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Antimicrobial and Antineoplasic Activity of *Pleurotus ostreatus*

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Abstract The objectives of this work were to evaluate the antimicrobial and antineoplasic activity of *Pleurotus ostreatus* DSM 1833. To study the antimicrobial activity, the following extracts were prepared: water infusion of the fresh fruiting bodies (B1), dehydrated fruiting bodies (B2), fresh mycelium (M1), and dehydrated mycelium (M2). Polysaccharides from the fresh mycelium were also obtained by water infusion followed by ethanol treatment (EP). The extracts were tested against *Candida albicans*, *Escherichia coli*, and *Bacillus subtilis*. To investigate the antineoplasic effect of *P. ostreatus*, the culture broth *in natura*, the extract from the culture broth (ECB), and the extract from the fruiting bodies were tested in female Swiss albino mice inoculated with the Ehrlich ascitic tumor (EAT). B1, B2, and M1 showed more than 50.0% inhibition against *C. albicans.* M2 presented a high degree of inhibition against *E. coli* (87.5%) and *B. subtilis* (57.5%), while EP was not effective. All the tested substances inhibited the development of EAT at levels near 70% when injected intraperitoneally in mice. The highest tumor inhibition (76%) was achieved for the treatment with ECB. The intragastric treatment did not promote any reduction in tumor cell development, independent of the test substance.

Keywords *Pleurotus ostreatus* · Antimicrobial activity · Antineoplasic activity · Solid cultivation · Submerged cultivation · Extraction processes

Introduction

Fungi of the *Pleurotus* genus have an important place among the commercially employed mushrooms because of their gastronomic, nutritional, and medicinal properties. They also require shorter growth time when compared to other edible fungi and demand fewer environmental controls. Diseases and pests rarely attack their fruiting bodies, and they can be cultivated in a simple and economical way [1, 2]. Fruiting bodies obtained from

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Pleurotus cultivated on solid substrates contain high amounts of proteins, carbohydrates, minerals (calcium, phosphorus, and iron), and vitamins (thiamin, riboflavin, and niacin), as well as low fat contents [3–6].

Besides the studies in solid culture for the purpose of producing fruiting bodies, the submerged culture of the *Pleurotus* genus has also been studied for the production of extracellular enzymes [7, 8], flavoring agents [9], β-glucosidases [10], antimicrobials [11–13], vitamins [14], polysaccharides [15, 16], etc. These polysaccharides represent the major constituent that determines the rigidity and morphological properties of the fungal cell wall and, depending on the culture conditions, they can also be excreted to the culture medium. Among the polysaccharides produced by *Pleurotus* spp., β-(1,3)- and β-(1,6)-glucans play an important role due to their number of medicinal properties.

These properties have gained much interest from the scientific community because of their hypoglycemic, antithrombotic, antitumor, antiinflammatory, and antimicrobial activities, as well as for possessing the ability to modulate the immune system and to reduce arterial pressure and blood cholesterol [17, 18].

According to data from the National Cancer Institute [19], cancer is the second cause of death through disease in Brazil. Its treatment can be performed through surgery, radiotherapy, and chemotherapy or through a combination of these therapies [20, 21]. However, new therapy options, whether as a complement or not to the existing ones, for the purpose of establishing more precise prognoses in the treatment of this pathology have been studied [22].

Investigations using polysaccharides from fruiting bodies and basidiomycete mycelium have shown that *Pleurotus ostreatus* [23], *Pleurotus sajor-caju* [24], and *Pleurotus citrinopileatus* [17] present antineoplasic activities, which can be attributed to the polysaccharides of the cell wall components.

Furthermore, different studies have also been conducted with *Pleurotus* genus in order to evaluate its antimicrobial activity [12, 13, 25].

The aim of the present work was to evaluate the antimicrobial activity of aqueous extracts of *P. ostreatus* fruiting bodies and mycelial biomass against *Candida albicans*, *Escherichia coli*, and *Bacillus subtilis* and to investigate the antineoplasic capacity of the fruiting bodies and culture broth obtained from the solid and the submerged cultivation, respectively, on the Ehrlich ascitic tumor (EAT).

