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# Cytotoxicity of *Agaricus sylvaticus* in non-tumor cells (NTH/3T3) and tumor (OSCC-3) using tetrazolium (MTT) assay

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**Abstract**

**The purpose of this study was to assess the cytotoxic effect of the non-fractionated aqueous extract of *A. sylvaticus* mushroom in cultures of non-tumor cells (NIH3T3) and tumor cells (OSCC-3). The cells were maintained in DMEN cell culture medium added of 10% of fetal bovine serum and 1% antibiotic. For the cytotoxicity test we prepared the aqueous mushroom extract at concentrations of 0.01 mg.ml$^{-1}$, 0.02 mg.ml$^{-1}$, 0.04 mg.ml$^{-1}$, 0.08 mg.ml$^{-1}$, 0.16 mg.ml$^{-1}$, and 0.32 mg.ml$^{-1}$. For the culture, 2 x 10$^{5}$ cells/ml was deposited in 96-well microplates during 24 hour incubation with subsequent exchange of medium by another containing the mushroom concentrations. After 24 hour incubation the medium was discarded and 100 ml of tetrazolium blue (MTT) was added at a concentration of 5 mg.ml$^{-1}$. The microplates were incubated for 2 h at 37° C. Spectrophotometric analysis was performed using 570 nm wavelength. From the values of the optical densities we determined the drug concentration capable of reducing cell viability by 50%. Therefore, the mushroom *A. sylvaticus*, at all concentrations tested, did not show cytotoxic effects, once the inhibitory concentration ($IC_{50}$) obtained for tumor cells OSCC-3 was 0.06194 mg.ml$^{-1}$, and the $IC_{50}$ checked for non-tumor cells NIH3T3 was 0,06468 mg.ml$^{-1}$. This test made it possible to determine that *A. sylvaticus* mushroom has no cytotoxic effects, suggesting its use safe for human consumption.**

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Key words: *Toxicity. Food safety.* Agaricus sylvaticus.

**CITOTOXICIDAD DE *AGARICUS SYLVATICUS* EN CÉLULAS NO TUMORALES (NIH/3T3) Y EL TUMOR (CCCA-3) USANDO TETRAZOLIO (MTT)**

**Resumen**

**El objetivo de este estudio fue evaluar el efecto citotóxico de un extracto acuoso no fraccionado de la seta *A. sylvaticus* en cultivos de células no tumorales (NIH3T3) y tumorales (OSCC-3). Se mantuvo a las células en un medio de cultivo celular DMEN al que se añadió suero de ternera fetal al 10% y antibiótico al 1%. Para la prueba de toxicidad, se preparó el extracto acuoso del hongo a concentraciones 0,01 mg.ml$^{-1}$, 0,02 mg.ml$^{-1}$, 0,04 mg.ml$^{-1}$, 0,08 mg.ml$^{-1}$, 0,16 mg.ml$^{-1}$ y 0,32 mg.ml$^{-1}$. Para el cultivo, 2 x 105 células/ml se depositaron en microplacas de 96 pocillos durante 24 horas de incubación con el subsiguiente recambio del medio por otro que contenía las concentraciones del hongo. Tras 24 horas de incubación, se desechó el medio y se añadieron 100 ml de azul de tetrazolio (MTT) a una concentración de 5 mg.ml$^{-1}$. Se incubaron las microplacas durante 2 h a 37° C. Se realizó un análisis espectrofotométrico con una longitud de onda de 570 nm. A partir de los valores de las densidades ópticas, se determinó la concentración de la droga capaz de reducir la viabilidad celular en un 50 %. La seta *A. sylvaticus*, a todas las concentraciones probadas, no mostró efectos citotóxicos una vez que la concentración inhibitoria ($IC_{50}$) obtenida para las células tumorales OSCC-3 fue de 0,06194 mg.ml$^{-1}$ y la $IC_{50}$ comprobada para las células no tumorales NIH3T3 fue de 0,06468 mg.ml$^{-1}$. Esta prueba consiguió determinar que la seta *A. sylvaticus* no posee efectos citotóxicos lo que sugiere un uso seguro para el consumo humano.**

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Palabras clave: *Toxicidad. Seguridad alimentaria.* Agaricus sylvaticus.

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## Abbreviations

*A. sylvaticus: Agaricus sylvaticus.*
CaCo-2: Colon cancer cells.
DMEM: Dulbecco's Modified Eagle Medium.
EDTA: Ethylenediamine-tetraacetic acid.
Hep-2 cell line: Human epithelial cells derived from a larynx carcinoma.
$IC_{50}$: Inhibitory concentration.
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
NIH/3T3: Non-tumoral fibroblasts cell line.
OSCC-3: Oral squamous cell carcinoma.

## Introduction

The mushrooms of the genus Agaricus have long been considered functional foods for their rich chemical composition and high amount of bioactive compounds, bringing many benefits to the health of those who consume it, besides the absence of toxicity (Orsine et al., 2012a).

