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Agaricus blazei Murill on tissue damage caused by Ehrlich tumor

Agaricus blazei Murill no dano tecidual causado pelo tumor de Ehrlich

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Abstract: *Agaricus blazei* Murill (ABM) is commercialized worldwide as a medicinal food with anticancer potential. The study investigated the effects of different ABM extracts on a mouse model of transplatable Ehrlich tumor. Different extracts were produced using a solution with pH 4 and 7, water bath or ultrasonic bath, with polysaccharide solution or supernatant. 192 mice were randomly separated into 4 groups for assessment of the 4 extraction methods. Each extraction group consisted of 2 groups with or without a tumor, further separated into 4 treatment groups. Evaluations included organ weight and histology of the spleen, kidney, lymph nodes, liver, and tumor. Ehrlich's tumor leads to an increase in the relative weight of the spleen, but the use of ABM supernatant at 60ºC at pH 7 decreases the weight of the spleen. Liver weight was reduced with extract ABM at 60ºC in pH 4. Histology findings for the spleen showed an increase in the number of macrophages and, in some cases, mild white pulp hypoplasia. In animals treated with ABM supernatant solution (60ºC and ultrasonic bath), when compared to animals treated with ABM polysaccharide solution (60ºC and ultrasonic bath), less tumor cellularity, smaller distance between the epidermis and the musculature, can be observed. Free areas of tumor cells in the epidermis of the foot padsand smaller areas of necrosis and cellular infiltration were observed, demonstrating less tumor growth in these animals. The findings indicate that ABM extract at 60ºC at pH 7 produced through an ultrasonic bath has the most therapeutic potential that should be further explored.

Keywords: mouse; polysaccharides; cancer; mushroom.

Resumo: *Agaricus blazei* Murill (ABM) é comercializado mundialmente como alimento medicinal com potencial anticancerígeno. O estudo investigou os efeitos de diferentes extratos de ABM em um modelo de camundongo com tumor de Ehrlich. Diferentes extratos foram produzidos utilizando solução com pH 4 e 7, banho-maria ou banho ultrassônico, com solução de polissacarídeo ou sobrenadante. 192 camundongos foram separados aleatoriamente em 4 grupos para avaliação dos 4 métodos de extração. Cada grupo de extração consistiu em 2 grupos com ou sem tumor, separados em 4 grupos de tratamento. As avaliações incluíram peso dos órgãos e histologia do baço, rim, linfonodos, fígado e tumor. O tumor de Ehrlich leva ao aumento do peso relativo do baço, mas o uso do sobrenadante de ABM a 60ºC em pH 7 diminui o peso do baço. O peso do fígado foi reduzido com extrato ABM a 60ºC em pH 4. Os achados histológicos do baço mostraram aumento no número de macrófagos e, em alguns

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casos, discreta hipoplasia de polpa branca. Nos animais tratados com solução sobrenadante de ABM (60ºC e banho ultrassônico), quando comparados aos animais tratados com solução polissacarídica de ABM (60ºC e banho ultrassônico), pode-se observar menor celularidade tumoral, menor distância entre a epiderme e a musculatura. Áreas livres de células tumorais na epiderme das patas, menores áreas de necrose e infiltração celular, demonstrando menor crescimento tumoral nestes animais. Os resultados indicam que o extrato de ABM a 60ºC em pH 7 produzido através de banho ultrassônico tem o maior potencial terapêutico que deve ser mais explorado.

Palavras-chave:camundongo; polissacarídeos; câncer; cogumelos.

1. Introduction

Agaricus blazei Murill also known as the sun mushroom is a Brazilian species from Basidiomycetes family commercialized worldwide as a medicinal food [1]. Previous reports indicate therapeutic potential regarding anticancer activity in cultured cells [2] and *in vivo* models [3], with antiviral properties [4], antioxidant activity, immunoregulation, and antidiabetic effect [5]. It also has an anti-inflammatory effect evidenced by a reduction of blood cytokine levels in patients with chronic inflammatory diseases [6].

The major chemical compounds consist of polysaccharides, lipids including ergosterol, sterols, proteins, vitamins B, C, and D, and phenolic compounds [7]. The complex composition results in different mechanisms of action. One of the most relevant is the anticancer potential, which includes the induction of apoptosis in cells and rodent models of different cancer types, such as fibrosarcoma, sarcoma, ovarian cancer, lung cancer, and leukemia, among others [6]. Therapeutic actions on cancer cells are associated with immune system modulation, as well as activation ofnatural killer (NK) cells and induction of apoptosis [3].

Although chemotherapy remains the gold standard for most cancer treatments, severe adverse reactions and tumor resistance are frequent [8]. For this reason, adjuvant anticancer strategies, such as the use of low-toxicity natural subproducts and extracts as supplements, are promising modalities [9]. In this regard, the solid Ehrlich tumor is a known fast-growth breast cancer model with low cost and easy monitoring [10]. Therefore, this study aimed to describe the effects of different *A. blazei* extracts towards an animal model or Ehrlich tumor, in addition to organ weight evaluation and histology analysis of the tumor, liver, kidney, spleen, and lymph nodes.

2. Material and methods

2.1 Preparation of A. blazei Muril (ABM) Extracts.

Substances with probable antineoplastic activity, such as the active hemicellulose compound (AHCC) andpolysaccharide–protein complex (designed as ATOM) were obtained from the cultured mycelia of *A. blazei.* Aqueous extractions are not yet capable of promoting the complete rupture of this barrier and, consequently, do not make such substances available for direct activity (*in vitro*) or for absorption by the digestive system (*in vivo*). The use of temperatures exceeding 100ºC, or the insertion of acids during the extraction process,

provides greater concentrations of active ingredients. This fact may be correlated to the rupture of the mushroom's hemicellulose barrier [11]. Therefore, different forms of extraction from *Agaricus blazei* Murrill can determine a greater or lesser concentration of the active pharmacological components with antineoplastic action present in the mushroom.

Extracts were obtained from 3 kg of dehydrated ABM purchased from a commercial producer in São José do Rio Preto City, São Paulo, Brazil. A sample was deposited in the herbarium of the Institute of Biological Sciences of the Federal University of Minas Gerais (BHCB 97946).

Extracts were prepared on four extraction methods [11]. Two main extracts were produced with neutral (7.0) and acidic (4.0) pH. Aliquots of 150 g of dried ABM were mixed into 1000 mL distilled water or 2% glacial acetic acid, producing neutral (pH 7) or acidic (pH 4) suspensions, respectively. The process was repeated, and each suspension was held in an ultrasonic bath at +37°C for 30 min. Following extraction, each solution was filtered (Table 1).

