganisms were cultured in Mueller-Hinton broth (MHB), following the protocol by Proaño [32], until obtaining $1x10^6$ CFU/mL. Subsequently, the microorganisms were transferred to different 96-well plates, which contained 2 μL of each peptide at different concentrations. For controls, 200 μL of MHB and 2 μL of PBS were used instead of synthetic peptides. The plates were incubated at 37 °C for 18 h. The absorbance was measured and recorded by establishing the optical density at 600 nm. Finally, 10 μL of the concentrations from each well that presented antimicrobial activity were added to Petri dishes with Mueller-Hinton agar (MHA). The plates were incubated at 37 °C for 24 h. The concentrations that did not show any microorganism growth were considered as MBC.

2.6. Anti-Leishmania effects

2.6.1. Leishmania spp.

L. amazonensis (MHOM/BR/1973/M2269) and *L. braziliensis* (MHOM/BR/75/2903) promastigotes were obtained from LEB-IL–UNICAMP (Laboratório de Estudos de Biologia da Infecção por *Leishmania*). Parasites were cultured on M199 medium, supplemented with 10% of FBS, 10 mM adenine, 5 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Only *L. braziliensis* cultures were supplemented with 2% human male sterile urine.

2.6.2. Balb/c mice

Female Balb/c mice (4–8 weeks old, weighing 20–25 g) were obtained from CEMIB–UNICAMP (Centro Multidisciplinar para Investigação Biológica). This investigation was approved by the Ethical Committee for Animal Experimentation from the Biology Institute − UNICAMP, logged under protocol number #4951-1/2018.

2.6.3. Evaluation of effects on promastigotes

The anti-promastigote activity was accessed according to the protocol presented by Mendes et al. [34]. $5x10^6$ early-logarithmic promastigotes/mL of both *Leishmania* strains were placed into a 96-well plate with Medium 199. The parasites were incubated with increasing peptide concentrations (0–200 μM) at 26 °C for 24 h. Control groups consisted of parasites with only medium. The effect of the peptide treatment on cell viability was determined by the metabolic activity using the MTT assay. Absorbance values, determined spectrophotometrically at 600 nm, were recorded to calculate the half maximal effective concentration (EC_{50}) using the GraphPad Prism 8 statistics package. All assays were performed in triplicate and the results were expressed in function of the mean ± SD.

2.6.4. Toxicity to macrophages and effect on intracellular amastigotes

To evaluate the toxicity to macrophages and determine the ability of peptides to reduce the number of amastigotes in this host cell, bone marrow-derived macrophages (BMDMs) were obtained from tibia and femur of individual Balb/c mice, based on the methodology of Miguel et al. [35]. For cytotoxicity assays, $5x10^5$ BMDMs/well were placed in a 96-well plate with RPMI medium and incubated at 37 °C and 5% CO_2 for 3 h. An additional incubation was carried out with increasing concentrations of peptides for 24 h under the same conditions. MTT assay was performed for the determination of the 50% cytotoxic concentration (CC_{50}). This parameter was calculated using the GraphPad Prism 8 statistics package. All assays were performed in triplicate and the results were expressed as mean ± SD.

The selectivity was estimated based on CC_{50}/EC_{50} (selectivity index). The peptides action on intra BMDMs infection was performed following the procedure used by Parra et al. [36]. Cells were infected in a 10:1 ratio (parasite: BMDM) and incubated at 34 °C and 5% CO_2 for 24 h. A wash step with PBS was performed to remove promastigotes that were not internalized and fresh RPMI medium was added. Subsequently, the

cells were incubated with increasing concentrations of peptides for 24 h under the same conditions. Infected cells were fixed in coverslips and stained with the Instant Prov kit (Newprov, Pinhais, Brazil). Amastigotes were calculated by counting 300 infected macrophages in triplicate, using the Leica LAS Core Microscope System. Infection index was calculated by multiplying the number of infected cells by the number of amastigotes per 100 BMDMs. Negative control groups consisted in infected cells not incubated with peptides.

2.7. Statistical analysis

The results were analyzed by one-way analysis of variance (ANOVA) and Tukey's test, using the GraphPad Prism 8 statistics package. A significance of $p < 0.05$ was considered as significant.

3. Results

3.1. Bioinformatic analysis

The selected PLA_2 toxins presented molecular regions with a high probability of inducing antimicrobial effect. According to the AMPA analysis, the fragments with the highest probability of antimicrobial activity were located in the area close to the C-terminus of each PLA_2 toxin (Fig. 1, Supplementary material Fig. 1**S**). Our Asp49 PLA_2 derived-peptides presented a high identity, in terms of their primary structure, in comparison with other short peptides also derived from these membranolytic enzymes, which contain experimentally validated antibacterial and leishmanicidal action (Supplementary material, Table 1**S**). This high sequence similarity suggested that the bioinformatically identified peptides could possibly act as antimicrobial or cytotoxic agents against cancer cells.