Studies have been conducted in an effort to utilize mushrooms of the genus Agaricus in the treatment of various ailments. The *Agaricus blazei Murill* mushroom showed antinociceptive and anti-inflammatory effects in Wistar rats (Carvalho et al., 2011); protective effect against lethal infection with *Streptococcus pneumoniae* in mice (Bernadshaw et al., 2005); reducing effect on the degree of edema and hemorrhagic halo in bothropic poisoning in experimental rabbits (Ferreira et al., 2003) further to high potential use in the treatment of leishmaniasis (Valadares et al., 2012). A dietary supplementation with *A. sylvaticus* was able to improve gastrointestinal disorders in post-surgery patients with colorectal cancer as well as the quality of life of these patients (Fortes et al., 2010). The *Agaricus bisporus* mushroom stimulated the production of immunoglobulin A in saliva samples of healthy volunteers, suggesting that its use was responsible for developing immunity (Jeong et al. 2012).

However, there are few toxicological studies on edible mushrooms and food safety tests. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test has often been used to investigate cytotoxicity caused by medicinal plants (Shoeb et al. Jan 2012; Talib and Mahasneh, 2010) and fungi with antimicrobial activity (Joel and Bhimba, 2012).

The principle of the MTT technique consists in the absorption of yellow tetrazolium salts by mitochondrial reductases of metabolically active cells, resulting in a product called formazan. This product accumulated intracellularly, is extracted by adding an appropriate solvent. This is a low-cost method, yielding fast results in 48 hours (Mosmann, 1983).

The purpose of this study was to perform cytotoxicity screening of the aqueous extract of *A. sylvaticus* mushroom in non-tumoral fibroblasts cell line (NIH/3T3) and oral squamous cell carcinoma (OSCC-3), using the MTT reduction test.

## Materials and methods

### *Obtaining the sample*

The *A. sylvaticus* mushroom was obtained from a producer in Minas Gerais, Brazil, in 2010. The sample was dried and milled.

### *Preparation of extract*

We weighed 10 g of dehydrated minced mushroom, and diluted it in 100 ml of distilled water. The solution was stirred in a mechanical shaker for 30 minutes and was then filtered through filter paper.

The filtered solution was then distributed into eppendorfs 1mL previously weighed and identified, frozen, and subsequently taken to a liophylization chamber. After complete sublimation of water, we weighed again the eppendorfs containing the soluble solids in mushroom *A. sylvaticus'* water.

We prepared the non fractionated aqueous extract of the mushroom *A. sylvaticus* at concentrations: 0.33 $mg.ml^{-1}$, 0.16 $mg.ml^{-1}$, 0.08 $mg.ml^{-1}$, 0.04 $mg.ml^{-1}$, 0.02 $mg.ml^{-1}$, and 0.01 $mg.ml^{-1}$.

### In vitro *study*

*In vitro* studies were carried out following the methodology proposed by Saldanha (2007), from the MTT assay.

### *Culture and proliferation of non-tumor fibroblast cell line (NIH/3T3) and oral squamous cell carcinoma (OSCC-3)*

Cell lines NIH/3T3 (non-tumor fibroblasts) and OSCC-3 (immortalized cells in culture from a human oral squamous cell carcinoma) were maintained separately in culture medium DMEM (Dulbecco's Modified Eagle Medium), GIBCO-BRL, supplemented with 10% fetal bovine serum (GIBCO-BRL) and 1% of antibiotics (penicillin-streptomycin).

The cultures were set up from an initial passage of $2 \times 10^5$ cells in 75 $cm^2$ culture flasks, maintained in an incubator at 37 °C with saturated humidity of 5 % $CO_2$ atmosphere. Upon reaching 80-90% confluence, cells were released from the bottom of the flask by treatment with 0.125% trypsin solution/0.02% EDTA (ethylenediamine-tetraacetic acid) for two minutes, centrifuged at 1,000 rpm for three minutes, using Neubauer counting chamber and transferred to a new culture flask.

*Treatment of NIH/3T3 cells and OSCC-3 with non-fractioned aqueous extract of mushroom* A. sylvaticus

After 24 hours of cultivation in the presence of non-fractioned aqueous extract of mushroom *A. sylvaticus* sample, cells were subjected to MTT test to determine viability of the isolated cells. Concentrations of the non-fractioned aqueous extract were added to the cultures, which were maintained for 24 hours under the conditions described in section 2.3.1. We used solution DMEN only as negative control. The NIH/3T3 cells and OSCC-3 were maintained at the Nanobiotechnology laboratory, Genetics and Morphology Department, Brasilia University.

*Analysis of cell viability*

Cell viability was assessed after two hours contact of NIH/3T3 cells and OSCC-3 with MTT in spectrophotometer. For the reading we used wavelength of 570 nm. The result obtained indicates the optical density, since the darker the color obtained, the greater the MTT metabolism of the cells under study. Consequently, a higher optical density results in less toxicity of the extract tested. We used the Prism Graph Software to analyze the results.

The cytotoxicity of each concentration of the non-fractionated aqueous extract of the mushroom *A. sylvaticus* was expressed by cell death, calculated in relation to negative control, according to the methodology proposed by Zhang et al. (2004).

$$\text{Dead cells (\%)} = \frac{\text{Absorbance of negative control - Absorbance of test x 100}}{\text{Absorbance of negative control}}$$

The data generated were used to plot a dose-response curve which determines the extract concentration capable of killing 50% of the cell population tested, indicating $IC_{50}$ (inhibitory concentration).

