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Leishmanicidal activity of the *Agaricus blazei* Murill in different *Leishmania* species

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ABSTRACT

Leishmaniasis is a major public health problem, and the alarming spread of parasite resistance underlines the importance of discovering new therapeutic products. The present study aims to investigate the *in vitro* leishmanicidal activity of an *Agaricus blazei* Murill mushroom extract as compared to different *Leishmania* species and stages. The water extract proved to be effective against promastigote and amastigote-like stages of *Leishmania amazonensis*, *L. chagasi*, and *L. major*, with IC_{50} (50% inhibitory concentration) values of 67.5, 65.8, and 56.8 μg/mL for promastigotes, and 115.4, 112.3, and 108.4 μg/mL for amastigotes-like respectively. The infectivity of the three *Leishmania* species before and after treatment with the water extract was analyzed, and it could be observed that 82%, 57%, and 73% of the macrophages were infected with *L. amazonensis*, *L. major*, and *L. chagasi*, respectively. However, when parasites were pre-incubated with the water extract, and later used to infect macrophages, they were able to infect only 12.7%, 24.5%, and 19.7% of the phagocytic cells for *L. amazonensis*, *L. chagasi*, and *L. major*, respectively. In other experiments, macrophages were infected with *L. amazonensis*, *L. chagasi*, or *L. major*, and later treated with the aforementioned extract, presented reductions of 84.4%, 79.6%, and 85.3% in the parasite burden after treatment. A confocal microscopy revealed the loss of the viability of the parasites within the infected macrophages after treatment with the water extract. The applied extract presented a low cytotoxicity in murine macrophages and a null hemolytic activity in type O^+ human red blood cells. No nitric oxide (NO) production, nor inducible nitric oxide syntase expression, could be observed in macrophages after stimulation with the water extract, suggesting that biological activity may be due to direct mechanisms other than macrophage activation by means of NO production. In conclusion, the results demonstrate that the *A. blazei* Murill water extract can potentially be used as a therapeutic alternative on its own, or in association with other drugs, to treat Visceral and Cutaneous Leishmaniasis.

1. Introduction

Protozoan parasites of the *Leishmania* genus are the etiological agents of a vector-borne disease that has presented high morbidity and mortality throughout the world. Leishmaniasis has affected around 12 million people and is present in 88 countries, mainly in tropical and subtropical areas. The approximately 2 million new cases per year and the nearly 350 million people living in endemic regions reveal the importance of this neglected disease [1,2].

Historically, chemotherapy to treat leishmaniasis has been based on the use of pentavalent antimonial drugs. One of the available anti-leishmanial drugs, meglumine antimoniate (Glucantime®), is clinically unsatisfactory, once that it is not very effective and may have severe side effects. Furthermore, a high number of relapses of the disease have been described [3,4]. Pentamidine, another anti-leishmanial drug, is unsuitable as a first line treatment due to its toxicity. Amphotericin B and its liposomal formulation are effective, though such drugs are expensive and their use requires hospitalization. Reported clinical results using oral miltefosine treatment are encouraging; however, this drug is linked to potential teratogenicity and should not be given to pregnant women or to those of childbearing age [5]. The number of reported VL cases is increasing. Moreover, VL has emerged as an opportunistic infection in human immunodeficiency virus-infected (HIV) patients [6]. Therefore, the development of cost-effective alternative therapeutic strategies has become a high-priority.

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Prior research has been carried out concerning natural products and their biological effects, such as fungicidal, antimicrobial, antimalarial, antimycobacterial, and antiviral activities. However, only a few studies have investigated the biological potential of Brazilian mushrooms. *Agaricus blazei* Murill is a commonly found mushroom in Brazil. Its use has been associated with folk medicine in the treatment of some diseases, including diabetes mellitus and arterial hypertension [7–13]. This mushroom presents compounds, such as β-D-glucans, glycoproteins, cerebrosides, polysaccharides, steroids, ergosterol, and graxs acids, which can activate and/or modulate the immune response of the hosts [14–17].

The present study assessed the *in vitro* leishmanicidal activity of an *A. blazei* Murill water extract against *L. amazonensis*, *L. chagasi*, and *L. major* promastigote and amastigote-like stages. Studies were extended to establish their minimum inhibitory concentrations (IC_{50}), leishmanicidal effects on intra-macrophages *Leishmania* stages, nitric oxide (NO) production, inducible nitric oxide syntase (iNOS) expression, as well as their cytotoxic effects on murine macrophages and human red blood cells.

2. Material and methods

2.1. A. blazei Murill water extract

The *A. blazei* Murill water extract was prepared by macerating 50 g of fresh mushrooms in 50 mL of sterile milli-Q water using a Waring–Blendor homogenizer. Next, the macerated mixture was centrifuged at 10.000 × *g* for 20 min at 4 °C (Sorval, LC5C model). The supernatant was then sterilized by passing through a 0.22 μm membrane in a laminar flow hood under sterile conditions and stored at −20 °C, until use. This procedure has been patented at the Federal University of Minas Gerais (UFMG) (PI 014100001550/CTI&T).

