Chemico-Biological Interactions 351 (2022) 109713

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Botryosphaeran, [(1 → 3)(1 → 6)-β-D-glucan], induces apoptosis-like death in promastigotes of *Leishmania amazonensis*, and exerts a leishmanicidal effect on infected macrophages by activating NF-kB and producing pro-inflammatory molecules

Amanda Cristina Machado Carloto [a,*], Bruna Taciane da Silva Bortoleti [a,b], Ana Carolina Jacob Rodrigues [a,c], Taylon Felipe Silva [a], Fernanda Tomiotto-Pellissier [a,d], Danielle Lazarin Bidóia [a], Manoela Daiele Gonçalves [e], João Paulo Assolini [f], Robert F. H. Dekker [g,h], Aneli M. Barbosa-Dekker [g,h], Idessania Nazareth Costa [a], Ivete Conchon-Costa [a], Milena Menegazzo Miranda-Sapla [a], Wander Rogério Pavanelli [a]

[a] *Laboratory of Immunoparasitology of Neglected Diseases and Cancer, Department of Pathological Sciences, Center of Biological Sciences, State University of Londrina, 86057-970, Londrina, Paraná, Brazil*
[b] *Gonçalo Moniz Institute (FIOCRUZ/Bahia), 40296-710, Salvador, Bahia, Brazil*
[c] *Carlos Chagas Institute (ICC/FIOCRUZ/Paraná), 81310-020, Curitiba, Paraná, Brazil*
[d] *Department of Medical Pathology, Health Sciences Sector, Federal University of Paraná, 80060-240, Curitiba, Paraná, Brazil*
[e] *Biotransformation and Phytochemistry Laboratory, Chemistry Department, Exact Sciences Center, State University of Londrina, 86057-970, Londrina, Paraná, Brazil*
[f] *Alto Vale University of Rio Do Peixe, 89500-000, Caçador, Santa Catarina, Brazil*
[g] *Postgradute Program in Environmental Engineering, Paraná Technological University, Londrina Campus, 86036-370, Londrina, Paraná, Brazil*
[h] *Beta-Glucan Pharmaceuticals EIRELI, Lote 24A, Zirconia Block, Paraná Technological University, Londrina Campus, Avenue João Miguel Caram 731, 86036-700, Londrina, Paraná, Brazil*

ARTICLE INFO

Keywords:
1 → 3)(1 → 6)-β-D-glucan
Leishmaniasis
Cytotoxicity
Apoptosis

ABSTRACT

Leishmaniasis is an infectious-parasitic disease caused by the protozoan *Leishmania* spp. The available treatments are based upon expensive drugs bearing adverse side-effects. The search for new therapeutic alternatives that present a more effective action without causing adverse effects to the patient is therefore important. The objective of this study was to evaluate the *in vitro* effect of botryosphaeran, a (1 → 3)(1 → 6)-β-D-glucan, on the promastigote and intracellular amastigote forms of *Leishmania amazonensis*. The direct activity of botryosphaeran on promastigote forms was evaluated *in vitro* and inhibited proliferation, the IC_{50} 7 μg/mL in 48 h was calculated. After 48 h treatment, botryosphaeran induced nitric oxide production (NO), caused mitochondrial membrane hyperpolarization, increased reactive oxygen species (ROS), and accumulation of lipid vesicles in promastigotes, resulting in apoptosis, necrosis and autophagy, and was accompanied by morphological and ultrastructural changes. The range of concentrations used did not alter the viability of peritoneal macrophages from BALB/c mice and erythrocytes of sheep. Botryosphaeran was able to reduce the number of infected macrophages and the number of amastigotes per macrophage at 12.5 μg/mL (50.75% ± 6.48), 25 μg/mL (55.66% ± 3.93) and 50 μg/mL (72.9% ± 6.98), and IC_{50} 9.3 μg/mL (±0.66) for intracellular amastigotes forms. The leishmanicidal effect was due to activation of NF-κB and promoted an increase in pro-inflammatory cytokines (TNF-α and IL-6), iNOS and microbial-derived ROS and NO, in addition to decreasing the levels of SOD. Based upon the data obtained, we infer that botryosphaeran exerted an active leishmanicidal and immunomodulatory effect, acting on promastigotes through autophagic, apoptotic and necrosis processes, and in the intracellular amastigote form, through the action of ROS and NO.

* Corresponding author. Department of Pathological Sciences, Laboratory of Immunoparasitology of Neglected Diseases, State University of Londrina. Celso Garcia Cid Highway, PR-445 Km-380, 86057-970, Londrina, PR, Brazil.
E-mail addresses: amanda.carloto@gmail.com (A.C.M. Carloto), wanderpavanelli@yahoo.com.br (W.R. Pavanelli).

https://doi.org/10.1016/j.cbi.2021.109713
Received 11 July 2021; Received in revised form 30 September 2021; Accepted 20 October 2021
Available online 23 October 2021

1. Introduction

Leishmaniasis is a neglected tropical disease caused by species of the protozoan of the genus *Leishmania* transmitted to mammals through the sand fly vector [1]. This disease has several clinical manifestations; the cutaneous form being the most common. According to the World Health Organization, an estimated 600,000 to 1 million new cases of cutaneous leishmaniasis occur annually with the majority of cases occurring in Afghanistan, Algeria, Brazil, Colombia and Syria [2].

Abbreviations

FBS	fetal bovine serum
AmB	amphotericin B
NO	nitric oxide
TMRE	tetramethylrhodamine methyl ester
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
ROS	reactive oxygen species
DCF	2′,7′-dichlorofluorescein
PI	propidium iodide
SEM	scanning electron microscopy
TEM	transmission electron microscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor-*kappa* B
TNF-α	necrosis factor-*alpha*
IL	interleukins
TGF-β	Transforming growth factor-*beta*
MCF-7	breast carcinoma cell line
ITAM	tyrosine-based immunoreceptor
MAP	mitogen kinases
NFAT	nuclear factor of activated T cells
TLRs	toll-like receptors

The first choice of treatment for leishmaniasis is based on the use of the pentavalent antimonials, such as sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®). Despite their proven efficacy, these drugs have adverse side effects due to the long period of treatment required, as well as their toxicity in the heart, kidneys, and liver [3,4]. In cases of contra-indications to antimonials, alternative treatments such as amphotericin B®, paromomycin, pentamidine, and miltefosine can be used. These second-choice drugs, however, also exert high toxicity and cause several side effects on the patient that includes gastrointestinal disorders, nephrotoxicity and pancreatitis, as well as leading to the development of resistant strains of *Leishmania* spp [5]. The search for alternative drugs therefore becomes an important and urgent issue, and studies have shown the beneficial effects of natural products derived from bacteria, fungi and plants.

Carbohydrate biopolymers (polysaccharides) that include the β-glucans are a class of natural products considered alternative and complementary medicinal compounds. They can be produced as exopolysaccharides in submerged fermentation by several microorganisms (basidiomycetes and filamentous fungi, among which are yeasts), or are found in the fungal cell wall, from which they can be extracted chemically [6–8]. The β-glucans have attracted wide attention for their ability to induce innate and adaptive immune responses in the host [9,10]. Among the fungal β-glucans, is the exopolysaccharide botryosphaeran from the ascomyceteous fungus *Botryosphaeria rhodina* MAMB-05. Botryosphaeran is a mixed-linked, branched (1 → 3)(1 → 6)-β-D-glucan consisting of a β-(1 → 3)-linked D-glucan backbone chain with glucose and gentiobiose appendages via β-(1 → 6)-glucosidic linkages [11]. Previous studies from our group [12] have demonstrated several *in vitro* and *in vivo* biological activities for botryosphaeran that include: antiproliferative, pro-apoptotic, antimutagenic and chemopreventive properties on tumor cells (V79, HTC, Jurkat and MCF-7 cell lines) [13–16]; antioxidant [17]; antiviral (*Herpes simplex* and dengue fever) [18]; anticoagulant [19], and in studies on mice and rats has presented anticlastogenic activities [20,21], hypoglycemia and hypocholesterolemia [21,22], anti-obesogenicity [23] and anti-tumor activity in rats bearing Walker-256 cancer cells [24].

A β-glucan from *Lentinus edodes* was reported to exhibit an immunomodulatory effect on *Leishmania*-infected J774A.1 macrophages, increasing the levels of pro-inflammatory cytokines (IL-12 and TNF-α) and decreasing anti-inflammatory interleukins (IL-10 and TGF-β) [25]. Other β-glucans from *Saccharomyces cerevisiae* and *Aureobasidium pullulans* have been reported to prevent the *in vivo* progression of cutaneous leishmaniasis infection by *L. major* and *L. amazonensis* in BALB/c [26] and C57BL/6 mice [27], respectively.

Although some studies have demonstrated the activities of β-glucans against *Leishmania* infections [25–27], nothing is known about the microbiocidal and immunomodulatory mechanisms involved in the death of the promastigote and amastigotes forms of *L. amazonensis* on treatment with the (1 → 3)(1 → 6)-β-D-glucan, botryosphaeran. The objective of this study was to evaluate the *in vitro* effect of botryosphaeran on the free forms of promastigotes and intracellular amastigotes of *L. amazonensis*, as well as to elucidate a mechanism of action on the death of these parasitic forms.

2. Materials and methods

2.1. Culture of leishmania (leishmania) amazonensis promastigotes

The promastigote form of *Leishmania* (*L.*) *amazonensis* (MHOM/BR/1989/166MJO) was maintained in culture medium 199 (GIBCO Invitrogen, New York, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO Invitrogen), 1.0 M HEPES medium, 1% human urine, 1% L-glutamine, streptomycin (10 U/mL) with penicillin (10 μg/mL) (GIBCO Invitrogen) and 10% sodium bicarbonate, and cultured as described by Tomiotto-Pellissier et al. (2018) [28]. Cell cultures were maintained in a B.O.D incubator at 25 °C, in 25-cm^2 culture flasks. Promastigotes at the stationary phase of growth (5-day culture) were used in all experiments.

2.2. Animals and Ethics Committee

Male BALB/c mice weighing 25–30 g aged 6–12 weeks were kindly donated by Dr. Giovanny Mazzarotto of Instituto Carlos Chagas/Fiocruz, Curitiba, Brazil. The mice were maintained and used according to the protocol approved by the Animal Use Ethics Committee of Universidade Estadual de Londrina (n°. 88/2018).

2.3. Production of botryosphaeran

Botryosphaeran was produced by *Botryosphaeria rhodina* isolate MAMB-05 grown by submerged fermentation on nutrient media containing 6% sucrose, and isolated from the cell-free fermentation broth by precipitation with absolute ethanol (3 vol) according to the procedure described by Barbosa et al. (2003) [11]. The precipitate was dissolved in water (heating at 60 °C for 2 h), and then exhaustively dialyzed against several changes of distilled water over a 48-h period. The dialyzate containing botryosphaeran was autoclaved (120 °C for 20 min) and stored at 4 °C until required. The concentration of botryosphaeran was determined by the phenol-sulfuric acid method [29].

2.4. Botryosphaeran activity on promastigote forms of Leishmania amazonensis

The promastigote forms of *L. amazonensis* (106 cells/mL) were

treated with different concentrations of botryosphaeran (6.25; 12.5; 25; 50; 100 μg/mL). This concentrations was chosen according to a previously study of botryosphaeran [16]. The parasites were counted in a Neubauer chamber after 24, 48, and 72 h of treatment (25 °C). As a negative control, promastigote forms maintained in the untreated culture medium (medium 199) were used. Parasites treated with 1 μM amphotericin B (AmB) (União Química, São Paulo/São Paulo, Brazil) were used as a positive control. From the generated dose-response curve, the IC50 (concentration capable of inhibiting the growth of parasites by 50%) and 2x IC50 values were calculated by non-linear regression analysis using the statistical software GraphPad Prism (GraphPad Software, Inc., USA, v. 5). The IC50 and 2x IC50 were chosen for use in the following experiments with promastigotes as previously described [30].

