

# Screening for In Vitro and In Vivo Antitumor Activities of the Mushroom *Agaricus Blazei*

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We have investigated the in vitro antitumor activity of the mushroom *Agaricus blazei* Murill on human cancer cell lines as well as its potential anticancer activity in a model of rat colon carcinogenesis. The in vitro anticancer analysis was performed using 9 human cancer cell lines incubated with organic and aqueous extracts of *A. blazei*. Antitumor activity was observed with the dichloromethane/methanol and hexanic extracts of *A. blazei* at 250 µg/ml for all cancer cell lines tested. No antiproliferative/cytotoxic activities were detected for the aqueous, methanol, ethyl acetate, or *n*-butanolic extracts. In the in vivo analysis, crude *A. blazei* was given orally after carcinogen treatment in a rat medium-term study (20 weeks) of colon carcinogenesis using aberrant crypt foci (ACF) as biomarker. Male Wistar rats were given dimethylhydrazine (DMH) and then were fed *A. blazei* at 5% in the diet until Week 20. ACF were scored for number and crypt multiplicity. *A. blazei* intake did not suppress ACF development or crypt multiplicity induced by DMH. No differences in tumor incidence in the colon were observed among the DMH-treated groups. Our results indicate that employing *A. blazei* in the diet does not have a suppressive effect on colon carcinogenesis.

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## INTRODUCTION

*Agaricus blazei* Murill, a mushroom native to Brazil, has been attracting attention as a health food and for its use as complementary and alternative medicine by cancer patients (1–3). A broad spectrum of biological activities has been attributed

to this edible mushroom including antiviral, antibacterial, antiparasitic, immunological, and antitumoral actions, among others (4–6). Various polysaccharides, such as beta-glucans, and protein-bound polysaccharides isolated from mycelia and fruiting bodies have shown antitumor activity both in vitro (7–9) and in tumor-bearing mice (10,11). The anticancer activity has been postulated to be mainly through immunomodulation (6,10,11). Recently, some studies have shown a direct antitumor activity of crude *A. blazei* or its specific compounds on human or murine tumor cell lines (8,9,12).

In contrast to the well-established information on the biological activities of this mushroom provided by in vitro and transplantable tumor models, there are limited data on experimental models of chemically induced carcinogenesis in rodents. We previously demonstrated a hepatoprotective effect of crude extracts of *A. blazei* on liver toxicity, as seen by decreased serum transaminase levels, and on the initiation stage of hepatocarcinogenesis induced by diethylnitrosamine in the rat (13). Similar chemopreventive results were observed when *A. blazei* was administered orally to the rats in a medium-term rat liver carcinogenesis assay (14). Also, aqueous and organic extracts of *A. blazei* provided significant protection against mutagenicity induced both in vivo by cyclophosphamide (15) and in vitro by methyl methanesulphonate and 2-aminoanthracene (16,17). These data suggest that this edible mushroom could be effective in reducing cancer incidence in other target organs like the colon. Little information is available on the potential beneficial effects of *A. blazei* in experimental models of chemically induced colon carcinogenesis.

Colorectal cancer is one of the most common malignancies of Western countries. Primary prevention by dietary intake of fruits, vegetables, and other natural compounds is of great

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Submitted 14 May 2008; accepted in final form 4 August 2008.

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interest in the control of this leading neoplastic disease (18–20). Experimental models of chemically induced colon cancer have shown that the development of tumors is a multistep process involving initiation, promotion, and progression (21). These stages of colon carcinogenesis can be expressed as pathological alterations ranging from discrete microscopic mucosal lesions, like aberrant crypt foci (ACF), to macroscopic malignant tumors (22–24). ACF are considered to be the earliest preneoplastic lesions because they are induced in rodents by colon carcinogens including 1,2-dimethylhydrazine (DMH) and are found in human colon at high risk for cancer development (25,26). ACF have been used as biomarkers in the identification of chemopreventive agents of colon cancer in rodents (27–30).

