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ANTINEOPLASIC ACTIVITY OF *Agaricus brasiliensis* BASIDIOCARPS ON DIFFERENT MATURATION PHASES

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ABSTRACT

The fungus *Agaricus brasiliensis* is a Basidiomycete studied because of its immunomodulation and/or antitumor substances. The objective of this study was to verify the *Agaricus brasiliensis* antineoplasic activity *in vivo* on different basidiocarp maturation phases on Sarcoma 180 cells implanted in mice. Sarcoma cells were implanted in mice and after seven days mice were divided in three groups. The first group was treated with saline solution, the second group was treated with closed basidiocarp extract solution and the third group was treated with opened basidiocarp extract solution. After 30 days of being daily orally treated with these three solutions all animals suffered euthanasia, and the splenic index, tumor mass and volume were determined. No significant differences of the tumor growth inhibition in function of the different basidiocarp maturation phases for the *Agaricus brasiliensis* strain were observed. The *in vivo* basidiocarp antineoplasic average activity was 89.22%.

Key words: *Agaricus blazei*; basidiome; active principle; antitumor; fungus extract.

INTRODUCTION

Agaricus brasiliensis Wasser *et al*. (*A. blazei* Murrill ss. Heinemann) (25), also denoted *A. subrufescens* Peck (10), is a Basidiomycete that has been reported because of its antitumoral substances (2, 9, 16). Reports have demonstrated that the use of *Agaricus blazei* basidiocarp aqueous extract induced the production of specific cytotoxic cells such as T lymphocytes and natural killer cells as well as gamma-interferon what consequently inhibited tumor growth in rodents (22, 27). Menoli *et al*. (14) reported a decrease of the micronuclear frequency in the presence of *A. blazei* basidiocarp aqueous extract and Rodrigues *et al*. (21) reported a bioantimutagenic action of *A. blazei* suggesting an action on one or more DNA damage-repair systems. Liu *et al*. (12) studied the immunostimulatory activity of *A. brasiliensis* basidiocarp extract in human volunteers and observed a natural killer activity increase on the immunological system of patients.

A. brasiliensis antineoplasic activity on Sarcoma 180 tumor cells has been reported from vegetative mycelium (16) as well as reproductive mycelium, the basidiocarp (18). However it is usual to find out variations among strains of the same species as well as among different growth phases of the

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basidiocarp in the same strain. Each *A. brasiliensis* basidiocarp growth phase shows biochemical differences such as different protein and beta-D-glucan concentrations which are the substances considered to be responsible for the antineoplasic effect (4).

A. brasiliensis has absence of universal veil and presence of inner veil which characterize basidiocarp maturation. Before the rupture of the inner veil the basidiocarp is immature and spores are in development. At this phase the basidiocarp is called "closed cap". After the rupture of the inner veil the basidiocarp is mature and spores are developed, ready to be spread and the senescence phase begins. At that phase the basidiocarp is called "opened cap" (4, 15).

Commercially the basidiocarps are cropped before the rupture of the veil (closed cap) mainly due to sensory characteristics and firmness what makes cropping easier and reduces fragmentation during the processing in Brazil (2). Opened caps are considered visually less attractive for consumers because of the dark spores released which gives a dirty appearance to mushrooms. Other commercial claim in Brazil is that closed caps have a better biological activity than opened caps so they get a higher price even when the final purpose is to make capsules. However Camelini *et al*. (4) and Firenzuoli *et al*. (7) suggest that opened caps are likely to have better biological activity because they have higher concentration and diversity of glucans and proteins.

Whereas active principles of some Basidiomycetes such as *Lentinula edodes* (8), *Pleurotus ostreatus* (1) and *Agaricus blazei* (4) are extracted from the vegetative mycelium or immature basidiocarps, others such as *Ganoderma lucidum* have their active principles extracted mainly from spores, where higher concentrations of these substances are found (13). Pinheiro *et al*. (20) evaluated chemoprevention in rat liver cells using different *A. blazei* strains and basidiocarps with closed or opened caps and reported that the fungus protective activity depends on strain and period of basidiocarp crop. However there are no conclusive reports on the *in vivo* antineoplasic activity of *A. brasiliensis* basidiocarps on different maturation phases on sarcoma 180 cells.

