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# International Journal of Medicinal Mushrooms

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# International Journal of Medicinal Mushrooms

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# Aqueous Extract of Culinary-Medicinal Royal Sun Mushroom, *Agaricus brasiliensis* S. Wasser et al. (Agaricomycetideae) Effects on Immunodepression in Mice

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**ABSTRACT:** *Agaricus brasiliensis* S. Wasser et al. (=*Agaricus blazei* Murrill sensu Heinem.) has received attention in folk medicine due to its possible medicinal values. This study evaluated the effects of an *A. brasiliensis* aqueous extract on weight gain; bacterial translocation; intestinal levels of total sIgA; serum levels of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin-10 (IL-10); splenic index; and histology of the ileum, spleen, and liver of mice experimentally immunodepressed with cyclophosphamide. *A. brasiliensis* aqueous extract was administrated *ad libitum* and by intragastric gavage in mice aged 6 to 7 weeks, 2 weeks before the first dose of cyclophosphamide and until the 23rd day of the experiment. Experimental immunodepression was confirmed by body-weight loss during the last week of the experiment, reduction of the splenic index, and altered splenic histology. However, Gram-negative bacteria translocation was not observed in the immunodepressed animal groups during the experimental period. Alterations due to immunodepression treatment and cited above were not influenced by oral treatment with *A. brasiliensis* extract. Similarly, treatment with *A. brasiliensis* extract did not influence intestinal IgA levels or TNF-α, IFN-γ, and IL-10 levels in serum. Under the conditions used in the present study, alterations obtained by experimental immunodepression in mice were not reduced by oral treatment with *A. brasiliensis* extract.

**KEY WORDS:** medicinal mushrooms, Royal Sun Agaricus, *Agaricus brasiliensis*, cyclophosphamide, immunodepression, IgAs, cytokines, bacterial translocation

## ABBREVIATIONS

**BHI:** brain heart infusion; **ELISA:** enzyme-linked immunosorbent assay; **GF:** germ-free; **IFN-γ:** interferon-γ; **IgA:** immunoglobulin A; **IL-1:** interleukin-1; **IL-8:** interleukin-8; **IL-1β:** interleukin-1β; **IL-10:** interleukin-10; **i.p.:** intraperitoneal; **MIP-2:** mouse IL-8 analogue; **MLN:** mesenteric lymph nodes; **PBS:** phosphate buffered saline; **SI:** splenic index; **sIgA:** secretory immunoglobulin A; **SPF:** specific-pathogen free; **TNF-α:** tumor necrosis factor-α

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## I. INTRODUCTION

Mushrooms have been studied for nutritional and medical purposes, and various potential anti-tumoral and immunomodulatory substances, mainly polysaccharides, have been identified.[1] For medical purposes, mushrooms have been consumed to prevent cancer and cardiac diseases, to improve blood circulation, and to reduce cholesterol.[2] Some of these mushrooms have also been used for the treatment of physical and emotional stress, osteoporosis, gastric ulcers, and chronic hepatitis; for the improvement of the quality of life of patients with diabetes; and especially for the stimulation of immunity.[3–7]

The basidiomycete *Agaricus brasiliensis* S. Wasser et al. (=*Agaricus blazei* Murrill sensu Heinem.) is an edible mushroom that is native to southeastern Brazil. It is popularly known as the "Royal Sun mushroom," and is produced and consumed on a large scale as a foodstuff and mainly as an infusion.[3,4,8–10] Since 1965, this lineage has been exported from Brazil to Japan, where it is popularly known as "himematsutake" or "kawariharatake" and is currently widely cultivated and studied.[3]