The discovery of bioactive compounds, including antitumoral substances, has stirred a growing interest in such mushrooms from industry, the media, and the scientific community (4–6,31). Considering that the popular consumption of *A. blazei* is in food or tea form, for both preventive and therapeutic purposes, we examined whether daily oral administration of crude *A. blazei* given after carcinogen treatment would suppress the development of ACF and tumors during the promotion stage of colon carcinogenesis induced by DMH in a medium-term bioassay (20 wk) in rats. Furthermore, we investigated the in vitro cytotoxic/antiproliferative activities of organic and aqueous extracts of this mushroom on human cancer cell lines.

## MATERIAL AND METHODS

The fruiting bodies of the mushroom *Agaricus blazei* (strains 99/26) were kindly provided by Dr Augusto Ferreira da Eira from the Department of Vegetable Production at the Faculty of Agronomic Sciences (UNESP Botucatu, Sao Paulo [SP], Brazil). *Agaricus blazei* dichloromethane/methanol extract (DME), methanol extract (ME), hexanic extract (HE), ethyl acetate extract (EAE), *n*-butanolic extract (BE), and aqueous extract (AE) were graciously provided by Dr Edson Rodrigues Filho and Ms Ana Paula Terezan from the Chemistry Department at Universidade Federal de Sao Carlos (Sao Carlos, SP, Brazil), as previously described (32).

### In Vitro Anticancer Analysis

Experiments were performed using the following human cancer cell lines: HT-29 (colon), K-562 (leukemia), MCF-7 (breast), NCI-ADR (breast expressing the multidrug resistance phenotype), NCI-460 (lung), UACC-62 (melanoma), OVCAR (ovary), PC-03 (prostate), and 786-0 (kidney), obtained from the National Cancer Institute (NCI) (33). Tumor cell lines were cultured in 25-cm<sup>2</sup> flasks (Nunc Brand Products, Roskilde, Denmark) containing 5 ml of RPMI-1640 culture medium (Gibco BRL, Life Technologies, Sao Paulo, Brazil) supplemented with 5% fetal bovine serum (Gibco BRL, Life Technologies) and 50 µg/ml gentamicin (Sigma Chemical Co., St. Louis, MO) (34).

Seeding densities varied among the human cancer cell lines as follows:  $6.0 \times 10^4$  for K562 and MCF-7;  $5 \times 10^4$  for NCI-ADR,

HT-29, and 786-0;  $4.5 \times 10^4$  for PC-03; and  $4 \times 10^4$  for NCI-460 and UACC62 cells/ml. After stabilization, microtiter plates containing tumor cells were incubated with the *A. blazei* extracts (0.25, 2.5, and 250 µ/ml) for 48 h in a humidified atmosphere of 5% CO<sub>2</sub>, at 37°C. The sulforhodamine B (SRB) assay was performed according to the method previously described (35). The tumor cell lines were fixed by means of protein precipitation with 50% trichloroacetic acid (TCA) (Sigma Chemical Co.) at 4°C (50 µl/well, final concentration = 10%) for 1 h. The supernatant was discarded, and the plates were washed 5 times with tap water. The cells were stained for 30 min with 0.4% SRB (Sigma Chemical Co.) dissolved in 1% acetic acid (50 µl/well; Sigma Chemical Co.) and subsequently washed 4 times with 1% acetic acid to remove unbound stain. The plates were air dried, and bound protein stain was solubilized with 150 µl of 10 mM Trizma buffer (Sigma Chemical Co.). The optical density was read on an automated spectrophotometer plate reader at 540 nm. Each assay was performed in triplicate. For cells growing in suspension (e.g., leukemia), the same method was employed, but the TCA concentration was 80% to fix the cells to the bottom.