2.5. Determination of nitrite as an estimate of NO levels in promastigote and amastigotes forms

Nitric oxide (NO) was determined as nitrite by the Griess assay procedure as described by Gonçalves et al., 2018 [31]. Briefly, aliquots of 60 μL of the promastigote form at concentrations of botryosphaeran of IC_{50} and 2x IC_{50} (7 and 14 μg/mL, respectively) were taken at 48 h, as well as aliquots of the supernatant from the 24-h anti-amastigote assay (5×10^5 cells/mL - infected and treated at concentrations of botryosphaeran of 12.5, 25 and 50 μg/mL). Griess reagent (60 μL), 1% sulfanilamide (Labsynth, Diadema/São Paulo, Brazil) and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Labsynth, Diadema/São Paulo, Brazil) were added in 5% H_3PO_4 and incubated at 25 °C for 10 min. A standard curve was constructed using serial dilutions of $NaNO_2$ (0–250 μM), and the absorbance measured at 550 nm in a microplate reader (Thermo Scientific, Multiskan GO, USA). Medium 199 was used as a negative control.

2.6. Determination of mitochondrial membrane potential

Promastigote forms (1×10^6 cells/mL) were treated with botryosphaeran at IC_{50} and 2x IC_{50} values (7 and 14 μg/mL, resp.) for 48 h to assess the membrane potential of the internal mitochondrial membrane. For this purpose, the probe tetramethylrhodamine methyl ester (TMRE) (Sigma-Aldrich, St. Louis, MO, USA) was used. The parasites to be treated were washed and incubated in 2.5 μM TMRE solution for 30 min at 25 °C and then analyzed by fluorometry using an excitation wavelength of 480 nm and an emission wavelength of 580 nm in a PerkinElmer Victor X3 fluorometer (PerkinElmer, MA, USA). Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was used as the positive control, and medium 199 as negative control.

2.7. Generation of reactive oxygen species (ROS) in promastigotes and infected macrophages

The production of ROS was evaluated by the conversion of non-fluorescent H2DCFDA to the highly fluorescent 2′,7′-dichlorofluorescein (DCF, Sigma-Aldrich, USA) on the promastigote forms of *L. amazonensis* (1×10^6 cells/mL) treated with IC_{50} and 2x IC_{50} (7 and 14 μg/mL) of botryosphaeran for 48 h, and on macrophages infected with *L. amazonensis* (5×10^5 cells/mL - infected and treated under the same conditions as described in the anti-amastigote test) according to Bortoleti et al. (2018) [32]. This was followed by washing the cells in PBS (pH 7.4), and they were then loaded with the permeant probe DCF diluted in DMSO (10 μM for promastigotes and 2 μM for amastigotes, resp.), and the cells incubated in the dark for 45 min for the promastigote forms, and 30 min for infected macrophages, at 37 °C. Hydrogen peroxide (H_2O_2) was used as the positive control, and medium 199 was used as negative control. The ROS generated were assayed fluorimetrically at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

2.8. Assessment of the presence of lipid droplets by Nile red staining

Promastigote forms (1×10^6 cells/mL) were treated with botryosphaeran (7 and 14 μg/mL) for 48 h. Afterwards, the cells were washed twice in PBS (pH 7.4) and then reacted with an aqueous solution of Nile red (10 μg/mL; Sigma-Aldrich, USA) for 30 min at 25 °C. The dyed cytoplasmic lipid droplets of the parasites were quantitated fluorometrically at wavelengths of 530 nm (emission) and 635 nm (excitation). PBS was used as a positive control.

2.9. Assessment of the presence of autophagic vacuoles

To assess the presence of autophagic vacuoles, promastigote forms were treated with IC_{50} and 2x IC_{50} (7 and 14 μg/mL) of botryosphaeran for 48 h. The parasites were washed twice in PBS (pH 7.4), and then incubated with 5 μL of monodansylcadaverine (Sigma-Aldrich, USA; a specific marker of autophagy vacuoles) solution (4.97 nM) at 25 °C for 1 h, followed by fluorometric analysis at an excitation and emission wavelengths of 380 nm and 525 nm, respectively. PBS was used as the positive control.

2.10. Co-determination of annexin-V and propidium iodide

Cellular apoptosis, necrosis or late apoptotic cells were evaluated using a combination of two stains - annexin V-FITC and propidium iodide (PI) (Santa Cruz Biotechnology, USA). Promastigote forms of *L. amazonensis* (1×10^6 cells/mL) were treated with IC_{50} and 2x IC_{50} (7 and 14 μg/mL) of botryosphaeran for 48 h at 25 °C. The parasites were then washed and resuspended in 100 μL of binding buffer (Santa Cruz Biotechnology, Dallas, TX, USA), followed by adding a mixture of 0.5 μL of annexin V-FITC and 0.5 μL of PI. Data acquisition and analysis were performed using a BD Accuri™ C6-Plus flow cytometer (BD Biosciences, San Jose, CA, USA). The cells were analyzed according to the procedure of Miranda-Sapla et al. (2019) [33].

2.11. Evaluation of morphological and ultrastructural changes of parasitic cells by electron microscopy

Promastigote forms treated for 48 h with 7 and 14 μg/mL of botryosphaeran were collected by centrifugation and washed in 0.01 M PBS (pH 7.2). Scanning electron microscopy (SEM) was used to assess morphological changes of the parasite cells. The parasites were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer followed by washing with 0.1 M sodium cacodylate buffer. The treated parasites were then allowed to adhere to glass coverslips and covered with a layer of poly-L-lysine solution. The adhered cells were next washed with 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated using increasing concentrations of ethanol (30–100%), then subjected to drying at the critical point with carbon dioxide, metalized with gold, and analyzed in a high-resolution double-beam electron microscope (FEI SCIOS, Oregon, USA).

Transmission electron microscopy (TEM) was used to detect ultrastructural changes. The parasites were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide solution and 0.8% potassium ferrocyanide at room temperature and protected from light. Afterwards, the cells were dehydrated using increasing concentrations of acetone (50–100%), followed by adding EPON™ resin, and polymerized in an oven at 60 °C for 72 h. Ultrathin sections made on an ultramicrotome (PowerTomer BMC – Germany) were deposited on a copper grid and contrasted with uranyl acetate (5%) and lead citrate (2%) for 20 and 10 min, respectively. TEM analysis was performed on a JEOL JEM 1400 instrument (Tokyo, Japan).

2.12. Peritoneal macrophage viability analysis

The MTT test using 3-(4,5-dimethylthiazol-2yl)-2,5-

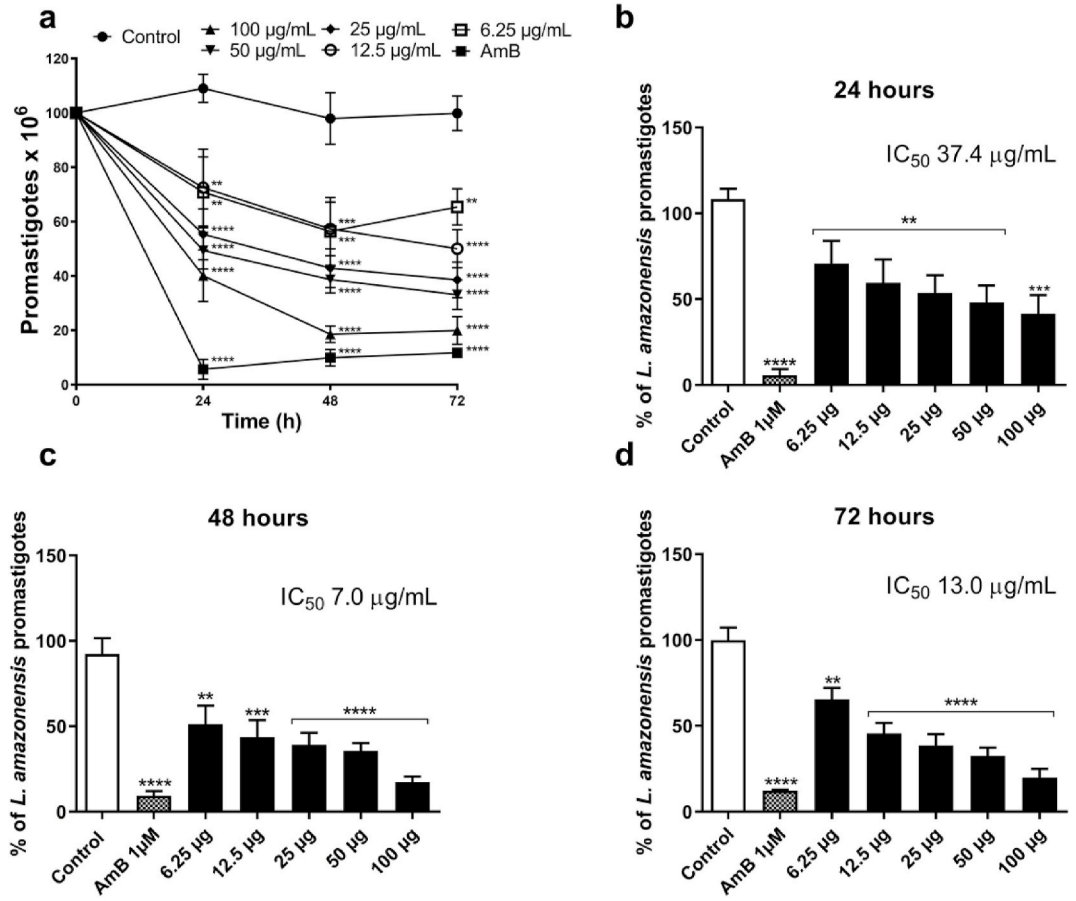

Fig. 1. Profiles showing leishmanicidal activity of botryosphaeran on promastigote forms of *L. amazonensis*. Promastigote forms were treated with different concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) of botryosphaeran, M199 medium (control) and 1 µM amphotericin B (AmB, positive control), and parasite proliferation was analyzed at the time of 24, 48 and 72 h (**a**). From the proliferation assays, concentrations of botryosphaeran that inhibited 50% of the promastigote forms were determined at 24 (**b**), 48 (**c**) and 72 h (**d**). The values represent the mean ± SEM of three independent experiments carried out in triplicate. ** Significant difference compared to the control group ($p \leq 0.05$), *** ($p \leq 0.0005$), **** ($p \leq 0.0001$).

diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) was performed according to Assolini et al. (2020) [34] to evaluate possible cytotoxic effects of botryosphaeran on peritoneal cells in BALB/c mice. Briefly, macrophages (5×10^5 cells/mL) were recovered from the peritoneal cavity of BALB/c mice with cold PBS supplemented with 3% of FBS and then cultured in 24-well plates with 500 µL of RPMI 1640 medium 10% FBS for 24 h (37 °C, 5% CO_2). Subsequently, the wells were washed with PBS to remove the non-adherent cells, and then treated with botryosphaeran (6.25; 12.5; 25; 50 µg/mL) for 24 h under the same conditions. MTT reagent (0.33 mg/mL) was added to the cells and incubated for 4 h. The MTT product (formazan crystals) was solubilized with DMSO, and read on a spectrophotometer (Thermo Scientific, Multiskan GO, USA) at 550 nm. As a negative control, untreated macrophages were used. The results were expressed as a percentage of viability compared to the control group.

