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ORIGINAL PAPER

Microgramma vacciniifolia Frond Lectin: *In Vitro* Anti-leishmanial Activity and Immunomodulatory Effects Against Internalized Amastigote Forms of *Leishmania amazonensis*

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Abstract

Purpose The treatment of leishmaniasis, an anthropozoonosis caused by *Leishmania* protozoa, is limited by factors, such as adverse effects, toxicity, and excessive cost, which has highlighted the importance of novel drugs. In this context, natural products have been considered as sources of antileishmanial agents. This study investigated the leishmanicidal activity of *Microgramma vacciniifolia* frond lectin (MvFL) on promastigotes and amastigotes of *Leishmania amazonensis*.

Methods The effects of MvFL on promastigote proliferation and macrophage infection by amastigotes were evaluated and mean inhibitory concentrations (IC_{50}) were calculated. As a safety assessment, the hemolytic capacity of MvFL (6.25–200 µg/mL) against mouse and human erythrocytes was determined. Additionally, the ability of MvFL (6.25–100 µg/mL) to modulate lysosomal and phagocytic activities and the nitric oxide (NO) production by murine peritoneal macrophages was also investigated.

Results After 24 h, MvFL inhibited the proliferation of *L. amazonensis* promastigotes, with an IC_{50} of 88 µg/mL; however, hemolytic activity was not observed. MvFL also reduced macrophage infection by amastigotes with an IC_{50} of 52 µg/mL. Furthermore, treatment with MvFL reduced the number of amastigotes internalized by infected murine peritoneal macrophages by up to 68.9% within 48 h. At a concentration of 25 µg/mL, MvFL stimulated lysosomal activity of macrophages within 72 h, but did not alter phagocytic activity or induce NO production at any of the tested concentrations.

Conclusion MvFL exerts antileishmanial activity and further studies are needed to assess its therapeutic potential in *in vivo* experimental models of leishmaniasis.

Keywords Antileishmanial · Immunomodulation · Lectin · *Microgramma vacciniifolia*

Introduction

Leishmaniasis is a cosmopolitan public health concern affecting more than 98 countries, with an estimated 900,000 to 1,600,000 new cases occurring annually, mainly in Brazil, Ethiopia, Somalia, South Sudan, and Sudan [1, 2]. Leishmaniasis is a complex of infectious parasitic diseases caused by protozoa of the genus *Leishmania* (family Trypanosomatidae), which are transmitted through bites of female phlebotomine vectors, and are associated with lesions in different organs [3].

According to the clinical manifestations of the disease, Leishmaniasis is divided into: cutaneous leishmaniasis, with 95% of the cases occurring in the Americas, Mediterranean Basin, Middle East, and Central Asia; mucocutaneous

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leishmaniasis, with 90% of the cases occurring in Bolivia, Brazil, and Peru; and visceral leishmaniasis, with more than 90% of the cases occurring in seven countries, including Brazil [4, 5]. *Leishmania amazonensis* is one of the pathogenic species distributed throughout South America, with a high prevalence in Brazil, and is associated with different clinical forms of leishmaniasis. It is also the main causative agent of diffuse cutaneous leishmaniasis in America, a rare clinical form developed by individuals infected with this parasite and that is commonly refractory to currently available treatments [6, 7]. To survive inside host cells and spread into host organs, the parasites trigger mechanisms of immune silencing and evasion to escape oxidative stress induced by hydrolases, lysosomal attack inside macrophage vacuoles, and intracellular calcium, which facilitates alteration of the cytoskeleton [8, 9].

The first prescription option for leishmaniasis is the pentavalent antimonial drugs; N-methyl-glucamine antimoniate is the first-choice drug in Brazil against cutaneous, mucocutaneous, and visceral leishmaniasis whilst amphotericin B (Amp-B) and pentamidine are the alternative options [10, 11]. However, the choice of drug depends on the *Leishmania* species and factors intrinsic to the patient (comorbidities, pregnancy and aging). Nonetheless, these drugs are potentially toxic, with several kinds of side effects, and low effective, which leads many patients to choose not to undergo treatment [12].

Thus, there is a need to develop novel antileishmanial compounds with pharmacological potential and reduced toxicity using alternative methods that are reliable, have lower cost, and are easily accessible. In this context, plant species have been considered as promising sources of substances with antileishmanial activity as plants in general contain diverse bioactive compounds that made them promising sources for development of novel drugs [13, 14]. Lectins, a heterogeneous group of proteins that can recognize and interact with carbohydrates present on the surface of different cell types, are among these bioactive compounds [15]. These biomolecules possess several biological properties, including antimicrobial [16], anti-inflammatory [17], antitumor [18], immunomodulatory [19], and leishmanicidal [20, 21] properties. The ability of lectins to interact with target glycans on cells plays a significant role in the immunological defense against pathogens [22].