Materials and Methods

Microorganisms and Maintenance

P. ostreatus was obtained from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" under the code DSM 1833. The culture was kept in Petri dishes containing WDA medium (1 L wheat extract, 20.0 g dextrose, and 15.0 g agar) at 4 °C [26].

The evaluation of antimicrobial activity was carried out against *C. albicans* CCT 0776, *E. coli* CCT 1371, and *B. subtilis* CCT 1940 obtained from the "Fundação Tropical de Pesquisas e Tecnologia André Tosello, São Paulo, Brazil". *C. albicans* was kept in yeast–malt–agar medium, composed of 3.0 g L^{-1} yeast extract, 3.0 g L^{-1} malt extract, 5.0 g L^{-1} peptone, 10.0 g L^{-1} glucose, and 15.0 g L^{-1} agar. *E. coli* and *B. subtilis* were kept in nutrient–agar medium composed of 5.0 g L^{-1} peptone, 3.0 g L^{-1} meat extract, and 15.0 g L^{-1} agar.

Animals and Maintenance

The animals used were female Swiss albino mice (*Mus musculus*) weighing 25±5 g obtained from the "Instituto de Tecnologia do Paraná" (TECPAR), Curitiba, Brazil. The animals were fed with standard food and water ad libitum at 22±1 °C, with 12 hourly light/dark cycles, as described in literature [27, 28].

Tumor and Maintenance

The tumor used in this study was the EAT derived from a spontaneous murine mammary adenocarcinoma. EAT was obtained in vivo and maintained through weekly intraperitoneal injections in mice according to Pagno et al. [29].

Antimicrobial Activity Experiments

Preparation of Extracts

The extracts to be evaluated were obtained by water infusion (3.0 g dry mass for 27.0 mL water at 100 °C for 1 h) of fresh fruiting bodies (B1), dehydrated fruiting bodies (B2), fresh mycelium (M1), and dehydrated mycelium (M2). Polysaccharides from the fresh mycelium [ethanol treatment (EP)] were also obtained by boiling water extraction for 4 h according to the methodology proposed by Berovic et al. [30] modified. The resulting filtrate was treated with ethanol using a volume ratio of 4:1 (ethanol/sample). After this extraction (48 h at 8 °C), the sample was centrifuged at 5,000×*g* for 10 min. The polysaccharides were dissolved in water to reach a concentration of 1.0 g L^{-1}. The concentration of polysaccharides was determined by the phenol–sulphuric method [31].

For fruiting body production, banana straw was packed in polypropylene bags, supplemented with rice bran, sterilized, and inoculated using 10% solid inoculum. The first step of the process, the mycelial growth, was carried out at 25 °C, with light for 20 days with 60% relative air humidity. The second step, induction of fruiting body formation, was achieved by perforating the plastic bags to increase air exchange and by exposing them to light for a period of 12 h a day and relative air humidity equal to 90% [32].

The mycelium was produced by submerged culture in wheat extract/dextrose medium consisting of wheat extract supplemented with 40.0 g L^{-1} glucose and 20.0 g L^{-1} corn steep liquor at pH 4.0, 30 °C, and initial $K_L a$ equal to 10.2 h^{-1}.

The fruiting body and the mycelium were used in two forms: fresh and dehydrated. The fruiting body dehydration was carried out at 40 °C for 24 h, and the mycelium was dehydrated at 60 °C for 48 h, as previously determined in our laboratory (results not published).

Antimicrobial Activity Evaluation

Ten test tubes were prepared with 45.5% nutrient or yeast–malt medium twice concentrated, 45.5% B1, B2, M1, M2, or EP extract and 9.0% active microorganisms, and incubated at 28 °C (*C. albicans* and *B. subtilis*) or 37 °C (*E. coli*). These tubes were shaken at 120 min^{-1} for 24 h. Antimicrobial activity was estimated spectrophotometrically at 460 nm based on the cell growth inhibition of the tested microorganisms compared to the control culture prepared without extracts.