2.13. Hemolysis assay

The hemolytic action of botryosphaeran was evaluated according to Bortoleti et al. (2018) [32] using sheep erythrocytes (Ethics Committee on Animal Experimentation at the Universidade Estadual de Londrina: N°. 8286/2016.60). Blood was collected and treated with heparin, and the erythrocytes were washed 3 times with PBS (pH 7.4) (centrifuged at 1000 rpm for 10 min). A 2% erythrocyte suspension was prepared with PBS and mixed in a 96-well plate with botryosphaeran (1:1) added in a final volume of 200 µL to obtain final concentrations of (6.25, 12.5, 25 and 50 µg/mL). The microplates were then incubated for 3 h at 37 °C under 5% CO_2. PBS was used as a negative control, and distilled water as a positive control for assessing hemolysis. The 96-well plates were centrifuged at 162×*g* for 10 min, and the supernatants collected to determine the absorbance at 550 nm, with hemoglobin measured as an indication of erythrocyte lysis.

2.14. Anti-amastigote cell test

The anti-amastigote cell test was evaluated according to Assolini et al. (2020) [34]. Peritoneal macrophages (5×10^5 cell/mL) from BALB/c mice were allowed to adhere to the walls in 24-well plates containing 13-mm glass coverslips, followed by the addition of 500 µL of RPMI 1640 medium, and the contents incubated for 24 h at 37 °C under 5% CO_2. Adherent macrophages were then infected with the promastigote form of *L. amazonensis* (2.5×10^6 cells/mL) for 3 h. After infection, non-internalized promastigote forms were removed by washing with PBS (pH 7.4), and the infected cells treated with botryosphaeran (6.25; 12.5; 25 and 50 µg/mL), RPMI 1640 medium (negative control) or 1 µM amphotericin (positive control) for 24 h (37 °C, 5% CO_2). The cells were then stained with Leishman's stain (Inlab, SP, Brazil) and 20 fields analyzed under an optical microscope (Olympus Bx41, Olympus Optical Co., Ltd., Tokyo, Japan) (1000x magnification) to determine the percentage of infected macrophages, and the number of intracellular amastigotes of macrophages after 24 h of treatment. The IC_{50} against amastigote forms was calculated by non-linear regression analysis using the statistical software GraphPad Prism (GraphPad Software, Inc., USA, v. 5).

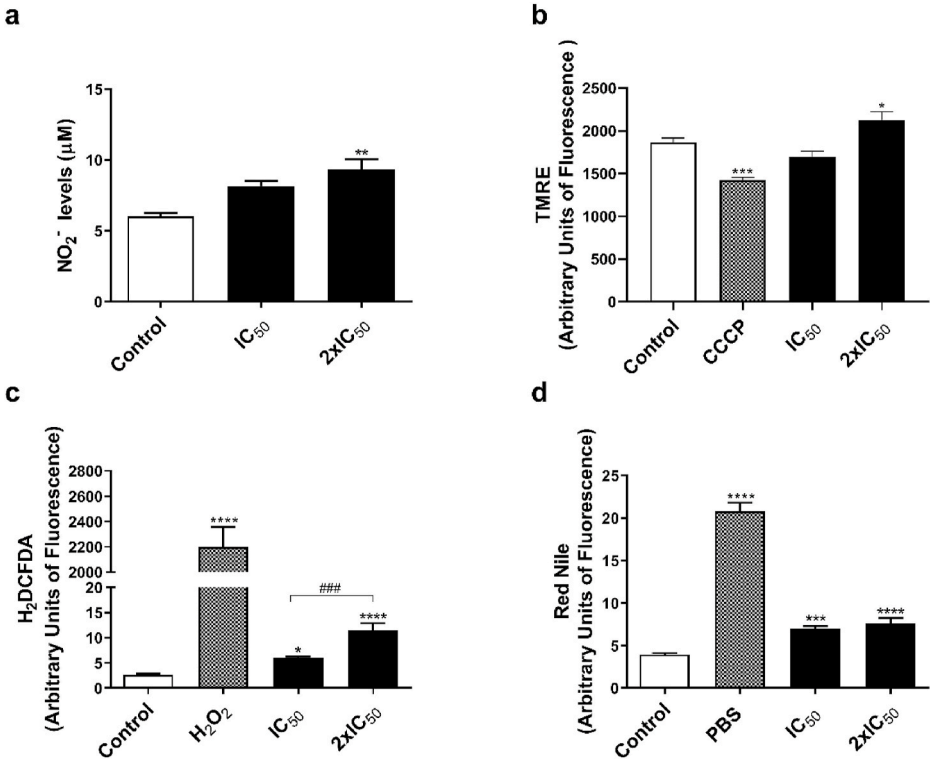

Fig. 2. Mechanisms of action of botryosphaeran on promastigote forms of *L. amazonensis*. The supernatant from the direct assay on the promastigote forms of *L. amazonensis* was collected to measure NO levels at concentrations of botryosphaeran of IC_{50} (7 μg/mL) and 2x IC_{50} (14 μg/mL) within 48 h (**a**). The parasites were treated with IC_{50} (7 μg/mL) and 2x IC_{50} (14 μg/mL) of botryosphaeran for 48 h and analyzed by the TMRE fluorometric method (**b**), reactive oxygen species were measured using the H2DCFDA probe (**c**), and the presence of lipid bodies was analyzed by the Nile red method (**d**). The values represent the mean ± SEM of three independent experiments carried out in triplicate. * Significant difference compared to the control ($p \leq 0.05$), ** ($p \leq 0.001$), *** ($p \leq 0.0005$), **** ($p \leq 0.0001$) and significant difference between the treatments ### ($p \leq 0{,}0005$).

2.15. Immunofluorescence analysis of phosphorylated nuclear factor-kappa B (NF-κB)

L. amazonensis-infected macrophages, as described in the anti-amastigote assay were treated with 12.5, 25 and 50 μg/mL of botryosphaeran, and analyzed for the presence of phosphorylated NF-κB. Briefly, after cell culture and treatment, cover slips containing the attached cells were fixed with 4% paraformaldehyde for 15 min, washed three times with PBS (pH 7.4) and permeabilizated for 15 min with PBS containing 0.5% of Triton X-100 (TBS) and 1% albumin bovine serum for 30 min at room temperature. Then, phospho NF-κB p65 primary antibody (Santa Cruz Biotech, USA) diluted (1:200) was added and incubated overnight at 4 °C. Subsequently, a universal solution of secondary antibodies with biotinylated anti-rabbit, anti-mouse, and anti-goat IgG (LSAB + System-HRP, DAKO) was added and left for 1 h at room temperature. After three washes with TBS, the slides containing the samples were incubated with the avidin-FITC complex (eBioscience™, ThermoFischer, GO, USA) for 1 h at room temperature, and then washed five times with TBS. Slides were mounted using mounting medium of glycerol-PBS, pH 8.5 (9:1) containing 0.5 μg/mL 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and 1 mM Trolox (both Sigma-Aldrich, USA). The slides were evaluated under fluorescence microscopy (EVOS FL Auto 2, Thermo Scientific, USA) using the appropriate filters. Twenty consecutive fields of the cover slip were digitally acquired, and the fluorescence intensity was measured by Image J software (NIH, USA), and normalized by cell number.

2.16. Measurement of cytokines

The supernatants from the anti-amastigote cell assay were used to measure levels of tumor necrosis factor-*alpha* (TNF-α), interleukins (IL)-6 and IL-10 by an ELISA procedure, according to the manufacturer's instructions (Thermo Fischer Scientific, Multiskan, GO, USA). The plates were read at 450 nm using an ELISA plate reader (Thermo Fischer Scientific, USA), and the results were expressed in pg of the cytokines/mL based on a standard curve.

2.17. Immunofluorescence analysis of inducible nitric oxide synthase (iNOS)

Peritoneal macrophages (1×10^6 cell/mL) were adhered in 24-well plates and infected with the promastigote form of *L. amazonensis* (1×10^7 cells/mL). Under the same conditions described in the anti-amastigote assay were treated with 12.5, 25 and 50 μg/mL of botryosphaeran, and analyzed for the presence of iNOS. Briefly, after cell culture and treatment, the cells were trypsinized for 15 min at 37 °C under 5% CO_2 and were put in Eppendorf. The pellet was fixed with methanol for 15 min, three washes with PBS (pH 7.4) and permeabilizated for 1 h with PBS containing 3% of albumin bovine serum at room temperature. Then, iNOS primary antibody (Santa Cruz Biotech, USA) diluted (1:200) was added and incubated overnight at 4 °C. Subsequently, a universal solution of secondary antibodies with biotinylated anti-rabbit, anti-mouse, and anti-goat IgG (LSAB + System-HRP, DAKO) was added and left for 1 h at room temperature. After three washes with PBS, the samples were incubated with avidin-FITC complex (eBioscience™, ThermoFischer, GO, USA) for 1 h at room temperature and then washed three times with PBS. Data acquisition and analysis were performed using a BD Accuri™ C6-Plus flow cytometer (BD Biosciences, San Jose, CA, USA).

2.18. Determination of superoxide dismutase (SOD) levels

L. amazonensis-infected macrophages, as described in the anti-amastigotes assay were treated with 12.5, 25 and 50 μg/mL of botryosphaeran, and measured the SOD levels. Briefly, after cell culture and treatment, the cells submitted a cycle of freezing at −80 °C and thawing at 37 °C for 30 min each. The method used for SOD is based on the enzyme's ability to inhibit the autoxidation of pyrogallol, as described by Marklund and Marklund (1974) [35].