*Statistical analysis*

Data were expressed as the mean percentage of toxicity. Significance levels among concentrations of non-fractionated aqueous extract of *A. sylvaticus* mushroom tested were analyzed using analysis of variance (ANOVA), with Software Graphpad PRISM® 4.0. For multiple comparisons among groups, control group and intra-group, we used the Newman-Keuls test, with significance set at $p < 0.05$.

## Results

*Agaricus sylvaticus* mushroom have a rich chemical composition, highlighting the variety and quantity of minerals as well as its high protein content (Orsine et al., 2012b). But, to be approved in the *in vitro* cytotoxicity assays, the sample to be tested must not cause cell death nor affect its cellular functions. Therefore, tests using cell culture can detect cell lysis, growth inhibition and other effects that can be triggered onto these cells (Daguano et al., 2007).

In figure 1 we presented the results for the OSCC-3 cells treated with different concentrations of mush-

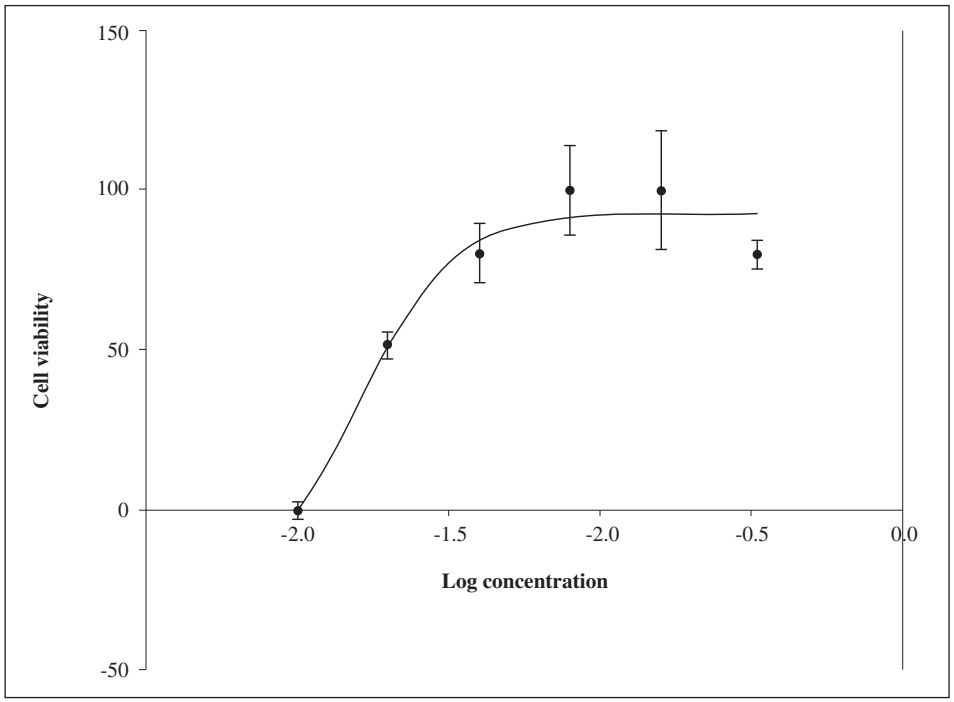


*Fig. 1.—Toxicity of mushroom A. sylvaticus in OSCC-3 cells by the MTT assay at concentrations 0.01, 0.02, 0.04, 0.08, 0.16, 0.33 mg.ml$^{-1}$.*

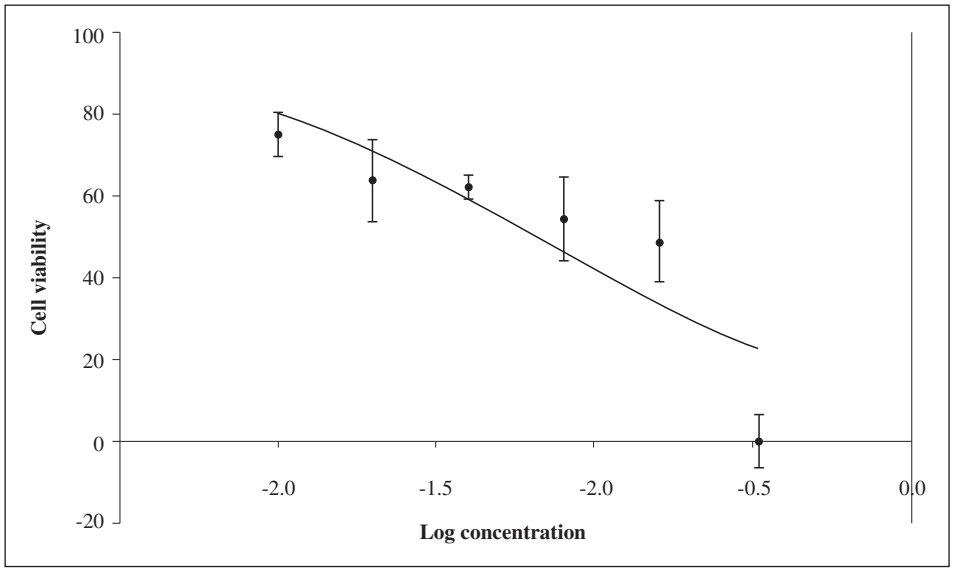


*Fig. 2.—Toxicity of mushroom A. Sylvaticus in NIH/3T3 cells by the MTT assay at concentrations 0.01, 0.02, 0.04, 0.08, 0.16, 0.33 mg.ml$^{-1}$.*

room *A. sylvaticus*. The $IC_{50}$ determined was of 0.06194 mg.ml$^{-1}$, that is, the *A. sylvaticus* non-fractionated water extract does not show toxicity in tumor cells used in this study.