Antineoplasic Activity Experiments

Culture Broth Preparation

Culture broth in natura After 14 days of submerged cultivation of *P. ostreatus* in POL medium (5.0 g L^{-1} $(NH_4)_2SO_4$; 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$; 1.0 g L^{-1} K_2HPO_4; 2.0 g L^{-1} yeast extract; 1.0 g L^{-1} peptone, 20 g L^{-1} glucose, pH 6.5–7.0) in Erlenmeyer flasks at 30 °C and 120 min^{-1} [13, 33], the culture broth was separated from biomass and maintained at 4 °C for further use in antineoplasic evaluation tests. This was called "culture broth *in natura*" (CBIN).

Extract from the culture broth The culture broth *in natura* was treated with acetone cooled down to 8 °C using a volume ratio acetone/culture broth of 3:1 according to Maziero [34]. After 24 h under refrigeration at 4 °C, for extracellular polysaccharide precipitation, the mixture was centrifuged at 4,500×*g* for 5 min and washed twice with an acetone/ethanol/distilled water (3:1:1) solution [35]. The extract obtained was lyophilized and called "extract from the culture broth" (ECB).

Fruiting Body Preparation

P. ostreatus fruiting bodies were obtained through solid-state cultivation of banana leaves, as described in "Preparation of extracts" section [32]. Extraction of the polysaccharides from the fruiting bodies based on the methodologies described by Zhuang et al. [24] was modified as follows: 900 g of fresh fruiting bodies were heated to 100 °C in water for 4 h; the extract was obtained by simple filtration and added to 96% ethanol (1:5 *v*/*v*); the precipitate was dialyzed in a Sigma diagnostics membrane for 20 h to remove sugars with low molecular weight and lyophilized. This extract was called "extract from the fruiting bodies" (EFB).

Tumor Induction

Tumor induction was performed intraperitoneally in each mouse from the test groups, as well as from the tumor control groups, by injecting 200 μL of a cell suspension containing 25×10^6cells mL^{-1}, representing an inoculation ratio of 5×10^6 tumor cells per animal [36].

In vivo treatments

Treatments involved the intraperitoneal (i.p.) or intragastric (i.g.) application of the test substances for six consecutive days starting 24 h after tumor implantation, based on the methodology of Pagno et al. [29] and Zhang et al. [36] modified. The test substance CBIN was administered in a daily dose of 10 mL per kilogram of body weight, while the test substances ECB and EFB were given in a daily dose of 10 mg per kilogram of body weight.

Four kinds of animal groups were used for each experiment:

1. Test groups—The test groups were carried out in triplicate, and each replicate was composed of six animals with tumor induction treated i.p. or i.g. with CBIN, ECB, or EFB. The purpose of these groups was to evaluate the capacity of the tested substance

to inhibit tumor development through the comparison of these groups with the tumor control group.

2. Tumor control group (positive control)—The tumor control group consisted of six animals with tumor induction and treated i.p. or i.g. with 0.01 M phosphate-buffered saline (PBS). Although these animals only received a placebo, they suffered the same daily stress as the treated animals.
3. Substance control groups—The substance control groups comprised of six animals without tumor induction treated i.p. or i.g. with CBIN, ECB, or EFB. The objective of these control groups was to qualitatively evaluate the possible toxicity of the test substances CBIN, ECB, and EFB on the healthy animals. This evaluation was possible through the comparison with the negative control group.
4. Negative control group—The negative control group consisted of six animals without tumor induction and treated i.p. or i.g. with 0.01 M PBS. The animals of this group suffered the same daily stress as the substance control groups.

Analysis

Tumor development was assessed through the determination of ascitic liquid volume in the peritoneal cavity and through counting the number of neoplasic cells present in the ascitic liquid [27, 29].

To determine the volume of ascitic liquid, 4 mL of PBS 0.01 M (8.0 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 0.12 g L^{-1} KH_2PO_4, 0.91 g L^{-1} Na_2HPO_4, pH 7.2) was inserted into the peritoneal cavity and, after careful massaging, the liquid was suctioned out and the volume measured.

To determine the number of neoplasic cells present in the ascitic liquid, the Trypan blue dye exclusion method was used: 20 μL ascitic liquid was added to 180 μL Trypan blue solution and 200 μL PBS 0.01 M. The cells were later counted in a Neubauer hemocitometer [37].