2.19. Statistical analysis

Data from three independent experiments performed in triplicate

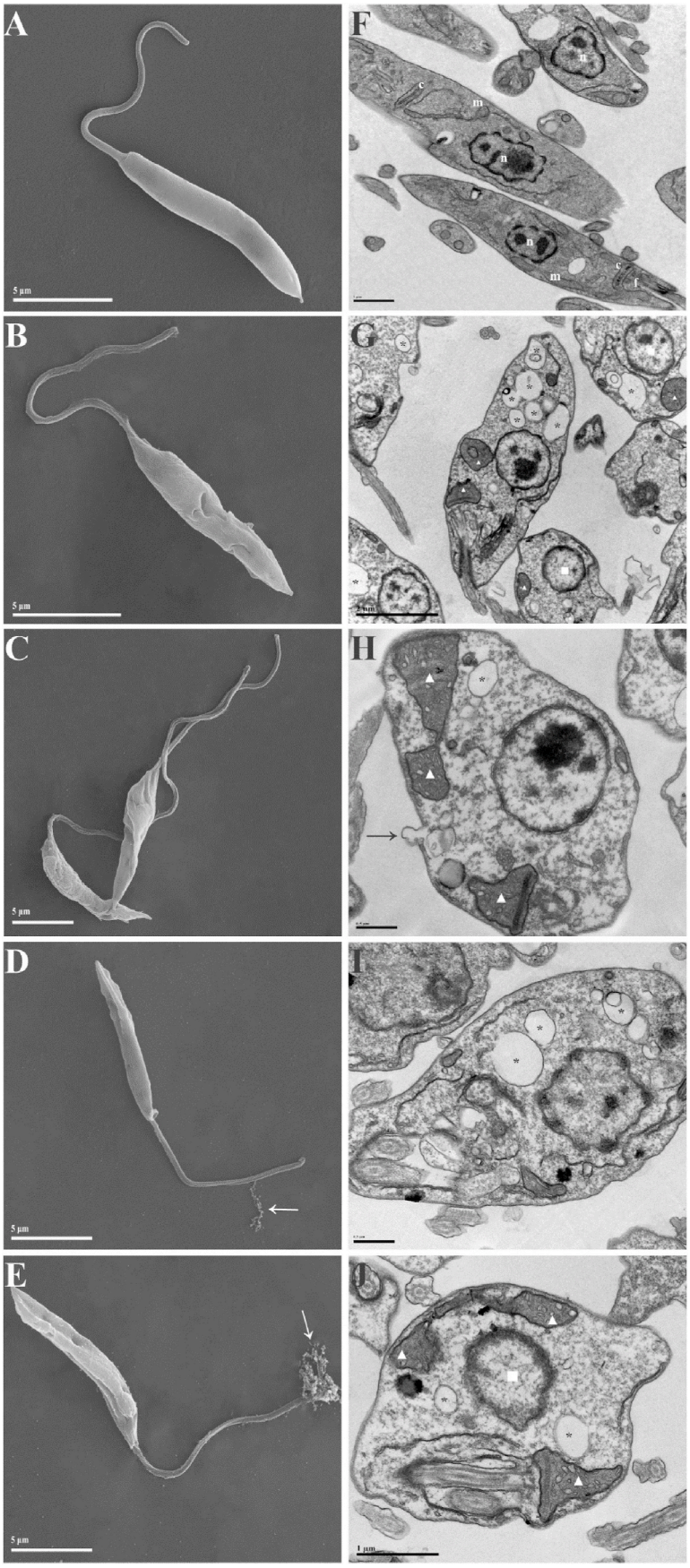

Fig. 3. Morphological and ultrastructural analysis of *L. amazonensis* promastigotes. Promastigote forms were treated with 7 and 14 µg/mL of botryosphaeran for 48 h and analyzed by scanning and transmission electron microscopy. (A and F) Control. (B, C, G and H) IC_{50}. (D, E, I, J) 2x IC_{50}. (A–E) Scanning electron microscopy. (F–K) Transmission Electron Microscopy. (white arrow) cell leakage; (black arrow), change in plasma membrane; ▲, mitochondrial swelling; *, autophagic vacuoles; ■, nuclear alteration; c, kinetoplast; f, scourge; m, mitochondria; n, nucleus. Bars = 5 µm (A–E), 2 µm (G), 1 µm (F, J) and 0.5 µm (H, I).

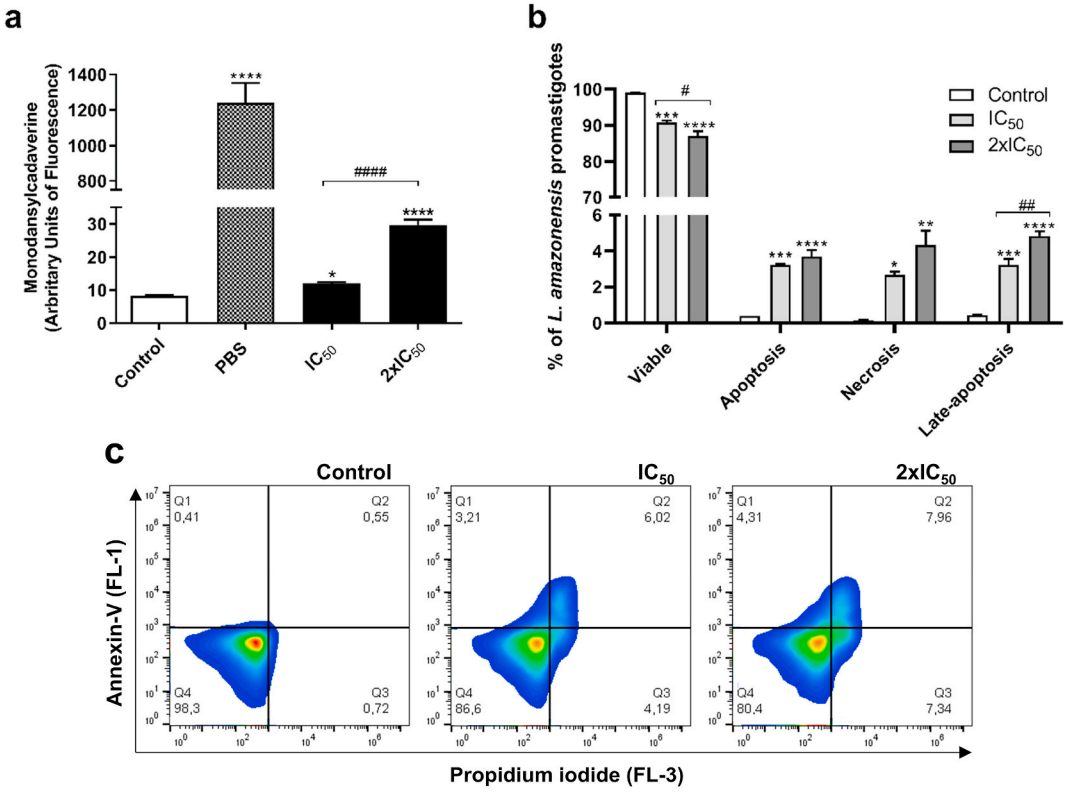

Fig. 4. Autophagic and apoptotic processes induced by botryosphaeran in promastigote forms of *L. amazonensis*. The parasites were treated for 48 h with IC_{50} (7.0 μg/mL) and 2x IC_{50} (14 μg/mL) and analyzed using monodansylcadaverine for quantification of autophagic vacuoles by fluorimetry (a), quantitative analysis of apoptosis, necrosis and late apoptosis by annexin-V, propidium iodide (PI) and double labeling, respectively (b), and control (untreated parasites) and promastigotes treated with botryosphaeran in the IC_{50} (7 μg/mL) and 2x IC_{50} (14 μg/mL), stained with annexin V-FITC and PI and analyzed by flow cytometry (**c**). Apoptosis was considered as Annexin V-FITC + /PI-, necrosis as Annexin V-FITC-/PI+, and late-apoptosis as Annexin V-FITC + /PI +. The values represent the mean ± SEM of three independent experiments carried out in triplicate. * Significant difference compared to the control ($p \leq 0.05$), ** ($p \leq 0.001$), *** ($p \leq 0.0005$), **** ($p \leq 0.0001$) and between treatments # ($p \leq 0.05$), ## ($p \leq 0.001$), #### ($p \leq 0.0001$).

and expressed as the mean ± standard error of the mean were used. The data were analyzed using GraphPad Prism® statistical software (GraphPad Software, Inc., USA, v. 500.288). Significant differences between treatments were determined by ANOVA, followed by the Tukey test for multiple comparisons. The differences were considered significant when $p \leq 0.05$.

3. Results and discussion

3.1. Botryosphaeran reduces proliferation of L. amazonensis

Botryosphaeran at concentrations (6.25, 12.5, 25, 50 and 100 μg/mL) and the times of treatment (24, 48, and 72 h), showed a significant reduction in the proliferation of the promastigote form of *L. amazonensis* in a concentration-time dependent relationship compared to the control (Fig. 1a) ($p \leq 0.01$) since the control presented an increase in the proliferation of the parasites at all tested times. Treatment with 1 μM AmB significantly reduced the proliferation of parasites compared to the control ($p \leq 0.001$) at all of the times tested. From the data presented, the IC_{50} value was calculated, and established the concentrations of botryosphaeran of 37.4; 7.0 and 13.0 μg/mL, respectively, for 24, 48 and 72 h of treatment (Fig. 1b, c, d). Also observed was the direct effect on parasites at dose-time dependency. We therefore chose to continue the experiments at the time when botryosphaeran exhibited the lowest effective concentration, i.e., 7.0 μg/mL (±0.21) at 48 h. No literature study too date has elucidated the possible mechanisms of direct action of a β-glucan on the promastigote form, nor on the immunomodulatory effects in the infection by *L. amazonensis*.

3.2. Mitochondrial hyperpolarization and oxidative stress

Having foreknowledge on the leishmanicidal effect of botryosphaeran, we analyzed what possible causes could be involved in the death of the promastigote form. We found that botryosphaeran treatment at 2x IC_{50} value (i.e., 14 μg/mL) was able to increase the levels of nitric oxide (NO) in the promastigotes after 48 h of treatment ($p \leq 0.001$) (Fig. 2a). NO, in addition to being a potent microbicide, is an important modulator of mitochondrial metabolism, affecting the crucial functions of mitochondria and generating hyperpolarization of mitochondrial membranes [36].

In this sense, we observed by fluorimetry that botryosphaeran (2x IC_{50}) caused mitochondrial hyperpolarization ($p \leq 0.05$) (Fig. 2b) which was reinforced by TEM images, showing swollen mitochondria (see below). Mitochondria are essential for the survival of trypanosomatids, such as *Leishmania* spp., as these parasites have a single mitochondria that is responsible for the generation of cellular ATP [37,38]. Mitochondrial hyperpolarization, in turn, is directly related to the synthesis of superoxide anion radical ($O_2^{-\cdot}$) and, consequently, raises the levels of intracellular ROS [37]. We found that concentrations of botryosphaeran at IC_{50} and 2x IC_{50} both caused an increase in ROS levels as measured by the H2DCFDA probe ($p \leq 0.05$), and the presence of lipid bodies ($p \leq 0.0005$) at 48 h of treatment is shown in Fig. 2b–d.

It is well-known that the hyperpolarization of mitochondrial can result in changes in the permeability of this organelle's membrane and induce the opening of non-selective and high conductance, transcription pores in the internal mitochondrial membrane. This phenomenon leads to mitochondrial swelling and eventually cell death due to apoptosis or necrosis [39]. Besides, Queiroz et al. (2015) [16] showed that

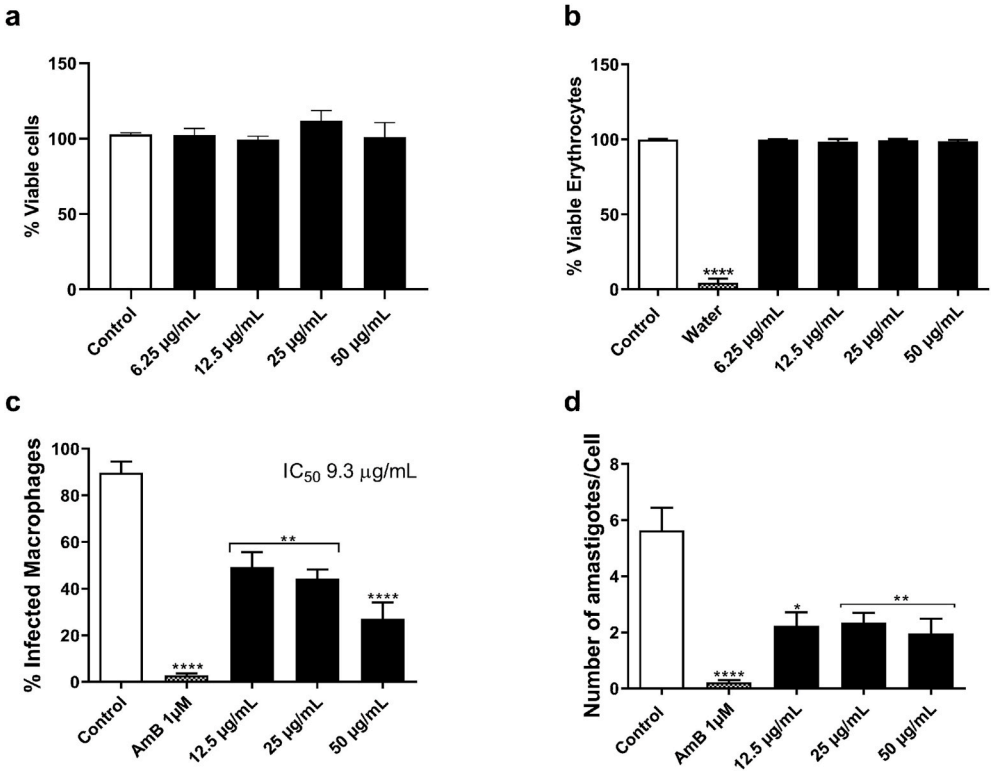

Fig. 5. Botryosphaeran does not alter the viability of peritoneal macrophages and sheep erythrocytes and has leishmanicidal effect on macrophages infected with *L. amazonensis*. Peritoneal macrophages (**a**) and sheep erythrocytes (**b**) were treated with botryosphaeran at concentrations of 6.25, 12.5, 25 and 50 µg/mL for 24 and 3 h, respectively. Positive control (PBS) and negative control (RPMI medium). Peritoneal macrophages were infected with *L. amazonensis* and treated with botryosphaeran (12.5, 25 and 50 µg/mL) for 24 h or Amphotericin B (1 µM) and analyzed the percentage of infected macrophages (**c**), and the number of amastigotes per macrophage (**d**). IC_{50} 9.3 µg/mL (±0.66) was calculated for the amastigotes forms at 24 h. The values represent the mean ± SEM of three independent experiments carried out in triplicate. * Significant difference compared to the control ($p \leq 0.05$) ** ($p \leq 0.01$) **** ($p \leq 0.0001$).

botryosphaeran was able to increase the production of ROS in the breast carcinoma cell line (MCF-7) in 48 h. Oxidative stress causes damage to genetic material, proteins, and lipids, which can cause the deposition of lipids in the cytosol [40,41]. The presence of lipid vacuoles as we observed in promastigotes of *L. amazonensis*, may in turn, be indicative of cell death [42].