Table 1 Final solutions of *Agaricus blazei* extracts

Solution	Kind of extract	pH	Part of the Solution
1 – A	Water bath 60°C / 3 h	Neutral (pH 7)	Total solution
1 – B	Water bath 60°C / 3 h	Neutral (pH 7)	Polysaccharides (precipitate)
1 – C	Water bath 60°C / 3 h	Neutral (pH 7)	Supernatant
2 – A	Water bath 60°C / 3 h	Acidic (pH 4)	Total solution
2 – B	Water bath 60°C / 3 h	Acidic (pH 4)	Polysaccharides (precipitate)
2 – C	Water bath 60°C / 3 h	Acidic (pH 4)	Supernatant
3 – A	Ultrasonic bath / 30 min	Neutral (pH 7)	Total solution
3 – B	Ultrasonic bath / 30 min	Neutral (pH7)	Polysaccharides (precipitate)
3 – C	Ultrasonic bath / 30 min	Neutral (pH 7)	Supernatant
4 – A	Ultrasonic bath / 30 min	Acidic (pH 4)	Total solution
4 – B	Ultrasonic bath / 30 min	Acidic (pH 4)	Polysaccharides (precipitate)
4 – C	Ultrasonic bath / 30 min	Acidic (pH 4)	Supernatant

In each 500 mL of extract was added to 2000 mL of ethanol to precipitate polysaccharides. Following precipitation, 50 mL of each sample was homogenized and centrifuged for 10 min. at +20°C at 300 g. The sediment was separated and desiccated at ambient temperature, weighed, and the percent polysaccharides determined.

The sediments were dissolved in 2 % acetic acid solution or distilled water to a volume of 500 mL to create solutions of *A. blazei* polysaccharides. The solutions were concentrated under reduced pressure at 80°C, for removal of the ethanolic fraction. Distilled water or 2 % acetic acid was added to make up the volume to 500 mL and to create the *A. blazei* extracts for use in the experiments. Total extract, supernatant, and polysaccharide solutions were created for each pH (4.0 or 7.0) of extraction, to obtain six solutions. Monosaccharides present in the polysaccharide solutions were determined by gas chromatography of alditol acetates produced by hydrolysis with trifluoroacetic acid, reduction with sodium borohydride

sodium, and acetylation. Thus, the ABM solute and relative content of glucose and mannose was determined.

The size of the polysaccharide molecules was measured by liquid chromatography using size exclusion or gel permeation chromatography, using standard polystyrene column GPC-803D 300 x 8 mm, dimethylformamide as mobile phase, flow rate of 1.0 mL/min, ultraviolet detector at 270 nm, and shot volume of 20 µL.

2.2 Extract Composition

150g of dried mushroom, *in natura*, crushed to a granulometry of 40 mesh, was used for evaluation of the levels of crude protein (CP), total dry matter (TDM), detergent fiber neutral (DFN), and acid detergent fiber (ADF). And finally, 600 g of dried mushroom, *in natura*, ground to a granulometry of 40 mesh, were destined to four different processes of aqueous extraction and subsequent fractionation into three solutions each: total solution, supernatant and precipitated extracts. A sample of each of the B solutions (of polysaccharides) was investigated for its constitution in monosaccharides. After hydrolysis with trifluoroacetic acid, reduction with sodium borohydride, and acetylation, the alditols acetates formed were analyzed by gas chromatography (GC), determining the levels of glucose (GL) and mannose (MA) present, as well as the relative value between these components. These samples were also evaluated in Size Exclusion Liquid Chromatography (STC) (Gel Permeation Chromatography). It was used to evaluate the size of the polysaccharide molecules present in the ABM samples. These evaluations were carried out by High-Performance Liquid Chromatography (HPLC), with a GPC-803D 300 x 8 mm column, and with the mobile phase of dimethylformamide, a flow rate of 1.0 ml per minute, an ultraviolet detector at 270 nm and an injection volume of 20 µL. For the Size Exclusion Chromatography evaluations, the standard used for the comparison and evaluation of the molecular weight of the polysaccharides was polystyrene.

2.3 Experimental Design

Animals

All the animal procedures were performed with the approval of the Animal Care and Use Committee of Federal University of Minas Gerais State. 192 Non-isogenic female Swiss mice (*Mus musculus*) were used in the experiments, weighing between 25 and 30 g, 60 days old. All were kept under standard conditions (12 hours light and dark cycles) at +22 ± 2 °C, fed with laboratory rodent diet and water *ad libitum*. Mice were randomly separated into 4 groups (n=48) for assessment of the 4 extraction methods. Each extraction group consisted of 2 groups (n=24), with or without a tumor, designated GI and GII, respectively, further separated into 4 treatment groups (n=6) (Table 2).

Ehrlich solid tumor

To prepare for the induction of a solid tumor, Ehrlich tumor cells were maintained in ascitic form in Swiss albino mice, and transferred weekly into healthy animals to preserve

the Ehrlich tumor *in vivo*. On day 7 post-inoculation, 3 mL of ascitic fluid was collected from the donor mouse in a disposable syringe. The ascitic fluid was placed in a microtube and centrifuged at 300 g for 3 min to separate the supernatant. The volume was increased to 3 mL with 0.9 % saline at room temperature and stirred slowly, and the procedure was repeated three times until the supernatant became translucent [11,12].

For analysis of tumor viability, 1.98 mL of 0.9 % saline solution was added to 0.02 mL of cell suspension from the ascitic fluid and homogenized. Aliquots of 0.1mL of this mixture were added to an equal volume of 0.1 % trypan blue. A cell count was performed in four external quadrants of a Neubauer hemocytometer. Counts showed 95.04 % of cells were viable (translucent) and 4.69 % nonviable (blue stained), yielding 5 x 10^8 viable tumor cells per 1.0 mL suspension.

The animals were inoculated in the plantar cushion of the left posterior hind limb with an injection (50µL) containing 2.5 x 10^6 of Ehrlich tumor cells taken from ascites from Swiss mice.

Treatments

Over 21 days, distilled water, total extract, supernatant, or polysaccharide solution were administered to mice daily by gavage, after 3 hours without food or water, at 2.5 g/kg of body weight (BW).

Table 2 Distribution of 192 mice without (GI) or with (GII) Ehrlich solid tumor groups (n = 24) and in treatment groups (n = 6) treated for 21 days with *Agaricus blazei* extracts produced in a water bath or ultrasonic bath at pH 4 or pH 7, to total solution, polysaccharide solution or supernatant or distilled water.