Microgramma vacciniifolia (Langsd. & Fisch) Copel. (family Polypodiaceae) is an epiphyte pteridophyte that has ornamental uses, is used as a medicinal plant for intestinal and respiratory infections, and hemorrhages, and promotes expectoration [23, 24]. Two lectins have been identified and isolated from *M. vacciniifolia*: one from the rhizome (MvRL) [25] and the other from the frond (MvFL) [26]. MvFL is a multifunctional protein that exhibits lectin activity and has been reported to exert immunomodulatory effects on human lymphocytes, which may be linked to its antitumor properties [18, 26].

There are no reports on the antileishmanial effects of the lectins derived from *M. vacciniifolia*. Considering the increasing need for alternatives sources of bioactive compounds that can facilitate the development of new drugs, this study aimed to investigate the antileishmanial and immunomodulatory activities of MvFL against promastigotes and internalized amastigote forms of *L. amazonensis in vitro*. Furthermore, the hemolytic activity of MvFL against mouse and human erythrocytes was evaluated.

Materials and Methods

Plant Material and Isolation of MvFL

Fronds of *M. vacciniifolia* were collected at the campus of the *Universidade Federal de Pernambuco* (UFPE) (Recife, Brazil) with authorization (number 36,301) from *the Instituto Chico Mendes de Conservação da Biodiversidade*. Access was recorded (A9D147B) at the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado*. Taxonomic identification was done at the herbarium Dárdano de Andrade Lima, at the *Instituto Agronômico de Pernambuco* (Recife, Brazil), where a voucher specimen (number 63,291) was deposited.

MvFL was isolated as described by Patriota *et al*. [26]. Briefly, fronds of *M. vacciniifolia* were washed with tap and distilled water and then dried at 28 °C for 7 days before being powdered using a blender. The powder was extracted with 0.15 M NaCl (10%, w/v) for 16 h at 25 °C with constant agitation using a magnetic stirrer. The mixture was filtered and centrifuged at 9000 × *g* for 15 min at 4 °C, and the supernatant was collected and used as the frond extract in experiments. Frond extract (containing 3.0 mg protein) was loaded onto a Sephadex G-75 (GE Healthcare Life Sciences, Marlborough, MA, USA) column (30.0 cm × 1.5 cm) equilibrated with distilled water. The fractions corresponding to the first absorbance peak at 280 nm were collected, lyophilized, resuspended in 0.1 M Tris–HCl (pH 8.0), and loaded (2.5 mg protein) onto a DEAE-Sephadex (GE Healthcare Life Sciences) ion exchange column (3.0 cm × 2.0 cm) previously equilibrated with the same buffer. The column was washed with Tris buffer and the adsorbed proteins (MvFL) were eluted with 0.1 M Tris–HCl (pH 8.0) containing 1.0 M NaCl, dialyzed with distilled water for 4 h, and lyophilized.

Protein Concentration and Hemagglutination Activity

The concentration of proteins was estimated according to the method described by Lowry *et al*. [27] using bovine serum

albumin (31.25–500 µg/mL) for the standard curve. The sample (0.2 mL) was incubated for 10 min at 25 °C with 1 mL alkaline copper solution (1 mL of 0.5% [w/v] copper sulfate in 1% [w/v] sodium citrate added to 50 mL of 2% [w/v] sodium carbonate solution in 0.1% [w/v] sodium hydroxide). After incubation, 0.1 mL of–Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:1 in water was added, and after 30 min incubation at 25 °C, the absorbance was measured at 720 nm.

The carbohydrate-binding ability of MvFL was assessed using a hemagglutination activity (HA) assay according to the protocol described by Patriota *et al*. [26]. Briefly, 50 µL of the sample was serially diluted twice in 0.15 M NaCl and mixed with 50 µL of 2.5% v/v suspension of rabbit erythrocytes fixed with glutaraldehyde [28] in each well of V-bottomed 96-well microplates. The plates were then incubated at 27 °C for 45 min. Erythrocyte suspension incubated in the absence of a sample was used as the negative control. HA was expressed as the reciprocal (titer^{-1}) of the highest dilution of the sample that was able to promote agglutination. Specific HA was calculated as the ratio of HA to protein concentration (mg/mL). HA inhibitory assay was also performed in the presence of 0.5 mg/mL fetuin as described by Patriota *et al*. [26]. The Ethics Committee on Animal Use of UFPE approved the method used to collect rabbit erythrocytes (process 23076.033782/2015-70).

Anti-leishmanial Assay in Promastigote Forms of *L. amazonensis*

For conducting *in vitro* assays to assess antileishmanial activity, promastigote forms of *L. amazonensis* (IFLA/BR/67/PH8) maintained at the *Laboratório de Atividade Antileishmania*, *Núcleo de Pesquisas em Plantas Medicinais, Universidade Federal do Piauí* were used. Promastigote forms *of L. amazonensis* were cultured in Schneider media (Sigma-Aldrich) supplemented with 10% bovine fetal serum (BFS) (Sigma-Aldrich) and 10,000 IU/10 mg/mL penicillin–streptomycin (Sigma-Aldrich) at 26 °C in a greenhouse of biological oxygen demand (BOD) [29, 30]. Promastigotes in the logarithmic growth phase (1×10^6 leishmania/100 µL medium) were seeded in 96-well culture plates containing supplemented Schneider's—medium, and then serial dilutions of MvFL (6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/mL) were added to the wells and the plates were incubated at 26 °C for 42 h in a BOD incubator.