### In Vivo Study for Colon Carcinogenesis

**Animals and diets.** Male 4-wk-old Wistar rats were obtained from CEMIB (UNICAMP Campinas, SP, Brazil). They were kept in polypropylene cages (5 animals/cage) covered with metallic grids in a room maintained at  $22 \pm 2^\circ\text{C}$ ,  $55 \pm 10\%$  humidity under a 12-h light-dark cycle. They were fed basal diet (NUVILAB-CR-1, NUVITAL, Curitiba, PR, Brazil) and water ad libitum for a 2-wk acclimation period before beginning the experiment. Fifty grams of powdered dry fruiting bodies of *A. blazei* was added to 1,000 g of the basal diet. The diet containing the mushroom was freshly prepared every 2 wk, stored at 4°C and offered to the rats daily ad libitum. The selected route of administration was oral (dietary) because this mushroom is an edible fungus intended for human consumption. The selection of 5% maximum dose level in our feeding study was due to the absence of toxicity of the *Ab* mushroom in rat prechronic and chronic studies (36,37).

**Experimental design.** The protocol used was consistent with the Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation. The animals were randomly allocated into 4 groups of 10 rats each: Groups 1 and 2 were given 4 subcutaneous injections of DMH (Sigma Chemical Co.), 40 mg/kg body weight, twice a week for 2 wk; Groups 3 and 4 received similar injections of EDTA solution (DMH vehicle, 37 mg/100ml, pH 6.0, distilled water). At the third week, after initiation of colon carcinogenesis with DMH, the animals were fed a diet with 5% *A. blazei* (Groups 2 and 3) or basal diet (Groups 1 and 4), both ad libitum, until the end of the experiment at Week 20. Food and water consumption levels were measured twice a week, and the animals were weighed once a week throughout the experimental period.

*Necropsy and tissue preparation for aberrant crypt foci counting.* At necropsy, the entire colon was removed, longitudinally opened, rinsed with 0.9% NaCl solution, and fixed flat in buffered formalin for 24 h. For ACF counting, the middle and distal portions of the colon were stained with 1% methylene blue dissolved in phosphate-buffered salt solution (PBS) for one minute. Analysis and quantification of ACF were performed according to Bird and Good's (22) criteria. The number of ACF/colon and the number of aberrant crypts in each ACF were determined under light microscopy at  $\times 40$  magnification. Colonic crypts overlying lymphoid follicles were excluded from the score because normal crypts in this area can occasionally be confused with aberrant crypts. After ACF analysis, samples of the middle and distal colon were processed for paraffin embedding, cut *en face* into 4–5  $\mu\text{m}$  thick sections, and stained with hematoxylin-eosin (H.E) for histological analysis. The material was analyzed for the presence and degree of dysplasia according to Siu et al. (38). The number, size, and location of tumors along the colon was registered. Samples were processed, sectioned at 4–5  $\mu\text{m}$ , and stained with H.E for histological analysis.

### Statistical Analysis

The statistical analysis was performed using Jandel Sigma Stat software (Jandel Corporation, San Rafael, CA). For in vivo analysis, the body weight gain, food consumption, ACF number,

and multiplicity were analyzed by the ANOVA or Kruskal–Wallis tests. Significant differences were assumed for  $P < 0.05$ .

## RESULTS

### In Vitro Anticancer Analysis

Methanol (ME), ethyl acetate (EAE), *n*-butanolic (BE) and aqueous (AE) extracts did not present any antiproliferative/cytotoxic activities in the 9 human cancer cell lines tested (data not shown). Antitumor activity was observed with dichloromethane/methanol (DME) and hexanic (HE) extracts at 250  $\mu\text{g}/\text{ml}$  for all cancer cell lines tested. A better selective effect was observed for DME extract with an antiproliferative effect on all human cancer cell lines tested and a cytotoxic effect on K-562, NCI-ADR, NCI-460, UACC62, OVCAR, HT-29, and 786-0 cell lines (Fig. 1).

### In Vivo Study for Colon Carcinogenesis

All rats survived until the end of the experiment at the 20th week. DMH initiation and *A. blazei* treatment were well accepted by the animals. Dietary *A. blazei* at 5% did not change body weight gain (Table 1). At the lifetime of the postinitiation period (Weeks 3–20), food consumption in the *A. blazei*-treated groups (Group 2 [G2] and G3) did not differ from the respective control groups (G1 and G4) as shown in Table 1.

