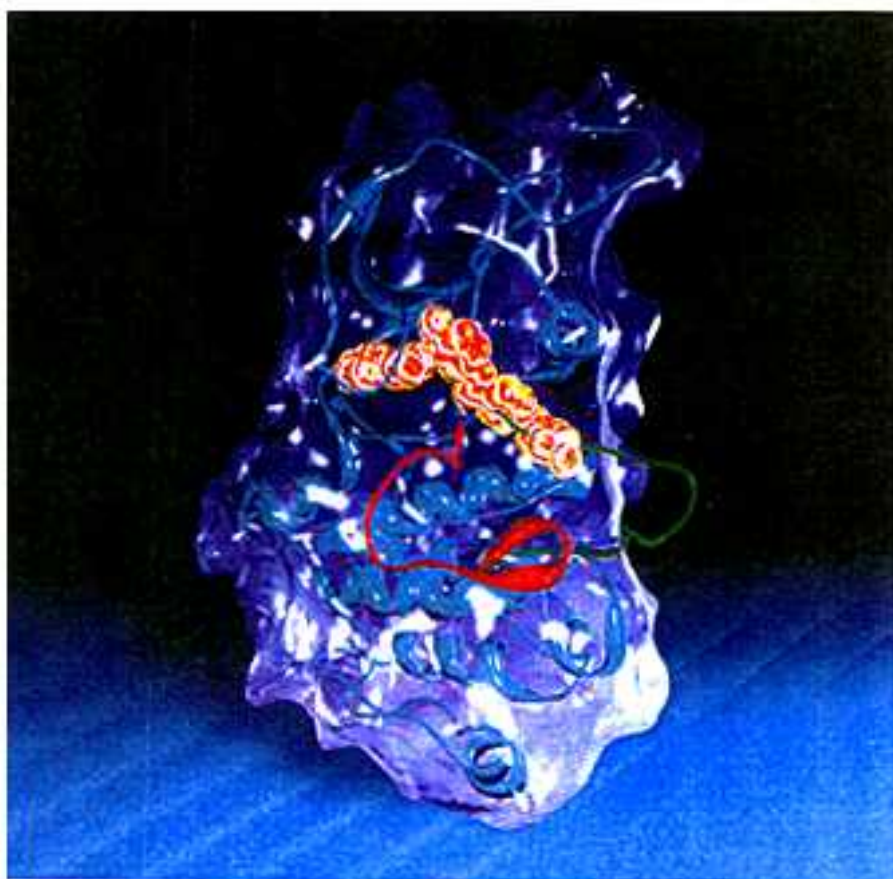


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Proceedings

Analysis of Carcinogens and the Safety of *Agaricus blazei Murill* (ABMK)

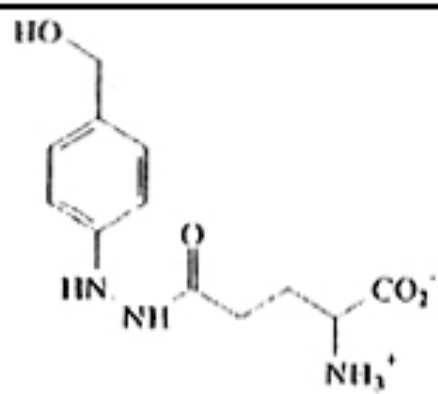
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Cancer death rates among mushroom consumers versus non-consumers in the same geographical region are 0.8 and 1.3% ($p < 0.05$), respectively. This significant difference in cancer death was attributed to enhancement of immunity against tumor growth by NK cell activation. Currently, *Agaricus blazei Murill* (ABMK) has been consumed worldwide, however, safety of this mushroom has not been determined previously. Since some edible mushrooms, such as *Agaricus bisporus* (AB), have been shown to contain potent carcinogens, we have undertaken a two-year chronic cancer bioassays (US FDA guidelines) to evaluate the safety of long term consumption, and also to analyze ABMK for the possible presence of mushroom related carcinogens. The results of a two year cancer bioassay showed that there are no significance differences in the frequency of spontaneous incidence of tumors in F344 rats, while the survival rates in the treatment groups were significantly increased by 33% of controls ($p < 0.05$). Concurrently, three separate lots of ABMK were examined for the presence of Agaritine (β -N-(γ -L(+)-glutamyl)-4-hydroxymethylphenylhydrazine), the presumed biosynthetic precursor of Agaritine, β -N-(γ -L(+)-glutamyl)-4-carboxyphenylhydrazine (CGPH) and two arenediazonium ions that have been previously observed to form from Agaritine and CGPH, 4-hydroxymethylbenzene diazonium ion (HMBD) and 4-carboxybenzenediazonium ion (CBD), respectively. Specifically, we used a combination of HPLC and mass spectrometry to detect Agaritine or CGPH and chemical derivatization with naphthol coupled with HPLC analysis to detect the presence of either HMBD or CBD, as there aryl azo- β -naphthol derivative. We did not detect the presence of Agaritine, CGPH, HMBD, or CBD in the samples examined (with the limits of detection being approximately 100 ng for Agaritine and GCPH and 10 ng for the naphthol adducts). Based on these results, ABMK could be used as a replacement AB and would likely reduce the cancer risk associated with AB.