Next, 20 µL of 1 mM resazurin (Sigma-Aldrich) was added to each well, and the plate was incubated for a further 6 h in the BOD incubator. Therefore, the total incubation time was 48 h. The absorbance was read at 550 nm using a BioTek ELx800 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Schneider medium with 0.2% DMSO was used as the negative control and its absorbance value was considered as representing 100% viability of the parasites. Amp-B at a concentration of 2 µg/mL was used as the positive control to validate the experiment. A blank read for each concentration and control was performed to subtract the absorbance resulting from the interaction of the medium with the tested plant materials. All experiments were performed in triplicate.

Hemolysis Assay

The hemolytic activity of MvFL was evaluated using mouse and human erythrocytes, according to the protocol described by Pita *et al*. [31], with some modifications. Swiss females (25–30 g, 6–8 weeks of age) were obtained from the bioterium of the *Laboratório de Imunopatologia Keiso Asami* (LIKA-UFPE). The animals were maintained under a 12 h light/dark cycle at a temperature of 23 ± 2 °C), with free access to water and food (Presence Nutrição Animal, São Paulo, Brazil). The mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (150 mg/kg) and xylazine (15 mg/kg) and subjected to brachial blood collection, which was immediately mixed with the anticoagulant ethylenediamine tetraacetic acid (EDTA). Similarly, approximately 4 mL of peripheral venous blood was collected in vials containing EDTA from voluntary donors with blood A^+, B^+, AB^+, and O^+ types who signed the free and informed consent form. All experiments performed in this study were approved by the Animal Research Ethics Committee of UFPE (permission No. 0037/2020) and by the Human Research Ethics Committee of UFPE (process 33550320.1.0000.5208).

To isolate erythrocytes, 2 mL of whole blood was diluted with 8 mL of phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 300 g for 5 min. The supernatant and buffy coat were then removed by gentle aspiration, and the above process was repeated twice. Erythrocytes were then resuspended in PBS to make a 1% suspension (v/v) for use in hemolysis assay. Various concentrations of MvFL (6.25, 12.5, 25, 50, 100, and 200 µg/mL) dissolved in PBS was added to 1 mL of the erythrocytes suspension. The MvFL-erythrocyte mixtures were incubated in a mixer for 60 min at 25 °C and then centrifuged at 3000 rpm for 5 min. The absorbance of the supernatants was determined at 540 nm using a microplate reader to measure the extent of red blood cell lysis and determine the concentration that produces 50% hemolysis (HC_{50}). Positive (100% hemolysis) and negative control (0% hemolysis) were also used and were determined by incubating erythrocytes with 1% Triton X-100 in PBS and PBS alone, respectively. All experiments were performed in triplicate.

Antileishmanial Activity of MvFL Against Intramacrophage Amastigotes

Male and female BALB/c mice also obtained from LIKA-UFPE, aged between 4 and 5 weeks, weighing 25–30 g, were used for the present experiment. Mice were intraperitoneally injected with 1.5 mL of 3% thioglycolate medium, and five days later, the mice were sacrificed, and peritoneal macrophages were collected. Animals were treated according to the Guiding Principles (NIH publication #85-23, revised in 1985) for care and use. All experiments performed in this study were approved by the Animal Research Ethics Committee of UFPE (permission No. 0037/2020).

Murine macrophages were cultured in 24-well plates containing sterile 13 mm diameter round coverslips in each well, at a concentration of 1×10^6 cells/mL in RPMI medium (Sigma-Aldrich) supplemented with 10% heat inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The culture plates were incubated at 37 °C in a 5% CO_2 incubator for 3 h to facilitate cell adhesion. Adhered macrophages were then incubated with fresh medium containing 2×10^6 promastigote forms in stationary growth phase at a ratio of 10 promastigotes to 1 macrophage and incubated at 37 °C in a 5% CO_2 incubator for 4 h. The supernatant was subsequently aspirated to remove non-internalized parasites, and the wells were washed with 0.1 M PBS.

Then, the infected macrophages were incubated with MvFL at 22.5, 45 and 90 µg/mL (concentrations non-toxic to cells), or 0.2 µg/mL Amp-B for 48 h. Subsequently, the coverslips were removed and stained using Panoptic fast staining technique (Laborclin, Curitiba, Brazil). The number of infected macrophages (%) and survival index (n) (number of amastigotes recovered per macrophage) were determined by counting the number of parasites in 100 macrophages in a Neubauer chamber. These values were used to calculate the half-maximal inhibitory concentration (IC_{50}) of internalized amastigotes. RPMI containing 0.2% DMSO was used as the negative control. Three independent experiments were performed in triplicate [30].

