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RESEARCH ARTICLE

In vitro pharmacological screening of macrofungi extracts from the Brazilian northeastern region

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Abstract

Although the use of macrofungi in popular medicine is very common, especially in East Asia, the knowledge about their pharmacological properties is poorly investigated. The aim of this work was to evaluate the pharmacological potential of six species of macroscopic fungi: *Phellinus rimosus* (Berk.) Pilát (Hymenochaetaceae), *Pycnoporus sanguineus* (L.) Murrill (Poyporaceae), *Hymenochaete rheicolor* (Mont.) Lév. (Hymenochaetaceae), *Hexagonia papyracea* Berk. (Polyporaceae), *Datronia caperata* (Berk.) Ryvarden (Polyporaceae), and *Lepiota* sp. (Agaricaceae), collected in the state of Bahia, in north-eastern Brazil. Extracts of these species were obtained and tested to determine their cytotoxicity in normal mouse spleen cells. Immunomodulatory, antineoplasic and antiparasitic activities were investigated. Four macrofungi extracts (*Phellinus rimosus*, *Hymenochaete rheicolor*, *Lepiota* sp., and *Datronia caperata*) inhibited the lymphoproliferative response stimulated by concanavalin A in 65–96% of inhibition in mitogen-induced lymphoproliferation assay, three (*Hymenochaete rheicolor*, *Pycnoporus sanguineus*, and *Lepiota* sp.) inhibited 60–70% of production of nitric oxide by J774 activated by IFN-γ and LPS, and two (*Phellinus rimosus* and *Pycnoporus sanguineus*) had antimalarial activity against chloroquine-resistant *Plasmodium falciparum* (over 60%). We did not find inhibition greater than 60% to the growth of both *Leishmania amazonensis* and *Trypanosoma cruzi*. To our knowledge, this is the first description of immunomodulatory activity of *Hymenochaete rheicolor* (HR), *Datronia caperata* (DC) and *Pycnoporus sanguineus* (PS). These results indicate that macrofungi species from the Brazilian north-east have pharmacological activity and are thus a potential source of natural products with medicinal interest.

Keywords: *Basidiomycota; antiparasitic activity; immunomodulation; pharmacological screening*

Introduction

The use of mushrooms in traditional oriental therapies has a well established history. Modern clinical practice in Japan, China, Korea and other East Asian countries continues to rely on mushroom-derived preparations (Zaidman et al., 2005). Several pharmacologically active compounds have been identified and isolated from Basidiomycota with a wide spectrum of biological activities, including immunomodulatory, antineoplasic, antibacterial, antiviral, and anti-inflammatory activities (Bobek et al., 1998; Gunde-Cimerman, 1998; Kino et al., 1989; Jang et al., 2004; Lee et al., 2005; Shon et al., 2003; Wasser, 2002; Zhuang & Mizuno, 1999; Zjawiony, 2004).

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The state of Bahia is situated in north-eastern Brazil and has an area of 567,295.3 km^2. The climate is very complex due to its diverse physiographic and rainfall patterns. Almost all of the Brazilian tropical biomes are represented in this state: Atlantic forest, mangroves, cerrado (woodland savannah) and caatinga (dry scrub forest), although more than 50% of its area is situated in the semi-arid region. These biomes display a rich fungal diversity with a significant lack of information on its pharmacological potential. The aim of this work was to assess the pharmacological potential of six species of macrofungi using *in vitro* assays. The investigation included native polypore and agaric mushrooms collected in Bahia, Brazil.

Materials and methods

Macrofungi

Six macrofungi species (Basidiomycota) were studied and their basidiomata were collected from private properties, with permission of each owner, in different localities in Bahia (Table 1). Specimens were identified following a descriptive study of external and internal morphology and using a key for neotropical polypores (Ryvarden, 2004). Specimen identifications were authenticated by A. Góes Neto, Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, Bahia, Brazil. Voucher specimens were deposited at the Herbarium of Universidade Estadual de Feira de Santana (HUEFS).

Preparation of extracts

Basidiomata were washed with tap water and sliced into small pieces, dried, and powdered. Powdered material of all species was extracted in a hydroalcoholic solution of 80% methanol. Macrofungi extracts were filtered and dried on a rotatory evaporator under reduced pressure. Masses of powdered macrofungi utilized, and the extract yields obtained, are shown in Table 1.