Although *A. brasiliensis* is popularly used to treat several diseases, including cancer, its complete chemical composition has not yet been established. Kawagishi et al.[11] showed that a (1→6)-β-D-glucan protein complex obtained from the *A. brasiliensis* fructification body was the most active fraction against Sarcoma 180 in mice. Mizuno et al.[12] observed an increase in mouse T-cell subpopulations after oral administration of α-1,6-glucan and α-1,4-glucan isolated from *A. brasiliensis*. Studies also showed that, *in vitro*, *A. brasiliensis* ethanolic fractions activated macrophages, antibody production, and mRNA synthesis for various cytokines (IL-8, TNF-α, IL-6, and IL-1β).[13,14] These observations suggest that a possible effect of medicinal mushroom extracts against cancer could be due to the stimulation of the immune system and not to a direct effect on neoplastic cells. Bernardshaw et al.[15] also observed an increase of MIP-2 and TNF-α levels in the serum of mice treated with *A. brasiliensis* extract, which simultaneously protected them against a *Streptococcus pneumoniae* systemic infection. Ishibashi et al.[16] demonstrated that oral administration of *A. brasiliensis* increased the anti-β-glucan antibody titer, and suggested that it induced a β-glucan-specific response via the mucosal immune system in humans.

Although various *in vitro* reports have described an activation of the immunologic system by *A. brasiliensis* fractions obtained through different extraction processes, to our knowledge there is no study to date that confirms this effect *in vivo*. In particular, research is lacking that examines testing of the extracts obtained through their popular method of preparation. In the present study, an *A. brasiliensis* aqueous extract was evaluated for its effect on weight gain; bacterial translocation; intestinal levels of total sIgA; serum levels of TNF-α, IFN-γ, and IL-10; splenic index; and histology of the small intestine, spleen, and liver of mice experimentally immunodepressed with cyclophosphamide.

## II. MATERIALS AND METHODS

### A. Mice

Male Swiss mice, aged 6 or 7 weeks, from the Central Animal House, Federal University of Viçosa, Viçosa, Brazil, were used in these experiments. The animals were fed a commercial diet for rodents (Purina, Paulínia, SP, Brazil) *ad libitum*, and were kept under constant temperature and humidity conditions with a 12-h dark/light cycle. Animals received water or *A. brasiliensis* aqueous extract *ad libitum* depending on the group. All procedures involving mice were conducted according to the Guide for the Care and Use of Laboratory Animals recommendations. The local ethics committee for animal experimentation approved this project.

### B. Experimental Design

The animals were separated into the following 8 groups (9 mice per group): 1) *Ad libitum* water and intraperitoneal (i.p.) injection of saline solution; 2) *Ad libitum* water and i.p. injection of cyclophosphamide; 3) *Ad libitum A. brasiliensis* aqueous extract and i.p. injection of saline solution; 4) *Ad libitum A. brasiliensis* aqueous extract and i.p. injection of

cyclophosphamide; 5) intragastric gavage water and i.p. injection of saline solution; 6) intragastric gavage water and i.p. injection of cyclophosphamide; 7) intragastric gavage *A. brasiliensis* aqueous extract and i.p. injection of saline solution; and 8) intragastric gavage *A. brasiliensis* aqueous extract and i.p. injection of cyclophosphamide. All animals were weighed at the beginning of the experiment and once every week for 24 days. At the end of the experiment, the animals were sacrificed for organ and blood collection.

### C. Preparation of *A. brasiliensis* Extract

Dehydrated samples of *A. brasiliensis* were acquired from the Mushroom Producers Association north of the Minas and Jequitinhonha Valley (APROCONOVA, Montes Claros, Brazil). The aqueous extract was prepared according to the methods of Delmanto et al.[9] and Barbisan et al.[10] Briefly, dehydrated mushrooms were pounded into a powder. The extract was prepared by adding 2.5 g of the powder into 100 mL of distilled water, which was then kept at room temperature for 2 h before filtration. The filtrate, referred to as *A. brasiliensis* aqueous extract, was prepared daily and is considered the popular form of use.[10]

### D. Treatment with *A. brasiliensis* Extract

In groups 3 and 4 receiving the extract *ad libitum*, recipients were covered with aluminum paper to prevent light-mediated decomposition.[10] In groups 7 and 8, mice received 0.6 mL extract $day^{-1}$ $animal^{-1}$ by intragastric gavages.[9] Control groups received water following the same schedule as their respective experimental groups.