Evaluation of Macrophage Activation Parameters

Lysosomal activity

Murine macrophages (2×10^5 cells/well) were cultured in 96-well plates and incubated with MvFL (6.25, 12.5, 25, 50, and 100 µg/mL) at 37 °C in a 5% CO_2 incubator. After 48 h, 10 µL of 2% neutral red stack solution in DMSO was added to each well and the plates were incubated for 30 min. Next, the supernatant was washed with 0.9% NaCl at 37 °C, and the neutral red trapped in lysosomal vesicles was solubilized by addition of 100 µL of extraction solution (1% [v/v] glacial acetic acid and 50% [v/v] ethanol (v/v) in distilled water). After 30 min at 25 °C on constant agitation, the absorbance was measured on a microplate reader at 550 nm [32].

Phagocytosis Assay

Murine macrophages (2×10^6 cells/well) were incubated with MvFL (6.25, 12.5, 25, 50, or 100 µg/mL) at 37 °C in a 5% CO_2 incubator. After 48 h of incubation, 10 µL of neutral red-stained zymosan solution was added per well and the plate was incubated for 30 min at 37 °C. Following incubation, the plates were washed with saline 0.9% (w/v) and 100 µL of Baker's fixative (4% [v/v] formaldehyde, 2% [w/v] sodium chloride, and 1% [w/v] calcium acetate in distilled water) was added to stop phagocytosis of zymosan. Then, 100 µL of extraction solution was added to each well, the plate was incubated for 30 min with constant agitation, and the absorbance was measured on a microplate reader at 550 nm [33].

NO Synthesis

Murine macrophages (2×10^6 cells/well) were plated in a 96-well plate and incubated with MvFL (6.25, 12.5, 25, 50, and 100 µg/mL) in the presence or absence of *L. amazonensis* promastigote forms (1×10^6 cells/well). After 24 h of incubation at 37 °C in a 5% CO_2 incubator, the supernatants were transferred to another 96-well plate for nitrite dosing. A standard curve was prepared with sodium nitrite in RPMI medium at concentrations of 1, 5, 10, 25, 50, 75, 100, and 150 µM. After 24 h of incubation with nitrate, the supernatants were transferred and incubated with equal amount of Griess reagent (Sigma-Aldrich). The absorbance was read on a microplate reader at 550 nm. The results are expressed as the percentage of nitrite production. RPMI medium containing 0.5% DMSO was used as the negative control [34, 35].

Statistical Analyses

All data are presented as the mean ± standard error and all experiments were performed in triplicate. IC_{50} was calculated with 95% confidence limit using the Probit regression model of SPSS 22.0 software (IBM, Armonk, NY, USA). One-way analysis of variance followed by Tukey's test was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) to assess statistical significance. Differences were considered statistically significant at $p < 0.05$.

Results

Effect of MvFL Against Promastigote Forms of *L. amazonensis*

M. vacciniifolia frond extract was subjected to chromatography on a Sephadex G-75 column, and MvFL was recovered in the first peak [26] with a specific HA of 12,100. The carbohydrate-binding ability of MvFL was neutralized in the presence of fetuin.

The inhibitory activity of MvFL against promastigote forms of *L. amazonensis* was also evaluated. Incubation with MvFL for 24 h inhibited the growth of promastigotes in a dose-dependent manner (Fig. 1), and maximum inhibition was observed at 800 µg/mL. After nonlinear regression analysis, at 24 h, an IC_{50} value at of 88 µg/mL (1.63 µM) was obtained.

Hemolytic Activity Assay

The cytotoxic effect of MvFL against mouse and human erythrocytes is shown in Fig. 2. MvFL was found to be safe for mouse and human erythrocytes, owing to its low toxicity against these cells. The maximum hemolytic activity of 200 µg/mL MvFL (the highest concentration evaluated) against mouse erythrocytes was $14.3 \pm 0.11\%$, and $1.66 \pm 0.36\%$, $2.53 \pm 0.08\%$, $1.67 \pm 0.08\%$, and $3.86 \pm 0.08\%$ for human erythrocytes isolated from blood types A^+, B^+, AB^+, and O^+, respectively.

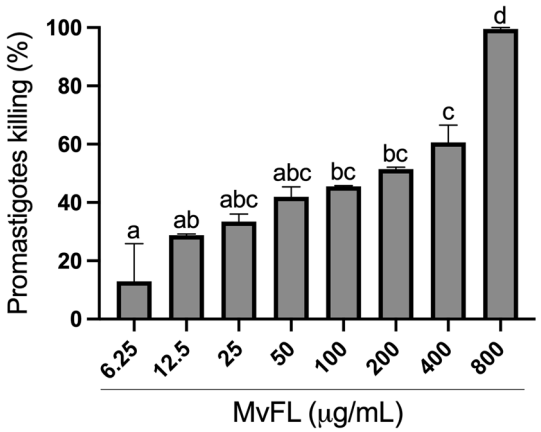

Fig. 1 Effect of *Microgramma vacciniifolia* frond lectin (MvFL) on promastigote forms of *Leishmania amazonensis* after 24 h exposure. The graphs represent the mean ± standard error of mean (SEM) of three independent experiments, considering the control (0.2% DMSO) as 100% viability. One-way ANOVA was used to compare the inhibition observed at different concentrations of MvFL with the control. Different letters (a, b, c and d) indicate significant differences between different concentrations of lectin ($p < 0.05$)

Effect of MvFL on Macrophages Infected by *L. amazonensis*

The effect of treatment with MvFL and the reference drug Amp-B on infected macrophages is shown in Fig. 3. MvFL at concentrations of 22.5, 45, and 90 µg/mL reduced both the number of infected macrophages (Fig. 3A) and internalized amastigotes (Fig. 3B) compared to the negative control. MvFL significant inhibited macrophage infection at 48 h at all three tested concentrations ($p < 0.05$), with an IC_{50} of 52 µg/mL. Furthermore, 90 µg/mL MvFL reduced the number of internalized amastigotes by $68.9 \pm 0.53\%$.