In figure 2 the results were expressed regarding NIH3T3 cell culture treated with different concentrations of mushroom *A. sylvaticus*. The $IC_{50}$ found was 0.06468 mg.ml$^{-1}$, that is, the *A. sylvaticus* non-fractionated water extract showed no toxicity in non-tumor cells analyzed.

## Discussion

This study investigated the mushroom *A. sylvaticus* and its safe use in food. These results may contribute towards research done with *A. sylvaticus*, toxicity testing and food safety, supplement, or as an adjunct in cancer treatment, since very low toxicity of the extract was observed in two types of cells tested.

Mushrooms of the genus Agaricus have been widely studied by several authors, in search of answers to their toxicity (Chang et al., 2012; Orsine et al., 2012c; Bellini et al., 2008; Novaes et al., 2007; Singi et al. 2006; Sugui et al. 2006; Kuroiwa et al. 2005; Costa et al. 2003).

Table I presents studies on the toxicity of edible mushrooms of different genres, performed worldwide in the period from 2003 to 2012 in order to support the discussion of this work.

Plants used in folk medicine in Jordan were tested for cytotoxic effects using the MTT assay on Vero cell line. The *Rosa damascena* plant showed $IC_{50}$ value of 454.11 mg.ml$^{-1}$, whereas the *Ononis hirta* plant showed $IC_{50}$ of 72.50 mg.ml$^{-1}$ (Talib and Mahasneh, 2010).

The cytotoxicity of five strains of fungus *Penicillium thiomii* (named as IR-1, IR-2, IR-4, IR-6 and IR-7) isolated from the medicinal plant *Terminalia chebula* Retz, in Bangladesh, was evaluated by the MTT assay. The ethyl acetate extract of the fungus strains inhibited the growth of colon cancer cells CaCo-2. Values were obtained for the $IC_{50}$ ranging from 44 to 67 mg.ml$^{-1}$ (Shoeb et al. 2012).

The cytotoxicity caused by the extract of fungi *Pestalotiopsis Microspora* VB5 was screened using the MTT test. As a result, the authors observed that the concentration of the extract tested was inversely proportional to Hep-2 cell line (human epithelial cells derived from a larynx carcinoma) growth (Joel and Bhimba, 2012).

## Conclusion

The non-fractionated aqueous extract of the mushroom *A. sylvaticus* showed no cytotoxic effect on tumor cells OSCC-3 and non-tumor cells NIH/3T3, showing to be safe for use in food and/or dietary supplementation.

## Acknowledgements

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**Table I**

*Studies on the toxicity of edible mushrooms and/or medicinal. Period 2003-2012*

| *References* | *Type of study* | *Mushroom* | *Type of toxicity* | *Objectives* | *Methods and materials* | *Results* |
|---|---|---|---|---|---|---|
| Chang et al. (2012)[15] | Experimental | *Agaricus blazei* Murrill | Genotoxicity | To evaluate the safety and tolerance of *A. blazei* Murrill in toxicology studies using the Ames test. | Doses of 0.1 and 10.5mg/rat of *A. blazei* Murrill daily were administered to 10 mice by gavage for 28 days. | There was no significant change in brain, heart, kidneys, liver, spleen, adrenal glands, ovaries or testicles histologically or macroscopically. With increasing doses, male and female rats did not show a gradual rise in serum concentration in any of the items examined, with the exception of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in females, which were significantly abnormal in comparison with the control group. The Ames test, pathology determinations, biochemical analysis and routine blood parameters were normal, except for AST and ALT in females. The results showed that statistic differences observed in one sex was not observed in the others and were not dose dependent. |
| Motoi and Ohno (2012)[23] | | *Agaricus brasiliensis* S. Wasser | Genotoxicity | Asses the genotoxicity of *A. brasiliensis* through bacterial reverse mutation tests, micronucleus and mouse lymphoma. | The reverse mutation test used five bacterial strains including *Salmonella typhimurium* and *Escherichia coli*. For the rat micronucleus test, we used the ratio of polychromatic erythrocyte and normochromatic as indicators of bone marrow cell growth inhibition. For the mutagenicity test we used L5178Y/TK+/- mouse lymphoma assay-Thymidine Kinase (TK), which detects mutations in the TK locus caused by changes in pairs, substitution of a single base pair and small deletions. The toxicity of test agent was indicated by a decrease in efficiency of colony formation, whereas the mutagenicity by the increase in the mutation frequency based on the number of mutants and adjusted for survival fraction of cells. | In the bacterial reverse mutation test, no toxicity was observed up to a dose of 5000 ug / plate. In the mouse micronucleus assay, no toxicity was observed up to a dose of 1 g/kg body weight. In mouse lymphoma assay, the frequency of mutation was similar both in the presence and absence of *Agaricus brasiliensis*. Supporting the long history of human consumption of *A. brasiliensis*, the data derived from this study strongly indicate the safety of this mushroom. |
| Orsine et al. (2012) | Experimental | *Agaricus sylvaticus* | Cytotoxicity | Evaluate the $CL_{50}$ of mushroom *A. sylvaticus*, through the hemolytic activity test on human erythrocytes. | Different concentrations of aqueous extract of the mushroom *A. sylvaticus* were tested against a suspension of human erythrocytes (Negative A Blood) at 2% and hemolytic activity determined in hemolysis percentage. A concentration curve was built (µg of *A. sylvaticus* mushroom) *versus* percentage of hemolysis and the concentration of the aqueous extract of the mushroom *A. sylvaticus* required to produce 50% haemolysis, known as 50% hemolytic concentration or 50% effective concentration (EC50). | We obtained the $CL_{50}$ value of 9.213 mg/mL, indicating the very low toxicity of the mushroom *A. sylvaticus* on human erythrocytes, proving to be safe for consumption. |