3.3. Botryosphaeran induces different types of death in L. amazonensis promastigotes

For organisms such as *Leishmania* spp., autophagy plays a fundamental role, participating in several effects, from survival to the depletion of nutrients to the differentiation of its morphology during the biological cycle [43]. Studies have suggested that autophagy may precede, or even trigger, apoptosis [44].

We also observed morphological and ultrastructural changes by SEM and TEM (see Fig. 3), when compared to the controls (Fig. 3a–f) whereby the promastigotes treated with IC_{50} and 2x IC_{50} of botryosphaeran showed a roughened appearance of the cell surface (Fig. 3b–e). Atypical nuclei with chromatin marginalization and changes in the plasma membrane were also be observed (Fig. 3g and h). Treatment with 2x IC_{50} botryosphaeran showed extravasation of cellular content in the region of the flagellum, with necrosis becoming more evident (Fig. 3d and e).

Our next objective was to identify the type of cell death triggered by botryosphaeran in the treated promastigote forms. First, we confirmed that treatments with IC_{50} and 2x IC_{50} of botryosphaeran for 48 h were able to cause an accumulation of autophagic vacuoles in the parasite (Fig. 4a) ($p \leq 0.05$), as was also observed by TEM (see Fig. 3g and h). Our results showed an increase in the annexin-V (apoptosis), propidium iodide (necrosis) and double staining parasite population (apoptosis-like), demonstrating that the treatment was capable of inducing both apoptosis and necrosis ($p \leq 0.0005$) (Fig. 4b).

An apoptosis-like effect is described in trypanosomatids, as they share morphological, biochemical and molecular aspects typical of metazoan apoptosis, however, they do not produce canonical mediators as members of the Bcl-2 family, caspases and TNF-related family of receptors [45,46]. In *Leishmania* spp., cell rounding and shrinkage, changes in the plasma membrane but maintaining its integrity, mitochondrial modification, chromatin condensation, nuclear and DNA fragmentation, may be present [47].

In cases where apoptotic cells persist due to an overload of dead cells, these early apoptotic cells will progress to late apoptotic cells, where the plasma membrane becomes more permeable, and is observed by the double annexin-V/PI (Annexin V-FITC + /PI +) labelling [48]. When the lesions become extensive, the cells can accidently die from necrosis. This is morphologically characterized by a gain in cell volume, swelling of the organelle, loss of integrity of the plasma membrane and, consequently, loss of intracellular content. This sequence of events has been described as a potential inducer of regulated cell death [47,49].

Several studies have demonstrated the importance of inducing this type of cell death with compounds possessing leishmanicidal action [28,32,33,50,51]. Botryosphaeran treatment of breast carcinoma MCF-7 cells was demonstrated to increase the levels of apoptosis that was associated with oxidative stress and activation of AMPK and FOXO3a [16].

3.4. Botryosphaeran eliminates amastigote forms without causing cell toxicity

Knowing the effect of botryosphaeran on promastigote forms, we investigated its potential action on intracellular amastigotes. First, we evaluated if botryosphaeran exerted cytotoxic effects on primary cells and observed that at all of the concentrations tested botryosphaeran was not toxic to both peritoneal macrophages of BALB/c mice and sheep erythrocytes (Fig. 5a and b). Corroborating our results, Roudbary et al. (2015) demonstrated that a β-glucan from *Saccharomyces cerevisiae*, did not present cytotoxicity on peritoneal macrophages [52]. Furthermore, Choromanska et al. (2018) demonstrated that hemolysis of human erythrocytes by oat β-glucan (a β-(1 → 3)(1 → 4)-linked glucan) occurred at concentrations above 300 µg/mL in hypotonic saline solutions [53].

Next, we evaluated the treatment of botryosphaeran on peritoneal macrophages infected with *L. amazonensis*. Macrophages are the main

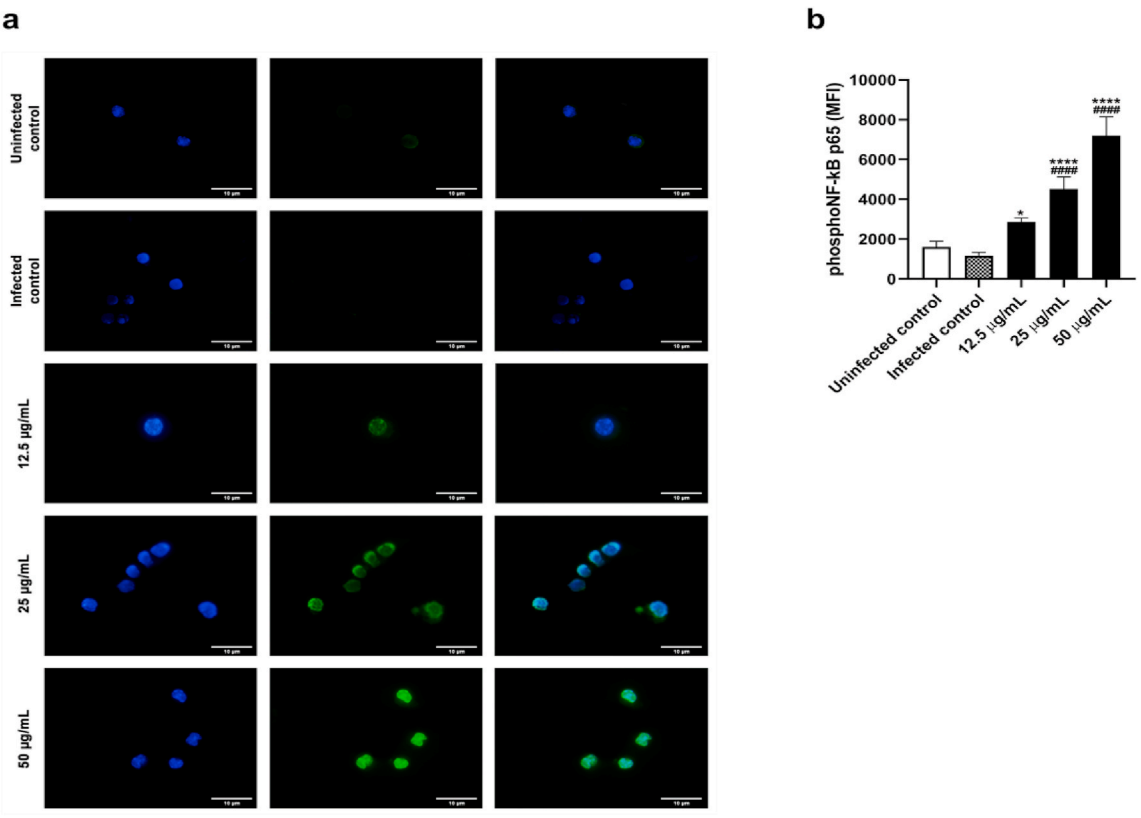

Fig. 6. Botryosphaeran induces activation of phosphorylated NF-κB. Peritoneal macrophages were infected with *L. amazonensis* and treated with botryosphaeran for 24 h at concentrations of 12.5, 25 and 50 μg/mL. After culture, the coverslips containing adhered cells were labeled with the primary antibody and subsequently added secondary antibodies. Then the slides were mounted using glycerol-PBS pH 8.5 (9:1) mouting medium containing DAPI and Trolox (Sigma, USA) and analyzed using an EVOS FL Auto 2 fluorescence microscope (Thermo Scientific, USA). Twenty consecutive fields were acquired digitally, the fluorescence intensity was measured using Image J software (NIH, USA) and normalized by the number of cells. The values represent the mean ± SEM of three independent experiments carried out in triplicate. * Significant difference compared to infected and treated control ($p \leq 0.05$), **** ($p \leq 0{,}0001$) and significant difference between uninfected control and treated #### ($p \leq 0{,}0001$).

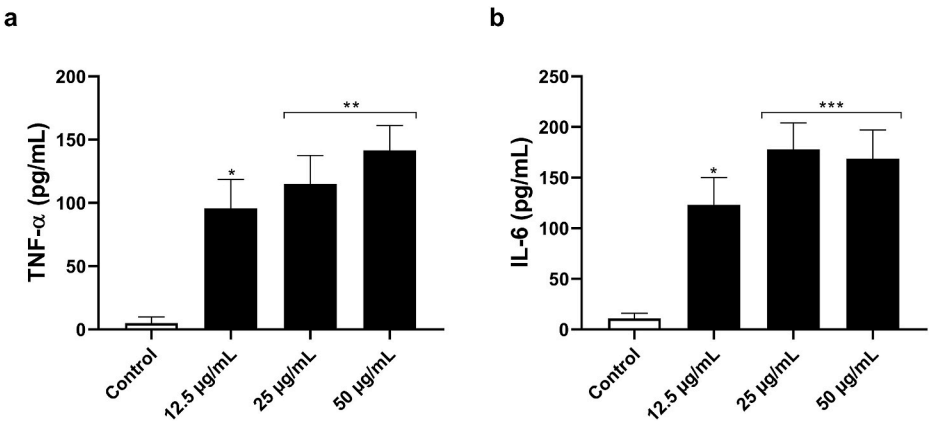

Fig. 7. Botryosphaeran increasing the levels of cytokines. Peritoneal macrophages were infected by *L. amazonensis* and subjected to treatment with botryosphaeran at concentrations of 12.5, 25 and 50 μg/mL for 24 h. Cytokines TNF-α (**a**) and IL-6 (**b**) levels in the culture supernatant was measured by ELISA. The values represent the mean ± SEM of three independent experiments carried out in triplicate. ** Significant difference compared to the control ($p \leq 0.01$), *** ($p \leq 0.0005$).

host cells for *Leishmania* spp., which are fundamental in the immune response against these parasites [54]. Our results showed that botryosphaeran treatment was able to reduce the percentage of infected macrophages at concentrations of 12.5 (50.75% ± 6.48), 25 (55.66% ± 3.93) and 50 μg/mL (72.9% ± 6.98), when compared to the control ($p \leq 0.01$) (Fig. 5c). A reduction in the number of amastigotes by macrophages treated with botryosphaeran at concentrations of 12.5; 25 and 50 μg/mL ($p \leq 0.05$) was also observed (Fig. 5d). Also, an IC_{50} of 9.3 μg/mL (±0.66) was calculated for the amastigotes forms at 24 h. A similar observation was reported by Shivahare et al. (2016) in evaluating the activity of lentinan, a β-glucan of the β-(1 → 3)(1 → 6)-linked type from shiitake (*Lentinula edodes*), against macrophages infected by *L. donovani*, recording an inhibition of 55.8% ± 3.8 of the parasitic load at the highest concentration examined (20 μg/mL) [25].