Effect of MvFL on Lysosomal Activity, Phagocytosis, and NO Production in Murine Macrophages

The effect of MvFL on macrophage activation-related parameters, such as lysosomal activity and phagocytic capacity, were assessed based on the retention of neutral red and phagocytosis of neutral red-stained zymosan by murine macrophages. NO production was assessed by nitrite measurement in murine macrophages that were incubated with the sample prior to nitrate treatment. Treatment with 25 µg/mL MvFL significantly ($p < 0.05$) increased lysosomal activity compared to the control (Fig. 4A). However, MvFL did not affect phagocytic capacity of macrophages at any of the tested concentrations (6.25–100 µg/mL) (Fig. 4B). Additionally, MvFL did not significantly affect NO levels compared to the control, with only a slight reduction in NO levels noted at 6.25 µg/mL (Fig. 5), suggested that MvFL does not directly interfere with nitric oxide synthase (NOS) activity or any other stage of NO synthesis in macrophages.

Discussion

Parasite resistance and substantial risk of toxicity with drugs used for the treatment of leishmaniasis have increased the need for new antileishmanial drugs [36–38]. In recent years, novel bioactive molecules from natural sources that can be obtained in a sustainable manner, are affordable, and have low toxicity, have been the focus of the pharmaceutical industry and researchers [39–41]. Several plant species have been reported to be important sources of bioactive compounds that can potentially be used as alternative treatments for various diseases, including leishmaniasis [13, 42, 43]. Among these chemical compounds are lectins, whose specific carbohydrate interactions have been the focus of several studies, mainly in relation to host–pathogen recognition [44], modulation of the host immune system [45], and inhibition of parasitic development [46]. Thus, understanding the nature of these interactions is not only useful for elucidating their biological function, but can also be applied