To follow the weight gain during the experiments, the animals were weighed before tumor implantation and then daily until they were sacrificed.

Statistical Analysis

Statistical analysis was done with the replicates, and the values were accepted or not through Dixon's "*Q*" test with 95% confidence level [38]. The mean difference between the control culture and the test cultures was determined using ANOVA test, with 5% significance.

Results and Discussion

Antimicrobial Activity

Figure 1 shows the growth inhibition of the tested microorganisms obtained by the antimicrobial action of B1, B2, M1, M2, and EP extracts. All extracts showed inhibition against some of the microorganisms tested. M2 extract denoted high growth inhibition (87.3%) against *B. subtilis*, but this bacterium presented less than 40.0% inhibition by the other extracts.

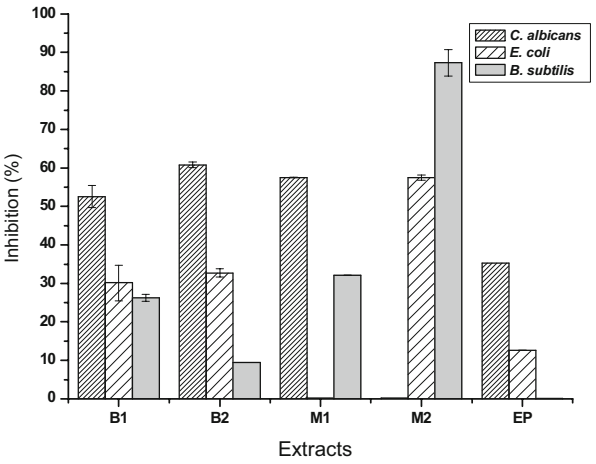

Fig. 1 Growth inhibition (%) of *C. albicans*, *E. coli*, and *B. subtilis* by *B1* (fresh fruiting bodies), *B2* (dehydrated fruiting bodies), *M1* (fresh mycelium), *M2* (dehydrated mycelium), and *EP* (polysaccharides from the fresh mycelium) extracts from *P. ostreatus*

B1 and B2 extracts showed efficient antimicrobial activity against *C. albicans*, with 52.5 and 60.5% growth inhibition, respectively. These extracts also presented some degree of inhibition against *E. coli* and *B. subtilis*.

Fresh mycelium (M1) extract inhibited 57.5% of *C. albicans* growth, which was also observed by Garcia et al. [25] using fresh mycelium from *P. ostreatus* grown on wheat grain. However, *C. albicans* was not inhibited by M2 (dehydrated mycelium) extract, indicating that some active properties could be lost by the dehydration process. On the other hand, the dehydrated mycelium extract M2 showed the highest inhibition levels against *E. coli* (57.5%) and *B. subtilis* (87.3%) growth. Comparing these results with those obtained with fresh mycelium M1, we can suppose that the dehydration process favors the formation of a new compound with bacteriostatic potential, but further investigation has to be made.

The EP extract containing polysaccharides from the fresh mycelium showed an inhibition degree lower than 40.0% against *C. albicans* and *E. coli* and no effect against *B. subtilis*. This may be associated with the loss of substances of low molecular weight with potential antimicrobial activity during the polysaccharide extraction process. Okamoto et al. [39] reported the formation of *p*-anisaldehyde by *P. ostreatus*, which showed to be an effective small compound with antimicrobial activity against *B. subtilis* [39].

Antineoplasic Activity

Table 1 shows the results obtained for the treatment of the Ehrlich ascitic tumor by *P. ostreatus* CBIN, as well as the respective control tests. As we can observe, the i.p. test group presented the lowest values of weight gain, ascitic liquid volume, and number of neoplasic cells when compared to the other groups. The reduction in the number of neoplasic cells obtained with i.p. treatment was about 70% (mean value), and considering the standard error, the minimal value that could be obtained for this parameter would be 59%. On the other hand, i.g. treatment was not effective.

The differences between the weight variation profiles with time for i.p. and i.g. treatments can be seen in Fig. 2a and b, respectively. While the weight variation profiles were quite similar for the test and the control groups submitted to i.g. treatment (Fig. 2b), the i.p. test group (Fig. 2a) presented lower weight gain along the treatment when compared to the control groups.