**Table I** *(cont.)*
*Studies on the toxicity of edible mushrooms and/or medicinal. Period 2003-2012*

| *References* | *Type of study* | *Mushroom* | *Type of toxicity* | *Objectives* | *Methods and materials* | *Results* |
|---|---|---|---|---|---|---|
| Savi et al. (2011)[24] | Experimental | *Agaricus brasiliensis* | Mutagenicity/ Genotoxicity | Asses the genotoxic activity and antigenotoxic of *A. brasiliensis* in *D. melanogaster in vivo test* from somatic mutation and recombination test (SMART). | Larvae with secondary markers for the third recessive chromosome, corresponding to multiple wings (mwh), trans-heterozygous, in its early stage of development, were pretreated for 24 hours with aqueous extract of *A. brasiliensis*. Then the larvae in the third stage of development were treated for 48 h with methyl methane alkylating agent (MMS). The frequency of mutation to replace the wing blade (number of wing spots of different sizes) induced in somatic cells was determined by a genetic change in parameter of the wing discs. | Results showed that the extract of the mushroom A. brasiliensis do not cause any genotoxic or mutagenic effects. However, no antigenotoxic effect and/or protection against mutations induced by MMS were observed. Instead, a frequency of mitotic recombination by MMS was seen after pretreatment with larvae extract of *A. brasiliensis*. |
| Kim et al. (2011)[25] | Experimental | *Agaricus blazei* | Cytotoxicity | Investigate where the extract of A. blazei has antiproliferative effects and apoptosis in human leukemic THP-1, using the MTT test (3-[4,5-dimethyl-2-thiazolyl] -2,5-diphenyl-2H-tetrazolium). | Human leukemic cells THP-1 were maintained in culture medium containing 10% fetal bovine serum inactivated by heat and 1% penicillin-streptomycin. Cell viability was determined by MTT assay of mitochondrial membrane and monitored by measuring the absorption of 3,3-Dihexyloxacarbocyanine iodide (DiOC6) and then analyzed by flow cytometry. Protease caspase activity was measured by spectrophotometric detection of the p-nitroaniline (pNA) molecule. The cell extracts were separated on polyacrylamide gels at 8 or 10% and then transferred to nitrocellulose membranes, where tests were developed using enhanced chemiluminescence system (ECL) Western blot method. | We observed that apoptosis induced by *Agaricus blazei* extract is associated with the mitochondrial pathway, which is mediated by reactive oxygen species (ROS), which are generated and prolonged by activation of c-Jun N-terminal kinase (JNK). Furthermore, treatment with *Agaricus blazei* extract resulted in the accumulation of cytochrome c into the cytoplasm, increased caspase activity, and up-regulation of pro-apoptotic proteins Bax and Bad. From these results, it was found that the decrease in *Agaricus blazei* extract resulted in activation of nuclear factor kappa B (NF-kB) and gene regulator products of NF-kB such as antibody PAI-1 and -2. We concluded that the extract of *Agaricus blazei* induces apoptosis through ROS-dependent JNK activation and constitutive activated NF-kB inhibitors in THP-1 cells. |
| Postemsky et al. (2011)[26] | Experimental | *Grifola gargal* Singer | Mutagenicity | To evaluate the protective effects of medicinal mushroom *Grifola gargal* Singer after induction of DNA damage in *D. melanogaster* by using DMBA (7-12-dimethyl-benz (α) anthracene) through somatic mutation and recombination test in Drosophila melanogaster (SMART). | Heterozygous larvae were grown in media with different concentrations of DMBA. *Grifola gargal* fruit bodies (*Gg*FB), or mycelia from liquid culture (*Gg*LC) or from solid culture (*Gg*WG), that is, biotransformed wheat kernel flour, were later added to the culture medium in combined treatments with DMBA. | The addition of *Gg*FB, *Gg*LC, or *Gg*WG produced a protective effect of 25 µmol/vial DMBA-induced mortality. Mutations observed in SMART as light spot (LS) 100 per eyes (eyes LS/100) increased with increasing dose of DMBA; this is also true when considering the occurrence of mutation expressed as percentage of eyes exhibiting light spots (% eyes with LS). Interestingly, mycelia from *Gg*FB, *Gg*LC or *Gg*WG in the presence of 25 µmol/vial DMBA showed lower values in SMART, both in total rate of LS/100 eyes as the percentage of eyes with LS. Thus, the *Grifola gargal* materials were not only non toxic, but in combination with 25 µmol/vial DMBA reduced induced-mortality through pro-mutagenic and showed antimutagenic effects. *G. gargal* protective effects against DMBA are discussed in terms of desmutagenic and/or bio-antimutagenic detoxifying mechanisms in the host organism, probably due to some bioactive compounds present in superior mushrooms. |