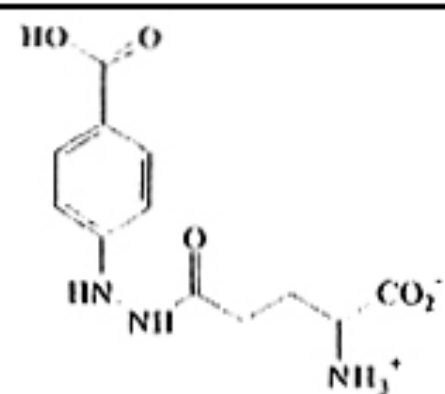
Introduction

The mushroom *Agaricus blazei* Murill extract (ABMK, Royal), a dietary supplement prepared from the edible mushroom *Agaricus blazei* Murill, has the potential for use as an immunomodulating agent, which enhances quality of life among cancer patients undergoing cancer chemotherapy. In this study, the freeze dried mushroom was assayed for the presence of four agents believed to be carcinogens and found in the related mushroom, *Agaricus bisporus* (AB), namely Agaritine (1, β -N-(γ -L(+)-glutamyl)-4-hydroxymethylphenylhydrazine), GCPH, (2, β -N-(γ -L(+)-glutamyl)-4-carboxyphenylhydrazine), 4-hydroxymethyl benzenediazonium ion (3) and 4-carboxybenzenediazonium ion (4). The analysis were performed by HPLC with both UV and MS detection and the analysis for 1-4 was negative within the limits of detection.

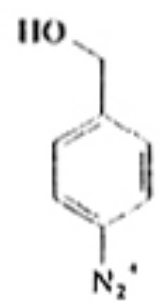
The ABMK extract was further examined in several biological assays including an oral toxicity study in both Sprague-Dawley Rats and Beagle Dogs, a genotoxicity study, a reproductive toxicity study, a neurotoxicity study in Fischer rats, and immunotoxicity study, and a two-year cancer bioassay study, also in Fischer rats. The results of these studies demonstrated that ABMK extract causes no reproductive toxicity, genotoxicity, neurotoxicity, immunotoxicity, nor longer term carcinogenesis. Based on these results, ABMK could be used as a replacement AB and would likely reduce the cancer risk associated with AB.



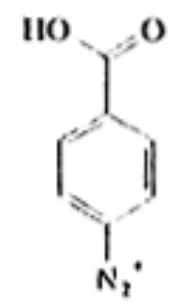
Agaritine
(1)



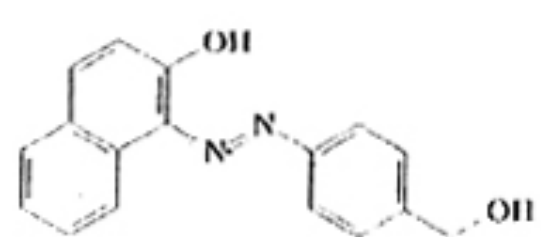
β -N(γ -L(+)-glutamyl)-4-carboxy-phenylhydrazine
(2)