Treatments for 4 experiments: without tumor and with Ehrlich tumor, with 4 treatments
Experiment 1: GI – without tumor
(A) Distilled water
(B) Total solution at pH 7 extracted in a water bath
(C) Polysaccharide solution at pH 7 extracted in a water bath
(D) Supernatant solution at pH 7 extracted in a water bath
Experiment 1: GII – with Ehrlich tumor
(A) Distilled water
(B) Total solution at pH 7 extracted in a water bath
(C) Polysaccharide solution at pH 7 extracted in a water bath
(D) Supernatant solution at pH 7 extracted in a water bath
Experiment 2: GI – without tumor
(E) Distilled water
(F) Total solution at pH 4 extracted in a water bath
(G) Polysaccharide solution at pH 4 extracted in a water bath
(H) Supernatant solution at pH 4 extracted in a water bath
Experiment 2: GII – with Ehrlich tumor
(E) Distilled water
(F) Total solution at pH 4 extracted in a water bath
(G) Polysaccharide solution at pH 4 extracted in a water bath
(H) Supernatant solution at pH 4 extracted in a water bath
Experiment 3: GI – without tumor
(I) Distilled water
(J) Total solution at pH 7 extracted in an ultrasonic bath
(K) Polysaccharide solution at pH 7 extracted in an ultrasonic bath
(L) Supernatant solution at pH 7 extracted in an ultrasonic bath
Experiment 3: GII – with Ehrlich tumor
(I) Distilled water
(J) Total solution at pH 7 extracted in an ultrasonic bath
(K) Polysaccharide solution at pH 7 extracted in an ultrasonic bath
(L) Supernatant solution at pH 7 extracted in an ultrasonic bath

Experiment 4: GI – without tumor
(M) Distilled water
(N) Total solution at pH 4 extracted in an ultrasonic bath
(O) Polysaccharide solution at pH 4 extracted in an ultrasonic bath
(P) Supernatant solution at pH extracted in an ultrasonic bath
Experiment 4: GII – with Ehrlich tumor
(M) Distilled water
(N) Total solution at pH 4 extracted in an ultrasonic bath
(O) Polysaccharide solution at pH 4 extracted in an ultrasonic bath
(P) Supernatant solution at pH 4 extracted in an ultrasonic bath

2.4 Samples Weight and Histopathology

Mice were euthanized by cervical dislocation. The necropsy exam was conducted, and samples from the spleen, liver, kidneys, tumor, and popliteal lymph nodes of the animals were taken. The material was weighed in the electronic balance with 5 decimals of precision, fixed in formol 10 %. Calculations of relative values of the organs collected (organ weight/animal weight) were performed to minimize the effects of weight differences between animals. From the total weight of the limb with tumor (left posterior) the weight of the tumor-free limb (right posterior) of each animal was subtracted. The values obtained were divided by the weight of the animal, obtaining the values considered as the relative weight of the tumor of each of the animals in group GII.

After collection and weighing, the spleen, liver, and lymph nodes were sectioned. Tumor masses were separated from the limbs and cut transversely. All of these were separately accommodated in plastic cassettes for histopathological inclusion, and fixed in 10 % neutral buffered formalin. Subsequently, they were dehydrated in increasing alcoholic solutions, cleared in xylol, and included in histological paraffin. The paraffin blocks were sectioned in a micrometer, obtaining 4 µm cuts, for subsequent deparaffinization in xylol, hydration in decreasing concentrations of ethyl alcohol, staining using the Hematoxylin and Eosin technique, new dehydration in increasing concentrations of alcohols, and mounting the slides and were evaluated through optical microscopy [13,14].

2.5 Statistical Analyses

Statistical analyses were performed, evaluating the normality of the variables using the Kolmogorov-Smirnov method. Parametric variables were subjected to analysis of variance using the ANOVA method and Duncan Test, with a significance level of $p<0.05$, which provides robustness to the study's conclusions. Non-parametric ones were analyzed with analysis of variance by the ANOVA method and the Kruskal-Wallis test [15], except for the results obtained from the descriptive evaluations, observed in the readings of the histological slides.

3. Results

3.1 Mushroom Identification

The mushroom was identified as *Agaricus blazei* Murill. Its aliquot was deposited at the Department of Botany of the Institute of Biological Sciences of the Federal University of Minas Gerais, under number BHCB: 97946.

3.2 Composition of the Extracts

After the extractions, there was a reduction to 21.4 % and 27.9 % of the original CP values, in the extractions in a water bath with neutral and acid pH, respectively. In ultrasonic bath extractions, the reduction was 16.91 % and 20.92 %, at neutral and acid pH respectively. The results indicate that the extracts can have up to 78.6 g; 72.1 g; 83.1 g and 79.08 g of CP from the ABM for every 100 mL of extracts in a neutral and acid water bath and in a neutral and acid ultrasonic bath, respectively. In this way, it was observed that the extraction in an ultrasonic bath at pH 7 was more efficient in extracting the CP, as well as in decreasing the NDF and ADF values. The lowest levels of DFN and ADF indicate the best breakdown of the mushroom's cellulose and hemicellulose barriers. This factor is of great relevance since authors indicate that parts of the mushroom's active principles may be trapped by these barriers. In this way, the ultrasonic bath is more efficient in breaking this barrier, and pH 7 is more efficient than pH 4.

Figure 1 shows the percentages of precipitates obtained in the different ABM extractions. It can be observed that the extraction in an ultrasonic bath with neutral pH provided the highest amount of precipitate (16.42%). Likewise, it is observed that the extraction in an ultrasonic bath with pH 4 (10.61%) was similar to that of 60ºC with pH 7 (10.83%) and better than the extraction at 60ºC with pH 4 (8.46 %).

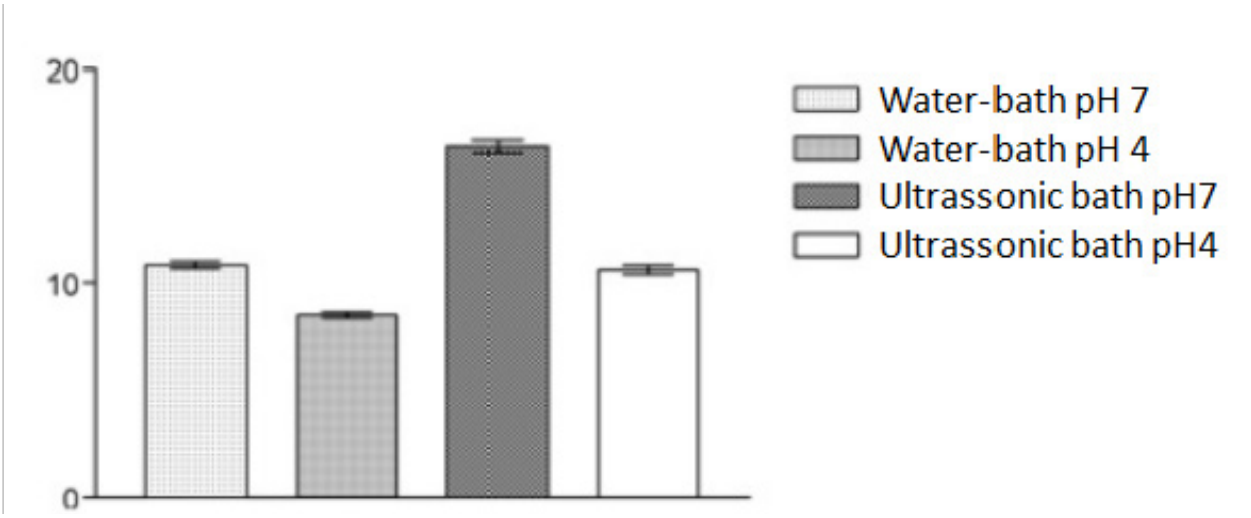

Figure 1 Percentage values of precipitate obtained from mushroom *Agaricus blazei*, after extractions at 60ºC for 3 hours at pH 7.0 and pH 4.0, and ultrasonic bath for 30 minutes at pH 7.0 and pH 4.0.