Table 1 Weight gain, ascitic liquid volume, and number of neoplasic cells for intraperitoneal and intragastric administration of culture broth of *P. ostreatus in natura* (CBIN) to the test animals.

Groups	Weight gain (g)	Ascitic liquid volume (mL)	Number of neoplasic cells ($\times 10^6$ cells mL^{-1})
Negative control[a] (i.p.)	10.6±0.5	–	–
Tumor control[a] (i.p.)	14.2±1.4	5.7±0.7	680±115*a*
Test[b] (i.p.)	3.05±0.7	3.8±0.4	206±28*b*
Substance control[a] (i.p.)	11.7±0.7	–	–
Negative control[a] (i.g.)	11.7±0.6	–	–
Tumor control[a] (i.g.)	15.1±0.6	5.4±1.4	422±63*a*
Test[b] (i.g.)	15.6±0.7	5.4±0.6	558±64*a*
Substance control[a] (i.g.)	11.6±0.6	–	–

Different letters in column 4 represent the existence or not of significant differences between the test and the tumor control groups.

i.p. Intraperitoneal, *i.g.* intragastric

[a] Mean values ± standard error

[b] Mean values between 3 test groups ± standard error.

Based on the poor results obtained using i.g. treatment with CBIN, this test was not carried out for the extract obtained from the culture broth (ECB). As previously observed for CBIN, i.p. treatment with ECB allowed the lowest values of weight gain, ascitic liquid volume, and number of neoplasic cells when compared to the control groups (Table 2). In

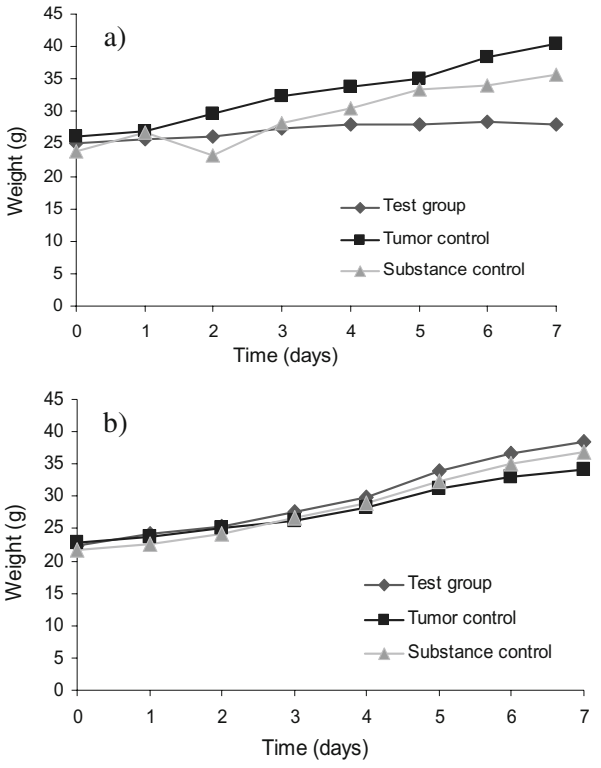

Fig. 2 Weight variation (g) with time (days) in i.p. groups (**a**) and i.g. groups (**b**) treated with *P. ostreatus* CBIN

Table 2 Weight gain, ascitic liquid volume, and number of neoplasic cells for intraperitoneal administration of extract from the culture broth of *P. ostreatus* (ECB) to the test animals.

Groups	Weight gain (g)	Ascitic liquid volume (mL)	Number of neoplasic cells ($\times 10^6$ cells mL^{-1})
Negative control[a] (i.p.)	10.6±0.5	–	–
Tumor control[a] (i.p.)	12.9±0.9	11.0±0.8	353±44*a*
Test[b] (i.p.)	9.0±0.8	6.75±0.6	84±12*b*
Substance control[a] (i.p.)	12.9±0.8	–	–

Different letters in column 4 represent the existence or not of significant differences between the test and the tumor control groups.

i.p. Intraperitoneal, *i.g.* intragastric

[a] Mean values ± standard error

[b] Mean values between 3 test groups ± standard error.

this case, the reduction in the number of neoplasic cells was about 76% (mean value), and considering the standard error, the minimal value that could be obtained for this parameter would be 69%. The weight gain evolutions with time (Fig. 3) were similar for the test and the control groups.