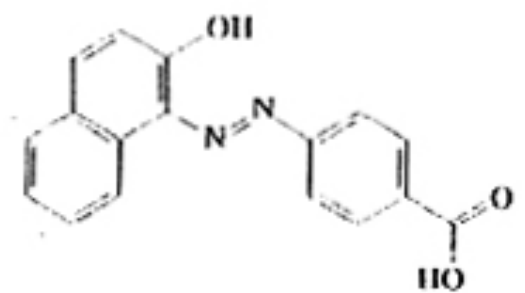
3



4



5



6

Figure 1. Structures of Agaritine (1), β -N(γ -L(+)-glutamyl)-4-carboxy-phenylhydrazine (2), 4-hydroxymethylphenylbenzene diazonium ion (3), 4-carboxybenzene diazonium ion (4), and the β -naphthol adducts of 3 and 4, 5 and 6, respectively.

Experimental Methods

Agaritine (1) and β -N-(γ -L(+)-glutamyl)-4-carboxyphenylhydrazine (2) (β -N-(γ -L(+)-glutamyl)-4-hydroxymethylphenylhydrazine, 1, and β -N-(γ -L(+)-glutamyl)-4-carboxyphenylhydrazine, 2, were prepared as previously described.¹

Synthesis of arenediazonium ion tetrafluoroborates of 3 and 4: The arenediazonium ions were prepared from the corresponding aromatic amine and nitrosyl tetrafluoroborate. The products were isolated by filtration in 90% yield.²

Synthesis of 2-hydroxy-1-naphthaleneazo-4'-arylbenzenes 5 and 6: The azo standards were prepared by the reaction of 2-naphthol and an equimolar solution of the tetrafluoroborate 3 or 4 in water, under basic conditions and at 5°C. The product was recrystallized from ethanol (~95% yield).³

Sample preparation for HPLC/MS of 1 and 2. The literature methods for HPLC analysis were used,^{4,5} and the eluant split between a UV detector and the mass spectrometer. An HPLC trace of a sample extract of the freeze-dried mushroom, spiked with agaritine, is shown in Figure 2. The mushroom extract was prepared by sonicating 1 g of dry powder with methanol (70 mL) and 1% sodium bisulfite (2 mL, omitted when analyzing for 3 and 4) for 1 h. The resulting slurry was filtered, and diluted to 100 mL. A 10 mL portion of this extract was concentrated to dryness, re-suspended in 0.005 N $\text{NH}_4\text{H}_2\text{PO}_4$ (10 mL, pH 4.25) and sonicated for 10 min. Four mL of the resulting solution was passed through a C18 reverse-phase SepPak washing with 0.005 N $\text{NH}_4\text{H}_2\text{PO}_4$ (1 mL, pH 4.25) and aliquots of the resulting eluant (25 μL) were analyzed by HPLC. Spiked samples were prepared by weighing 10 mg of 1 or 2 and dissolving in methanol (10 mL). Aliquots were then transferred to a flask, freeze-dried mushroom added (1 g), and treated as described above. In addition to using the 'spiked' sample data to determine recovery, reproducibility, and linearity of the method, they also were used to demonstrate the 1 and 2 could be detected by HPLC and mass spectrometry.

HPLC and MS: Agaritine (1) and 2 were chromatographed on a Partisil 10-SCX 25 cm x 4.6 mm i.d. column, 0.005 $\text{NH}_4\text{H}_2\text{PO}_4$ buffer (pH 4.25) as eluant, 0.6 mL/min flow rate, detection at 237 nm. Under these conditions, the approximate retention times were 1, 14 min, 2, 10.1 min. The azo compounds 5 and 6 were analyzed by HPLC on a C18 reverse-phase column, 25 cm x 4.5 mm, methanol/water eluant, flow rate 1 mL/min, detection wavelength 274 nm.

Genotoxicity Studies: Genotoxicity studies were performed according to ICH Harmonized Tripartite guidelines including the Ames assay using 4 strains Salmonella typhimurium and *E. coli*, chromosomal aberration assay using CHO cells, and the Mouse micronuclei test.

Toxicity Studies: A 28-day oral toxicity study of ABMK extract in Sprague-Dawley rats and Beagle dogs was conducted. Doses in rats (1x, 10x, and 20x the anticipated daily human dose) and in dogs (1x, 5x, or 10x the anticipated daily human dose) were administered. Rat toxicity data was evaluated in 40 male and 40 female rats (10 rats/sex/dose/group) and in 12 male and 12 female dogs (3 dogs/sex/treatment group). A rats and dogs survived to the scheduled necropsy on Day 29.