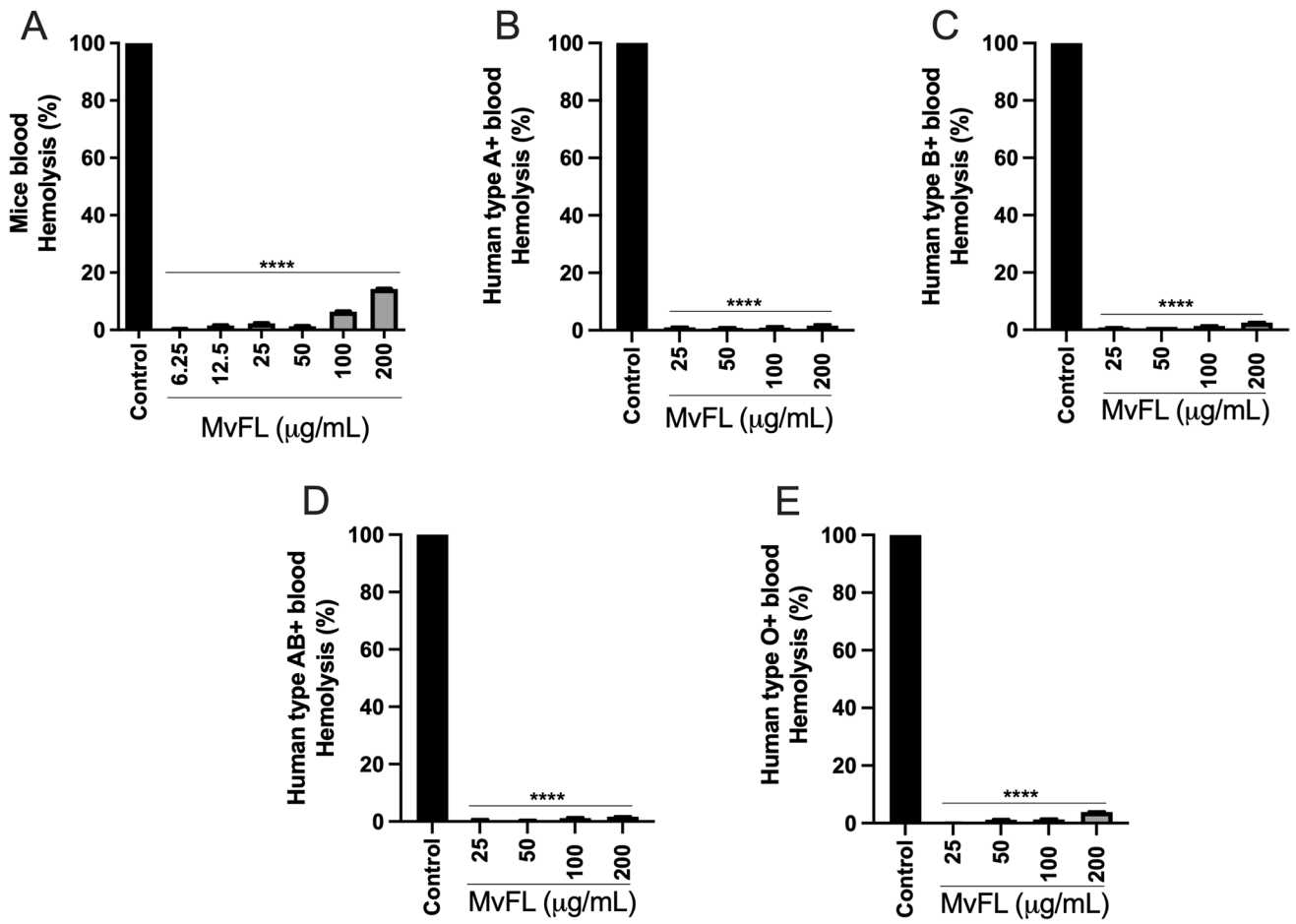

Fig. 2 Cytotoxic effects of MvFL against mouse (**A**) and human erythrocytes with blood type A + (**B**), B + (**C**), AB + (**D**) and O + (**E**). Data are expressed as the mean ± SEM of three independent experiments performed in triplicate, considering the control group (1% Triton X-100 in PBS) as 100% hemolysis; (*) $p < 0.005$ vs control; (**) $p < 0.01$ vs control; (***) $p < 0.001$ vs control

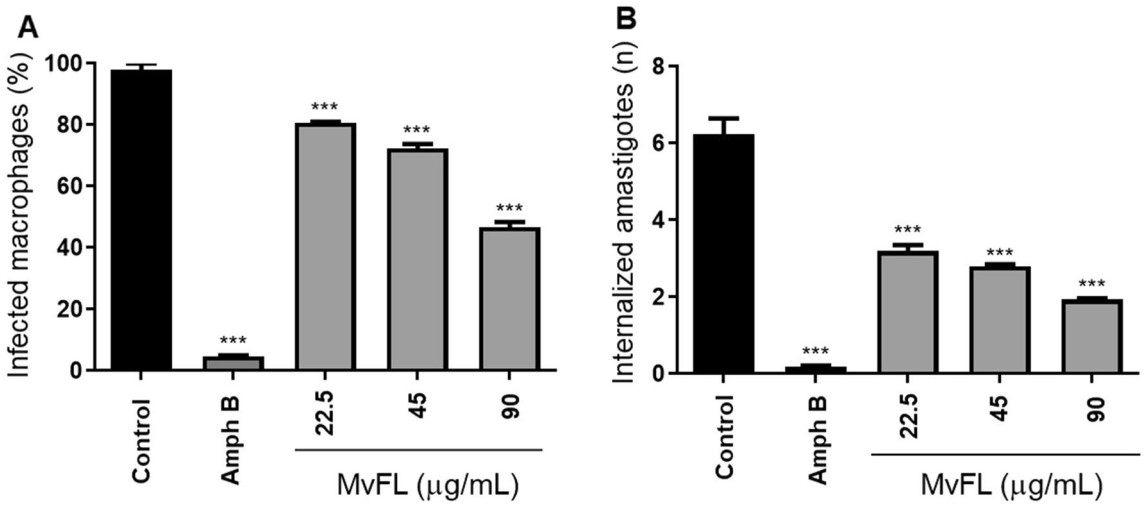

Fig. 3 Effects of MvFL and reference drug amphotericin B (Amp-B) on infected macrophages (**A**) and survival index of BALB/c murine macrophages infected with *L. amazonensis* (B). Cells infected with *L. amazonensis* were treated with MvFL or Amp-B for 48 h. Data are presented as the mean ± SEM of three independent experiments, considering the control as 100% viability. (***) $p < 0.001$ vs control