Cell lines and parasite cultures

Human chronic myeloid leukemia cell K562 and human Burkitt's lymphoma cell Daudi were maintained in RPMI 1640 (Life Technologies, GIBCO-BRL, Gaithersburg, MD). Murine macrophage cell line J774 was maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT). Both media were supplemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, Brazil) and 40 μg/mL gentamycin (Novafarma, Anápolis, Brazil). Cell cultures were maintained at 37ºC and 5% CO_2.

Plasmodium falciparum (strain W2, chloroquine-resistant, mefloquine-sensitive) was maintained in human A^+ erythrocytes in RPMI 1640 (GIBCO-BRL) supplemented with 10% human plasma at 37ºC in a 5% CO_2-air mixture.

Trypanosoma cruzi (epimastigote form of Y strain) and *Leishmania amazonensis* (promastigote form of MHOM/BR88/BA-125 Leila strain) were maintained at 25ºC in liver infusion tryptose medium (Difco, Detroit, MI) supplemented with 10% FBS, 1% hemin (Sigma, St. Louis, MO), 1% R9 medium (Hyclone) and 5% sterile human urine.

Animals

BALB/c mice (6-8 weeks old) were used as spleen donors for cytotoxicity and lymphoproliferation assays. Animals received water and food ad libitum. This work was approved by the Ethics Committee for Animal Use of the Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz.

Table 1. Macrofungi species collected in the State of Bahia, Brazil.

Species (Family)	Extract	Locality	Voucher number (HUEFS)	Mass of powdered basidiomata in g (yield, in %)
Phellinus rimosus (Berk.) Pilát (Hymenochaetaceae)	PR	Fazenda Nova Favela, Cachoeira, Bahia, Brazil12º34'S, 38º59'W (100 m)	106114	208.7 (8.43)
Pycnoporus sanguineus (L.) Murrill (Poyporaceae)	PS	Sauípe, Mata de São João, Bahia, Brazil12°22'S; 37°56'W (50 m)	102966	3.74 (7.75)
Hymenochaete rheicolor (Mont.) Lév. (Hymenochaetaceae)	HR	Fazenda Nova Favela, Cachoeira, Bahia, Brazil12º34'S, 38º59'W (100 m)	106118	9.42 (3.82)
Lepiota sp. (Agaricaceae)	LP	Fazenda Conceição das Flores, Conceição do Coité, Bahia, Brazil11º33' 39º17' (440 m)	115874	525.17 (3.33)
Hexagonia papyracea Berk. (Polyporaceae)	HP	Fazenda Belmonte, Coração de Maria, Bahia, Brazil12°15'S; 38°48'W (230 m)	105888	1.38 (5.80)
Datronia caperata (Berk.) Ryvarden (Polyporaceae)	DC	Fazenda Belmonte, Coração de Maria, Bahia, Brazil12°15'S; 38°43'W (230 m)	102916	14.5 (6.83)

Assessment of cytotoxicity

BALB/c spleen cells obtained from normal mice were used to determine non-toxic concentrations. Cells were plated at 5×10^6 cells/well to 96-well plates with or without macrofungi extracts and 1 μCi/well [^{3}H]-thymidine (Amersham, Little Chalfont, UK). Saponin (Sigma) was used as a positive control. Plates were incubated at 37°C and 5% CO_2 for 24 h. Cells were harvested using a Filtermate 196 cell harvester and the radioactivity in dried filters was counted using a β-counter (Packard, Meriden, CT). Mean values of the triplicates expressed in counts per minute (CPM) were calculated and cytotoxicity was given as the percentage of control values. Concentrations used were 1, 10, and 100 μg/mL and extract concentrations with values smaller or equal to 30% were considered non-toxic (Leite et al., 2006).

Mitogen-induced lymphoproliferation assay

BALB/c spleen cells obtained from normal mice were used in this assay. Cells were added at 5×10^6 cells/well to 96-well plates with or without macrofungi extracts, in non-toxic concentrations, and in the presence of concanavalin A (2 μg/mL, Sigma). Plates were incubated for 48 h at 37°C and 5% CO_2. One μCi of ^{3}H-thymidine was added to each well, and plates were incubated for additional 18 h. Uptake of ^{3}H-thymidine was determined as described above. Mean values of the triplicates expressed in CPM were calculated, and lymphoproliferation inhibition was given as the percentage of control values (Soares et al., 2006).