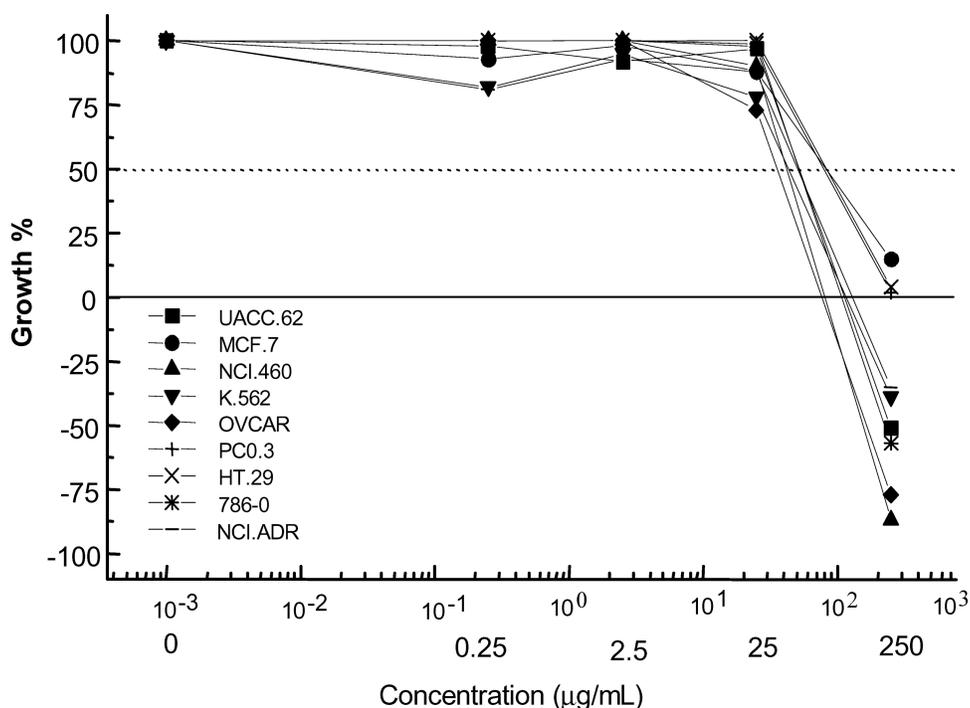


FIG. 1. Antiproliferative/cytotoxic effects of dichloromethane/methanol (DME) extract on human cancer cell lines. Because the extract is considered to be active if its inhibition is  $>50\%$ , a dashed line was placed in the figure (growth between 0% and 50% indicate a cytostatic effect, whereas growth  $<0\%$  indicate a cytotoxic effect). NCI cell lines tested: UACC62 (melanoma), MCF-7 (breast), NCI-460 (lung), K562 (leukemia), OVCAR (ovary), PC-03 (prostate), HT-29 (colon), 786-0 (kidney), and NCI-ADR (breast expressing the multidrug resistance phenotype). For details, see **Materials and Methods** section.

TABLE 1  
Initial and final body weight, body-weight gain, food, and *Agaricus blazei* (*Ab*) consumption<sup>a</sup>

Group/Treatment <sup>b</sup>	No. Rats	Initial Body Weight (g)	Final Body Weight (g)	Body-Weight Gain (g) <sup>c</sup>	Food Consumption (g/Rat/Day) <sup>c</sup>	Mushroom Consumption (g/Rat/Day) <sup>c</sup>
(G1) DMH	10	228.6 ± 15.9	470.3 ± 29.5	242.0 ± 32.7	24.6 ± 1.8	—
(G2) DMH+ 5% <i>Ab</i>	10	224.9 ± 13.3	465.4 ± 29.7	240.5 ± 27.4	25.8 ± 2.1	1.29 ± 0.1
(G3) 5% <i>Ab</i>	10	226.0 ± 11.7	472.0 ± 15.8	246.0 ± 5.2	25.6 ± 1.7	1.28 ± 0.2
(G4) Control	5	230.4 ± 7.4	466.4 ± 41.3	237.8 ± 30.6	24.8 ± 1.6	—

<sup>a</sup>Values are mean ± SD. Abbreviations are as follows: DMH, 1,2 dimethylhydrazine; *Ab*, *Agaricus blazei*.