Reproductive Toxicity Study: The effects of long term dietary feeding of ABMK powder for 6, 9, and 12 months, with respect to male reproductive function, was evaluated. Animals were observed for potential affects on rate of mating, fertility, pregnancy, weight changes among pregnant rats, parturition, gestational periods, number of live and stillbirth, sex ratio of live litters, dietary changes in newborn and autopsy of all female rats for abnormalities.

Neurotoxicity Study: Fischer rats were fed dietary concentrations of ABMK, powder, of 0, 6,250, 12,500, and 25,000 ppm *ad libitum*. Each experimental group consisted of 10 males and 10 females. After 6 months of dietary feeding, spontaneous locomotor activity, Morris water maze and passive avoidance tests were made to evaluate the dietary effects of ABMK, powder.

Immunotoxicity Study: T-cell dependent antibody response in all ABMK dietary treated animals were evaluated by challenging animals with sheep red blood cells (SRBC) 4 days prior to sacrifice. The spleen was removed and spleen cells were assayed for antibody forming capability. Antibody plaque assay was expressed as both spleen cell numbers and spleen weight.

Two-year Cancer Bioassay: A two-year (104 weeks) oral chronic cancer bioassay of ABMK was made with 4 groups of Fischer rats, both male (50) and female (50) animals. The diet contained 0 (VC), 6,250 (T₁), 12,500 (T₂) and 25,000 (T₃) ppm and given *ad libitum* for the entire 104 weeks.

Results

Analysis for Agaritine (1) and (2)

Three separate lots of ABMK powder were examined and three samples from each lot were made for the analysis of agaritine (1) and 2 for a total of 9 samples (Figure 1). Each sample was examined by HPLC for the presence of both 1 and 2. A representative HPLC trace is shown in Figure 2. Each sample was spiked with either 1 and 2 as a positive control. In addition to the use of UV detection, we also examined the eluants for 1 and 2 by MS. Regardless of the detection method or sample, we were unable to detect the presence of either 1 and 2 (limit of detection is approximately 100 ng for 1 and 2.^{4,5}

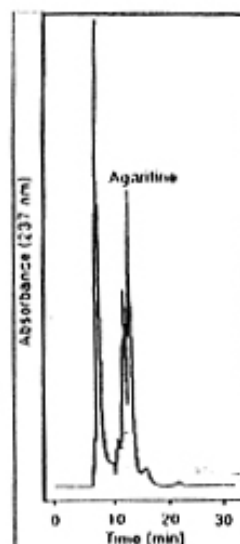


Figure 2. HPLC trace of Agaritine spiked from dried *Agaricus Blazei Murill*

Analysis for the Arenediazonium Ions (3) and (4)

The analysis of 3 and 4 were made by conversion (if present) to their corresponding azo-naphthol 2-naphthol adducts 5 and 6, respectively (Figure 1). This method had been used previously to demonstrate the presence of arenediazonium ions in *Agaricus bisporus*.³ As in the case of the analysis for 1 and 2, 3 separate lots of ABMK were examined and each 3 separate samples from each lot were prepared. HPLC analysis on C18 was performed on these sample using both UV and MS detection. Samples were spiked with 5 and 6 to insure they would be detected if present. In no case were we able to detect compounds either 5 or 6 and therefore conclude that neither 3 nor 4 were present in any of the lots of *Agaricus Blazei Murill* we analyzed, within the level of detection (10 ng). It is possible that any of the compounds 1-4 are in *Agaricus Blazei Murill* but they would have to be present at concentrations below the noted limits of detection.

Genotoxicity Data Summary for ABMK

FDA recommended genotoxicity studies were performed according to ICH Harmonized Tripartite guidelines that include Ames test using 4 strains *Salmonella typhimurium* and *E. coli*, chromosomal aberration assay using CHO cells, and Mouse micronuclei test to evaluate ABMK. Genotoxicity studies with ABMK showed all negative results demonstrating that ABMK was not a genotoxicant.

