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JOICE VINHAL COSTA ORSINE

COMPOSIÇÃO QUÍMICA, TOXICIDADE, GENOTOXICIDADE E ANTIGENOTOXICIDADE DO COGUMELO *Agaricus sylvaticus* (COGUMELO DO SOL)

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Tese apresentada ao Curso de Pós-Graduação em Ciências da Saúde, Faculdade de Ciências da Saúde, Universidade de Brasília como requisito parcial à obtenção do título de Doutor em Ciências da Saúde.

Orientadora: Prof[a.] Dr[a.] Maria Rita C. Garbi Novaes

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Tese apresentada ao Curso de Pós-Graduação em Ciências da Saúde da Faculdade de Ciências da Saúde da Universidade de Brasília para obtenção do título de Doutor.
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“Fiz a escalada da montanha da vida
removendo pedras e plantando flores.”
(Cora Coralina)

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RESUMO

Orsine JVC. **Composição química, toxicidade, genotoxicidade e antigenotoxicidade do cogumelo *Agaricus sylvaticus* visando à segurança alimentar.** 2013. 198 folhas. Tese [Doutorado] – Programa de Pós-Graduação em Ciências da Saúde, Universidade de Brasília. Orientadora: Profa Dra Maria Rita Carvalho Garbi Novaes.

Cogumelos da espécie *Agaricus sylvaticus* têm sido amplamente utilizados como suplemento dietético, apesar de que ainda não foram completamente estudados, o que gera a necessidade de pesquisas acerca da segurança quanto ao seu uso. O objetivo deste estudo foi determinar a composição química, atividade antioxidante, citotoxicidade, genotoxicidade e antigenotoxicidade do cogumelo *Agaricus sylvaticus*. Foram avaliados umidade, proteínas, lipídeos, carboidratos, minerais, vitamina C e vitaminas lipossolúveis. Foi determinada a atividade antioxidante dos extratos aquoso, etanólico e etéreo do cogumelo *Agaricus sylvaticus,* utilizando-se o teste do "2,2-difenilpicril-hidrazila". A citotoxicidade do cogumelo em estudo foi avaliada por meio do teste de hemólise em eritrícitos humanos, e teste do azul de tetrazólio utilizando-se linhagens de células tumorais e não-tumorais. A genotoxicidade e antigenotoxicidade dos extratos aquosos do cogumelo *Agaricus sylvaticus* foram avaliadas através do teste SMART, em asa de *Drosophila melanogaster*, e pelo teste do micronúcleo, utilizando-se camundongos *Mus musculus* (Swiss Webster). O estudo foi aprovado pelo Comitê de Ética em Pesquisa em Animais, da Universidade Federal de Goiás. Observou-se que o cogumelo *Agaricus sylvaticus* apresenta rica composição química, além de interessante atividade antioxidante. Os resultados acerca de sua toxicidade mostram que o cogumelo *Agaricus sylvaticus* não apresenta-se tóxico em eritrócitos humanos, células não tumorais (NIH3-T3) e células tumorais (OSCC-3). Através dos resultados do teste SMART foi possível observar que o cogumelo *Agaricus sylvaticus* não apresenta efeito genotóxico, e apresenta fraco efeito protetor contra danos provocados pela mitomicina, nas concentrações avaliadas. Quando avaliados os efeitos genotóxicos e antigenotóxicos do cogumelo *Agaricus sylvaticus* por meio do teste do micronúcleo, foi possível observar que o cogumelo *A. sylvaticus* apresentou atividade genotóxica e antigenotóxica em todas as concentrações testadas, nos tempos de 24 e 48 horas, indicando o efeito Janus do composto. Dessa forma, estudos clínicos randomizados são necessários para elucidar as consequências no uso terapêutico e/ou efeitos benéficos dos achados.

Palavras-chave: *Agaricus sylvaticus*, *Agaricaceae*, cogumelos medicinais, atividade antioxidante, genotoxicidade, antigenotoxicidade.

ABSTRACT

Orsine JVC. **Chemical composition, toxicity, genotoxicity and antigenotoxicity of *Agaricus sylvaticus* aimed at food security.** 2013. 198 pages. PhD Dissertation - Program in Health Sciences. Brasilia University. Advisor: Prof. Dr. Maria Rita Carvalho Garbi Novaes.

Agaricus sylvaticus mushroom have been widely used as nutritional complement, inspide does not exist so many studies about them, what causes the necessity of more studies about its safe use. The purpose of the present study was to determine the chemical composition, antioxidants activity, citotoxicity, genotoxicity and antigenotoxic of the *A. sylvaticus* mushroom. It was evaluate the moisture, proteins, ash, lipids, total dietary fiber, carbohydrates, fat-soluble vitamins and vitamin C. It was also observed the antioxidant potential of the aqueous, alcoholic and ethereal *A. sylvaticus* mushroom extracts, by the 2,2-difenilpicril-hidrazila assay. The citotoxicity of the aqueous extract of *A. sylvaticus* mushroom was estimate on human erythrocytes, and tetrazolium assay, in cultures of non-tumor cells and tumor cells. The genotoxic and antigenotoxic action of *A. sylvaticus* extract was evaluated by the somatic mutation and recombination test (SMART) in *Drosophila melanogaster* and by the frequency of micronucleated polychromatic erythrocytes in *Mus musculus* (Swiss Webster). The study was approved by the Animal Ethics Committee of the Federal University of Goiás. Through this study it was able to observe the rich chemical composition of *A. sylvaticus* and its great antioxidant potential. The toxicity results suggest that *A. sylvaticus* mushroom has no toxicity on human erythrocytes**,** non-tumor cells (NIH3-T3) and tumor cells (OSCC-3). By SMART test, we observed that *A. sylvaticus* mushroom was not genotoxic, and the co-treatments with mitomicin demonstrated that the mushroom extract have some anti-mutagenic activity in all concentrations evaluated. The results obtained from the evaluation of mutagenicity and antimutagenicity of this mushroom, using the micronucleus assay, showed that *A. sylvaticus* mushrrom has both mutagenic and antimutagenic effect in all doses, at 24 and 48 hours, suggesting the Janus effect of the extract. Thus, clinic randomized studies comes important to prove the consequences of the terapeutic and/or the positive effects found.

Keywords: *Agaricus sylvaticus*, *Agaricaceae*, medicinal mushroom, antioxidant activity, genotoxicity, antigenotoxicity.

1 INTRODUÇÃO

Existem diversos estudos realizados com o cogumelo *Agaricus sylvaticus*, comercialmente conhecido como Cogumelo do Sol que sugerem benefícios a saúde de pacientes oncológicos devido à presença de substâncias bioativas em sua composição (Fortes e Novaes 2006; Taveira & Novaes, 2007; Novaes et al., 2007; Fortes et al., 2010; Fortes et al., 2011).

O objetivo geral do trabalho foi analisar a composição química e os possíveis efeitos citototóxicos, genotóxicos e antigenotóxicos do cogumelo *A. sylvaticus*, cultivado no Brasil de forma a determinar a segurança no consumo humano para fins alimentares e terapêuticos.

Os objetivos específicos deste trabalho foram:

- Realizar a caracterização físico-química do cogumelo com a determinação da umidade, de proteínas, de lipídeos, de carboidratos, de fibras, de minerais e de vitaminas;
- Avaliar a atividade antioxidante dos extratos etéreo, aquoso e alcoólico do cogumelo *A. sylvaticus*;
- Avaliar a citotoxicidade do cogumelo *A. sylvaticus* por meio dos testes *in vitro* de hemólise em eritrócitos humanos e teste do MTT (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina) em células tumorais e não-tumorais;
- Avaliar o efeito mutagênico e antimutagênico da administração do cogumelo *A. sylvaticus* em asa de *Drosophila melanogaster*.
- Avaliar a ação genotóxica e antigenotóxica do cogumelo *A. sylvaticus* em eritrócitos policromáticos da medula óssea de camundongos.

Os experimentos deste trabalho foram conduzidos no período de 2010 a 2013 sendo que os resultados dos estudos foram apresentados na forma de artigos científicos.

Em um primeiro momento foram elaborados dois artigos de revisão. O artigo **Cogumelos Comestíveis: Uso, Conservação, Características Nutricionais e Farmacológicas** publicado na **Revista do Hospital das Clínicas de Porto Alegre e da Faculdade de Medicina da Universidade Federal do Rio Grande do Sul 2012; 32(4): 452-60**, periódico Científico indexado nas Bases Lilacs e Latindex, classificado pelo Programa da CAPES - Qualis Medicina II como B4, que aborda as características gerais de cogumelos comestíveis, englobando diversas espécies, os métodos de

conservação empregados para evitar a deterioração dos cogumelos, seu emprego como ingrediente de outros alimentos, a forma de consumo, os estudos recentes acerca das substâncias bioativas presentes nos cogumelos, os testes relacionados à toxicidade de cogumelos, entre outros.

O segundo artigo de revisão intitulado **Mushrooms of the Genus Agaricus as Functional Foods** foi publicado na revista **Nutrición Hospitalaria 2012; 27(4):1017-24**, periódico indexado nas bases de dados Medline, Index Medicus, Embase, Excerpta Médica, Cancerlit, Toxline, Aidsline, Health Planning and Administration, Índice Médico Español (IME), Índice Bibliográfico Español en Ciencias de la Salud (IBECS), SENIOR), classificado pelo Programa da CAPES - Qualis Medicina II como B2. Este artigo relaciona os cogumelos do Gênero Agaricus com os conceitos e abordagens de um alimento funcional, de acordo com diversos autores e legislação de alimentos.

Foram elaborados três artigos originais que tratam da determinação da composição bromatológica do cogumelo *A. sylvaticus*, a presença de ácido ascórbico, vitaminas lipossolúveis e minerais, além do potencial antioxidante dos extratos etéreo, etanólico e aquoso, e o teor de compostos fenólicos, no sentido de contribuir para o conhecimento das características físico-químicas do Cogumelo do Sol.

(1) O artigo intitulado: **Nutritional value of *Agaricus sylvaticus* mushroom grown in Brazil** foi publicado na revista **Nutrición Hospitalaria 2012; 27(2): 449-455**.

(2) O artigo intitulado: **Chemical and Antioxidant Potential of *Agaricus sylvaticus* Mushroom Grown in Brazil** foi publicado no periódico **Journal of Bioanalysis & Biomedicine 2011; 3(2): 49-54**, indexado nas bases Gale, Hinari, Scopus e Embase, podendo também ser encontrado no Google Scholar, Scientific Commons, Index Copernicus e EBSCO.

(3) o artigo intitulado: **"Determination of chemical antioxidants and phenolic compounds in the Brazilian mushroom *Agaricus sylvaticus"*** foi aceito para publicação no periódico **West Indian Medical Journal**, indexado nas bases de dados MedCarib, Lilacs e Medline e classificado pelo Programa da CAPES - Qualis Medicina II como B2.

Foram elaborados outrosdois artigos originais que abordam a toxicidade do extrato aquoso não fracionado do cogumelo *A. sylvaticus in vitro*.

(1) O artigo intitulado **The acute cytotoxicity and lethal concentration (LC_{50}) of *Agaricus sylvaticus* through hemolytic activity on human erythrocyte** foi

publicado no periódico**International Journal of Nutrition and Metabolism 2012; 4(11):19-23**, indexado em Chemical Abstract.

(2) O artigo intitulado **Cytotoxicity of *A. sylvaticus* in non-tumor cells (NIH/3T3) and tumor (OSCC-3) using Tetrazolium (MTT) assay** foi aceito para publicação no periódico **Nutrición Hospitalaria**, no ano de 2013.

Por último, foi elaborado outro artigo original relacionado à atividade genotóxica e antigenotóxica do extrato aquoso não fracionado do cogumelo *A. sylvaticus*, em concentrações variadas, utilizando-se dois testes *in vivo*, intitulado **Genotoxicidade e antigenotoxicidade do cogumelo *Agaricus sylvaticus* em *Drosophila melanogaster* por meio do teste de mutação e recombinação somáticas (SMART) e em *Mus musculus* (Swiss Webster) por meio do teste do micronúcleo.** Este último artigo descrito nesta tese encontra-se em fase de redação final e tradução para submissão a uma revista científica.

ARTIGO 1 – ARTIGO DE REVISÃO

Versão publicada em português:

Cogumelos Comestíveis: Uso, Conservação, Características Nutricionais e Farmacológicas. Orsine JVC, Brito LM, Novaes MRCG. Revista HCPA. 2012;32(4):452-460.

2 ARTIGO DE REVISÃO

COGUMELOS COMESTÍVEIS: USO, CONSERVAÇÃO, CARACTERÍSTICAS NUTRICIONAIS E FARMACOLÓGICAS

EDIBLE MUSHROOMS: USE, CONSERVATION, NUTRITIONAL AND PHARMACOLOGICAL CHARACTERISTICS

Resumo

É crescente o interesse na produção e consumo de cogumelos devido às suas qualidades nutricionais e terapêuticas, o que tem estimulado sua utilização como alimento funcional e como coadjuvante no tratamento de enfermidades como o câncer. O presente trabalho tem por objetivo discutir o uso de cogumelos como alimento e com fins medicinais pela população através da apresentação de trabalhos publicados, considerando a composição química e nutricional, aspectos farmacológicos e tóxicos para o uso seguro em seres humanos. A coleta de dados foi realizada por meio de pesquisa em bases eletrônicas Lilacs, Sciello, Medline, PubMed e Cochrane. Foi possível verificar que os cogumelos apresentam interessantes características nutricionais devido ao alto teor de proteínas e fibras alimentares, baixo teor de lipídeos e fonte considerável de sais minerais. Possuem diversas substâncias com atividade antioxidante, como a Vitamina C, Vitamina E e polifenóis. Dentre as substâncias com interesse na medicina, está o ergosterol, precursor da Vitamina D, que possui ação em enfermidades ósseas, como raquitismo e osteoporose. Na profilaxia e tratamento do câncer, foram observados possíveis efeitos anticarcinogênicos e antimutagênicos, proporcionados por glucanas, arginina, proteoglucanas, glutamina, lectina. Como não estão incluídos nas práticas alimentares da maioria da população do Brasil, muitos estudos estão sendo realizados no intuito de desenvolver formulações com adição de cogumelos, tornando os alimentos mais saudáveis.

Palavras-chave: alimento funcional, suplementos dietéticos, hábitos alimentares.

Abstract

The increasing interest in the production and consumption of mushrooms is due to its nutritional and therapeutic qualities which have encouraged the use of mushrooms as functional food and as adjuvant in the treatment of diseases like cancer. The objective of this article is to discuss the use of mushrooms as food and with medicinal purposes. For that, we searched for published works that consider their chemical and nutritional composition as well as their pharmacological and toxicological aspects for safe use in humans. Data collection was performed by a research on the electronic databases LILACS, SciELO, MEDLINE, PubMed, and Cochrane. The analysis of published studies showed that mushrooms have interesting nutritional characteristics due to high protein and dietary fiber, low lipid content, and it is also a substantial source of dietary minerals. They have several substances with antioxidant activity, such as Vitamin C, Vitamin E, and polyphenols. Within the group of substances of medicinal interest is ergosterol, a precursor of Vitamin D, which acts on bone diseases such as rickets and osteoporosis. In the prophylaxis and treatment of cancer, we observed some possible anticarcinogenic and antimutagenic effects provided by glucan, arginine, proteoglucans, glutamine, and lectin. However, mushrooms are not part of most Brazilians' diet yet. For this reason, there are many ongoing studies to develop formulations with addition of mushrooms to make food healthier.

Keywords: Functional food; dietary supplements; food habits

2.1 INTRODUÇÃO

Os cogumelos são muito apreciados desde a idade antiga. Acreditava-se no elevado valor nutritivo e no potencial medicinal, além de serem considerados uma especiaria nobre na culinária. São conhecidas no mundo aproximadamente 140.000 espécies de cogumelos, sendo 2000 comestíveis, e 700 com propriedades farmacológicas comprovadas. Destas, 25 são cultivadas comercialmente (1).

De acordo com o *Codex Alimentarius*, os cogumelos comestíveis são alimentos pertencentes ao grupo Funghi, os quais podem crescer em estado silvestre ou serem

cultivados, e que depois de sua elaboração estarão apropriados para serem utilizados como alimentos (2).

O crescente interesse comercial e científico em cogumelos para uso na gastronomia ou na terapêutica clínica estimulou o aprimoramento de técnicas de cultivo, e a introdução de novas espécies (1). Sendo assim, informações sobre a composição dos cogumelos são essenciais para avaliar a sua qualidade. Uma vez que os cogumelos desempenham funções importantes no organismo humano, a comprovação da rica composição química tem grande valor e tem se tornado uma preocupação de profissionais da área de saúde e de alimentos (3).

O objetivo deste trabalho é discutir o uso de cogumelos como alimentos e com fins medicinais pela população através da apresentação dos trabalhos publicados, considerando a composição química, aspectos farmacológicos e toxicológicos para o uso seguro em seres humanos.

2.2 MÉTODO

Dos 230 artigos encontrados, foram selecionados 56 artigos publicados entre 2000 e 2012, nas bases de dados Scielo, Lilacs, Medline, Pubmed e Biblioteca Cochrane, nos idiomas inglês, português e espanhol. Foram aplicados os seguintes critérios de inclusão: artigos originais que apresentassem composição dos cogumelos terapêuticos, os resultados e benefícios do uso na alimentação. Foi utilizado o Mesh/DECS - descritores em Ciências da Saúde - para definir os termos de busca: “Agaricales” e “Cogumelo” aplicando-os nos critérios de inclusão dos artigos pesquisados.

2.3 RESULTADOS

2.3.1 Aspectos químicos e nutricionais de cogumelos comestíveis

Quando analisada sua composição bromatológica, os cogumelos são indicados para dietas balanceadas, em razão da baixa concentração de gordura e de energia, bem como da alta concentração de fibras alimentares e proteínas (4) (Tabela 1).

Tabela 1. Composição química de alguns cogumelos comestíveis. Estudos selecionados nas bases de dados Lilas, Medline, Sciello, Cochrane. Período de 2000 a 2012.

Referência	Espécie de cogumelo	Substâncias presentes
COSTA et al. (2011) (4)	*Agaricus sylvaticus*	- Carboidratos (36,21%), Proteínas (41,16%), Cinzas (7,38%), Lipídios (6,60%), Fibras (2,34%). - Ferro (0,72690%), Cálcio (0,00135%), Zinco (0,54925%), Cobalto (0,00775%), Magnésio (0,02119%), Sódio (0,25534%), Potássio (0,61303%), Manganês (0,02318%) e Cobre (0,27666%). - Vitamina C (0,01265%), Vitamina A (0,000001%), Vitamina D2 (0,000018%), Vitamina E (0,000020%) e Vitamina K2 (0.000001%).
CHARALO et al. (2007) (25)	*Agaricus blazei*	- 29,23% de ácido palmítico (16:0), 7,46% de ácido esteárico (18:0), 10,84% de ácido oléico (18:1-n9), 49,68% de ácido linoléico (18:2-n6), 2,34% de ácido aracdônico (20:4n-6)
FULLANI et al. (2007) (3)	*Lentinula edodes*	- Proteína 19%, em base seca, cerca de 4,4% de lipídios e fibra alimentar em torno de 41,9%, fósforo aproximadamente 0,0894%
FULLANI et al. (2007) (3)	*Agaricus bisporus*	- Teor de proteínas próximo a 28% em relação à base seca, fibras alimentares (20,4%) e baixo teor de lipídeos (5,4%), fósforo, valores médios de 0,1133%.
FULLANI et al. (2007) (3)	*Pleorotus spp*	- Proteínas 22%, fibras alimentares (39,6%) e lipídios (4,30%), fósforo de 0,1097%.

2.3.2 Estocagem e cuidados pós-colheita de cogumelos

Os cogumelos do gênero Pleurotus são mais delicados e sensíveis do que os do gênero Agaricus e deterioram-se mais rapidamente após a colheita. Uma vez deteriorados, podem causar severas intoxicações gastro-intestinais (5).

O cogumelo, depois de colhido, tem no máximo dez dias de vida útil, tendo sua temperatura de armazenamento interferência direta sobre a qualidade do produto. Sob refrigeração a 2ºC, o cogumelo tem vida de prateleira de aproximadamente nove dias. Quando armazenado a 18ºC, observa-se a redução da vida útil para apenas três dias (6).

2.3.3 Conservação e preservação das características nutricionais de cogumelos

Devido a seu elevado conteúdo de água, os cogumelos são altamente perecíveis. Quando não consumidos em curto intervalo de tempo após a colheita na forma fresca, devem passar por algum tipo de tratamento para evitar sua deterioração (7) (Tabela 2).

Tabela 2. Formas de aplicação de métodos de conservação de alimentos sobre cogumelos.

Referência	Método de conservação	Resultados encontrados
MC DONALD e SUN (2000) (26)	Resfriamento a vácuo	· A técnica a vácuo promove a aceleração do resfriamento, mas pode causar alguns efeitos desagradáveis na qualidade dos cogumelos, como problemas relacionados à perda de massa.
BURTON et al. (1987) (27)	Resfriamento e refrigeração a vácuo	· Não foram encontradas diferenças na estrutura dos cogumelos resfriados a vácuo e convencionalmente. · Após 102 horas estocados a 5ºC não foi detectado escurecimento significativo, porém os cogumelos resfriados a vácuo tiveram menor escurecimento do que os resfriados convencionalmente. · Quando os cogumelos foram estocados a 18ºC houve um aumento linear no escurecimento com o tempo de estocagem. · A perda de massa dos cogumelos estocados a 5ºC foi consideravelmente menor do que aqueles estocados a 18ºC.
APATI (2004) (28)	Secagem	· A melhor temperatura de desidratação é de 40ºC, levando em consideração a melhor capacidade de reidratação (por meio de imersão em água a temperatura ambiente, por um período de 30 minutos) dos cogumelos desidratados nesta temperatura. · O tempo de secagem é aproximadamente duas vezes superior, se comparado à secagem realizada a 60ºC e umidade relativa do ar de aproximadamente 75%.
MARTÍNEZ-SOTO et al. (2001) (29)	Branqueamento com metabissulfito de sódio ou ácido cítrico antes da secagem	· Cogumelos que sofreram branqueamento ficaram mais escuros depois da secagem do que aqueles que não foram submetidos ao branqueamento. · Os cogumelos liofilizados apresentaram maior capacidade de reidratação e cor mais próxima à dos cogumelos *in natura* do que os cogumelos secos por ar quente ou a vácuo. · O aroma e o sabor dos cogumelos secos por ar quente foram estatisticamente semelhantes aos apresentados pelos cogumelos liofilizados.
GEORGE e DATTA	Liofilização	- Tempo final de desidratação dos cogumelos de cinco horas, porém a liofilização não é um processo viável economicamente para o

(2002) (30) processamento industrial de cogumelos.

* O branqueamento é utilizado como pré-tratamento no processamento de alimentos, devendo ser seguido de um método de conservação adequado.

2.3.4 Formas de utilização de cogumelos comestíveis

No Brasil, os cogumelos ainda não fazem parte do cardápio da maioria da população, que oferece certa resistência com relação ao seu consumo, podendo este fato ser explicado pela falta de conhecimento quanto à disponibilidade de diferentes espécies e ao seu preparo (8).

O grau de escolaridade entre os consumidores de cogumelos representa uma parcela muito bem informada da população, e a espécie mais consumida é o tradicional Champignon de Paris, seguida pelo Shiitake e o Shimeji. As formas de consumo de cogumelos mais utilizadas são em molhos, cogumelo fresco e seco, em sopa e refogado, em conserva, acompanhando pizzas, massas e risotos (9).

O uso do chá de cogumelos é uma das práticas mais populares da medicina tradicional chinesa relacionada à prevenção ou ao tratamento de várias doenças humanas (10), sendo a forma mais comum para o seu preparo a infusão e fervura do fungo desidratado (11).

Em relação às formas de preparo, uma questão ainda a ser considerada é o efeito do processamento dos cogumelos sobre as suas propriedades. O cozimento dos cogumelos comestíveis pode afetar os nutrientes termolábeis. Porém, o uso de altas temperaturas tem efeito positivo na maior parte dos minerais que ativam o sistema imunológico, que se tornam mais disponíveis ao organismo humano após o cozimento. Já as fibras são parcialmente quebradas e as proteínas são afetadas sem, no entanto, ter seu valor nutricional reduzido (8).

Em alguns casos, como o cogumelo Shitake, suas propriedades nutricionais são ressaltadas após cozimento. Quando submetido a processo de fritura leve, tem preservados os nutrientes instáveis. A maior parte dos constituintes ativos, como os polissacarídeos, está associada a estruturas da parede celular e, em processo de ebulição, é liberada. Outros constituintes ativos como os terpenos são também melhor solubilizados em água quente, sendo relativamente estáveis ao calor (8).

2.3.5 Aspectos farmacológicos de cogumelos comestíveis

Diversas substâncias bioativas com propriedade farmacológica como glucanas, proteoglucanas, lecitinas, ergosterol e arginina têm sido identificadas e isoladas em numerosas espécies de fungos medicinais (12).

A exemplo dos cogumelos *A. sylvaticus*, *Lentinula edodes* e *A. blazei* são relatados vários polissacarídeos com atividade imunomodulatória, anticancerígena, antiinflamatória e antioxidante (13).

Acredita-se que a principal substância que responde pelos atributos funcionais dos fungos medicinais são as β -glucanas, fibras alimentares solúveis capazes de atuar eficazmente na redução do colesterol e de outros lipídeos plasmáticos (14). Aumenta as funções imunológicas através do estímulo à expansão clonal de células T, Natural Killer (NK), linfócitos B e células complementares, aumentando o número de macrófagos e monócitos, promovendo a proliferação e/ou produção de anticorpos e de várias citocinas e, dessa forma, evitam a regeneração e a metástase do câncer (15).

Fibras como as β-proteoglucanas, heteroglucanas, quitina, peptideoglucanas, atuam como imunomoduladores (16). A composição da fração fibra dos cogumelos é composta principalmente por β-glicanas, quitina e hemicelulose, as quais apresentam propriedades antitumorais e antimutagênicas por estimularem o sistema imune (17).

As vitaminas do *A. blazei* Murill estão relacionadas à antiangiogênese, que corresponde à nova formação vascular. Apresentam efeito sobre o crescimento da microcirculação, a vitamina D3 e a vitamina D2 (ergosterol), que também apresenta um efeito antiangiogênico potente. O responsável por esse efeito é o ergosterol presente no extrato do cogumelo, que possui ação na redução do volume e inibição do crescimento tumoral, em ratos com sarcoma 180, sem efeitos adversos geralmente causados pelos quimioterápicos. Seu mecanismo de ação ocorre através da inibição da neovascularização. O ergosterol, precursor do ergocalciferol, é, sobretudo, uma substância antiangiogênica, explicando em parte seu efeito antitumoral (18).

Em estudo realizado por FORTES et al. (14), os autores observaram a redução significativa dos níveis plasmáticos de CT e LDL-c durante todo o período de suplementação dietética com *A. sylvaticus*, sendo sugeridas a presença de substâncias bioativas nesses fungos, capazes de reduzir frações lipídicas: colesterol total, LDL-c e triglicérides, apresentando efeitos benéficos no metabolismo lipídico e, conseqüentemente, no prognóstico dos pacientes.

Já a lectina exerce propriedade antitumoral, antimutagênica e hemaglutinizante através de sua propriedade indutora de apoptose nas células tumorais, mecanismo primário contra as neoplasias malignas (18).

Outros estudos experimentais conduzidos em animais de laboratório têm comprovado que a administração de determinadas espécies de fungos medicinais é capaz de promover redução significativa do CT, LDL-c (4, 5, 17-20), VLDL-c (5, 17), TG (16-20), fosfolipídio, índice aterogênico e da atividade da enzima 3-hidroxi-3-metilglutarilcoenzima A redutase (HMG-CoA redutase), além do aumento do HDL-c (20). O mecanismo pelo qual fungos medicinais capazes de reduzir os níveis lipídicos é explicado por meio do aumento da excreção fecal de ácidos biliares e de colesterol, especificamente, por aumentar o receptor hepático LDL. As lovastatinas, inibidoras da enzima HMG-CoA redutase, que catalisam a síntese do mevalonato, atuam conjuntamente como responsáveis pelos efeitos observados. Também já foi identificada uma substância denominada eritadenina, agente hipolipidêmico, capaz de reduzir os níveis de colesterol e outros lipídeos por meio da excreção do colesterol ingerido e de sua decomposição metabólica (14).

A arginina é descrita como estimuladora do hormônio de crescimento hipofisário e está relacionada com o aumento da atividade das células NK, células T *helper* e com o estímulo da produção de citocinas tais como IL-1, IL-2 e IL-6. Estudos indicam que o aumento da imunidade promovida pela arginina é obtido pela estimulação da liberação do hormônio de crescimento, estímulo na produção de oxido nítrico, hidroxiprolina, citocinas e poliaminas (18).

Já as proteoglucanas têm seu mecanismo de ação baseado na estimulação das funções imunológicas, da atividade fagocitária de macrófagos e melhoria das funções do sistema retículo-endotelial, amenizando assim os sintomas associados à quimioterapia, além de melhorar a infiltração tumoral pelas células T citotóxicas (18).

Por fim, a glutamina age aumentando a função imune e intestinal, reduz a bacteremia e os danos na mucosa associados à quimioterapia, mantendo a integridade intestinal, melhora o equilíbrio nitrogenado, contribui com a não elevação de citocinas pró-inflamatórias, possui capacidade antioxidante, e melhora a preservação da musculatura esquelética. Seu mecanismo de ação se justifica por ser fonte de energia preferencial à glicose por todas as células de divisão rápida, como os enterócitos, células do sistema imunológico e nervoso. Prolonga a sobrevida no tratamento do câncer, diminuindo o catabolismo debilitante (20).

2.3.6 Estudos do efeito de cogumelos comestíveis em pacientes oncológicos

Após suplementação dietética com fungos *A. sylvaticus*, Fortes et al. (15) observaram que este cogumelo é capaz de melhorar as alterações gastrointestinais de pacientes no pós-operatório de câncer colorretal, promovendo melhoria na qualidade de vida desses pacientes.

Foi realizado um estudo por Fortes et al. (21), com o objetivo de avaliar os efeitos da suplementação dietética com fungos *A. sylvaticus* em pacientes no pós-operatório de câncer colorretal, após seis meses de tratamento, a respeito dos indicadores da qualidade de vida - sedentarismo, tabagismo, etilismo, distúrbios do sono, alterações na disposição e no humor e presença de dores - que acometem principalmente os pacientes com câncer. Os resultados encontrados pelos autores sugerem que a suplementação dietética com este cogumelo é capaz de melhorar a qualidade de vida de pacientes com câncer colorretal em fase pós-operatória por reduzir significativamente os efeitos deletérios ocasionados pela própria enfermidade e pelo tratamento convencional da mesma.

Com o objetivo de avaliar os efeitos da suplementação dietética com fungos *A. sylvaticus* no perfil lipídico de pacientes com câncer colorretal em fase pós-operatória, Fortes et al. (14) verificaram que a suplementação dietética com fungos *Agaricus sylvaticus* é capaz de reduzir o colesterol total, LDL-c e triglicérides, apresentando efeitos benéficos no metabolismo lipídico e, conseqüentemente, no prognóstico desses pacientes.

Pacientes com câncer de mama com metástase pulmonar foram submetidos a tratamento com o cogumelo comestível *A. sylvaticus*, sendo o tratamento realizado como complemento da tradicional quimioterapia, radioterapia e cirurgia. O sucesso evolutivo observado foi atrribuído ao aumento das células "Natural Killer" do paciente (22).

2.3.7 Elaboração de produtos alimentícios utilizando-se cogumelos

Alguns autores observaram em seus estudos efeitos benéficos na dieta de indivíduos que consumiram, em um período de quatro dias, uma média de 419,9kcal e

30,83g de gordura a menos nos pratos preparados com cogumelo quando comparados aos pratos que utilizaram carne em sua formulação. Foi verificado ainda que a aceitação dos pratos com cogumelo foi similar aos pratos com carne, mostrando o potencial de utilização deste tipo de substituição (23).

Trabalhos têm sido realizados com o objetivo de avaliar a aceitabilidade do cogumelo *A. brasiliensis* em pratos culinários como referência para o desenvolvimento de tecnologias de preparo deste cogumelo visando impulsionar o seu uso na alimentação (24).

Em outro estudo foi desenvolvido e caracterizado um produto análogo a hambúrguer a base de cogumelo *A. brasiliensis* e comparado suas características com uma formulação controle, na qual o cogumelo foi substituído por carne moída de patinho, e com produtos comerciais: um à base de carne bovina e outro à base de proteína vegetal. Considerando-se os resultados obtidos neste trabalho, o hambúrguer de cogumelo *A. brasiliensis* demonstrou ser uma alternativa mais saudável ao produto tradicional, pois além das propriedades nutricionais e gastronômicas, o cogumelo apresenta inúmeras propriedades medicinais, além do alto teor de fibras (9).

Em outro estudo foi verificado que molhos de tomate com adição do cogumelo *Agaricus brasiliensis* possuíam quantidade de polifenóis maior em relação aos molhos sem o extrato do cogumelo (13).

O extrato de cogumelo obtido em estudos apresentou-se efetivo na proteção do óleo de soja adicionado de cogumelo, podendo ser considerado um potencial antioxidante natural promissor. Os autores concluíram que é fundamental a investigação da sua ação em diferentes concentrações para que o cogumelo seja mais competitivo no mercado (25).

2.3.8 Toxicidade de cogumelos comestíveis

Infelizmente, são escassos os dados na literatura acerca da toxicidade de cogumelos. Em trabalho realizado por Orsine et al. (2012), os autores verificaram que o cogumelo *A. sylvaticus* não apresenta toxicidade, comprovando ser seguro para o consumo humano. Nesse estudo, foram realizados testes utilizando-se o extrato aquoso não fracionado do cogumelo, e a toxicidade foi avaliada observando-se qual a

concentração letal (CL50) por meio de atividade hemolítica em eritrócitos humanos (27).

Yoshkoda et al. (2010) avaliaram a toxicidade do extrato obtido a partir do micélio do cogumelo *Lentinula edodes* em ratos Wistar, com doses diárias de 2000 mg/kg, durante 28 dias. Os autores observaram que não ocorreram mortes ou mudanças de comportamento dos animais. Porém, foram reduzidos o peso corporal e o consumo de alimentos, em particular no caso de ratos do sexo masculino, embora o grau de diminuição não tenha sido tão proeminente no final da administração. Nenhum efeito toxicológico significativo foi observado nos exames de hematologia, bioquímica sérica, peso dos órgãos absolutos e relativos, autópsia e histopatologia. Consequentemente, o nível sem efeitos adversos observados para o cogumelo *L. edodes* foi considerado como mais de 2.000 mg/kg/dia nas condições do presente estudo (28).

Em 2008, Bellini et al. (2008) observaram que as frações metanólicas do cogumelo *A. blazei* testadas não ofereceram proteção química e que todas as frações apresentaram-se potencialmente mutagênicas no teste de HGPRT (hypoxanthine-guanine phosphoribosyl transferase locus). Sendo assim, os autores concluíram que mais testes são necessários para uma investigação dos efeitos biológicos dos extratos metanólico e aquoso do *A. blazei*, além de outras interações com o metabolismo das células antes de recomendar o seu largo uso pela população, o que já ocorre em diversos países. Este estudo indica que os extratos metanólicos do fungo não devem ser utilizados em função de sua genotoxicidade e que se deve ter cuidado no uso de *A. blazei* pela população antes que a caracterização bioquímica deste fungo seja completa (29).

Novaes et al. (2007) observaram que a administração do extrato aquoso do cogumelo *A. sylvaticus* em doses superiores às usadas nos protocolos terapêuticos em humanos, apresenta toxicidade muito baixa, quando realizados testes de toxicidade clínica, bioquímica e histopatológica em ratos saudáveis (30).

Costa e Nepomuceno (2003), objetivando avaliar os possíveis efeitos protetores do chá de *A. blazei* (62,5 g.L-1) contra a ação genotóxica do uretano (10 mM), não observaram aumento estatisticamente significativo nas frequências de manchas mutantes em larvas expostas ao chá de *A. blazei*, no teste SMART (Somatic Mutation And Recombination Test). Quando o cogumelo A. blazei foi associado ao uretano, foi observada uma redução estatisticamente significativa nas frequências das manchas

mutantes. Os resultados sugerem que o *A. blazei* não é genotóxico e exerce um efeito protetor contra a ação genotóxica do uretano (31).

2.4 CONCLUSÃO

Cogumelos são alimentos com excelentes características nutricionais, com alto teor de proteínas e fibras alimentares, além do baixo teor de lipídeos e fonte considerável de minerais e vitaminas. É relatada ainda a presença de diversas substâncias bioativas com propriedades farmacológicas como glucanas, proteoglucanas, lectinas, ergosterol e arginina que podem ser acrescidas aos hábitos alimentares normais e usuais da população.

São diversas as formas de inclusão dos cogumelos na dieta, porém, ainda não são aderidas por toda a população. Muitas pesquisas têm sido desenvolvidas no intuito de avaliar os efeitos dos métodos de conservação de alimentos sobre as características nutricionais dos produtos, e também no desenvolvimento de novos produtos, contendo cogumelos em sua formulação, de forma a aumentar o valor nutritivo dos alimentos ou até mesmo atender consumidores com dietas que restringem certos grupos de alimentos, como produtos de origem animal.

Neste contexto abre-se a possibilidade de utilizar alimentos industrializados que contenham cogumelos adicionados de forma a atender ao mercado consumidor com vantagens nutricionais, como o desenvolvimento de molho de tomate e de hambúrguer contendo cogumelo *A. brasiliensis* em suas formulações e do óleo de soja enriquecido com *A. blazei*. O desafio da indústria de alimentos é desenvolver tecnologias compatíveis para a preservação das propriedades nutritivas e a estabilidade de vitaminas e aminoácidos dos alimentos formulados com cogumelos durante todo o período de armazenamentos dos produtos. Devem ainda ser avaliadas a eficiência das embalagens dos alimentos contendo cogumelos em sua formulação, reduzindo ao máximo as perdas nutricionais durante a estocagem destes alimentos.

Além dos benefícios da ingestão de alimentos ricos em nutrientes como forma de suprir as necessidades do organismo, deve-se atentar para o fornecimento de produtos com características sensoriais satisfatórias, além da garantia da qualidade e segurança, que podem ser obtidas utilizando-se as Boas Praticas de Fabricação desde a obtenção das matérias-primas até a distribuição do produto final. A aplicação dos

cuidados pós-colheita evita possíveis contaminações por micro-organismos deteriorantes e patogênicos, além de reduzirem reações enzimáticas, responsáveis por alterações na cor, textura, sabor e aroma dos cogumelos.

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ARTIGO 2 –ARTIGO DE REVISÃO

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3 ARTIGO DE REVISÃO

MUSHROOMS OF THE GENUS AGARICUS AS FUNCTIONAL FOODS

HONGOS DEL GÉNERO *AGARICUS* COMO ALIMENTOS FUNCIONALES

Abstract

Mushrooms of the genus *Agaricus* are noted for their pharmacological and culinary properties. In this study, it was performed a critical literature review, focusing primarily on aspects of the chemical composition of these mushrooms whose pharmacological properties and nutritional composition characterize them as functional foods. It was also discussed articles conducted *in vitro* and *in vivo* proving the high antioxidant potential of the *Agaricaceae* family, in addition to articles which emphasize the toxicity characteristics and safety for its use in therapy or in human nutrition. These mushrooms exhibit numerous bioactive substances as well as safety regarding toxicity, which characterize them as functional foods. Despite the countless beneficial effects on human health, mushrooms of the genus *Agaricus* are little known by the population, making it necessary partnership and combined efforts among producers, industries and researchers in order to disseminate, research and consumption of these foods.

Key words: Agaricaceae. *Health. Medicinal foods.*

Resumen

Hongos del género *Agaricus* son conocidos por sus propiedades farmacológicas y culinarias. En este estudio, se realizó una revisión crítica de la literatura, centrándose principalmente en los aspectos de la composición química de estos hongos, cuyas propiedades farmacológicas y composición nutricional caracterizarlos como alimentos funcionales. También se discutió artículos realizados *in vitro* e *in vivo* demostrando el potencial antioxidante de alta de la familia *Agaricaceae*, además de los artículos que hacen hincapié en las características de toxicidad y seguridad para su uso en terapia o en la nutrición humana. Estos hongos presentan numerosas sustancias bioactivas, así como

la seguridad en relación con la toxicidad, lo que les caracterizan como alimentos funcionales. A pesar de los innumerables efectos beneficiosos sobre la salud humana, las setas del género *Agaricus* son poco conocidos por la población, por lo que es colaboración necesaria y El trabajo conjunto entre productores, industrias e investigadores con el fin de difundir, la investigación y el consumo de estos alimentos.

Palabras clave: Agaricaceae. *Salud. Alimentos funcionales.*

3.1 INTRODUCTION

Edible mushrooms belong to the Funghi group, which can grow in the wild or be cultivated, and after properly prepared, will be suitable for use as food.[1]

In accordance with Resolution RDC no 272/05 of the Anvisa (National Health Surveillance Agency), edible mushrooms are classified as products obtained from species of edible fungi, traditionally used as food, and can be prepared in different ways such as dried, whole, fragmented, ground or preserved, subject to drying, smoked, cooked, salted, fermented or any other technical process deemed safe for food production.[1]

The term functional food attributed to edible mushrooms is due to its rich nutritional value and therapeutic properties described by several researchers, but regulation is permitted only after proof of its healthy physiological effects. To be classified as functional foods they should be included in daily eating habits, providing consumers with specific physiological benefits, thanks to its components capable of causing physiological sound effects.[2]

To be considered functional food, conditions of use and nutritional value, chemical composition or molecular characterization or the product formulation must be registered. Biochemical, nutritional and/or physiological, and/or toxicological tests in experimental animals should also be submitted, further to epidemiological studies, clinical trials, and comprehensive evidence of scientific literature; accredited by international health organizations and international laws recognized under properties and characteristics of the product; proven to be of traditional use by the population having no associationwith adverse health effects.[3,4,5]

The study of functional foods is very important, since they have beneficial results for the increase in life expectancy of the population. Often times there are cases of chronic diseases such as obesity, atherosclerosis, hypertension, osteoporosis, diabetes and cancer. These ailments have been of great concern both for the population as well as public agencies related to health, and are part of their agenda to discuss solutions for better eating habits.[6]

According to Araújo,[7] health-conscious consumers are increasingly looking for foods that help control their own health and well-being. This growing search for a balanced diet in maintaining health has contributed to encourage research into new biologically active natural components and has changed our understanding of the importance of diet in good health.