<sup>b</sup>DMH is 4 × 40 mg/kg, subcutaneous; *Ab* is 5.0% in basal diet.

<sup>c</sup>From the 1st to the 20th experimental week.

The numbers of ACF and crypt multiplicities in the middle and distal colon in the different groups are summarized in Table 2. All DMH-initiated animals (G1 and G2) developed ACF. No significant differences in the number of ACF/cm<sup>2</sup> were observed between the DMH-initiated groups. *A. blazei* at 5% given by diet after carcinogen treatment did not suppress ACF development or crypt multiplicity induced by DMH in the middle and distal colon when compared with the respective DMH-initiated group (G1; Table 2).

The histopathological analysis of the colon showed lower numbers of dysplastic aberrant crypt foci in the group fed *A. blazei* in the diet (G2) than in the respective DMH-treated group (G1), although these differences were not statistically significant (0.05 < *P* < 0.1).

There were no differences in tumor incidence or multiplicity in the colon among the DMH-treated groups at the end of 20 wk. Four tumors were found in three DMH-treated rats and two tumors were identified in two rats from the group that

received *A. blazei* in the diet. Histologically, all tumors were well-differentiated adenocarcinomas.

## DISCUSSION

The present study was undertaken to investigate the potential anticancer activity of the *A. blazei* mushroom in two different bioassays. Our in vitro assay results showed that hexanic and dichloromethane/methanol (DME) extracts of *A. blazei* were able to induce significant inhibition of cell growth in all the examined human cancer cell lines. These findings are in agreement with recent reports on the anticancer activity directly induced by *A. blazei* extracts in human cancer cell lines (8,9,12). It has been demonstrated that in vitro *A. blazei*-mediated apoptosis occurs by modulation of Bcl-2 protein levels and proteolytic activation of caspase-3 (8). Gao et al. (9) reported that the mechanism of apoptosis induction by the FA 2b beta fraction of *A. blazei* in a leukemia cell line was through the combined effect of down-regulation of telomerase activity and upregulation of caspase-3

TABLE 2  
Effect of dietary *Agaricus blazei* (*Ab*) on aberrant crypt (AC) foci (ACF) and crypt multiplicity (crypt/ACF) in the middle and distal colon<sup>a</sup>

Group (G)/Treatment	No. Rats	Number of Aberrant Crypts per ACF				Total Number AC	Total Number ACF	Crypt/ACF
		1 Crypt	2 Crypts	3 Crypts	>4 Crypts			
Middle Colon								
(G1) DMH	10	73.9 ± 44.4	56.3 ± 29.1	42.3 ± 18.5	59.4 ± 30.3	604.9 ± 248.3	231.9 ± 96.4	2.65 ± 0.5
(G2) DMH + 5% <i>Ab</i>	10	71.2 ± 53.5	52.8 ± 25.6	46.8 ± 21.9	64.7 ± 31.2	636.4 ± 284.1	235.5 ± 109.9	2.68 ± 0.3
(G3) 5% <i>Ab</i>	5	0	0	0	0	0	0	0
(G4) Control	5	0	0	0	0	0	0	0
Distal Colon								
(G1) DMH	10	49.8 ± 29.8	55.5 ± 35.3	36.6 ± 21.2	37.7 ± 23.9	446.5 ± 214.9	179.6 ± 87.9	2.52 ± 0.4
(G2) DMH + 5% <i>Ab</i>	10	43.6 ± 36.0	50.9 ± 32.1	40.4 ± 19.6	32.2 ± 18.1	412.8 ± 204.6	167.1 ± 90.1	2.46 ± 0.4
(G3) 5% <i>Ab</i>	5	0	0	0	0	0	0	0
(G4) Control	5	0	0	0	0	0	0	0

<sup>a</sup>Values are mean ± SD. Abbreviations are as follows: DMH, 1,2 dimethylhydrazine; *Ab*, *Agaricus blazei*.