Toxicity Data Summary for ABMK

ABMK has been proposed as being of potential use as an immunomodulating agent, which enhances quality of life among cancer patients undergoing cancer chemotherapy. Therefore, it was necessary to obtain toxicity data. As described in the Experimental section, toxicity data was obtained on a total of 80 rats and 24 beagle dogs. All survive the 28 day testing period. No test related clinical signs of toxicity were observed in either group of animals. No test data indicated any significant effects on body weights, food consumption, hematology, clinical chemistry, gross necropsy findings, or histopathology. The NOEL doses in Sprague-Dawley rats and beagles were > 17.6 ml/kg/day (20x the daily human dose) and >2.63 ml/kg/day (10x the daily human dose) respectively.

Reproductive Toxicity Data Summary of ABMK

The effects of long term dietary feeding of ABMK for 6, 9, and 12 months with respect to male reproductive function was evaluated. The key points are:

- 1) The results showed no dietary dose related effect on the rates of mating, fertility, and pregnancy.
- 2) Neither dietary dose related general clinical abnormalities nor pregnancy related abnormalities were observed during the entire period of pregnancy.
- 3) No dietary dose related body weight changes among pregnant rats in the treatment groups were observed.
- 4) No dietary dose related effect on parturition, gestational periods, the number of live and stillbirth, and the sex ration of live litters were observed and no statistically different changes in all treatment groups were demonstrated from that of the control.
- 5) No dietary dose related changes of newborn of all treatment groups were observed.
- 6) Autopsy of all females showed no abnormalities either in the control or treated groups.

These studies demonstrate that a long term dietary feeding of ABMK in male rats causes no reproductive toxicity.

Neurotoxicity Studies

Neurotoxicity studies were conducted as described in the Experimental section with Fischer rats. The key results are summarized below.

- 1) Spontaneous locomotor activity between the control and the treated-groups of both males and females following a 6 month dietary feeding of the test material showed no statistical difference, despite the test groups showed a tendency to have increased spontaneous locomotor activity (Table 1, below).
- 2) Morris water maze test results with male and female test groups receiving 6,250 (5x the anticipated daily human dose), 12,500 (10x the anticipated daily human dose), and 25,000ppm (20x the anticipated daily human dose) ABMK for 12 months. Male groups receiving the 12,500 and 25,000ppm demonstrated to significant increase of cognitive and memory function, however, it was not statistically significant. The female test group receiving the same doses showed statistically significant increase of both cognitive and memory function as compared to that of the control group.

Table 1. Spontaneous Locomotor Activity, Cognitive, and Memory Function in Male and Female Rats

Group	Dose (ppm)	Time (min)		Mean Response latency	
		M	F	M	F
Control	0	349.7±56.2	404.9±65.3	7.37±1.04	7.47±1.62
T ₁	6,250	398.0±45.3	453.0±62.8	8.53±1.29	4.93±0.40
T ₂	12,500	438.9±58.7	445.6±58.1	5.70±0.97	4.24±0.48*
T ₃	25,000	437.9±33.6	557.3±40.2	5.60±0.55	4.39±0.42*

*Significantly different from control group ($p < 0.05$). Each value represents the Mean±SEM(n=10).

From the results obtained from the experimental groups after 6, 12, and 22 months of dietary feeding of the ABMK, effects on spontaneous locomotor activity, cognitive, and memory function were not affected, but rather demonstrated to enhance spontaneous motor activity, cognitive, and memory function.

Immunotoxicity Data

Experimental results showed that in male groups, there was a trend of dose dependent increase in antibody formation per unit spleen cells in all ABMK treatment groups as compared to that of control. However, these increases were not statistically significant. Likewise, Cytoxan-induced reduction in antibody forming capability per unit spleen cells appeared to have increased in a dose dependent manner in ABMK treatment groups, however, the increases were not statistically significant.

In female ABMK treatment groups demonstrated a statistically significant increase of antibody forming capability in dose responsive manner per unit spleen cells by 30% ($p < 0.01$). Likewise, increase of antibody forming capability in ABMK group expressed as unit spleen was also statistically significant, however, total cells per unit spleen was not increased. Cytoxan-induced suppression of antibody forming capability in all ABMK-treatment group was protected significantly from immunosuppressive effects of cytotoxic agents, Cytoxan. The magnitude of the protection from Cytoxan-induced suppression of antibody forming capability was statistically protected in all ABMK treatment groups either expressed as per unit spleen cells or as per unit spleen.