Mushrooms are very rich in proteins, vitamins and minerals, and have been used worldwide as nutraceuticals in the prevention and treatment of various diseases.[8]

The objective of this study was to perform a critical review of the literature, highlighting aspects of the chemical composition of these mushrooms responsible for the pharmacological properties and nutritional composition which characterize them as functional foods. It was also discussed articles conducted *in vitro* and *in vivo* attesting the antioxidant potential of the *Agaricaceae* family, besides articles that emphasize the toxicity characteristics and safety for the use in therapy or human nutrition.

3.2 MATERIALS AND METHODS

A review of articles published in Data Bases Medline, Lilacs, PubMed, from 1990 to 2012 was done, crossing data between the descriptors in Health Sciences: mushrooms, functional foods, Agaricaceae, in Portuguese, English and Spanish.

3.3 RESULTS AND DISCUSSION

It was found 60 papers and given the reduced number of articles, all of them have been selected in this review. The mushrooms showed numerous bioactive substances and safety for toxicity, which characterize them as functional foods. Some

species of the genus *Agaricus* have shown chemical and nutritional composition suitable for human consumption, as well as a flavor much appreciated for culinary purposes.

In 2007 the Brazilian production of mushrooms of the genus *Agaricus* reached around 40 tons of dehydrated mushrooms, 95% of which destined for export to the Japanese market. In order to increase their profits, many businessmen and farmers started looking for these mushrooms as a new alternative source of income. For this reason, several companies and cooperatives have produced and marketed the inoculum (seed or *spawn)* of *A. blazei* or the colonized compost itself. But little is known about the origin and genetic variability of these products.[9]

The identification and classification of species of *Agaricus* mushrooms have been based on morphological and physiological characteristics or by genetic methods, molecular and biochemical. The genetic variability of the genus *Agaricus,* native or cultivated throughout the world is enormous. Generally these differences are in color, shape and size of microscopic structures and fruiting bodies (spores, plates, and cystides).[10]

To talk about *A. sylvaticus* is the same as to talk about *A. blazei.* When there are small differences in morphology, it does not justify creating a new species. Therefore, mushrooms *A. sylvaticus* and *A. brasiliensis* are synonyms of *A. blazei.*[10]

In a study conducted by Tominazawa et al.,[9] the authors investigated nine isolates of *A. blazei* obtained from different regions in Brazil (São Paulo, Espírito Santo, Minas Gerais, Rio Grande do Sul), through the use of molecular markers to assess genetic similarity among them. The authors concluded that six of the nine isolates showed high genetic similarity and are considered the same origin or clones.

A. sylvaticus mushroom is a Brazilian fungus found natively in the countryside in Brazil. Its popular name is "Sun Mushroom". This mushroom is ranked as Eukaryotic superkingdom, Fungi kingdom, Metazoa group, Phylum Basidiomycota, class Hymenomycetes, subclass Homobasidiomycetes, order Agaricales, family Agaricaceae.[11]

3.3.1 Chemical composition of mushrooms of the genus Agaricus

Through knowledge of the chemical composition of a product, it is possible to recognize its nutritional value and perform analysis of the proportion of homogeneous

groups of substances in 100 g of food analyzed. The homogeneous groups of substances considered are those present in all foods, such as water, lipids, protein, fiber, minerals and sugars.[12]

Determination of the chemical composition of mushrooms shows the nutritional value of the food under consideration and can be used as a source of information for nutritional tables on the labels, since several companies that commercialize mushrooms do not display the chemical composition on the Nutrition Facts label of their product.[13] The high water content in fresh commercialized mushrooms, limits its nutritional value when analyzing a portion of 15 g commonly used on labels. Information on food composition is critical to assess their quality.[13]

There are several factors which directly influence the bromatological characteristics of mushrooms. Among these, species, lineage, post-harvest processing, development stage of the basidiome, the part of the basidiome analyzed and substrate,[14] in addition to genetic factors, environmental characteristics, intrinsic attributes, season and growing conditions, substrate composition, handling, storage and transportation.[13]

According to Braga et al.,[15] other determinants for the characteristics of mushrooms, especially when measured protein content are: age, environment and area of cultivation. This fact can be observed when analyzing young mushrooms, which have higher protein content than the more mature ones. According to Shibata et al.,16 larger mushrooms are higher in carbohydrates mainly in the strain; smaller mushrooms have more protein, concentrated mainly in the pileus part.

3.3.2 Composition and health benefits

For a food to be considered functional it should have beneficial effects; reach one or more functions or actions in the human body. It should also provide well-being, quality of life, health, and reduce the risk of disease[17] as in the case of chronic degenerative diseases.[18]

Only with the development of more accurate techniques for isolation and purification of chemicals, was it possible to prove scientifically the therapeutic action of some mushrooms, isolating both antibacterial and antitumoral substances.[19]

Agaricales mushrooms and other medicinal fungi exert essential nutritional and pharmacological effects, which can be used as adjuvant in cancer therapy. The

mechanisms of action of bioactive substances present in mushrooms are not yet completely understood. But there seems to be clear scientific evidence suggesting that these substances contribute to modulate both the initiation and promotion/ progression stages of carcinogenesis, thus propitiating benefits to individuals with various cancers, mainly by immunostimulatory activity.[20]

Several studies have also revealed that *A. sylvaticus* mushroom potentially reduces tumor growth, stimulates the immune system and even contributes to a better prognosis of these patients improving their quality of life.[21]

In folk medicine the *A. brasiliensis* mushroom has been used to fight physical and emotional stress, treat and prevent illnesses such as diabetes, osteoporosis and gastric ulcer, digestive and circulatory problems in addition to reducing cholesterol.[21]

The main group of inhibitory agents of carcinogenesis is represented by antioxidant and free radicals blockers,[21] substances capable of slowing oxidation rate. In this way, they inhibit free radicals and prevent diseases, hence contributing to longevity, helping maintain the essential balance between free radicals and antioxidant defense system of the body.[23]

3.3.3 Antioxidant activity

In a study by Costa et al.[24] observation noted that the alcoholic extract of the mushroom A. sylvaticus has great antioxidant potential (74.6%), suggesting that most of the antioxidant compounds present in mushrooms can be diluted more easily by alcohol. However, aqueous and ether fractions showed reduced antioxidant potential (14.6% each) when compared to the alcoholic fraction, since they were less able to hijack the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical after 20 minutes reaction.

On the other hand the antioxidant potential of different extracts of the *A. blazei* mushroom, through the DPPH method by Silva et al.,[25] showed a higher antioxidant activity (28.6%) in methanol extracts: aqueous (1:1).

According to Tsai et al.,[26] mushrooms of the genus *Agaricus* may have their antioxidant properties associated with a high concentration of tocopherols. Percário et al.[27] researched the antioxidant capacity of different molecules of the *A. sylvaticus* mushroom, and found results of 72 mg/g for β-glucan in the liquid suspension and 14.1 mg/g in the form of compressed tablets. As for flavonoids, he found values of 0.88 mg/g

in liquid suspension and 0.63 mg/g for tablets. For total phenols he found values of 0.1 mg/g for the liquid suspension and 3.4 mg/g for tablets. The authors suggested that the antioxidant activity of *A. sylvaticus* mushroom is attributable to the number of molecules present, not to a specific component, and these molecules are easily degraded when exposed to industrial processes, which reduces its antioxidant capacity.

3.3.4 In vitro studies

In a study by Angeli et al.,[28] the authors suggested that -glucan present in *A. blazei* has no genotoxic or mutagenic effect, but protects the damaged DNA (Deoxyribonucleic acid) caused by benzopyrene in test protocols. Results indicate that the beta-glucan works through a link with benzopyrene by capturing free radicals during their activation.

In the clastogenicity test performed by Mantovani et al.,[21] the authors discovered that concentrations of 0.2% and 0.4% of *A. brasiliensis* mushroom were not damage-inducing, unlike a higher concentration of (0.6%). On the genotoxic treatments in SCGE (single cell gel electrophoresis), the concentration of 0.2% of the mushroom extract showed no genotoxic activity, as opposed to concentrations of 0.4% and 0.6% that proved to be effective DNA damage-inducing. Anticlastogenicity results indicated that, in most treatments, the aqueous extract of *A. brasiliensis* showed no protective activity against DNA damage induced by Ara-C (Arabinofuranosyl Cytidine) and Ara-C + MMS (methyl methanesulfonate.) Through SCGE, the *A. brasiliensis*, in the three concentrations tested, showed no activity anti-genotoxic. The data suggest caution in the consumption and ingestion of *A. brasiliensis* by humans, particularly at high concentrations.

3.3.5 *In vivo studies*

In a study by Fortes et al.,[29] the authors found that dietary supplementation with *A. sylvaticus* can provide metabolic benefits when analyzing biochemical, enzymatic and blood pressure of patients with colorectal cancer in post-operative phase.

Carvalho et al.,[30] aiming at verifying the antinociceptive and anti-inflammatory activity of *A. blazei* Murill in Wistar rats, through modified formalin test, found results showing that *A. blazei* acts on nociceptive response and in acute inflammation, because rats treated with this mushroom made fewer movements with paws during phase III, this most likely being related to pain caused by mediators of acute-phase inflammation.

Ishii et al.[31] demonstrated in their studies that *A. blazei* mushroom has no genotoxic activity but, rather, anti-genotoxic activity. Results derived from these data propose that *A. blazei* may act as a functional food capable of promoting immunomodulation which can account for the destruction of cells with DNA alterations correlated with the development of cancer. Therefore, supplementation with *A. blazei* mushroom can be an effective method for the prevention of cancer as well as being an important co-adjuvant treatment in chemotherapy.

In works carried out by Fortes et al.,[32] the authors suggested that dietary supplementation with *A. sylvaticus* mushroom showed to be beneficial in improving well-being and quality of life of patients with colorectal cancer in post-surgery phase.

In a study by Padilha et al.,[33] the authors studied the action of *A. blazei* extract on chronic inflammatory diseases in male albino Wistar rats. Results found indicated that *A. blazei* extract was active in experimental animals, this response is consistent, since the D-glucan compound is present in the extract.

Fortes et al.34 conducted a study to assess the effects of dietary supplementation with *A. sylvaticus* in the lipid profile of patients with colorectal cancer in postsurgery phase. The experiment revealed that dietary supplementation with *A. sylvaticus* fungi is capable of reducing total cholesterol, LDL-C (low-density lipoprotein cholesterol) and triglycerides, with beneficial outcome on lipid metabolism and, consequently, the prognosis of these patients.

Fortes et al.[35] also found that dietary supplementation with *A. sylvaticus* fungi acts in regulating fasting blood glucose levels of patients after colorectal cancer surgery. A dietary supplementation with these fungi was found to be successful in reducing blood sugar levels of patients in post-surgery phase, providing beneficial effects on the carbohydrate metabolism of these patients. However, the authors emphasize the importance of studying other clinical conditions to determine the benefits of using *A. sylvaticus.*

Hi et al.,[36] with the purpose of assessing the effects of *A. sylvaticus* extract in supplemented mice inoculated with pristane (2,6,10,14-tetrametilpentadecano), attested

the carcinogen nature of this drug and that the extract of *A. sylvaticus* mushroom has immunomodulatory activity, without producing toxic effects in test animals.

Hsu et al.[37] obtained results that indicate the potential benefits of supplementation with *A. blazei* Murill fungus to normalize liver function in patients with hepatitis B after 12 months of clinical observations.

Taveira et al.[38] conducted a study to determine the effects of *A. sylvaticus* extract on anaemia and C-reactive protein (CRP) levels in rats inoculated with Walker 256 solid tumor. Results suggest that treatment with *A. sylvaticus* mushroom has positive outcome in animals with Walker 256 tumor. Observation noted that the fungus is capable of reducing anaemia in animals, obtaining results close to those obtained for healthy pets.

Hsu et al.[39] observed in their studies that supplementation with A. Murill *blazei* improves insulin resistance in patients with type 2 diabetes. The beneficial effects assessed were due to increase in AdipoQ (adiponectin) concentration from adipose tissue with anti-inflammatory and antiteratogenic effect after ingestion of the mushroom for 12 weeks.

Bernardshaw et al.[40] observed an increase in the concentrations of cytokines MIP-2 (macrophage inflammatory protein 2) and TNF-α (tumor necrosis factor alphal) in the serum of mice supplemented with *A. blazei* extract, resulting in protection against systemic infection by *Streptococcus pneumonieae* owing to involvement of the innate immune system.

Miglinski[41] intending to evaluate the immunomodulatory effect of dry *A. blazei* Murill extract on the growth and differentiation of hematopoietic precursors of granulocytes-macrophage (CFU-GM), in bone marrow and spleen of BALB/c mice infected with *Lysteria monocytogenes,* obtained results demonstrating that *A.* Murill *blazei* has potent immunomodulatory activity able to increase survival of animals infected with a lethal dose of *L. monocylogenes,* likely due to the ability of this extract to restore marrow and spleen hematopoiesis.

In a study by Verçosa-Junior et al.[42] whose purpose was to evaluate the use of *A. blazei* in the form of filtered and full aqueous suspension (10 mg/animal) in the treatment of mice bearing Ehrlich solid tumor testing its anti-cancer activity, the authors found that animals treated daily with A. blazei showed higher values of haematological parameters (erythrogram and leukogram), and final relative spleen weight compared to the control group (distilled water), but with no significant difference ($p > 0.05$).

In works carried out by Ferreira et al.,[43] whose purpose was to evaluate the use of *A. blazei* Murrill mushroom (5%) in topical therapy of experimental poisoning of rabbits by Bothrops alternatus, aiming to antagonize the local effects (oedema, hemorrhage and necrosis) caused by this poison, the outcome showed a lower degree of swelling and bleeding halo in the treated group compared to the control group (saline). They also noticed that in the group treated with *A. blazei* Murrill (5%) there was no death.

Delmanto et al.[44] investigated the probable antimutagenic potency of *A. blazei* in rats, assayed its effect on clastogenicity induced by cyclophosphamide. Results derived from this study suggest that in some circumstances *A. blazei* exhibits antimutagenic activity that probably contributes to the anticarcinogenic effects observed.

Takaku et al.[45] observed the action of ergosterol isolated from the lipid fraction of *A. blazei* as being responsible for antitumor action against sarcoma 180 in mice. According to the authors, tumor regression activity may be related to direct inhibition of angiogenesis, resulting in death of tumor cells.

3.3.6 Eating habits and use of mushrooms

Among the characteristics necessary for food to be framed as functional food, is that these should be conventional foods consumed in normal and usual diet.[17]

In Brazil, mushrooms are not part of the diet of most people, being restricted to economic and cultural groups most favored.46 According to Shibata et al.,[16] the greatest barriers to the use of mushrooms in Brazil are linked to popular belief in their poisonous nature, expensive, eating habits and poor availability of product on the market.

The low consumption of mushrooms can also be explained by its recent cultivation in the country, still low productivity compared to its commercialization potential. With the development of new cultivation techniques, the market for these products has become an expensive culture, and their popularity depends on reducing the selling price. This could be achieved through increased production or imports, particularly from countries like China.[47]

According to Ishii et al.,[31] further researches must be carried out on the functional characteristics of the genus *Agaricus* mushrooms. Brazil should also pursue

a policy of effective use of these foods; enable their consumption by a new target public in the quest for continuous improvement of quality of life and prevention of diseases, mainly cancer.

In research performed by Lemos,[48] the author concluded that different ways of consumption most used with mushrooms are in sauces, followed by fresh or dry form in soup. Mushroom sauté, pickled, on pizzas, pastas and risottos was also mentioned. However, due to its nutraceutical characteristics, the *A. blazei* mushroom can also be consumed as tea or in capsules containing lyophilized extract.[15]

3.3.7 Studies on the addition of mushrooms in functional foods

Bassan et al.[49] developed a gluten-free cake, sponge like, with *A. brasiliensis* mushroom. The authors obtained positive results in this study because the product reached a high level of acceptance (83.22%).

Mesomo et al.[50] determined the chemical composition of *A. blazei* residue obtained after aqueous extraction of β-glucans and analyzed the shelf life of cheese bread made with this byproduct. Observation revealed that *A. blazei* Murrill residue is an excellent source of nutrients and its addition in the cheese bread formulation did not cause significant changes in the visual aspect of the product. For all attributes evaluated by the authors, the sample with the largest storage time had good sensory acceptance, which shows the product can be stored for about 30 days without major changes in taste, texture and appearance.

Escouto et al.[51] noted that there is a diversity of studies on the *A. brasiliensis* mushroom, but realized that there are no literature accounts on the use of this mushroom as food appreciated for its sensory characteristics, nor studies to assess its acceptance. Therefore, we conducted a survey of the acceptance of this mushroom taking a rice dish as reference for developing preparation techniques to boost its use in food. The global average grade obtained in the hedonic scale was 6.14 (liked slightly) and global acceptance rate was 68.3%.

Lemos[48] developed and characterized a product similar to burger based on the *A. brasiliensis* mushroom and compared their characteristics with a control formulation in which the mushroom was replaced with ground beef and commercialized products: one with bovine meat and another one with vegetable protein. The sensory analysis showed

that the mushroom-based product was well accepted by consumers when their attitude and intention to purchase were tested. The formulation that had 12% of mushroom stood out among the others, presenting high protein content (20.31%), carbohydrates (27.84%), dietary fiber (24.47%) and ash (6.12%), higher than the commercial burgers also evaluated in the work, and lipid content (1.60%) was much lower.

In another study headed up by Miller,[52] it was found that tomato sauces with *A. brasiliensis* mushroom had higher amounts of polyphenols in relation to sauces without the extract. The results obtained by the author indicated that *A. brasiliensis* contributed to increase polyphenols in tomato sauces. Glucan complex, lycopene, β-carotene present in this mushroom, meant that when added to tomato sauce they present β-glucan and increased levels of carotenoids and lycopenes.

A study was developed by Silva et al.,[25] aiming at assessing the antioxidant activity of different extracts of mushroom *A. blazei*, as well as the oxidative stability of soybean oil added with mushroom extract. Results demonstrated that mushroom extract is effective in preserving the oil, and could be considered a promising natural potential antioxidant ingredient. The authors concluded that further research on its role at different concentrations is fundamental so that mushrooms might be more competitive in the food market.

3.3.8 Toxicity of mushrooms

Despite the fact that mushrooms are considered a functional food, they may also present some type of toxicity.[10] However, for a food to be considered functional, there should be no risk or toxic effects for the consumer.[5]

The substrate exerts direct influence on the chemical composition of mushroom, because nutrients are removed by hyphae which are in direct contact with this material. Consequently, they absorb essential elements, but together with these they can accumulate toxic metals such as lead, mercury, cadmium, arsenic and others.[53] In this sense, some species of mushrooms have been used as bioindicators of environmental pollution. Knowing that chemical composition of mushrooms may be related to the substrate, it stands to reason that a polluted region will produce mushrooms with high levels of metals. This fact was observed by Kalac et al.[54] when they presented different species of mushrooms such as *A. sylvaticus*, with high levels of accumulated cadmium.

In a study performed by Moura[55] it was detected the presence of arsenic in mushrooms of the genus *Agaricus.* But this fact was not considered indicative of risk to human health, since the concentration of this element in the samples analyzed by the author was rather low.

Bellini et al.[56] observed that the methanolic fractions of *A. blazei* tested in their study did not provide chemical protection, being potentially mutagenic according to results in HGPRT test. For the authors, the methanol extracts of this mushroom should not be used widely by individuals because of the possibility of their genotoxicity. Therefore, care must be taken in the use of *A. blazei* by the population as long as a comprehensive assessment of the biochemical characterization of this fungus is not complete.

In a study conducted by Sugui,[57] the outcome indicates no mutagenic, genotoxic or carcinogenic effects on rats tested with the aqueous solution of the *A. brasiliensis.* Nevertheless, an antimutagenic effect against the mutagenicity of ENU (N-ethyl-N-nitrosourea) was observed in bone marrow cells, in addition to a significant reduction in the number of aberrant crypts per focus (4-6 crypts/focus) induced by DMH (1,2-dimethylhydrazine) in the colon of animals posttreated with the aqueous solution of the mushroom. In this context, results suggest that the aqueous solution of *A. brasiliensis* possesses compounds that can significantly reduce the frequency of micronucleated cells from bone marrow of rats, and that they can act at a later stage of carcinogenesis initiation.

In study carried out by Singi et al.[58] results revealed that the concentration of 1.25 mg/kg of *A. blazei* mushroom did not cause significant changes in mean arterial pressure (MAP) or heart rate (HR).The concentration of 2.50 mg/kg of mushroom caused decreased MAP to 15s ($p < 0.01$) and HR to 30s ($p < 0.001$) and of 5.00 mg/kg decreased MBP to 15s ($p < 0.001$) and HR at 15 and 30s ($p < 0.001$).

Costa et al.,[59] aiming at evaluating the possible protective effects of *A. blazei* tea against the urethane genotoxic action in somatic cells of *Drosophila melanogaster*, noted that no increase was statistically significant in the frequency of mutant spots in larvae exposed to *A. blazei* tea. However, when this mushroom was associated with urethane, we observed a reduction statistically significant in the frequency of mutant spots. The results imply that A. blazei is not genotoxic and has a protective effect against the genotoxicity of urethane.

With the intent of investigating effects of acute toxicity of *A. sylvaticus* aqueous extract by clinical, biochemical and histopathological on healthy mice, Novaes et al.[11] verified that both the administration of the aqueous extract as well as the placebo, caused a temporary rise of apathy, piloerection and respiratory changes, which were slightly more persistent in the group treated with the fungus. Biochemical and histopathological changes were not statistically significant between groups. The authors determined that administration of *A. sylvaticus* aqueous extract showed very low toxicity.

In a study by Ishii et al.,[31] the researchers concluded that the *Agaricus blazei* mushroom offers no genotoxic consequences, but made it possible to visualize the antigenotoxic effects. The results suggested that the fungus acted as functional food, capable of promoting immunomodulation when the destruction of cells with DNA damage correlated with cancer development was observed. Therefore, the Sun mushroom had a preventive effect against colorectal neoplastic lesions assessed.

Orsine et al.[60] observed that *A. sylvaticus* extract has no toxicity proving to be safe for human use.

3.4 CONCLUSIONS

To be included in the group of functional foods, mushrooms should bring benefits to human health, do not present themselves toxic and be included in the daily eating habits. Thus, the beneficts of eating mushrooms of the genus *Agaricus* are shown in several papers. Currently there are many researchers working in order to spread the advantages of the consumption of mushrooms of the genus *Agaricus.*

It has been shown in some studies the rich nutritional composition of mushrooms of the genus *Agaricus,* and the presence of substances that act on the human body, being widely used in therapy against cancer. Also low toxicity was observed in different studies using different toxicological methods evaluation.

Despite the countless beneficial effects on human health, mushrooms of the genus *Agaricus* are little known by the population, making it necessary partnership and combined efforts among producers, industries and researchers in order to disseminate, research and consumption of these foods.

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ARTIGO 3 – ARTIGO ORIGINAL

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4 ARTIGO ORIGINAL

NUTRITIONAL VALUE OF *AGARICUS SYLVATICUS;* MUSHROOM GROWN IN BRAZIL

EL VALOR NUTRITIVO DE *AGARICUS SYLVATICUS;* SETAS CULTIVADAS EN BRASIL

Abstract

The bromatological characterization of the *Agaricus sylvaticus* species *(A. sylvaticus),* known as the Sun Mushroom and cultivated in Brazil, is necessary to determine substances with pharmacological and nutritional potential, in view its safe use in food and in human medicine. The purpose of the present study was to determine the chemical composition of the *A. sylvaticus* mushroom grown in Brazil. Mushrooms were obtained in dehydrated form from a producer in Minas Gerais State. Through this study it was able to observe the fungus' rich chemical composition, highlighting the variety and quantity of minerals as well as its high protein content. There are many components of this mushroom that have medicinal properties, which are recognized as excellent antioxidants. Results also proved that the composition of *A.sylvaticus* presented differences when compared to the chemical composition of other *Agaricaceae* fungi.

Key words: Therapeutic fungi. Chemical composition. Protein. Mushroom. Cancer.

Resumen

En la caracterización bromatológica del género *Agaricus sylvaticus (A. sylvaticus),* conocido como la seta del sol y cultivado en Brasil, es necesario determinar las sustâncias con potencial farmacológico y nutritivo con el objetivo de un uso seguro en la alimentación y la medicina humana. El objetivo de este estudio fue determinar la composición química de la seta *A. sylvaticus* cultivada en Brasil. Se obtuvieron las setas en su forma deshidratada de un cultivador del estado de Minas Gerais. A través de este estudio pudimos observar la rica composición química del hongo, destacando la

variedad y cantidad de minerales así como su alto contenido en proteínas. Esta seta contiene muchos componentes con propiedades medicinales, que se sabe que son excelentes antioxidantes. Los resultados también muestran que la composición de *A. sylvaticus* mostraba diferencias al compararla con la composición química de otros hongos de la familia *Agaricaceae.*

Palabras clave: Hongos terapéuticos. Composición química. Proteínas. Setas. Cáncer.

4.1 INTRODUCTION

Due to their high nutritional value, mushrooms have been widely consumed by people seeking a healthier and more nutritional diet. Some mushrooms are considered nutraceuticals, that is, functional foods, being that in addition to their high protein content, low concentration of total fats, added to a significant concentration of vitamins and minerals, they contain antioxidants that are extremely important in the cure, treatment, and prevention of various diseases, including cancer.[1]

In Brazil, the consumption of mushrooms by the population is still considered low, but mushrooms of the *Agaricus* genus are becoming very popular owing to their attributed medicinal properties, often associated to the presence of bioactive compounds with medicinal value, such as phenolic compounds, polyketides, terpenes and steroids, which are recognized as excellent antioxidants.[2]

Several investigations related to dietary supplementation with *A. sylvaticus* mushroom have shown positive results in patients with colorectal cancer in postoperative phase reducing the deleterious effects caused by the disease itself and by conventional treatment,[3] also in the improvement of gastrointestinal changes of these patients.[4,5]

According to Furlani & Godoy,[6] the concentration of macro and micronutrients in food is directly related to the benefits they play in humans and animals. The aim of this study was to evaluate the chemical composition of the *A. sylvaticus* fungus (Sun Mushroom) with respect to protein, lipids, carbohydrates, dietary fiber, minerals, fat soluble vitamins and Vitamin C.

4.2 MATERIALS AND METHODS

4.2.1 Obtainment of sample of A. sylvaticus mushroom (Sun Mushroom)

A sample of dehydrated *A. sylvaticus* mushroom (Sun mushroom), was obtained from a producer in Minas Gerais State. To allow greater extraction of its components, the mushroom was mashed up in a Willey type (Model ET-648, Tecnal Brand mill). The physical and chemical analysis were performed at the Physical Chemistry Laboratory of the Food Research Center, School of Veterinary Medicine (accredited by MAPA - Ministerio da Agricultura, Pecuaria e Abastecimento) and the Laboratory of Food Biochemistry, Pharmacy School, both from Universidade Federal de Goias - UFG, from March to June 2010.

4.2.2 Chemical characterization

The whole analysis, in duplicate, has followed the official methods established by MAPA, by the Association of Official Analytical Chemists (AOAC).[7-10] Moisture analysis were performed using a kiln at 105^{o} C for 24 hours and total ash by means of sample calcination in a muffle furnace at 550 ^{o}C for 12 hours. The Kjedahl method was utilized for protein determination, using a 6.25 correction factor. Sample fat content was detected by continuous "Soxhlet" device type extraction. Determination of total dietary fiber was based on sequential enzymatic digestion of the dried mushroom sample with alphaamylase thermo-stable; protease and amyloglucosidase. The determination of carbohydrates was calculated by the difference, using rates obtained by moisture analysis, fixed mineral residue, proteins and lipids.

4.2.3 Evaluation of minerals

The determination of minerals was performed by means of atomic absorption spectrometry (spectrometer GBC Brand, Model 932AA), in duplicate. The search for iron, zinc, manganese, sodium, potassium, cobalt, copper, calcium and magnesium

made was possible, as the laboratory where these tests were performed only contained specific cathode lamps for each of these minerals.

4.2.4 Evaluation of fat-soluble vitamins

Fat-soluble vitamins were determined by high performance liquid chromatography (HPLC), in duplicate. This analysis was used to determine the oil extracted lipids, stored at 10 °C for conservation. Gilson brand liquid chromatography was used with a stationary phase column E-18, column 10 cm/4.6 mm and 5 micras particles. Methanol was used for the mobile phase, utilizing an isocratic working system with 100% methanol and 1 mL/min flow. Variable wavelength was used for each vitamin studied.

4.2.5 Evaluation of Vitamin C

The determination of Vitamin C was performed in triplicate, following the Tillmans Method with titration of standard solution of ascorbic acid and oxalic acid solution with DCFI solution (2, 6-dichlorophenol indophenol sodium), and the solutions used were prepared as described by Instituto Adolfo Lutz1[1] for Tillmans Method. To determine Vitamin C it was obtained an aqueous, non fractioned extract of *A. sylvaticus* mushroom from diluted dehydrated mushrooms ground in water, kept under agitation at room temperature for one hour.

4.3 RESULTS AND DISCUSSION

4.3.1 Chemical composition of Agaricus sylvaticus

The nutritional value of food is commonly expressed according to the chemical composition or percentage of homogeneous groups of substances in one hundred grams of food, which are: moisture, lipids, proteins, carbohydrates, fiber and ash[11] (Table I)

shows the results found by analyzing the chemical composition of dehydrated *A. sylvaticus* mushroom.

Table I. Bromatological composition (% per 100g) of dehydrated *A. sylvaticus* mushroom cultivated in Brazil in 2010.

Analysis	**Humidity**	**Ash**	**Protein**	**Lipids**	**Carbohydrates**	**Fibers**
A. sylvaticus	6.31	7.38	41.16	6.60	36.21	2.34

* Results are shown in % in 100g sample.
* The chemical analysis of this study was performed in duplicate.
* The methodology of the chemical analysis used with dehydrated *A. sylvaticus* mushroom is described by AOAC: Moisture (kiln 105ºC), ash (muffle furnace at 550°C), proteins (Kjedahl), lipids (Soxhlet), Carbohydrate (difference from the other constituents of 100%), and dietary fiber (by enzymatic digestion of the sample).

As they have high nutritional value, mushrooms have been identified as alternatives for a healthier diet rich in proteins. They are highly recommended in countries with high rates of malnutrition,[13] or for people who need a high protein diet with low lipid content.[14] Observation noted that the *A. sylvaticus* mushroom grown in Brazil contains high protein content (41.16%). However, although some authors compare the nutritional value of mushrooms to that of beef (approximately 14.8%),[15] it should be taken into account the biological utilization of protein, since the *Agaricus brasiliensis* mushroom presented, in some studies,[16] low concentrations of essential amino acids necessary for animal growth in experiments, as well as other native cultivated mushrooms in the far east.[17]

In 2005 a survey was conducted on the chemical composition of *A. sylvaticus* grown in Brazil by the Japan Food Research Laboratories.[18] For the dehydrated mushroom, were found values of 4.4 g/100 g of moisture, 39.4 g/100 g of protein, 3.0 g/100 g of lipid, 45.6 g/100 g of carbohydrate and 7.6 g/100 g of minerals. The *A. sylvaticus* mushroom grown in Brazil in 2010 showed higher values of moisture content (6.31%), lipids (6.60%) and protein (41.16%), which can be explained taking into account the differences in growing region, climate, genetic mutations,[18] conditions which are probably better in the areas cultivated today.

According to Minhoni et al.,[20] the qualitative characteristics of mushrooms are also influenced by species, strain, post-harvest processing, the basidiomata development stage, part of basidiomata and substrate. Braga et al.,[21] highlight age, environment and locality, as factors influencing the variations in protein content of mushrooms. According to these authors, young mushrooms are richer in protein than the more

mature and open ones. In works performed by Shibata & Demiate,[22] the authors observed that smaller mushrooms have higher protein content, mainly at the pileus.

In addition to high-protein content, the *A. sylvaticus* mushroom contains high biological value, since it presents all the essential amino acids,[23] as shown by research conducted by the Japan Food Research Laboratories[18] on the *A. sylvaticus* grown in Brazil. Such research detected 1.71 g/100 g levels of arginine, 1.55 g/100g levels of lysine, 0.62 g/100 g levels of histidine, 1.11 g/100 g levels of phenylalanine, 0.83 g/100 g levels of tyrosine, 1.72 g/100 g levels of leucine, 1.01 g/100 g levels of isoleucine, 0.39 g/100 g levels of methionine, 1.28 g/100 g levels of valine, 1.75 g/100 g levels of alanine, 1.25 g/100 g levels of glycine, 1.26 g/100 g levels of proline, 5.73 g/100 g levels of glutamic acid, 1.20 g/100 g levels of serine, 1.2 g/100 g levels of threonine, 2.35 g/100 g levels of aspartic acid, 0.43 g/100 g levels of tryptophan and 0.36 g/100 g levels of cystine. According to Henriques et al.,[16] it is important to check the standards set by FAO/WHO (Food and Agriculture Organization/World Health Organization) for essential amino acid contents such as lysine and leucine, so that the mushroom protein will not be considered as low-quality protein and digestibility. In such case, this mushroom should not be indicated as the only source of protein to ensure satisfactory growth levels.

The we alth of nutrients from the *A. sylvaticus* mushroom is of great importance in terms of public health, since the Brazilian population has a high number of obese people.[14] According to results related to amounts of protein and lipids in the present study, *A. sylvaticus* mushroom can be presented as an important alternative for healthy food, assisting those who seek better quality of life. The *A. sylvaticus* mushroom could be used as food in a mixed diet with other protein sources, or be added to other foods in the hope of enriching the product, as suggested by Monteiro,[24] in adding the *A. brasiliensis* mushroom to tomato sauce.

With respect to the lipid content in this study, 6.60% of this nutrient was detected in the *A. sylvaticus* mushroom. According to Borchers et al.,[25] although mushrooms contain small quantities of total fat, they have a high percentage of polyunsaturated fatty acids (PUFA) and low content of saturated fatty acids and cholesterol.

According to Novaes & Novaes,[16] crude fat of mushrooms consists of several classes of lipids, including free fatty acids, mono-di and triglycerides, sterols, terpenoids and phospholipids, especially lecithin.

The amount of carbohydrates found in the *A. sylvaticus* mushroom was 36.21%. According to Shibata & Demiate,[22] carbohydrate content increases when the strain of mushrooms has increased size, and upon analyzing the carbohydrate content of the pileus, a lower concentration of this nutrient is presented when compared to the strain.
In a study by Copercom,26 the chemical composition of other mushrooms of the *Agaricus* genus, *A. brasiliensis* in dried state showed the following results: water (7.5%), protein (36.6%), lipids (3.4%), fiber (6.8%), ash (7.3%), and carbohydrates (38.3%). Comparing these results with those of the present work, we see that only the ash content of the fungi studied was similar.

On aiming to analyze the chemical composition of two strains of *Agaricus Blazei Murrill*, Shibata & Demiate,[22] protein values of 34.80% to 39.80%, fiber values of 7.35% to 9.65%, ash values of 6.99 % to 7.89%, lipid values of 0.80% to 3.68% and carbohydrate values of 46.22% to 41.41% were found, which also differ from those results presented in this paper.

A study on *A. sylvaticus* mushroom detected an amount of 2.34% of dietary fiber. According to Novaes & Novaes,[16] the dietary fiber contained in mushrooms has adverse physical action on the absorption of toxic, harmful and carcinogenic substances. Numerous studies show that the fibers are associated to a lower incidence of colorectal cancer, since it accelerates faecal excretion by laxative action, reducing time spent in the intestines.

By studying the chemical composition of edible mushrooms, Andrade et al.[27] observed that crude fiber content varies depending on the part of the mushroom like the stalk, pileus or the whole basidiomata.

4.3.2 Characterization of minerals present in the Agaricus sylvaticus mushroom

Table II presents the mineral composition of nine minerals researched in *A. sylvaticus* fungus according to the conditions and limitations of the laboratory used in this study.

Among micronutrients, substances required by the body in small quantities for normal operation are zinc, copper, selenium, manganese, chromium, molybdenum and iron.[28] Significant amounts of iron were found (726.90 mg/100 g) in the *A. sylvaticus*,

which makes the mushroom a rich source of this mineral. According to Crichton et al.,[29] iron works in oxygen transport, DNA synthesis, redox reactions in the electron transport chain, and is part of the molecular chain of several proteins and enzymes.

Table II. Determination of minerals in *A. sylvaticus.*

Minerals	***A. sylvaticus* (mg/100g)**	**Recommended Daily Intake (RDI) for adults (ANVISA, 1998)**
Iron	726.90	14mg
Calcium	1.35	800mg
Zinc	549.25	15mg
Cobalt	7.75	-
Magnesium	21.19	300mg
Sodium	255.34	-
Potassium	613.03	-
Manganese	23.18	5mg
Copper	276.66	3mg

* Analyses of minerals were performed by atomic absorption spectrometry.

Results also showed 1.35 g/100 g of calcium in the *A. sylvaticus.* Calcium is very important for bone mineralization, maintaining the structure and rigidity of the skeleton.[30]

A. sylvaticus mushroom has also presented an important source of zinc (549.25 g/100 g). Zinc has an important physiological role, acting as an antioxidant, preventing lipid peroxidation.[31] Zinc, found in significant concentrations in *A. sylvaticus* grown in Brazil in 2010, has been the object of studies in various researches related to the performance of this mineral in the human body. Studies have shown that children supplemented with zinc have lower incidence of diarrhea, pneumonia and malaria, when compared with children not receiving zinc.[32-33]

Magnesium acts as a cofactor of both enzymes responsible for various metabolic activities and in innate and acquired immune response, in addition to the important role of tissues maintenance and lymphoid cells.[34] It was found, 21.19 g/100 g of this mineral in the *A.sylvaticus*.

In this study, it was found high values for sodium content in *A. sylvaticus* mushroom. According to Amazonas Mala,[23] these mushrooms have significant amounts of sodium.

Copper is an essential trace element involved in multiple enzyme systems including the immune response[35] and high concentration is present in the *A. sylvaticus* mushroom (276.66 g/100 g).

In the 2005 research, the Japan Food Research Laboratories,[18] also conducted an analysis of sodium (4.2 mg/100 g), iron (21.2 mg/100 g), calcium (35.7 mg/100 g), potassium (3.15 mg/100 g) magnesium (100 mg/100 g), copper (8.24 mg/100 g), zinc (6.61 mg/100 g), manganese (0.65 mg/100 g), selenium (36 g/100 g), cobalt (0.13 ppm). Neither molybdenum nor boron was detected. Comparing these results with those of the present study, one may observe the difference between results for most minerals, which come in higher concentrations in this work. According to Urben,[19] this variation in minerals can be explained by the type of crop, climate, region, genetic mutations among others, which are possibly more favorable regarding the techniques used to cultivate *A. sylvaticus* mushroom today.

Borchers et al.[25] also observed the presence of potassium, calcium, phosphorus, magnesium, iron and zinc. In a study by Copercom,[26] the mineral composition of the dehydrated *A. brasiliensis* mushroom showed the following results for phosphorus, iron and calcium: 939 mg/100 g, 18.2 mg/100 g and 41.6 mg/100 g, respectively.

Oliveira et al.,14 upon studying the *A. blazei* fungus, found high levels of minerals such as potassium (2.34%), phosphorus (0.87%), calcium (0.07%), magnesium (0.08%), sulfur (0.29%), copper (61.88 mcg), zinc (86.90 mcg), iron (79.63 mcg).

4.3.3 Characterization of vitamins present in the Agaricus sylvaticus mushroom

Table III shows the vitamins composition in *A. sylvaticus* fungus according to the conditions and limitations of the laboratories used in this study to develop the analysis.

As seen in Table III, Vitamin C was detected in samples of *A. sylvaticus* analyzed in this study, which disagrees with results presented by the Japan Food Research Laboratories[18] in 2005.

Vitamin C acts on cicatrizing wounds, collagen synthesis, skin lightener.[36] Photoprotection increases and improves the antioxidant defenses.[37] The recommended

daily dose for maintaining Vitamin C saturation level in the body is approximately 100 mg. Higher doses are necessary in cases of infections, pregnancy and breastfeeding.[38] According to Lederer,[39] the importance of Vitamin C is associated to several types of cancer, since daily doses administered to cancer patients provided improved survival. Vitamin A deficiency causes night blindness, rough and peeling skin, dry mucous membranes, growth inhibition, reduced resistance to infections, defects in bone development and modulation.[40] In the *A. sylvaticus* fungus Vitamin A was found only in the form of retinol (0.001 mg/100 g).

Table 3. Determination of fat-soluble vitamins and Vitamin C in the *Agaricus sylvaticus* mushroom cultivated in Brazil.

Vitamins	***A. sylvaticus***	**Recommended Dietary Allowances (RDA) for adults (ANVISA, 1998)**
Ascorbic acid (Vitamin C)	12.65mg/100g	60mg
A complex	- Retinol: 0.001mg/100g (Retinol acetate, retinol palmitate and retinol propionate were not detected).	800μg
Vitamin D2	0.018mg/100g	5mg
E complex	- Alpha tocopherol: 0.020 mg/100g (Tocopherol acetate, Beta tocopherol, Delta tocopherol and Gamma tocopherol were not detected)	10mg
K Complex	- Menaquinone (K2): 0.001mg/100g (Phylloquinone (K1), Menadione (K3) and Naftoquinona were not detected (K4)).	80μg

* The determination of fat-soluble vitamins was performed in duplicate, using liquid chromatography from oil obtained in the lipid analysis of *A. sylvaticus* mushroom.