Due to the functional importance of this fungus and the absence of published researches comparing the *in vivo* biological activity of the fungus on different basidiocarp maturation phases on sarcoma 180 the objective of this study was to verify the *Agaricus brasiliensis* antineoplasic activity on different basidiocarp maturation phases on sarcoma 180 cells implanted in mice.

MATERIALS AND METHODS

The research was approved by the Animal Ethics Committee at the Paranaense University. *Agaricus brasiliensis* (*Agaricus blazei* 97/11) obtained from the Molecular Biology Laboratory at Paranaense University was used in the research. Mushrooms were cropped on different maturation phases: before the rupture of the inner veil (closed cap) and after its rupture (opened cap). Mushrooms were washed with a sponge under flowing tap water and dehydrated in an oven with air circulation at 65 ^{o}C. After that they were grounded, placed in a double polypropylene bag that was thermically sealed and kept in a freezer at -70 °C.

A mix of grounded mushrooms and ultrapure water in a proportion of 1:10 was kept in a glass flask with lid and immersed at 90 °C for 12 h. After this period the solution was filtered with Qualy brand filter paper 80 g/m^2, thickness 205 µm, 14 µm pores. The filtered solution was stored in a refrigerator at 4 °C in individual aliquots to be used daily.

The strain TG180 of murine sarcoma cells were obtained from the peritoneum of mice previously inoculated and kept for 10 days according to Desmonts and Remington (5). Cell concentration was determined in a Newbauer chamber and it was adjusted to 10^6 with isotonic saline solution (NaCl 0.18%).

Female Swiss mice with 21 days of age and weight of 25 g ± 5 g from the Biotery at Paranaense University were subcutaneously inoculated on their backs with 10^6 sarcoma

cells. Animals were kept in conventional plastic boxes in a room with controlled temperature at 24 ^{o}C ± 2 ^{o}C in a cycle of 12 h of light per 12 h of dark regime. After seven days the rodents were divided into three groups: "Control" group with eight animals that received NaCl 0.18%, "Closed" group with eight animals that received closed cap extracted solution and "Opened" group with eight animals that received opened cap extracted solution. Extracted solutions were taken from the refrigerator and kept at room temperature (around 26 °C) for 30 minutes before use. Each mouse received a daily oral dose of 0.1 mL.

After 30 days of the beginning of the treatments the animals were killed by anesthetic overdose in a chamber saturated of ether vapor. Spleen and tumoral mass were removed and measured. Tumor volume was measured by a millimeter ruler and calculated considering its minimum (a) and maximum (b) diameter (tumor volume = 4/3 π (a^2 b)/2). The inhibition rate was calculated considering the volume of the tumor in the Control group as 100% (11).The splenic index was calculated considering the spleen mass per animal body mass. Comparison among groups was evaluated by variance analysis and significant differences were determinate by Tukey's test with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

One mouse in the Closed group and two mice in the Opened group did not show any subcutaneous tumoral mass, thus they were not considered in the experiment. Results of the splenic index and tumoral mass (width, length and volume) are shown in Table 1.

Table 1. Evaluation of neoplasm growth in Swiss mice inoculated with sarcoma 180 cells and treated with oral saline solution 0.18% (Control) or with Closed or Opened *Agaricus brasiliensis* basidiocarp extract.