**Table I** *(cont.)*
*Studies on the toxicity of edible mushrooms and/or medicinal. Period 2003-2012*

| *References* | *Type of study* | *Mushroom* | *Type of toxicity* | *Objectives* | *Methods and materials* | *Results* |
|---|---|---|---|---|---|---|
| Yoshkoda (2010)[27] | Experimental | *Lentinula edodes* | Toxicity in rats | To evaluate the toxicological safety of extract of *L. edodes* | Mycelia *L. edodes* were cultivated and extracts prepared (LEM) with filtration, concentration, sterilization and liophylization. 25 females and 25 male rats were used in the experiment, 10 being the control group. The animals received 2,000 mg/kg/day of LEM for 28 days. The mice were observed and hematological, biochemical and histological tests were performed. | There were no deaths or behavioral changes in animals. Body weight and food consumption dropped, particularly in the case of male mice, although the reduction wasn't relevant after completing the administration. No significant effect was observed in toxicological tests of hematology, serum biochemistry, organ weights relative and absolute, necropsy and histopathology. Consequently, the no observed adverse effect level (NOAEL) of LEM was considered over 2.000 mg/kg/day in the conditions of this study. |
| Gill (2008)[28] | Experimental | *Ganoderma lucidum* | Cell Toxicity | To determine the effects of low and high concentrations of three different extracts of *Ganoderma lucidum* (GL, and PSGL Reishi) on the viability of T. lymphoblast cell line Jurkat E6.1, LG2 cells, a human B lymphoblast derived from a lymph node metastasis and peripheral blood mononuclear cells (PBMC) isolated from healthy adults, healthy children and pediatric patients Chemotherapy | The cells were maintained in culture medium RPMI -1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and streptomycin (P/S) and 1% L-glutamine in a humidified chamber at 37° C and 5% CO2. Complete blood count was a obtained from five healthy adults, five healthy children, and 6 pediatric patients undergoing chemotherapy and suffering from acute lymphoblastic leukemia. The extracts used were: a crude extract of *G. lucidum* (GL), a polysaccharide extract of *G. lucidum* (PSGL), a commercially available extract of *G. lucidum* (Reishi) in capsules of Chinese herbal supplements purchased at supermarkets. The extracts were dissolved in culture media of cells specific for the cell type being used. Cells were incubated with both low concentrations of extracts from 1 µg/mL and 50 g/mL, to determine immunostimulatory effects, and concentrations between 50 g/mL and 350 g/mL, to determine toxicity. Following incubation, 25 uL of 5 mg/mL MTT (3 - [4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide). The absorption was measured using a spectrophotometer Molecular Devices at a wavelength of 590 nm. | When cells of study individuals (Jurkat E6.1 and LG2) were treated with increasing concentrations of the extracts, decreases cell viability. However, when cells PBMCs were treated with the same extracts, the results were variable. Although there was no standard toxicity, toxicity was observed in PBMCs cells. |

**Table I** *(cont.)*
*Studies on the toxicity of edible mushrooms and/or medicinal. Period 2003-2012*

| *References* | *Type of study* | *Mushroom* | *Type of toxicity* | *Objectives* | *Methods and materials* | *Results* |
|---|---|---|---|---|---|---|
| Bellini et al. (2008)[17] | Experimental | *Agaricus blazei* | Toxicity ovary cell clone of Chinese hamster (CHO K1) | Review the mutagenic and protective capacity of mushroom *A. blazei* | Different fractions of the methanol extract of the mushroom *A. blazei* were tested with clastogenicity cytokinesis-blocking micronucleus (CBMN) and the test hypoxanthine guanine phosphoribosyl transferase locus (HGPRT) gene mutation in both cell clone using Chinese hamster ovary K1 (CHO-K1). | The methanolic fractions of *A. blazei* mushroom tested did not provide chemical protection and all fractions showed to be potentially mutagenic in hgprt test. It was evident that more tests are needed to investigate the biological effects of methanolic and aqueous extracts of *A. blazei,* and other interactions with the metabolism of the cells before recommending its widespread use by the population, which is already happening in many countries. These findings indicate that the methanol extracts of the fungus should not be used on account of its genotoxicity and that one should be careful in the use of *A. blazei* by the population before the biochemical characterization of this fungus is complete. |
| Nieto (2008)[29] | Experimental | *Pleurotus ostreatus* and *Pleurotus pulmonarius* | Toxicity to *Artemia salina* | Provide information on the toxicity of three species of basidiomycetous fungi of the Order Agaricales. | Solutions were obtained with seven different concentrations of mushrooms *P. ostreatus* and *P. pulmonarius.* Eggs of *Artemia salina* (Arthropoda, Crustacea, Anostraca) were placed in one liter of culture medium, nauplii hatched. 5 ml of each solution were added to ten nauplii. After 24 hours of exposure, the dead nauplii were counted in each test tube. Five replicates were performed by dilution. | For both *Pleurotus* species tested, no concentration of 50% mortality reached the nauplii. In the case of *P. pulmonarius,* concentrations below 1.000 mg/ml did not affect 25% of the population, while for *P. ostreatus* was achieved by 45%.The results suggested that the biologically active metabolites in extracts *P.* and *P ostreatus. pulmonarius* have low toxicity, rendering them safer for use as nutraceuticals. |
| Novaes et al. (2007)[18] | Laboratory, double-blind trial | *Agaricus sylvaticus* | Toxicity clinical, biochemical and histopathological. | To evaluate the effects of acute toxicity of aqueous extract of *A. sylvaticus* (AAS) by clinical, biochemical and histopathological findings in healthy mice. | Aqueous extract was obtained by infusion. The animals were fed by gavage (esophageal) 1.5 g/kg over 24 hours. The biochemical sample was collected 15 days after administration in cardiac puncture. The histopathological study was conducted in the lungs, intestines, kidneys, stomach and liver. | Signs of apathy and respiratory changes occurred more often in groups of male and female animals treated with AAS. Dosages of biochemistry elements showed no differences statistically significant. There were no cellular morphological changes. Changes found were correlated with later studies with presence of phenol in the mushroom, a substance that acts on the central nervous system, initially causing stimulation followed by depression. Administration of A. sylvaticus at doses greater than those used in human therapeutic protocols, showed very low toxicity. |