Vitamin K acts as a cofactor for carboxylation of specific glutamic acid residues to form gamma carboxyglutamic acid (Gla), amino acid found in coagulation factors, which appears related to calcium and may regulate the disposal of the mineral matrix bone as part of osteocalcin.[41] In the *A. sylvaticus* mushroom, we detected the presence of Vitamin K2, menaquinone, at 0.001 mg/100 g concentration.

Vitamin E helps protect the long-chain polyunsaturated fatty acid of cell membranes and lipoproteins against oxidation in the body.[42] Among fat-soluble vitamins, alpha tocopherol appeared in higher concentration (0.020 mg/100 g) in the *A. sylvaticus* mushroom. Vitamin D regulates the metabolism of calcium and phosphorus, maintaining serum calcium and phosphorus able to provide normal conditions for most metabolic functions, including bone mineralization.[43] It was detected 0.018 mg/100 g of Vitamin D2 in the *A. sylvaticus.*

Among the *A. sylvaticus* vitamins exhibited in the survey by the Japan Food Research Laboratories[18] in 2005, the following substances were not detected in the sample: α-carotene, β-carotene and Vitamin C. However, there were findings of 1.21 mg/100 g of thiamine (Vitamin B1), 3.41 mg/100 g of riboflavin (Vitamin B2), 0.83 mg/100 g of Vitamin B6, 0.17 μg of Vitamin B12, 5.8 μg of calciferol (Vitamin D), 0.36 mg/100 g of folic acid, 39.4 mg/100 g of pantothenic acid, 201 mg/100 g of inositol and 39.9 mg/100 g of niacin.

According to Soares,[44] the accumulation of compounds such as vitamins is dependent on the handling, processing and maturity of mushroom at harvest. Tocopherol acetate and retinol acetate, obtained only synthetically, were not detected in this sample of dehydrated *A. sylvaticus*, as shown in Table II.

According to Borchers et al.,[25] mushrooms contain significant amounts of niacin, thiamin, riboflavin, biotin, ascorbic acid and pro-vitamins A and D. According to Eira & Braga,[45] knowledge of the chemical composition of mushrooms is very important, and in Brazil the genetic and physiological studies, basic and applied, can be extended aiming to select more stable and productive lineages in addition to establishing more appropriate physiological conditions for the production of mushrooms in order to attain a desired standard of quality .

Clinical and experimental studies demonstrate that dietary supplementation with Agaricales mushrooms and other medicinal fungi exert positive nutritional, medicinal and pharmacological effects and can be used as an adjuvant in cancer therapy. The mechanisms of action of bioactive compounds present in mushrooms are yet to be fully elucidated in the literature, but scientific evidence suggests that these substances are able to modulate carcinogenesis not only at early stages, but also at more advanced ones, providing benefits to individuals with various types of cancer, mainly by stimulating the immune system.[46] It was observed that dietary supplementation with this medicinal fungus can significantly reduce fasting glycemia levels of colorectal cancer

patients in post-surgery phase[47] and is capable of improving the life quality of these patients.[48]

4.4 CONCLUSIONS

Through this study it was able to observe the fungus' rich chemical composition, highlighting the variety and quantity of minerals as well as its high protein content. There are many components of this mushroom that have medicinal properties, which are recognized as excellent antioxidants.

Results also proved that the composition of *A. sylvaticus* presented differences when compared to the chemical composition of other *Agaricaceae* fungi.

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ARTIGO 4 – ARTIGO ORIGINAL

Versão aprovada para publicação em inglês:

Determination of chemical antioxidants and phenolic compounds in the Brazilian mushroom *Agaricus sylvaticus.* Orsine JVC, Novaes MRCG, Asquieri ER, Cañete R. Aprovado para publicação na revista West Indian Medical Journal 2013.

5 ARTIGO ORIGINAL

DETERMINATION OF CHEMICAL ANTIOXIDANTS AND PHENOLIC COMPOUNDS IN THE BRAZILIAN MUSHROOM *Agaricus sylvaticus*

Abstract

Agaricus sylvaticus mushroom has been widely studied because of its high nutritional value and medicine properties. The objective of this study was to evaluate the antioxidant potential of both, alcoholic and aqueous extracts of *Agaricus sylvaticus*, and quantify their total polyphenol content. The antioxidant activity was performed by the 2, 2-difenilpicril-hydrazyl radical scavenging capacity and total polyphenol content was assessed by colorimetric method. Observation also noted the great antioxidant potential of aqueous, alcoholic and ethereal extracts (14.6%, 75.6% and 14.6%, respectively) of the *Agaricus sylvaticus* mushroom, highlighting the alcoholic extract, which demonstrates the extraordinary benefits of this mushroom in the diet, since antioxidants prevent against premature aging and various types of cancer.

Keywords: *Agaricus sylvaticus*, antioxidants, medicinal fungus, phenolic compounds, sun mushroom.

5.1 INTRODUCTION

Appropriate nutrition is essential to maintaining health, contributing to risk reduction of disease but also for the restoration of homeostasis in cases of illness. Through nutrition it is possible to promote recovery, rehabilitation, detoxification and repair of cells, providing greater vitality to organs and tissues [(1)].

For more than two thousand years natural products have been used empirically in the treatment of various diseases such as cancer. Mushrooms are fungi known from ancient times when man used them as a food of high nutritional and therapeutic value [(2)]. Mushrooms have high genetic diversity that represents a source of protein essential to human health [(1)].

Despite the great biodiversity of fungi existing in Brazil and the great potential still to be explored, there are few data related to its antioxidant activity [3]. That activity is very important because antioxidants have the ability to sequester free radicals harmful to human health [4].

Antioxidants are able to slow down oxidation rate, inhibiting free radicals and preventing the onset of diseases, thus contributing to greater longevity, making essential the balance between free radicals and the antioxidant defence system [5]. Among the various classes of naturally antioxidants, phenolic compounds have received much attention in recent years, especially by inhibiting *in vitro* lipid peroxidation and lipooxygenase [6].

As the human antioxidant defense system is not complete without dietary antioxidants [7], the main way of getting antioxidants in the body is the ingestion of compounds with this activity through the diet. The main dietary antioxidants are some vitamins, carotenoids and phenolic compounds [8].

The *Agaricus sylvaticus* mushroom (Sun Mushroom) has nutritional, anti-mutagenic, antitumor, antiviral, antithrombotic, hypocholesterolemic, hypolipidemic properties and antioxidant activities that are related to the presence of esters, oleic and linoleic acid, proteins, enzymes, vitamins and polysaccharides [9,10].

Study of the characteristics and effects of the medicinal *A. sylvaticus* mushroom is relevant in the context of public health, given that the population has used it as a nutritional supplement, either in dry form, capsules or as tea [11]. Its suggested that dietary supplementation with *Agaricus sylvaticus* fungus is able to promote beneficial effects on energy metabolism, blood pressure, biochemical parameters and enzyme activities [12] and improve the life quality of patients with colorectal cancer in the postsurgical phase [13].

Based on the numerous benefits provided by this mushroom, the objective of this study was to evaluate the antioxidant potential and the amount of total polyphenols in ethereal, alcoholic and aqueous extracts obtained from it.

5.2 METHODS

5.2.1 Obtaining the sample

A sample of the dehydrated *A. sylvaticus* mushroom was obtained from a producer in Minas Gerais state. The sample remained stored at room temperature until the time of analysis. First, the mushroom was processed in a Willey mill type, Model TE-648, Brand Tecnal in order to obtain higher extraction of its components. All the analyses were performed at the Laboratory of Food Biochemistry, Pharmacy School, Universidade Federal de Goiás (UFG).

5.2.2 Evaluation of antioxidant potential

The antioxidant potential of *A. sylvaticus* mushroom was determined following the methodology used by Borguini [(14)]. The entire experiment was conducted using aluminum foil to reduce any possibility of interference of light in the sample. It was obtained the ether, alcoholic and aqueous extracts of the mushroom. First it was obtained the ether extract from the initial dilution of 2.5g mushroom ground in 50mL of ethyl ether. From non-filtered residue and therefore not ether-soluble, it was obtained the alcoholic extract with the addition of ethanol at a ratio of 1:20 (residue weight: volume of alcohol). And finally, it was obtained the aqueous extract from addition of water to the non-filtered residue from the previous step, also adding distilled water at a ratio of 1:20 (residue weight: water volume).

In the experiment it was used BHT (Butylated hydroxytoluene) as standard antioxidant and DPPH (2, 2-difenilpicril-hydrazyl) as oxidant. The antioxidant activity of mushroom extracts was determined by DPPH described by Brand-Williams et al [(15)]. The DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule and, in this way, is reduced in the presence of an antioxidant.

To evaluate the antioxidant activity, the extracts were reacted with the stable DPPH radical in ethanol solution. According to Neves et al., [(16)] in radical form, DPPH has a characteristic absorption at 517nm, which disappears after reduction by hydrogen pulled from an antioxidant compound.

The reduction of DPPH radical was measured by reading absorbance at 517nm in a spectrophotometer Model SP-220, Brand Biospectro at intervals of 0, 1, 2, 3, 4, 5, 10, 15 and 20 minutes of reaction. The values observed in the spectrophotometer were

converted to a percentage scale, which 0% indicates no inhibition by the production of free radicals, and 100% indicates complete inhibition of them.

The antioxidant activity was expressed according to Equation 1, Mensor et al., [17] described below.

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\} \quad (1)$$

5.2.3 Quantification of total polyphenols

The concentration of total polyphenols was determined by the colorimetric method, [15] using the Folin Ciocalteau, which is based on the reduction of acids and fosfomolibdic fosfotungstic in alkaline solution. The blue color produced by reduction of the Folin Ciocalteau phenol is measured spectrophotometrically at a wavelength of 765nm.

For quantification of total polyphenols of sample it was used a standard curve of gallic acid solution at concentrations of 0.01 mg/mL to 0.06 mg/mL. It was calculated a correlation coefficient (R^2), resulting $R^2 = 0.99775$ to the level significance of 5%. The result was expressed as milligrams of gallic acid equivalents per gram of extract. (mg/g).

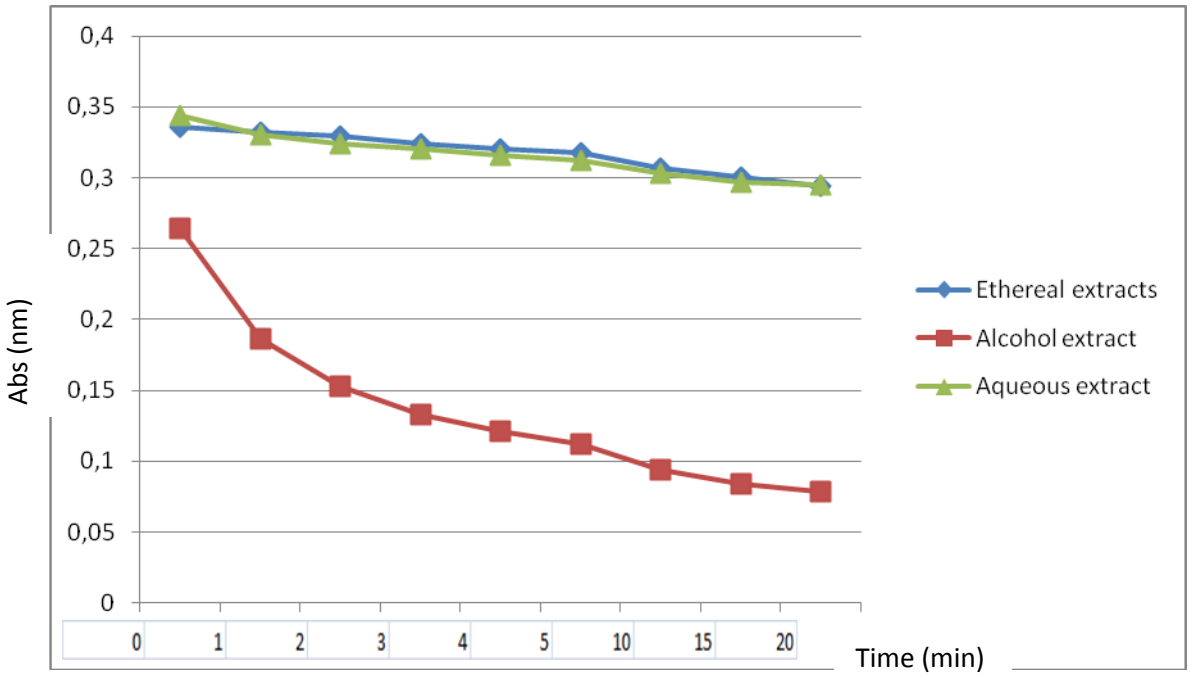
The analysis of total polyphenols was performed in triplicate, from the use of ether extracts, alcoholic and aqueous sample, the same concentration used for the standard solution of gallic acid previously reported. The readings were taken in a spectrophotometer Model SP-220, Brand Biospectro to 750 nm.

5.3 RESULTS AND DISCUSSION

5.3.1 Potential antioxidant and total amount of polyphenols

The aqueous, ethereal and ethanolic extracts of *A. sylvaticus* mushroom showed the DPPH inhibition percentage of 14.6%, 75.6% and 14.6%, respectively. The value obtained for the synthetic antioxidant (BHT), used in this study for comparison, was 80.06%.

The antioxidant effect of aqueous, ethanol and ether of the mushroom *A. sylvaticus* was shown in Figure 1 by the decrease of absorbance observed at 0, 1, 2, 3, 4, 5, 10, 15 and 20 minutes.



* The antioxidant potential of the *A. sylvaticus* mushroom was observed from spectrophotometric analysis of three extracts from the sample, being that we used as standard the DPPH as oxidant.

Figure 1. Antioxidant potential of ether, alcoholic and aqueous extracts of the *A. sylvaticus* mushroom.

The mean percentage of total polyphenol extracts, ethereal and ethanolic mushroom *Agaricus sylvaticus* were shown in Table 1.

Table 1. Amount of polyphenol extracts of ether, alcoholic and aqueous extracts of *A. Sylvaticus* mushroom.

Polyphenols (%)	**Ethereal extract**	**Ethanolic extract**	**Aqueous extract**
	4.11±1.40	9.42±2.45	0.98±0.31

* The Folin-Ciocalteou reagent was used in a spectrophotometer at 750nm.

* We calculated the mean and standard deviation of the results obtained for each extract analyzed.

Regarding the antioxidant activity, results showed that the alcoholic extract of the *A. sylvaticus* mushroom has great antioxidant potential (74.6%), suggesting that most antioxidant compounds present in this mushroom can be more easily diluted in alcohol. As for the aqueous and ether fractions, they showed reduced antioxidant potential (14.6% each), when compared to the alcoholic fraction, since it had less ability in kidnapping the DPPH radical after 20 minutes of reaction.

Lately the interest in the study of phenolic compounds has increased greatly, mainly due to the ability of these antioxidant substances in kidnapping free radicals, which are harmful to human health [(4)].

Comparing the results of this study to the results reported by Percário et al. [(19)] for the mushroom in liquid suspension (50%), the aqueous fraction of this study obtained reduced antioxidant potential (14.6%), which can be explained by the fact that the antioxidants components had already been extracted by ether and by alcohol before the analysis of the antioxidants in aqueous extract.

The biological properties of phenolic compounds are related to the antioxidant activity each phenol exerts on a given medium. The activity of antioxidants, in turn, depends on their chemical structure and it can be determined by the action of the molecule as a reducing agent, represented by the rate of inactivation of free radical reactivity with other antioxidants and metal chelation potential [(20)].

Epidemiological studies revealed correlation between the increased consumption of phenolic compounds with antioxidant activity [(21)] and reduced risk of cardiovascular disease as well as certain types of cancer [(20)].

Phenolic compounds appear to be the main components responsible for antioxidant activity of extracts from mushrooms [(22)]. According to Tsai et al. [(23)] the genus Agaricus mushrooms may have antioxidant properties associated with its high concentration of tocopherols.

Polyphenols make a heterogeneous group, composed of several classes of substances with antioxidant capacity, among which phenolic acids and flavonoids stands out. The antioxidant activity of polyphenols is mainly due to its reducing properties, whose intensity of antioxidant activity exhibited by these phytochemicals is notably different since it fundamentally depends on the number and position of hydroxyl groups present in the molecule [(24)].

In this study it was determined the amount of total polyphenol for the etheric, alcoholic and aqueous extracts. It was noticed that the alcoholic extract concentrates the

biggest amount of polyphenols (9.43 mg/100g) followed by etheric extract (4.11 mg/100g), and aqueous extract (0.98 mg/100g). The use of ethanol made possible the extraction of a higher content of polyphenols as the alcoholic extract of the sample *A. sylvaticus* mushroom exhibited higher total phenolic content if compared to the aqueous and ethereal, which have lower levels of these constituents.

The significant antioxidant capacity, but the low total polyphenol extracts in ether, alcoholic and aqueous indicates that antioxidants other than polyphenols, are the bioactive compounds of the *A. sylvaticus* mushroom.

Aiming at evaluating the antioxidant capacity of the *A. sylvaticus* mushroom in different forms of preparation (liquid suspension, fresh, dry and tablets), Percário et al. [19] evaluated the ability of samples to inhibit in vitro the formation of free radicals by ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid-diamonic) over a period of 90 seconds, resulting in decreased absorbance at 600nm. The authors observed excellent antioxidant activity (%) in all forms of preparation of *A. sylvaticus* at concentrations of 1mg sample. The also emphasized that the temperatures used in the preparation of the samples were 60° C for the dried mushroom and liquid suspension, since high temperatures can inactivate most molecules with antioxidant properties. According to the authors, these molecules are easily degraded when exposed to industrial processes, which makes its antioxidant capacity reduced. According to Barros et al. [25] the cooking processes are responsible for the reduction of nutrients with antioxidant capabilities in several mushrooms analyzed in Portugal.

Percário et al. [19] researched different molecules with antioxidant capacity in *A. sylvaticus*, and found results of 72mg/g for β-Glucan in the liquid suspension and 14.1mg/g in tablet form. For flavonoids, values of 0.88mg/g were found in liquid suspension and 0.63mg/g in tablet form. For total phenols values of 0.1mg/g were found in the liquid suspension and 3.4mg/g for tablets. The authors suggested that the antioxidant activity of mushroom *A. sylvaticus* is by virtue of the number of molecules present, not for a specific component.

In a study performed by Silva et al. [3] the antioxidant potential of different extracts of the mushroom *Agaricus blazei* was evaluated by the DPPH method. The authors also observed a higher antioxidant activity (28.6%) in methanol extract: aqueous (1:1), with extraction time of six hours. In results presented in the present work for *A. sylvaticus*, the best antioxidant activity was observed in the alcoholic fraction

(74.6%), which shows that components with antioxidant properties of this mushroom are more easily soluble in alcohol.

It was observed that some authors used the mushroom extracts under analysis as ingredients of some foods, in order to find out the antioxidant effect in the processed product. Silva et al. [3] added the methanol: water extract (1:1) to soybean oil and obtained good results, since it showed a protective effect (20.4 h of oxidative stability) and the activity of the extract of *A. blazei* more efficient than the synthetic antioxidant BHT (100mg/kg) and less efficient than the TBHQ (tert-Butylhydroquinone) (50mg/kg).

Silva et al.,[3] evaluating the mushroom *A. blazei*, had a concentration of 15mg/g of total phenolic compounds in methanol extract: water extract (1:1). The content of total phenolic compounds exhibited by the *A. blazei* was also assessed by Tsai et al. (2007), who obtained 5.67mg/g of phenolic compounds in the aqueous extract of this mushroom. In this study, the values of total polyphenols were lower. The alcoholic extract of the mushroom *A. sylvaticus* has 9.43mg/100g of phenolic compounds. The aqueous and ether extracts show 4.11 and 0.98 mg/100g mg/100g respectively.

In a study conducted by Cruz et al., [2] the authors found positive results in tests for pharmacognostic tannins, flavonoids glycosides and essential oils, indicating the antioxidant capacity of *A. sylvaticus.*

Chemical studies have revealed that the high concentration of nutrients and active ingredients in mushrooms is directly related to the type of lineage used, which requires specific conditions or several factors, such as: A) Nutritional factors (substances essential for development: carbon, nitrogen, vitamins and minerals); B) abiotic factors (moisture content of compost and cover, temperature, light, oxygen, chemicals in the air, CO_2); C) Biotic (virus, bacteria, actinomycetes, fungi, nematodes, insects, mites and genetic); D) Genetic factors (natural or artificial); E) factors of processing (harvest, drying/dehydration and storage) [26].

According to Neves et al., [16] the market demand for functional foods has grown considerably; the consumer expects to reduce spending on various diseases that affect the population. During the last decade of the twentieth century, consumers in western countries have shown great interest in functional foods, including in this category all food products or ingredients, whether conventional or not, capable of providing health benefits. Among the benefits of eating *A. sylvaticus* mushroom, are the nutritional and antioxidant properties, [10] which is why this is considered an excellent functional food.

The relevance of *A. sylvaticus* researches in Brazil, as a developing country, is to increase this medicinal mushroom production and processing. The results show that *A. sylvaticus* fungus has a great antioxidant potential can prove that this mushroom can be used as a functional food, being a supporting actor for the cancer combating. This way, Brazil producers can expand the therapeutics mushrooms' market, leading benefits to many parts of the world.

5.4 CONCLUSIONS

Through the results obtained in this work, we can conclude that the *A. sylvaticus* mushroom is an excellent source of antioxidants. It was observed its great antioxidant potential particularly in alcoholic extract when compared to concentrations obtained in aqueous and ethereal extracts, which demonstrates the extraordinary benefits of this mushroom as preventive medicine, inasmuch as antioxidants fight free radicals produced in various metabolic situations mainly as consequence of countless diseases.

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ARTIGO 5 – ARTIGO ORIGINAL

Versão publicada em inglês:

Chemical and antioxidant potential of *Agaricus sylvaticus* mushroom grown in Brazil. Costa JV, Novaes MRCG, Asquieri ER. J Bioanal Biomed 2011, 3(2):49-54.

6 ARTIGO ORIGINAL

CHEMICAL AND ANTIOXIDANT POTENTIAL OF *Agaricus sylvaticus* MUSHROOM GROWN IN BRAZIL

Abstract

The chemical characterization of *Agaricus sylvaticus (A. sylvaticus)* cultivated in Brazil is necessary to determine nutritional and pharmacological substances in order to guarantee its safe use as food or herbal medicine. The objective of this study was to determine the chemical composition and assess the antioxidant potential of *A. sylvaticus* fungi grown in Brazil. Through this study it was able to observe the rich chemical composition of *A. sylvaticus,* highlighting the variety and amount of minerals as well as the high protein content of this fungus. It was also observed the great antioxidant potential of the aqueous, alcoholic and ethereal *A. sylvaticus* mushroom extracts, emphasizing the alcoholic extract, which testifies the extraordinary benefits of this fungus in diet, since antioxidants prevent premature aging and various types of cancer as well. The composition of *A. sylvaticus* mushroom displayed differences when compared to the chemical composition of the same fungus in other studies and with other *Agaricales* fungi.

Keywords: Chemical composition; Medicinal mushroom; Potential antioxidant.

6.1 INTRODUCTION

Mushrooms are considered nutraceuticals or functional foods by many clinicians and researchers, a fact that has also stimulated the search by Brazilian producers for more advanced production techniques along with introduction of new species [1].

According to Urben [3], there is great genetic variety of native *Agaricus* genus mushrooms cultivated throughout the world. Strains produced by these mushrooms result from the kind of substrate or compost used, climatic conditions, cultivation area and genetic mutation that can occur naturally or artificially. Mushrooms are highly nutritious foods, having high amounts of protein, equivalent to meat, eggs and milk,

much higher than vegetables and fruits. They contain vitamins such as thiamine, riboflavin, ascorbic acid (Vitamin C), erbocalciferol (Vitamin D2), and a high percentage of minerals like calcium, iodine and phosphorus, besides considerable amounts of fiber [2].

Chemical studies have revealed that the high concentration of nutrients and active ingredients in mushrooms is directly related to the type of lineage used, which requires specific conditions or several factors, such as: A) nutritional factors (substances essential for development: carbon, nitrogen, vitamins and minerals), B) abiotic factors (moisture content of compost and cover, temperature, light, oxygen, chemicals in air, CO_2), C) and biotic factor (virus, bacteria, actinomycetes, fungi, nematodes, insects, mites and genetic), D) genetic factors (natural or artificial); E) processing factors (harvest, drying/dehydration and storage) [3].

Mushrooms have been used for therapeutic prevention of various diseases, in the form of drugs and/or functional foods [4]. In Brazil, despite the low consumption of mushrooms by the population, *Agaricus* genus fungi are becoming very popular due to attributed medicinal properties. There are several studies that report the effects of *A. sylvaticus* (Sun mushroom) on various diseases and these properties may also be associated to the presence of bioactive compounds with medicinal value, such as phenolic compounds, polyketides, terpenes and steroids recognized as excellent antioxidants [5].

According to Elmastas et al. [6], phenolic compounds seem to be the main component responsible for the antioxidant activity in mushroom extracts. According to Tsai et al. [7], the antioxidant properties of *Agaricus blazei* may be associated with its high concentration of tocopherols.

The aim of this study was to evaluate the chemical composition of dehydrated *A. sylvaticus* fungus with respect to protein, lipids, carbohydrates, dietary fiber, minerals, liposoluble vitamins and vitamin C as well as determine the antioxidant potential of ether, alcoholic and aqueous extracts obtained from this mushroom.

6.2 MATERIALS AND METHODS

6.2.1 Evaluation of chemical composition

In this laboratory based experimental study, samples of dehydrated *A. sylvaticus* (Sun mushroom) mushroom were obtained from a producer in the State of Minas Gerais. Mushrooms were crushed in a Willey type grinder, Model ET-648, Brand Tecnal to allow greater extraction of components. Physical and chemical analysis was performed at the Physical Chemistry Laboratory of "Centro de Pesquisa em Alimentos", School of Veterinary Medicine (accredited by the Ministry of Agriculture, Livestock and Supply) and the Food Biochemistry Laboratory, School of Pharmacy, Universidade Federal de Goias - UFG from March to June 2010.

6.2.2 Moisture evaluation

Moisture evaluation was performed in duplicate with dehydrated *A. sylvaticus* fungus, applying the official method for moisture rating, using a kiln at 105.C ± 3°C for 24 hours, established by the Ministry of Agriculture, Livestock and Supply, determined by the Association of Official Analytical Chemists [8]. This methodology quantifies the water withdrawn from the product by heating process, whereas the moisture content is calculated by the weight difference of the sample at the beginning (100%) and at the end of the process (100% -% water evaporated at 105.C). This difference reflects the moisture of the sample under analysis. First the sample was weighed (approximately 5g) and placed in a kiln at 105.C until its weight remained constant. After two weightings at intervals of five hours each, weight was observed to be constant. Next the sample remained in a desiccator in order to lower the temperature (up to room temperature) and was then weighed to check moisture content.

6.2.3 Ash evaluation

Ash evaluation of dehydrated *A. sylvaticus* fungus was performed by calcining the sample in furnace FDG Brand, Model 3P-S 7000, at 550°C for 12 hours, according to the official method of AOAC [8]. Through this technique it is possible to determine the total ash produced using the heat in a muffle furnace, where there is total destruction of organic matter present in the sample, leaving only those minerals present.

A sample of approximately 2g of *A. sylvaticus* mushroom was weighed in a porcelain crucible, which had previously been incinerated with the aid of Bunsen burner, cooled and weighed. Then the set (sample + crucible) was incinerated in a muffle furnace, first at lower temperature and then at 550°C. After incineration, the set was removed from the flask, placed in a desiccator to cool off and weighed when it reached room temperature. The amount of ash in the sample was detected from the weight difference between the weight of the set and the weight of the empty crucible. The mushroom ash sample served as a starting point for analyzing specific minerals.

6.2.4 Evaluation of minerals

To determine the minerals, an atomic absorption spectrometry was used in spectrometer GBC Brand, Model 932AA. Duplicate analyses were performed. The principle of this technique is based on measuring the absorption of electromagnetic radiation intensity, from a primary source of radiation by gaseous atoms in ground state. It was possible to search for iron, zinc, manganese, sodium, potassium, cobalt, copper, calcium and magnesium, as these tests were performed in a laboratory where there were specific cathode lamps for each of these minerals.

6.2.5 Protein evaluation

For protein grading the Kjedahl method was used following the AOAC [8] methodology. Total nitrogen was obtained from the sample which, through calculation was transformed into protein Nitrogen considering that each 100g of protein contains an average 16g of nitrogen. Therefore we used a 6.25 correction factor, which was multiplied by the total Nitrogen percentage of the sample, which corresponded to the protein percentages [9]. To develop this methodology we used a Nitrogen distiller Brand Tecator, Kjeltec System Model 1026. Protein analysis involved three phases. In the first phase the nitrogen in the sample was transformed into ammonium (NH_4+) through acid digestion of organic matter, starting from 0.1 g of Degreased Dry Matter. In the second phase, separation was obtained by means of distillation and in the third phase, dosage by titration with HCl 0.02 N.

6.2.6 Evaluation of lipids

The amount of lipids present in the sample of the *A. sylvaticus* mushroom was obtained through continuous extraction with a Soxhlet device, Brand Gerhardt, Soxtherm Model 2000, using sulfuric ether as solvent, which has a boiling point of approximately 35.C. After extraction, the solvent was evaporated using a Rotavapor and lipid fraction was determined gravimetrically. After 24 hours, we obtained the average weight of lipid fraction. The extracted oil was stored at 10°C for later chromatographic analysis of fat soluble vitamins.

6.2.7 Evaluation of total dietary fiber

The methodology for the evaluation of total dietary fiber of *A. sylvaticus* fungus was proposed by AOAC [10], whose principle is based on the sequential enzymatic digestion of dehydrated mushroom sample, in duplicate, with thermostable alpha-amylase, protease and amyloglucosidase. The digested sample was then treated with alcohol to precipitate the soluble fiber before filtering, and the residue was washed with alcohol and acetone, dried and weighed.

6.2.8 Carbohydrate evaluation

The evaluation of carbohydrates was calculated by the difference, using rates obtained by the analysis of moisture, fixed mineral residue, proteins and lipids, following methodology recommended by AOAC [11].

6.2.9 Evaluation of fat-soluble vitamins

Fat-soluble vitamins were determined by high performance liquid chromatography (HPLC), and the performance of duplicate analysis. The principle of this technique evaluates the extraction of active compounds of vitamins studied and their conversion in free form in chloroform solution for later evaluation. For this

analysis, it was used as sample the oil obtained in lipid analysis through Soxhlet extraction. It was used liquid chromatography, Gilson brand, with a stationary phase column E-18, column 10 cm/4.6 mm and particles of 5micras. For the mobile phase was used a methanol and isocratic working system with 100% of methanol and 1mL/min flow. Variable wavelengths (l) were used for each vitamin studied, as shown in Table 3.

6.2.10 Vitamin C cvaluation

Vitamin C evaluation was performed in triplicate, following the Tillmans Method starting from titration of a standard solution of ascorbic acid and oxalic acid solution with DCFI solution (2, 6-dichlorophenol indophenol sodium), and the solutions used were prepared as described by the Adolfo Lutz Institute (1995) for the Tillmans Method. To determine Vitamin C, it was obtained an aqueous, non fractioned extract of *A. sylvaticus* mushroom by diluting dried mushrooms ground in water, kept under agitation at room temperature for one hour.

6.2.11 Evaluation of antioxidant potential

The antioxidant potential of *A. sylvaticus* mushroom was determined following the methodology used by Borguini [12]. In order to avoid interference of light in the sample, the experiment was conducted using material covered with aluminum foil. It was obtained the ether, alcoholic and aqueous extracts from the mushroom. First it was obtained the ether extract by diluting 2.5g of ground mushroom in 50mL of ethyl ether. From non-filtered residue and therefore ether-insoluble, it was obtained the alcoholic extract by adding ethanol at 1:20 ratio (residue weight: volume of alcohol). And finally, it was obtained the aqueous extract by adding water to the non-filtered residue from the previous step and also adding distilled water at 1:20 ratio (residue weight: water volume). BHT was used as a standard antioxidant and DPPH as an oxidant.

The antioxidant activity of mushroom extracts was determined by DPPH (2.2-difenilpicril-hydrazyl) described by BRAND-WILLIAMS et al. [13]. DPPH is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule, and thus, is reduced in the presence of an antioxidant.

Absorbance decrease was monitored at 517nm in a spectrophotometer Model SP-220, Biospectro brand, at intervals of 0, 1, 2, 3, 4, 5, 10, 15 and 20 minutes of reaction. The values observed in the spectrophotometer were converted to a percentage scale, which indicates 0% - no inhibition of free radical production, and 100% indicates complete inhibition of the same.

6.2.12 Quantification of total polyphenols

Concentration of total polyphenols was determined by colorimetric method described by Singleton and Rossi [14], using the Folin Ciocalteau reagent. For quantification of total polyphenols in the sample, a standard curve of gallic acid solution at concentrations of 0.01mg/mL to 0.06mg/ mL was used. The correlation coefficient (R^2) was calculated, resulting in $R^2 = 0.99775$ to a 5% level of significance. This test was performed in triplicate, by using the ether, alcoholic and aqueous extracts of sample at the same concentrations utilized for the standard solution of gallic acid. The reading was performed with spectrophotometer Model SP-220, brand Biospectro at 750nm.

6.3 RESULTS

6.3.1 Chemical composition

Table 1 shows the results found by analyzing the chemical composition of *A. sylvaticus* dehydrated mushroom. One can observe the high protein content (41.16%), followed by carbohydrates (36.21%).

Table 1. Chemical composition of dehydrated *A. sylvaticus*.

Constituent Composition (% in 100g)	**Constituent Composition (% in 100g)**
Hmidity	6.31
Ash	7.38
Protein	41.16
Lipids	6.60
Carbohydrates	36.21
Dietary Fiber	2.34

* The chemical analysis was performed in duplicate. * The methods of chemical analysis of dehydrated *A. sylvaticus* mushroom are described by AOAC: Moisture (kiln at 105ºC), ash (muffle furnace at 550°C),

proteins (Kjedahl), lipids (Soxhlet), Carbohydrate (difference from the other constituents of 100%), and dietary fiber (by enzymatic digestion of the sample).

Table 2 shows values found for rating minerals in dehydrated *A.sylvaticus* fungus, including iron, zinc, calcium, cobalt, magnesium, sodium, potassium, manganese and copper. It was not possible to determine the dosage of other minerals performed in the laboratory owing to operational reasons.

Table 2. Evaluation of minerals in dehydrated *A. sylvaticus*.

Constituent Composition (% in 100g)	**Constituent Composition (% in 100g)**
Iron	726.90 mg/100g
Zinc	549.25 mg/100g
Magnesium	21.19 mg/100g
Potassium	613.03 mg/100g
Copper	276.66 mg/100g
Calcium	1.35 mg/100g
Cobalt	7.75 mg/100g
Sodium	255.34 mg/100g
Manganese	23.18 mg/100g

*Analyses of minerals was performed by atomic absorption spectrometry.

The quantities of liposoluble vitamins and vitamin C found in the mushroom *A. sylvaticus* are shown in Table 3. Liquid chromatography analysis enabled the analysis of vitamin A in acetate form, palmitate and propionate in addition to its pure form; of vitamin E in acetate form, alpha, beta, delta and gamma tocopherol; of vitamin K in the K1, K2, K3 and K4 form; however, vitamin D2 was detected by titration.

Table 3. Composition of vitamins of *A. sylvaticus* mushroom.

Vitamin	**Composition**	**Wavelength (ʎ)**
Ascorbic acid (Vitamin C)	12.65 mg/100g	-
Retinol acetate (Vitamin A)	0.000 mg/100g	460nm
Retinol (Vitamin A)	0.001 mg/100g	460nm
Retinol palmitate (Vitamin A)	0.000 mg/100g	460nm
Propionate, retinol (Vitamin A)	0.000 mg/100g	460nm
Vitamin D2	0.018 mg/100g	460nm
Tocopherol acetate (Vitamin E)	0.000 mg/100g	295nm
Alpha tocopherol (Vitamin E)	0.020 mg/100g	295nm
Beta Tocopherol (Vitamin E)	0.000 mg/100g	295nm
Delta Tocopherol (Vitamin E)	0.000 mg/100g	295nm
Gamma tocopherol (Vitamin E)	0.000 mg/100g	295nm
Phylloquinone (vitamin K1)	0.000 mg/100g	350nm
Menaquinone (vitamin K2)	0.001 mg/100g	280nm
Menadione (Vitamin K3)	0.000 mg/100g	460nm
Naftaquinone (Vitamin K4)	0.000 mg/100g	350nm

* The analysis of liposoluble vitamins was performed in duplicate, using liquid chromatography of the oil obtained from the lipids' analysis of *A. sylvaticus* fungus.
* The analysis for detecting vitamin C was performed in triplicate by titration from the non fractioned aqueous extract of *A. sylvaticus* mushroom.

6.3.2 Antioxidant potential

The antioxidant potential of ether, alcoholic and aqueous extracts obtained from *A. sylvaticus* mushroom is shown in Table 4.

Table 4. Antioxidant potential of ether, alcoholic and aqueous of *A. sylvaticus* fungus extracts.

Extract	**Antioxidant potential (%)**
Alcoholic	75.6
Ethereal	14.6
Aqueous	14.6

* The antioxidant potential of *A. sylvaticus* mushroom was observed from spectrophotometric analysis of three extracts from the sample. As oxidant we used the DPPH as standard.

6.3.3 Total polyphenols

The amount of polyphenols detected in the ether, alcoholic and aqueous extracts are shown in Table 5.

Table 5. Quantification of total polyphenol of ether, alcoholic and aqueous extracts of *A. sylvaticus* fungus.

Extract	**Total polyphenols (%)**
Alcoholic	4.11
Ethereal	9.43
Aqueous	0.98

* Total polyphenols research was performed using the Folin-Ciocalteou in spectrophotometer at 750nm.

6.4 DISCUSSION

In this study we observed that the protein content of *A.sylvaticus* (41.16%) is superior when compared to the protein content of beef (approximately 14.8%), as well as of other mushrooms from the *Agaricales* family [15].

In addition to the high-protein content, protein from mushroom *A. sylvaticus* has high biological value, since it exhibits all the essential amino acids [16], as shown by research conducted by the Japan Food Research Laboratories [14] on *A. sylvaticus* grown in Brazil.

The following levels were detected at the time: 1.71g/100 g of arginine, 1.55g/100g of lysine, 0.62g/100g of histidine, 1.11g/100g of phenylalanine, 0.83g/100g of tyrosine, 1.72g/100g of leucine, 1.01g/100g of isoleucine, 0.39g/100g of methionine, 1.28g/100g of valine, 1.75g/100g of alanine, 1.25g/100g of glycine, 1, 26g/100g of proline, 5.73g/100g of glutamic acid, 1.20g/100g of serine, 1.21g/100g of threonine, 2.35g/100g of aspartic acid, 0.43g/100g of tryptophan and 0,36g/100g of cystine.

Because they are high-protein food, mushrooms are highly recommended for those who need a high protein diet, or for those whose diet has restrictions on lipids. This fact is of great importance regarding public health, since research reveals that the Brazilian population includes a large number of overweight or obese individuals. This is certainly already causing public health concern, upon considering a population whose consumption profile has considerably changed, especially during the 80's, due to economic factors and the related social consequences [18].

According to results on the amounts of protein and lipids in the present study, *A. sylvaticus* mushroom can also be suggested as an important alternative health food.

In the 2005 survey conducted by the Japan Food Research Laboratories on the *A Sylvaticus* grown in Brazil, values found for dehydrated mushroom were 4.4 g/100g of moisture, 39.4 g/100g of protein, 3.0g/100g of lipid, 45.6g/100g of carbohydrate and 7.6/100g of minerals. Comparing the above results with the present study, *A. sylvaticus* mushroom grown in Brazil in 2010 in dried state, shows higher values of moisture content (6.31%), lipids (6.60%) and protein (41.16%), which can be explained if taking into account differences in farming technique, region, climate, genetic mutations [3], conditions which are probably better in the areas where the mushroom is currently cultivated.

In a study by Copercon, cited by Eira [19], the chemical composition of other mushrooms of the genus *Agaricus*, *A. brasiliensis* in dried state, showed the following results: water (7.5%), protein (36.6%), lipids (3.4%), fiber (6.8%), ash (7.3%), and carbohydrates (38.3%). Comparing these results with those of the present work, we see that only the ash content of the fungi studied was similar.

The present study revealed 2.34% value of dietary fiber. According to Novaes and Novaes [15], the dietary fibers contained in mushrooms can absorb toxic, harmful and carcinogenic substances. Countless studies show fibers being associated to lower incidence of colorectal cancer, since it accelerates faecal excretion by laxative action, reducing the time spent in the intestines.

With respect to the lipid content, we detected 6.60% of this nutrient in the *A. sylvaticus* fungus. According to Borchers et al. [20], although mushrooms contain small quantities of total fat, they have a high percentage of polyunsaturated fatty acids (PUFA) and low content of saturated fatty acids and cholesterol. According to Novaes and Novaes [15], crude fat mushrooms consists of several classes of lipids, including free fatty acids, mono- di- and triglycerides, sterols, terpenoids and phospholipids, especially lecithin.

The Japan Food Research Laboratories also performed analysis of sodium (4.2mg/100g), iron (21.2mg/100g), calcium (35.7mg/100 g), potassium (3.15mg/100g) magnesium (100mg/100g), copper (8.24 mg/100 g), zinc (6.61mg/100g), manganese (0.65mg/100 g), selenium (36μ g/100g), and cobalt (0.13ppm). Neither molybdenum nor boron was detected. Comparing these results with this study, we can observe the discrepancy between results for the most researched minerals, which come in higher concentrations in this work. According to Urben [3], this variation in minerals can also be explained by the type of crop, climate, region, and genetic mutations, among others, found more favorable in techniques used at present to cultivate the genus *A.sylvaticus* mushroom.