Tumor measurements*	Control group	Closed group	Opened group
Splenic index	$0.0134^{a} \pm 0.0033$	$0.0064^{a} \pm 0.0004$	$0.0108^{a} \pm 0.0014$
Mass (g)	$3.0400^{a} \pm 1.1492$	$0.2180^{b} \pm 0.0557$	$0.9333^{b} \pm 0.4228$
Width (mm)	$1.4400^{a} \pm 0.2298$	$0.7200^{b} \pm 0.0567$	$0.7017^{b} \pm 0.0744$
Length (mm)	$2.3286^{a} \pm 0.3519$	$1.1040^{b} \pm 0.0793$	$1.3667^{ab} \pm 0.2297$
Volume (mm^3)	$13.9248^{a} \pm 5.0425$	$1.2804^{b} \pm 0.2953$	$1.7213^{b} \pm 0.7219$

*Means followed by different letters indicate significant differences among the groups according to Tukey's test ($p < 0.05$)

There was no significant difference in the splenic index among the experimental groups (Table 1). The tumor volume of Closed and Opened groups treated with *A. brasiliensis* extracts was significantly ($p < 0.05$) smaller than Control group. However there was no significant difference between Closed and Opened groups (Table 1). The rate of tumor growth inhibition compared with Control group was 90.80% on Closed group and 87.64% on Opened group, with average of 89.22% between them. Based on these data closed caps have the same biological activity than opened caps. Thus the claim in the Brazilian market about higher prices for closed caps based on higher biological activity has no confirmation on this study. However other commercial advantages related to sensory and processing characteristics remains for that choice. Another relevant aspect is that because there is no difference of active principle concentration between closed

and opened caps it is suggest that the main active principle is not concentrated in spores as occurs in *Ganoderma lucidum* (13). Thus the production of vegetative mycelium in liquid medium is likely to be more advantageous for active principle production in large scale than basidiocarps.

Camelini *et al*. (4) and Firenzuoli *et al*. (7) reported differences in the concentration of *A. brasiliensis* glucans and proteins at different basidiocarp growth phase (closed or opened cap), suggesting that mushrooms with opened cap would have higher quantities of antitumoral active principles and probably higher antineoplasic activity. However according to our results the differences in the basidiocarp maturation were not enough to affect fungal antineoplasic activity *in vivo* (Table 1). It suggests that beta-glucan even found in higher amounts in opened caps (4) may not be the only or the main responsible substance for the biological activity for this fungus. It is possible that other factors may play a concomitant role either helping or interfering on the activity of antitumor substances of *A. brasiliensis*.

Zang *et al*. (26) reported that the presence of protein or mannose produced higher immunomodulatory action against sarcoma 180 neoplasms. Mizuno *et al*. (17), Wang *et al*. (24) and Eo *et al*. (6) reported several biologically active compounds obtained from fungi such as polysaccharides, glycoproteins and glycopeptides acting synergistically in the immunomodulatory activity. On the other hand Walton *et al*. (23) reported that basidiocarps of the genus *Agaricus* have a substance called *Agaritine* which shows cancerigenous activity when extracted by cold infusion. However it was not found reports about this substance in the vegetative mycelium.

Camelini *et al*. (4) reported higher quantity and structural diversity of glucans in opened basidiocarps. Other authors (3, 19) reported that quantity and structural diversity of fungal glucans are related to their biological activity in the immune system. They suggest that opened caps would be more indicated for finding out glucans with higher antineoplasic activity. However according to Mizuno *et al*. (16) the antitumoral activity is related to a higher amount of water soluble glucans but it is not necessarily related to a specific basidiocarp maturation phase. Besides that our results did not confirm a better *in vivo* antineoplasic activity on opened basidiocarps (Table 1). Apparently there is a tendency of decreasing the antineoplasic activity *in vivo* of opened basidiocarps what is the opposite of Camelini *et al*. (4) and Firenzuoli *et al*. (7) suggestions. New studies should be carried out in order to verify the association between *in vivo* biological activity and beta-glucan, protein and other substances such as triterpens concentration, in different basidiocarp maturation phases and in different *A. brasiliensis* strains in order to amplify the knowledge in this area.

In conclusion, our results suggest that there are no significant differences of tumor growth inhibition in function of different basidiocarp maturation phases for the *Agaricus brasiliensis* strain, being the *in vivo* basidiocarp antineoplasic activity average of 89.22%. These findings will contribute to the development of the biological activity research on *Agaricus brasiliensis* strains.