3.3 Chromatographic Profile of Monosaccharides from ABM Extracts

Figure 2 shows the chromatograms obtained by evaluating a sample of each of the solutions B (of polysaccharides), regarding its constitution in monosaccharides, analyzed by gas chromatography (GC). GC evaluations revealed that the extracted polysaccharides are not only constituted of glucopyranosides (β-glucan) as expected, but also present mannopyranosides (mannan) and/or glucomanopyranosides (glucomannans).

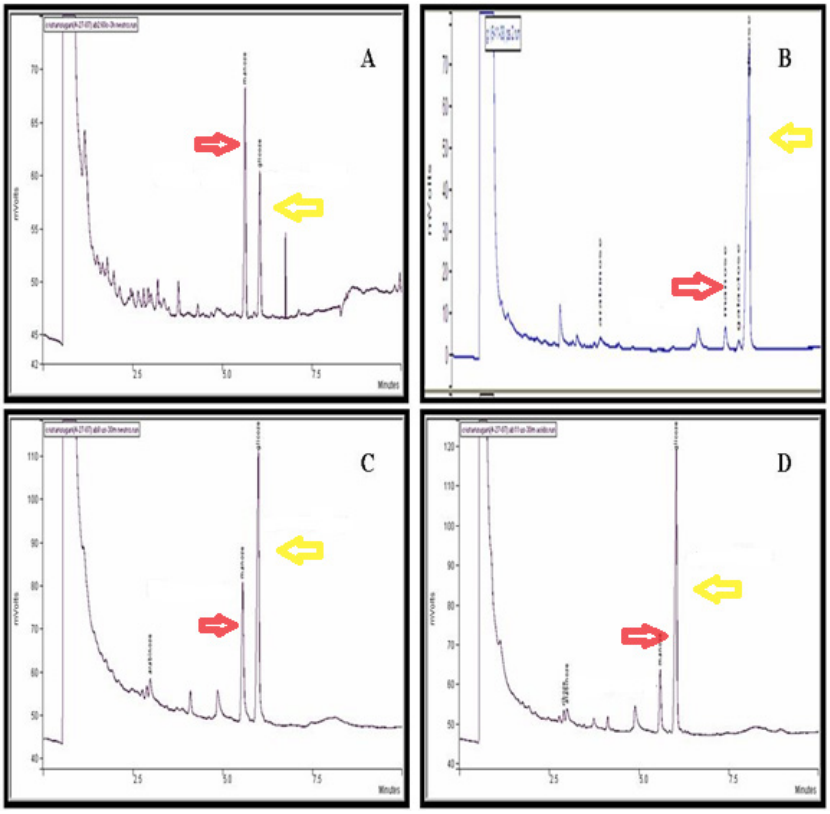

Figure 2 Chromatographic profile of monosaccharides analyzed by gas chromatography (GC), demonstrating the levels of glucose (GL – yellow arrows) and mannose (MA – red arrows) present in the polysaccharide extracts (B) from aqueous extraction of the mushroom *Agaricus blazei*, after extractions at 60ºC for 3h at pH 7.0 and pH 4.0 and extractions in an ultrasonic bath for 30 min at pH 7.0 (C) and pH 4.0 (D).

3.4 Assessments of Internal Organs Weight

Figures 3-5 show the mean values and standard deviation of the relative weight of the spleen, liver, and kidney of the animals in the four evaluated experimental blocks. Regarding the spleen, in the first experimental group, there was a statistical difference in spleen weight between animals from GI-A (inoculated with saline and treated with water), and GII-A (with tumor and treated with water), demonstrating that Ehrlich's tumor leads to an increase in the relative weight of the spleen. The use of ABM supernatant at 60ºC at pH 7 influences the weight of the spleen, which may be related to the lower production of cytokines in the body.

In the second experimental moment, animals were treated with solutions and ABM at 60ºC at pH 4, and no significant difference was observed. When using ABM extracts in an ultrasonic bath at pH 4, a statistical difference was found between the values of the relative weight of the spleen of mice in the GI group (healthy), except for those in the GI-P subgroup (healthy and treated with ABM supernatant in an ultrasonic bath at pH 4), in relation to the animals in group GII (with tumor).

Liver assessment showed that animals treated with ABM solutions at 60ºC in pH 7, the subgroup GI-C (healthy and treated with polysaccharides) presented the highest value, different from the subgroup GII-A (with tumor and treated with water). The treatments with different extracts of ABM at 60ºC in pH 4, caused statistical differencesin weight reduction in the animals of the subgroup GI-F (healthy) and GII-F (carriers of tumor).

In the third experimental phase, where the animals were treated with ABM solutions in an ultrasonic bath at pH 7, no statistical difference was observed between the evaluated subgroups. However, in the treatment with extraction in an ultrasonic bath at pH 4, a statistical reduction could be verified in the values of the subgroups GII-O (with tumor and treated with ABM polysaccharides in an ultrasonic bath atpH 4), and animals from subgroups GII-P (inoculated with tumor and treated with ABM supernatant solution in an ultrasonic bath at pH 4).

When the average values of relative weight of the liver of animals treated with ABM extracts at 60ºC at pH 4 were evaluated, it was observed that animals with tumors and treated with total solution had lower average values of relative weight.

No alterations were observed in the values of the relative weight of the kidneys in the different subgroups of the four experimental blocks evaluated. The presence of the tumor or the use of different treatments with ABM did not alter these values.

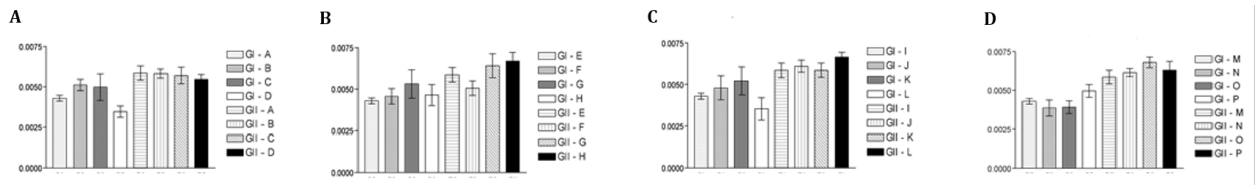

Figure 3 - Mean values of relative weight (organ weight/animal weight) of the spleen (A-D), on the 21st day of the experiment, in healthy mice (GI) and with solid Ehrlich Tumor (GII), treated with different *Agaricus blazei* extracts.