According to [16], mushrooms have significant amounts of sodium. The presence of potassium, calcium, phosphorus, magnesium, iron and zinc was also observed by Borchers et al. [20].

In a study by Copercon, cited by Eira [19], the mineral composition of the dehydrated mushroom *A. brasiliensis* showed the following results for phosphorus, iron and calcium: 939mg/100g, 18.2mg/100g and 41.6mg/100g, respectively.

Olivera et al. [18], studying the fungus *A. blazei,* found high levels of minerals such as potassium (2.34%), phosphorus (0.87%), calcium (0.07%), magnesium (0.08%), sulfur (0.29%), copper (61.88 mcg), zinc (86.90 mcg), iron (79.63 mcg).

Among the vitamins exhibited by *A. sylvaticus* surveyed by the Japan Food Research Laboratories in 2005, the following substances were not detected in the sample: α-carotene, β-carotene and Vitamin C. However, values found were

1.21mg/100g of thiamine (Vitamin B1), 3.41mg/100g of riboflavin (Vitamin B2), 0.83mg/100g of Vitamin B6, 0,17μg of Vitamin B12, 5,8μg of calciferol (Vitamin D), 0.36mg/100g of folic acid, 39.4mg/100g of pantothenic acid, inositol 201mg/100g and 39.9mg/100g of niacin.

As seen in Table 3, vitamin C was detected in samples of *A. sylvaticus* analyzed in this study, which disagrees with the results presented by the Japan Food Research Laboratories [17]. According to Lederer [21], the importance of vitamin C is associated with several types of cancer, and daily doses administered to patients with cancer have improved their survival.

Among the surveyed liposoluble vitamins, alpha tocopherol within the D complex, retinol, within the A complex and menaquinone from K Complex were detected. According to Soares [22], the accumulation of these compounds is dependent on the handling, processing and maturity of mushroom at harvest.

Because they are obtained synthetically, tocopherol acetate and retinol acetate were not detected in samples of dehydrated *A. sylvaticus* mushroom. According to Borchers et al. [20], mushrooms contain significant amounts of niacin, thiamin, riboflavin, biotin, ascorbic acid and pro-vitamins A and D. According to Eira and Braga [23], knowledge of the chemical composition of mushrooms is very important, and in Brazil the genetic and physiological studies, basic and applied, can be expanded aiming at selecting more stable and productive lineages, establishing more appropriate physiological conditions for the cultivation of mushrooms so as to attain the desired standard of quality.

According to Silva et al. (24), despite the high biodiversity of mushrooms found in Brazil and great exploitation potential, there is little data on the antioxidant activity of mushroom extracts, since antioxidants have the ability to scavenge free radicals, which are harmful to human health [25].

Antioxidants are able to slow oxidation rate, inhibiting free radicalsand preventing the onset of diseases, thus contributing to greaterlongevity, making the balance between free radicals and the antioxidantdefense system essential [26].

Clinical and experimental studies demonstrate that dietary supplementation with *Agaricales* mushrooms and other medicinal fungi exert positive nutritional, medicinal and pharmacological effects and can be used as an adjuvant in cancer therapy. The mechanisms of action of bioactive compounds found in mushrooms are yet to be fully elucidated in the literature, but scientific evidence suggests that these substances are

able to modulate carcinogenesis not only at early stages, but at more advanced phases of disease progression as well, providing benefits to individuals with various types of cancer, mainly by stimulating the immune system [27].

Regarding antioxidant activity it was observed that the alcoholicextract of the mushroom *A. sylvaticus* has great antioxidant potential (74.6%), suggesting that most antioxidant compounds present in thismushroom can be more easily diluted in alcohol. However, the aqueousand ether fractions showed lower antioxidant potential (14.6% each) when compared to alcoholic fraction. The aqueous fraction presented reduced antioxidant potential (14.6%) compared to results reported by Percario et al. [28] for the fungus in liquid suspension (50%), since in this work, antioxidant compounds had already been extracted by ether and by alcohol.

Polyphenols make a heterogeneous group, composed of several classes of substances with antioxidant capacity, among which phenolic acids and flavonoids stand out. The antioxidant activity of polyphenols is mainly due to its reducing properties, whose intensity of antioxidant activity exhibited by these phytochemicals is notably differentiated because it depends fundamentally on the number and position of hydroxyl groups present in the molecule [29].

In this study we determined the amount of total polyphenol for the etheric, alcoholic and aqueous extracts. We noticed that the largest amount of alcoholic extract is concentrated in polyphenols (9.43mg/100g) followed by etheric extract (4.11mg/100g), and aqueous extract (0.98mg/100g). The use of ethanol made possible the extraction of a higher content of polyphenols, since the alcoholic extract of the *A. sylvaticus* sample exhibited higher total phenolic content than the aqueous and ethereal which hold lower levels of these constituents.

Aiming to evaluate the antioxidant capacity of the *A. sylvaticus* mushroom in different forms of preparation (liquid suspension, fresh, dry and tablets), Percario et al. [28] assessed the ability of samples to inhibit *in vitro* the formation of free radicals by ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid-diamonic) over a period of 90 seconds, resulting in decreased absorbance at 600nm.The authors observed excellent antioxidant activity (%) in all forms of preparation of *A. sylvaticus* at concentrations of 1mg sample. The authors emphasized that the temperatures used in the preparation of the samples were 60°C for the dried mushroom and liquid suspension, since high temperatures can inactivate most molecules with antioxidant properties present in *A. sylvaticus* According to the authors, these molecules are easily degraded when exposed

to industrial processes, which reduces their antioxidant capacity. According to Barros et al. [30], the cooking processes are responsible for the reduction of nutrients with antioxidant capabilities in several mushrooms analyzed in Portugal.

Percario [28] researched different molecules with antioxidant capacity in *A. sylvaticus* fungus, and found results of 72mg/g for β-Glucan in the liquid suspension and 14.1mg/g in tablet form. For flavonoids, values of 0.88mg/g were found in liquid suspension and 0.63mg/g in tablet form. For total phenols, values were 0.1mg/g for liquid suspension and 3.4mg/g for tablet form. The author suggested that the antioxidant activity of *A. sylvaticus* mushroom is due to the entirety of molecules it contains, and not a specific component only.

In a study performed by Silva et al. [24] the antioxidant potential of different extracts of the mushroom *A. blazei* was evaluated by the DPPH method. The authors also observed a higher antioxidant activity (28.6%) in methanol extract: aqueous (1:1), with extraction time of six hours. Results displayed in the present work, confirmed that the best antioxidant activity for *Agaricus sylvaticus* extract was in the alcoholic fraction (74.6%), which shows that components with antioxidant properties of this mushroom are more easily soluble in alcohol.

Some authors utilized the researched mushroom extracts as ingredients in some foods in order to find out the antioxidant effect in processed products. Silva et al. [24] added the methanol: water extract (1:1) to soybean oil and obtained good results. Results showed effective protection (20.4 h of oxidative stability), and the activity of *A. blazei* extract was more efficient than the synthetic antioxidant BHT (100mg/kg) and less efficient than the TBHQ (50mg/kg).

Silva et al. [24], evaluating the *A. blazei* mushroom, obtained concentration of 15mg/g of total phenolic compounds in methanol extract: water extract (1:1). The content of total phenolic compounds present in *A. blazei* was also assessed by Tsai et al. [7], who obtained 5.67mg/g of phenolic compounds in the aqueous extract of this mushroom. In this study, the values of total polyphenols were lower. The alcoholic extract of the mushroom *A. sylvaticus* showed 9.43mg/100g of phenolic compounds. The aqueous and ether extracts showed 4.11 and 0.98mg/100g respectively.

6.5 CONCLUSION

Through this study we were able to observe the rich chemical composition of *A. sylvaticus,* highlighting the variety and quantity of minerals and the high protein content of this mushroom. It was also found that the chemical composition of the mushroom showed differences when compared to the composition of the same mushroom in other studies and other mushrooms of the *Agaricales* genus. It was also observed the great antioxidant potential of aqueous, alcoholic and ethereal extracts of the *A. sylvaticus* mushroom, emphasizing the alcoholic extract, which demonstrated the extraordinary benefits of this mushroom in diet, considering that antioxidants prevent against premature aging and various types of cancer.

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ARTIGO 6 – ARTIGO ORIGINAL

Versão publicada em inglês:

The acute cytotoxicity and lethal concentration (LC_{50}) of *Agaricus sylvaticus* through hemolytic activity on human erythrocyte. Orsine JVC, Costa RV, Silva RC, Santos MFMA, Novaes MRCG. Int J Nutr Metab 2012, 4 (11):19-23.

7 ARTIGO ORIGINAL

THE ACUTE CYTOTOXICITY AND LETHAL CONCENTRATION (LC_{50}) OF *Agaricus sylvaticus* THROUGH HEMOLYTIC ACTIVITY ON HUMAN ERYTHROCYTE

Abstract

There is limited information regarding acute toxicity and lethal concentration of edible and medicinal mushrooms. The objective of this paper is to estimate the cytotoxicity of the aqueous extract of *Agaricus sylvaticus* mushroom on human erythrocytes by determining the lethal average concentration (LC_{50}). Six concentrations of the mushroom (17, 8.5, 4.25, 2.125, 1.0625 and 0.5312 mg/mL) were submitted for evaluation of hemolytic activity *in vitro,* using a suspension of blood. Through the Prism GraphPad Software, using the Tukey test for statistical analysis ($p < 0.05$), a curve was constructed with values of *A. sylvaticus* mushroom concentrations versus the values determined by absorbance spectrophotometry at 540 nm. Results of hemolytic activity for the aqueous extract were fitted using nonlinear regression and the equation: $Y_i = ax_i / (b + X_i)$. We used values of y as hemolytic activity and x as log of *A. sylvaticus* mushroom concentration. The coefficient for determining the curve (R^2) was 0.95 of the original data. The percentage of haemolysis increased in a concentration-dependent manner of *A. sylvaticus* extract used. The LC_{50} value obtained was 9.213 mg/mL. Results derived from this experiment suggest that this mushroom extract has very low toxicity proving to be safe for human use.

Key words: Lethal concentration, *Agaricus sylvaticus*, hemolytic activity, sun mushroom.

7.1 INTRODUCTION

Chemicals used in therapy should be effective and provide safety (Goodman and Gilman, 2007). Unfortunately, any substance can be a toxic agent and cause undesirable effects (Goodman and Gilman, 2007; Oga, 2003), depending on the dose administered

or absorbed, time and frequency of exposure and routes of administration (Oga, 2003). Highly toxic substances cause death at concentrations equivalent to a fraction of a microgram. In others, low toxicity may be almost harmless in concentrations of several grams or more (Goodman and Gilman, 2007; Oga, 2003).

The toxicity of a substance to an organism refers to its ability to cause serious injury or death. In therapy, the concentration of a substance should be enough to achieve the desired effect and achieve it well with the lowest concentration, and as much as possible, without producing adverse reactions or side effects (Oga, 2003).

The safety of drugs and foods should be determined through the analysis of several factors related not only to the individual characteristics of the organism, but also considering the physic-chemical, pharmacodynamic and pharmacokinetic of each substance, the various routes of exposure and different methods of administration (Silva, 2006).

Depending on the cultivation and composting, mushrooms can have varying levels of toxicity and risk to human health, although preliminary studies suggest that experimental use of *Agaricus sylvaticus* may present low toxicity. The use of this mushroom in folk medicine began in ancient peoples and between indigenous communities (Novaes et al., 2007).

The assessment of exposure can be performed by measuring the concentration of a substance administered to a particular organism (Oga, 2003). The study of concentration-response or concentration-effect in toxicology is essential and is used to determine the median lethal concentration (LC_{50}) of drugs and other chemicals (Goodman and Gilman, 2007).

The concentration-response curve is represented by the Gaussian theory, rarely found in practice. This curve is calculated statistically from observations of mortality after exposure related to concentrations of the substance to be tested, and it is widely used to calculate the 50% lethal concentration (LC_{50}). The LC_{50} is thus a statistical index which indicates the concentration of a chemical agent capable of causing death in 50% of organisms in a population with defined experimental conditions (Oga, 2003).

To know the effects of a toxic substance and classify them according to their potential lethality or toxicity and concentration-response curve, one needs to perform toxicological tests (Oga, 2003).

Mushrooms of the genus *Agaricus* have been widely studied for their nutritional characteristics and many medicinal properties they exhibit. The *A. sylvaticus* mushroom

(Sun Mushroom) has been reported to have rich nutritional composition, with high protein content (41.16%), carbohydrates (36.21%), low lipid content (6.60%), considerable amounts of fiber (2.34%) and minerals (7.38%), besides having excellent antioxidant activity (Costa et al., 2011).

A. sylvaticus has been widely used as nutritional supplement for cancer patients, with likely effects of growth inhibition, tumor regression and stimulation of the immune system of patients.4 According to recent studies there seems to be clear evidence of its immunomo-dulatory activity and efficacy against carcinogenic activity of the drug pristine (Hi et al., 2008).

There is also indication that dietary supplementation with *Agaricus sylvaticus* may reduce total cholesterol, LDL-C and triglycerides, with favorable outcome on lipid metabolism and, consequently, on the prognosis of patients with colorectal cancer in post-operative phase (Fortes et al., 2008). Furthermore, it has contributed to improve the quality of life of these patients by significantly reducing the harmful effects caused by the disease itself (Fortes et al., 2007).

The safety and effectiveness of medicinal plants and fungi are dependent on various factors, of these the quality of the product commercialized can be highlighted. Effectiveness and low toxicity to humans should be verified as well (Arnous et al., 2005).

In this context, the objective of this study is to evaluate the acute toxicity of *A. sylvaticus* mushroom aqueous extract *in vitro*, from the determination of lethal concentration (LC50) through its hemolytic activity on human erythrocytes so as to refer the determination of toxicity parameters for human use.

7.2 METHODS

The experiment, in triplicate, was performed at the Nanotechnology Institute Laboratory of Biological Sciences, University of Brasilia, Brazil, in January and February 2011.

7.2.1 Obtaining the sample

The sample of dried *A. sylvaticus* mushroom (Sun Mushroom) was obtained from a producer in Minas Gerais State, Brazil.

7.2.2 Preparation of the solution containing the A. sylvaticus mushroom

We weighed 9.0 g of dehydrated *A. sylvaticus* mushroom and added to the sample 105 mL of distilled water. The solution was stirred for 20 min at room temperature, filtered through paper filter, and then 1000 μL of the solution was distributed into previously weighed Eppendorf tubes. The solution was lyophilized and the Eppendorf tubes were then weighed again, in order to obtain the average weight of the mushroom dissolved in water (17 mg/mL).

Serial dilutions were performed resulting in six concentrations for study: 17, 8.5, 4.25, 2.125, 1.0625 and 0.5312 mg/mL.

7.2.3 Preparation of erythrocyte suspension at 2% (human blood A-)

Erythrocytes were obtained from fresh A Negative type human blood. For erythrocyte suspension, 1 mL of blood was centrifuged for five minutes at 14000 rpm. Next 9.8 mL of saline solution (NaCl 150 mm) and 200 μL of the erythrocytes precipitate were added to the tube. The tube was then centrifuged for ten minutes at 2000 rpm. The supernatant was discarded and the process repeated three more times. Finally, the tube was shaken with the erythrocyte suspension ready for use.

7.2.4 Testing of hemolytic activity - Dose relation/hemolytic activity

Samples with 3 mL of saline solution + 500 μL of erythrocyte suspension + 500 μL of *Agaricus sylvaticus* extract were prepared in six different concentrations. The tubes were stirred manually and incubated at 35°C/60 min. After this interval, the tubes were centrifuged at 2500 rpm for ten minutes. The absorbance of the supernatant was read at 540 nm. The negative control (no haemolysis) was prepared only with saline

solution and erythrocyte suspension, and the positive control (100% haemolysis) with 3 mL of distilled water + 500 µL of mushroom extract and a reading taken after 60 min.

We built graphics were built of the kinetics and of the dose-response relationship with mean values and standard deviation (SD). Data were expressed as percentage of viability in control wells, through the GraphPad Prism software, using the Tukey test for statistical analysis ($p < 0.05$).

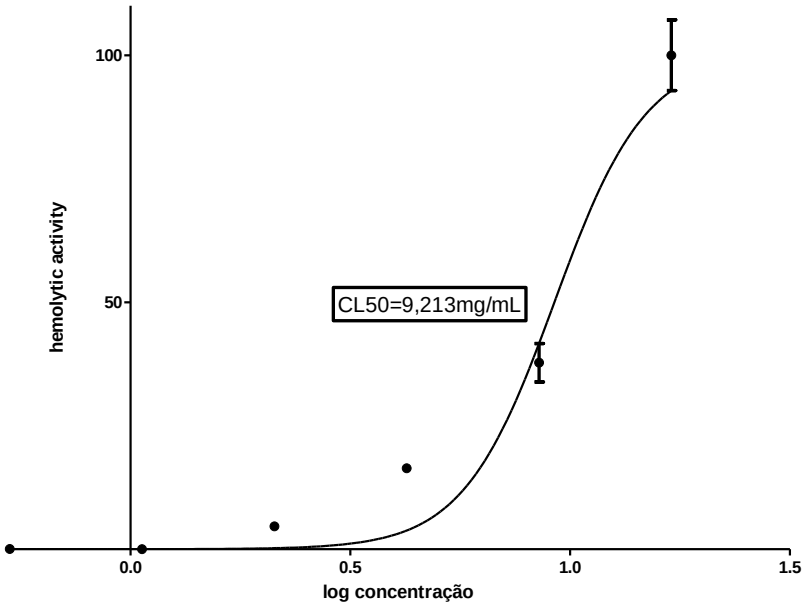

Figure 1. *In vitro* hemolytic activity presented by the aqueous extract of the mushroom *A. sylvaticus* at a 2% suspension of human erythrocytes incubated at 35°C for 60 minutes. The results presented correspond to the average of a test in triplicate.

The assessment of cytotoxicity through hemolytic activity tests has proved to be an alternative screening method for simple toxicity. It is fast, reproducible and inexpensive to evaluate erythrocyte hemolytic activity against concentrations of aqueous extract of *A. sylvaticus*, a fact making it possible to reduce the use of laboratory animals for *in vivo* tests, helping reach the goal to decrease, refine and replace studies conducted with animals.

The intent of reducing animals in the research and development of new methodologies in Brazil is timid and will require further discussion with participation of educational institutions and research laboratories together with the industry and

regulatory agencies, since this reality affects all those involved in research, registration and approval of new substances.

As the focus of this article is to observe the acute cytotoxicity of mushroom extract, further studies are still necessary to investigate the mechanism of action of this extract and the possible organs or systems sensitive to the same, as well as additional studies on sub-acute and chronic toxicity, mutagenic and teratogenic activity, embriotoxicity and special studies particularly regarding the choice of concentrations of the extract, so as to validate its safety.

7.3 RESULTS

Evaluation of toxicity is paramount when considering a safe treatment. Haemolysis is characterized by erythrocytes rupturing with the release of hemoglobin. The *in vitro* haemolysis test is used as a method for substance toxicity screening, estimating any likely *in vivo* damage (Aparício et al., 2005).

Different aqueous extract concentrations of the *A. sylvaticus* mushroom were tested on a suspension of human erythrocytes at 2% and hemolytic activity deter-mined as haemolysis percentage. We built a curve of concentration (μg of *A. sylvaticus* mushroom) versus percentage of haemolysis and concentration of the mushroom aqueous extract required to produce 50% haemolysis, known as 50% hemolytic concentration or 50% effective concentration (EC_{50}).

Test results of the hemolytic activity in tubes for the aqueous extract of *A. sylvaticus* mushroom were then adjusted using nonlinear regression, through the equation:

$$Yi = axi/(b + Xi)$$

The statistical analysis (Tukey test) was defined according to nonlinear fitting model using the Prism Software. To determine the curve we used the values of y as the hemolytic activity and x as the log of *A. sylvaticus* mushroom concentration. The coefficient for determining the curve (R^2) was 0.95 of the original data.

The percentage of haemolysis increased in a dependent-concentration manner of the extract of *A. sylvaticus* used. The LC_{50} value obtained in this experiment was 9.213 mg/mL.

The curve obtained (Figure 1) represents the hemolytic activity of aqueous extract of the *A. sylvaticus* mushroom on the solution of human erythrocytes at 2%.

7.4 DISCUSSION

Several authors suggest that the exact calculation of LC50 is valid only for substances that pose a lethal concentration of 1 and 5000 mg/kg. However, regulatory international institutions of chemical composition toxicity recommend a limit of 2000 mg/kg for the LC_{50} test (Larini, 1997).

By determining the LC_{50} of aqueous extract from the *A. sylvaticus* mushroom, it was observed that this extract has low toxicity, since many grams are needed to cause cellular damage.

No study has been found in the literature using methods of cytotoxicity *in vitro* so that the extracts of this mushroom could be evaluated and compared. Nevertheless, the present results corroborate the results found by Novaes et al. (2007), where the effects of acute toxicity of the aqueous extract of this mushroom were assessed by clinical, biochemical and histopathological parameters in healthy mice, showing very low toxicity.

The low toxicity of this aqueous extract on erythrocytes may be related to the low toxicity of this extract found in animals, suggesting its potential for therapeutic purposes. But there are few studies in the literature regarding comparative sensitivity between these two methods (Cruz et al., 1998).

In 1927, Trevan suggested that lethal concentration should be considered when it kills 50% of the animals (LC_{50}) since the LC_{50} values vary less than those of LD_1 and LD_{99} (dosage required to kill 1 or 99% respectively of the test population) (Silva, 2006). Many toxicity tests currently used for assessment of toxic agents still employ laboratory animals (Harbell et al., 1997). However, the LC_{50} tests advocated by Trevan have been the subject of several reviews and discussions, especially of ethical nature, owing to the large number of animals sacrificed, the suffering caused during some tests, the imprecision of values obtained and the information it fails to provide (Silva, 2006; Cazarin et al., 2004).

Therefore, the completion of toxicological studies in animals with *in vitro* tests is a global trend (Cazarin et al., 2004). The development of new methods for *in vitro*

toxicity testing and its recognition by international organi-zations such as the FDA (Food and Drug Administration) in 1983 and the OECD (Organization for Economic Cooperation and Development) in 1987 has fostered the replacement of tests using laboratory animals (Cruz et al., 1998; Cazarin et al., 2004).

These two organizations, further to promoting the improvement of toxicity tests, have been engaged in reducing costs and time spent in studies, decreasing and replacing animal use (Cazarin et al., 2004).

In this sense, there has been growing demand for *in vitro* tests, which do not sacrifice animals. The evaluation of *in vitro* hemolytic action has been used as screening methodology for various toxic agents (Kublik et al., 1996; Mehta et al., 1984). *In vitro* haemolysis tests have also been employed by several authors for the toxicological evaluation of different plants (Gandhi et al., 2000).

According to Queiroz (2009), laboratory experiments with cells reproduce the conditions and even reactions similar to those occurring in the body, and are thus able to observe and quantify changes undergone by cells from a particular product or medicament, as well as the behavior of each cell component separately, restricting the number of variables.

Ralph et al. (2009) through testing for hemolytic activity rated the degree of *in vitro* toxicity according to the observed mortality rate: 0 to 9% = non-toxic, 10 to 49% = slightly toxic, 50 to 89% = toxic; 90 to 100% = highly toxic. Therefore, for new studies to be conducted, the use of non-toxic concentrations (LC_{0-9}) is suggested.

Arguing that the chemical and the pharmaceutical industry perform the LC_{50} test simply because it is required by authorities, in which case without any scientific justification, some authors propose replacing the LC_{50} with maximum non-lethal concentration (MNLC). The MNLC of a substance is defined as the maximum concentration which does not cause any mortality in a number of animals.

This indicator has been proposed as being more useful than the LC_{50} for evaluating the risk/safety of a product by the fact that it uses the non-occurrence of deaths (most severe of toxic effects) as analytical criterion (Larini, 1997). The maximum concentration is defined as the highest dose tolerated without toxic symptoms. The maxi-mum lethal concentration refers to the smallest amount of drug capable of producing death. The therapeutic dose or effective dose is between the minimum and maximum therapeutic dose (Silva, 2006).

Silva et al. (2009) considering that a safe drug cannot cause injury to the plasma membrane of healthy cells, either by forming pores or breaking down the cell, evaluated the cytotoxic activity of triazoles on human erythrocytes. On the other hand, Ralph et al. (2009) evaluated the cytotoxicity of synthetic naphthoquinones on human erythrocytes, demonstrating the possibility of its use for therapeutic purposes, since it had no cytotoxicity on the human erythrocyte membrane.

The hemolytic activity test was also used by Maia et al. (2009), who evaluated the hemolytic activity of dry extract from the bark of *Maytenus guianensis*, verifying that this species did not cause haemolysis on human erythrocytes and may be used for pharmacological purposes.

Furthermore, Schulz et al. (2005) found positive values of the cytotoxic effect from crude extract of *Bacillus amyloliquefaciens* against sheep erythrocytes.

Vieira et al. (2002) in turn, using the hemolytic activity test to investigate the cytotoxic outcome of chloroform on human lymphocytes, found results that do not prove the cytotoxic action of chloroform, but its genotoxic con-sequences, since it is capable of causing DNA damage without affecting the normal activity of cells.

Laranjeira et al. (2010) with the purpose of evaluating the hemolytic activity of ethanol extract from *Croton grewioides* leaves on erythrocytes from mice, found results that prove the absence of hemolytic activity on erythrocytes from these animals, suggesting that the cytotoxicity of the extract under analysis was not related to membrane damage, but rather related to apoptosis.

A study by Pita (2010) evaluated the cytotoxicity of natural products utilized in therapy against cancer, obtained from essential oil of *X. langsdorffiana* leaves (trachylobano-360 and OEX) on erythrocytes from mice. The author found values that show the reduced cytotoxic activity of these products.

Cazarini et al. (2004) points out that the *in vitro* alternative tests validated and accepted with regulatory purposes in substitution to methods performed on animals, are still much more a goal than a reality.

The scarcity of literature data to discuss the results and evaluation of acute cytotoxicity *in vitro*, reasserts the need for scientific research of this nature considering that they contribute greatly towards the safe use of such substances by humans.

Results derived from this experiment suggest that this mushroom extract has very low toxicity proving to be safe for human use. Further study on the safety of using

mushroom are needed, since *A. sylvaticus* has now been used for several diseases, including in therapy against cancer.

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ARTIGO 7 – ARTIGO ORIGINAL

Versão aprovada para publicação em inglês.

Cytotoxicity of *Agaricus sylvaticus* in non-tumor cells (NIH/3T3) and tumor (OSCC-3) using Tetrazolium (MTT) assay. Orsine JVC, Brito LM, Silva RC, Santos MFMA, Novaes MRCG. Aprovado para publicação na revista Nutr Hosp 2013.

8 ARTIGO ORIGINAL

CYTOTOXICITY OF *A. sylvaticus* IN NON-TUMOR CELLS (NIH/3T3) AND TUMOR (OSCC-3) USING TETRAZOLIUM (MTT) ASSAY

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

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 (NIH/3T3) 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 NIH/3T3 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.

Keywords: toxicity, food safety, *Agaricus sylvaticus*

8.1 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.

8.2 MATERIALS AND METHODS

8.2.1 Obtaining the sample

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

8.2.2 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 $mg.ml^{-1}$.

8.2.3 In vitro study

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

8.2.4 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 x 10^5 cells in 75 cm^2 culture flasks, maintained in an incubator at 37 $^{\circ}C$ 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 1000 rpm for three minutes, using Neubauer counting chamber and transferred to a new culture flask.

8.2.5 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.

8.2.6 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).

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

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).

8.2.7 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 $p < 0.05$.

8.3 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 mushroom *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.

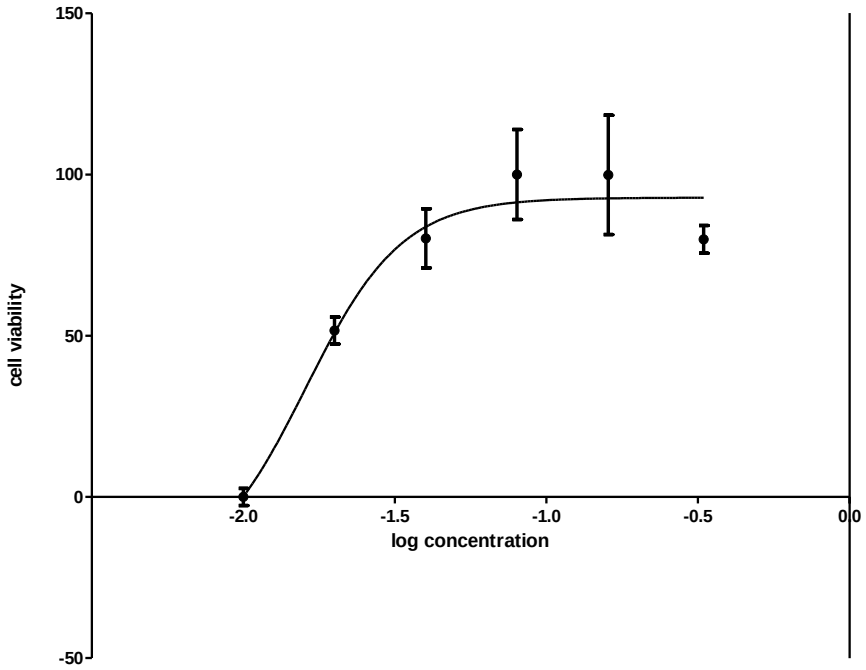

Figure 1. Toxicity of mushroom A. sylvaticus in OSCC-3 cells by the MTT assay at concentrations 0.01, 0.02, 0.04, 0.08, 0.16, 0.33 mg.ml^{-1}.

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.

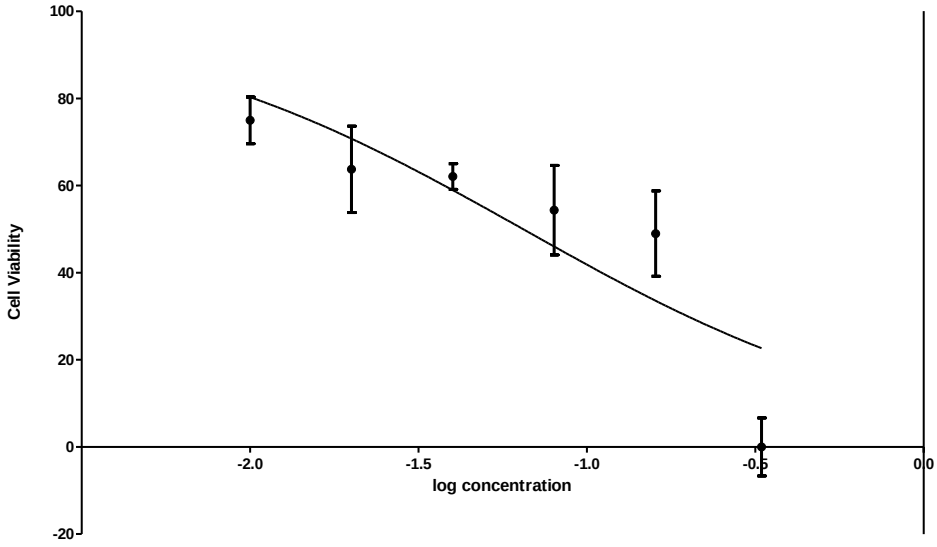

Figure 2. Toxicity of mushroom *A. sylvaticus* in NIH/3T3 cells by the MTT assay at concentrations 0.01, 0.02, 0.04, 0.08, 0.16, 0.33 mg.ml^{-1}.

8.4 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 1 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.

Table 1. 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	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	We obtained the CL_{50} value of 9.213mg/mL, indicating the very low toxicity of the mushroom *A. sylvaticus* on human erythrocytes, proving to be safe for consumption.

				human erythrocytes.	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 (EC_{50}).	
Savić 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 48h 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	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.

					10% and then transferred to nitrocellulose membranes, where tests were developed using enhanced chemiluminescence system (ECL) Western blot method.	
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 (GgLC) or from solid culture (GgWG), 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.
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 2000mg/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 2.000mg/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	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 obtained	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.

				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	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 from1 μg/mL and 50 μg/mL, to determine immunostimulatory effects, and concentrations between 50 μg/mL and 350 μg/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.	
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	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.

					added to ten nauplii. After 24 hours of exposure, the dead nauplii were counted in each test tube. Five replicates were performed by dilution.	
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.
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 dissolving. 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 R*attus 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 120s 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 15s ($p < 0.01$) and in HR at 30s ($p < 0.001$) and the 5.00 mg/kg decreased the MAP at 15s to ($p < 0.001$) and HR at 15 and 30s ($p < 0.001$). 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 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.
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 20g of dried mushroom and ground into 200mL 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	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.

					dehydrated and crushed, dissolved in dimethylsulfoxide (DMSO) at a ratio of 5mg/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.	
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 (2654 mg/kg body weight/day to male rats and 2965 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.

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).

8.5 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.

8.6 REFERENCES

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ARTIGO 8 – ARTIGO ORIGINAL

Versão em fase de tradução para posterior publicação.

Genotoxicidade e antigenotoxicidade do cogumelo *Agaricus sylvaticus* em *Drosophila melanogaster* por meio do teste de mutação e recombinação somáticas (SMART) e em *Mus musculus* (Swiss Webster) por meio do teste do micronúcleo. Orsine JVC, Oliveira, AC, Silva CR, Guimarães NN, Silva KC, Chen Chen L, Novaes MRCG. Em fase de tradução para publicação.

9 ARTIGO ORIGINAL

GENOTOXICIDADE E ANTIGENOTOXICIDADE DO COGUMELO *Agaricus sylvaticus* EM *Drosophila melanogaster* POR MEIO DO TESTE DE MUTAÇÃO E RECOMBINAÇÃO SOMÁTICAS (SMART) E EM *Mus musculus* (Swiss Webster) POR MEIO DO TESTE DO MICRONÚCLEO.

Resumo

O objetivo deste trabalho foi avaliar os efeitos genotóxicos e angenotóxicos do cogumelo *Agaricus sylvaticus* (cogumelo do sol). O estudo é experimental, laboratorial, *in vivo.* Para o Teste de Mutação e Recombinação Somáticas (SMART) foram usadas diferentes concentrações do extrato aquoso não fracionado do cogumelo (31,25; 62,5; 125,0 e 200 $mg.mL^{-1}$), como controle negativo foi usada a água destilada e como controle positivo foi usada a mitomicina-C.Os tricomonas presentes nas asas dos indivíduos adultos de *Drosophila melanogaster* foram analisados para identificar e quantificar as alterações fenotípicas. Os resultados de cada experimento foram testados pelo teste binomial condicional ($p \leq 0,05$), no qual os dados dos diferentes tratamentos foram comparados com o controle negativo (água destilada). Para o teste do micronúcleo em medula óssea de camundongos da espécie *Mus musculus* (Swiss Webster), os animais foram tratados com três diferentes doses do extrato do cogumelo (100, 200 e 300 mg / Kg de peso corpóreo) enquanto que para a avaliação da atividade antimutagênica foram administradas as mesmas doses (100, 200 e 300 mg /Kg de peso corpóreo) concomitantemente a uma dose de 4 mg / Kg de mitomicina-C p.c., seguido da avaliação da genotoxicidade através da medição da freqüência de eritrócitos policromáticos micronucleados (EPCMN). Os resultados obtidos no teste SMART demonstram que o cogumelo *A. sylvaticus* possui fraco efeito antimutagênico em todas as concentrações testadas, além de não apresentar efeito mutagênico em células somáticas de *D. melanogaster*. Por meio do teste do micronúcleo pôde-se observar que todas as doses do extrato aquoso do cogumelo *A. sylvaticus* aumentaram significativamente o número de EPCMN ($p \leq 0,05$) dos animais quando comparados com o grupo controle negativo, e que todas as doses administradas aos animais reduziram significativamente a freqüência de EPCMN, em relação ao grupo controle positivo ($p \leq 0,05$). Os resultados dos experimentos *in vivo* sugerem que o cogumelo *A. sylvaticus*

apresentou efeito Janus, sendo evidenciadas ambas as atividades genotóxica e antigenotóxica do cogumelo nas concentrações testadas, superiores a dose letal em animais.

Palavras-chave: mutagenicidade; antimutagenicidade; cogumelo do sol.

9.1 INTRODUÇÃO

Os corpos de frutificação e o micélio de cogumelos possuem elevado valor nutricional, podendo ser utilizados como ingredientes na formulação de diversos alimentos, incluindo os alimentos funcionais (Ulziijargal e Mau, 2011).

O cogumelo *Agaricus sylvaticus* (*A. sylvaticus*) cultivano em Minas Gerais, em 2010, apresentou, na forma desidratada, 42,16% de proteínas; 6,6% de lipídios, 36,21% de carboidratos; 2,34% de fibras; 7,38% de minerais e 6,31% de água, além da presença significativa de ácido ascórbico, ferro, potássio e zinco (Costa et al., 2011; Orsine et al., 2012a) . Por seu crescente uso popular, pode ser encontrado na forma de chás, cápsulas, tabletes, pó (Santa, 2010) ou até mesmo ser utilizado nas práticas dietéticas, por apresentar fragrância adocicada e excelente textura (Bellini et al., 2003).

É comum o uso empírico e sem prescrição médica de produtos naturais, como os cogumelos, com fins medicinais pela população por acreditarem que são isentos de efeitos nocivos ou efeitos adversos à saúde humana (Silva et al., 2005).

A segurança alimentar relacionada ao consumo de cogumelos medicinais tem sido amplamente estudada em diversos tipos de cogumelos, utilizando-se diferentes testes. Orsine et al. (2012b) avaliaram a toxicidade do cogumelo *A. sylvaticus* em eritrócitos humanos e verificaram que este cogumelo apresenta baixíssima toxicidade. Novaes et al. (2007) avaliaram os efeitos de toxicidade aguda do extrato aquoso do *A. sylvaticus*, mediante parâmetros clínicos, bioquímicos e histopatológicos em ratos saudáveis e observaram que administração de *A. sylvaticus* em doses superiores às usadas nos protocolos terapêuticos em humanos, apresentou toxicidade muito baixa. Já o cogumelo *Agaricus brasiliensis*, testado por Masuno e Ohno (2012), não apresentou toxicidade nos testes de mutação reversa bacteriana, ensaio de micronúcleo em ratos, e no teste de linfoma de ratos. Porém, em pesquisa conduzida por Mantovani et al. (2006), os autores observaram atividade genotóxica e clastogênica do cogumelo

Agaricus blazei através do teste cometa, sugerindo cuidado na ingestão do chá do cogumelo, em elevada concentração. Bellini et al. (2008) também atentaram a população quanto aos cuidados acerca da ingestão do extrato metanólico do *A. blazei*, por ter apresentado em seus estudos efeito genotóxico.

As substâncias contidas na composição química de cogumelos (Bellini et al., 2008) podem estar relacionadas a atividades mutagênicas, teratogênicas e/ou carcionogênicas. Uma vez presentes, os componentes genotóxicos podem intercalar-se com a molécula de DNA, provocando danos genéticos em regiões muito importantes para o controle do ciclo celular e apoptose, favorecendo ou acelerando o processo neoplásico, tornando-se necessárias avaliações toxicológicas e genotóxicas em compostos naturais para assegurar o uso alimentar e terapêutico em seres humanos (Santos et al., 2008).

O objetivo deste trabalho foi avaliar os efeitos genotóxicos e antigenotóxicos do extrato aquoso do cogumelo *A. sylvaticus* em *D. melanogaster* utilizando-se o teste SMART, e em eritrócitos policromáticos da medula óssea de animais da espécie *Mus musculus* (Swiss Webster) através do teste do micronúcleo em medula óssea.

9.2 MATERIAL E MÉTODOS

9.2.1 Obtenção das amostras e preparação do extrato

As amostras do cogumelo *A. sylvaticus* desidratado foram obtidas de um produtor do Estado de Minas Gerais. Procedeu-se a moagem do cogumelo em moinho tipo Wiley, com posterior pesagem. Adicionou-se água, e após 30 minutos sob agitação, filtrou-se o material. O extrato aquoso obtido foi desidratado em estufa a 105ºC, obtendo-se um concentrado.

Para o teste SMART, as concentrações do extrato aquoso não fracionado do cogumelo *A. sylvaticus* preparadas foram: 31,25 $mg.mL^{-1}$; 62,5 $mg.mL^{-1}$; 125 $mg.mL^{-1}$; 250 $mg.mL^{-1}$; 500 $mg.mL^{-1}$ e 1000 $mg.mL^{-1}$.

Para o teste do micronúcleo, foram preparadas três concentrações distintas: 100, 200 e 300 mg⁄Kg de peso corpóreo do animal.

9.2.1 Teste SMART

9.2.1.1 Obtenção das larvas de D. melanogaster

As larvas foram obtidas no estoque do laboratório de Toxicologia Genética do Instituto de Biologia da Universidade Federal de Goiás, UFG. Foi utilizado o cruzamento padrão (ST) do teste SMART, com larvas de terceiro estágio, originadas do cruzamento entre linhagens mutantes de *D. melanogaster* (machos mwh e fêmeas virgens *flr*^{3}). As moscas, representadas por 80 fêmeas e 40 machos para cada cruzamento, foram mantidas por três dias em vidros contendo meio de cultura padrão, elaborado a partir de farinha de milho (75mL), açúcar (67,5mL), fermento biológico (37,5g), água (750mL), ágar (7,5mL) e antifúngico (3,75mg). Após este período, os casais foram transferidos para frascos contendo meio de ovoposição, elaborado a partir de fermento biológico fresco, onde permaneceram por oito horas, sendo em seguida descartados. Após 72 ±4 horas do início do período de ovoposição, foi realizada a coleta das larvas de terceiro estágio, com auxílio de água corrente.

Neste cruzamento padrão foram produzidos dois tipos de progênie:

- Indivíduos trans-heterozigotos para os genes marcadores (MH), com constituição genotípica *mwh* + / + *flr*^{3};
- Indivíduos heterozigotos para o cromossomo TM_3 (BH), constituídos por mwh + / + TM_3, Bd^S.