The sequences with the best scores determined by AMPA analysis, pCergo, pBmTxJ and pBmje, have a high density of hydrophobic and cationic amino acids, which is reflected in the high values of hydrophobicity, charge, and pI (Table 2). The bioinformatic tools predicted the possible activities of these peptides. This *in silico* analysis indicated a low probability of the selected peptides to induce hemolysis and toxicity, and high cytotoxic potential against cancer cells. Based on this prediction, these three Asp49 PLA_2s fragments were chosen for synthesis, *in vitro* assays and structural characterization. NetWheels (Supplementary material Fig. 2**S**) and I-TASSER analysis (Supplementary material Fig. 3**S**) suggested that pCergo, pBmTxJ and pBmje display an alpha-helical structure with an "imperfect" amphipathicity.

Table 2
Physicochemical characteristics of Asp49 PLA_2-derived peptides. pI, charge and hydrophobicity of the selected peptides were determined using PepDraw.

Peptides	Length (aa)	pI	Charge	Hydrophobicity (Kcal*mol-1)	Mass (Da)
pCergo	12	10.45	+5	+12.46	1680.8896
pBmTxJ	15	10.45	+7	+25.16	1878.0453
pBmje	17	10.30	+7	+30.89	2171.1751

3.2. Synthesis and characterization of peptides

After the chemical synthesis process 149.1 mg of pCergo, 169 mg of pBmTxJ, and 190.5 mg of pBmje were obtained. The crude peptides presented purity percentages of 68.76% for pCergo, 77.25% for pBmTxJ, and 67.77% for pBmje. The purification of the three peptides by FLASH chromatography (Supplementary material Fig. 4**S**) allowed a purity percentage of 90.05% for pCergo, 99.7% for pBmTxJ, and 97.17% for pBmje, which was determined by RP-HPLC (Fig. 2). These results coincided with the mass spectra of the three peptides (Fig. 2), where a signal in *m/z* corresponding to pCergo, pBmTxJ, and pBmje was observed, confirming the identities and monomeric nature of these molecules (Supplementary material Fig. 5**S**).

3.3. Biological assays

3.3.1. Asp49 PLA_2-derived peptides' hemolytic activity

The hemolytic activity of pCergo, pBmTxJ, and pBmje was screened *in vitro* and *in silico* (Fig. 3). None of the three peptides presented hemolytic activity greater than 3% in any of the concentrations evaluated, coinciding with the bioinformatic analyzes determined by the HemoPI tool.

3.3.2. Activity of peptides against cancer cells

Three adherent cell lines were used to evaluate the cell-killing activities of pCergo, pBmTxJ, and pBmje. Among the peptides evaluated, pCergo showed the highest cytotoxic effect against MCF–7 cells (Fig. 4**A**), with an EC_{50} in micromolar range (464.85 μM). On the other hand, none of the three peptides showed a cytoxicity greater than 50% at 250 μM towards Caco–2 and Hep G2 cells (Fig. 4**B and 4C**).