2.2. Parasites and mice

Leishmania amazonensis (IFLA/BR/1967/PH-8), *L. chagasi* (MHOM/BR/1970/BH46), and *L. major* (MHOM/IL/1980/Friedlin) were used in this study. Parasites were grown at 24 °C in Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 20% heat-inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at a 7.4 pH [18]. Stationary-phase promastigote and amastigote-like of *Leishmania* used in this work were prepared as described [19]. Murine macrophages were collected from peritoneal cavities of female BALB/c mice (6-weeks old), which were purchased from Institute of Biological Sciences of UFMG. The Animal Use Committee of the UFMG approved experimental protocols.

2.3. Leishmanicidal in vitro activity

Inhibition of cell growth was assessed *in vitro* by cultivating promastigote and amastigote-like (4×10^5, each one) of *L. amazonensis*, *L. chagasi*, and *L. major* in the presence of different individual concentrations (25 to 200 μg/mL) of an *A. blazei* Murill water extract in 96-well culture plates (Corning Life Sciences, Corning, NY, USA), for 48 h at 24 °C. Amphotericin B was used as a positive control (10 μg/mL). Cell viability was assessed by measuring the cleavage of 2 mg/mL of MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] (Sigma). Absorbances were measured by using a multi-well scanning spectrophotometer (LABTRADE, model 660) at a wavelength of 570 nm. Analyses were performed in triplicate, and results were expressed as the mean percentage reduction of the parasites compared to non-treated control wells. The 50% inhibitory concentration (IC_{50}) was determined by applying the sigmoidal regression of the concentration–response curves. Data shown are representative of three different experiments, performed in triplicate, which presented similar results.

2.4. Chemical characterization of the A. blazei Murill water extract

Chemical analysis of the water extract was performed on chromatography plates coated with silica gel GF 254® (Merck, Darmstadt, Germany) for the detection of tannins, coumarins, flavonoids, anthraquinones, triterpenes, steroids, saponins, cardiotonic glycosides, and alkaloids. Different mobile phases and detection reagents were used in accordance with the protocol described by Wagner et al. [20]. Proteins were detected in SDS-PAGE 10% gels by silver staining, while glycoproteins presence were demonstrated in SDS-PAGE 10% stained by periodic acid Schiff. The presence of carbohydrates was investigated using a phenol-sulphuric acid method [21].

2.5. Inhibition of infection in phagocytic cells

The inhibitory effect of the *A. blazei* Murill water extract on the *Leishmania* invasion of macrophages was evaluated in promastigotes of *L. amazonensis*, *L. chagasi*, and *L. major*. Parasites were pre-incubated with the water extract (25 μg/mL), for 1 h at 24 °C. Next, cells were washed three times with RPMI 1640 medium and further incubated for 4 h with murine macrophages at a ratio of 10 *Leishmanias* per 1 macrophage. After, cells were again washed, set, and stained, to determine the percentages of infected macrophages by counting 100 cells in triplicate. Additionally, an optical microscopy was used to view the *Leishmania* infection profiles within the murine macrophages.

2.6. Treatment of infected macrophages

Macrophages were plated on round glass coverslips inside the wells of a 24-well culture plate at a concentration of 5×10^5 cells per coverslip in an RPMI 1640 medium supplemented with 20% FBS, 2 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at a 7.4 pH. After 2 h of incubation at 37 °C in 5% CO_2; promastigotes of *L. chagasi*, *L. major*, and *L. amazonensis* 5×10^6 were added to the wells and the cultures were incubated for 4 h at 37 °C, 5% CO_2. Next, free parasites were removed by extensive washing with an RPMI 1640 medium, and infected macrophages were treated for 48 h with the *A. blazei* Murill water extract (50 μg/mL). Cells were washed in RPMI 1640 and incubated with 4% paraformaldehyde for 15 min, at which time they were treated with 70% ethanol in an ice-bath for 4 h, and again washed three times with sterile PBS. The percentage of the inhibition of *Leishmania* intra-macrophages viability was determined by counting 100 cells in triplicate and comparing this to the infected and non-treated cells. Additionally, a confocal microscopy was performed to view the inhibition of *Leishmania* viability within intra-macrophages. For this, an RNaseA solution was added (200 μg/mL), and incubation occurred for 30 min at 37 °C, after which time, a solution comprised of 370 μL of 0.1 M HCl in 148 μL of PBS 1× was added per well. After 45 s, 1.5 mL of an acridine orange solution (3 μg/mL, pH 5.0) was added and incubation occurred for 5 min, at which time the cells were analyzed. Data shown are representative of three separate experiments, performed in triplicate, which presented similar results.

2.7. Nitric oxide (NO) production and iNOS Western blot

To visualize the macrophage activation via NO production after treatment with an *A. blazei* Murill water extract, cells (4×10^5) were incubated alone in an RPMI 1640 medium (background control) or separately stimulated with the water extract (50 μg/mL) or concanavalin A (ConA; 5 μg/mL), at 37°C in 5% CO_2 for 24, 48, and 72 h. Following incubation, 100 μL of culture supernatant was mixed with an equal volume of Griess reagent (Sigma). After an incubation of 30 min at room temperature, nitrite concentration was calculated using a standard curve of known concentrations. Data were expressed as μM per 4×10^5 cells. Data shown are representative of three different experiments, performed in triplicate, which presented similar results.