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REFERENCES

1. Alarcón, J.N.; Águila, S.; Avila-Arancibia, P.; Fuentes, O.; Zamorano-Ponce, E.; Hernández, M. (2003). Production and purification of statins from *Pleurotus ostreatus* (Basidiomycetes) strains. *Z. Naturforsch. [C]* 58, 62-64.
2. Braga, G.C.; Eira, A.F.; Celso, P.G.; Colauto, N.B. (1998). *Manual do cultivo de* Agaricus blazei *Murril "Cogumelo-do-sol"*. FEPAF, Botucatu.
3. Brown, G.; Gordon, S. (2003). Fungal β-glucans and mammalian immunity. *Immunity* 19, 311-315.
4. Camelini, C.M.; Maraschin, M.; Mendonça, M.M.; Zucco, C.; Ferreira, A.G.; Tavares, L.A. (2005). Structural characterization of β-glucans of *Agaricus brasiliensis* in different stages of fruiting body maturity and their use in nutraceutical products. *Biotechnol. Lett.* 27, 1295-1299.

5. Desmonts, G.; Remington, J.S. (1980). Direct agglutination test for diagnosis of Toxoplasma infection: method for increasing sensitivity and specificity. *J. Clin. Microbiol.* 11, 562-568.
6. Eo, S.K.; Kim, Y.S.; Lee, C.K.; Han, S.S. (2000). Possible mode of antiviral activity of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* on herpes simplex viruses. *J. Ethnopharmacol.* 72, 475-481.
7. Firenzuoli, F.; Gori, L.; Lombardo, G. (2008). The medicinal mushroom *Agaricus blazei* Murril: review of literature and pharmaco-toxicological problems. *Evid. Based Complement. Alternat. Med.* 5, 3-15.
8. Hassegawa, R.H.; Kasuya, M.C.M.; Vanetti, C.D. (2005). Growth and antibacterial activity of *Lentinula edodes* in liquid media supplemented with agricultural wastes. *Electron. J. Biotechnol.* 8, 94-99.
9. Kawagishi, H.; Kanao, T.; Inagaki, R.; Mizuno, T.; Shimura, K.; Ito, H.; Hagiwaram, T.; Nakamura, T. (1990). Formolysis of a potent antitumor (1->6) – D – glucan – protein complex from *Agaricus blazei* fruiting bodies and antitumor activity of the resulting products. *Carbohydr. Polym.* 12, 393-403.
10. Kerrigan, R.W. (2005). *Agaricus subrufescens*, a cultivated edible and medicinal mushroom and its synonyms. *Mycologia* 97, 12-24.
11. Lee, Y.L.; Kim, H.J.; Lee, M.S.; Kim, J.M.; Han, J.S.; Hong, E.K.; Kwon, M.S.; Lee, M.J. (2003). Oral administration of *Agaricus blazei* (H1 strain) inhibited tumor growth in a Sarcoma 180 inoculation model. *Exp. Anim.* 52, 371-375.
12. Liu, Y.; Fukuwatari, Y.; Okumura, K.; Takeda, K.; Ishibashi, K.I.; Furukawa, M.; Ohno, N.; Mori, K.; Gao, M.; Motoi, M. (2008). Immunomodulating activity of *Agaricus brasiliensis* KA21 mice and in human volunteers. *Evid. Based Complement. Alternat. Med.* 5, 205-219.
13. Lu, Q.Y.; Sartippour, M.R.; Brooks, M.N.; Zhang, Q.; Hardy, M.; Go, V.L.; Li, F.P.; Heber, D. (2004). *Ganoderma lucidum* spore extract inhibits endothelial and breast cancer cells *in vitro*. *Oncol. Rep.* 12, 659-662.
14. Menoli, R.C.R.N.; Mantovani, M.S.; Ribeiro, L.R.; Speit, G.; Jordão, B.Q. (2001). Antimutagenic effects of the mushroom *Agaricus blazei* Murril extracts on V79 cells. *Mutat. Res.* 496, 5-13.