3.3.3. Inhibition of microbial growth

Activity against *E. coli* and *S. aureus* was not observed for pBmje,

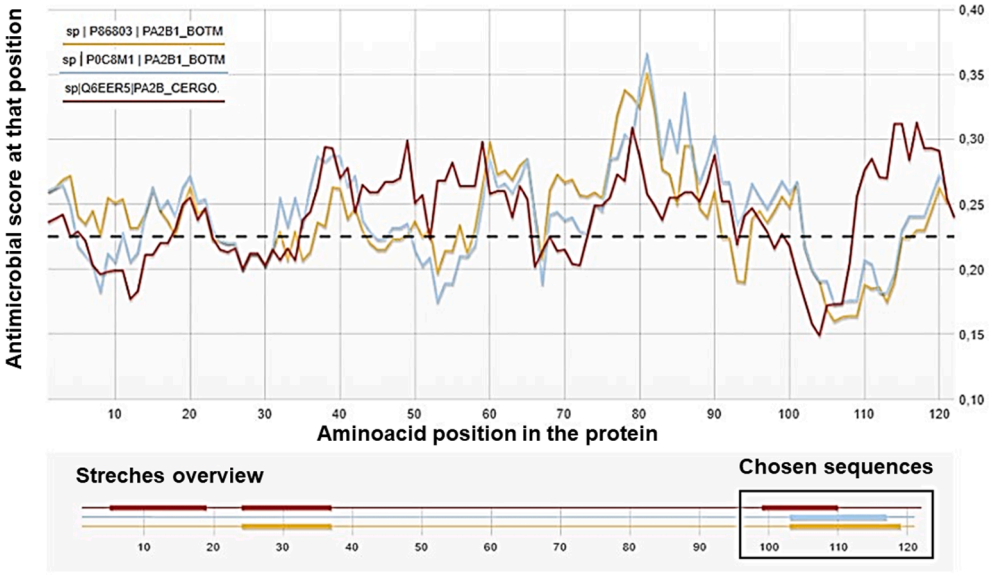

Fig. 1. Antimicrobial regions from precursor Asp49 PLA_2s identified by AMPA web application. The primary structures analysis of the membrane damaging enzymes: Cergo (red), BmTxJ (blue) and Bmje (yellow), showed antimicrobial stretches. These toxins presented 3, 1, and 2 antimicrobial stretches, respectively. Among all the possible bioactive regions identified, those that presented the best scores according to the characteristics of AMPA were evaluated *in silico* for toxicity to host cells, hemolysis and activity against cancer cells, and selected for the synthesis. In the three proteins analyzed, the fragment with the best index was located on the C-terminal sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