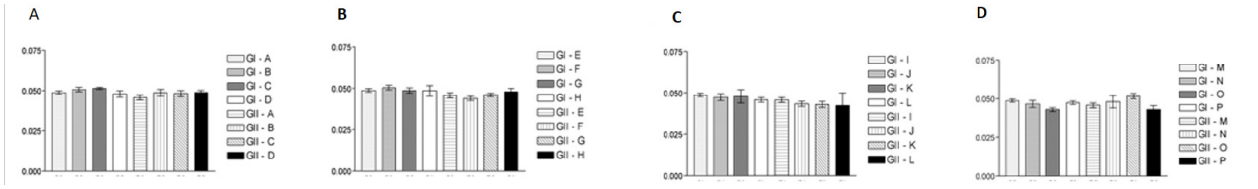

Figure 4 Mean values of relative weight (organ weight/animal weight) of the liver (A-D), on the 21st day of the experiment, in healthy mice (GI) and with solid Ehrlich Tumor (GII), treated with different *Agaricus blazei* extracts.

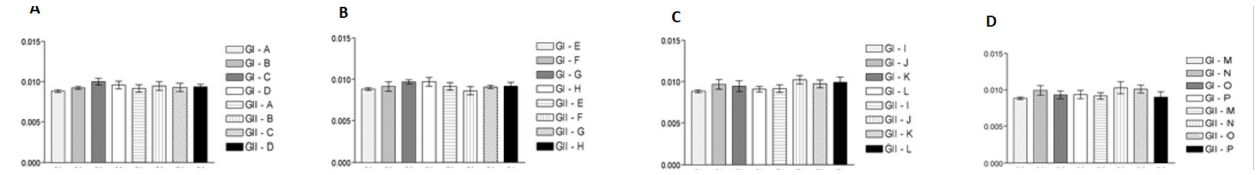

Figure 5 Mean values of relative weight (organ weight/animal weight) of the kidneys (A-D), on the 21st day of the experiment, in healthy mice (GI) and with solid Ehrlich Tumor (GII), treated with different *Agaricus blazei* extracts.

3.5 Histology of internal organs

During the necropsy of the animals, no noteworthy macroscopic alterations were observed in any evaluated group (Figures, 6, 7, 8 and 9). On microscopic examination, no noteworthy changes were observed in animals from the GI (healthy) groups.

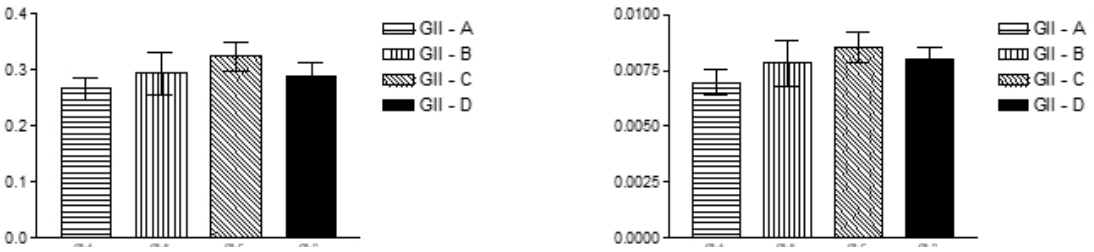

Figure 6 Mean values of Ehrlich tumor (weight of the left hind limb – weight of the right hind limb) on the 21st day of the experiment, of mice with solid Ehrlich Tumor (GII), treated with water (A) and extracts of *Agaricus blazei* at 60°C at pH 7: total solution (B), polysaccharides (C) and supernatant (D).

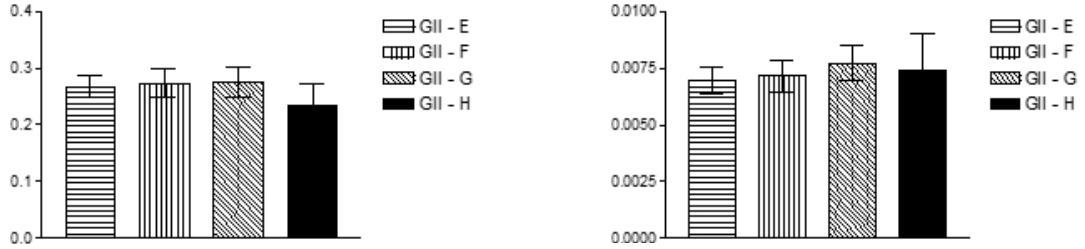

Figure 7 Mean values of Ehrlich tumor (weight of the left hind limb – weight of the right hind limb) on the 21st day of the experiment, of mice with solid Ehrlich Tumor (GII), treated with water (E) and extracts of *Agaricus blazei* at 60°C at pH 4: total solution (F), polysaccharides (G) and supernatant (H).

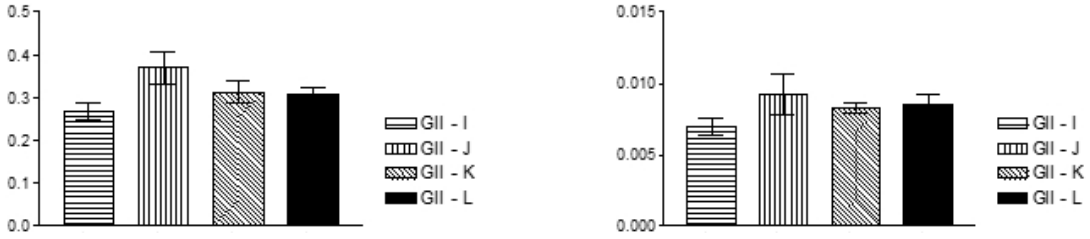

Figure 8 Mean values of the Ehrlich tumor (weight of the left hind limb – weight of the right hind limb) on the 21st day of the experiment, of mice with solid Ehrlich Tumor (GII), treated with water (I) and extracts of *Agaricus blazei* in ultrasonic bath and pH 7: total solution (J), polysaccharides (K) and supernatant (L).

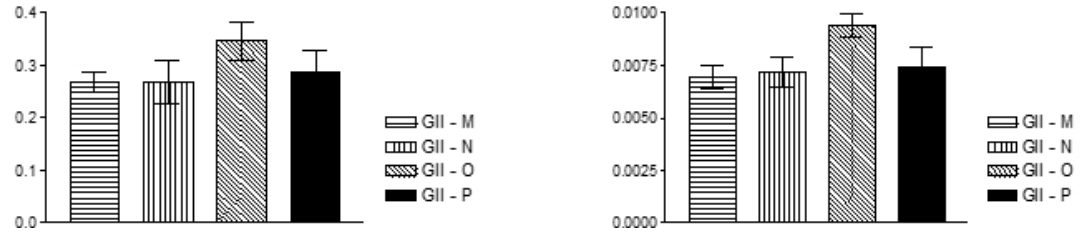

Figure 9 Mean values of the Ehrlich tumor (weight of the left hind limb – weight of the right hind limb) on the 21st day of the experiment, of mice with solid Ehrlich Tumor (GII), treated with water (M) and extracts of *Agaricus blazei* in ultrasonic bath and pH 4: total solution (N), polysaccharides (O) and supernatant (P).