### E. Immunodepression Treatment

Cyclophosphamide (Genuxal®; ASTA MÉDICA AG, Frankfurt, Germany) was diluted in a sterile saline solution to 0.85%, according to the producer's instructions. Intraperitoneal administration (i.p.) of 200 mg $kg^{-1}$ of body weight was performed on days 15, 17, 19, and 21 of the *A. brasiliensis* extract treatment. Control groups received i.p. administration of an 0.85% sterile saline solution following the same schedule as their respective experimental groups. Immunodepression due to cyclophosphamide administration was confirmed by total leukocyte counts ranging from 450 to 100 cells $mL^{-1}$ in blood from treated mice. In immunocompetent mice, these values vary from 1000 to 5000 cells $mL^{-1}$.[17]

### F. Determination of Bacterial Translocation

After sacrifice, the liver, spleen, and mesenteric lymph nodes (MLNs) were aseptically removed, weighed, and homogenized in glass tubes containing an 0.85% saline solution and, when necessary, serial decimal dilutions were performed. Aliquots of 0.1 mL from adequate dilutions were plated onto MacConkey agar (Merck, Darmstadt, Germany) and incubated at 37°C for 48 h for the Enterobacteriaceae count.

### G. Determination of Splenic Index

Spleens from the animals were weighed and the splenic index (SI) was expressed as organ weight (mg) per unit of body weight (g).[18]

### H. Determination of Intestinal (sIgA) Levels

Levels of small intestine total sIgA were determined by enzyme-linked immunosorbent assay (ELISA), according to Rodrigues et al.[19] After sacrifice, the small intestine contents were removed, weighed, and diluted in phosphate buffered saline (PBS) in a proportion of 500 mg of intestinal content to 2.0 mL of PBS. After centrifugation at 2000 × *g* for 30 min, the supernatant was collected and kept at –70°C until use. Intestinal fluid samples were diluted 1:10 in PBS and serial dilutions were added in triplicate to 96-well ELISA plates. Total intestinal fluid sIgA was determined using goat anti-mouse IgA (A90-103A; Bethyl Laboratories, Montgomery, TX, USA) and goat anti-mouse IgA conjugated with peroxidase (A90-103P; Bethyl Laboratories). Color development was performed using *o*-phenylene-

diamine (1,2-benzenediamine) (Fast® OPD; Sigma, St. Louis, MO, USA), and absorbance measurements were conducted at 420 nm with an ELISA reader (Thermoplater–TP reader). The total sIgA concentration was determined using a standard curve obtained with purified standard mouse IgA (106-01; Southern Biotechnology Associates, Birmingham, AL, USA).

### I. Determination of Cytokines in Serum

Concentrations of TNF-α, IFN-γ, and IL-10 in serum were determined by capture ELISA. Blood samples were collected by cardiac puncture and centrifuged at 1000 × *g* for 10 min. The supernatant was then frozen at −70°C until further analysis. Cytokine measurements were determined by using commercial kits and following the manufacturer's instructions (DuoSet; R & D Systems, Minneapolis, MN, USA).

### J. Histological Examination

After sacrifice, tissue samples from the ileum, spleen, and liver were fixed in a Karnovsky solution and processed for paraffin embedding. Histological sections (3–5 μm) were stained with hematoxylin and eosin. The slides were examined by a single pathologist (Dr. Rosa M.E. Arantes), who was unaware of the experimental conditions of each group.

### K. Statistical Analyses

Variance and regression analysis were used. A Tukey test was used for means comparison. The level of significance was set at $P < .05$.

## III. RESULTS

### A. Animal Weight

Figure 1 shows that animals submitted to the different treatments during the initial 3 weeks of the experiment experienced a similar weight gain. However, on the fourth week, a similar body weight loss was observed for all of the immunodepressed