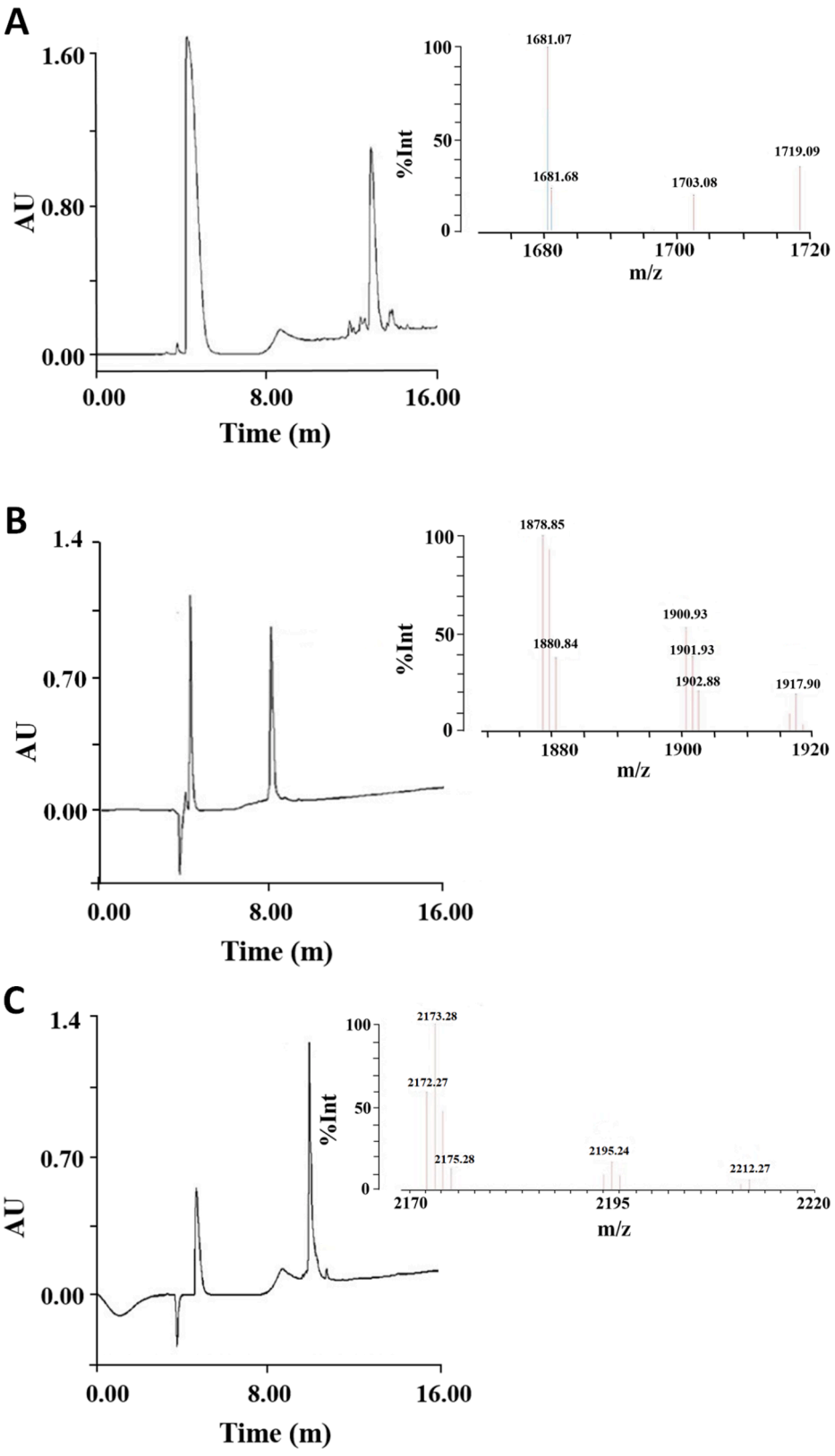

Fig. 2. RP-HPLC profile and mass spectra of synthetic peptides after the purification process with FLASH chromatography. (**A**) pCergo has a retention time of 13.5 min, 90.05% purity; and $m/z = 1681.07$ Da. (**B**) pBmTxJ eluted at a retention time of 7.97 min with a purity of 99.7% and $m/z = 1878.85$ Da. (**C**) pBmje eluted in a retention time of 10.02 min showing a purity of 97.17% and $m/z = 2173.28$ Da.

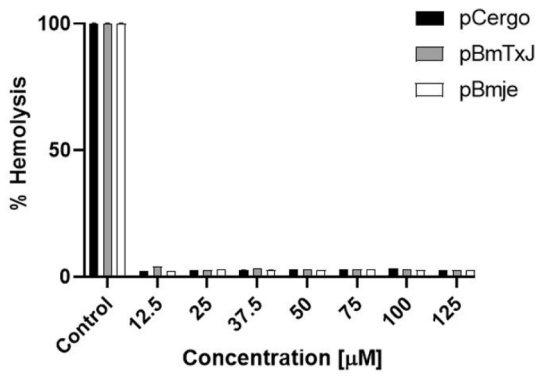

Fig. 3. Hemolytic activity of pCergo, pBmTxJ and pBmje. None of the peptides exceeded 3% cellular disruption. Positive control (Triton X-100) was considered as 100% of hemolysis. Therefore, the three peptides are not hemolytic at the evaluated concentrations.

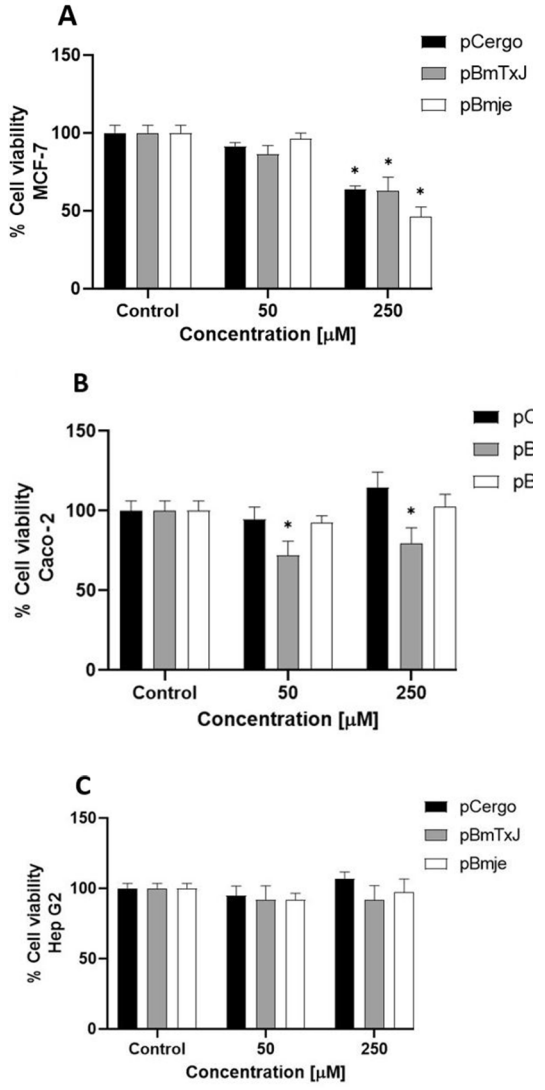

Fig. 4. Effect of Asp49 PLA_2-derived peptides on three different cancer cells. (A) MCF–7, **(B)** Caco–2, and **(C)** Hep G2 cell cultures were incubated with 50 μM and 250 μM of the peptides. (*) There is a significant difference compared to the negative control determined by ANOVA analysis ($p < 0.05$).

even though it was the peptide with the most significant cytotoxic action on cancer cells. In contrast, pCergo showed an MIC of 75 μM and MBC of 150 μM against *E. coli*. Finally, pBmTxJ showed an antimicrobial activity against *S. aureus* with an MIC of 37.5 μM (Table 3).