In the spleen of animals from subgroup GII (tumor carriers) an increase in the number of macrophages with displacement of the nucleus to the periphery and the presence of moderate rarefaction of white pulp lymphoid follicleswere observed (Figure 10-A).

The liver evaluation revealed that in all four experimental groups, in animals from subgroup GII (carriers of Ehrlich's tumor regardless of the treatment used) (Figure 10-C), cells with granular cytoplasm were observed, characteristic of turbid degeneration of hepatocytes, located mainly close to the capsule of the organs, and areas with vacuoles and nuclei displaced to the periphery, indicative of the beginning of a hepatic steatosis process. No alterations were observed in any animal from the GI group (healthy), regardless of the treatment used.

No macroscopic or microscopic alterations were observed in the kidneys of animals in the four experimental blocks, inoculated or not with Ehrlich's tumor, and treated with placebo or different ABM solutions.

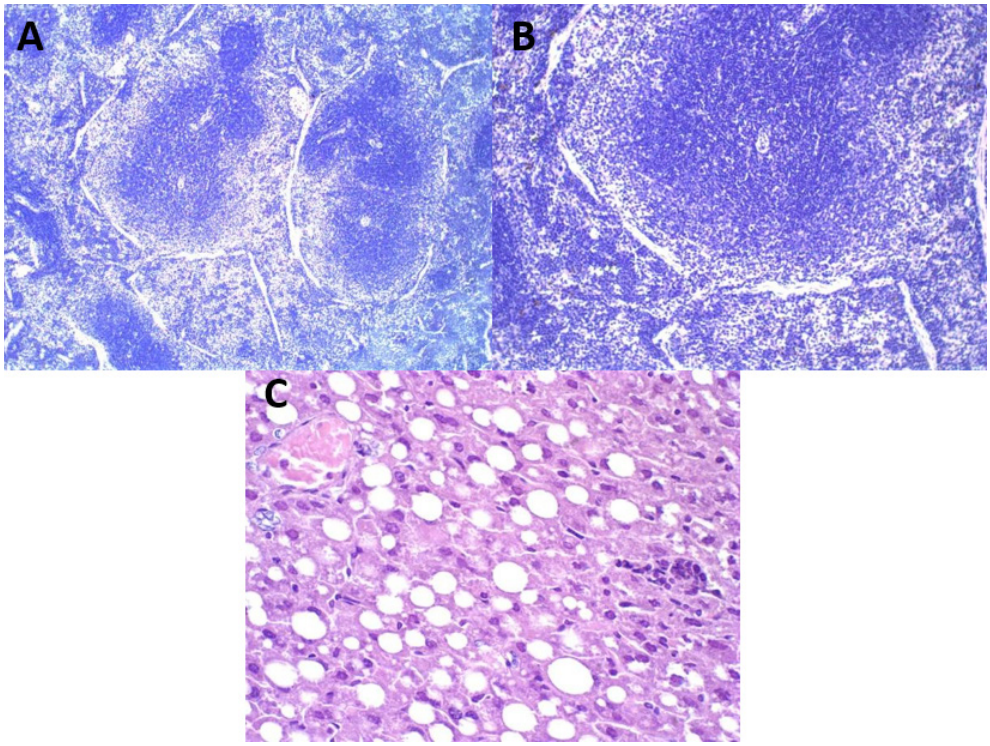

Figure 10. A and B: Section of the spleen of mice from the group inoculated with solid Ehrlich Tumor and treated with water (GI-A) (HE, 10 and 20x, respectively), demonstrating the presence of moderate rarefaction of white pulp lymphoid follicles; c - Histological slides of mouse liver from the group inoculated with tumor and treated with water (GI-A) (HE, 60x), demonstrating the presence of turbid degeneration, moderate lymphocytosis and vacuoles with displacement of the nucleus to the periphery of the cells.

3.6 Assessment of tumor weight and histology

Weights were calculated from the subtraction of the value obtained with the weight of the left hind limb (inoculated with neoplasia) from the value of the weight of the right hind limb (without neoplasia). No statistical differences were observed in the values of tumor weight or relative tumor weight in the subgroups evaluated in the four experimental blocks.

During the necropsy, the macroscopic evaluation, the tumors of the animals of all the subgroups of the four experimental blocks evaluated were similar, with whitish coloration and quite infiltrated in the bones of the region. Although no morphometry evaluations were performed, differences were observed in the morphology of the neoplasms of the evaluated animals (Figures 11 and 12).

Areas of ulceration can be observed in the epidermis of the footpad in animals (100% of animals) from the groups inoculated with tumor and treated with water, a fact that did not occur in any of the animals treated with the different ABM solutions. In animals treated with ABM supernatant solution (60ºC and ultrasonic bath), when compared to animals treated with ABM polysaccharide solution (60ºC and ultrasonic bath), less tumor cellularity, smaller distance between the epidermis and the musculature, can be observed. free areas of tumor cells in the epidermis of the foot pads, smaller areas of necrosis and cellular infiltration, demonstrating less tumor growth in these animals. Such factors may indicate the antineoplastic activity of ABM supernatant solutions.