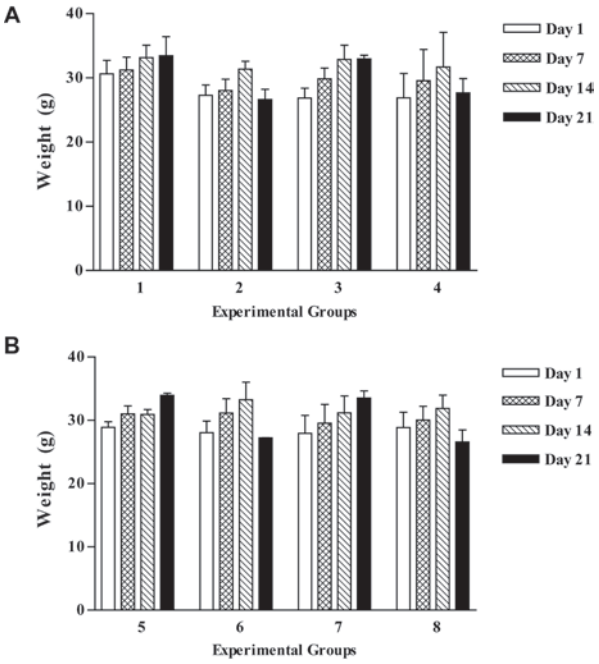


**FIGURE 1**. Influence of immunodepression, *Agaricus brasiliensis* treatment, and method of administration on body weight evolution of mice. Conditions used for each experimental group are described in the Materials and Methods.

mice (groups 2, 4, 6, and 8), independent of both the administration of the *A. brasiliensis* aqueous extract and the means of administration (*ad libitum* or intragastric gavage). Mortality was not observed during the experimental period.

### B. Splenic Index

Figure 2 shows a significant reduction ($P < .05$) of SI values in mice treated with cyclophosphamide. However, the SI value did not increase in immunodepressed animals treated with the *A. brasiliensis* aqueous extract (groups 4 and 8). Furthermore, the SI value did not increase in animals treated with the *A. brasiliensis* aqueous extract (groups 3 and 7) when compared with control animals (groups 1 and 5).

### C. Bacterial Translocation

Translocation of autochthonous Gram-negative bacteria to the MLNs, liver, or spleen was not observed in any of the evaluated groups (data not shown).

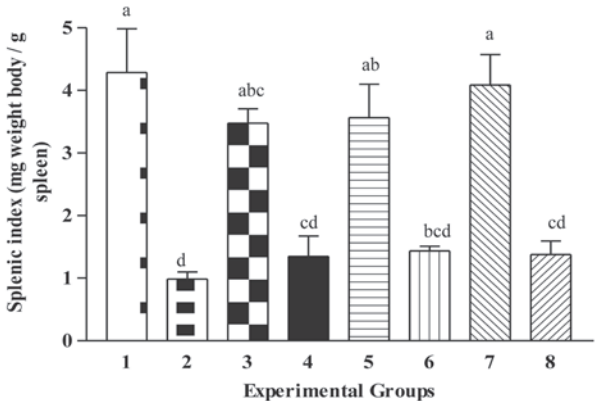


**FIGURE 2**. Influence of immunodepression, *A. brasiliensis* treatment, and method of administration on the splenic index of mice. Conditions used for each experimental group are described in the Materials and Methods. Data are expressed as average ± standard deviation. [a–d]Different letters indicate a statistically significant difference as analyzed by the Tukey test ($P < .05$).

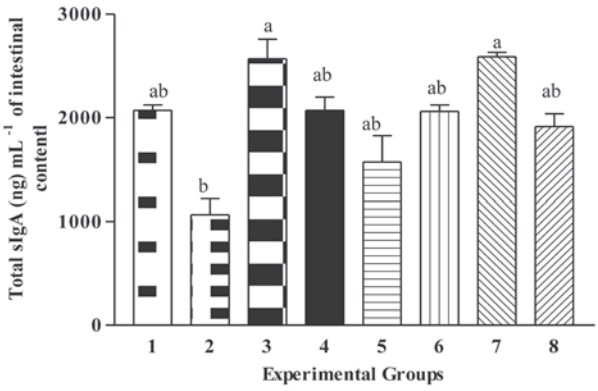


**FIGURE 3**. Influence of immunodepression, *A. brasiliensis* treatment, and method of administration on total intestinal sIgA levels of mice. Conditions used for each experimental group are described in the Materials and Methods. Data are expressed as average ± standard deviation. [a,b]Different letters indicate a statistically significant difference as analyzed by the Tukey test ($P < .05$).

### D. Total Small Intestine (sIgA) Secretion

Figure 3 shows a significant decrease of total sIgA in intestinal contents ($P < .05$) only in the immunodepressed mice receiving water *ad libitum* (group 2) compared to the respective control (group 1). *Ad libitum* ingestion and intragastric gavage of *A. brasiliensis* aqueous extract significantly increased the intestinal total sIgA production ($P < .05$) in immunodepressed animals (groups 4 and 8). However, because a similar increase was observed in immunodepressed mice receiving water by intragastric gavage (group 6), a stimulatory effect of *A. brasiliensis* aqueous extract cannot be suggested.