3.3.4. Biological assays on Leishmania spp.

The inhibitory potential of synthetic Asp49 PLA_2-derived peptides on promastigotes was evaluated. Incubation of parasites with increasing concentrations of peptides resulted in decreased cell viability (Fig. 5). pCergo was the most active for both species, with an $EC_{50} = 93.69$ μM for *L. braziliensis* and 110.40 μM for *L. amazonensis*, whereas the other peptides showed higher values (pBmTxJ: 264.24 μM for *L. braziliensis* and 142.88 μM for *L. amazonensis*; pBmje: 125.31 μM for *L. braziliensis* and 184.50 μM for *L. amazonensis*). Viability reduction of promastigotes was observed by microscopy. In photomicrographs, changes in the morphology and viability of *L. braziliensis* at a concentration of 100 μM of pCergo were shown, in comparison with the control group (Fig. 5**C**). In addition, we observed alterations in the shape of promastigotes, which were characterized by enveloped flagellum, reduction of cell volume, and rounded body.

Given the location of amastigotes in the macrophages of the infected host, it is imperative to access the cytotoxicity towards BMDMs of new potential therapeutic candidates against leishmaniasis. Table 4 shows the CC_{50} values obtained after incubation of BMDMs with increasing concentrations of the peptides. These values are higher than the EC_{50} values. Table 4 also lists the selectivity index, which is a ratio of the CC_{50} to the EC_{50} values. The index showed that Asp49 PLA_2-derived peptides are selective to the promastigotes of both species, with pCergo being the most selective peptide mainly against *L. braziliensis*.

The three synthetic peptides showed a significant dose-dependent decrease in intracellular amastigote survival (Fig. 6). Once again, pCergo stands out in *Leishmania* infection in murine macrophages. Photomicrographs (Fig. 6**C**) showed that pCergo had the highest activity in comparison to the control group, generating a significant reduction in the number of amastigotes per macrophage and thus, in the infective progression.

4. Discussion

pCergo, pBmTxJ, and pBmje constitute the C-terminal fragments from each selected Asp49 PLA_2 sequences. These cationic-hydrophobic regions of PLA_2s have been defined as the protagonist agents and possible antimicrobial compounds, especially due to the presence of several lysine residues and the capacity to mimic the pharmacological action of the parent proteins [10,11,32,37–39]. Some studies have confirmed the antimicrobial effects of the peptide specifically derived from the end of Lys49 PLA_2s [12,27,39] .

Although much less studied, Asp49 isoforms are effective on different cell targets. Assays using pepMTX–I showed a significant multifunctional inhibition of leukemia cells, bacteria, and *Leishmania* [24]. The low interest in Asp49 PLA_2s, far from being a disadvantage, represents an important opportunity due to its similar properties and composition with the Lys49 C-terminal isoforms. For example, pCergo, pBmTxJ, and pBmje have a high lysine amount in their sequences,

Table 3
Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of synthetic peptides derived from Asp49 PLA_2s. Synthetic peptides were evaluated against Gram-positive and Gram-negative bacteria. (-) Not determined.

Peptides	MIC (μM)		MBC (μM)
	E. coli	*S. aureus*	*E. coli*
pCergo	75	–	150
pBmTxJ	–	37.5	–
pBmje	–	–	–