Table 3 and Fig. 4a and b show the results achieved when the animals were treated with the extract obtained from the fruiting bodies (EFB). I.p. treatment promoted 71% (mean value) inhibition of EAT development, and considering the standard error, the minimum tumor inhibition would be about 60%. After 4-day treatment, the weight gain profile of the i.p.-treated group was very similar to that of the substance control group, while the weight gain of the tumor control group increased. The i.g. treatment was not effective.

We can observe that treatments, which promoted the highest values of tumor inhibition (i.p. treatments), i.e. the best activation of the immune system, also promoted the lowest body weight gain. Benjamini et al. [40] stated that the activation of the animal immune system generates weight loss due to the energy spent for defense cell production. Considering that the body weight determined in this work includes the tumor weight, the results of our investigations confirm the observations of Benjamini et al. [40].

These observations also agree with Pagno et al. [29]. The authors evaluated i.p. and oral treatments of male inbred Swiss mice inoculated i.p. (5×10^6 cells per animal) with EAT. After animals were treated for 7 days with different concentrations of a dichloromethane fraction from *Vernonia scorpioides* (DCM), they observed that the body weight of mice treated with a saline solution (tumor control group) increased, while the body weight of

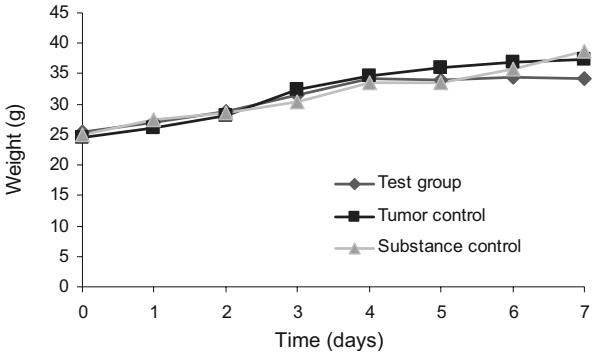

Fig. 3 Weight variation (g) with time (days) in i.p. groups treated with extract obtained from *P. ostreatus* culture broth (ECB)

Table 3 Weight gain, ascitic liquid volume, and number of neoplasic cells for intraperitoneal and intragastric administration of extract obtained from the fruiting bodies of *P. ostreatus* (EFB) to the test animals.

Groups	Weight gain (g)	Ascitic liquid volume (mL)	Number of neoplasic cells ($\times 10^6$ cells mL^{-1})
Negative control[a] (i.p.)	10.6±0.5	–	–
Tumor control[a] (i.p.)	13.0±0.1	1.5±1.0	575±100*a*
Test[b] (i.p.)	2.1±0.4	1.8±0.3	167±24*b*
Substance control[a] (i.p.)	7.3±0.8	–	–
Negative control[a] (i.g.)	11.7±0.6	–	–
Tumor control[a] (i.g.)	10.7±0.9	2.3±0.6	546±64*a*
Test[b] (i.g.)	14.2±0.6	5.0±0.7	551±58*a*
Substance control[a] (i.g.)	9.1±0.3	–	–

Different letters in column 4 represent the existence or not of significant differences between the test and the tumor control groups.

i.p. Intraperitoneal, *i.g.* intragastric

[a] Mean values ± standard error

[b] Mean values between 3 test groups ± standard error.

animals treated with DCM decreased. The authors also remarked that treatment with lower doses of the test substance caused smaller loss of body weight.

The highest tumor inhibition (mean value equal to 76%) attained in this work is still lower than those observed by Pagno et al. [29]. The authors concluded that oral treatment was not effective, which is in agreement with the results presented in this work.