Nitric oxide production assay

Nitric oxide (NO) production was estimated by the levels of nitrite determined by the Griess method (Padgett & Pruett, 1992). J774 macrophages were added at 1×10^5 cells/well to 96-well plates with or without macrofungi extracts, in non-toxic concentrations, in the presence of 5 ng/mL gamma-interferon (IFN-γ, BD Biosciences PharMingen, San Diego, CA) and 1 μg/mL lipopolysaccharide (LPS, *Escherichia coli* serotype 0111:B4, Sigma). Plates were incubated at 37°C and 5% CO_2 and, after 24 h, 50 μL aliquots of supernatants were collected and mixed with an equal volume of Griess reagent in 96-well plates. Absorbance was measured at 570 nm using microplate reader Spectramax 190 (Molecular Devices, Sunnyvale, CA). Quantitative analysis was performed by optical density (OD) comparison with standard solutions freshly prepared $NaNO_2$ in culture medium and mean values of the triplicates were calculated and inhibition of the nitric oxide production was given as percentage of control values (Morazzoni et al., 2005).

Antineoplasic assay

Cancer cells were added to 96-well plates (Daudi at 5×10^4 cells/well and K562 at 1×10^5 cells/well) with or without macrofungi extracts. Positive control used in the half-maximal inhibitory response (IC_{50}) determination was doxorubicin. Plates were initially incubated at 37°C and 5% CO_2 for 24 h. Then, [^{3}H]-thymidine was added and plates were incubated for additional 18 h. Uptake of ^{3}H-thymidine was determined as described above. Mean values of triplicates expressed in CPM were calculated and the growth inhibition of the neoplasic cells was given as percentage of control values.

Antimalarial assay

Antimalarial effect of macrofungi extracts was measured by the [^{3}H]-hypoxanthine incorporation assay. Trophozoite stages at 1% to 2% parasitemia and 2.5% hematocrit were incubated with or without macrofungi extracts, in non-toxic concentration, in culture medium without hypoxanthine; a mefloquine control (as a reference antimalarial drug) was used in IC_{50} determination. Parasites were harvested using a Packard Filtermate 196 cell harvester and the radioactivity in dried filters was counted using a β-counter. Mean values of the triplicates expressed in CPM were calculated and growth inhibition was given as the percentage of control values (Andrade-Neto et al., 2004; Zalis et al., 1998).

Anti-Leishmania amazonensis and anti-Trypanosoma cruzi assays

Epimastigotes of *T. cruzi* and promastigotes of *L. amazonensis* were plated in 96-well plates at 1×10^7 and 5×10^6 parasites/well, respectively, with or without macrofungi extracts, in non-toxic concentrations to mouse spleen cells. After incubation at 25°C for 24 h, the number of viable parasites was evaluated by counting in a Neubauer chamber using a light microscope. Mean values of the triplicates were calculated and growth inhibition was given as the percentage of control values.

Data analyses

All bioassays were performed in triplicate in three independent experiments and inhibition percentage was calculated as follows:

$$\% \text{ inhibition} = 100 - (\text{extract value} \times 100)/\text{control value}$$

where extract value corresponds to CPM, OD or number of parasites in the presence of extracts, and control value corresponds to CPM, OD or number of parasites in the absence of extracts. Extracts with inhibition percentage

greater than 60% were used for the IC_{50} calculation by curve-fitting.

All data were expressed as mean ± SD of three independent experiments. ANOVA followed by Tukey´s multiple comparison test were used to assess statistical significance and $P < 0.01$ was considered significant. All analyses were conducted in Prism 4 for Windows (Graphpad Software Inc., San Diego, CA).

Results

Cytotoxicity

Macrofungi extracts were tested at 1, 10, and 100 μg/mL in mouse spleen cell cultures (Table 2). Concentrations causing an inhibition greater than 30% were arbitrarily considered cytotoxic. Thus, macrofungi extracts were subsequently used in the bioassays in the following concentrations: extracts PR, HR, HP and DC were tested at 100 μg/mL, whereas PS and LP were tested at 10 μg/mL.

Immunomodulatory activity

Inhibition of proliferation of splenocytes and nitric oxide production by macrophages were used as indicators of immunosuppressive activity of the macrofungi extracts (Table 3). In lymphoproliferation assays, the macrofungi extracts PR, HR, LP and DC had inhibitory activities greater than 65%, while PS and HP inhibited below 50%. The former extracts were then tested to determine the IC_{50} values (Table 4). LP had the lowest IC_{50} value (2.6 μg/mL). The inhibition of nitric oxide production was above 60% only when PS, HR and LP were added to the culture, with IC_{50} of 1.7, 34.4, and 2.2 μg/mL, respectively (Table 4).