Two-year Cancer Bioassay

As described in the Experimental section, feeding studies were conducted for 104 weeks with four groups consisting of 50 male and 50 female groups. Diet at 4 different levels of ABMK were studied. The total incidence of tumors in all animals, in dead and moribund animals and, in terminal sacrifice animals are shown in Tables 2-4, below.

Table 2. Incidence of Tumors in All Animals

Description	Male				Historical data Range(%)	Female				Historical data Range(%)
	VC	T ₁	T ₂	T ₃		VC	T ₁	T ₂	T ₃	
Total animals per Group	50	50	50	50		50	50	50	50	
Total animals with tumors	47 (94%)	48 (96%)	49 (98%)	50 (100%)	90-100%	31 (62%)	32 (64%)	29 (58%)	32 (64%)	64-98%
Total animals with malignant tumors	24 (48%)	25 (50%)	22 (44%)	26 (52%)	38-82%	7 (14%)	14 (28%)	9 (18%)	9 (18%)	18-56%
Total animals with benign tumors	41 (82%)	42 (84%)	45 (90%)	49 (98%)	82-100%	28 (56%)	23 (46%)	27 (54%)	27 (54%)	58-86%

Boorman, GA, Montgomery, CA, MacKenzie, WF Pathology of the Fischer Rat: Reference & Atlas. Acad. Press, Inc. 1993.

Table 3. Incidence of Tumors in Dead and Moribund Animals

Description	Male				Female			
	VC	T ₁	T ₂	T ₃	VC	T ₁	T ₂	T ₃
Total animals per Group	24	14	8	15	5	9	7	5
Total animals with tumors	22 (92%)	13 (93%)	8 (100%)	15 (100%)	3 (60%)	8 (89%)	5 (71%)	4 (80%)
Total animals with malignant tumors	19 (79%)	12 (86%)	5 (63%)	11 (73%)	2 (40%)	6 (67%)	3 (43%)	4 (80%)
Total animals with benign tumors	17 (71%)	9 (64%)	6 (75%)	14 (93%)	2 (40%)	2 (22%)	4 (57%)	0 (0%)

Table 4. Incidence of Tumors in Terminal Sacrificed Animals

Description	Male				Female			
	VC	T ₁	T ₂	T ₃	VC	T ₁	T ₂	T ₃
Total animals per Group	26	36	42	35	45	40	43	45
Total animals with tumors	25 (96%)	35 (97%)	41 (98%)	35 (100%)	28 (62%)	24 (59%)	24 (56%)	28 (62%)
Total animals with malignant tumors	5 (19%)	13 (36%)	17 (40%)	15 (43%)	5 (11%)	8 (20%)	6 (14%)	5 (11%)
Total animals with benign tumors	24 (92%)	33 (92%)	39 (93%)	35 (100%)	26 (58%)	21 (51%)	19 (44%)	27 (60%)

Statistics: Animal survival rate analyses - Log-Rank test; Dose response test - Both Logistic Regression test, Fisher's exact test, and Cochran-Armitage trend test. For the Cancer risk rat, Poly 3 method was applied.

Conclusions

- 1) **ABMK** does not contain Agaritine (1), GCPH (2) or the arenediazonium ions 3 and 4 at detectable levels, unlike the related mushroom **AB**.
- 2) In a variety of genotoxicity studies, **ABMK** was found to be lacking any detectable genotoxicity.
- 3) **ABMK**, in a 28-day feeding study in Sprague-Dawley rats or beagle dogs gave no evidence of toxicity.
- 4) No evidence of reproductive toxicity was observed in long term feeding studies in rats.
- 5) Feeding studies of **ABMK** in Fischer rats were negative for neurotoxicity.
- 6) Immunotoxicity studies showed some increase in antibody formation but was statistically significant only in females. **ABMK** was protective against Cyclophosphamide-induced suppression of antibody formation.
- 7) Long term feeding studies showed no significant increase in tumor formation relative to control. Notably, female tumor rates were relatively in the low range of historical data.
- 8) **ABMK** appears to be a non-toxic, non-carcinogenic food-stuff that could serve as a substitute for **AB**. In addition, **ABMK** may have immunoprotective properties.

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