### E. Seric Cytokine Secretion

There were no differences in cytokine secretion between the TNF-$\alpha$, IFN-$\gamma$, and IL-10 levels in the serum of the tested groups (data not shown).

### F. Histopathological Alterations

Histological examination of the ileum and liver did not show any differences between the groups. However, a reduction in the lymph node follicles and the depletion of lymphocytes were observed in the spleen of immunodepressed animals, confirming the efficiency of the cyclophosphamide treatment. These alterations were not influenced by the administration of *A. brasiliensis* aqueous extract (Fig. 4).

## IV. DISCUSSION

In the present study, an immunodepressed pattern was obtained 1 week after treatment with four doses of 200 mg $kg^{-1}$ cyclophosphamide spread over a 7-day period, as demonstrated by weight loss, reduction of blood lymphocytes and splenic index, as well as by spleen histological alterations. Previous experiments have shown that this was the ideal dose for our animal model. In the literature, lower doses have been used, such as those cited by Péret-Filho et al.,[20] who obtained immunodepression with four doses of 100 mg $kg^{-1}$ cyclophosphamide, or Hou et al.,[21] who observed immunodepression with daily doses of 10 mg $kg^{-1}$ cyclophosphamide.

The significant decrease in SI values observed in the group of immunodepressed animals is a well-known effect that has been previously reported in literature. Anton[22] observed a decrease in SI values in lineage C57BL/6 and DBA/2 mice 1 day after the administration of one dose of 300 mg $kg^{-1}$ cyclophosphamide. Chen et al.[23] also verified that the administration of one dose of 150 mg $kg^{-1}$

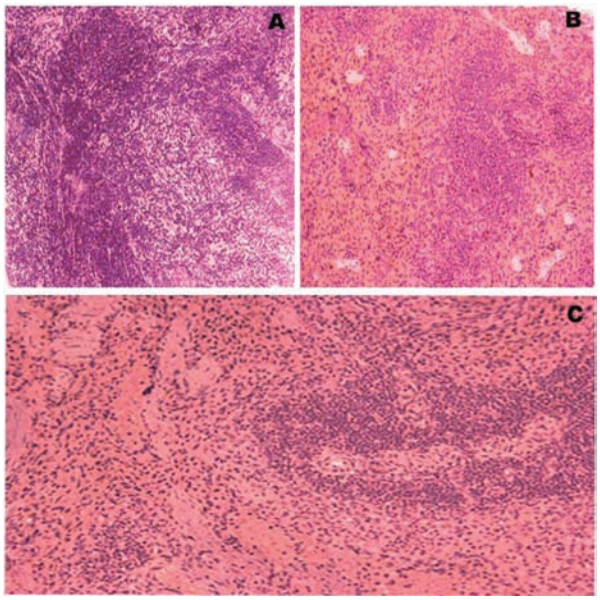


**FIGURE 4**. Histopathological aspects of spleen parenchyma of experimental mice. (**A**) immunocompetent mice (G1); (**B**) immunodepressed mice (G2); (**C**) immunodepressed mice treated with aqueous extract of *Agaricus brasiliensis* (G4). Hematoxylin and eosin (100×).

cyclophosphamide significantly reduced the SI values of mice compared with animals from a non-immunodepressed control group. A reduction of SI values was also related to the splenocyte number reduction, and not only to the fluid volume loss reduction or connective tissue of this organ.[24]