Table 2. Cytotoxicity of macrofungi extracts in normal spleen cells.

	% of inhibition in concentrations tested (μg/mL)		
Extracts	1	10	100
PR	37.4 ± 15.1	23.5 ± 18.1	29.4 ± 10.7
PS	0	32.0 ± 32.0	39.5 ± 13.9
HR	41.3 ± 18.4	24.1 ± 21.7	29.6 ± 11.3
LP	0	0	51.2 ± 12.1
HP	35.2 ± 24.4	25.1 ± 25.1	0
DC	34.9 ± 25.7	28.1 ± 22.7	13.0 ± 7.6
SAP[a]	79.0 ± 6.6	–	–

SAP[a]: saponin at 1%. Values are mean ± SEM of 3 independent experiments. PR: Phellinus rimosus; PS: Pycnoporus sanguineus; HR: Hymenochaete rheicolor; LP: Lepiota sp.; HP: Hexagonia papyracea; DC: Datronia caperata.

Table 3. Pharmacological activity of the macrofungi extracts in different assays.

	% of inhibition in tested assays						
	Immunomodulatory activity		Antineoplasic activity		Antiparasitic activity		
Extracts	Lymphoproliferation	NO production	K562	Daudi	*T. cruzi*	*L. amazonensis*	*P. falciparum*
PR	95.8 ± 3.6 *	36.0 ± 3.6	12.0 ± 12.0	0	50.2 ± 1.4 **	1.7 ± 0.8	87.9 ± 0.4 **
PS	32.1 ± 18.0	60.8 ± 7.0 **	82.5 ± 8.4 **	30.0 ± 12.0	59.0 ± 0.8**	8.7 ± 1.6	60.5 ± 2.6 **
HR	88.1 ± 11.3 *	67.3 ± 9.0 **	77.1 ± 4.9 **	63.9 ± 7.1 *	0	0	41.2 ± 0.6
LP	65.9 ± 33.0	60.3 ± 12.4 **	15.0 ± 1.8	32.5 ± 2.9	36.6 ± 0.5	0.3 ± 0.2	0
HP	35.8 ± 19.5	41.8 ± 6.7	61.3 ± 8.1 **	60.4 ± 4.1 *	50.1 ± 2.4**	10.8 ± 1.7	4.3 ± 2.2
DC	68.5 ± 8.1	46.7 ± 4.3	50.4 ± 9.2	44.0 ± 0.4	42.9 ± 2.4	0	39.7 ± 3.8

Values are mean ± SEM of three independent experiments; * $P < 0.01$, ** $P < 0.001$ compared to control. NO: Nitric oxide; PR: Phellinus rimosus; PS: Pycnoporus sanguineus; HR: Hymenochaete rheicolor; LP: Lepiota sp.; HP: Hexagonia papyracea; DC: Datronia caperata.

Table 4. IC_{50} of macrofungi extracts with inhibition percentage greater than 60% in different assays.

	IC_{50} (μg/ml) in tested assays				
	Immunomodulatory activity		Antineoplasic activity		Antiparasitic activity
Extracts	Lymphoproliferation	NO production	K562	Daudi	*P. falciparum*
PR	40.0 ± 0.6	–	–	–	4.4 ± 0.2
PS	–	1.7 ± 0.1	4.1 ± 0.3	–	0.3 ± 0.1
HR	62.1 ± 1.1	34.4 ± 3.4	30.9 ± 6.0	40.8 ± 8.4	–
LP	2.6 ± 1.4	2.2 ± 1.4	–	–	–
HP	–	–	39.1 ± 1.3	–	
DC	65.6 ± 17.9	–	–	–	–
DOX	–	–	2.8 ± 0.02	1.9 ± 0.3	–
MFQ	–	–	–	–	0.0003 ± 10^{-5}

Values are mean ± SEM of three independent experiments. NO: Nitric oxide; PR: Phellinus rimosus; PS: Pycnoporus sanguineus; HR: Hymenochaete rheicolor; LP: Lepiota sp.; HP: Hexagonia papyracea; DC: Datronia caperata; DOX: doxorubicin; MFQ: mefloquine.