15. Miles, P.G.; Chang, S.T. (1997). *Mushroom biology: concise basics and current development*. World Scientific, London.
16. Mizuno, T.; Hagiwara, T.; Nakamura, T.; Ito, H.; Shimura, K.; Sumiya, T.; Asakura, A. (1990). Antitumor activity and some properties of water-soluble polysaccharides from "himematsutake," the fruiting body of *Agaricus blazei* Murril. *Agric. Biol. Chem.* 54, 2889-2896.
17. Mizuno, T.; Suzuki, E.; Maki, K.; Tamaki, H. (1985). Fractionation, chemical modification and antitumor activity of water-soluble polysaccharides of the fruiting body of *Ganoderma lucidum*. *Nippon Nogeikagaku Kaishi* 59, 1143-1151.
18. Ohno, N.; Furukawa, M.; Miura, N.N.; Adachi, Y.; Motoi, M.; Yadomae, T. (2001). Antitumor beta-glucan from the cultured fruit body of *Agaricus blazei*. *Biol. Pharm. Bull.* 24, 820-828.
19. Pelosi, L.; Imai, T.; Chanzy, H.; Heux, L.; Buhler, E.; Bulone, V. (2003). Structural and morphological diversity of (1→3)-β-D-glucans synthesized *in vitro* by enzymes from *Saprolegnia monoïca*. Comparison with a corresponding *in vitro* product from blackberry (*Rubus fruticosus*). *Biochemistry*, 42, 6264-6274.
20. Pinheiro, F.; Faria, R.R.; Camargo, J.L.V.; Spinardi-Barbisan, A.L.T.; Eira, A.F.; Barbisan, L.F. (2003). Chemoprevention of preneoplastic liver foci development dietary mushroom *Agaricus blazei* Murril in the rat. *Food. Chem. Toxicol.* 41, 1543-1550.
21. Rodrigues, S.B.; Jabor, I.A.S.; Marques-Silva, G.G.; Rocha, C.L.M.S.C. (2003). Avaliação do potencial antimutagênico do cogumelo do sol (A*garicus blazei*) no sistema MethG1 em *Aspergillus* (=*Emericella*) *nidulans*. *Acta Sci.* 25, 513-517.
22. Takimoto, H.; Wakita, D.; Kawaguchi, K.; Kumazawa, Y. (2003). Potentiation of cytotoxic activity in naive and tumor-bearing mice by oral administration of hot-water extracts from *Agaricus blazei* fruiting bodies. *Biol. Pharm. Bull.* 27, 404-406.
23. Walton, K.; Coombs, M.M.; Catteral, F.S.; Ioanides, C. (1997). Bioactivation of the mushroom hydrazine, agaritine, to intermediates that bind covalently to proteins and induce mutations in the Ames test. *Carcinogenesis* 18, 1603–1608.
24. Wang, H.X.; Liu, W.K.; Ng, T.B.; Ooi, V.E.; Chang, S.T. (1995). Immunomodulatory and antitumor activities of a polysaccharide-peptide complex from a mycelial culture of *Tricholoma* sp., a local edible mushroom. *Life Sci.* 57, 269-281.
25. Wasser, S.P.; Diduk, M.Y.; Amazonas, M.A.L.A.; Nevo, E.; Stamets, P.; Eira, A.F. (2002). Is a widely cultivated culinary-medicinal royal sun *Agaricus* (the himematsutake mushroom) indeed *Agaricus blazei* Murril? *Int. J. Med. Mushrooms* 4, 267-290.
26. Zhang, M.; Cui, S.W.; Cheung, P.C.K.; Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends Food Sci. Technol.* 18, 4-19.
27. Zhong, M.; Tai, A.; Yamamoto, I. (2005). *In vitro* augmentation of natural killer activity an interferon gama production in murine spleen cells with *Agaricus blazei* fruiting body fractions. *Biosci. Biotechnol. Biochem.* 60, 2466-2469.