9.2.1.2 Teste de sobrevivência de D. melanogaster

Inicialmente foram preparados tubos contendo 900mg de purê de batata desidratado. Em cada tubo, foram adicionadas 100 larvas para o teste de sobrevivência, e 3mL das diferentes concentrações do extrato aquoso não fracionado do cogumelo *A. sylvaticus* previamente preparado. Para o controle negativo, utilizou-se um tubo de tratamento contendo o purê de batata e 3mL de água.

As larvas permaneceram em tratamento por aproximadamente dez dias, o que caracteriza o tratamento crônico do ensaio, até atingirem o estágio de pupa. Os adultos que eclodiram das pupas após os dez dias de tratamento, foram contados e conservados

em álcool 70%, até a montagem das lâminas. O número de moscas sobreviventes por tratamento forneceu uma indicação da toxicidade do extrato (DL_{70}).

9.2.1.3 Atividade mutagênica e antimutagênica

Para avaliação das atividades mutagênica e antimutagênica, foram realizados os mesmos procedimentos descritos anteriormente. Porém foram utilizadas somente as concentrações do extrato do cogumelo *A. sylvaticus* que apresentaram crescimento maior que 30 moscas no teste de sobrevivência (DL_{70}).

Para o teste de antimutagenicidade do *A. sylvaticus* foi utilizado, como controle positivo, a mitomicina-C (MMC, *Bristol-Myers Squibb*), substância conhecidamente mutagênica, na concentração de 0,02mM, adicionada concomitantemente ao extrato aquoso não fracionado do cogumelo *A. sylvaticus* em diferentes concentrações.

9.2.1.4 Análise microscópica e avaliação tóxico-genética

Para avaliação das atividades mutagênica e antimutagênica, foram utilizados quarenta indivíduos de *D. melanogaster* mwh para cada análise realizada, sendo que 50% pertenciam ao sexo feminino e 50% ao sexo masculino, para cada concentração do extrato do cogumelo. Porém, quarenta indivíduos de *D. melanogaster* flr^3 também foram utilizados na avaliação da atividade antimutagênica, no sentido de avaliar a atividade recombinogênica do extrato.

As lâminas das asas dos adultos tratados foram montadas utilizando-se solução de Faure [goma arábica (30g), glicerol (20mL), água (50mL) e hidrato de cloral (50g)] e, após secagem, foram analisadas em microscópio óptico com aumento de 400 vezes (Graf et al., 1984).

A análise dos tricomas, presente nas superfícies dorsal e ventral das asas, permitiu a identificação de manchas de pêlos mutantes que podem ser classificadas como:

- Simples pequenas (com uma ou duas células mutantes) ou simples grandes (com três ou mais células mutantes): expressando o fenótipo mutante *mwh* ou flr^3, indicando a ocorrência de mutações gênicas, alterações cromossômicas e recombinação mitótica;

- Gêmeas: formadas por células adjacentes *mwh* e *flr*^{3}, originadas exclusivamente por recombinação, o que significa que este tipo de mancha pode fornecer indicações da ação recombinogênica do extrato aquoso não fracionado do cogumelo *A. sylvaticus*.

9.2.1.5 Análise estatística para o teste SMART

Foi realizada uma comparação entre as concentrações do extrato aquoso não fracionado do cogumelo *A. sylvaticus* e o controle negativo, quando pôde ser observada se havia ou não diferença significativa ($p \leq 0,05$) na ocorrência de manchas de pelos mutantes. Foi utilizado o teste binomial condicional, de acordo com metodologia proposta por Frei e Würgler (1988).

9.2.3 Teste do micronúcleo

O procedimento experimental seguiu metodologia proposta por Schmid (1975). Foram utilizados 80 animais da espécie *Mus musculus* (Swiss Webster) *out bred*, do sexo masculino, com peso médio de 35g, idade de sete a 12 semanas, procedentes do Biotério Central da Universidade Federal de Goiás. Os animais foram divididos em 16 grupos com cinco animais por grupo, conforme demonstrado na Tabela 1.

Tabela 1. Condições experimentais dos testes de genotoxicidade e antigenotoxicidade do cogumelo *A. sylvaticus* em camundongos *Mus musculus*.

Genotoxicidade	**Antigenotoxicidade**
Controle negativo	Controle negativo
Controle positivo (MMC-c)	Controle positivo (MMC-c)
100 mg / Kg p. c. (24h)	100 mg / Kg p. c. + MMC-c (24h)
100 mg / Kg p. c. (48h)	100 mg / Kg p. c. + MMC-c (48h)
200 mg / Kg p. c. (24h)	200 mg / Kg p. c. + MMC-c (24h)
200 mg / Kg p. c. (48h)	200 mg / Kg p. c. + MMC-c (48h)
300 mg / Kg p. c. (24h)	300 mg / Kg p. c. + MMC-c (24h)
300 mg / Kg p. c. (48h)	300 mg / Kg p. c. + MMC-c (48h)

* Para cada experimento (genotoxicidade e anti-genotoxicidade) foram avaliadas três doses do extrato aquoso não fracionado do cogumelo *A. sylvaticus*.
** Foram utilizados cinco animais por grupo, totalizando 80 animais.
*** Dose padrão de mitomicina-c: 4 mg / Kg p.c..

O projeto de pesquisa foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Goiás, e seguiram-se os princípios de boas práticas laboratoriais e monitoramento dos testes utilizando-se substâncias químicas da OECD (*Organization for Economic Cooperation and Development Council*).

Os animais foram mantidos em gaiolas de polipropileno, devidamente identificadas, por sete dias que antecedeu o experimento visando à ambientação dos animais. As gaiolas, de dimensão de 40x30x16 cm com cinco animais cada, eram forradas com maravalha trocadas diariamente.

Os animais foram identificados individualmente em cada gaiola através da pintura de parte da cauda com símbolos diferentes, com tinta atóxica e resistente a água. Os grupos experimentais e controles permaneceram sob idênticas condições ambientais, em lugar arejado e em temperatura ambiente (equivalente à média local para a época do ano de aproximadamente 25ºC), no escuro e à luz artificial durante ciclos alternados de 12 horas, alimentados com ração comercial (Albina, Ecibra Ltda) e água filtrada, ambos oferecidos "*ad libitum"*.

Para avaliação da atividade mutagênica do cogumelo *A. sylvaticus*, grupos distintos contendo cinco animais, conforme descrito na Tabela 1, foram submetidos via oral, com procedimento de gavagem esofágica, a administração do extrato aquoso do cogumelo no período de 24 e 48 horas, conforme protocolo n. 474 da OECD (Guideline for the testing of chemicals). O grupo controle negativo foi tratado com água destilada filtrada e o grupo controle positivo recebeu uma dose padrão de mitomicina-C (MMC – c), de 4 mg / Kg p.c..

Para avaliação da antimutagenicidade foram administradas as mesmas concentrações do extrato aquoso do cogumelo *A. sylvaticus*, concomitantemente com a mesma dose de MMC–c do controle positivo. Os animais foram sacrificados por deslocamento cervical e os respectivos fêmures foram retirados. A medula óssea foi lavada com 1mL de soro fetal bovino na temperatura de 37°C. Após homogeneização da medula no soro, esta foi centrifugada a 1000 rpm durante cinco minutos. O sobrenadante foi parcialmente descartado. O precipitado de células foi homogeneizado com pipeta Pasteur. Uma gota de suspensão celular foi transferida para a lâmina de vidro onde foi feito o esfregaço celular. Após secagem das lâminas, estas foram fixadas em metanol absoluto durante cinco minutos e coradas em soluções de Giemsa tamponada com pH 6,8 por um período de 15 minutos (Heddle, 1973). Após este

período, as lâminas foram lavadas em água corrente e deixadas secar em temperatura ambiente.

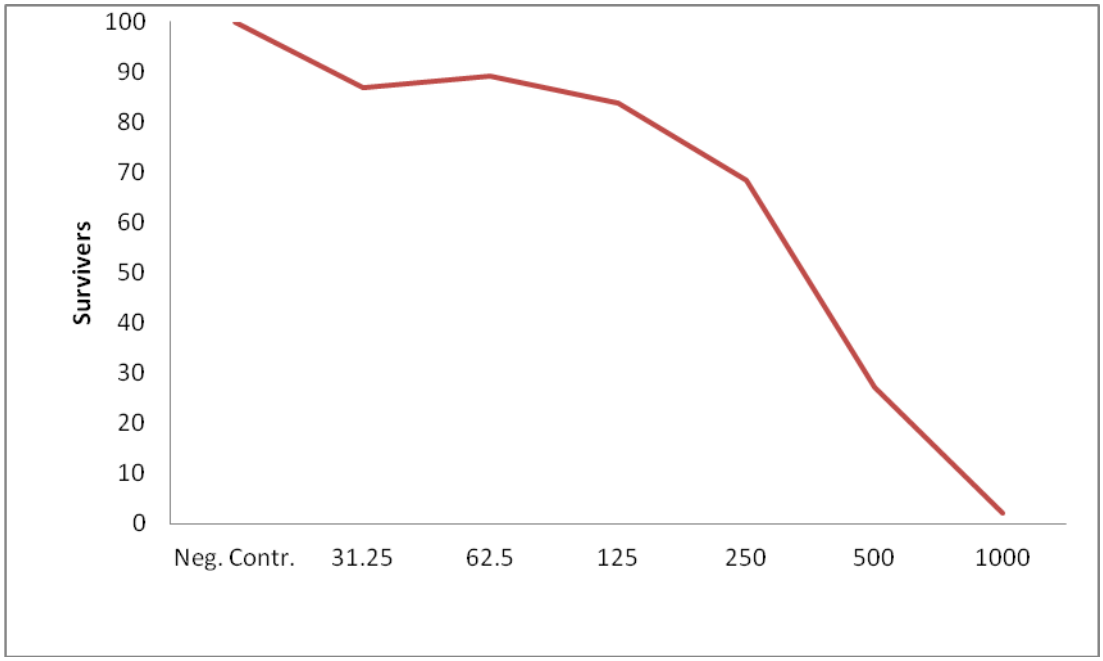
A análise das lâminas foi realizada em microscópio óptico comum (Olympus BH-2) com a finalidade de se detectar possíveis alterações e/ou perdas cromossômicas (micronúcleos) nos eritrócitos da medula óssea dos animais submetidos aos diferentes tratamentos. As freqüências de eritrócitos policromáticos micronucleados (EPCMN) em 2000 eritrócitos policromáticos (EPC) de cada animal de cada grupo foram comparadas em relação ao grupo controle negativo ou positivo pelo teste qui quadrado e foram considerados significativos valores de $p \leq 0,05$.

9.3 RESULTADOS E DISCUSSÃO

9.3.1 Teste SMART

9.3.1.1 Curva de Sobrevivência

Foi observada a toxicidade das concentrações de 500 $mg.mL^{-1}$ e 1000 $mg.mL^{-1}$ do extrato aquoso não fracionado do cogumelo *A. sylvaticus,* uma vez que apresentaram crescimento de indivíduos em número menor que 30, conforme Figura 1. Dessa forma, estas concentrações não foram utilizadas para a realização dos testes de mutagenicidade e antimutagenicidade.



*n = 100 **DL_{70}

Figura 1. Curva de sobrevivência de *Drosophila melanogaster* no meio depurê de batata desidratado adicionado de diferentes concentrações do extrato aquoso não fracionado do cogumelo *A. sylvaticus*.

Através da Figura 1, observa-se que as concentrações de 31,25, 62,5, 125 e 250mg.mL^{-1} não comprometeram o desenvolvimento de larvas de *D. melanogaster*, sugerindo que o cogumelo não apresentoum toxicidade nas condições experimentais testadas.

9.3.1.2 Atividade mutagênica

Os resultados obtidos para o extrato aquoso não fracionado do cogumelo *A. sylvaticus* foram comparados a frequência de manchas mutantes de cada grupo tratado (31,25; 62,5; 125 e 250 mg.mL^{-1}) com o controle negativo, no qual foi utilizada apenas água destilada.

Não foi observado aumento estatisticamente significativo nas frequências totais de manchas mutantes dos indivíduos trans-heterozigotos tratados com as diferentes concentrações do extrato, o que indica que o extrato do cogumelo testado não induz alterações no DNA de células somáticas de *D. melanogaster* relacionadas com mutação

e/ou recombinação, não apresentando, portanto, efeito mutagênico, conforme mostrado na Tabela 2. Por não apresentar genotoxicidade, não foi necessária a avaliação dos indivíduos do genótipo *mwh*/TM3.

Tabela 2. Avaliação da mutagenicidade e/ou efeitos recombinogênicos do extrato aquoso do cogumelo *Agaricus sylvaticus* em células somáticas de larvas de *Drosophila melanogaster* de cruzamento padrão.

Genótipos e concentração	Número de indivíduos (N)	Manchas por indivíduo (nº de manchas) Diagnóstico estatístico[a]				Total manchas *mwh*[cd]
		MSP (1-2 cels)[b] m = 2	MSG (> 2 cels)[b] m = 5	MG m = 5	TM m = 2	n = 5
			A. sylvaticus mwh/flr^3			
Cont. Negativo	40	0,40 (16)	0,13 (05)	0,00 (00)	0,53 (21)	21
31,25	40	0,20 (08) -	0,18 (07) i	0,00 (00) i	0,38 (15) -	13
62,5	40	0,23 (09) -	0,05 (02) -	0,00 (00) i	0,28 (11) -	11
125	40	0,23 (09) -	0,08 (03) i	0,00 (00) i	0,30 (12) -	11
250	40	0,33 (13) -	0,03 (01) -	0,00 (00) i	0,35 (14) -	14

[a] Diagnóstico estatístico de acordo com Frei e Würgler (1988): +, positivo; -, negativo; i, inconclusivo. m, fator de multiplicação para a avaliação de resultados significativamente negativos. Níveis de significância 0,05. [b] Incluindo manchas simples *flr*^{3} raras. [c] Considerando os clones mwh para as manchas simples mwh e para as manchas gêmeas. [d] C = 48.000, isto é, número aproximado de células examinadas por indivíduo. [*] Calculado de acordo com Frei et al. (1992). [*] Apenas manchas simples mwh podem ser observadas nos indivíduos heterozigotos mwh/TM3, já que o cromossomo balanceador TM3 não contém o gene mutante *flr*^{3}. *MSP: manchas simples pequenas; MSG: manchas simples grandes; MG: manchas gêmeas; TM: total de manchas. ** Controle negativo: utilizado apenas água destilada.

9.3.1.3 Atividade antimutagênica

Para realização da análise dos resultados obtidos para o extrato aquoso não fracionado do cogumelo *A. sylvaticus*, foi comparada a frequência de manchas mutantes de cada grupo tratado (31,25; 62,5; 125; 250 mg.mL^{-1} e controle positivo) com o controle negativo, no qual foi utilizada apenas água destilada. O teste de antimutagenicidade possibilita verificar se o extrato em avaliação é capaz de bloquear ou alterar mutações em células somáticas, que poderiam conduzir à formação de neoplasias. Caso isso acontecesse, o extrato poderia ser utilizado na terapia preventiva para indivíduos em tratamento anticâncer, já que o extrato apresentaria proteção contra os efeitos colaterais de mutações em células normais, provocados por radiação ou drogas.

O resultado da análise antimutagênica mostra efeito fraco positivo do extrato sobre a indução de manchas simples grandes (MSG) em todas as concentrações e sobre as manchas simples pequenas (MSP) nas três menores concentrações (31,25; 62,5; 125 $mg.mL^{-1}$). Este resultado indica que o extrato do cogumelo *A. sylvaticus* foi capaz de inibir a manifestação de eventos genotóxicos nas células, desde os primeiros ciclos até o final das divisões mitóticas dos discos imaginais das asas. O diagnóstico fraco positivo (f+) indica que o efeito foi moderado, ou seja, não foi *m* vezes maior que o apresentado pelo controle negativo, de acordo com as análises estatísticas definidas por Frei e Würgler (1988) para o teste de SMART. Com relação às manchas gêmeas (MG), apenas na concentração de 62,5 $mg.mL^{-1}$ foi diagnosticado o resultado fraco positivo (f+). No total de manchas todas as concentrações demonstraram moderada atividade antimutagênica.

Uma vez que os indivíduos *mwh*/TM_3 apresentaram diagnóstico negativo, pode-se inferir que a atividade do extrato se dá sobre os eventos recombinogênicos, como pode ser observado na Tabela 3.

Tabela 3. Avaliação dos efeitos antimutagênicos e/ou antirecombinogênicos do extrato aquoso do cogumelo *Agaricus sylvaticus* em células somáticas de larvas de *Drosophila melanogaster* procedentes de cruzamento padrão.

Genótipos e concentração	**N. de indivíduos**	**Manchas por indivíduo (nº de manchas) Diagnóstico estatístico[a]**				**Total manchas *mwh*[cd]**	**Recom-binação (%)**
	(N)	**MSP (1-2 cels)[b] m = 2**	**MSG (> 2 cels)[b] m = 5**	**MG m = 5**	**TM m = 2**	**n = 5**	
A. sylvaticus mwh/flr³							
C. Negativo	40	5,58 (223)	17,30 (692)	7,40 (296)	30,28 (1211)	1211	
31,25 + MMC-c	40	8,45 (338)f+	21,08 (843) f+	7,48 (299) -	37,00 (1480) f+	1480	93,65
62,5 + MMC-c	40	7,10 (284) f+	20,43 (817) f+	8,58 (343) f+	36,10 (1444) f+	1444	93,49
125 + MMC-c	40	7,83 (313) f+	19,03 (761) f+	7,75 (310) -	34,60 (1384) f+	1384	97,85
250 + MMC-c	40	6,35 (254) -	19,05 (762) f+	8,33 (333) -	33,73 (1349) f+	1349	87,87
C. Positivo (MMC-c)	40	0,13 (0,5) -	0,03 (01) -	0,03 (01) -	0,18 (07) -	07	
mwh/TM3							
C. Negativo	40	1,28 (51)	1,68 (67)	*	2,95 (118)	118	*
31,25 + MMC-c	40	0,90 (36) -	1,58 (63) -		2,48 (99) -	99	
62,5 + MMC-c	40	1,55 (62) -	1,70 (68) -		3,25 (130) -	130	
125 + MMC-c	40	1,35 (54) -	1,55 (62) -		2,90 (116) -	116	
250 + MMC-c	40	1,73 (69) -	1,55 (62) -		3,28 (131) -	131	
C. Positivo (MMC-c)	40	0,28 (11) -	0,05 (02) -		0,33 (13) -	13	

[a] Diagnóstico estatístico de acordo com Frei e Würgler (1988): +, positivo; -, negativo; i, inconclusivo. m, fator de multiplicação para a avaliação de resultados significativamente negativos. Níveis de significância 0,05. [b] Incluindo manchas simples *flr*^{3} raras. [c] Considerando os clones mwh para as manchas simples mwh e para as manchas gêmeas. [d] C = 48.000, isto é, número aproximado de células examinadas por indivíduo. [*] Calculado de acordo com Frei et al. (1992). [*] Apenas manchas simples mwh podem ser observadas nos

indivíduos heterozigotos mwh/TM3, já que o cromossomo balanceador TM3 não contém o gene mutante *flr*^{3}. **MSP: manchas simples pequenas; MSG: manchas simples grandes; MG: manchas gêmeas; TM: total de manchas. *** Controle negativo: utilizado apenas água destilada; Controle positivo: utilizado o extrato do cogumelo *A. sylvaticus* concomitantemente à mitomicina-c.

Como pode ser observado na Tabela 2, o aumento da concentração do extrato aquoso não fracionado do cogumelo *A. sylvaticus* não induziu aumentos significativos nas frequências totais de manchas mutantes. Segundo Ribeiro et al. (2003), o efeito mutagênico é a conseqüência de danos genéticos causados por agentes físicos, químicos e biológicos, induzido por mutações nas células de um organismo.

A análise de mutagenicidade do extrato aquoso não fracionado do cogumelo *A. sylvaticus*, mostrou que as freqüências de manchas mutantes das séries tratadas ficaram abaixo das freqüências espontâneas induzidas pelo controle negativo (Tabela 2). Isto indica que além de não exercer efeito mutagênico, o extrato pode estar interferindo em algum mecanismo protetor do metabolismo celular, inibindo a indução de eventos mutacionais. Deste modo, procedeu-se com a análise de antimutagenicidade para verificar se o extrato possui esta ação.

As mutações estão sempre ocorrendo em um organismo, no qual são conhecidas como as recombinações genéticas, capacidade natural do DNA de se recombinar com outras moléculas. Por serem realizadas naturalmente, não são chamadas de mutações (Silva et al., 2003). Ao contrário destas, estão as mutações causadas por fatores exógenos ou endógenos que podem ser classificadas como gênicas, quando referem-se a mudanças de uma ou poucas sub-unidades do DNA, alterando apenas o funcionamento de um gene, por substituição, perda ou ganho destas sub-unidades. Podem ser também do tipo cromossômicas quando há reorganização dos cromossomos, por translocação, inversão, ou mesmo ganho ou perda de parte maior destes cromossomos (Ghiffiths et al., 2002).

O aumento do contato da população com novos compostos sintéticos ou naturais obtidos a partir de plantas ou fungos indica que é preciso avaliar estes compostos devido aos possíveis efeitos genotóxicos, mutagênicos e carcinogênicos (Barret, 1993).

Costa e Nepomuceno (2003) utilizaram o teste SMART para avaliar o efeito genotóxico do chá de *A. blazei* em *D. melanogaster*. Os autores não observaram aumento, estatisticamente significativo, nas freqüências de manchas mutantes, em larvas expostas ao chá de *A. blazei, na concentração* de 62,5 g.L^{-1}, demonstrando que este cogumelo não apresenta efeito genotóxico. Porém, quando o chá do cogumelo *A. blazei*

foi associado ao uretano, os autores observaram uma redução estatisticamente significativa nas freqüências das manchas mutantes. Dessa forma, os resultados encontrados por estes autores corroboram com os resultados do presente estudo, uma vez que o cogumelo *A. blazei* exerceu um efeito protetor contra a ação genotóxica do uretano.

Postemsky et al. (2011) avaliaram os efeitos protetivos do cogumelo medicinal *Grifola gargal* Singer após indução de dano no DNA provocado por DMBA (7-12-dimethyl-benz(α)anthracene), em *D. melanogaster*, utilizando-se o teste SMART. A adição dos extratos de *G. gargal* produziram efeito protetivo quando administrados concomitantemente a 25 μmol de DMBA. Pelo teste SMART pôde ser observado que o cogumelo *G. gargal*, além de não ter apresentado toxicidade, quando em combinação de 25 μmol/vial DMBA reduziu a mortalidade induzida pelo pró-mutagênico, mostrando efeito antigenotóxico, assim como o cogumelo *A. sylvaticus* do presente estudo, que não se mostrou genotóxico, e exerceu uma fraca atividade antigenotóxica sobre a mitomicina-c.

Em estudo realizado por Rodrigues et al. (2003), os autores não utilizaram nenhuma substância mutágena em pesquisa sobre os efeitos antimutagênicos do cogumelo *A. blazei* no sistema metionina em *Aspergillus nidulans*, analisando apenas mutações espontâneas. Os autores verificaram que o cogumelo preparado em temperatura ambiente foi capaz de reduzir a frequência de mutação espontânea, apresentando efeito bioantimutágeno, com ação em um ou mais sistemas de reparo de danos no DNA. No presente trabalho, foram utilizadas temperaturas de 100ºC para o preparo do extrato aquoso do cogumelo *A. sylvaticus*, nas concentrações conhecidas. Segundo Delmanto et al. (2001), a temperatura de preparo pode influenciar a eficiência do cogumelo, pois quando muito elevadas são capazes de afetar os princípios ativos e, desta maneira, o cogumelo pode não se mostrar tão eficiente como deveria.

9.3.2 Teste do micronúcleo

Na Tabela 4 foram apresentados os resultados dos testes de genotoxicidade e antigentoxicidade do extrato aquoso não fracionado do cogumelo *A. sylvaticus*, em células de medula óssea de camundongos.

Tabela 4. Efeito da administração do extrato do cogumelo *Agaricus sylvaticus* por gavagem esofágica em animais da espécie *Mus musculus* (Swiss Webster) e controles.

Tratamentos	Tempo (h)	Eritrócitos policromáticos micronucleados		EPC/ ENC	Atividade mutagênica	Atividade citotóxica
		Dados individuais	$\bar{X} \pm S$			
Água destilada (C-)*	24	5,4,2,4,6	4,2 ± 1,48 [a]	1,19 ± 0,09 [a]		
MMC (C+)**	24	31,35,39,32,40	35,4 ± 4,04 [b]	0,72 ± 0,05 [b]		
MMC (C+)**	48	14,12,9,13,7	11,0 ± 2,91 [b]	0,53 ± 0,04 [b]		
100 mg/Kg p.c. EAS	24	13, 18, 21, 21, 18	18,2 ± 3,27[b]	1,56 ± 0,07 [b]	+	+
200 mg/Kg p.c. EAS	24	19, 18, 21, 22, 18	19,6 ± 1,82 [b]	1,79 ± 0,27 [b]	+	+
300 mg/Kg p.c. EAS	24	24, 16, 20, 17, 20	19,4 ± 3,13 [b]	1,71 ± 0,17 [b]	+	+
100 mg/Kg p.c. EAS	48	18, 27, 17, 27, 21	22,0 ± 4,80 [b]	1,89 ± 0,42 [b]	+	+
200 mg/Kg p.c. EAS	48	23, 24, 21, 19, 25	22,4 ± 2,41 [b]	1,59 ± 0,02 [b]	+	+
300 mg/Kg p.c. EAS	48	20, 23, 17, 20, 21	20,2 ± 2,17 [b]	1,53 ± 0,12 [b]	+	+

[a] P> 0,05; [b] P< 0,05. Todos os resultados foram comparados ao grupo controle negativo. * Controle negativo: água destilada; ** Controle positivo: 4 $mg.Kg^{-1}$ p.c. de MMC. *** Em 2000 eritrócitos policromáticos por animal. **** EPC/ ENC : razão de eritrócitos policromáticos por eritrócitos normocromáticos

Pode-se observar a partir dos resultados apresentados na Tabela 4 que a atividade mutagênica exercida pelo extrato aquoso do cogumelo *A. sylvaticus* não foi dose-dependente.

A atividade antigenotóxica do extrato aquoso do cogumelo *A. sylvaticus* foi apresentada na Tabela 5.

Tabela 5. Efeito da administração do extrato do cogumelo *Agaricus sylvaticus* por gavagem esofágica + MMC i.p. em animais da espécie *Mus musculus* (Swiss Webster) e controles.

Tratamentos	Tempo (h)	Eritrócitos policromáticos micronucleados		EPC/ ENC	Atividade antimutagênica	Atividade anticitotóxica
		Dados individuais	$\bar{X} \pm S$			
Água destilada (C-)*	24	5,4,2,4,6	4,2 ± 1,48 [a]	1,55 ± 0,13 [a]		
MMC (C+)**	24	31,35,39,32,40	35,4 ± 4,04 [b]	0,72 ± 0,05 [b]		
MMC (C+)**	48	14,12,9,13,7	11 ± 2,91 [b]	0,53 ± 0,04 [b]		
100 mg/Kg p.c. EAS	24	20, 24, 20, 21, 21	21,2 ± 1,64[b]	1,55 ± 0,06 [b]	+	+
200 mg/Kg p.c. EAS	24	19, 21, 20, 18, 24	20,4 ± 2,30[b]	1,73 ± 0,16 [b]	+	+
300 mg/Kg p.c. EAS	24	24, 20, 19, 23, 18	20,8 ± 2,59[b]	1,54 ± 0,07 [b]	+	+
100 mg/Kg p.c. EAS	48	20, 18, 19, 20, 18	19,0 ± 1,00 [b]	1,59 ± 0,10 [b]	+	+
200 mg/Kg p.c. EAS	48	19, 23, 24, 22, 22	22,0 ± 1,87 [b]	1,69 ± 0,17 [b]	+	+
300 mg/Kg p.c. EAS	48	19, 24, 20, 22, 21	21,2 ± 1,92 [b]	1,45 ± 0,08 [b]	+	+

[a] P> 0,05; [b] P< 0,05. Os resultados de cada grupo foram comparados com o grupo controle positivo em concordância com o respectivo tempo. * Controle negativo: água destilada; ** Controle positivo: 4 $mg.Kg^{-1}$ p.c. de MMC. *** Em 2000 eritrócitos policromáticos por animal. MMC: Mitomicina C. EAS: Extrato de *Agaricus sylvaticus.* **** EPC/ ENC : razão de eritrócitos policromáticos por eritrócitos normocromáticos

O teste do micronúcleo é capaz de detectar danos genotóxicos em células em estágio de interfase. A presença de micronúcleos indica dano aneugênico, quando compromete todo o cromossomo, ou dano clastogênico, quando provoca a quebra do cromossomo (Doherty, 2012). Na investigação do potencial mutagênico do cogumelo *A. sylvaticus* expressa pela média das frequências de EPCMN (Tabela 4), pode-se verificar que para os tratamentos de 24 horas, os animais apresentaram uma média de 18,2; 19,6 e 19,4 EPCMN/2000 EPC para as doses de 100, 200 e 300 mg / Kg p. c. respectivamente, enquanto que o grupo controle negativo apresentou uma média de 4,2 EPCMN/2000 EPC. Sendo assim, pôde-se observar que foi possível detectar diferença significativa para todas as doses testadas ($p<0,05$), quando comparadas ao controle negativo. Porém, verificou-se que não houve diferença significativa ($p >0,05$) quando comparadas as doses de 100, 200 e 300 mg / Kg p. c.. Os mesmos resultados foram obtidos para os tratamentos de 48 horas com o extrato do cogumelo *A. sylvaticus*, quando os animais apresentaram uma média de 22,0; 22,4 e 20,2 EPCMN/2000 EPC para as doses de 100, 200 e 300 mg / Kg p. c., respectivamente. Dessa forma, também pôde-se observar que houve diferença significativa para as diferentes doses do extrato e o grupo controle negativo ($p>0,05$). A partir desses resultados, verifica-se a mutagenicidade do extrato aquoso do cogumelo *A. sylvaticus*, observando-se a importância de mais estudos relacionados às substâncias responsáveis pela ação mutagênica deste cogumelo.

O teste do micronúcleo também permite detectar o potencial citotóxico, utilizando-se a razão entre EPC/ENC. Quando a proliferação normal de células da medula óssea é afetada por um agente tóxico, ocorre uma redução do número de eritrócitos policromáticos (EPC) em relação ao número de eritrócitos normocromáticos (ENC) e a razão EPC/ENC decresce (Rabello-Gay et al., 1991). Sendo assim, os resultados encontrados na Tabela 4, para 24 horas e 48 horas após o tratamento com o cogumelo *A. sylvaticus*, mostraram atividade citotóxica em todas as doses testadas, uma vez que diferiram significativamente ($p >0,05$) do controle negativo.

Os resultados do presente estudo se diferem dos resultados encontrados por Motoi e Ohno (2012). Os autores também utilizaram o teste do micronúcleo em ratos para avaliar o efeito genotóxico do cogumelo *A. brasiliensis*, e através de seus resultados negativos para genotoxicidade em doses acima de 1 g/kg peso animal, foi sustentada a segurança de seu consumo, para fins alimentares e terapêuticos.

Os resultados apresentados na Tabela 5 podem indicar uma ação moduladora da atuação da MMC-c pelo extrato aquoso do cogumelo *A. sylvaticus*, demonstrando assim, a ação genotóxica desse fungo. Segundo Ghoneun (1995), os polissacarídios extraídos de cogumelos do gênero Agaricus aumentam as ligações β (1→6) (1→4) D-glucano, aumentando a população de linfócitos, bem como, a atividade das células NK (*Natural Killer cells*), podendo estes cogumelos ser considerados como modificadores da resposta biológica para o tratamento do câncer.

Assim como no presente trabalho, Oliveira et al. (2002), puderam verificar o efeito protetor do cogumelo *A. blazei* nos tratamentos simultâneo com metil metanosulfonato (MMS) e simultâneo com pré-incubação de extratos aquosos, no ensaio do micronúcleo em células V79, *in vitro*. Os autores fundamentaram-se em Kuroda et al. (1992), sugerindo que os extratos dos cogumelos do gênero Agaricus apresentam atividade desmutagênica, agindo através da inativação química ou enzimática da substância mutagênica.

Em pesquisa realizada por Primo et al. (2010), os autores avaliaram a ação mutagênica e antimutagênica de um biopolímero de glucose extraído da *Agrobacterium radiobacter*, que continha elevada quantidade de β-glucanas em sua composição. O agente indutor de danos no DNA utilizado pelos autores foi a ciclofosfamida. O teste de micronúcleo foi aplicado em células do sangue periférico de camundongos *Swiss* 24 e 48 horas após a aplicação das substâncias-teste. Os autores observaram que o biopolímero não possui atividade mutagênica e que é efetivo em prevenir danos no DNA. As porcentagens de redução de danos nos grupos de antimutagenicidade foram de 83,9%, 89,1% e 103,1% em 24 horas e 101,24%, 98,14% e 120,64% em 48 horas para as doses de 75, 150 e 300 mg/kg (*p.c.*), respectivamente. A alta porcentagem de redução de danos associada à ausência de efeitos mutagênicos indicou, além da atividade quimioprotetora, a possibilidade do biopolímero ser um alimento funcional candidato à utilização como co-adjuvante na quimioterapia para prevenir efeitos colaterais.

Os resultados do presente trabalho, que indicam a atividade antigenotóxica do cogumelo *A. sylvaticus* não corroboram com os resultados encontrados por Mantovani et al. (2006). Os autores observaram que o extrato do cogumelo *A. blazei* não apresentou atividade protetora quando associado a Ara-C e 3DeoT, nas concentrações testadas, de 0,2; 0,4 e 0,6%, quando associado aos inibidores Ara-C e 3DeoT no ensaio Cometa, não apresentando atividade antigenotóxica, visto que, a redução do número de células com dano não foi significativa.

O cogumelo *A. sylvaticus* apresenta intensa atividade antioxidante e significativa quantidade de polifenóis (Costa et al., 2011). Segundo Kong e Lillei (1998), as substâncias com atividade antioxidante exercem três efeitos nas linhas de defesa orgânica contra as espécies reativas de oxigênio: i) atua na prevenção, caracterizando-se pela proteção contra a formação de substâncias agressoras; ii) atua na interceptação, uma vez que os antioxidantes interceptam os radicais livres, os quais uma vez formados iniciam suas atividades destrutivas; iii) atua no sistema de reparo que ocorre quando as duas primeiras linhas não foram completamente efetivas e os produtos de destruição pelas espécies reativas de oxigênio estão sendo continuamente formados e podem se acumular no organismo.

Como foi possível verificar nas Tabelas 4 e 5, o cogumelo *A. sylvaticus* apresentou tanto atividade genotóxica quanto atividade antigenotóxica, apresentando-se como os compostos Janus (Zeiger, 2003), nome que faz referência ao nome do deus romano Janus, descrito como um deus que apresenta em uma cabeça, duas faces distintas. Segundo Bhattacharya (2011), compostos vegetais que possuem em sua composição diversas substâncias imunomoduladoras podem apresentar efeitos genotóxicos e antigenotóxicos.

9.4 CONCLUSÃO

O cogumelo *A. sylvaticus* não apresenta ação genotóxica ou recombinogênica nas concentrações testadas que variaram de 31,25 a 250mg.mL^{-1}com a aplicação do teste SMART. Como os resultados foram positivos para a atividade antimutagênica contra a forte ação genotóxica da mitomicina-C, sugere-se que o extrato aquoso do cogumelo *A. sylvaticus,* nas condições experimentais testadas, possa agir como um agente desmutagênico extracelular, impedindo que a mitomicina C atue sobre o DNA celular.

Porém, o cogumelo *A. sylvaticus* apresentou atividade genotóxica e antigenotóxica em todas as concentrações testadas no teste do micronúcleo, nos tempos de 24 e 48 horas sugerindoa ocorrência do efeito Janus do composto e pode estar relacionado a .....Dessa forma, estudos clínicos randomizados são necessários para elucidar as consequências no uso terapêutico e/ou efeitos benéficos dos achados.

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10 CONCLUSÕES

No presente trabalho foram realizadas a determinação da composição química, a caracterização de minerais e vitaminas e o potencial antioxidante do cogumelo *A. sylvaticus*. Além disso, foram realizados dois testes *in vitro*, de hemólise em eritrócitos humanos e do MTT em células tumorais e não tumorais, com objetivo de verificação da citotoxicidade provocada pelo cogumelo *A. sylvaticus*, e sua concentração letal (CL_{50}). Foram realizados ainda, dois testes in vivo, com objetivo de avaliar os efeitos genotóxicos e antigenotóxicos do cogumelo *A. sylvaticus* em asa de *D. melanogaster*, pelo teste SMART e em camundongos, por meio do teste do micronúcleo em células de medula óssea. Com os resultados obtidos, concluiu-se que:

- O cogumelo *A. sylvaticus* possui elevado teor de proteínas e carboidratos, além de minerais, com destaque para o ferro, zinco, sódio, potássio e cobre. Este fungo terapêutico apresenta ainda, em sua composição, a Vitamina C, diferenciando-se dos demais cogumelos do gênero Agaricus.
- O cogumelo *A. sylvaticus* apresenta-se como uma rica fonte em compostos antioxidantes, dentre estes os polifenóis totais, detectados principalmente no extrato etanólico deste fungo terapêutico, indicando que a maioria dos componentes antioxidantes presentes neste cogumelo podem ser diluídos, mais facilmente, pelo álcool.
- O cogumelo *A. sylvaticus* não apresentou toxicidade em eritrócitos humanos, uma vez que a CL_{50} observada foi baixíssima, de 9,213mg.ml^{-1}.
- O cogumelo *A. sylvaticus* não apresentou toxicidade *in vitro*, pelo teste do MTT, quando avaliado seu efeito em diferentes doses em células tumorais OSCC-3 e células não-tumorais NIH3/T3.
- O cogumelo *A. sylvaticus* apresentou fraco efeito antimutagênico em todas as concentrações testadas no teste SMART, *in vivo*, além de não apresentar efeito mutagênico em células somáticas de *Drosophila melanogaster*.
- O cogumelo *A. sylvaticus* apresentou efeito genotóxico em todas as concentrações testadas no teste do micronúcleo, em células de medula óssea de *Mus musculus* (Swiss Webster) e ao mesmo tempo, apresentou efeito antigenotóxico quando ministrado concomitantemente a uma substância mutagênica.

Com base nos estudos realizados, sugerimos que o Cogumelo *Agaricus sylvaticus* é seguro para o uso alimentar em humanos. Ensaios clínicos randomizados são necessários para avaliar quais seriam as enfermidades, agravos e condições clínicas em que o cogumelo poderia ser utilizado com finalidade terapêutica.

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ANEXOS

Anexo A – Documento de Aprovação do Comitê de Ética

Anexo B – Termo de Consentimento Livre e Esclarecido

Anexo C – Carta de aprovação da Revista West Indian Medical Journal

Anexo D – e-mail de aprovação da Revista Nutricion Hospitalaria

1 via.

Termo de Consentimento Livre e Esclarecido – TCLE

O Senhor está sendo convidado a participar do projeto: **Citotoxicidade aguda e concentração letal (CL_{50}) do cogumelo *Agaricus sylvaticus* por meio da atividade hemolítica em eritrócitos humanos**.

O nosso objetivo é estimar a citotoxicidade do extrato aquoso do cogumelo *A. sylvaticus* em eritrócitos humanos através da determinação da concentração letal.

O senhor receberá todos os esclarecimentos necessários antes e no decorrer da pesquisa e lhe asseguramos que seu nome não aparecerá sendo mantido o mais rigoroso sigilo através da omissão total de quaisquer informações que permitam identificá-lo.

A sua participação será através da doação de 1mL de sangue na data combinada. Informamos que o Senhor pode desistir de participar da pesquisa em qualquer momento sem nenhum prejuízo para o senhor.

Os resultados da pesquisa irão compor um capítulo de uma tese de doutorado, e podem ainda ser publicados posteriormente, na forma de artigo científico. Os dados e materiais utilizados na pesquisa ficarão sobre a guarda do pesquisador.

Se o Senhor tiver qualquer dúvida em relação à pesquisa, por favor telefone para a pesquisadora principal Joice Vinhal Costa Orsine, doutoranda do Programa de Pós-Graduação em Ciências da Saúde da Universidade de Brasília, telefone: 62-92355342, em horário comercial, das 8:00 as 18:00h.

Este documento foi elaborado em duas vias, uma ficará com o pesquisador responsável e a outra com o sujeito da pesquisa.

Clarice Marçal Faria
Nome / assinatura:

_____
Pesquisador Responsável
Nome e assinatura:

Brasília, 04 de novembro de 2010

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Faculty of Medical Sciences
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July 17, 2012

Dr M Novaes
SHIS-QI-09-conj
06-cs 14 – Lago Sul
Brasilis – DF
Brazil cep 71.625.060

Dear Dr Novaes,

Re: your manuscript numbers **2011-216** entitled:

"Determination of Chemical Antioxidants and Phenolic Compounds in the Brazilian Mushroom Agaricus Sylvaticus"

The above captioned paper has been accepted for publication in the West Indian Medical Journal.

If you wish to have reprints of your papers' please give us a written order immediately, on receipt of this letter. Fifty (50) is the minimum number of reprints that can be bought. The cost of 50 reprints of 1-4 pages is US$100.00 (most articles are between 1-4 pages). Articles exceeding 4 pages will cost US$150.00.

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Yours sincerely,

M. Melbourne
for **Professor EN Barton**
Editor-in-Chief

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Apreciado Autor

Su artículo "6461-Citotoxicidad de A. sylvaticus en células no tumorales (NIH/3T3) y el tumor (CCCA-3) usando tetrazolio (MTT)" ha sido finalmente aprobado para su publicación en Nutrición Hospitalaria.

Antes de iniciar el proceso, deberá abonar 150 € (más IVA en el caso de los residentes en España excepto Canarias, Ceuta y Melilla) en concepto de contribución parcial al coste del proceso (ver carta adjunta del director de Nutrición Hospitalaria al final de este documento).