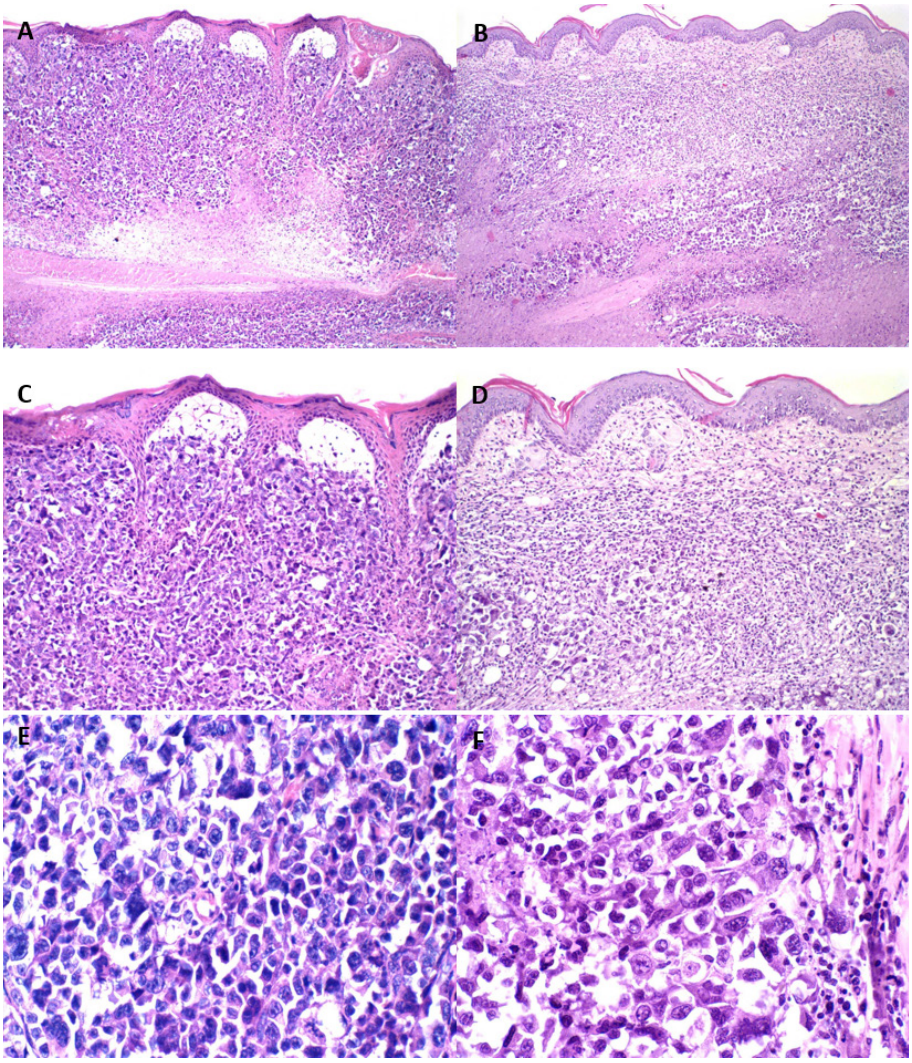

Figure 11. Skin: Mice inoculated with Ehrlich's tumor. Observe infiltrative pleomorphic neoplastic cells in animals treated both with water (1) and with a total ABM solution at 60ºC in pH 7; HE staining and A and B-10x magnification; C and D-20x and E and F-60x.

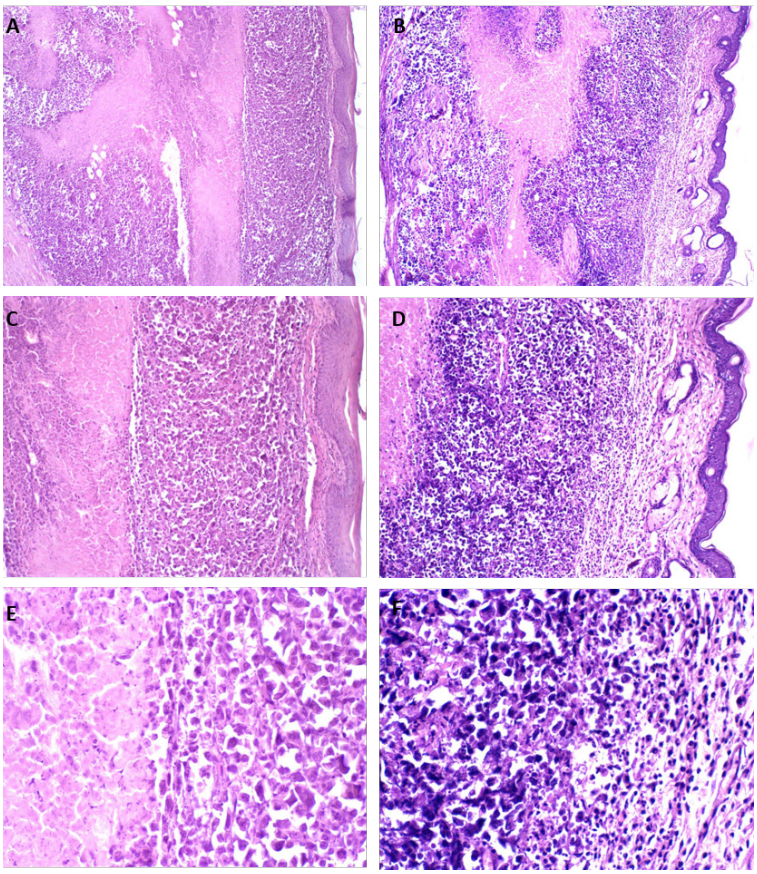

Figure 12. Skin: Mice inoculated with Ehrlich's tumor and treated with 1- ABM polysaccharide solution at 60°C in pH 4 and 2- ABM supernatant solution at 60°C in pH4; HE stain and A and B -10x magnification; C and D- 20x and E and F- 60x demonstrating greater length between the epidermis and the musculature in A due to the greater amount of tumor cells, inflammatory infiltrate and areas of necrosis, in relation to B; larger areas of necrosis in C and E compared to D and F.

3.7 Evaluation of the popliteal lymph nodes.

Metastasis was observed in the left popliteal lymph nodes in all animals with neoplasia, regardless of the use of different ABM extracts. Neoplastic cells were visualized at similar intensity in all subgroups, being found mainly in subcapsular regions, grouped or in the form of cords.

4. Discussion

In the present study, the anticancer potential of the sun mushroom, *A. blazei*, was investigated in a mice model of Ehrlich tumor. *A. blazei*, is known for its anticancer [3] and hepatoprotective activity [16]. Ehrlich tumor is a well-established animal model for rapid cancer development. Ehrlich tumor is traditionally used as a murine experimental oncology model to investigate chemotherapeutic agents. Metastasis is common at regional lymph nodes and systemic repercussions are also well known. Several natural extracts have been used in the research for adjuvant cancer therapies that could improve clinical outcome [17,18], including mushroom extracts [11].

A. brasiliensis Murill or *A. blazei* Murill mushroom, also known as the sun mushroom, is a Brazilian species that has been commercialized worldwide. Different methodologies have been used to extract its active components [19]. In our study, following botanical confirmation and residue screening, four approaches were made considering extraction with water bath, ultrasonic bath, pH 7, and pH 4, reaching the temperature of 60ºC. ABM extract in ultrasonic bath and pH 7, provided higher PB values in the final solution, as well as lower NDF and ADF values in relation to the initial values, indicating greater breakdown of cellulose barriers, in relation to the other extractions of the ABM mushroom. This provides a greater amount of polysaccharides in the final solution and reveals high protein values. It was reported values between 27.8 to 43.19 g of CP per 100 g of mushroom. In the *in natura* sample, a 31.31 g% CP content was observed, confirming that the evaluated sample has high levels of this substance [20].