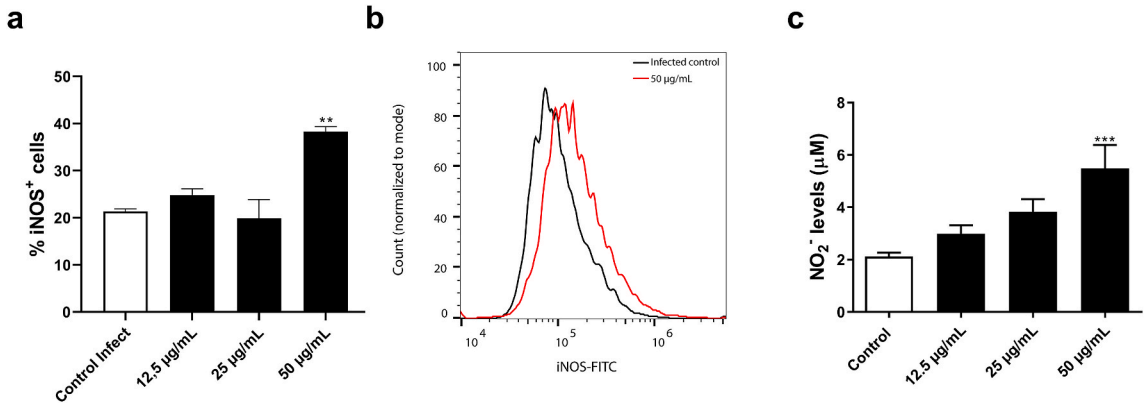

Fig. 8. Botryosphaeran increasing the levels of iNOS and NO. Peritoneal macrophages were infected by *L. amazonensis* and subjected to treatment with botryosphaeran at concentrations of 12.5, 25 and 50 μg/mL for 24 h. After culture, the cells were labeled with the primary antibody and subsequently added secondary antibodies. The iNOS fluorescence intensity was analyzed by flow cytometer (**a, b**). NO levels in the culture supernatant were measured by Griess method (**c**). The values represent the mean ± SEM of three independent experiments carried out in triplicate. ** Significant difference compared to the control ($p \leq 0.01$), *** ($p \leq 0.0005$).

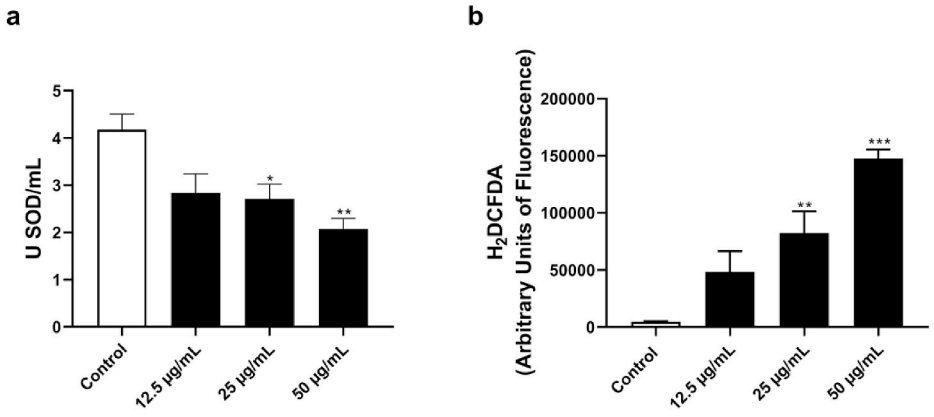

Fig. 9. Botryosphaeran decreases the levels of SOD and increases ROS levels. Peritoneal macrophages were infected by *L. amazonensis* and subjected to treatment with botryosphaeran at concentrations of 12.5, 25 and 50 μg/mL for 24 h. SOD levels were measured by autoxidation of pyragallol (**a**). The H2DCFDA probe was used to measure ROS (**b**). The values represent the mean ± SEM of three independent experiments carried out in triplicate. * Significant difference compared to the control ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.0005$).

3.5. Mechanisms of elimination of amastigote forms by botryosphaeran

Once the effect of botryosphaeran on infected macrophages was known, we investigated possible mechanisms by which the elimination of amastigote forms occurred. Our results showed that treatment with botryosphaeran was able to activate NF-κB (phosphorylated NF-κB) (Fig. 6) and the levels of cytokines TNF-α and IL-6 increased at all of the concentrations evaluated (Fig. 7). The IL-10 levels did not reach the level of detection (data not shown).

Furthermore, botryosphaeran is able to increase the production of iNOS (Fig. 8a and b) and, as a consequence, the production of NO at a concentration of 50 μg/mL (Fig. 8c), when compared to the control ($p \leq 0.01$).

We also observed an increase in ROS production and a decrease in SOD levels at concentrations of 25 and 50 μg/mL, when compared to the control ($p \leq 0.01$) (Fig. 9).

Literature studies have reported that β-glucans can act as immunomodulators, activating cellular and humoral components and increasing the activity of cells such as macrophages binding to cell surface receptor sites, such as dectin-1 [55–57]. The binding of β-glucans with dectin-1 triggers a series of intracellular signaling cascades. Dectin-1 has in its tail a motif for the activation of the tyrosine-based immunoreceptor (ITAM), which upon binding by β-glucan, the ITAM sequences (tyrosine) become phosphorylated by the kinases of the Src family, providing a site of connection for Syk anchoring, and initiating subsequent signaling events downstream. These events involve the CARD9 adapter, activation of protein activation by mitogen kinases (MAP), nuclear factor of activated T cells (NFAT), and NF-κB [57]. However, no studies are showing the receptor and botryosphaeran interaction, but since it is a β-glucan, it can present or trigger similar signaling pathways. Therefore, more research is needed.

L. amazonensis is known to escape the defense of the host by activating the NF-κB repressor complex, leading to a reduction in NO synthesis [58]. However, in macrophages stimulated with β-glucan, there is cooperation between dectin-1 and toll-like receptors (TLRs) acting both in a dependent and collaborative manner, triggering inflammatory and microbicidal responses [59]. Gantner et al. (2003) demonstrated that the binding of β-glucan to dectin-1 results in the phosphorylation of ITAM-like, generating intracellular signals that mediate phagocytosis and the activation of NADPH oxidase, a mitochondrial enzyme important in the synthesis of ROS. In addition, TLR stimulation leads to the activation of NF-κB and the production of pro-inflammatory cytokines such as TNF-α, IL-12 and iNOS [60,61].

For the production of pro-inflammatory cytokines, dectin-1 requires cooperative signaling from other TLRs coupled to Myd88, resulting in improved translocation of NF-κB to the nucleus, and consequently resulting in the induction of multiple cytokines that include TNF-α, IL-6, IL-10, IL-12, IL-23, among others [56,57,62–64]. Furthermore, iNOS expression can be induced by proinflammatory cytokines, and consequently, there is an increase in NO production in large amounts in macrophages activated by external stimuli [65].

Lentinan from L. *edodes* is of the (1 → 3)(1 → 6)-β-D-glucan type, which has a similar chemical structure to botryosphaeran, but where the side-appendages of both β-glucans differ [11]. In the model of

L. donovani, Shivahare et al. (2016) observed the immunomodulatory effect of lentinan potentiated pro-inflammatory responses, such as IL-12, TNF-α and NO [25].

In the context of leishmaniasis, a positive regulation of pro-inflammatory cytokines, such as TNF-α and IL-6, is associated with the induction of a protective response manifested by the production of ROS and NO, which together combine to "kill" the pathogen, mainly through direct oxidative damage [66,67]. In addition, *Leishmania* developed efficient protection against ROS, inducing the expression of the enzyme superoxide dismutase (SOD), whose antioxidant function is capable of converting $O_2^{-\bullet}$ into molecular oxygen (O_2) [68,69].

Thus, the development of a drug with immunomodulatory properties that induces the elimination of the parasite would be an ideal tool in the fight against infections caused by *Leishmania* spp, and botryosphaeran could become a lead compound for new prototypes of antileishmanial drugs.

4. Conclusion

Altogether, our results showed that botryosphaeran demonstrated leishmanicidal activity in promastigote forms of *Leishmania (L.) amazonensis*, acting in autophagic, necrotic and apoptotic processes. In *Leishmania*-infected macrophages, an immunomodulatory effect was observed, with an increase in NF-κB levels, induced the production of TNF-α, IL-6, iNOS, NO and ROS, and decreased levels of SOD, contributing to the elimination of intracellular amastigote forms. It is important to highlight that these data are the first to determine the mechanisms for the elimination of *L. amazonensis in vitro* when treated with a β-glucan, more specifically botryosphaeran. As a result, botryosphaeran becomes a potential compound for use *in vivo* assays.

CRediT authorship contribution statement

Amanda Cristina Machado Carloto: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing – original draft. Bruna Taciane da Silva Bortoleti: Methodology, Formal analysis, Investigation, Data Curation, Writing – original draft. Ana Carolina Jacob Rodrigues: Methodology, Investigation, Formal analysis, Writing – original draft. Taylon Felipe Silva: Methodology, Investigation, Formal analysis, Writing – original draft. Fernanda Tomiotto-Pellissier: Methodology, Formal analysis, Investigation, Data Curation, Writing – original draft. Danielle Lazarin-Bidóia: Methodology, Investigation, Formal analysis. Manoela Daiele Gonçalves: Methodology, Investigation, Writing – original draft. João Paulo Assolini: Methodology, Investigation, Writing – original draft. Robert Frans Huibert Dekker: Methodology, Writing – review & editing. Aneli Mello Barbosa-Dekker: Conceptualization, Methodology, Resources. Idessania Nazareth Costa: Resources, Writing – review & editing, Project administration, Funding acquisition. Ivete Conchon-Costa: Resources, Writing – review & editing, Project administration, Funding acquisition. Milena Menegazzo Miranda-Sapla: Conceptualization, Methodology, Investigation, Writing – review & editing, Project administration. Wander Rogério Pavanelli: Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Funding

This study was supported by Conselho Nacional de Pesquisa (CNPq, Brazil), Coordenadoria de Aperfeiçoamento Pessoal de Nível Superior (CAPES, Brazil), and Fundação Araucária - Governo do Estado do Paraná (Brazil).

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.

Acknowledgments

The authors gratefully acknowledge Dr[a] Danielle Lazarin-Bidóia, Laboratório de Microscopia Eletrônica de Varredura, Laboratório de Microscopia Eletrônica de Transmissão and Complexo de Centrais de Apoio à Pesquisa (COMCAP) at the Universidade Estadual de Maringá (UEM) for the images of scanning and transmission electron microscopy.