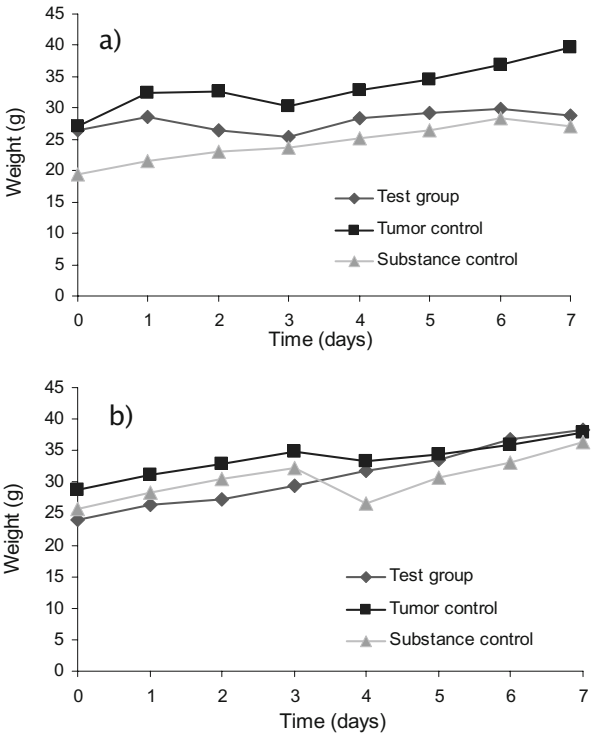

Fig. 4 Weight variation (g) with time (days) in i.p. groups (**a**) and i.g. groups (**b**) treated with the extract obtained from *P. ostreatus* fruiting bodies (EFB)

Nevertheless, i.p. administration of DCM promoted suppression in 100% of tumor cells and ascitic liquid volume. Furthermore, the tumor control group presented a larger volume of ascitic liquid, which was also observed in this work for most of the i.p. tests.

Matsuzaki et al. [27] observed that the volume of ascitic liquid was significantly lower when the Balb/cICB mice inoculated with EAT were treated by gavage with *Pfaffia paniculata* powdered roots. Ajith and Janardhanan [41] detected an increase in life span and EAT inhibition after oral treatment (25 or 50 mg per kilogram body weight) of male Swiss albino mice, with different extracts obtained from the polypore macrofungus *Phellinus rimosus.* Ninety-six percent inhibition of the solid tumor was achieved after 10-day treatment of the animals with 50 mg of ethyl-acetate extract per kilogram body weight.

Benkovic et al. [42] investigated the anticancer drug irinotecan combined or not with ethanolic extract of propolis, a water-soluble derivative of propolis (WSDP) and polyphenolic compounds (quercitin and naringin) against EAT inoculated i.p. (1×10^6 cells/animal) in male Swiss albino mice. Weight of mice was used as a measure of proliferation of the tumor cells in the peritoneal cavity. A synergistic action of irinotecan and EPP or WSDP was observed in the surviving time. Forty-four percent of the animals submitted to the treatments with these combined substances survived more than 50 days. The authors did not report data about tumor reduction.

The comparison of the results obtained in this work with literature suggests that the daily dose used was not high enough to inhibit tumor growth by i.g. treatment and that i.p. treatment may be improved when increasing the daily dose and extending the treatment time.

Conclusions

B. subtilis was the microorganism more affected by the extracts evaluated in this work, with 87.3% inhibition by the extract obtained from dehydrated mycelium (M2). The fresh fruiting bodies (B1), dehydrated fruiting bodies (B2), and fresh mycelium (M1) extracts showed inhibition of more than 50.0% against *C. albicans*, and the dehydrated mycelium (M2) extract was the most effective against *E. coli* with 57.5% inhibition. The polysaccharides extracted from the fresh mycelium (EP) did not show to be an efficient antimicrobial agent as both *C. albicans* and *E. coli* were inhibited at a low level, and *B. subtilis* growth was not affected by this extract.

All the substances tested inhibited the development of the Ehrlich ascitic tumor at levels near 70% (mean inhibition values) when injected intraperitoneally in the female Swiss albino mice. The highest tumor inhibition (76%) was achieved for the treatment carried out with the extract from *P. ostreatus* culture broth (ECB). The intragastric treatment did not promote any reduction in tumor cell development, independent of the test substance.

These results prove that *P. ostreatus* DSM 1833 is a promising microorganism for the production of antineoplasic substances, which represents the novelty of this paper.

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