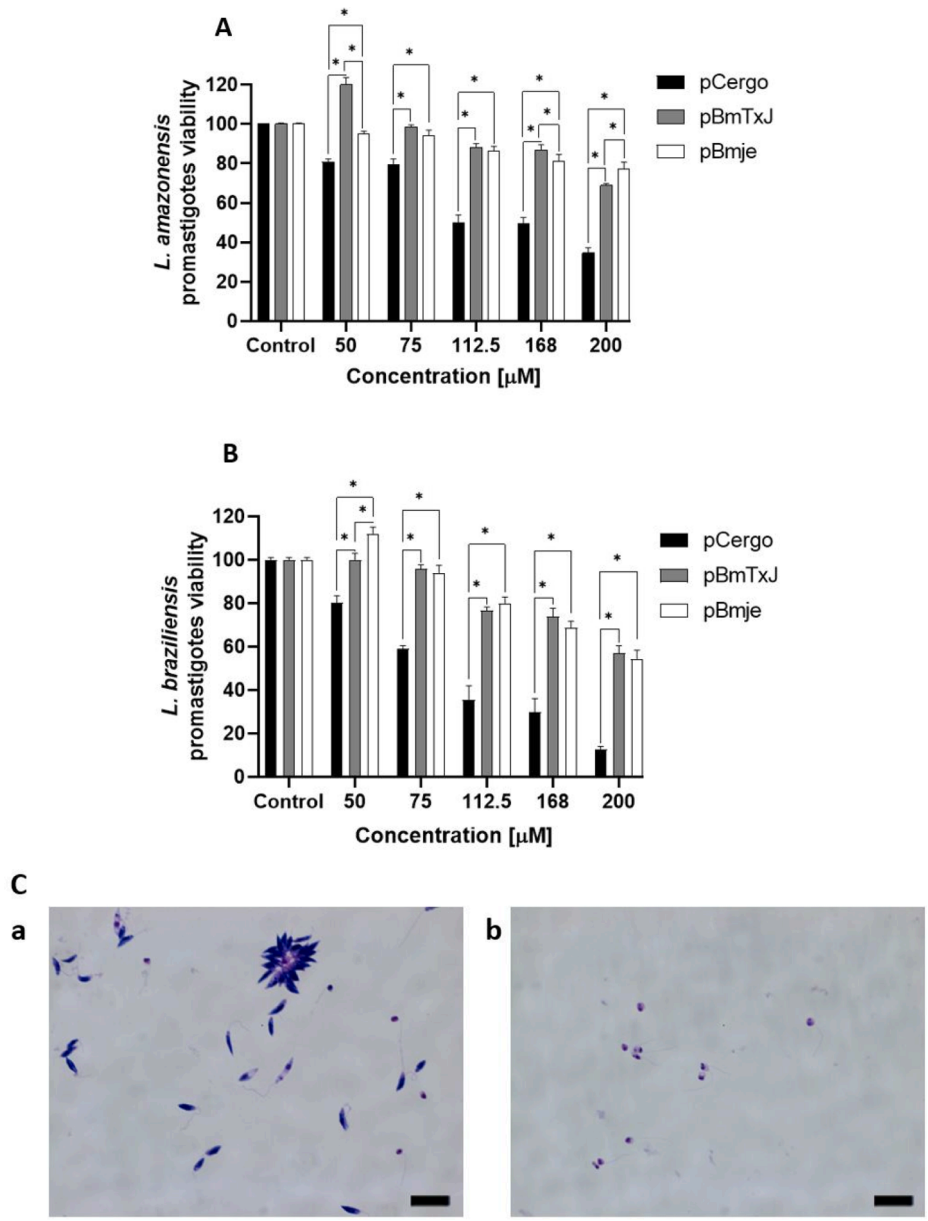

Fig. 5. Antiprotozoal effects of Asp49 PLA_2-derived peptides on *Leishmania* promastigotes. Flagellated extracellular promastigotes of (**A**) *L. amazonensis* and (**B**) *L. braziliensis* were incubated with increasing concentrations of peptides (0–200 μM) for 24 h. The parasite viability was assessed by MTT assay. Control groups (untreated) were considered as 100% cell viability. The results are presented as the mean viability ± SD. Asterisks indicate significant differences ($p < 0.05$). (**C**) Photomicrographs of *L. braziliensis* promastigotes were obtained using the Leica LAS Core microscope system; (**a**) the control group and (**b**) the treatment with the most active peptide (pCergo 100 μM) are shown. Bar = 4 μm. Morphological alterations such as rounded shapes, enveloped flagellum, and reduction in cell volume were observed.

Table 4
Cytotoxicity in murine macrophages and selectivity index of Asp49 PLA_2-derived peptides. The index was defined by the ratio between the CC_{50} and EC_{50} values, which were obtained in experiments against BMDMs and extracellular forms of *Leishmania* spp., respectively.

Peptides	CC_{50} BMDMs (μM)	Selectivity index (CC_{50}/EC_{50})	
		L. amazonensis	*L. braziliensis*
pCergo	448	4.05	4.78
pBmTxJ	492	3.44	1.86
pBmje	550.83	2.98	4.39

similar to other peptides from Lys49 toxins such as pEM–2 [40], p–Acl [25], p–AppK [25], pC–CoaTxII [28], pMTX–II [24], and p–BthTX–1 [41]. Hydrophobicity and cationicity are intrinsic properties of the C-terminal end of PLA_2s [24] and of a high percentage of antimicrobial and anticancer peptides, such as pMTX-I (pI = 10.55; net charge = +5; hydrophobicity = +15.35 Kcal*mol-1), BP100 (pI = 10.79; net charge = +5; hydrophobicity = +14.61 Kcal*mol-1) and PepC (pI = 9.90; net charge = +5; hydrophobicity = +18.49 Kcal*mol-1) [10,42].

Hemolytic activity has been portrayed as one of the critical challenges in the clinical application of peptides [7]. In fact, some peptides tend to present both antimicrobial and hemolytic activities [1,7], which substantially reduce their translational potential. For instance, the peptides melittin (HC_{50} = 1.7 μM), magainin–H1 (HC_{50} = 2.9 μM), and Pis–1 (HC_{50} = 11 μM) [41] are highly toxic to red blood cells. However, pCergo, pBmTxJ, and pBmje did not show hemolytic activity, highlighting the successful use of computational-aided design of non-hemolytic AMPs derived from PLA_2s.