**Table I** *(cont.)*
*Studies on the toxicity of edible mushrooms and/or medicinal. Period 2003-2012*

| *References* | *Type of study* | *Mushroom* | *Type of toxicity* | *Objectives* | *Methods and materials* | *Results* |
|---|---|---|---|---|---|---|
| Luo (2007)[30] | Experimental | *Coprinus comatus* | Toxicity in nematodes | Obtain evidence of nematicidal activity of *C. comatus* mushrooms. | We performed a bioassay of exposure of nematodes *Panagrellus redivivus* to the mushroom with the regeneration plates of mushrooms, with organic solvent extracted from prickly balls; with purified and crushed from prickly balls. The extract was subjected to thin layer chromatography (TLC) in silica gel to extract the toxin. We conducted a spectrum analysis and nematicidal assay of compounds. | 73.7% and 98.3% of the nematodes were immobilized by strain *Comatus c*.LHA-7 and 75.7 and 98.9%, were immobilized by C-1 after 15 and 30 min. 75% and 93.8% of strain LHA-7e 76.9 and 92.3% of strain C-1 were immobilized by the prickly balls after 5 and 10 min. The results of tests with prickly balls extracts were similar to that of efficiency immobilization produced by normal balls. However, none of the extracts obtained showed any obvious effect on the nematodes tested. Compounds 1 and 2, determined by a spectrophotometer, were the most nematotoxic of the seven extracts with 90% lethal dose (LD90) values of 200 g/mL against both *M. incognita* and *P. redivivus*. The other compounds isolated from *C. comatus* also showed nematicidal activity, with higher doses (400 to 800 g/ml). |
| Singi et al. (2006)[19] | Experimental | *Agaricus blazei* | The clinic | To evaluate the acute effect of intravenous injection of A. blazei Murrill on mean arterial pressure (MAP) and heart rate (HR) of anesthetized rats, creating the possibility of studying its chronic use. | Aqueous extract of the mushroom was prepared by drying, crushing and disolving. We administered concentrations of 1.25 mg/kg 2.50 mg/kg and 5.00 mg/kg of aqueous extract volume of 0.2 ml in six rats *Rattus norvegicus albinus* anesthetized with sodium thiopental, through tracheostomy and cannulated via the jugular vein and carotid artery. The values of mean arterial pressure (MAP) and heart rate (HR) were obtained in control and in 15, 30, 45, 60 and 120 s after application of the extracts. | A concentration of 1.25 mg/kg caused no significant change in MAP or HR; the 2.50 mg/kg caused a decrease in MAP at 15 s ($p < 0.01$) and in HR at 30 s ($p < 0.001$) and the 5.00 mg/kg decreased the MAP at 15 s to ($p < 0.001$) and HR at 15 and 30 s ($p < 0.001$). The aqueous extract of *A. blazei* reduced MAP in a concentration-dependent manner. The HR also suffered decline, but not in concentration-dependent. Correlating with other studies, the authors attributed the decrease in MAP to gamma-aminobutyric acid (GABA) found in *A. blazei,* which by direct action on blood vessels, by ganglionic blockade with consequent inhibition of the the release of transmitters in the sympathetic nerve terminals, would reduce the MAP. Previous studies have also cited the explanation that high levels of potassium and calcium in *A. blazei* would cause hyperpolarization and relaxation of vascular smooth muscle leading to decreased blood pressure. |
| Sugui et al. (2006)[20] | Experimental | *Agaricus brasiliensis* | Genotoxicity | To evaluate the protective effect of an aqueous solution of A.brasiliensis (AB strain 99/29) in bone marrow, peripheral blood, bladder, colon and liver of Wistar rats. | Different experimental protocols (micronucleus test, comet assay and testing of aberrant crypt foci) were used for a broader assessment of the chemopreventive effect of *A. brasiliensis*. The animals were treated with the aqueous solution (60° C) of strain AB 99/29, and with agents target organ N-ethyl-N-nitrosourea (ENU), N-Methyl-N-nitrosourea (MNU), 1, 2-dimethylhydrazine (DMH) and diethylnitrosamine (DEN). | The aqueous solution *A. brasiliensis* under the conditions tested, showed no mutagenic, genotoxic or carcinogenic effects. However, an antimutagenic effect against the mutagenicity of ENU was observed in bone marrow cells and a significant reduction in the number of aberrant crypts per focus (4-6 crypts/focus) in DMH-induced colon of animals post-treated with aqueous solution of the mushroom. In this context, the results suggested that the aqueous solution of *A. brasiliensis* may have compounds that significantly reduce the frequency of micronucleated cells in the bone marrow of rats, and that they may act at a later stage of the carcinogenesis process. |