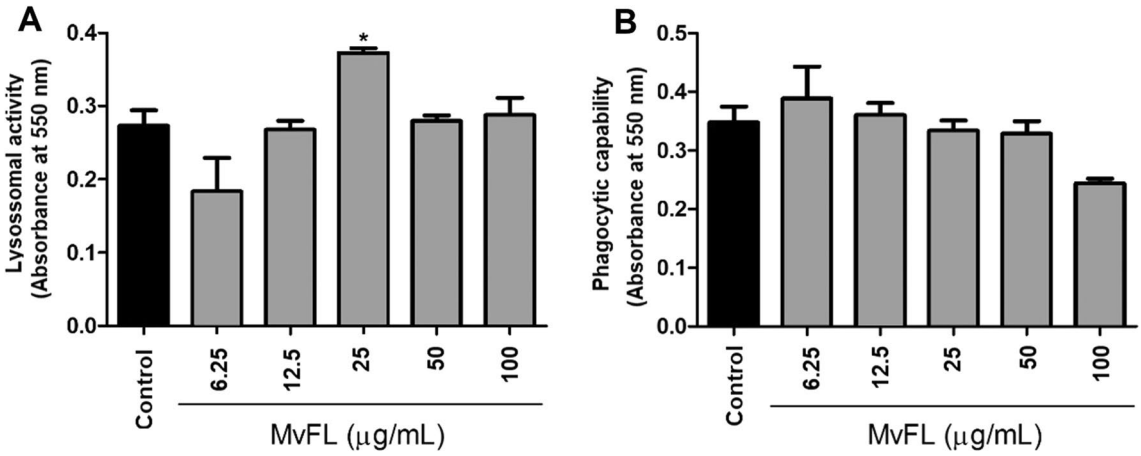

Fig. 4 Effects of MvFL on lysosomal activity (**A**) and phagocytic capacity (**B**). Murine peritoneal macrophages were treated at ranging concentrations for 48 h. Lysosomal activity and phagocytic capacity were assessed by quantification of neutral red (NR). Phagocytic capability was determined as the ratio of incorporation of zymosan to NR, solubilized by the extraction solution. (*) $p < 0.001$ vs control (untreated cells)

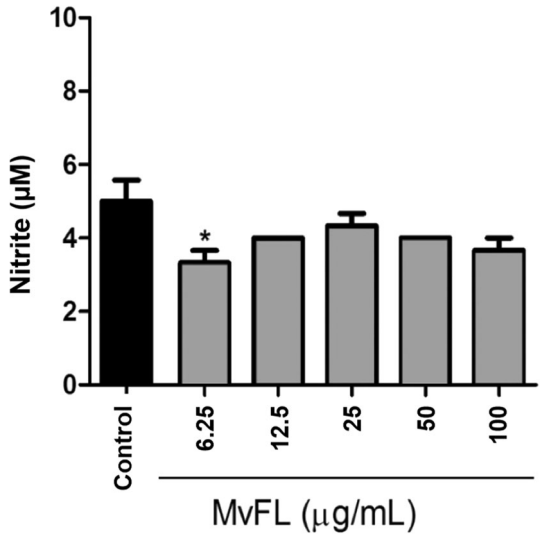

Fig. 5 Nitrite measurement in infected or non-infected murine peritoneal macrophages treated with MvFL for 24 h. The culture supernatant was mixed with equal parts of the Griess reagent. Data are presented as the mean ± SEM of three experiments performed in triplicate. *$p < 0.05$ vs control (0.5% DSMO)

to different areas of research, such as immunology, drug development and delivery, and diagnostics [44].

In the present study, the lectin MvFL, with high HA, was isolated following the protocol described previously. As expected, HA of MvFL was inhibited by fetuin, confirming the presence of lectin molecules with functional carbohydrate-binding sites [26]. Then, we investigated the effect of MvFL on the interaction between *L. amazonensis* and macrophages. We first evaluated whether MvFL exerts leishmanicidal activity against promastigote forms and observed that MvFL inhibited the growth of promastigote forms at 24 h in a dose-dependent manner. Other natural compounds have also been reported to exert inhibitory effect against promastigote forms of *L. amazonensis*. Verbascoside, a phenylethanoid glycoside, was reported to have antileishmanial activity and inhibited *L. amazonensis* promastigotes with an IC_{50} of 19 µM [47]. Garcinielliptone FC obtained from the seeds of *Platonia insignis* and bioflavone from the flowers promoted *in vitro* growth inhibition of promastigotes with IC_{50} values of 14.06 µM and 47.71 µg/mL, respectively [42, 48].

Promastigotes are the flagellated forms of *Leishmania* spp., which infect humans through bites of infected sand flies, and are phagocytosed by immune cells, transforming into amastigotes. To prevent infection of host cells by the parasite and subsequent development of leishmaniasis, it is important to inhibit the growth of promastigotes to prevent their proliferation and transformation into intracellular amastigote forms [49, 50].

Lectins have been described in the literature as promising agents against promastigote forms of *Leishmania* spp. Similarly, a lectin isolated from the venom of *Bothrops leucurus* (BLL) inhibited *L. amazonensis* promastigote growth and viability, with an IC_{50} value of 1.5 µM [20]. Carneiro *et al.* [21] reported that *Parkia pendula* lectin (PpeL) significantly inhibited the growth of *Leishmania infantum* promastigotes with an IC50 value of 4.9 ± 0.05 µM at 48 h, through its carbohydrate recognition domain, suggesting an interaction between PpeL and glycans from *L. infantum*. Sousa *et al.* [51] reported that a galectin isolated from the marine sponge *Chondrilla caribensis* exerted a leishmanicidal effect on *L. infantum* promastigote forms with an IC_{50} value of 1.2 ± 0.094 µM at 24 h through its interaction with parasite glycans.