Cancer cells have typically been characterized by the overexpression of anionic molecules in their membranes [43,44], making it possible to develop strategies based on cationic peptides [25]. In line with this, pBmje showed cytotoxic activity against MCF–7 cells at a higher concentration compared to other anticancer peptides [45]. FBS used in MTT assay may affect the stability and activity of pBmje, given the presence of proteins, salts and proteolytic enzymes. To illustrate this, Wen-Hung Tang et al. [46] showed a loss of antimicrobial activity of a peptide, when it bound to the albumin-α1-antitrypsin complex. Collectively, the selectivity and null hemolytic activity presented by pBmje indicated an

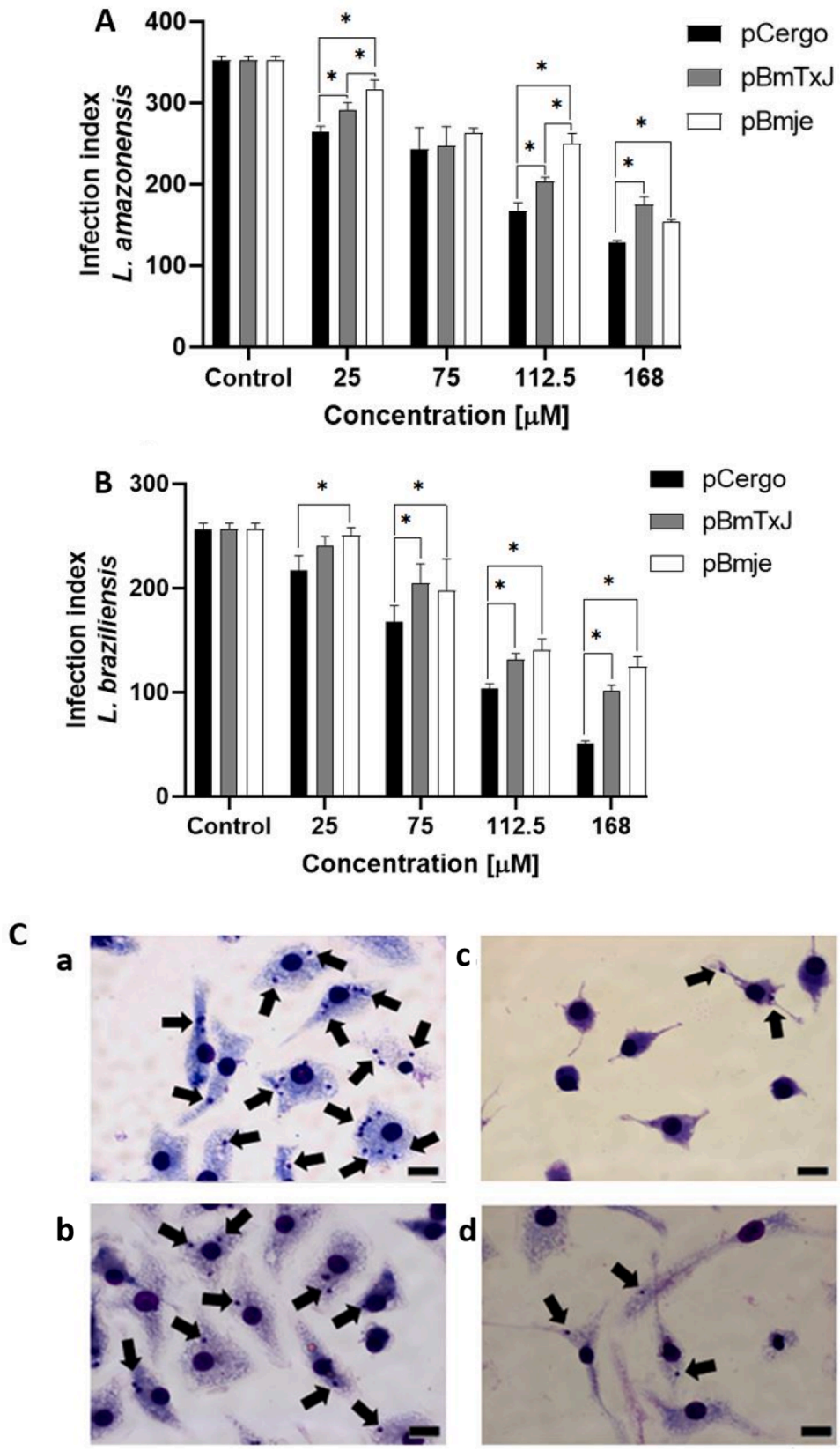

Fig. 6. Effects of Asp49 PLA_2-derived peptides on infected macrophages by *Leishmania* parasites. BMDMs and promastigotes of (**A**) *L. amazonensis* and (**B**) *L. braziliensis* were incubated in a 10:1 ratio with the peptides (0–168 μM) for 24 h. BMDMs and amastigotes were fixed and stained for the determination of infection index using microscopy analysis. The results are presented as mean ± SD. Asterisks represent significant differences ($p < 0.05$). (**C**) Representative photomicrographs obtained using the Leica LAS Core microscope system show untreated BMDMs infected with amastigotes (**a**) *L. amazonensis* and (**b**) *L. braziliensis*. pCergo (168 μM) reduced intramacrophage survival of amastigotes (**c**) *L. amazonensis* and (**d**) *L. braziliensis*. Black arrows highlight intracellular amastigotes. Bar = 10 μm.

important starting point for further anticancer drug research. In addition, pBmje could be combined with a known antineoplastic drug [47] or conjugated with nanoparticles [48]. On the other hand, pBmTxJ and pCergo did not show high toxicity to cancer cell lines. Although the three peptides are similar, specially pBmje and pBmTxJ, their subtle differences have a relevant impact on their functions, which are also dependent on the heterogeneity of the chemical, structural, and biophysical patterns of the target cancer cells [44,49].

Despite being the peptide that showed the highest cytotoxicity in cancer cells, pBmje did not display any activity against *E. coli* and *S. aureus*. In contrast, pCergo was active against *E. coli*, while pBmTxJ showed antimicrobial activity against *S. aureus*. These data introduce questions about the role of amino acids and how their position and chemical nature affect the antimicrobial potency [13]. For example, pBmje contains an aspartate (D) residue that consequently could affect its cationic character. Furthermore, the three peptides have asparagine (N) which, similarly to aspartate, could influence their capacity to establish electrostatic interactions with negative membranes.

Leishmaniasis is the second most serious neglected parasitic disease, causing around 500 k deaths annually and affecting people in the most disadvantaged social classes [50]. The species used in this work, *L. amazonensis* and *L. braziliensis*, are the causative agents of cutaneous leishmaniasis in America, which may progress into diffuse or mucocutaneous leishmaniasis, respectively [51]. Antileishmanial drugs have been widely questioned due to their side effects and the recent evidence of parasite drug resistance [52].