<sup>b</sup>DMH at 4 × 40mg/kg body weight subcutaneously. *Ab* at 5.0 % in basal diet.

expression. These findings indicate that the mitochondrial pathway may be the target for *A. blazei*-mediated apoptosis. Given that mitochondria has been proposed as a potential target for apoptosis induction in chemotherapy (39,40), treatment with *A. blazei extracts* might be a promising adjuvant approach for cancer therapy.

On the other hand, we did not observe antiproliferative or cytotoxic activities resultant from aqueous extracts of crude *A. blazei* in all the examined human cancer cell lines even at the highest dose levels. These results may be of value because *A. blazei* is usually consumed as an infusion for prevention and treatment of cancer. As aqueous extracts from crude mushroom may contain very low concentrations of pharmacologically active compounds, our results suggest that the extraction of bioactive compounds by more refined methods could be a better way to prepare *A. blazei* extracts for therapeutic purposes.

In contrast to the anticancer activity directly observed in the in vitro assay, no beneficial effects of crude *A. blazei* given by diet were detected on the in vivo, medium-term experiment using a postinitiation protocol for colon carcinogenesis induced by DMH in the rat. Our data show that dietary *A. blazei* given after carcinogen treatment was not able to suppress the development of preneoplastic (ACF) and neoplastic lesions induced by DMH in the colonic mucosa. We have previously observed that giving *A. blazei* through the diet 2 wk before and during carcinogen treatment did not protect the epithelial cells of the colonic mucosa against the genotoxic damage induced by DMH (41). The possibility exists that the pharmacologically active fractions with potential anticancer activities could not reach the target cells in the colon and exert their beneficial effects due to digestion of the mushroom by the small intestinal enzymes. Similarly, in vivo lack of efficacy of oral supplementation with beta-glucan extracted from *A. blazei* has been recently reported in an experimental model of lung metastasis (12). In fact, we have also observed in a previous study that oral treatment with *A. blazei* aqueous extracts failed to protect against the development of preneoplastic lesions during the postinitiation stage of rat liver carcinogenesis (42).

The antitumor activity of *A. blazei* reported in the in vivo tumor transplantable models has been attributed to the immunopotentiating activity of the polysaccharide fractions (10–12). They appear to modulate both innate and adaptive immunity by enhancing the number and/or function of natural killer cells, the cytolytic T lymphocyte, and macrophage activities (7,43). Interestingly, we did not find on the histological examination of the colon tumor-infiltrating lymphocytes or lymphoid aggregates near the neoplastic or preneoplastic lesions, which could represent, at the morphological level, the mushroom-induced immunological reaction of the host against the tumor. These observations further support our finding that *A. blazei* lacks a protective effect in this experimental model of colon carcinogenesis.

In conclusion, our in vitro and in vivo results on the antitumor activity of crude *A. blazei* extracts show that beneficial effects

were obtained in vitro against fully transformed malignant cells, but no suppressive effect on the development of preneoplastic (ACF) and neoplastic lesions was observed in the experimental model of colon carcinogenesis. This may probably be due to the lack of opportunity of the bioactive fractions to reach their targets in the colon. Therefore, crude *A. blazei*, as commonly consumed through the diet, does not present an in vivo protective effect at the promotion stage of colon carcinogenesis.

## ACKNOWLEDGMENTS

We are grateful to Drs. Luciana K Kohn and João Ernesto de Carvalho from Universidade Estadual de Campinas (Unicamp, Campinas, SP, Brazil) for helping with the in vitro experiments. This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), Brazil, Núcleo de Avaliação Toxicogenética e Cancerígena (Toxican) Botucatu, SP, UNESP, Brazil.

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