References

[1] S. Burza, S.L. Croft, M. Boelaert, Leishmaniasis, Lancet 392 (2018) 951–970, https://doi.org/10.1016/S0140-6736(18)31204-2.
[2] (n.d. Leishmaniasis (accessed March 8, 2021), https://www.who.int/news-room/fact-sheets/detail/leishmaniasis.
[3] L.s. Anversa, M.G.S. Tiburcio, V. nia B, Richini-Pereira, L.E. Ramirez, Human leishmaniasis in Brazil: a general review, Rev. Assoc. Med. Bras. 64 (2018) 281–289, https://doi.org/10.1590/1806-9282.64.03.281.
[4] S. Sundar, B. Singh, Emerging therapeutic targets for treatment of leishmaniasis, Expert Opin. Ther. Targets 22 (2018) 467–486, https://doi.org/10.1080/14728222.2018.1472241.
[5] M. Ghorbani, R. Farhoudi, Leishmaniasis in humans: drug or vaccine therapy? Drug Des. Dev. Ther. 12 (2018) 25–40, https://doi.org/10.2147/DDDT.S146521.
[6] H.-K.A. Jung, Seung-Chun Park, A.E. Byung-Kwon Park, A.E. Joo-Heon Hong, Physiological Activities of a B-Glucan Produced by Panebacillus Polymyxa, 2008, https://doi.org/10.1007/s10529-008-9732-3.
[7] S. Mahapatra, D. Banerjee, M. Banerjee, Fungal exopolysaccharide: production, composition and Applications, Microbiol. Insights (2013) 6–7, https://doi.org/10.4137/MBI.S10957.
[8] V. Vetvicka, R. Fernandez-Botran, Minireview β-Glucan and parasites, Helminthologia 55 (2018) 177–184, https://doi.org/10.2478/helm-2018-0021.
[9] J. Chen, R. Seviour, Medicinal importance of fungal β-(1→3), (1→6)-glucans, Mycol. Res. 111 (2007) 635–652, https://doi.org/10.1016/j.mycres.2007.02.011.
[10] H.-J. Graubaum, R. Busch, H. Stier, J. Gruenwald, A double-blind, randomized, placebo-controlled nutritional study using an insoluble yeast beta-glucan to improve the immune defense system, Food Nutr. Sci. 3 (2012) 738–746, https://doi.org/10.4236/fns.2012.36100.
[11] A.M. Barbosa, R.M. Steluti, R.F.H. Dekker, M.S. Cardoso, M.L. Corradi Da Silva, Structural characterization of Botryosphaeran: a (1→3;1→6)-β-D-glucan produced by the ascomyceteous fungus, Botryosphaeria sp, Carbohydr. Res. 338 (2003) 1691–1698, https://doi.org/10.1016/S0008-6215(03)00240-4.
[12] R.F.H. Dekker, E.A.I.F. Queiroz, M.A.A. Cunha, A.M. Barbosa-Dekker, Botryosphaeran – A Fungal Exopolysaccharide of the (1→3)(1→6)-β-D-Glucan Kind: Structure and Biological Functions, 2019, pp. 433–484, https://doi.org/10.1007/978-3-030-12919-4_11.
[13] L.E. Kerche-Silva, I.M.S. Cólus, M. Malini, M.P. Mori, R.F.H. Dekker, A.M. Barbosa-Dekker, In vitro protective effects of botryosphaeran, a (1 → 3;1 → 6)-β-D-glucan, against mutagens in normal and tumor rodent cells, Mutat, Res. - Genet. Toxicol. Environ. Mutagen. 814 (2017) 29–36, https://doi.org/10.1016/j.mrgentox.2016.12.003.
[14] M. Malini, M.S. Camargo, L.C. Hernandes, C.G. Vargas-Rechia, E.A. Varanda, A. M. Barbosa, R.F.H. Dekker, S.T. Matsumoto, L.M.G. Antunes, I.M.S. Cólus, Chemopreventive effect and lack of genotoxicity and mutagenicity of the exopolysaccharide botryosphaeran on human lymphocytes, Toxicol. Vitro 36 (2016) 18–25, https://doi.org/10.1016/j.tiv.2016.06.008.
[15] M. Malini, M.F. de Souza, M.T. de Oliveira, L.M.G. Antunes, S.G. de Figueiredo, A. M. Barbosa, R.F.H. Dekker, I.M. de S. Cólus, Modulation of gene expression and cell cycle by botryosphaeran, a (1→3)(1→6)-β-d-glucan in human lymphocytes, Int. J. Biol. Macromol. 77 (2015) 214–221, https://doi.org/10.1016/j.ijbiomac.2015.03.010.
[16] E.A.I.F. Queiroz, Z.B. Fortes, M.A.A. Da Cunha, A.M. Barbosa, N. Khaper, R.F. H. Dekker, Antiproliferative and pro-apoptotic effects of three fungal exocellular β-glucans in MCF-7 breast cancer cells is mediated by oxidative stress, AMP-activated protein kinase (AMPK) and the Forkhead transcription factor, FOXO3a, Int. J. Biochem. Cell Biol. 67 (2015) 14–24, https://doi.org/10.1016/j.biocel.2015.08.003.
[17] E.C. Giese, J. Gascon, G. Anzelmo, A.M. Barbosa, M.A.A. da Cunha, R.F.H. Dekker, Free-radical scavenging properties and antioxidant activities of botryosphaeran and some other β-D-glucans, Int. J. Biol. Macromol. 72 (2015) 125–130, https://doi.org/10.1016/j.ijbiomac.2014.07.046.
[18] B.A.L. Sacchelli, L.C. Faccin-Galhardi, V.Y. Ito, J.L. Lopes, R.F.H. Dekker, A. M. Barbosa-Dekker, A. Orsato, Botryosphaeran and sulfonated derivatives as novel antiviral agents for herpes simplex and dengue fever, Int. J. Biol. Macromol. 138 (2019) 334–339, https://doi.org/10.1016/j.ijbiomac.2019.07.084.
[19] S.F. Mendes, O. dos Santos, A.M. Barbosa, A.F.D. Vasconcelos, G. Aranda-Selverio, N.K. Monteiro, R.F.H. Dekker, M.S. Pereira, A.M.F. Tovar, P.A.d.S. Mourão, M. de L.C. da Silva, Sulfonation and anticoagulant activity of botryosphaeran from Botryosphaeria rhodina MAMB-05 grown on fructose, Int. J. Biol. Macromol. 45 (2009) 305–309, https://doi.org/10.1016/j.ijbiomac.2009.06.004.
[20] C.C.B.O. Miranda, R.F.H. Dekker, J.M. Serpeloni, E.A.I. Fonseca, I.M.S. Cólus, A. M. Barbosa, Anticlastogenic activity exhibited by botryosphaeran, a new