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APÊNDICES

Apêndice 1 – Artigo intitulado "Cogumelos comestíveis: uso, conservação, características nutricionais e farmacológicas" publicado na Revista HCPA. 2012; 32(4): 452-460

Apêndice 2 – Artigo intitulado "Mushrooms of the genus *Agaricus* as functional foods" publicado na revista Nutr Hosp. 2012; 27(4): 1017-1024.

Apêndice 3 – Artigo intitulado "Nutritional value of *Agaricus sylvaticus;* mushroom grown in Brazil" publicado na revista Nutr Hosp. 2012; 27(2): 449-455.

Apêndice 4 – Artigo intitulado "Chemical and Antioxidant Potential of *Agaricus sylvaticus* Mushroom Grown in Brazil" publicado na revista J Bioanal Biomed 2011; 3(2): 049-054.

Apêndice 5 - Artigo intitulado "The acute cytotoxicity and lethal concentration (LC_{50}) of *Agaricus sylvaticus* through hemolytic activity on human erythrocyte" publicado na revista International Journal of Nutrition and Metabolism 2012; 4(11): 19-23.

Cogumelos comestíveis: uso, conservação, características nutricionais e farmacológicas

Edible mushrooms: use, conservation, nutritional and pharmacological characteristics

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Revista HCPA. 2012;32(4):452-460

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RESUMO

É crescente o interesse na produção e consumo de cogumelos devido às suas qualidades nutricionais e terapêuticas, o que tem estimulado sua utilização como alimento funcional e como adjuvante no tratamento de enfermidades como o câncer. O presente artigo tem por objetivo discutir o uso de cogumelos como alimento e com fins medicinais. Para isso, buscamos trabalhos publicados que consideram a composição química e nutricional, bem como os aspectos farmacológicos e tóxicos para o uso seguro em seres humanos. A coleta de dados foi realizada por meio de pesquisa nas bases eletrônicas LILACS, SciELO, MEDLINE, PubMed e Cochrane. Foi possível verificar que os cogumelos apresentam características nutricionais interessantes devido ao alto teor de proteínas e fibras alimentares, baixo teor de lipídeos e fonte considerável de sais minerais. Possuem diversas substâncias com atividade antioxidante, como a Vitamina C, Vitamina E e polifenóis. Entre as substâncias com interesse na medicina, está o ergosterol, precursor da Vitamina D, que possui ação em enfermidades ósseas, como raquitismo e osteoporose. Na profilaxia e tratamento do câncer, foram observados possíveis efeitos anticarcinogênicos e antimutagênicos, proporcionados por glucanas, arginina, proteoglucanas, glutamina, lectina. Como não estão incluídos nas práticas alimentares da maioria da população do Brasil, muitos estudos estão sendo realizados no intuito de desenvolver formulações com adição de cogumelos, para tornar os alimentos mais saudáveis.

Palavras-chave: Alimento funcional; suplementos dietéticos; hábitos alimentares

ABSTRACT

The increasing interest in the production and consumption of mushrooms is due to its nutritional and therapeutic qualities which have encouraged the use of mushrooms as functional food and as adjuvant in the treatment of diseases like cancer. The objective of this article is to discuss the use of mushrooms as food and with medicinal purposes. For that, we searched for published works that consider their chemical and nutritional composition as well as their pharmacological and toxicological aspects for safe use in humans. Data collection was performed by a research on the electronic databases LILACS, SciELO, MEDLINE, PubMed, and Cochrane. The analysis of published studies showed that mushrooms have interesting nutritional characteristics due to high protein and dietary fiber, low lipid content, and it is also a substantial source of dietary minerals. They have several

substances with antioxidant activity, such as Vitamin C, Vitamin E, and polyphenols. Within the group of substances of medicinal interest is ergosterol, a precursor of Vitamin D, which acts on bone diseases such as rickets and osteoporosis. In the prophylaxis and treatment of cancer, we observed some possible anticarcinogenic and antimutagenic effects provided by glucan, arginine, proteoglucans, glutamine, and lectin. However, mushrooms are not part of most Brazilians' diet yet. For this reason, there are many ongoing studies to develop formulations with addition of mushrooms to make food healthier.

Keywords: Functional food; dietary supplements; food habits

Os cogumelos são muito apreciados desde a idade antiga. Acreditava-se no elevado valor nutritivo e no potencial medicinal, além de serem considerados uma especiaria nobre na culinária. Aproximadamente 140.000 espécies de cogumelos são conhecidas no mundo, sendo 2.000 comestíveis e 700 com propriedades farmacológicas comprovadas. Destas, 25 são cultivadas comercialmente (1).

De acordo com o *Codex Alimentarius,* os cogumelos comestíveis são alimentos pertencentes ao grupo *Funghi*. Eles podem crescer em estado silvestre ou serem cultivados e, após a sua elaboração, estarão próprios para serem utilizados como alimento (2).

O crescente interesse comercial e científico em cogumelos para uso na gastronomia ou na terapêutica clínica estimulou o aprimoramento de técnicas de cultivo e a introdução de novas espécies (1). Portanto, informações sobre a composição dos cogumelos são essenciais para avaliar a sua qualidade. Uma vez que os cogumelos desempenham funções importantes no organismo humano, a comprovação da rica composição química tem grande valor e tem se tornado uma preocupação de profissionais das áreas de saúde e de alimentos (3).

O objetivo deste trabalho é discutir o uso de cogumelos como alimento e com fins medicinais com base em trabalhos publicados, que consideram a composição química e os aspectos farmacológicos e toxicológicos para o uso seguro em seres humanos.

MÉTODOS

Dos 230 artigos encontrados, foram selecionados 56 artigos publicados entre 2000 e 2012, nas bases de dados SciELO, LILACS, Medline, Pubmed e Cochrane, nos idiomas inglês, português e espanhol. Foram aplicados os seguintes critérios de inclusão: artigos originais que apresentassem a composição dos cogumelos terapêuticos e os resultados e benefícios do uso na alimentação. Foi utilizado o Mesh/DECS - descritores em Ciências da Saúde - para definir os termos de busca: "Agaricales" e "Cogumelo" aplicando-os nos critérios de inclusão dos artigos pesquisados.

RESULTADOS

Aspectos químicos e nutricionais de cogumelos comestíveis

Quando analisada sua composição bromatológica, os cogumelos são indicados para dietas balanceadas em razão da baixa concentração de gordura e de energia, bem como da alta concentração de fibras alimentares e proteínas (4) (tabela 1).

Estocagem e cuidados pós-colheita de cogumelos

Os cogumelos do gênero *Pleurotus* são mais delicados e sensíveis do que os do gênero *Agaricus* e deterioram-se mais rapidamente após a colheita. Uma vez deteriorados, podem causar severas intoxicações gastrointestinais (5).

O cogumelo, depois de colhido, tem no máximo dez dias de vida útil, tendo a sua temperatura de armazenamento interferência direta sobre a qualidade do produto. Sob refrigeração a 2ºC, o cogumelo tem vida de prateleira de aproximadamente nove dias. Quando armazenado a 18ºC, observa-se a redução da vida útil para apenas três dias (6).

Conservação e preservação das características nutricionais de cogumelos

Devido ao seu elevado conteúdo de água, os cogumelos são altamente perecíveis. Quando não consumidos em curto intervalo de tempo após a colheita na forma fresca, devem passar por algum tipo de tratamento para evitar a sua deterioração (7) (tabela 2).

Tabela 1: Composição química de alguns cogumelos comestíveis. Estudos selecionados nas bases de dados LILACS, MEDLINE, PubMed, SciELO e Cochrane. Período de 2000 a 2012.

Referência	Espécie de cogumelo	Substâncias presentes
Costa et al. (2011) (4)	*Agaricus sylvaticus*	Carboidratos (36,21%), Proteínas (41,16%), Cinzas (7,38%), Lipídios (6,60%), Fibras (2,34%).
		Ferro (0,72690%), Cálcio (0,00135%), Zinco (0,54925%), Cobalto (0,00775%), Magnésio (0,02119%), Sódio (0,25534%), Potássio (0,61303%), Manganês (0,02318%) e Cobre (0,27666%).
		Vitamina C (0,01265%), Vitamina A (0,000001%), Vitamina D2 (0,000018%), Vitamina E (0,000020%) e Vitamina K2 (0.000001%).
Charalo et al. (2007) (25)	*Agaricus blaze*i	29,23% de ácido palmítico (16:0), 7,46% de ácido esteárico (18:0), 10,84% de ácido oleico (18:1-n9), 49,68% de ácido linoleico (18:2-n6), 2,34% de ácido aracdônico (20:4n-6).
Fullani et al. (2007) (3)	*Lentinula edodes*	Proteína 19%, em base seca, cerca de 4,4% de lipídios e fibra alimentar em torno de 41,9%, fósforo aproximadamente 0,0894%.
	Agaricus bisporus	Teor de proteínas próximo a 28% em relação à base seca, fibras alimentares (20,4%) e baixo teor de lipídeos (5,4%), fósforo, valores médios de 0,1133%.
	Pleorotus spp	Proteínas 22%, fibras alimentares (39,6%) e lipídeos (4,30%), fósforo de 0,1097%.

Tabela 2: Formas de aplicação de métodos de conservação de alimentos sobre cogumelos.

Referência	Método de conservação	Resultados encontrados
Mc Donald e Sun (2000) (26)	Resfriamento a vácuo	A técnica a vácuo promove a aceleração do resfriamento, mas pode causar alguns efeitos desagradáveis na qualidade dos cogumelos, como problemas relacionados à perda de massa.
Burton et al. (1987) (27)	Resfriamento e refrigeração a vácuo	Não foram encontradas diferenças na estrutura dos cogumelos resfriados a vácuo e convencionalmente.
		Após 102 horas estocados a 5ºC, não foi detectado escurecimento significativo, porém os cogumelos resfriados a vácuo tiveram menor escurecimento do que os resfriados convencionalmente.
		Quando os cogumelos foram estocados a 18ºC houve um aumento linear no escurecimento com o tempo de estocagem.
		A perda de massa dos cogumelos estocados a 5ºC foi consideravelmente menor do que aqueles estocados a 18ºC
Apati (2004) (28)	Secagem	A melhor temperatura de desidratação é de 40ºC, levando em consideração a melhor capacidade de reidratação (por meio de imersão em água a temperatura ambiente, por um período de 30 minutos) dos cogumelos desidratados nesta temperatura.
		O tempo de secagem é aproximadamente duas vezes superior, se comparado à secagem realizada a 60ºC e umidade relativa do ar de aproximadamente 75%.

Referência	Método de conservação	Resultados encontrados
Martinez-Soto et al. (2001) (29)	Branqueamento com metabissulfito de sódio ou ácido cítrico antes da secagem	Cogumelos que sofreram branqueamento ficaram mais escuros depois da secagem do que aqueles que não foram submetidos ao branqueamento. Os cogumelos liofilizados apresentaram maior capacidade de reidratação e cor mais próxima a dos cogumelos *in natura* do que os cogumelos secos por ar quente ou a vácuo. O aroma e o sabor dos cogumelos secos por ar quente foram estatisticamente semelhantes aos apresentados pelos cogumelos liofilizados.
George e Datta (2002) (30)	Liofilização	Tempo final de desidratação dos cogumelos de cinco horas, porém a liofilização não é um processo viável economicamente para o processamento industrial de cogumelos

* O branqueamento é utilizado como pré-tratamento no processamento de alimentos, devendo ser seguido de um método de conservação adequado.

Formas de utilização de cogumelos comestíveis

No Brasil, os cogumelos ainda não fazem parte do cardápio da maioria da população, que oferece certa resistência com relação ao seu consumo, podendo esse fato ser explicado pela falta de conhecimento quanto à disponibilidade de diferentes espécies e ao seu preparo (8).

O grau de escolaridade entre os consumidores de cogumelos representa uma parcela muito bem informada da população, e a espécie mais consumida é o tradicional Champignon de Paris (*Agaricus bisporus*), seguida pelo Shiitake (*Lentinula edodes*) e o Shimeji (*Pleurotus sp*). As formas de consumo de cogumelos mais utilizadas são em molhos, cogumelo fresco e seco, em sopa e refogado, em conserva, acompanhando pizzas, massas e risotos (9).

O uso do chá de cogumelos é uma das práticas mais populares da medicina tradicional chinesa relacionada à prevenção ou ao tratamento de várias doenças humanas (10), sendo a forma mais comum para o seu preparo a infusão e fervura do fungo desidratado (11).

Em relação às formas de preparo, uma questão ainda a ser considerada é o efeito do processamento dos cogumelos sobre as suas propriedades. O cozimento dos cogumelos comestíveis pode afetar os nutrientes termolábeis. Porém, o uso de altas temperaturas tem efeito positivo na maior parte dos minerais que ativam o sistema imunológico, que se tornam mais disponíveis ao organismo humano após o cozimento. Já as fibras são parcialmente quebradas e as proteínas afetadas sem, no entanto, ter seu valor nutricional reduzido (8).

Em alguns casos, como o cogumelo shiitake, suas propriedades nutricionais são ressaltadas após cozimento. Quando submetido a processo de fritura leve, os nutrientes são preservados instáveis. A maior parte dos constituintes ativos, como os polissacarídeos, está associada a estruturas da parede celular e, em processo de ebulição, é liberada. Outros constituintes ativos, como os terpenos, também são mais bem solubilizados em água quente, sendo relativamente estáveis ao calor (8).

Aspectos farmacológicos de cogumelos comestíveis

Diversas substâncias bioativas com propriedades farmacológicas, como glucanas, proteoglucanas, lecitinas, ergosterol e arginina têm sido identificadas e isoladas em numerosas espécies de fungos medicinais (12).

A exemplo dos cogumelos *Agaricus sylvaticus, Lentinula edodes e Agaricus blazei*, são relatados vários polissacarídeos com atividade imunomodulatória, anticancerígena, anti-inflamatória e antioxidante (13).

Acredita-se que a principal substância que responde pelos atributos funcionais dos fungos medicinais são as β-glucanas, fibras alimentares solúveis capazes de atuar de forma eficaz na redução do colesterol e de outros lipídeos plasmáticos (14). Elas aumentam as funções imunológicas por intermédio do estímulo à expansão clonal de células T, *Natural Killer* (NK),

linfócitos B e células complementares, aumentando o número de macrófagos e monócitos, promovendo a proliferação e/ou produção de anticorpos e de várias citocinas e, dessa forma, evitando a regeneração e a metástase do câncer (15).

Fibras como as β-proteoglucanas, heteroglucanas, quitina e peptideoglucanas atuam como imunomoduladoras (16). A composição da fração fibra dos cogumelos é composta principalmente por β-glicanas, quitina e hemicelulose, as quais apresentam propriedades antitumorais e antimutagênicas por estimularem o sistema imune (17).

As vitaminas do *A. blazei Murill* estão relacionadas à antiangiogênese, que corresponde à nova formação vascular. Apresentam efeito sobre o crescimento da microcirculação, a vitamina D3 e a vitamina D2 (ergosterol), que também apresenta um efeito antiangiogênico potente. O responsável por esse efeito é o ergosterol presente no extrato do cogumelo, que possui ação na redução do volume e inibição do crescimento tumoral, em ratos com sarcoma 180, sem os efeitos adversos geralmente causados pelos quimioterápicos. Seu mecanismo de ação ocorre pela inibição da neovascularização. O ergosterol, precursor do ergocalciferol é, sobretudo, uma substância antiangiogênica, explicando em parte seu efeito antitumoral (18).

Em estudo realizado por Fortes et al. (14), os autores observaram a redução significativa dos níveis plasmáticos de colesterol total (CT) e lipoproteína de baixa densidade (LDL colesterol/ LDL-c) durante todo o período de suplementação dietética com *A. sylvaticus*, sendo sugerido que a presença de substâncias bioativas nesses fungos apresentam efeitos benéficos no metabolismo lipídico e, consequentemente, no prognóstico dos pacientes.

Outros estudos experimentais conduzidos em animais de laboratório têm comprovado que a administração de determinadas espécies de fungos medicinais é capaz de promover redução significativa do colesterol total (CT); lipoproteína de baixa densidade LDL-c (4,5,17-20); lipoproteína de muito baixa densidade (VLDL colesterol/ VLDL-c) (5,17); triglicerídeos (TG) (16-20), fosfolipídio, índice aterogênico e da atividade da enzima 3-hidroxi-3-metilglutarilcoenzima A redutase (HMG-CoA redutase), além do aumento da lipoproteína de alta densidade (HDL colesterol/ HDL-c) (20). O mecanismo pelo qual fungos medicinais são capazes de reduzir os níveis lipídicos é explicado por meio do aumento da excreção fecal de ácidos biliares e de colesterol, especificamente, por aumentar o receptor hepático LDL. As lovastatinas, inibidoras da enzima HMG-CoA redutase, que catalisam a síntese do mevalonato, atuam conjuntamente como responsáveis pelos efeitos observados. Também já foi identificada uma substância denominada eritadenina, agente hipolipidêmico, capaz de reduzir os níveis de colesterol e outros lipídeos por meio da excreção do colesterol ingerido e de sua decomposição metabólica (14).

A arginina é descrita como estimuladora do hormônio de crescimento hipofisário e está relacionada ao aumento da atividade das células NK, células T helper e com o estímulo da produção de citocinas, tais como interleucina 1 (IL-1), interleucina 2 (IL-2), interleucina 6 (IL-6). Estudos indicam que o aumento da imunidade promovida pela arginina é obtido pela estimulação da liberação do hormônio de crescimento, estímulo na produção de óxido nítrico, hidroxiprolina, citocinas e poliaminas (18).

Já as proteoglucanas têm seu mecanismo de ação baseado na estimulação das funções imunológicas, da atividade fagocitária de macrófagos e melhoria das funções do sistema retículo-endotelial, amenizando, assim, os sintomas associados à quimioterapia, além de melhorar a infiltração tumoral pelas células T citotóxicas (18).

Dentre as numerosas moléculas bioativas que podem ser isoladas no corpo de frutificação de diversos fungos, a lectina, que é um fosfolipídeo, exerce propriedade antitumoral, antimutagênica e hemaglutinizante por intermédio de sua propriedade indutora de apoptose nas células tumorais, mecanismo primário contra as neoplasias malignas (18).

Por fim, a glutamina age aumentando a função imune e intestinal, reduz a bacteremia e os danos na mucosa associados à quimioterapia, mantendo a integridade intestinal, melhora o equilíbrio nitrogenado, contribui com a não elevação de citocinas pró-inflamatórias, possui capacidade antioxidante e melhora a preservação da musculatura esquelética. Seu mecanismo de ação se justifica por ser fonte de energia preferencial à glicose por todas as células de divisão rápida, como os enterócitos, células do sistema imunológico e nervoso. Prolonga a sobrevida no tratamento do câncer, diminuindo o catabolismo debilitante (20).

Estudos do efeito de cogumelos comestíveis em pacientes oncológicos

Após suplementação dietética com fungos *A. sylvaticus*, Fortes et al. (15) observaram que este cogumelo é capaz de melhorar as alterações gastrointestinais de pacientes no pós-operatório de câncer colorretal, promovendo melhoria na qualidade de vida desses pacientes.

Foi realizado um estudo por Fortes et al. (21), com o objetivo de avaliar os efeitos da suplementação dietética com fungos *A. sylvaticus* em pacientes no pós-operatório de câncer colorretal, após seis meses de tratamento, a respeito dos indicadores da qualidade de vida - sedentarismo, tabagismo, etilismo, distúrbios do sono, alterações na disposição e no humor e presença de dores - que acometem principalmente os pacientes com câncer. Os resultados encontrados pelos autores sugerem que a suplementação dietética com este cogumelo é capaz de melhorar a qualidade de vida de pacientes com câncer colorretal em fase pós-operatória por reduzir significativamente os efeitos deletérios ocasionados pela própria enfermidade e pelo tratamento convencional da mesma.

Com o objetivo de avaliar os efeitos da suplementação dietética com fungos *A. sylvaticus* no perfil lipídico de pacientes com câncer colorretal em fase pós-operatória, Fortes et al. (14) verificaram que a suplementação dietética com fungos *Agaricus sylvaticus* é capaz de reduzir o colesterol total, LDL-c e triglicérides, apresentando efeitos benéficos no metabolismo lipídico e, consequentemente, no prognóstico desses pacientes.

Pacientes com câncer de mama e metástase pulmonar foram submetidos a tratamento com o cogumelo comestível *A. sylvaticus*, como complemento da tradicional quimioterapia, radioterapia e cirurgia. O sucesso evolutivo observado foi atribuído ao aumento das células "*Natural Killer*" do paciente (22).

Para maiores esclarecimentos quanto aos efeitos adversos das espécies comestíveis são necessários mais estudos, pois os estudos existentes não demonstram haver toxicidade significativa com o uso dos cogumelos nas doses recomendadas (18). Na literatura, é possível encontrar, entretanto, alguns relatos de hipersensibilidade (1).

Elaboração de produtos alimentícios com a utilização de cogumelos

Alguns autores observaram, em seus estudos, os efeitos benéficos na dieta de indivíduos que consumiram, em um período de quatro dias, uma média de 419,9 kcal e 30,83 g de gordura a menos nos pratos preparados com cogumelo quando comparados aos pratos que utilizaram carne em sua formulação. Foi verificado ainda que a aceitação dos pratos com cogumelo foi similar aos pratos com carne, mostrando o potencial de utilização deste tipo de substituição (23).

Trabalhos têm sido realizados com o objetivo de avaliar a aceitabilidade do cogumelo *A. brasiliensis* em pratos culinários como referência para o desenvolvimento de tecnologias de preparo deste cogumelo, visando a impulsionar o seu uso na alimentação (24).

Em outro estudo foi desenvolvido e caracterizado um produto análogo a hambúrguer a base de cogumelo *A. brasiliensis* e comparado suas características com uma formulação controle, na qual o cogumelo foi substituído por carne moída de patinho, e com produtos comerciais: um a base de carne bovina e outro a base de proteína vegetal. Considerando-se os resultados obtidos neste trabalho, o hambúrguer de cogumelo *A. brasiliensis* demonstrou ser uma alternativa mais saudável ao produto tradicional, pois além das propriedades nutricionais e gastronômicas, o cogumelo apresenta inúmeras propriedades medicinais, além do alto teor de fibras (9).

Em outro estudo, foi verificado que molhos de tomate com adição do cogumelo *Agaricus brasiliensis* possuíam quantidade de polifenóis maior em relação aos molhos sem o extrato do cogumelo (13).

O extrato de cogumelo do gênero Agaricus apresentou-se como um agente antioxidante natural promissor, efetivo na proteção do óleo de soja. Porém, os autores afirmaram em seus estudos que é fundamental a investigação da atividade antioxidante do extrato do cogumelo em diferentes concentrações para que o produto possa se tornar mais competitivo no mercado (25).

Uma combinação purificada é muito diferente do cogumelo inteiro e, portanto, é inevitável questionar se comer o cogumelo inteiro tem valor preventivo ou terapêutico e, nesse caso,

quanto deveria ser consumido e de que forma. Para shiitake, os pesquisadores descobriram que os corpos de frutificação pulverizados dados a ratos como 10-20% da sua dieta inibiu tumores transplantados e estudos pequenos demonstraram redução do efeito ameaçador do consumo de lipídeos com 9 g de cogumelos secos ou 90 g de cogumelos frescos (26).

O conteúdo e potência de ingredientes bioativos podem diferir, dependendo da forma como o cogumelo é preparado e ingerido. Por exemplo, o conteúdo de tioprolina anticarcinógena varia de quantias indetectáveis em shiitake fresco, a 134 mg/100 g de shiitake seco, a 843 mg/100 g de shiitake fervido. Como é o caso para a maioria das plantas e ervas, a tensão específica, condições de crescimento e outros fatores ambientais também afetam significativamente o gosto, a forma, a substância do cogumelo e seu conteúdo bioativo (26).

Em outros trabalhos, algumas espécies de cogumelos comestíveis foram utilizadas nas seguintes dosagens em testes realizados em humanos: *Lentinus edodes*, 2 mg de lentinana após uma semana, quatro vezes por dois ou quatro intervalos semanais. *Agaricus bisporus*, 2,5 µL; 5 µL ou 10 µL de extrato liofilizado; mistura de polissacárides de seis cogumelos medicinais (*Agaricus blazei, Lentinus edodes, Grifola frondosa, Ganoderma lucidum, Coriolus versicolor, Cordyceps sinensis*) e poliactina A três vezes ao dia, representando um total de 6 g da mistura de cogumelos (18).

Toxicidade de cogumelos comestíveis

Infelizmente, são escassos os dados na literatura acerca da toxicidade de cogumelos. Em trabalho realizado por Orsine et al. (2012), os autores verificaram que o cogumelo *A. sylvaticus* não apresenta toxicidade, comprovando ser seguro para o consumo humano. Nesse estudo, foram realizados testes utilizando-se o extrato aquoso não fracionado do cogumelo, e a toxicidade foi avaliada observando-se qual a concentração letal (CL50) por meio de atividade hemolítica em eritrócitos humanos (27).

Yoshkoda et al. (2010) avaliaram a toxicidade do extrato obtido a partir do micélio do cogumelo *Lentinula edodes* em ratos Wistar, com doses diárias de 2000 mg/kg, durante 28 dias. Os autores observaram que não ocorreram mortes ou mudanças de comportamento dos animais. Porém, foram reduzidos o peso corporal e o consumo de alimentos, em particular no caso de ratos do sexo masculino, embora o grau de diminuição não tenha sido tão proeminente no final da administração. Nenhum efeito toxicológico significativo foi observado nos exames de hematologia, bioquímica sérica, peso dos órgãos absolutos e relativos, autópsia e histopatologia. Consequentemente, o nível sem efeitos adversos observados para o cogumelo *L. edodes* foi considerado como mais de 2.000 mg/kg/dia nas condições do presente estudo (28).

Em 2008, Bellini et al. (2008) observaram que as frações metanólicas do cogumelo *A. blazei* testadas não ofereceram proteção química e que todas as frações apresentaram-se potencialmente mutagênicas no teste de HGPRT (hypoxanthine-guanine phosphoribosyl transferase locus). Sendo assim, os autores concluíram que mais testes são necessários para uma investigação dos efeitos biológicos dos extratos metanólico e aquoso do *A. blazei*, além de outras interações com o metabolismo das células antes de recomendar o seu largo uso pela população, o que já ocorre em diversos países. Este estudo indica que os extratos metanólicos do fungo não devem ser utilizados em função de sua genotoxicidade e que se deve ter cuidado no uso de *A. blazei* pela população antes que a caracterização bioquímica deste fungo seja completa (29).

Novaes et al. (2007) observaram que a administração do extrato aquoso do cogumelo *A. sylvaticus* em doses superiores às usadas nos protocolos terapêuticos em humanos, apresenta toxicidade muito baixa, quando realizados testes de toxicidade clínica, bioquímica e histopatológica em ratos saudáveis (30).

Costa e Nepomuceno (2003), objetivando avaliar os possíveis efeitos protetores do chá de *A. blazei* (62,5 g.L-1) contra a ação genotóxica do uretano (10 mM), não observaram aumento estatisticamente significativo nas frequências de manchas mutantes em larvas expostas ao chá de *A. blazei,* no teste SMART (Somatic Mutation And Recombination Test). Quando o cogumelo A. blazei foi associado ao uretano, foi observada uma redução estatisticamente significativa nas frequências das manchas mutantes. Os resultados sugerem que o *A. blazei* não é genotóxico e exerce um efeito protetor contra a ação genotóxica do uretano (31).

CONCLUSÃO

Cogumelos são alimentos com excelentes características nutricionais, como alto teor de proteínas, fibras alimentares, minerais, vitaminas, diversas substâncias bioativas com propriedades farmacológicas e baixo teor de lipídeos, podendo ser acrescido aos hábitos alimentares normais e usuais da população.

São diversas as formas de inclusão dos cogumelos na dieta. Muitas pesquisas têm sido desenvolvidas para avaliar os efeitos dos métodos de conservação de alimentos nas características nutricionais dos produtos e, também, no desenvolvimento de novos produtos contendo cogumelos em sua formulação, de modo a aumentar o valor nutritivo das preparações ou até mesmo atender consumidores cujas dietas restringem certos grupos de alimentos, como produtos de origem animal.

Nesse contexto, abre-se a possibilidade de utilizar alimentos industrializados que contenham cogumelos adicionados, atendendo ao mercado consumidor com vantagens nutricionais, como o desenvolvimento de molho de tomate e de hambúrguer contendo cogumelo *A. brasiliensis* em suas formulações e do óleo de soja enriquecido com *A. blazei.* O desafio da indústria de alimentos é desenvolver tecnologias compatíveis com a preservação das propriedades nutritivas e a estabilidade de vitaminas e aminoácidos dos alimentos durante o período de armazenamento, reduzindo ao máximo as perdas nutricionais durante a estocagem desses produtos.

Além dos benefícios da ingestão de alimentos ricos em nutrientes para suprir as necessidades do organismo, deve-se atentar ao fornecimento de produtos com características sensoriais satisfatórias. A garantia da qualidade e segurança pode ser obtida utilizando-se as Boas Práticas de Fabricação desde a obtenção das matérias-primas até a distribuição do produto final. Também é importante a aplicação dos cuidados pós-colheita, evitando assim possíveis contaminações por microrganismos deteriorantes e patogênicos, reduzindo reações enzimáticas, responsáveis por alterações na cor, textura, sabor e aroma dos cogumelos.

Com relação à toxicidade dos cogumelos comestíveis, observou-se que ainda devem ser realizados estudos com o intuito de determinar as quantidades ideais para consumo humano, como forma de garantir a segurança alimentar quanto ao seu uso.

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Revisión

Mushrooms of the genus *Agaricus* as functional foods

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Abstract

Mushrooms of the genus *Agaricus* are noted for their pharmacological and culinary properties. In this study, it was performed a critical literature review, focusing primarily on aspects of the chemical composition of these mushrooms whose pharmacological properties and nutritional composition characterize them as functional foods. It was also discussed articles conducted in vitro and in vivo proving the high antioxidant potential of the *Agaricaceae* family, in addition to articles which emphasize the toxicity characteristics and safety for its use in therapy or in human nutrition. These mushrooms exhibit numerous bioactive substances as well as safety regarding toxicity, which characterize them as functional foods. Despite the countless beneficial effects on human health, mushrooms of the genus *Agaricus* are little known by the population, making it necessary partnership and combined efforts among producers, industries and researchers in order to disseminate, research and consumption of these foods.

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Key words: Agaricaceae. *Health. Medicinal foods.*

HONGOS DEL GÉNERO *AGARICUS* COMO ALIMENTOS FUNCIONALES

Resumen

Hongos del género *Agaricus* son conocidos por sus propiedades farmacológicas y culinarias. En este estudio, se realizó una revisión crítica de la literatura, centrándose principalmente en los aspectos de la composición química de estos hongos, cuyas propiedades farmacológicas y composición nutricional caracterizarlos como alimentos funcionales. También se discutió artículos realizados in vitro e in vivo demostrando el potencial antioxidante de alta de la familia *Agaricaceae,* además de los artículos que hacen hincapié en las características de toxicidad y seguridad para su uso en terapia o en la nutrición humana. Estos hongos presentan numerosas sustancias bioactivas, así como la seguridad en relación con la toxicidad, lo que les caracterizan como alimentos funcionales. A pesar de los innumerables efectos beneficiosos sobre la salud humana, las setas del género *Agaricus* son poco conocidos por la población, por lo que es colaboración necesaria y el trabajo conjunto entre productores, industrias e investigadores con el fin de difundir, la investigación y el consumo de estos alimentos.

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Palabras clave: Agaricaceae. *Salud. Alimentos funcionales.*

Abbreviations

A. blazei: Agaricus blazei.
A. brasiliensis: Agaricus brasiliensis.
A. sylvaticus: Agaricus sylvaticus.
AdipoQ: Adiponectin.
Anvisa: National Health Surveillance Agency.
CFU-GM: Granulocytes-macrophage.
CRP: C-reactive protein.
DMH: 1,2-dimethylhydrazine.
DNA: Deoxyribonucleic acid.
DPPH: 2, 2-diphenyl-1-picrylhydrazyl.
ENU: N-ethyl-N-nitrosourea.
HR: Heart rate.
LDL-C: Low-density lipoprotein cholesterol.
MAP: Mean arterial pressure.
MIP-2: Macrophage inflammatory protein 2.
Pristane: 2,6,10,14-tetrametilpentadecano.
SCGE: Single cell gel electrophoresis.
TNF-α: Tumor necrosis factor alphal.

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Introduction

Edible mushrooms belong to the Funghi group, which can grow in the wild or be cultivated, and after properly prepared, will be suitable for use as food.[1]

In accordance with Resolution RDC no 272/05 of the Anvisa (National Health Surveillance Agency), edible mushrooms are classified as products obtained from species of edible fungi, traditionally used as food, and can be prepared in different ways such as dried, whole, fragmented, ground or preserved, subject to drying, smoked, cooked, salted, fermented or any other technical process deemed safe for food production.[1]

The term functional food attributed to edible mushrooms is due to its rich nutritional value and therapeutic properties described by several researchers, but regulation is permitted only after proof of its healthy physiological effects. To be classified as functional foods they should be included in daily eating habits, providing consumers with specific physiological benefits, thanks to its components capable of causing physiological sound effects.[2]

To be considered functional food, conditions of use and nutritional value, chemical composition or molecular characterization or the product formulation must be registered. Biochemical, nutritional and/or physiological, and/or toxicological tests in experimental animals should also be submitted, further to epidemiological studies, clinical trials, and comprehensive evidence of scientific literature; accredited by international health organizations and international laws recognized under properties and characteristics of the product; proven to be of traditional use by the population having no association with adverse health effects.[3,4,5]

The study of functional foods is very important, since they have beneficial results for the increase in life expectancy of the population. Often times there are cases of chronic diseases such as obesity, atherosclerosis, hypertension, osteoporosis, diabetes and cancer. These ailments have been of great concern both for the population as well as public agencies related to health, and are part of their agenda to discuss solutions for better eating habits.[6]

According to Araújo,[7] health-conscious consumers are increasingly looking for foods that help control their own health and well-being. This growing search for a balanced diet in maintaining health has contributed to encourage research into new biologically active natural components and has changed our understanding of the importance of diet in good health.

Mushrooms are very rich in proteins, vitamins and minerals, and have been used worldwide as nutraceuticals in the prevention and treatment of various diseases.[8]

The objective of this study was to perform a critical review of the literature, highlighting aspects of the chemical composition of these mushrooms responsible for the pharmacological properties and nutritional composition which characterize them as functional foods. It was also discussed articles conducted *in vitro* and *in vivo* attesting the antioxidant potential of the *Agaricaceae* family, besides articles that emphasize the toxicity characteristics and safety for the use in therapy or human nutrition.

Materials and methods

A review of articles published in Data Bases Medline, Lilacs, PubMed, from 1990 to 2012 was done, crossing data between the descriptors in Health Sciences: mushrooms, functional foods, Agaricaceae, in Portuguese, English and Spanish.

Results and discussion

It was found 60 papers and given the reduced number of articles, all of them have been selected in this review. The mushrooms showed numerous bioactive substances and safety for toxicity, which characterize them as functional foods. Some species of the genus *Agaricus* have shown chemical and nutritional composition suitable for human consumption, as well as a flavor much appreciated for culinary purposes.

In 2007 the Brazilian production of mushrooms of the genus *Agaricus* reached around 40 tons of dehydrated mushrooms, 95% of which destined for export to the Japanese market. In order to increase their profits, many businessmen and farmers started looking for these mushrooms as a new alternative source of income. For this reason, several companies and cooperatives have produced and marketed the inoculum (seed or *spawn)* of *A. blazei* or the colonized compost itself. But little is known about the origin and genetic variability of these products.[9]

The identification and classification of species of *Agaricus* mushrooms have been based on morphological and physiological characteristics or by genetic methods, molecular and biochemical. The genetic variability of the genus *Agaricus,* native or cultivated throughout the world is enormous. Generally these differences are in color, shape and size of microscopic structures and fruiting bodies (spores, plates, and cystides).[10]

To talk about *A. sylvaticus* is the same as to talk about *A. blazei.* When there are small differences in morphology, it does not justify creating a new species. Therefore, mushrooms *A. sylvaticus* and *A. brasiliensis* are synonyms of *A. blazei.*[10]

In a study conducted by Tominazawa et al.,[9] the authors investigated nine isolates of *A. blazei* obtained from different regions in Brazil (São Paulo, Espírito Santo, Minas Gerais, Rio Grande do Sul), through the use of molecular markers to assess genetic similarity among them. The authors concluded that six of the nine isolates showed high genetic similarity and are considered the same origin or clones.

A. sylvaticus mushroom is a Brazilian fungus found natively in the countryside in Brazil. Its popular name is "Sun Mushroom". This mushroom is ranked as Eukaryotic superkingdom, Fungi kingdom, Metazoa group, Phylum Basidiomycota, class Hymenomycetes, subclass Homobasidiomycetes, order Agaricales, family Agaricaceae.[11]

Chemical composition of mushrooms of the genus Agaricus

Through knowledge of the chemical composition of a product, it is possible to recognize its nutritional value and perform analysis of the proportion of homogeneous groups of substances in 100 g of food analyzed. The homogeneous groups of substances considered are those present in all foods, such as water, lipids, protein, fiber, minerals and sugars.[12]

Determination of the chemical composition of mushrooms shows the nutritional value of the food under consideration and can be used as a source of information for nutritional tables on the labels, since several companies that commercialize mushrooms do not display the chemical composition on the Nutrition Facts label of their product.[13]

The high water content in fresh commercialized mushrooms, limits its nutritional value when analyzing a portion of 15 g commonly used on labels. Information on food composition is critical to assess their quality.[13]

There are several factors which directly influence the bromatological characteristics of mushrooms. Among these, species, lineage, post-harvest processing, development stage of the basidiome, the part of the basidiome analyzed and substrate,[14] in addition to genetic factors, environmental characteristics, intrinsic attributes, season and growing conditions, substrate composition, handling, storage and transportation.[13]

According to Braga et al.,[15] other determinants for the characteristics of mushrooms, especially when measured protein content are: age, environment and area of cultivation. This fact can be observed when analyzing young mushrooms, which have higher protein content than the more mature ones. According to Shibata et al.,[16] larger mushrooms are higher in carbohydrates mainly in the strain; smaller mushrooms have more protein, concentrated mainly in the pileus part.

Composition and health benefits

For a food to be considered functional it should have beneficial effects; reach one or more functions or actions in the human body. It should also provide well-being, quality of life, health, and reduce the risk of disease[17] as in the case of chronic degenerative diseases.[18]

Only with the development of more accurate techniques for isolation and purification of chemicals, was it possible to prove scientifically the therapeutic action of some mushrooms, isolating both antibacterial and antitumoral substances.[19]

Agaricales mushrooms and other medicinal fungi exert essential nutritional and pharmacological effects, which can be used as adjuvant in cancer therapy. The mechanisms of action of bioactive substances present in mushrooms are not yet completely understood. But there seems to be clear scientific evidence suggesting that these substances contribute to modulate both the initiation and promotion/ progression stages of carcinogenesis, thus propitiating benefits to individuals with various cancers, mainly by immunostimulatory activity.[20]

Several studies have also revealed that *A. sylvaticus* mushroom potentially reduces tumor growth, stimulates the immune system and even contributes to a better prognosis of these patients improving their quality of life.[21]

In folk medicine the *A. brasiliensis* mushroom has been used to fight physical and emotional stress, treat and prevent illnesses such as diabetes, osteoporosis and gastric ulcer, digestive and circulatory problems in addition to reducing cholesterol.[21]

The main group of inhibitory agents of carcinogenesis is represented by antioxidant and free radicals blockers,[21] substances capable of slowing oxidation rate. In this way, they inhibit free radicals and prevent diseases, hence contributing to longevity, helping maintain the essential balance between free radicals and antioxidant defense system of the body.[23]

Antioxidant activity

In a study by Costa et al.[24] observation noted that the alcoholic extract of the mushroom A. sylvaticus has great antioxidant potential (74.6%), suggesting that most of the antioxidant compounds present in mushrooms can be diluted more easily by alcohol. However, aqueous and ether fractions showed reduced antioxidant potential (14.6% each) when compared to the alcoholic fraction, since they were less able to hijack the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical after 20 minutes reaction.

On the other hand the antioxidant potential of different extracts of the *A. blazei* mushroom, through the DPPH method by Silva et al.,[25] showed a higher antioxidant activity (28.6%) in methanol extracts: aqueous (1:1).

According to Tsai et al.,[26] mushrooms of the genus *Agaricus* may have their antioxidant properties associated with a high concentration of tocopherols.

Percário et al.[27] researched the antioxidant capacity of different molecules of the *A. sylvaticus* mushroom, and found results of 72 mg/g for β-glucan in the liquid suspension and 14.1 mg/g in the form of compressed tablets. As for flavonoids, he found values of 0.88 mg/g in liquid suspension and 0.63 mg/g for tablets. For total phenols he found values of 0.1 mg/g for the liquid suspension and 3.4 mg/g for tablets. The authors suggested that the antioxidant activity of *A. sylvaticus* mushroom is attributable to the number of molecules present, not to a specific component, and these molecules are easily degraded when exposed to industrial processes, which reduces its antioxidant capacity.

In vitro *studies*

In a study by Angeli et al.,[28] the authors suggested that -glucan present in *A. blazei* has no genotoxic or mutagenic effect, but protects the damaged DNA (Deoxyribonucleic acid) caused by benzopyrene in test protocols. Results indicate that the beta-glucan works through a link with benzopyrene by capturing free radicals during their activation.

In the clastogenicity test performed by Mantovani et al.,[21] the authors discovered that concentrations of 0.2% and 0.4% of *A. brasiliensis* mushroom were not damage-inducing, unlike a higher concentration of (0.6%). On the genotoxic treatments in SCGE (single cell gel electrophoresis), the concentration of 0.2% of the mushroom extract showed no genotoxic activity, as opposed to concentrations of 0.4% and 0.6% that proved to be effective DNA damage-inducing. Anti-clastogenicity results indicated that, in most treatments, the aqueous extract of *A. brasiliensis* showed no protective activity against DNA damage induced by Ara-C (Arabinofuranosyl Cytidine) and Ara-C + MMS (methyl methanesulfonate.) Through SCGE, the A. brasiliensis, in the three concentrations tested, showed no activity anti-genotoxic. The data suggest caution in the consumption and ingestion of *A. brasiliensis* by humans, particularly at high concentrations.