Antineoplasic activity

Antineoplasic potential of the macrofungi extracts was evaluated using human leukemic cells lines K562 and Daudi (Table 3). HR and HP extracts inhibited more than 60% of the proliferation of both K562 and Daudi cells, while PS extract inhibited only K562 cell growth. The IC_{50} values of extracts PS, HR and HP against K562 cells were 4.1, 30.9, and 39.1 μg/mL, respectively. HR inhibitory activity in Daudi cells was 40.8 μg/mL (Table 4).

Antiparasitic activity

Effects of the macrofungi extracts in cultures of erythrocytic forms of *P. falciparum*, promastigotes of *L. amazonensis* and epimastigotes of *T. cruzi* were evaluated (Table 3). The extracts tested did not inhibit more than 11% of the growth of *L. amazonensis*; however, extracts PR, PS and HP inhibited 50-58% the growth of *T. cruzi* (Table 3). Extracts PR and PS had antimalarial activity of 88% and 60%, respectively, while the others inhibited less than 42%. PR and PS extracts had IC_{50} of 4.4 and 0.3 μg/mL, respectively (Table 4).

Discussion

The results obtained in this work indicate that non-cultivated macrofungi from Bahia have pharmacological activities, as demonstrated using different bioassays *in vitro*. The evaluation of cytotoxicity in normal spleen cells prior to testing the extracts in the experimental models was important to determine non-toxic concentrations, warranting that the pharmacological activity was not due to toxic effects. Only *Pycnoporus sanguineus* (PS) and *Lepiota* sp. (LP) presented cytotoxicity greater than 30% at 100 μg/mL.

Mytogen-induced lymphoproliferation and nitric oxide production by macrophages stimulated with LPS and IFN-γ are assays commonly used to evaluate the immunomodulatory potential of natural products (Meselhy, 2003; Morazzoni et al., 2005; Nicholl et al., 2001; Soares et al., 2006). *Phellinus rimosus* (PR) extract had the most potent inhibitory activity on lymphoproliferation, although it had little effect on nitric oxide production. This suggests that the extract acts on lymphocytes but not on macrophages. It is also possible that its free radical scavenging properties interfere with the assay (Ajith & Janardhanan, 2001), considering the indirect measurement of nitric oxide by nitrite by the Griess method. To our knowledge, this is the first description of immunomodulatory activity of *Hymenochaete rheicolor* (HR), *Datronia caperata* (DC) and *Pycnoporus sanguineus* (PS).

Cell lines K562 (chronic myelogenic leukemia) and Daudi (Burkitt's lymphoma) are derived from neoplasies of different origins and are commonly used in the screening of new antineoplasic agents. This difference may explain the antineoplasic activity of *P. sanguineus* in K562 cells but not in Daudi cells, with an IC_{50} value of 4.1 μg/mL, which was close to the doxorubicin IC_{50} value (2.8 μg/mL; $P > 0.05$). The hydroalcoholic extract of *P. rimosus* did not have activity against the tumoral cell lines assayed, although, in a previous work of Ajith and Janardhanan (2003), methanol and ethyl acetate extracts of *P. rimosus* exhibited cytotoxic and antitumor activities against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines. To our knowledge, this is the first description of antineoplasic activity of the *Hexagonia papyraceae* (HP) and *Hymenochaete rheicolor* (HR).

Leishmanicidal, antibacterial, antiviral and cytotoxic activities of substances isolated from *P. sanguineus* have been described before (Correa et al., 2006; Smânia et al., 1995; Smânia Jr et al., 2003). Despite the activity against *L.* (Viannia) *panamensis* described by Correa et al. (2006), *P. sanguineus* (PS) did not show any inhibition to *L. amazonensis*, possibly due to differences between species of the parasite utilized in the assays and/or the collection site, since in the aforementioned study the fungus was obtained from Colombian mycota. In addition, we observed a moderate activity against *T. cruzi* and *P. falciparum*. *Hexagonia papyracea* (HP) extract had a significant anti-*T. cruzi* activity and *Phellinus rimosus* (PR) had an inhibition of more than 85% against *P. falciparum*, demonstrating for the first time the antiparasitic activity of this macrofungus.

In conclusion, our results suggest that extracts of the studied macrofungi collected in the Brazilian northeastern region have marked biological effects, including immunomodulatory, antiparasitic, and antineoplasic activities. Therefore, these macrofungi may be sources of natural products with medicinal interest, requiring chemical investigations in these extracts to identify the active principles.

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