In this study, the three peptides showed activity against promastigotes, with pCergo being the most effective on both species. The EC_{50} values obtained are in accordance with the range of concentrations for some leishmanicidal peptides derived from different natural sources and tested on the same species [53–56]. However, pCergo, pBmTxJ, and pBmje were less effective in contrast with other peptides derived from Lys49 PLA_2s [24,34]. This difference probably occurs because Lys49 toxins require effective protein regions to act on the membrane phospholipids, whereas Asp49 PLA_2s do not strictly depend on extreme C-terminus [10] by presenting a catalytic mechanism to disrupt cell membranes. Nevertheless, the interaction between the Asp49 enzymes and the membrane remains a key step, which validates the screening for short peptides inspired by these catalytic isoforms.

Although cationic charge is described as a determining factor for an initial membrane binding, it is not always related to the antimicrobial effects. For instance, Mangoni et al. [53] showed that in temporins, the positive charge does not play a primary role in the inhibition reported. Similarly, pCergo's charge (+5) seems to be an advantage over pBmje and pBmTxJ (+7) which, in complementarity with the alpha-helix secondary structure and hydrophobicity, could improve the effects on the promastigotes' membranes. Selectivity indices of Asp49 PLA_2-derived peptides indicate a molecular affinity for promastigotes. This selectivity could be related to differences in lipid profiles between parasitic and mammalian membranes [15,57].

Most investigations use promastigote forms as a model to evaluate the leishmanicidal activity [58]. The reduction of amastigotes within the macrophages is an experimental parameter that has not been performed by a large number of studies addressing the potential of peptides, which highlights the relevance of our findings [34,59–61]. The control of infective progression observed must occur by mechanisms similar to those generated by other cationic peptides [60,62,63], which are basically based on the host cell penetration and lysis of intracellular amastigotes, or modulation of intramacrophagic responses [64]. However, both hypotheses require future experimental studies in order to clarify the exact mode of action responsible for reducing the number of amastigotes within macrophages.

5. Conclusions

The chemical nature and biological interactions of precursor PLA_2s make them sources of peptides with different actions for the treatment of public health problems. This work is a small step in the discovery of the multiple potential of PLA_2 enzyme templates and peptides derived from them, as more in-depth investigations are needed to elucidate mechanisms of action of these molecules. New studies can improve the biological properties, reducing the EC_{50} values through the amino acid substitutions in parental AMPs sequences, modifications or combination with conventional drugs.

Declaration of Competing Interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.105041.

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