In vivo *studies*

In a study by Fortes et al.,[29] the authors found that dietary supplementation with *A. sylvaticus* can provide metabolic benefits when analyzing biochemical, enzymatic and blood pressure of patients with colorectal cancer in post-operative phase.

Carvalho et al.,[30] aiming at verifying the antinociceptive and anti-inflammatory activity of *A. blazei* Murill in Wistar rats, through modified formalin test, found results showing that *A. blazei* acts on nociceptive response and in acute inflammation, because rats treated with this mushroom made fewer movements with paws during phase III, this most likely being related to pain caused by mediators of acute-phase inflammation.

Ishii et al.[31] demonstrated in their studies that *A. blazei* mushroom has no genotoxic activity but, rather, anti-genotoxic activity. Results derived from these data propose that *A. blazei* may act as a functional food capable of promoting immunomodulation which can account for the destruction of cells with DNA alterations correlated with the development of cancer. Therefore, supplementation with *A. blazei* mushroom can be an effective method for the prevention of cancer as well as being an important co-adjuvant treatment in chemotherapy.

In works carried out by Fortes et al.,[32] the authors suggested that dietary supplementation with *A. sylvaticus* mushroom showed to be beneficial in improving well-being and quality of life of patients with colorectal cancer in post-surgery phase.

In a study by Padilha et al.,[33] the authors studied the action of *A. blazei* extract on chronic inflammatory diseases in male albino Wistar rats. Results found indicated that *A. blazei* extract was active in experimental animals, this response is consistent, since the D-glucan compound is present in the extract.

Fortes et al.[34] conducted a study to assess the effects of dietary supplementation with *A. sylvaticus* in the lipid profile of patients with colorectal cancer in post-surgery phase. The experiment revealed that dietary supplementation with *A. sylvaticus* fungi is capable of reducing total cholesterol, LDL-C (low-density lipoprotein cholesterol) and triglycerides, with beneficial outcome on lipid metabolism and, consequently, the prognosis of these patients.

Fortes et al.[35] also found that dietary supplementation with *A. sylvaticus* fungi acts in regulating fasting blood glucose levels of patients after colorectal cancer surgery. A dietary supplementation with these fungi was found to be successful in reducing blood sugar levels of patients in post-surgery phase, providing beneficial effects on the carbohydrate metabolism of these patients. However, the authors emphasize the importance of studying other clinical conditions to determine the benefits of using *A. sylvaticus.*

Hi et al.,[36] with the purpose of assessing the effects of *A. sylvaticus* extract in supplemented mice inoculated with pristane (2,6,10,14-tetrametilpentadecano), attested the carcinogen nature of this drug and that the extract of *A. sylvaticus* mushroom has immunomodulatory activity, without producing toxic effects in test animals.

Hsu et al.[37] obtained results that indicate the potential benefits of supplementation with *A. blazei* Murill fungus to normalize liver function in patients with hepatitis B after 12 months of clinical observations.

Taveira et al.[38] conducted a study to determine the effects of *A. sylvaticus* extract on anaemia and C-reactive protein (CRP) levels in rats inoculated with Walker 256 solid tumor. Results suggest that treatment with *A. sylvaticus* mushroom has positive outcome in animals with Walker 256 tumor. Observation noted that the fungus is capable of reducing anaemia in animals, obtaining results close to those obtained for healthy pets.

Hsu et al.[39] observed in their studies that supplementation with A. Murill *blazei* improves insulin resistance in patients with type 2 diabetes. The beneficial effects assessed were due to increase in AdipoQ (adiponectin) concentration from adipose tissue with anti-inflammatory and antiteratogenic effect after ingestion of the mushroom for 12 weeks.

Bernardshaw et al.[40] observed an increase in the concentrations of cytokines MIP-2 (macrophage inflammatory protein 2) and TNF-α (tumor necrosis factor alphal) in the serum of mice supplemented with *A. blazei* extract, resulting in protection against systemic infection by *Streptococcus pneumonieae* owing to involvement of the innate immune system.

Miglinski[41] intending to evaluate the immunomodulatory effect of dry *A. blazei* Murill extract on the growth and differentiation of hematopoietic precursors of granulocytes-macrophage (CFU-GM), in bone marrow and spleen of BALB/c mice infected with *Lysteria monocytogenes,* obtained results demonstrating that *A.* Murill *blazei* has potent immunomodulatory activity able to increase survival of animals infected with a lethal dose of *L. monocylogenes,* likely due to the ability of this extract to restore marrow and spleen hematopoiesis.

In a study by Verçosa-Junior et al.[42] whose purpose was to evaluate the use of *A. blazei* in the form of filtered and full aqueous suspension (10 mg/animal) in the treatment of mice bearing Ehrlich solid tumor testing its anti-cancer activity, the authors found that animals treated daily with A. blazei showed higher values of haematological parameters (erythrogram and leukogram), and final relative spleen weight compared to the control group (distilled water), but with no significant difference ($p > 0.05$).

In works carried out by Ferreira et al.,[43] whose purpose was to evaluate the use of *A. blazei* Murrill mushroom (5%) in topical therapy of experimental poisoning of rabbits by Bothrops alternatus, aiming to antagonize the local effects (oedema, hemorrhage and necrosis) caused by this poison, the outcome showed a lower degree of swelling and bleeding halo in the treated group compared to the control group (saline). They also noticed that in the group treated with *A. blazei* Murrill (5%) there was no death.

Delmanto et al.[44] investigated the probable antimutagenic potency of *A. blazei* in rats, assayed its effect on clastogenicity induced by cyclophosphamide. Results derived from this study suggest that in some circumstances *A. blazei* exhibits antimutagenic activity that probably contributes to the anticarcinogenic effects observed.

Takaku et al.[45] observed the action of ergosterol isolated from the lipid fraction of *A. blazei* as being responsible for antitumor action against sarcoma 180 in mice. According to the authors, tumor regression activity may be related to direct inhibition of angiogenesis, resulting in death of tumor cells.

Eating habits and use of mushrooms

Among the characteristics necessary for food to be framed as functional food, is that these should be conventional foods consumed in normal and usual diet.[17]

In Brazil, mushrooms are not part of the diet of most people, being restricted to economic and cultural groups most favored.[46] According to Shibata et al.,[16] the greatest barriers to the use of mushrooms in Brazil are linked to popular belief in their poisonous nature, expensive, eating habits and poor availability of product on the market.

The low consumption of mushrooms can also be explained by its recent cultivation in the country, still low productivity compared to its commercialization potential. With the development of new cultivation techniques, the market for these products has become an expensive culture, and their popularity depends on reducing the selling price. This could be achieved through increased production or imports, particularly from countries like China.[47]

According to Ishii et al.,[31] further researches must be carried out on the functional characteristics of the genus *Agaricus* mushrooms. Brazil should also pursue a policy of effective use of these foods; enable their consumption by a new target public in the quest for continuous improvement of quality of life and prevention of diseases, mainly cancer.

In research performed by Lemos,[48] the author concluded that different ways of consumption most used with mushrooms are in sauces, followed by fresh or dry form in soup. Mushroom sauté, pickled, on pizzas, pastas and risottos was also mentioned. However, due to its nutraceutical characteristics, the *A. blazei* mushroom can also be consumed as tea or in capsules containing lyophilized extract.[15]

Studies on the addition of mushrooms in functional foods

Bassan et al.[49] developed a gluten-free cake, sponge like, with *A. brasiliensis* mushroom. The authors obtained positive results in this study because the product reached a high level of acceptance (83.22%).

Mesomo et al.[50] determined the chemical composition of *A. blazei* residue obtained after aqueous extraction of β-glucans and analyzed the shelf life of cheese bread made with this byproduct. Observation revealed that *A. blazei* Murrill residue is an excellent source of nutrients and its addition in the cheese bread formulation did not cause significant changes in the visual aspect of the product. For all attributes evaluated by the authors, the sample with the largest storage time had good sensory acceptance, which shows the product can be stored for about 30 days without major changes in taste, texture and appearance.

Escouto et al.[51] noted that there is a diversity of studies on the *A. brasiliensis* mushroom, but realized that there are no literature accounts on the use of this mushroom as food appreciated for its sensory characteristics, nor studies to assess its acceptance. Therefore, we conducted a survey of the acceptance of this mushroom taking a rice dish as reference for developing preparation techniques to boost its use in food. The global average grade obtained in the hedonic scale was 6.14 (liked slightly) and global acceptance rate was 68.3%.

Lemos[48] developed and characterized a product similar to burger based on the *A. brasiliensis* mushroom and compared their characteristics with a control

formulation in which the mushroom was replaced with ground beef and commercialized products: one with bovine meat and another one with vegetable protein. The sensory analysis showed that the mushroom-based product was well accepted by consumers when their attitude and intention to purchase were tested. The formulation that had 12% of mushroom stood out among the others, presenting high protein content (20.31%), carbohydrates (27.84%), dietary fiber (24.47%) and ash (6.12%), higher than the commercial burgers also evaluated in the work, and lipid content (1.60%) was much lower.

In another study headed up by Miller,[52] it was found that tomato sauces with *A. brasiliensis* mushroom had higher amounts of polyphenols in relation to sauces without the extract. The results obtained by the author indicated that *A. brasiliensis* contributed to increase polyphenols in tomato sauces. Glucan complex, lycopene, β-carotene present in this mushroom, meant that when added to tomato sauce they present β-glucan and increased levels of carotenoids and lycopenes.

A study was developed by Silva et al.,[25] aiming at assessing the antioxidant activity of different extracts of mushroom *A. blazei,* as well as the oxidative stability of soybean oil added with mushroom extract. Results demonstrated that mushroom extract is effective in preserving the oil, and could be considered a promising natural potential antioxidant ingredient. The authors concluded that further research on its role at different concentrations is fundamental so that mushrooms might be more competitive in the food market.

Toxicity of mushrooms

Despite the fact that mushrooms are considered a functional food, they may also present some type of toxicity.[10] However, for a food to be considered functional, there should be no risk or toxic effects for the consumer.[5]

The substrate exerts direct influence on the chemical composition of mushroom, because nutrients are removed by hyphae which are in direct contact with this material. Consequently, they absorb essential elements, but together with these they can accumulate toxic metals such as lead, mercury, cadmium, arsenic and others.[53] In this sense, some species of mushrooms have been used as bioindicators of environmental pollution. Knowing that chemical composition of mushrooms may be related to the substrate, it stands to reason that a polluted region will produce mushrooms with high levels of metals. This fact was observed by Kalac et al.[54] when they presented different species of mushrooms such as *A. sylvaticus,* with high levels of accumulated cadmium.

In a study performed by Moura[55] it was detected the presence of arsenic in mushrooms of the genus *Agaricus.* But this fact was not considered indicative of risk to human health, since the concentration of this element in the samples analyzed by the author was rather low.

Bellini et al.[56] observed that the methanolic fractions of *A. blazei* tested in their study did not provide chemical protection, being potentially mutagenic according to results in HGPRT test. For the authors, the methanol extracts of this mushroom should not be used widely by individuals because of the possibility of their genotoxicity. Therefore, care must be taken in the use of *A. blazei* by the population as long as a comprehensive assessment of the biochemical characterization of this fungus is not complete.

In a study conducted by Sugui,[57] the outcome indicates no mutagenic, genotoxic or carcinogenic effects on rats tested with the aqueous solution of the *A. brasiliensis.* Nevertheless, an antimutagenic effect against the mutagenicity of ENU (N-ethyl-N-nitrosourea) was observed in bone marrow cells, in addition to a significant reduction in the number of aberrant crypts per focus (4-6 crypts/focus) induced by DMH (1,2-dimethylhydrazine) in the colon of animals post-treated with the aqueous solution of the mushroom. In this context, results suggest that the aqueous solution of *A. brasiliensis* possesses compounds that can significantly reduce the frequency of micronucleated cells from bone marrow of rats, and that they can act at a later stage of carcinogenesis initiation.

In study carried out by Singi et al.[58] results revealed that the concentration of 1.25 mg/kg of *A. blazei* mushroom did not cause significant changes in mean arterial pressure (MAP) or heart rate (HR).The concentration of 2.50 mg/kg of mushroom caused decreased MAP to 15s ($p < 0.01$) and HR to 30s ($p < 0.001$) and of 5.00 mg/kg decreased MBP to 15s ($p < 0.001$) and HR at 15 and 30s ($p < 0.001$).

Costa et al.,[59] aiming at evaluating the possible protective effects of *A. blazei* tea against the urethane genotoxic action in somatic cells of *Drosophila melanogaster,* noted that no increase was statistically significant in the frequency of mutant spots in larvae exposed to *A. blazei* tea. However, when this mushroom was associated with urethane, we observed a reduction statistically significant in the frequency of mutant spots. The results imply that A. blazei is not genotoxic and has a protective effect against the genotoxicity of urethane.

With the intent of investigating effects of acute toxicity of *A. sylvaticus* aqueous extract by clinical, biochemical and histopathological on healthy mice, Novaes et al.[11] verified that both the administration of the aqueous extract as well as the placebo, caused a temporary rise of apathy, piloerection and respiratory changes, which were slightly more persistent in the group treated with the fungus. Biochemical and histopathological changes were not statistically significant between groups. The authors determined that administration of *A. sylvaticus* aqueous extract showed very low toxicity.

In a study by Ishii et al.,[31] the researchers concluded that the Agaricus blazei mushroom offers no genotoxic

consequences, but made it possible to visualize the antigenotoxic effects. The results suggested that the fungus acted as functional food, capable of promoting immunomodulation when the destruction of cells with DNA damage correlated with cancer development was observed. Therefore, the Sun mushroom had a preventive effect against colorectal neoplastic lesions assessed.

Orsine et al.[60] observed that *A. sylvaticus* extract has no toxicity proving to be safe for human use.

Conclusions

To be included in the group of functional foods, mushrooms should bring benefits to human health, do not present themselves toxic and be included in the daily eating habits. Thus, the beneficts of eating mushrooms of the genus *Agaricus* are shown in several papers. Currently there are many researchers working in order to spread the advantages of the consumption of mushrooms of the genus *Agaricus.*

It has been shown in some studies the rich nutritional composition of mushrooms of the genus *Agaricus,* and the presence of substances that act on the human body, being widely used in therapy against cancer. Also low toxicity was observed in different studies using different toxicological methods evaluation.

Despite the countless beneficial effects on human health, mushrooms of the genus *Agaricus* are little known by the population, making it necessary partnership and combined efforts among producers, industries and researchers in order to disseminate, research and consumption of these foods.

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Original

Nutritional value of *Agaricus sylvaticus;* mushroom grown in Brazil

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Abstract

The bromatological characterization of the *Agaricus sylvaticus* species *(A. sylvaticus),* known as the Sun Mushroom and cultivated in Brazil, is necessary to determine substances with pharmacological and nutritional potential, in view its safe use in food and in human medicine. The purpose of the present study was to determine the chemical composition of the *A. sylvaticus* mushroom grown in Brazil. Mushrooms were obtained in dehydrated form from a producer in Minas Gerais State. Through this study it was able to observe the fungus' rich chemical composition, highlighting the variety and quantity of minerals as well as its high protein content. There are many components of this mushroom that have medicinal properties, which are recognized as excellent antioxidants. Results also proved that the composition of *A. sylvaticus* presented differences when compared to the chemical composition of other *Agaricaceae* fungi.

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Key words: *Therapeutic fungi. Chemical composition. Protein. Mushroom. Cancer.*

EL VALOR NUTRITIVO DE *AGARICUS SYLVATICUS;* SETAS CULTIVADAS EN BRASIL

Resumen

En la caracterización bromatológica del género *Agaricus sylvaticus (A. sylvaticus)*, conocido como la seta del sol y cultivado en Brasil, es necesario determinar las sustancias con potencial farmacológico y nutritivo con el objetivo de un uso seguro en la alimentación y la medicina humana. El objetivo de este estudio fue determinar la composición química de la seta *A. sylvaticus* cultivada en Brasil. Se obtuvieron las setas en su forma deshidratada de un cultivador del estado de Minas Gerais. A través de este estudio pudimos observar la rica composición química del hongo, destacando la variedad y cantidad de minerales así como su alto contenido en proteínas. Esta seta contiene muchos componentes con propiedades medicinales, que se sabe que son excelentes antioxidantes. Los resultados también muestran que la composición de *A. sylvaticus* mostraba diferencias al compararla con la composición química de otros hongos de la familia *Agaricaceae.*

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Palabras clave: *Hongos terapéuticos. Composición química. Proteínas. Setas. Cáncer.*

Abbreviations

A. brasiliensis: Agaricus brasiliensis.
A. sylvaticus: Agaricus sylvaticus.
AOAC: Association of Official Analytical Chemists.
DCFI: 2, 6-dichlorophenol indophenol sodium.
FAO: Food and Agriculture Organization.
Gla: Gamma carboxyglutamic acid.
HPLC: High performance liquid chromatography.
MAPA: Ministério da Agricultura, Pecuária e Abastecimento.
UFG: Universidade Federal de Goiás.
WHO: World Health Organization.

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Introduction

Due to their high nutritional value, mushrooms have been widely consumed by people seeking a healthier and more nutritional diet. Some mushrooms are considered nutraceuticals, that is, functional foods, being that in addition to their high protein content, low concentration of total fats, added to a significant concentration of vitamins and minerals, they contain antioxidants that are extremely important in the cure, treatment, and prevention of various diseases, including cancer.[1]

In Brazil, the consumption of mushrooms by the population is still considered low, but mushrooms of the *Agaricus* genus are becoming very popular owing to their attributed medicinal properties, often associ-

ated to the presence of bioactive compounds with medicinal value, such as phenolic compounds, polyketides, terpenes and steroids, which are recognized as excellent antioxidants.[2]

Several investigations related to dietary supplementation with *A. sylvaticus* mushroom have shown positive results in patients with colorectal cancer in postoperative phase reducing the deleterious effects caused by the disease itself and by conventional treatment,[3] also in the improvement of gastrointestinal changes of these patients.[4,5]

According to Furlani & Godoy,[6] the concentration of macro and micronutrients in food is directly related to the benefits they play in humans and animals.

The aim of this study was to evaluate the chemical composition of the *A. sylvaticus* fungus (Sun Mushroom) with respect to protein, lipids, carbohydrates, dietary fiber, minerals, fat soluble vitamins and Vitamin C.

Materials and methods

Obtainment of sample of A. sylvaticus *mushroom (Sun Mushroom)*

A sample of dehydrated *A. sylvaticus* mushroom (Sun mushroom), was obtained from a producer in Minas Gerais State. To allow greater extraction of its components, the mushroom was mashed up in a Willey type (Model ET-648, Tecnal Brand mill). The physical and chemical analysis were performed at the Physical Chemistry Laboratory of the Food Research Center, School of Veterinary Medicine (accredited by MAPA - Ministério da Agricultura, Pecuária e Abastecimento) and the Laboratory of Food Biochemistry, Pharmacy School, both from Universidade Federal de Goiás - UFG, from March to June 2010.

Chemical characterization

The whole analysis, in duplicate, has followed the official methods established by MAPA, by the Association of Official Analytical Chemists (AOAC).[7-10] Moisture analysis were performed using a kiln at 105 º C ± 3 °C for 24 hours and total ash by means of sample calcination in a muffle furnace at 550 ºC for 12 hours. The Kjedahl method was utilized for protein determination, using a 6.25 correction factor. Sample fat content was detected by continuous "Soxhlet" device type extraction. Determination of total dietary fiber was based on sequential enzymatic digestion of the dried mushroom sample with alpha-amylase thermo-stable; protease and amyloglucosidase. The determination of carbohydrates was calculated by the difference, using rates obtained by moisture analysis, fixed mineral residue, proteins and lipids.

Evaluation of minerals

The determination of minerals was performed by means of atomic absorption spectrometry (spectrometer GBC Brand, Model 932AA), in duplicate. The search for iron, zinc, manganese, sodium, potassium, cobalt, copper, calcium and magnesium made was possible, as the laboratory where these tests were performed only contained specific cathode lamps for each of these minerals.

Evaluation of fat-soluble vitamins

Fat-soluble vitamins were determined by high performance liquid chromatography (HPLC), in duplicate. This analysis was used to determine the oil extracted lipids, stored at 10 °C for conservation. Gilson brand liquid chromatography was used with a stationary phase column E-18, column 10 cm/4.6 mm and 5 micras particles. Methanol was used for the mobile phase, utilizing an isocratic working system with 100% methanol and 1 mL/min flow. Variable wavelength was used for each vitamin studied.

Evaluation of Vitamin C

The determination of Vitamin C was performed in triplicate, following the Tillmans Method with titration of standard solution of ascorbic acid and oxalic acid solution with DCFI solution (2, 6-dichlorophenol indophenol sodium), and the solutions used were prepared as described by Instituto Adolfo Lutz[11] for Tillmans Method. To determine Vitamin C it was obtained an aqueous, non fractioned extract of *A. sylvaticus* mushroom from diluted dehydrated mushrooms ground in water, kept under agitation at room temperature for one hour.

Results and discussion

Chemical composition of Agaricus sylvaticus

The nutritional value of food is commonly expressed according to the chemical composition or percentage of homogeneous groups of substances in one hundred grams of food, which are: moisture, lipids, proteins, carbohydrates, fiber and ash[11] table I shows the results found by analyzing the chemical composition of dehydrated *A. sylvaticus* mushroom.

As they have high nutritional value, mushrooms have been identified as alternatives for a healthier diet rich in proteins. They are highly recommended in countries with high rates of malnutrition,[13] or for people who need a high protein diet with low lipid content.[14] Observation noted that the *A. sylvaticus* mushroom grown in Brazil contains high protein content (41.16%). However,

Table I
Bromatological composition (% per 100 g) of dehydrated A. sylvaticus *mushroom cultivated in Brazil in 2010*

Analysis	*Humidity*	*Ash*	*Protein*	*Lipids*	*Carbohydrates*	*Fibers*
A. sylvaticus	6.31	7.38	41.16	6.60	36.21	2.34

*Results are shown in % in 100 g sample.
*The chemical analysis of this study was performed in duplicate.
*The methodology of the chemical analysis used with dehydrated A. sylvaticus mushroom is described by AOAC: Moisture (kiln 105 ºC), ash (muffle furnace at 550 °C), proteins (Kjedahl), lipids (Soxhlet), Carbohydrate (difference from the other constituents of 100%), and dietary fiber (by enzymatic digestion of the sample).

although some authors compare the nutritional value of mushrooms to that of beef (approximately 14.8%),[15] it should be taken into account the biological utilization of protein, since the *Agaricus brasiliensis* mushroom presented, in some studies,[16] low concentrations of essential amino acids necessary for animal growth in experiments, as well as other native cultivated mushrooms in the far east.[17]

In 2005 a survey was conducted on the chemical composition of *A. sylvaticus* grown in Brazil by the Japan Food Research Laboratories.[18] For the dehydrated mushroom, were found values of 4.4 g/100 g of moisture, 39.4 g/100 g of protein, 3.0 g/100 g of lipid, 45.6 g/100 g of carbohydrate and 7.6 g/100 g of minerals. The *A. sylvaticus* mushroom grown in Brazil in 2010 showed higher values of moisture content (6.31%), lipids (6.60%) and protein (41.16%), which can be explained taking into account the differences in growing region, climate, genetic mutations,[18] conditions which are probably better in the areas cultivated today.

According to Minhoni et al.,[20] the qualitative characteristics of mushrooms are also influenced by species, strain, post-harvest processing, the basidiomata development stage, part of basidiomata and substrate. Braga et al.,[21] highlight age, environment and locality, as factors influencing the variations in protein content of mushrooms. According to these authors, young mushrooms are richer in protein than the more mature and open ones. In works performed by Shibata & Demiate,[22] the authors observed that smaller mushrooms have higher protein content, mainly at the pileus.

In addition to high-protein content, the *A. sylvaticus* mushroom contains high biological value, since it presents all the essential amino acids,[23] as shown by research conducted by the Japan Food Research Laboratories[18] on the *A. sylvaticus* grown in Brazil. Such research detected 1.71 g/100 g levels of arginine, 1.55 g/100g levels of lysine, 0.62 g/100 g levels of histidine, 1.11 g/100 g levels of phenylalanine, 0.83 g/100 g levels of tyrosine, 1.72 g/100 g levels of leucine, 1.01 g/100 g levels of isoleucine, 0.39 g/100 g levels of methionine, 1.28 g/100 g levels of valine, 1.75 g/100 g levels of alanine, 1.25 g/100 g levels of glycine, 1.26 g/100 g levels of proline, 5.73 g/100 g levels of glutamic acid, 1.20 g/100 g levels of serine, 1.2 g/100 g levels of threonine, 2.35 g/100 g levels of aspartic acid, 0.43 g/100 g levels of tryptophan and 0.36 g/100 g levels of cystine.

According to Henriques et al.,[16] it is important to check the standards set by FAO/WHO (Food and Agriculture Organization/World Health Organization) for essential amino acid contents such as lysine and leucine, so that the mushroom protein will not be considered as low-quality protein and digestibility. In such case, this mushroom should not be indicated as the only source of protein to ensure satisfactory growth levels.

The wealth of nutrients from the *A. sylvaticus* mushroom is of great importance in terms of public health, since the Brazilian population has a high number of obese people.[14] According to results related to amounts of protein and lipids in the present study, *A. sylvaticus* mushroom can be presented as an important alternative for healthy food, assisting those who seek better quality of life. The *A. sylvaticus* mushroom could be used as food in a mixed diet with other protein sources, or be added to other foods in the hope of enriching the product, as suggested by Monteiro,[24] in adding the *A. brasiliensis* mushroom to tomato sauce.

With respect to the lipid content in this study, 6.60% of this nutrient was detected in the *A. sylvaticus* mushroom. According to Borchers et al.,[25] although mushrooms contain small quantities of total fat, they have a high percentage of polyunsaturated fatty acids (PUFA) and low content of saturated fatty acids and cholesterol. According to Novaes & Novaes,[16] crude fat of mushrooms consists of several classes of lipids, including free fatty acids, mono-di and triglycerides, sterols, terpenoids and phospholipids, especially lecithin.

The amount of carbohydrates found in the *A. sylvaticus* mushroom was 36.21%. According to Shibata & Demiate,[22] carbohydrate content increases when the strain of mushrooms has increased size, and upon analyzing the carbohydrate content of the pileus, a lower concentration of this nutrient is presented when compared to the strain.

In a study by Copercom,[26] the chemical composition of other mushrooms of the *Agaricus* genus, *A. brasiliensis* in dried state showed the following results: water (7.5%), protein (36.6%), lipids (3.4%), fiber (6.8%), ash (7.3%), and carbohydrates (38.3%). Comparing these results with those of the present work, we see that only the ash content of the fungi studied was similar.

On aiming to analyze the chemical composition of two strains of *Agaricus Blazei Murrill*, Shibata & Demiate,[22] protein values of 34.80% to 39.80%, fiber

values of 7.35% to 9.65%, ash values of 6.99 % to 7.89%, lipid values of 0.80% to 3.68% and carbohydrate values of 46.22% to 41.41% were found, which also differ from those results presented in this paper.

A study on *A. sylvaticus* mushroom detected an amount of 2.34% of dietary fiber. According to Novaes & Novaes,[16] the dietary fiber contained in mushrooms has adverse physical action on the absorption of toxic, harmful and carcinogenic substances. Numerous studies show that the fibers are associated to a lower incidence of colorectal cancer, since it accelerates faecal excretion by laxative action, reducing time spent in the intestines. By studying the chemical composition of edible mushrooms, Andrade et al.[27] observed that crude fiber content varies depending on the part of the mushroom like the stalk, pileus or the whole basidiomata.

Characterization of minerals present in the Agaricus sylvaticus *mushroom*

Table II presents the mineral composition of nine minerals researched in *A. sylvaticus* fungus according to the conditions and limitations of the laboratory used in this study.

Among micronutrients, substances required by the body in small quantities for normal operation are zinc, copper, selenium, manganese, chromium, molybdenum and iron.[28]

Significant amounts of iron were found (726.90 mg/100 g) in the *A. sylvaticus,* which makes the mushroom a rich source of this mineral. According to Crichton et al.,[29] iron works in oxygen transport, DNA synthesis, redox reactions in the electron transport chain, and is part of the molecular chain of several proteins and enzymes.

Results also showed 1.35 g/100 g of calcium in the *A. sylvaticus.* Calcium is very important for bone mineralization, maintaining the structure and rigidity of the skeleton.[30]

Table II
Determination of minerals in A. sylvaticus

Minerals	*A. sylvaticus (mg/100 g)*	*Recommended Daily Intake (RDI) for adults (ANVISA, 1998)*
Iron	726.90	14 mg
Calcium	1.35	800 mg
Zinc	549.25	15 mg
Cobalt	7.75	–
Magnesium	21.19	300 mg
Sodium	255.34	–
Potassium	613.03	–
Manganese	23.18	5 mg
Copper	276.66	3 mg

*Analyses of minerals were performed by atomic absorption spectrometry.

A. sylvaticus mushroom has also presented an important source of zinc (549.25 g/100 g). Zinc has an important physiological role, acting as an antioxidant, preventing lipid peroxidation.[31] Zinc, found in significant concentrations in *A. sylvaticus* grown in Brazil in 2010, has been the object of studies in various researches related to the performance of this mineral in the human body. Studies have shown that children supplemented with zinc have lower incidence of diarrhea, pneumonia and malaria, when compared with children not receiving zinc.[32-33]

Magnesium acts as a cofactor of both enzymes responsible for various metabolic activities and in innate and acquired immune response, in addition to the important role of tissues maintenance and lymphoid cells.[34] It was found, 21.19 g/100 g of this mineral in the *A. sylvaticus*.

In this study, it was found high values for sodium content in *A. sylvaticus* mushroom. According to Amazonas Mala,[23] these mushrooms have significant amounts of sodium.

Copper is an essential trace element involved in multiple enzyme systems including the immune response[35] and high concentration is present in the *A. sylvaticus* mushroom (276.66 g/100 g).

In the 2005 research, the Japan Food Research Laboratories,[18] also conducted an analysis of sodium (4.2 mg/100 g), iron (21.2 mg/100 g), calcium (35.7 mg/100 g), potassium (3.15 mg/100 g) magnesium (100 mg/100 g), copper (8.24 mg/100 g), zinc (6.61 mg/100 g), manganese (0.65 mg/100 g), selenium (36 g/100 g), cobalt (0.13 ppm). Neither molybdenum nor boron was detected. Comparing these results with those of the present study, one may observe the difference between results for most minerals, which come in higher concentrations in this work. According to Urben,[19] this variation in minerals can be explained by the type of crop, climate, region, genetic mutations among others, which are possibly more favorable regarding the techniques used to cultivate *A. sylvaticus* mushroom today.

Borchers et al.[25] also observed the presence of potassium, calcium, phosphorus, magnesium, iron and zinc. In a study by Copercom,[26] the mineral composition of the dehydrated *A. brasiliensis* mushroom showed the following results for phosphorus, iron and calcium: 939 mg/100 g, 18.2 mg/100 g and 41.6 mg/100 g, respectively.

Oliveira et al.,[14] upon studying the *A. blazei* fungus, found high levels of minerals such as potassium (2.34%), phosphorus (0.87%), calcium (0.07%), magnesium (0.08%), sulfur (0.29%), copper (61.88 mcg), zinc (86.90 mcg), iron (79.63 mcg).

Characterization of vitamins present in the Agaricus sylvaticus *mushroom*

Table III shows the vitamins composition in *A. sylvaticus* fungus according to the conditions and limi-

Table III
Determination of fat-soluble vitamins and Vitamin C in the Agaricus sylvaticus *mushroom cultivated in Brazil*

Vitamins	*A. sylvaticus*	*Recommended Dietary Allowances (RDA) for adults (ANVISA, 1998)*
Ascorbic acid (Vitamin C)	12.65 mg/100 g	60 mg
A complex	– Retinol: 0.001 mg/100 g (Retinol acetate, retinol palmitate and retinol propionate were not detected).	800 µg
Vitamin D2	0.018 mg/100 g	5 mg
E complex	– Alpha tocopherol: 0.020 mg/100 g (Tocopherol acetate, Beta tocopherol, Delta tocopherol and Gamma tocopherol were not detected)	10 mg
K Complex	– Menaquinone (K2): 0.001 mg/100 g [Phylloquinone (K1), Menadione (K3) and Naftoquinona were not detected (K4)].	80 µg

*The determination of fat-soluble vitamins was performed in duplicate, using liquid chromatography from oil obtained in the lipid analysis of A. sylvaticus mushroom.
*The wavelengths used in chromatography for the analysis of fat-soluble vitamins were mixed (varied) (λ = 460 nm for Complex A, Vitamin D2 and Vitamin K3; λ = 295 nm for Complex E; λ = 350 nm for vitamin K1 and K4; λ = 280 nm for Vitamin K2).
*The analysis for detecting Vitamin C was performed in triplicate by titration from the non fractioned aqueous of A. sylvaticus extract.

tations of the laboratories used in this study to develop the analysis.

As seen in table III, Vitamin C was detected in samples of *A. sylvaticus* analyzed in this study, which disagrees with results presented by the Japan Food Research Laboratories[18] in 2005.

Vitamin C acts on cicatrizing wounds, collagen synthesis, skin lightener.[36] Photoprotection increases and improves the antioxidant defenses.[37] The recommended daily dose for maintaining Vitamin C saturation level in the body is approximately 100 mg. Higher doses are necessary in cases of infections, pregnancy and breastfeeding.[38] According to Lederer,[39] the importance of Vitamin C is associated to several types of cancer, since daily doses administered to cancer patients provided improved survival.

Vitamin A deficiency causes night blindness, rough and peeling skin, dry mucous membranes, growth inhibition, reduced resistance to infections, defects in bone development and modulation.[40] In the *A. sylvaticus* fungus Vitamin A was found only in the form of retinol (0.001 mg/100 g).

Vitamin K acts as a cofactor for carboxylation of specific glutamic acid residues to form gamma carboxyglutamic acid (Gla), amino acid found in coagulation factors, which appears related to calcium and may regulate the disposal of the mineral matrix bone as part of osteocalcin.[41] In the *A. sylvaticus* mushroom, we detected the presence of Vitamin K2, menaquinone, at 0.001 mg/100 g concentration.

Vitamin E helps protect the long-chain polyunsaturated fatty acid of cell membranes and lipoproteins against oxidation in the body.[42] Among fat-soluble vitamins, alpha tocopherol appeared in higher concentration (0.020 mg/100 g) in the *A. sylvaticus* mushroom.

Vitamin D regulates the metabolism of calcium and phosphorus, maintaining serum calcium and phosphorus able to provide normal conditions for most metabolic functions, including bone mineralization.[43] It was detected 0.018 mg/100 g of Vitamin D2 in the *A. sylvaticus.*

Among the *A. sylvaticus* vitamins exhibited in the survey by the Japan Food Research Laboratories[18] in 2005, the following substances were not detected in the sample: α-carotene, β-carotene and Vitamin C. However, there were findings of 1.21 mg/100 g of thiamine (Vitamin B1), 3.41 mg/100 g of riboflavin (Vitamin B2), 0.83 mg/100 g of Vitamin B6, 0.17 µg of Vitamin B12, 5.8 µg of calciferol (Vitamin D), 0.36 mg/100 g of folic acid, 39.4 mg/100 g of pantothenic acid, 201 mg/100 g of inositol and 39.9 mg/100 g of niacin.

According to Soares,[44] the accumulation of compounds such as vitamins is dependent on the handling, processing and maturity of mushroom at harvest.

Tocopherol acetate and retinol acetate, obtained only synthetically, were not detected in this sample of dehydrated *A. sylvaticus*, as shown in table II.

According to Borchers et al.,[25] mushrooms contain significant amounts of niacin, thiamin, riboflavin, biotin, ascorbic acid and pro-vitamins A and D.

According to Eira & Braga,[45] knowledge of the chemical composition of mushrooms is very important, and in Brazil the genetic and physiological studies, basic and applied, can be extended aiming to select more stable and productive lineages in addition to establishing more appropriate physiological conditions for the production of mushrooms in order to attain a desired standard of quality .

Clinical and experimental studies demonstrate that dietary supplementation with Agaricales mushrooms

and other medicinal fungi exert positive nutritional, medicinal and pharmacological effects and can be used as an adjuvant in cancer therapy. The mechanisms of action of bioactive compounds present in mushrooms are yet to be fully elucidated in the literature, but scientific evidence suggests that these substances are able to modulate carcinogenesis not only at early stages, but also at more advanced ones, providing benefits to individuals with various types of cancer, mainly by stimulating the immune system.[46] It was observed that dietary supplementation with this medicinal fungus can significantly reduce fasting glycemia levels of colorectal cancer patients in post-surgery phase[47] and is capable of improving the life quality of these patients.[48]

Conclusions

Through this study it was able to observe the fungus' rich chemical composition, highlighting the variety and quantity of minerals as well as its high protein content. There are many components of this mushroom that have medicinal properties, which are recognized as excellent antioxidants.

Results also proved that the composition of *A. sylvaticus* presented differences when compared to the chemical composition of other *Agaricaceae* fungi.

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Chemical and Antioxidant Potential of *Agaricus sylvaticus* Mushroom Grown in Brazil

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Abstract

The chemical characterization of *Agaricus sylvaticus (A. sylvaticus)* cultivated in Brazil is necessary to determine nutritional and pharmacological substances in order to guarantee its safe use as food or herbal medicine. The objective of this study was to determine the chemical composition and assess the antioxidant potential of *A. sylvaticus* fungi grown in Brazil. Through this study it was able to observe the rich chemical composition of *A. sylvaticus,* highlighting the variety and amount of minerals as well as the high protein content of this fungus. It was also observed the great antioxidant potential of the aqueous, alcoholic and ethereal *A. sylvaticus* mushroom extracts, emphasizing the alcoholic extract, which testifies the extraordinary benefits of this fungus in diet, since antioxidants prevent premature aging and various types of cancer as well. The composition of *A. sylvaticus* mushroom displayed differences when compared to the chemical composition of the same fungus in other studies and with other *Agaricales* fungi.

Keywords: Chemical composition; Medicinal mushroom; Potential antioxidant

Abbreviations: %: Percentage; Wavelengths; *A. sylvaticus* : *Agaricus sylvaticus*; ABTS: 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid-diamonic; AOAC: Association of Official Analytical Chemists; BHT: di-terc-butil metil fenol; DPPH: 2.2-difenilpicril-hydrazyl; HCl: Chloridric acid; HPLC: High performance liquid chromatography; NH_4^+ : Ammonium; PUFA: Polyunsaturated fatty acids; R^2: Correlation coefficient; TBHQ: Terc butil hidroquinona

Introduction

Mushrooms are considered nutraceuticals or functional foods by many clinicians and researchers, a fact that has also stimulated the search by Brazilian producers for more advanced production techniques along with introduction of new species [1].

According to Urben [3], there is great genetic variety of native *Agaricus* genus mushrooms cultivated throughout the world. Strains produced by these mushrooms result from the kind of substrate or compost used, climatic conditions, cultivation area and genetic mutation that can occur naturally or artificially.

Mushrooms are highly nutritious foods, having high amounts of protein, equivalent to meat, eggs and milk, much higher than vegetables and fruits. They contain vitamins such as thiamine, riboflavin, ascorbic acid (Vitamin C), erbocalciferol (Vitamin D2), and a high percentage of minerals like calcium, iodine and phosphorus, besides considerable amounts of fiber [2].

Chemical studies have revealed that the high concentration of nutrients and active ingredients in mushrooms is directly related to the type of lineage used, which requires specific conditions or several factors, such as: A) nutritional factors (substances essential for development: carbon, nitrogen, vitamins and minerals), B) abiotic factors (moisture content of compost and cover, temperature, light, oxygen, chemicals in air, CO_2), C) and biotic factor (virus, bacteria, actinomycetes, fungi, nematodes, insects, mites and genetic), D) genetic factors (natural or artificial); E) processing factors (harvest, drying/dehydration and storage) [3].

Mushrooms have been used for therapeutic prevention of various diseases, in the form of drugs and/or functional foods [4]. In Brazil, despite the low consumption of mushrooms by the population, *Agaricus* genus fungi are becoming very popular due to attributed medicinal properties. There are several studies that report the effects of *A. sylvaticus* (Sun mushroom) on various diseases and these properties may also be associated to the presence of bioactive compounds with medicinal value, such as phenolic compounds, polyketides, terpenes and steroids recognized as excellent antioxidants [5].

According to Elmastas et al. [6], phenolic compounds seem to be the main component responsible for the antioxidant activity in mushroom extracts. According to Tsai et al. [7], the antioxidant properties of *Agaricus blazei* may be associated with its high concentration of tocopherols.

The aim of this study was to evaluate the chemical composition of dehydrated *A. sylvaticus* fungus with respect to protein, lipids, carbohydrates, dietary fiber, minerals, liposoluble vitamins and vitamin C as well as determine the antioxidant potential of ether, alcoholic and aqueous extracts obtained from this mushroom.

Materials and Methods

Evaluation of chemical composition

In this laboratory based experimental study, samples of dehydrated *A. sylvaticus* (Sun mushroom) mushroom were obtained from a producer in the State of Minas Gerais. Mushrooms were crushed in a Willey type grinder, Model ET-648, Brand Tecnal to allow greater extraction of components. Physical and chemical analysis was performed at the Physical Chemistry Laboratory of "Centro de Pesquisa em Alimentos", School of Veterinary Medicine (accredited

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by the Ministry of Agriculture, Livestock and Supply) and the Food Biochemistry Laboratory, School of Pharmacy, Universidade Federal de Goiás - UFG from March to June 2010.