exopolysaccharide produced by Botryosphaeria rhodina MAMB-05, Int. J. Biol. Macromol. 42 (2008) 172–177, https://doi.org/10.1016/j.ijbiomac.2007.10.010.
[21] G.G. Silva-Sena, M. Malini, J.M. Delarmelina, J.C.V. Dutra, S.V. Gervásio, M.A. S. Leal, T. de M. Costa Pereira, A.M. Barbosa-Dekker, R.F.H. Dekker, F. de Paula, M. do C.P. Batitucci, In vivo antimutagenic and antiatherogenic effects of the (1 → 3) (1 → 6)-β-D- glucan botryosphaeran, Mutat. Res. Genet. Toxicol. Environ. Mutagen 826 (2018) 6–14, https://doi.org/10.1016/j.mrgentox.2017.12.008.
[22] C.C.B.O. Miranda-Nantes, E.A.I. Fonseca, C.T.B.V. Zaia, R.F.H. Dekker, N. Khaper, I.A. Castro, A.M. Barbosa, Hypoglycemic and hypocholesterolemic effects of botryosphaeran from Botryosphaeria rhodina MAMB-05 in diabetes-induced and hyperlipidemia conditions in rats, MYCOBIOLOGY 39 (2011) 187–193, https://doi.org/10.5941/MYCO.2011.39.3.187.
[23] A.Z. Silva, F.P.L. Costa, I.L. Souza, M.C. Ribeiro, M.A. Giordani, D.A. Queiroz, R.A. M. Luvizotto, A.F. Nascimento, G.F. Bomfim, M.M. Sugizaki, R.F.H. Dekker, A. M. Barbosa-Dekker, E.A.I.F. Queiroz, Botryosphaeran reduces obesity, hepatic steatosis, dyslipidaemia, insulin resistance and glucose intolerance in diet-induced obese rats, Life Sci. 211 (2018) 147–156, https://doi.org/10.1016/j.lfs.2018.09.027.
[24] D. Geraldelli, M.C. Ribeiro, T.C. Medeiros, P.K. Comiran, K.O. Martins, M. F. Oliveira, G.A. Oliveira, R.F.H. Dekker, A.M. Barbosa-Dekker, P. Alegranci, E.A.I. F. Queiroz, Botryosphaeran, a (1 → 3)(1 → 6)-β-D-Glucan, Reduces Tumor Development and Cachexia Syndrome in Obese Male Rats by Increasing Insulin Sensitivity and FOXO3a Activity,, 2020, https://doi.org/10.1016/j.ijbiomac.2020.09.168.
[25] R. Shivahare, W. Ali, U.S. Singh, S.M. Natu, S. Khattri, S.K. Puri, S. Gupta, Immunoprotective effect of lentinan in combination with miltefosine on Leishmania-infected J-774A.1 macrophages, Parasite Immunol. 38 (2016) 618–627, https://doi.org/10.1111/pim.12346.
[26] R. Goldman, C.L. Jaffe, Administration of β-glucan following Leishmania major infection suppresses disease progression in mice, Parasite Immunol. 13 (1991) 137–145, https://doi.org/10.1111/j.1365-3024.1991.tb00270.x.
[27] L. Yatawara, S. Wickramasinghe, M. Nagataki, M. Takamoto, H. Nomura, Y. Ikeue, Y. Watanabe, T. Agatsuma, Aureobasidium-derived soluble branched (1,3-1,6) β-glucan (sophy β-glucan) enhances natural killer activity in Leishmania amazonensis-infected mice, Kor. J. Parasitol. 47 (2009) 345–351, https://doi.org/10.3347/kjp.2009.47.4.345.
[28] F. Tomiotto-Pellissier, D.R. Alves, M.M. Miranda-Sapla, S.M. de Morais, J. P. Assolini, B.T. da Silva Bortoleti, M.D. Gonçalves, A.H.D. Cataneo, D. Kian, T. B. Madeira, L.M. Yamauchi, S.L. Nixdorf, I.N. Costa, I. Conchon-Costa, W. R. Pavanelli, Caryocar coriaceum extracts exert leishmanicidal effect acting in promastigote forms by apoptosis-like mechanism and intracellular amastigotes by Nrf2/HO-1/ferritin dependent response and iron depletion: leishmanicidal effect of Caryocar coriaceum leaf exracts, Biomed. Pharmacother. 98 (2018) 662–672, https://doi.org/10.1016/j.biopha.2017.12.083.
[29] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric Method for Determination of Sugars and Related Substances, (n.d).
[30] C.A. Antwi, C.M. Amisigo, J.P. Adjimani, T.M. Gwira, In vitro activity and mode of action of phenolic compounds on Leishmania donovani, PLoS Neglected Trop. Dis. 13 (2019), e0007206, https://doi.org/10.1371/JOURNAL.PNTD.0007206.
[31] M.D. Gonçalves, B.T.S. Bortoleti, F. Tomiotto-Pellissier, M.M. Miranda-Sapla, J. P. Assolini, A.C.M. Carloto, P.G.C. Carvalho, E.T. Tudisco, A. Urbano, S. R. Ambrósio, E.Y. Hirooka, A.N.C. Simão, I.N. Costa, W.R. Pavanelli, I. Conchon-Costa, N.S. Arakawa, Dehydroabietic acid isolated from Pinus elliottii exerts in vitro antileishmanial action by pro-oxidant effect, inducing ROS production in promastigote and downregulating Nrf2/ferritin expression in amastigote forms of Leishmania amazonensis, Fitoterapia 128 (2018) 224–232, https://doi.org/10.1016/j.fitote.2018.05.027.
[32] B.T. da S. Bortoleti, M.D. Gonçalves, F. Tomiotto-Pellissier, M.M. Miranda-Sapla, J. P. Assolini, A.C.M. Carloto, P.G.C. de Carvalho, I.L.A. Cardoso, A.N.C. Simão, N. S. Arakawa, I.N. Costa, I. Conchon-Costa, W.R. Pavanelli, Grandiflorenic acid promotes death of promastigotes via apoptosis-like mechanism and affects amastigotes by increasing total iron bound capacity, Phytomedicine 46 (2018) 11–20, https://doi.org/10.1016/j.phymed.2018.06.010.
[33] M.M. Miranda-Sapla, F. Tomiotto-Pellissier, J.P. Assolini, A.C.M. Carloto, B.T. da S. Bortoleti, M.D. Gonçalves, E.R. Tavares, J.H. da S. Rodrigues, A.N.C. Simão, L. M. Yamauchi, C.V. Nakamura, W.A. Verri, I.N. Costa, I. Conchon-Costa, W. R. Pavanelli, trans-Chalcone modulates Leishmania amazonensis infection in vitro by Nrf2 overexpression affecting iron availability, Eur. J. Pharmacol. 853 (2019) 275–288, https://doi.org/10.1016/j.ejphar.2019.03.049.
[34] J.P. Assolini, T.P. da Silva, B.T. da Silva Bortoleti, M.D. Gonçalves, F. Tomiotto-Pellissier, C.S. Sahd, A.C.M. Carloto, P.E. Feuser, A.P. Cordeiro, C. Sayer, P. H. Hermes de Araújo, I.N. Costa, I. Conchon-Costa, M.M. Miranda-Sapla, W. R. Pavanelli, 4-nitrochalcone exerts leishmanicidal effect on L. amazonensis promastigotes and intracellular amastigotes, and the 4-nitrochalcone encapsulation in beeswax copaiba oil nanoparticles reduces macrophages cytotoxicity, Eur. J. Pharmacol. 884 (2020) 173392, https://doi.org/10.1016/j.ejphar.2020.173392.
[35] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469–474, https://doi.org/10.1111/J.1432-1033.1974.TB03714.X.
[36] G. Nagy, A. Koncz, D. Fernandez, A. Perl, Nitric oxide, mitochondrial hyperpolarization, and T cell activation, Free Radic. Biol. Med. 42 (2007) 1625–1631, https://doi.org/10.1016/j.freeradbiomed.2007.02.026.
[37] L.M. Fidalgo, L. Gille, Mitochondria and trypanosomatids: targets and drugs, Pharm. Res. (N. Y.) 28 (2011) 2758–2770, https://doi.org/10.1007/s11095-011-0586-3.
[38] L. Monzote, L. Gille, Mitochondria as a promising antiparasitic target, Curr. Clin. Pharmacol. 5 (2010) 55–66, https://doi.org/10.2174/157488410790410605.
[39] S. Javadov, X. Chapa-Dubocq, V. Makarov, Different approaches to modeling analysis of mitochondrial swelling, Mitochondrion 38 (2018) 58–70, https://doi.org/10.1016/j.mito.2017.08.004.
[40] P.D. Ray, B.W. Huang, Y. Tsuji, Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling, Cell. Signal. 24 (2012) 981–990, https://doi.org/10.1016/j.cellsig.2012.01.008.
[41] Y. Morita, O. Nascimento Silva, D. Bosco, B. Elmo Eduardo Almeida Amaral, I. Oswaldo Cruz, C. V Nakamura, D.B. Scariot, E.A. Britta, A.L. Moreira, H. Falzirolli, C.C. Silva, T. Ueda-Nakamura, B.P. Dias-Filho, Induction of Early Autophagic Process on Leishmania Amazonensis by Synergistic Effect of Miltefosine and Innovative Semi-synthetic Thiosemicarbazone, vol. 8, 2017, p. 255, https://doi.org/10.3389/fmicb.2017.00255.
[42] J. Boren, K.M. Brindle, Apoptosis-induced mitochondrial dysfunction causes cytoplasmic lipid droplet formation, Cell Death Differ. 19 (2012) 1561–1570, https://doi.org/10.1038/cdd.2012.34.
[43] S. Giri, C. Shaha, Leishmania donovani parasite requires Atg8 protein for infectivity and survival under stress, Cell Death Dis. 10 (2019), https://doi.org/10.1038/s41419-019-2038-7.
[44] G. Mariño, M. Niso-Santano, E.H. Baehrecke, G. Kroemer, Self-consumption: the interplay of autophagy and apoptosis, Nat. Rev. Mol. Cell Biol. 15 (2014) 81–94, https://doi.org/10.1038/nrm3735.
[45] W.R. Proto, G.H. Coombs, J.C. Mottram, Cell Death in Parasitic Protozoa: Regulated or Incidental?, 2012, https://doi.org/10.1038/nrmicro2929.
[46] D. Smirlis, K. Soteriadou, Trypanosomatid Apoptosis: "Apoptosis" without the Canonical Regulators, 2011, https://doi.org/10.4161/viru.2.3.16278.
[47] L. Basmaciyan, M. Casanova, La mort cellulaire chez Leishmania, Parasite 26 (2019) 71, https://doi.org/10.1051/parasite/2019071.
[48] I.K.H. Poon, M.D. Hulett, C.R. Parish, Molecular mechanisms of late apoptotic/necrotic cell clearance, Cell Death Differ. 17 (2010) 381–397, https://doi.org/10.1038/cdd.2009.195.
[49] R.F.S. Menna-Barreto, Cell death pathways in pathogenic trypanosomatids: lessons of (over)kill, Cell Death Dis. 10 (2019) 1234567890, https://doi.org/10.1038/s41419-019-1370-2.
[50] L.M.R. Antinarelli, I. de Oliveira Souza, P.V. Zabala Capriles, J. Gameiro, E. A. Britta, C.V. Nakamura, W.P. Lima, A.D. da Silva, E.S. Coimbra, Antileishmanial activity of a 4-hydrazinoquinoline derivative: induction of autophagy and apoptosis-related processes and effectiveness in experimental cutaneous leishmaniasis, Exp. Parasitol. 195 (2018) 78–86, https://doi.org/10.1016/j.exppara.2018.10.007.
[51] A. López-Arencibia, M. Reyes-Batlle, M.B. Freijo, I. Sifaoui, C.J. Bethencourt-Estrella, A. Rizo-Liendo, O. Chiboub, G. Mcnaughton-Smith, J. Lorenzo-Morales, T. Abad-Grillo, J.E. Piñero, In vitro activity of 1H-phenalen-1-one derivatives against Leishmania spp. and evidence of programmed cell death, (n.d.). https://doi.org/10.1186/s13071-019-3854-4.
[52] Immunomodulatory Effect of β-Glucan on Peritoneal Macrophages of Babl/c Mice – PJM ONLINE, (n.d.). βhttp://www.pjmonline.org/immunomodulatory-effect-of-β-glucan-on-peritoneal-macrophages-of-bablc-mice/(accessed April 5, 2021).
[53] A. Choromanska, J. Kulbacka, J. Harasym, R. Oledzki, A. Szewczyk, J. Saczko, High- and low-molecular weight oat beta-glucan reveals antitumor activity in human epithelial lung cancer, Pathol. Oncol. Res. 24 (2018) 583–592, https://doi.org/10.1007/s12253-017-0278-3.
[54] F. Tomiotto-Pellissier, B.T. da S. Bortoleti, J.P. Assolini, M.D. Gonçalves, A.C. M. Carloto, M.M. Miranda-Sapla, I. Conchon-Costa, J. Bordignon, W.R. Pavanelli, Macrophage polarization in leishmaniasis: broadening horizons, Front. Immunol. 9 (2018) 2529, https://doi.org/10.3389/fimmu.2018.02529.
[55] Z.Q. Chang, J.S. Lee, E. Gebru, J.H. Hong, H.K. Jung, W.S. Jo, S.C. Park, Mechanism of macrophage activation induced by β-glucan produced from Paenibacillus polymyxa JB115, Biochem. Biophys. Res. Commun. 391 (2010) 1358–1362, https://doi.org/10.1016/j.bbrc.2009.12.064.
[56] D.M. Reid, N.A. Gow, G.D. Brown, Pattern recognition: recent insights from Dectin-1, Curr. Opin. Immunol. 21 (2009) 30–37, https://doi.org/10.1016/j.coi.2009.01.003.
[57] S.V. Tsoni, G.D. Brown, β-Glucans and dectin-1, Ann. N. Y. Acad. Sci. 1143 (2008) 45–60, https://doi.org/10.1196/annals.1443.019.
[58] T.C. Calegari-Silva, R.M.S. Pereira, L.D.B. De-Melo, E.M. Saraiva, D.C. Soares, M. Bellio, U.G. Lopes, NF-κB-mediated repression of iNOS expression in Leishmania amazonensis macrophage infection, Immunol. Lett. 127 (2009) 19–26, https://doi.org/10.1016/j.imlet.2009.08.009.
[59] B.N. Gantner, R.M. Simmons, S.J. Canavera, S. Akira, D.M. Underhill, Collaborative induction of inflammatory responses by dectin-1 and toll-like receptor 2, J. Exp. Med. J. Exp. Med 197 (2003) 1107–1117, https://doi.org/10.1084/jem.20021787.
[60] S. Mukherjee, S. Karmakar, S.P.S. Babu, TLR2 and TLR4 mediated host immune responses in major infectious diseases: a review, Braz. J. Infect. Dis. 20 (2016) 193–204, https://doi.org/10.1016/j.bjid.2015.10.011.
[61] H.S. Kim, J.T. Hong, Y. Kim, S.-B. Han, Stimulatory effect of β-glucans on immune cells, Immune Netw 11 (2011) 191, https://doi.org/10.4110/IN.2011.11.4.191.
[62] G.D. Brown, Dectin-1 : a signalling non-TLR pattern-recognition receptor, Nat. Rev. Immunol. 6 (2006) 33–43, https://doi.org/10.1038/nri1745.
[63] Z.Q. Chang, J.S. Lee, E. Gebru, J.H. Hong, H.K. Jung, W.S. Jo, S.C. Park, Mechanism of macrophage activation induced by β-glucan produced from

Paenibacillus polymyxa JB115, Biochem. Biophys. Res. Commun. 391 (2010) 1358–1362, https://doi.org/10.1016/j.bbrc.2009.12.064.

[64] E.H. Roma, J. Pereira Macedo, G.R. Goes, J.L. Gonçalves, W. De Castro, D. Cisalpino, L.Q. Vieira, Impact of Reactive Oxygen Species (ROS) on the Control of Parasite Loads and Inflammation in Leishmania Amazonensis Infection, 2016, https://doi.org/10.1186/s13071-016-1472-y.

[65] S.A. Jang, S.C. Kang, E.H. Sohn, Phagocytic effects of β-glucans from the mushroom Coriolus versicolor are related to dectin-1, NOS, TNF-α signaling in macrophages, Biomol. Ther. 19 (2011) 438–444, https://doi.org/10.4062/BIOMOLTHER.2011.19.4.438.

[66] M.F. Horta, B.P. Mendes, E.H. Roma, F.S.M. Noronha, J.P. MacDo, L.S. Oliveira, M. M. Duarte, L.Q. Vieira, Reactive oxygen species and nitric oxide in cutaneous leishmaniasis, 2012, J. Parasitol. Res. (2012), https://doi.org/10.1155/2012/203818.

[67] M. Rossi, N. Fasel, How to master the host immune system? Leishmania parasites have the solutions!, Int. Immunol. 30 (2018) 103–111, https://doi.org/10.1093/intimm/dxx075.

[68] C.C.B. Brito, H.V.C. da Silva, D.J. Brondani, A.R. de Faria, R.M. Ximenes, I.M. da Silva, J.F.C. de Albuquerque, M.S. Castilho, Synthesis and biological evaluation of thiazole derivatives as LbSOD inhibitors, J. Enzym. Inhib. Med. Chem. 34 (2019) 333, https://doi.org/10.1080/14756366.2018.1550752.

[69] J.F. Turrens, Oxidative stress and antioxidant defenses: a target for the treatment of diseases caused by parasitic protozoa, Mol. Aspect. Med. 25 (2004) 211–220, https://doi.org/10.1016/J.MAM.2004.02.021.