The indiscriminate use of medicinal plants to prevent or cure diseases can cause cytotoxic and genotoxic changes that can potentially lead to the development of other pathologies. Therefore, it is important to understand the action of natural products at the cellular level to ensure their safety and to support further research [52, 53]. *In vitro* toxicological assays, such as hemolytic activity, are preliminary tests that are especially useful in cytotoxicity assessment of compounds to choose a safe concentration range for use in later stages of drug development [34, 54]. Our study showed that MvFL did not cause significant hemolysis of mouse erythrocytes or human erythrocytes derived from different blood types, suggesting that this lectin can be evaluated in animal models at the concentrations assessed in the present study.

Given the observed inhibitory activity of MvFL on promastigote proliferation, we investigated whether it could also reduce the percentage of macrophages infected by *L. amazonensis*. Experimental models using macrophages infected with amastigotes of *Leishmania* are used because these forms in the parasite life cycle are associated with the clinical manifestations of leishmaniasis [48, 55]. Furthermore, models with the intracellular form provide the most efficient method for correlating the *in vitro* activity of a drug with its effectiveness in an *in vitro* assay [56]. Based on the results of our experiments, MvFL is an inhibitor of promastigote growth and reduces the number of amastigotes internalized by macrophages.

Afonso-Cardoso *et al*. [57] evaluated the effect of latex lectin of *Synadenium carinatum* (ScLL) (100, 50, and 10 µg/mL) against infection of peritoneal macrophages of BALB/c mice by *L. amazonensis in vitro*, and noted infection rates of 55%, 65%, and 45%, respectively, compared to the control (100% infected cells). Aranda-Souza *et al*. [20] also analyzed the effect of BLL lectin against macrophages infected with *L. amazonensis* and *Leishmania braziliensis* amastigotes and reported that the infection rate in cells treated with 1.6 µM BLL was 28.5 ± 7.91% and 35 ± 8.83% for *L. amazonensis* and *L. braziliensis*, respectively.

Afonso-Cardoso *et al*. [58] analyzed whether association of ScLL with the soluble antigen of *L. amazonensis* would immunize BALB/c mice against promastigote forms of *L. amazonensis*. They observed that ScLL (100 µg/animal) promoted a 61.7% reduction in the parasite load inside macrophages compared to the control group, suggesting that the underlying mechanism responsible for the observed protection required further investigation. ArtinM, a lectin isolated from the seeds of *Artocarpus heterophyllus,* binds to D-mannose and has immunomodulatory properties due to its interaction with N-glycans of immune cells. ArtinM was reported to induce infected human neutrophils infected with *Leishmania major* promastigotes and reduced parasite viability by 50% when compared to untreated neutrophils [59].

In the present study, MvFL significantly reduced the number of intracellular amastigotes, in addition to reducing infection; however, the underlying mechanism is not clear as it could be due to direct effect on the parasite or activation of macrophage defense mechanisms. Macrophages play a vital role in modulating the immune response against *Leishmania* parasites through cytokine production and phagocytosis to destroy pathogens or repair lesions associated with inflammation [60]. Macrophages are activated on contact with parasites, which triggers changes in lysosomal activity, phagocytic capacity, and NO production [61]. To understand the mechanism underlying the decrease in the survival rate of intramacrophage amastigotes, the effect of MvFL on parameters associated with macrophage immune response were analyzed to determine whether changes in these parameters stimulated inhibition of parasites.

Phagocytosis and the lysosomal system are essential components that mediate the functions of activated macrophages in innate immune responses through antigen internalization and degradation. In macrophages, parasites are restricted to phagosomes, which fuse with lysosomes to form the parasitic vacuole, an inhospitable compartment with acid hydrolases, reactive oxygen, and NO species, which participate in pathogen degradation [62, 63]. However, our results suggest that the effect of MvFL on the number of amastigotes is not due to an effect of MvFL on phagocytic action; therefore, the observed effect of the lectin could be due to direct action on the parasite or inhibition of infection by promastigotes.

Like the findings of the present study, Afonso-Cardoso *et al*. [57] observed that ScLL did not induce NO production, suggesting that a NO-independent pathway engages in the decrease of the number of intracellular *L. amazonensis*. Thomazelli *et al*. [64] reported that concanavalin A lectin (Con-A) increased the phagocytic capacity of immune cells and promoted *L. amazonensis* elimination. Pretreatment with Con-A increased the synthesis of reactive oxygen species as well as the expression of iNOS enzyme, but did not affect NO production, perhaps because even though Con-A treatment induced production of pro-inflammatory cytokines, it also promoted the synthesis of anti-inflammatory cytokines. Our results suggest that the leishmanicidal activity of MvFL against promastigote and amastigote forms of *L. amazonensis* may be related to its proinflammatory potential, as previously described by Patriota *et al*. [26].

The findings of the present study showed that MvRL, a lectin isolated from the frond of *M. vacciniifolia* exerts significant *in vitro* antileishmanial activity against *L. amazonensis* promastigote replication and macrophage infection. This study also demonstrated that MvFL promoted the activation of macrophages by increasing lysosomal activity but did not affect NO production and phagocytosis. Our study paves the way for further studies to elucidate the immunological mechanisms involved in the leishmanicidal effects of

MvFL as well as investigating its effect in *in vivo* immunization models for leishmaniasis.

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Data availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of 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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