Moisture evaluation

Moisture evaluation was performed in duplicate with dehydrated *A. sylvaticus* fungus, applying the official method for moisture rating, using a kiln at 105ºC ± 3ºC for 24 hours, established by the Ministry of Agriculture, Livestock and Supply, determined by the Association of Official Analytical Chemists [8].

This methodology quantifies the water withdrawn from the product by heating process, whereas the moisture content is calculated by the weight difference of the sample at the beginning (100%) and at the end of the process (100% -% water evaporated at 105ºC). This difference reflects the moisture of the sample under analysis.

First the sample was weighed (approximately 5g) and placed in a kiln at 105ºC until its weight remained constant. After two weightings at intervals of five hours each, weight was observed to be constant. Next the sample remained in a desiccator in order to lower the temperature (up to room temperature) and was then weighed to check moisture content.

Ash evaluation

Ash evaluation of dehydrated *A. sylvaticus* fungus was performed by calcining the sample in furnace FDG Brand, Model 3P-S 7000, at 550°C for 12 hours, according to the official method of AOAC [8]. Through this technique it is possible to determine the total ash produced using the heat in a muffle furnace, where there is total destruction of organic matter present in the sample, leaving only those minerals present.

A sample of approximately 2g of *A. sylvaticus* mushroom was weighed in a porcelain crucible, which had previously been incinerated with the aid of Bunsen burner, cooled and weighed. Then the set (sample + crucible) was incinerated in a muffle furnace, first at lower temperature and then at 550°C. After incineration, the set was removed from the flask, placed in a desiccator to cool off and weighed when it reached room temperature. The amount of ash in the sample was detected from the weight difference between the weight of the set and the weight of the empty crucible.

The mushroom ash sample served as a starting point for analyzing specific minerals.

Evaluation of minerals

To determine the minerals, an atomic absorption spectrometry was used in spectrometer GBC Brand, Model 932AA. Duplicate analyses were performed. The principle of this technique is based on measuring the absorption of electromagnetic radiation intensity, from a primary source of radiation by gaseous atoms in ground state. It was possible to search for iron, zinc, manganese, sodium, potassium, cobalt, copper, calcium and magnesium, as these tests were performed in a laboratory where there were specific cathode lamps for each of these minerals.

Protein evaluation

For protein grading the Kjedahl method was used following the AOAC [8] methodology. Total nitrogen was obtained from the sample which, through calculation was transformed into protein Nitrogen considering that each 100g of protein contains an average 16g of nitrogen. Therefore we used a 6.25 correction factor, which was multiplied by the total Nitrogen percentage of the sample, which corresponded to the protein percentages [9].

To develop this methodology we used a Nitrogen distiller Brand Tecator, Kjeltec System Model 1026. Protein analysis involved three phases. In the first phase the nitrogen in the sample was transformed into ammonium (NH_4^+) through acid digestion of organic matter, starting from 0.1 g of Degreased Dry Matter. In the second phase, separation was obtained by means of distillation and in the third phase, dosage by titration with HCl 0.02 N.

Evaluation of lipids

The amount of lipids present in the sample of the *A. sylvaticus* mushroom was obtained through continuous extraction with a Soxhlet device, Brand Gerhardt, Soxtherm Model 2000, using sulfuric ether as solvent, which has a boiling point of approximately 35ºC. After extraction, the solvent was evaporated using a Rotavapor and lipid fraction was determined gravimetrically. After 24 hours, we obtained the average weight of lipid fraction. The extracted oil was stored at 10°C for later chromatographic analysis of fat soluble vitamins.

Evaluation of total dietary fiber

The methodology for the evaluation of total dietary fiber of *A. sylvaticus* fungus was proposed by AOAC [10], whose principle is based on the sequential enzymatic digestion of dehydrated mushroom sample, in duplicate, with thermostable alpha-amylase, protease and amyloglucosidase. The digested sample was then treated with alcohol to precipitate the soluble fiber before filtering, and the residue was washed with alcohol and acetone, dried and weighed.

Carbohydrate evaluation

The evaluation of carbohydrates was calculated by the difference, using rates obtained by the analysis of moisture, fixed mineral residue, proteins and lipids, following methodology recommended by AOAC [11].

Evaluation of fat-soluble vitamins

Fat-soluble vitamins were determined by high performance liquid chromatography (HPLC), and the performance of duplicate analysis. The principle of this technique evaluates the extraction of active compounds of vitamins studied and their conversion in free form in chloroform solution for later evaluation.

For this analysis, it was used as sample the oil obtained in lipid analysis through Soxhlet extraction. It was used liquid chromatography, Gilson brand, with a stationary phase column E-18, column 10 cm/4.6 mm and particles of 5micras. For the mobile phase was used a methanol and isocratic working system with 100% of methanol and 1mL/min flow. Variable wavelengths (λ) were used for each vitamin studied, as shown in Table 3.

Vitamin C cvaluation

Vitamin C evaluation was performed in triplicate, following the Tillmans Method starting from titration of a standard solution of ascorbic acid and oxalic acid solution with DCFI solution (2, 6-dichlorophenol indophenol sodium), and the solutions used were prepared as described by the Adolfo Lutz Institute (1995) for the Tillmans Method. To determine Vitamin C, it was obtained an aqueous, non fractioned extract of *A. sylvaticus* mushroom by diluting dried mushrooms ground in water, kept under agitation at room temperature for one hour.

Evaluation of antioxidant potential

The antioxidant potential of *A. sylvaticus* mushroom was determined following the methodology used by Borguini [12]. In

order to avoid interference of light in the sample, the experiment was conducted using material covered with aluminum foil. It was obtained the ether, alcoholic and aqueous extracts from the mushroom. First it was obtained the ether extract by diluting 2.5g of ground mushroom in 50mL of ethyl ether. From non-filtered residue and therefore **ether-insoluble**, it was obtained the alcoholic extract by adding ethanol at 1:20 ratio (residue weight: volume of alcohol). And finally, it was obtained the aqueous extract by adding water to the non-filtered residue from the previous step and also adding distilled water at 1:20 ratio (residue weight: water volume).

BHT was used as a standard antioxidant and DPPH as an oxidant. The antioxidant activity of mushroom extracts was determined by DPPH (2.2-difenilpicril-hydrazyl) described by BRAND-WILLIAMS et al. [13]. DPPH is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule, and thus, is reduced in the presence of an antioxidant.

Absorbance decrease was monitored at 517nm in a spectrophotometer Model SP-220, Biospectro brand, at intervals of 0, 1, 2, 3, 4, 5, 10, 15 and 20 minutes of reaction. The values observed in the spectrophotometer were converted to a percentage scale, which indicates 0% - no inhibition of free radical production, and 100% indicates complete inhibition of the same.

Quantification of total polyphenols

Concentration of total polyphenols was determined by colorimetric method described by Singleton and Rossi [14], using the Folin Ciocalteau reagent.

For quantification of total polyphenols in the sample, a standard curve of gallic acid solution at concentrations of 0.01mg/mL to 0.06mg/mL was used. The correlation coefficient (R^2) was calculated, resulting in $R^2 = 0.99775$ to a 5% level of significance. This test was performed in triplicate, by using the ether, alcoholic and aqueous extracts of sample at the same concentrations utilized for the standard solution of gallic acid.

The reading was performed with spectrophotometer Model SP-220, brand Biospectro at 750nm.

Results

Chemical composition

Table 1 shows the results found by analyzing the chemical composition of *A. sylvaticus* dehydrated mushroom. One can observe the high protein content (41.16%), followed by carbohydrates (36.21%).

Table 2 shows values found for rating minerals in dehydrated *A. sylvaticus* fungus, including iron, zinc, calcium, cobalt, magnesium, sodium, potassium, manganese and copper. It was not possible to determine the dosage of other minerals performed in the laboratory owing to operational reasons.

The quantities of liposoluble vitamins and vitamin C found in the mushroom *A. sylvaticus* are shown in Table 3. Liquid chromatography analysis enabled the analysis of vitamin A in acetate form, palmitate and propionate in addition to its pure form; of vitamin E in acetate form, alpha, beta, delta and gamma tocopherol; of vitamin K in the K1, K2, K3 and K4 form; however, vitamin D2 was detected by titration.

Antioxidant potential

The antioxidant potential of ether, alcoholic and aqueous extracts obtained from *A. sylvaticus* mushroom is shown in Table 4.

Constituent	Composition (% in 100g)
Humidity	6.31
Ash	7.38
Protein	41.16
Lipids	6,60
Carbohydrates	36.21
Dietary fiber	2.34

* The chemical analysis was performed in duplicate.
* The methods of chemical analysis of dehydrated *A. sylvaticus* mushroom are described by AOAC: Moisture (kiln at 105ºC), ash (muffle furnace at 550°C), proteins (Kjedahl), lipids (Soxhlet), Carbohydrate (difference from the other constituents of 100%), and dietary fiber (by enzymatic digestion of the sample).

Table 1: Chemical composition of dehydrated *A. sylvaticus*.

Constituent	Composition
Iron	726.90 mg/100g
Calcium	1.35 mg/100g
Zinc	549.25 mg/100g
Cobalt	7.75 mg/100g
Magnesium	21.19 mg/100g
Sodium	255.34 mg/100g
Potassium	613.03 mg/100g
Manganese	23.18 mg/100g
Copper	276.66 mg/100g

*Analyses of minerals was performed by atomic absorption spectrometry.

Table 2: Evaluation of minerals in dehydrated *A. sylvaticus*.

Vitamin	Composition	Wavelength (λ)
Ascorbic acid (Vitamin C)	12.65 mg/100g	-
Retinol acetate (Vitamin A)	0.000 mg/100g	460nm
Retinol (Vitamin A)	0.001 mg/100g	460nm
Retinol palmitate (Vitamin A)	0.000 mg/100g	460nm
Propionate, retinol (Vitamin A)	0.000 mg/100g	460nm
Vitamin D2	0.018 mg/100g	460nm
Tocopherol acetate (Vitamin E)	0.000 mg/100g	295nm
Alpha tocopherol (Vitamin E)	0.020 mg/100g	295nm
Beta Tocopherol (Vitamin E)	0.000 mg/100g	295nm
Delta Tocopherol (Vitamin E)	0.000 mg/100g	295nm
Gamma tocopherol (Vitamin E)	0.000 mg/100g	295nm
Phylloquinone (vitamin K1)	0.000 mg/100g	350nm
Menaquinone (vitamin K2)	0.001 mg/100g	280nm
Menadione (Vitamin K3)	0.000 mg/100g	460nm
Naftaquinone (Vitamin K4)	0.000 mg/100g	350nm

* The analysis of liposoluble vitamins was performed in duplicate, using liquid chromatography of the oil obtained from the lipids' analysis of *A. sylvaticus* fungus.
* The analysis for detecting vitamin C was performed in triplicate by titration from the non fractioned aqueous extract of *A. sylvaticus* mushroom.

Table 3: Composition of vitamins of *A. sylvaticus* mushroom.

Total polyphenols

The amount of polyphenols detected in the ether, alcoholic and aqueous extracts are shown in Table 5.

Discussion

In this study we observed that the protein content of *A.sylvaticus* (41.16%) is superior when compared to the protein content of beef (approximately 14.8%), as well as of other mushrooms from the *Agaricales* family [15].

In addition to the high-protein content, protein from mushroom *A. sylvaticus* has high biological value, since it exhibits all the essential

amino acids [16], as shown by research conducted by the Japan Food Research Laboratories [14] on *A. sylvaticus* grown in Brazil.

The following levels were detected at the time: 1.71g/100 g of arginine, 1.55g/100g of lysine, 0.62g/100g of histidine, 1.11g/100g of phenylalanine, 0.83g/100g of tyrosine, 1.72g/100g of leucine, 1.01g/100g of isoleucine, 0.39g/100g of methionine, 1.28g/100g of valine, 1.75g/100g of alanine, 1.25g/100g of glycine, 1, 26g/100g of proline, 5.73g/100g of glutamic acid, 1.20g/100g of serine, 1.21g/100g of threonine, 2.35g/100g of aspartic acid, 0.43g/100g of tryptophan and 0,36g/100g of cystine.

Because they are high-protein food, mushrooms are highly recommended for those who need a high protein diet, or for those whose diet has restrictions on lipids. This fact is of great importance regarding public health, since research reveals that the Brazilian population includes a large number of overweight or obese individuals. This is certainly already causing public health concern, upon considering a population whose consumption profile has considerably changed, especially during the 80's, due to economic factors and the related social consequences [18].

According to results on the amounts of protein and lipids in the present study, *A. sylvaticus* mushroom can also be suggested as an important alternative health food.

In the 2005 survey conducted by the Japan Food Research Laboratories on the *A Sylvaticus* grown in Brazil, values found for dehydrated mushroom were 4.4 g/100g of moisture, 39.4 g/100g of protein, 3.0g/100g of lipid, 45.6g/100g of carbohydrate and 7.6/100g of minerals. Comparing the above results with the present study, *A. sylvaticus* mushroom grown in Brazil in 2010 in dried state, shows higher values of moisture content (6.31%), lipids (6.60%) and protein (41.16%), which can be explained if taking into account differences in farming technique, region, climate, genetic mutations [3], conditions which are probably better in the areas where the mushroom is currently cultivated.

In a study by Copercon, cited by Eira [19], the chemical composition of other mushrooms of the genus *Agaricus*, *A. brasiliensis* in dried state, showed the following results: water (7.5%), protein (36.6%), lipids (3.4%), fiber (6.8%), ash (7.3%), and carbohydrates (38.3%). Comparing these results with those of the present work, we see that only the ash content of the fungi studied was similar.

The present study revealed 2.34% value of dietary fiber. According to Novaes and Novaes [15], the dietary fibers contained in mushrooms can absorb toxic, harmful and carcinogenic substances. Countless studies show fibers being associated to lower incidence of colorectal cancer, since it accelerates faecal excretion by laxative action, reducing the time spent in the intestines.

Extract	Antioxidant potential (%)
Alcoholic	75.6
Ethereal	14.6
Aqueous	14.6

* The antioxidant potential of *A. sylvaticus* mushroom was observed from spectrophotometric analysis of three extracts from the sample. As oxidant we used the DPPH as standard.

Table 4: Antioxidant potential of ether, alcoholic and aqueous of *A. sylvaticus* fungus extracts.

Extract	Total polyphenols (%)
Ethereal	4.11
Alcoholic	9.43
Aqueous	0.98

* Total polyphenols research was performed using the Folin-Ciocalteou in spectrophotometer at 750nm.

Table 5: Quantification of total polyphenol of ether, alcoholic and aqueous extracts of *A. sylvaticus* fungus.

With respect to the lipid content, we detected 6.60% of this nutrient in the *A. sylvaticus* fungus. According to Borchers et al. [20], although mushrooms contain small quantities of total fat, they have a high percentage of polyunsaturated fatty acids (PUFA) and low content of saturated fatty acids and cholesterol. According to Novaes and Novaes [15], crude fat mushrooms consists of several classes of lipids, including free fatty acids, mono- di- and triglycerides, sterols, terpenoids and phospholipids, especially lecithin.

The Japan Food Research Laboratories also performed analysis of sodium (4.2mg/100g), iron (21.2mg/100g), calcium (35.7mg/100 g), potassium (3.15mg/100g) magnesium (100mg/100g), copper (8.24 mg/100 g), zinc (6.61mg/100g), manganese (0.65mg/100 g), selenium (36µ g/100g), and cobalt (0.13ppm). Neither molybdenum nor boron was detected. Comparing these results with this study, we can observe the discrepancy between results for the most researched minerals, which come in higher concentrations in this work. According to Urben [3], this variation in minerals can also be explained by the type of crop, climate, region, and genetic mutations, among others, found more favorable in techniques used at present to cultivate the genus *A. sylvaticus* mushroom.

According to [16], mushrooms have significant amounts of sodium. The presence of potassium, calcium, phosphorus, magnesium, iron and zinc was also observed by Borchers et al. [20].

In a study by Copercon, cited by Eira [19], the mineral composition of the dehydrated mushroom *A. brasiliensis* showed the following results for phosphorus, iron and calcium: 939mg/100g, 18.2mg/100g and 41.6mg/100g, respectively.

Olivera et al. [18], studying the fungus *A. blazei,* found high levels of minerals such as potassium (2.34%), phosphorus (0.87%), calcium (0.07%), magnesium (0.08%), sulfur (0.29%), copper (61.88 mcg), zinc (86.90 mcg), iron (79.63 mcg).

Among the vitamins exhibited by *A. sylvaticus* surveyed by the Japan Food Research Laboratories in 2005, the following substances were not detected in the sample: α-carotene, β-carotene and Vitamin C. However, values found were 1.21mg/100g of thiamine (Vitamin B1), 3.41mg/100g of riboflavin (Vitamin B2), 0.83mg/100g of Vitamin B6, 0,17µg of Vitamin B12, 5,8µg of calciferol (Vitamin D), 0.36mg/100g of folic acid, 39.4mg/100g of pantothenic acid, inositol 201mg/100g and 39.9mg/100g of niacin.

As seen in Table 3, vitamin C was detected in samples of *A. sylvaticus* analyzed in this study, which disagrees with the results presented by the Japan Food Research Laboratories [17]. According to Lederer [21], the importance of vitamin C is associated with several types of cancer, and daily doses administered to patients with cancer have improved their survival.

Among the surveyed liposoluble vitamins, alpha tocopherol within the D complex, retinol, within the A complex and menaquinone from K Complex were detected. According to Soares [22], the accumulation of these compounds is dependent on the handling, processing and maturity of mushroom at harvest.

Because they are obtained synthetically, tocopherol acetate and retinol acetate were not detected in samples of dehydrated *A. sylvaticus* mushroom. According to Borchers et al. [20], mushrooms contain

significant amounts of niacin, thiamin, riboflavin, biotin, ascorbic acid and pro-vitamins A and D. According to Eira and Braga [23], knowledge of the chemical composition of mushrooms is very important, and in Brazil the genetic and physiological studies, basic and applied, can be expanded aiming at selecting more stable and productive lineages, establishing more appropriate physiological conditions for the cultivation of mushrooms so as to attain the desired standard of quality.

According to Silva et al. (24), despite the high biodiversity of mushrooms found in Brazil and great exploitation potential, there is little data on the antioxidant activity of mushroom extracts, since antioxidants have the ability to scavenge free radicals, which are harmful to human health [25].

Antioxidants are able to slow oxidation rate, inhibiting free radicals and preventing the onset of diseases, thus contributing to greater longevity, making the balance between free radicals and the antioxidant defense system essential [26].

Clinical and experimental studies demonstrate that dietary supplementation with *Agaricales* mushrooms and other medicinal fungi exert positive nutritional, medicinal and pharmacological effects and can be used as an adjuvant in cancer therapy. The mechanisms of action of bioactive compounds found in mushrooms are yet to be fully elucidated in the literature, but scientific evidence suggests that these substances are able to modulate carcinogenesis not only at early stages, but at more advanced phases of disease progression as well, providing benefits to individuals with various types of cancer, mainly by stimulating the immune system [27].

Regarding antioxidant activity it was observed that the alcoholic extract of the mushroom *A. sylvaticus* has great antioxidant potential (74.6%), suggesting that most antioxidant compounds present in this mushroom can be more easily diluted in alcohol. However, the aqueous and ether fractions showed lower antioxidant potential (14.6% each) when compared to alcoholic fraction. The aqueous fraction presented reduced antioxidant potential (14.6%) compared to results reported by Percario et al. [28] for the fungus in liquid suspension (50%), since in this work, antioxidant compounds had already been extracted by ether and by alcohol.

Polyphenols make a heterogeneous group, composed of several classes of substances with antioxidant capacity, among which phenolic acids and flavonoids stand out. The antioxidant activity of polyphenols is mainly due to its reducing properties, whose intensity of antioxidant activity exhibited by these phytochemicals is notably differentiated because it depends fundamentally on the number and position of hydroxyl groups present in the molecule [29].

In this study we determined the amount of total polyphenol for the etheric, alcoholic and aqueous extracts. We noticed that the largest amount of alcoholic extract is concentrated in polyphenols (9.43mg/100g) followed by etheric extract (4.11mg/100g), and aqueous extract (0.98mg/100g). The use of ethanol made possible the extraction of a higher content of polyphenols, since the alcoholic extract of the *A. sylvaticus* sample exhibited higher total phenolic content than the aqueous and ethereal which hold lower levels of these constituents.

Aiming to evaluate the antioxidant capacity of the *A. sylvaticus* mushroom in different forms of preparation (liquid suspension, fresh, dry and tablets), Percario et al. [28] assessed the ability of samples to inhibit *in vitro* the formation of free radicals by ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid-diamonic) over a period of 90 seconds, resulting in decreased absorbance at 600nm.The authors observed excellent antioxidant activity (%) in all forms of preparation of *A. sylvaticus* at concentrations of 1mg sample. The authors emphasized that the temperatures used in the preparation of the samples were 60°C for the dried mushroom and liquid suspension, since high temperatures can inactivate most molecules with antioxidant properties present in *A. sylvaticus* According to the authors, these molecules are easily degraded when exposed to industrial processes, which reduces their antioxidant capacity. According to Barros et al. [30], the cooking processes are responsible for the reduction of nutrients with antioxidant capabilities in several mushrooms analyzed in Portugal.

Percario [28] researched different molecules with antioxidant capacity in *A. sylvaticus* fungus, and found results of 72mg/g for β-Glucan in the liquid suspension and 14.1mg/g in tablet form. For flavonoids, values of 0.88mg/g were found in liquid suspension and 0.63mg/g in tablet form. For total phenols, values were 0.1mg/g for liquid suspension and 3.4mg/g for tablet form. The author suggested that the antioxidant activity of *A. sylvaticus* mushroom is due to the entirety of molecules it contains, and not a specific component only.

In a study performed by Silva et al. [24] the antioxidant potential of different extracts of the mushroom *A. blazei* was evaluated by the DPPH method. The authors also observed a higher antioxidant activity (28.6%) in methanol extract: aqueous (1:1), with extraction time of six hours. Results displayed in the present work, confirmed that the best antioxidant activity for *Agaricus sylvaticus* extract was in the alcoholic fraction (74.6%), which shows that components with antioxidant properties of this mushroom are more easily soluble in alcohol.

Some authors utilized the researched mushroom extracts as ingredients in some foods in order to find out the antioxidant effect in processed products. Silva et al. [24] added the methanol: water extract (1:1) to soybean oil and obtained good results. Results showed effective protection (20.4 h of oxidative stability), and the activity of *A. blazei* extract was more efficient than the synthetic antioxidant BHT (100mg/kg) and less efficient than the TBHQ (50mg/kg).

Silva et al. [24], evaluating the *A. blazei* mushroom, obtained concentration of 15mg/g of total phenolic compounds in methanol extract: water extract (1:1). The content of total phenolic compounds present in *A. blazei* was also assessed by Tsai et al. [7], who obtained 5.67mg/g of phenolic compounds in the aqueous extract of this mushroom. In this study, the values of total polyphenols were lower. The alcoholic extract of the mushroom *A. sylvaticus* showed 9.43mg/100g of phenolic compounds. The aqueous and ether extracts showed 4.11 and 0.98mg/100g respectively.

Conclusion

Through this study we were able to observe the rich chemical composition of *A. sylvaticus,* highlighting the variety and quantity of minerals and the high protein content of this mushroom. It was also found that the chemical composition of the mushroom showed differences when compared to the composition of the same mushroom in other studies and other mushrooms of the *Agaricales* genus.

It was also observed the great antioxidant potential of aqueous, alcoholic and ethereal extracts of the *A. sylvaticus* mushroom, emphasizing the alcoholic extract, which demonstrated the extraordinary benefits of this mushroom in diet, considering that antioxidants prevent against premature aging and various types of cancer.

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Full Length Research Paper

The acute cytotoxicity and lethal concentration (LC_{50}) of *Agaricus sylvaticus* through hemolytic activity on human erythrocyte

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There is limited information regarding acute toxicity and lethal concentration of edible and medicinal mushrooms. The objective of this paper is to estimate the cytotoxicity of the aqueous extract of *Agaricus sylvaticus* mushroom on human erythrocytes by determining the lethal average concentration (LC_{50}). Six concentrations of the mushroom (17, 8.5, 4.25, 2.125, 1.0625 and 0.5312 mg/mL) were submitted for evaluation of hemolytic activity *in vitro,* using a suspension of blood. Through the Prism GraphPad Software, using the Tukey test for statistical analysis ($p < 0.05$), a curve was constructed with values of *A. sylvaticus* mushroom concentrations versus the values determined by absorbance spectrophotometry at 540 nm. Results of hemolytic activity for the aqueous extract were fitted using nonlinear regression and the equation: Yi = axi / (b + Xi). We used values of y as hemolytic activity and x as log of *A. sylvaticus* mushroom concentration. The coefficient for determining the curve (R^2) was 0.95 of the original data. The percentage of haemolysis increased in a concentration-dependent manner of *A. sylvaticus* extract used. The LC_{50} value obtained was 9.213 mg/mL. Results derived from this experiment suggest that this mushroom extract has very low toxicity proving to be safe for human use.

Key words: Lethal concentration, *Agaricus sylvaticus,* hemolytic activity, sun mushroom.

INTRODUCTION

Chemicals used in therapy should be effective and provide safety (Goodman and Gilman, 2007). Unfortunately, any substance can be a toxic agent and cause undesirable effects (Goodman and Gilman, 2007; Oga, 2003), depending on the dose administered or absorbed, time and frequency of exposure and routes of administration (Oga, 2003). Highly toxic substances cause death at concentrations equivalent to a fraction of a microgram. In others, low toxicity may be almost harmless in concentrations of several grams or more (Goodman and Gilman, 2007; Oga, 2003).

The toxicity of a substance to an organism refers to its ability to cause serious injury or death. In therapy, the concentration of a substance should be enough to achieve the desired effect and achieve it well with the lowest concentration, and as much as possible, without producing adverse reactions or side effects (Oga, 2003).

The safety of drugs and foods should be determined through the analysis of several factors related not only to the individual characteristics of the organism, but also considering the physic-chemical, pharmacodynamic and pharmacokinetic of each substance, the various routes of exposure and different methods of administration

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(Silva, 2006).

Depending on the cultivation and composting, mushrooms can have varying levels of toxicity and risk to human health, although preliminary studies suggest that experimental use of *Agaricus sylvaticus* may present low toxicity. The use of this mushroom in folk medicine began in ancient peoples and between indigenous communities (Novaes et al., 2007).

The assessment of exposure can be performed by measuring the concentration of a substance administered to a particular organism (Oga, 2003). The study of concentration-response or concentration-effect in toxicology is essential and is used to determine the median lethal concentration (LC_{50}) of drugs and other chemicals (Goodman and Gilman, 2007).

The concentration-response curve is represented by the Gaussian theory, rarely found in practice. This curve is calculated statistically from observations of mortality after exposure related to concentrations of the substance to be tested, and it is widely used to calculate the 50% lethal concentration (LC_{50}). The LC_{50} is thus a statistical index which indicates the concentration of a chemical agent capable of causing death in 50% of organisms in a population with defined experimental conditions (Oga, 2003).

To know the effects of a toxic substance and classify them according to their potential lethality or toxicity and concentration-response curve, one needs to perform toxicological tests (Oga, 2003).

Mushrooms of the genus *Agaricus* have been widely studied for their nutritional characteristics and many medicinal properties they exhibit. The *A. sylvaticus* mushroom (Sun Mushroom) has been reported to have rich nutritional composition, with high protein content (41.16%), carbohydrates (36.21%), low lipid content (6.60%), considerable amounts of fiber (2.34%) and minerals (7.38%), besides having excellent antioxidant activity (Costa et al., 2011).

A. sylvaticus has been widely used as nutritional supplement for cancer patients, with likely effects of growth inhibition, tumor regression and stimulation of the immune system of patients.[4] According to recent studies there seems to be clear evidence of its immunomodulatory activity and efficacy against carcinogenic activity of the drug pristine (Hi et al., 2008).

There is also indication that dietary supplementation with *Agaricus sylvaticus* may reduce total cholesterol, LDL-C and triglycerides, with favorable outcome on lipid metabolism and, consequently, on the prognosis of patients with colorectal cancer in post-operative phase (Fortes et al., 2008). Furthermore, it has contributed to improve the quality of life of these patients by significantly reducing the harmful effects caused by the disease itself (Fortes et al., 2007).

The safety and effectiveness of medicinal plants and fungi are dependent on various factors, of these the quality of the product commercialized can be highlighted. Effectiveness and low toxicity to humans should be verified as well (Arnous et al., 2005).

In this context, the objective of this study is to evaluate the acute toxicity of *A. sylvaticus* mushroom aqueous extract *in vitro,* from the determination of lethal concentration (LC_{50}) through its hemolytic activity on human erythrocytes so as to refer the determination of toxicity parameters for human use.

METHODS

The experiment, in triplicate, was performed at the Nanotechnology Institute Laboratory of Biological Sciences, University of Brasilia, Brazil, in January and February 2011.

Obtaining the sample

The sample of dried *A. sylvaticus* mushroom (Sun Mushroom) was obtained from a producer in Minas Gerais State, Brazil.

Preparation of the solution containing the *A. sylvaticus* mushroom

We weighed 9.0 g of dehydrated *A. sylvaticus* mushroom and added to the sample 105 mL of distilled water. The solution was stirred for 20 min at room temperature, filtered through paper filter, and then 1000 µL of the solution was distributed into previously weighed Eppendorf tubes. The solution was lyophilized and the Eppendorf tubes were then weighed again, in order to obtain the average weight of the mushroom dissolved in water (17 mg/mL).

Serial dilutions were performed resulting in six concentrations for study: 17, 8.5, 4.25, 2.125, 1.0625 and 0.5312 mg/mL.

Preparation of erythrocyte suspension at 2% (human blood A-)

Erythrocytes were obtained from fresh A Negative type human blood. For erythrocyte suspension, 1 mL of blood was centrifuged for five minutes at 14000 rpm. Next 9.8 mL of saline solution (NaCl 150 mm) and 200 µL of the erythrocytes precipitate were added to the tube. The tube was then centrifuged for ten minutes at 2000 rpm. The supernatant was discarded and the process repeated three more times. Finally, the tube was shaken with the erythrocyte suspension ready for use.

Testing of hemolytic activity - Dose relation/hemolytic activity

Samples with 3 mL of saline solution + 500 µL of erythrocyte suspension + 500 µL of *Agaricus sylvaticus* extract were prepared in six different concentrations. The tubes were stirred manually and incubated at 35°C/60 min. After this interval, the tubes were centrifuged at 2500 rpm for ten minutes. The absorbance of the supernatant was read at 540 nm. The negative control (no haemolysis) was prepared only with saline solution and erythrocyte suspension, and the positive control (100% haemolysis) with 3 mL of distilled water + 500 µL of mushroom extract and a reading taken after 60 min.

We built graphics were built of the kinetics and of the dose-response relationship with mean values and standard deviation (SD). Data were expressed as percentage of viability in control wells, through the GraphPad Prism software, using the Tukey test for statistical analysis ($p < 0.05$).

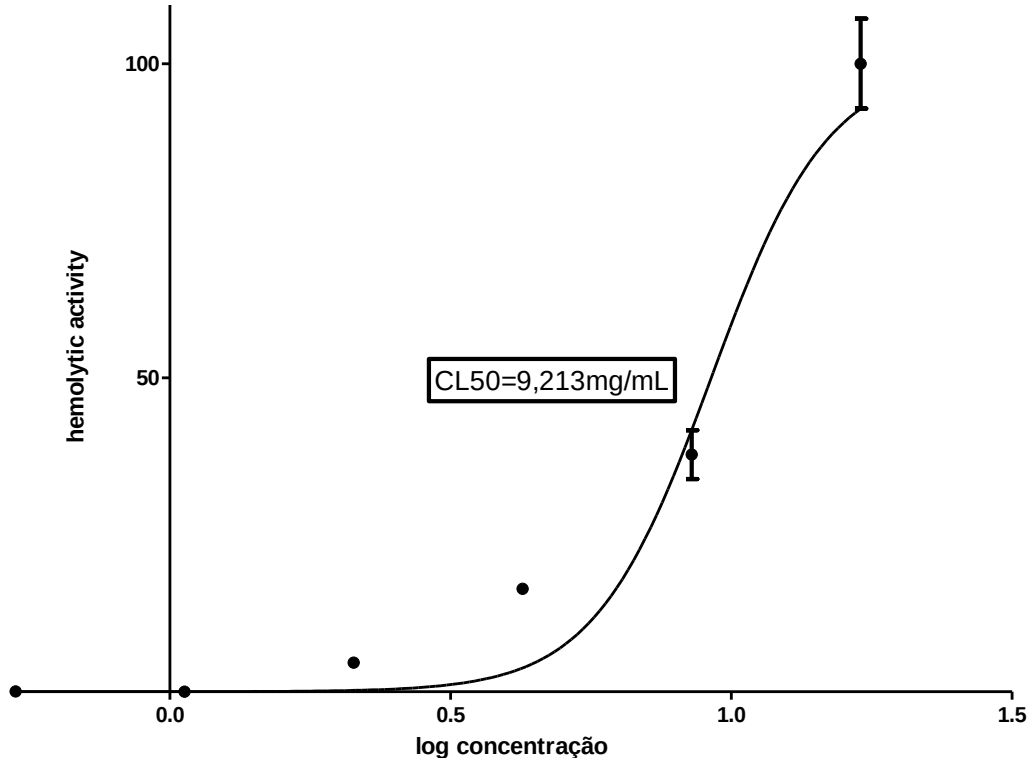

Figure 1. *In vitro* hemolytic activity presented by the aqueous extract of the mushroom *A. sylvaticus* at a 2% suspension of human erythrocytes incubated at 35°C for 60 minutes. The results presented correspond to the average of a test in triplicate.

The assessment of cytotoxicity through hemolytic activity tests has proved to be an alternative screening method for simple toxicity. It is fast, reproducible and inexpensive to evaluate erythrocyte hemolytic activity against concentrations of aqueous extract of *A. sylvaticus,* a fact making it possible to reduce the use of laboratory animals for *in vivo* tests, helping reach the goal to decrease, refine and replace studies conducted with animals.

The intent of reducing animals in the research and development of new methodologies in Brazil is timid and will require further discussion with participation of educational institutions and research laboratories together with the industry and regulatory agencies, since this reality affects all those involved in research, registration and approval of new substances.

As the focus of this article is to observe the acute cytotoxicity of mushroom extract, further studies are still necessary to investigate the mechanism of action of this extract and the possible organs or systems sensitive to the same, as well as additional studies on sub-acute and chronic toxicity, mutagenic and teratogenic activity, embriotoxicity and special studies particularly regarding the choice of concentrations of the extract, so as to validate its safety.

RESULTS

Evaluation of toxicity is paramount when considering a safe treatment. Haemolysis is characterized by erythrocytes rupturing with the release of hemoglobin. The *in vitro* haemolysis test is used as a method for substance toxicity screening, estimating any likely *in vivo* damage (Aparício et al., 2005).

Different aqueous extract concentrations of the *A. sylvaticus* mushroom were tested on a suspension of human erythrocytes at 2% and hemolytic activity determined as haemolysis percentage. We built a curve of concentration (µg of *A. sylvaticus* mushroom) versus percentage of haemolysis and concentration of the mushroom aqueous extract required to produce 50% haemolysis, known as 50% hemolytic concentration or 50% effective concentration (EC_{50}).

Test results of the hemolytic activity in tubes for the aqueous extract of *A. sylvaticus* mushroom were then adjusted using nonlinear regression, through the equation:

$$Yi = axi/(b + Xi).$$

The statistical analysis (Tukey test) was defined according to nonlinear fitting model using the Prism Software. To determine the curve we used the values of y as the hemolytic activity and x as the log of *A. sylvaticus* mushroom concentration. The coefficient for determining the curve (R^2) was 0.95 of the original data.

The percentage of haemolysis increased in a dependent-concentration manner of the extract of *A. sylvaticus* used. The LC_{50} value obtained in this experiment was 9.213 mg/mL.

The curve obtained (Figure 1) represents the hemolytic

activity of aqueous extract of the *A. sylvaticus* mushroom on the solution of human erythrocytes at 2%.

DISCUSSION

Several authors suggest that the exact calculation of LC_{50} is valid only for substances that pose a lethal concentration of 1 and 5000 mg/kg. However, regulatory international institutions of chemical composition toxicity recommend a limit of 2000 mg/kg for the LC_{50} test (Larini, 1997).

By determining the LC_{50} of aqueous extract from the *A. sylvaticus* mushroom, it was observed that this extract has low toxicity, since many grams are needed to cause cellular damage.

No study has been found in the literature using methods of cytotoxicity *in vitro* so that the extracts of this mushroom could be evaluated and compared. Nevertheless, the present results corroborate the results found by Novaes et al. (2007), where the effects of acute toxicity of the aqueous extract of this mushroom were assessed by clinical, biochemical and histopathological parameters in healthy mice, showing very low toxicity.

The low toxicity of this aqueous extract on erythrocytes may be related to the low toxicity of this extract found in animals, suggesting its potential for therapeutic purposes. But there are few studies in the literature regarding comparative sensitivity between these two methods (Cruz et al., 1998).

In 1927, Trevan suggested that lethal concentration should be considered when it kills 50% of the animals (LC_{50}) since the LC_{50} values vary less than those of LD_1 and LD_{99} (dosage required to kill 1 or 99% respectively of the test population) (Silva, 2006). Many toxicity tests currently used for assessment of toxic agents still employ laboratory animals (Harbell et al., 1997). However, the LC_{50} tests advocated by Trevan have been the subject of several reviews and discussions, especially of ethical nature, owing to the large number of animals sacrificed, the suffering caused during some tests, the imprecision of values obtained and the information it fails to provide (Silva, 2006; Cazarin et al., 2004).

Therefore, the completion of toxicological studies in animals with *in vitro* tests is a global trend (Cazarin et al., 2004). The development of new methods for *in vitro* toxicity testing and its recognition by international organizations such as the FDA (Food and Drug Administration) in 1983 and the OECD (Organization for Economic Cooperation and Development) in 1987 has fostered the replacement of tests using laboratory animals (Cruz et al., 1998; Cazarin et al., 2004).

These two organizations, further to promoting the improvement of toxicity tests, have been engaged in reducing costs and time spent in studies, decreasing and replacing animal use (Cazarin et al., 2004).

In this sense, there has been growing demand for *in vitro* tests, which do not sacrifice animals (13). The evaluation of *in vitro* hemolytic action has been used as screening methodology for various toxic agents (Kublik et al., 1996; Mehta et al., 1984). *In vitro* haemolysis tests have also been employed by several authors for the toxicological evaluation of different plants (Gandhi et al., 2000).

According to Queiroz (2009), laboratory experiments with cells reproduce the conditions and even reactions similar to those occurring in the body, and are thus able to observe and quantify changes undergone by cells from a particular product or medicament, as well as the behavior of each cell component separately, restricting the number of variables.

Ralph et al. (2009) through testing for hemolytic activity rated the degree of *in vitro* toxicity according to the observed mortality rate: 0 to 9% = non-toxic, 10 to 49% = slightly toxic, 50 to 89% = toxic; 90 to 100% = highly toxic. Therefore, for new studies to be conducted, the use of non-toxic concentrations (LC0-9) is suggested.

Arguing that the chemical and the pharmaceutical industry perform the LC_{50} test simply because it is required by authorities, in which case without any scientific justification, some authors propose replacing the LC_{50} with maximum non-lethal concentration (MNLC). The MNLC of a substance is defined as the maximum concentration which does not cause any mortality in a number of animals.

This indicator has been proposed as being more useful than the LC_{50} for evaluating the risk/safety of a product by the fact that it uses the non-occurrence of deaths (most severe of toxic effects) as analytical criterion (Larini, 1997). The maximum concentration is defined as the highest dose tolerated without toxic symptoms. The maximum lethal concentration refers to the smallest amount of drug capable of producing death. The therapeutic dose or effective dose is between the minimum and maximum therapeutic dose (Silva, 2006).

Silva et al. (2009) considering that a safe drug cannot cause injury to the plasma membrane of healthy cells, either by forming pores or breaking down the cell, evaluated the cytotoxic activity of triazoles on human erythrocytes. On the other hand, Ralph et al. (2009) evaluated the cytotoxicity of synthetic naphthoquinones on human erythrocytes, demonstrating the possibility of its use for therapeutic purposes, since it had no cytotoxicity on the human erythrocyte membrane.

The hemolytic activity test was also used by Maia et al. (2009), who evaluated the hemolytic activity of dry extract from the bark of *Maytenus guianensis*, verifying that this species did not cause haemolysis on human erythrocytes and may be used for pharmacological purposes.

Furthermore, Schulz et al. (2005) found positive values of the cytotoxic effect from crude extract of *Bacillus amyloliquefaciens* against sheep erythrocytes.

Vieira et al. (2002) in turn, using the hemolytic activity test to investigate the cytotoxic outcome of chloroform on

human lymphocytes, found results that do not prove the cytotoxic action of chloroform, but its genotoxic consequences, since it is capable of causing DNA damage without affecting the normal activity of cells.

Laranjeira et al. (2010) with the purpose of evaluating the hemolytic activity of ethanol extract from *Croton grewioides* leaves on erythrocytes from mice, found results that prove the absence of hemolytic activity on erythrocytes from these animals, suggesting that the cytotoxicity of the extract under analysis was not related to membrane damage, but rather related to apoptosis.

A study by Pita (2010) evaluated the cytotoxicity of natural products utilized in therapy against cancer, obtained from essential oil of *X. langsdorffiana* leaves (trachylobano-360 and OEX) on erythrocytes from mice. The author found values that show the reduced cytotoxic activity of these products.

Cazarini et al. (2004) points out that the *in vitro* alternative tests validated and accepted with regulatory purposes in substitution to methods performed on animals, are still much more a goal than a reality.

The scarcity of literature data to discuss the results and evaluation of acute cytotoxicity *in vitro,* reasserts the need for scientific research of this nature considering that they contribute greatly towards the safe use of such substances by humans.

Results derived from this experiment suggest that this mushroom extract has very low toxicity proving to be safe for human use.

Further study on the safety of using mushroom are needed, since *A. sylvaticus* has now been used for several diseases, including in therapy against cancer.

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