**Table I** *(cont.)*
*Studies on the toxicity of edible mushrooms and/or medicinal. Period 2003-2012*

| *References* | *Type of study* | *Mushroom* | *Type of toxicity* | *Objectives* | *Methods and materials* | *Results* |
|---|---|---|---|---|---|---|
| Mantovani et al. (2006)[31] | Experimental | *Agaricus brasiliensis* | Genotoxic and clastogenic. | To evaluate the genotoxic clastogenic effects and protective of aqueous extracts of A. brasiliensis prepared in different ways in cell culture of Chinese hamster ovary, CHO-k1. | Chinese hamster ovary cells were grown in culture medium F-12/DMEM supplemented with 10% fetal bovine serum. We tested two types of aqueous extracts of *A. brasiliensis*. The first concentration 10%, by dissolving 20 g of dried mushroom and ground into 200 mL of deionized water at room temperature (20° C), three concentrations: 0.2, 0.4 and 0.6% in culture. The second concentration was produced after extraction of organic compounds, prepared from the same mushroom dehydrated and crushed, dissolved in dimethylsulfoxide (DMSO) at a ratio of 5 mg/ml, and the final concentration in culture of 100 mg/mL, used in the Chromosomal Aberration assay (CA-II). Comet assay was also performed (SCGE) associated with two DNA blocking repair, Ara-C and 3DeoT in the presence or absence of an alkylating agent. | As for the clastogenicity test, we verified that the concentrations 0.2 and 0.4% of the aqueous extracts of *A. brasiliensis* did not induce damage, unlike the highest concentration (0.6%), which showed clastogenic activity. In genotoxicity treatments in SCGE the concentration of 0.2% of the extract showed no genotoxic activity, unlike concentrations of 0.4 and 0.6%, which were effective in inducing DNA damage. The 0.4% concentration was found to be damage inducing by comet assay. The anticlastogenicity results indicated that in most treatments, the aqueous extract of *A. brasiliensis* showed no protective activity against DNA damage induced by Ara-C and Ara-C + MMS. Through SCGE, A. brasiliensi in the three concentrations tested showed no antigenotoxic activity. The data suggest caution in the consumption and ingestion of *A. brasiliensis* by humans, especially at high concentrations, due to its genotoxic and clastogenic activity. |
| Kuroiwa et al. (2005)[21] | Experimental | *Agaricus blazei* | Clinical, hematological, serum biochemical parameters, histopathological. | Subchronic toxicity study in F344 rats seeking food safety, setting not observable adverse effect level (NOAEL). | We used 20 animals randomly distributed into five groups. The control group received the basal diet and the others fed the diet containing powdered aqueous extract of *A. blazei* Murrill at doses of 0.63, 1.25, 2.5 and 5% (maximum - according to preliminary study of two weeks) for 90 days. We performed hematological tests, biochemical and histopathological serum tests. | There were no significant changes in the general appearance and no deaths occurred in neither groups. Although urea nitrogen levels were slightly higher in male of groups 2.5% and 5%, histopathological changes were not observed in the kidneys. The serum creatinine levels were very low, suggesting that the increase in blood urea nitrogen has little toxicological significance. However, there was no evidence of hepatic toxicity in serum assays, organ weights and histopathology. Extract *A. blazei* Murrill demonstrated little or no significant toxicity, even at 5% dietary supplementation. Thus, the mushroom extract up to 5% in diet (2,654 mg/kg body weight/day to male rats and 2,965 mg/kg body weight/day for females) does not cause noticeable adverse effects in F344 rats. |
| Costa et al. (2003)[22] | Experimental | *Agaricus blazei* | Genotoxicity | To evaluate the possible protective effects of *A. blazei* against tea genotoxic action of urethane in somatic cells of *Drosophila melanogaster* | To evaluate the possible protective effects of *A. blazei* tea (62.5 g.l-1) against the urethane genotoxic action (10 mM) we used the Somatic Mutation and Recombination Detection and (Somatic Mutation and Recombination Test-SMART). We used larvae of 72 ± 4h, resulting from crosses and high standard metabolic bioactivation. | No increase was statistically significant in the frequencies of mutant spots in larvae exposed to tea *A. blazei*. When the mushroom *A. blazei* was associated with urethane, we observed a statistically significant reduction in the frequency of mutant spots. The results suggest that A. blazei is not genotoxic and exerts a protective effect against genotoxic action of urethane. |

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