Complementary evaluation by gas chromatography identified the presence of f glucopyranosides (β-glucan) as expected, but also present mannopyranosides (mannan) and/or glucomanopyranosides (glucomannans), also showing that different forms of extraction lead to changes in the mannose:glucose ratio. These results are of great importance since most authors mention only the existence of glucose in aqueous extracts of ABM. It was reported different values of β-glucan in different aliquots of mushroom, and warned that differences in cultivation methods can change the amounts of this substance in the ABM [21]. Such observations indicate that the different forms of extraction, in addition to interfering with the amount of polysaccharides, also lead to the extraction of different amounts of each type of element. Proving the existence of glucose and mannose, and the fact that the levels of these polysaccharides change with different extractions, is important for studies on treatments with ABM solution, since the β-glucan found in this mushroom is a polysaccharide with proven activity in modulating the immune system [21]. Thus, different ABM extracts can lead to different results regarding the activation of the immune system and its influence on the evaluated treatments. It is also important that most authors attribute ABM activity to polysaccharides, but the contribution of other components, such as proteins, triterpenes, steroids, nucleosides, and phenols cannot be discarded [22].

All animals that received tumor cells developed lesions, however the use of *A. blazei* was not able to stop the process of metastasis of the neoplasm in the popliteal lymph nodes, regardless of the different treatments, as described by Verçosa Junior et al. [23], who used total ABM extract for 17 and 57 days in mice with Ehrlich's tumor for 17 days. Neoplastic cells were visualized at similar intensity in all subgroups, being found mainly in subcapsular regions, grouped or in the form of cords. This finding reveals that the extracts did not have significant anticancer action, contradicting previous research [3]. Authors suggest that extraction at high temperatures, such as 60ºC, could possibly induce degradation of the active(s) principle(s) of the mushroom [24]. It was reported an increase in the relative weight of the spleen and alterations in the erythrogram and leukogram of mice inoculated with Ehrlich tumor treated with filtrate and total suspension of ABM (10mg/mL) [25]. Such results differ from those observed in the ABM extract at 60ºC at pH 7, in which the treatment with the total ABM

solution was similar to the treatment with placebo. Regarding spleen damage, the lowest mean values of relative weight of the spleen were seen in the two subgroups that received ABM supernatant at 60ºC at pH 7 and at 60ºC at pH 4.

The increase in the relative weight of the spleen observed in the different studied groups may be correlated with the greater hemocateretic or blood cell maturation action of this organ in mice and agree with the results described by Korekane et al. [26], who reported greater relative weight of the spleen in animals inoculated with tumor. According to the authors, the release of cytokines can lead to an increase in splenic volume and weight. Kidney evaluation did not reveal important findings, suggesting that liver and spleen are better samples for histology analysis.

Liver damage was observed, indicating degeneration and possible steatosis, in all cancer groups. It was not possible to verify hepatoprotective activities in animals treated with ABM extracts in this experiment, since lesions in liver cells were observed in animals inoculated with the tumor, regardless of the use or not of treatments with the mushroom. Likewise, hepatotoxic activities cannot be established for the extracts used in this experiment, as suggested by Mukai et al. [27], who reported liver dysfunction and increased morphological lesions in cancer patients who started using commercial ABM extracts. Such facts can be correlated with the acute and aggressive characteristicsof Ehrlich's tumor, both in terms of local growth and hepatotoxic activity. Some authors [26] also described changes in liver cells with impairment of functions in mice with ascitic Ehrlich tumor, caused by cytokines (IL-1 and TNFα) and others [28] reported cloudy degeneration of hepatocytes in mice with tumorsin solid and ascites form. It was reported hepatoprotective activity in patients with chronic hepatitis and inhibition of collagen fiber formation in human hepatocarcinoma with the use of ABM extracts [29,30].

Barbisan et al. analyzed the modifying influence of prior administration of an aqueous extract of the mushroom *A. blazei* on necrosis, proliferation, and on the development of glutathione S-transferase, placental form (GST-P) positive hepatocytes induced by different doses of diethylnitrosamine in Wistar rats,suggesting that the treatment with aqueous extract of *A. blazei* Murrill exerts a hepatoprotective effect on liver toxicity and on the initiation of hepatocarcinogenesis in an environment of moderate toxicity[31].Pinheiro et al. demonstrated a beneficial influence from a 10% powdered *Agaricus blazei* Murrill mushroom meal on rat chemically-initiated liver carcinogenesis. The beneficial influence of that diet was dependent on the strain and harvest period of the fungi (opened or closed basidiocarp) and, also was characterized by reduced cell proliferation and development of putative preneoplastic foci of altered hepatocytes[32].The liver is vulnerable to a wide variety of toxic and microbial substances. In most cases, hepatic involvement is secondary. Turbid swelling is a form that hepatocytes can assume before characterizing necrosis. The cytoplasm becomes irregularly compact with large clear spaces (grainy appearance) and basophilic [33].

Authors divide the degenerations by the chemical nature of the solutions that accumulate in the injured cells (accumulation of water, proteins, lipids, and glycides). Cloudy swelling

(due to water accumulation) can be caused by hypoxia, acute viral or bacterial infections, hyperthermia, and exogenous intoxication, in addition to circulatory alterations. Various aggressions occur, acting directly or indirectly on the cytoplasmic membrane, altering the exchange of ions and water that accumulates inside the cells. Such degenerations can cause an increase in the volume and weight of the organ, but most of the time, they do not compromise the basic functions of its cells [34]. These observations may be related to the metabolism of cytokines present in the circulation in the presence of Ehrlich's tumor.

5. Conclusion

In conclusion, our results suggest that ABM extract at 60ºC at pH 7 produced through ultrasonic bath has the most therapeutic potential that should be further explored. However, no significant hepatoprotection or anticancer activity was observed. Further studies must evaluate the relevance of extraction temperature in the stabilization of active components from *A. blazei* extract.

6. Final Considerations

Substances with probable antineoplastic activity are found within the mushroom's hemicellulose barriers. Aqueous extractions are not yet capable of promoting the complete rupture of this barrier and, consequently, do not make such substances available for direct activity (*in vitro*) or for absorption by the digestive system (*in vivo*). The use of temperatures exceeding 100ºC, or the insertion of acids during the extraction process, provides greater concentrations of active ingredients. This fact may be correlated to the rupture of the mushroom's hemicellulose barrier.

The determination of acid detergent fiber (ADF) evaluates the cellulose and lignin values of the sample, being used in evaluations of animal feed, and the evaluation of neutral detergent fiber (NDF) determines the levels of cellulose, hemicellulose and lignin in the analyzed material, In this method, pectin and tannins are also soluble. The lower NDF and FDA contents indicate better breakdown of the cellulose and hemicellulose barriers. In this way, such centesimal evaluations carried out prior to extractions and on the remaining post-extraction material, can be of great value in evaluating the breakdown of the barriers present in this mushroom.

Conflicts of Interest

The authors declare no conflict of interest.

Author contributions

CRediT authorship contribution statement

D. Verçosa Junior: Methodology, Formal analysis, Data curation. **A.F.M. Botelho**: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **G.D. Cassali**: Investigation, Methodology. **M.M. Melo**: Conceptualization, Resources, Project administration, Funding acquisition, Methodology, Formal analysis, Writing – review & editing.

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