On the other hand, the absence of autochthonous Gram-negative bacteria translocation in animals that received cyclophosphamide does not corroborate data obtained by Berg in a similar study.[25] Berg observed that a unique cyclophosphamide intraperitoneal injection, in different doses (100–400 mg $kg^{-1}$), promoted bacterial translocation from indigenous microbiota to the MLNs, spleen, and liver in specific-pathogen free (SPF) mice 1 week after administration. Berg[25] emphasized, however, that there was not a direct relationship between the immunosuppressive characteristics of other drugs also tested in the study and their capacity to promote bacterial translocation. According to Berg, drugs may promote toxic manifestations in the intestine, such as mucosa injury, which allow for autochthonous bacterial translocation. In the present work, histopathological alterations in ileum fragments were not observed in immunodepressed animals. On the basis of our results and on Berg's inference,[25] it may be presumed that the absence of translocation in immunodepressed mice could be partially explained by the preservation of the ileum architecture. Another relevant fact that must be highlighted is that the methodology used in the present study was different from that used by Berg.[25] This author performed a pre-enrichment step of collected organs in BHI broth, at 37°C for 48 h before plating in culture media, which can amplify a physiological translocation level to an apparent pathological one. Similar to the present study, Suzuky et al.[26] did not observe *Escherichia coli* C25 translocation to the MLNs of antibiotic-decontaminated SPF animals and of germ-free (GF) animals, which received different cyclophosphamide doses and treatments. These authors verified the reduction in the number of lymphoid cells, especially B cells, in the Peyer plates, MLNs, and spleen, and suggested that the lack of bacterial translocation may have been due to morphological and physiological changes in M cells, independent of immunologic function changes.

The apparent increase of IgA secretion in immunodepressed animals that received *A. brasiliensis* aqueous extract in comparison to immunodepressed animals that received water does not necessarily indicate a local protection of mouse mucosa, because IgA is the predominant antibody class in many external secretions and has many direct and indirect functions that are useful in protecting against infectious agents such as bacteria and viruses.[27,28] In addition, the majority of IgA secreted in intestinal mucosa is directed against commensal microbiota in a T lymphocyte independent manner, and is also considered part of the innate immune system.[29] According to Bollinger et al.[30] and Everett et al.,[31] secretory IgA (sIgA) is one of the main factors preventing bacterial translocation. Alverdy and Ayos[32] observed bacterial translocation to the MLNs of mice in which sIgA secretion was experimentally reduced by corticoid administration. An IgA increase was also related to a local immunity increase in studies of probiotic use.[19,33]

The similar TNF-α, IFN-γ, and IL-10 levels observed in the serum of all of the experimental and control groups did not corroborate with the *in vitro* data of Ellertsen et al.,[34] who reported that

commercial *A. brasiliensis* aqueous extract had an effect on cytokine gene expression in human monocyte cells. These authors observed that after 24 h of monocyte exposure to *A. brasiliensis* aqueous extract, there was a $T_H1$ response induction, as indicated by an increase in IL-8, IL-1β, and TNF-α expression. However, it must be noted that in the present study, blood was collected only at the end of the experimental period and that studies on cytokine expression are more sensitive and specific than secretion analyses.[14] Another factor that influences data is the variation in methodology to obtain the *A. brasiliensis* extract used in the experiments, making it difficult to directly compare our results with other groups. For example, Grind et al.[7] did not observe any systemic effects when *A. brasiliensis* aqueous extract was orally administrated to patients with hepatitis C virus. According to these authors, this possibly occurred because β-glucan, the supposed agent responsible for the *in vitro* cytokine secretion effects, was not absorbed by the patients' intestines. The hydrolysis of β-glucan by indigenous microbiota before its absorption could be another possibility.

The histological alterations observed in the spleen were also expected and compatible with the immunodepressed situation caused by the cyclophosphamide treatment.[35] With regard to the weight loss and SI reduction, no effect was observed for this histological parameter when mice were previously treated with the *A. brasiliensis* extract.

The *A. brasiliensis* extract did not promote SI reduction, histopathological alterations, or IgA and cytokine secretion in the studied animal model, which does not correspond with some data in the literature that describe *in vivo* and *in vitro* effects of *A. brasiliensis* extracts on tumors,[11,18] immunologic system parameters,[8,13,14,34] and induced mutations.[9,3,4] In addition, the absence of such effects using *A. brasiliensis* aqueous extract was also reported in other works, such as those by Luiz et al.[36] and Guterrez et al.[4] on anti-mutagenic activity *in vitro* and Grind et al.[7] on the treatment of patients with hepatitis C. In contrast, some reports describe a strong causal relationship between severe hepatic dysfunction and the use of *A. brasiliensis* aqueous extracts in patients with cancer.[37]

In conclusion, the experimental animal model used in this study was adequate to investigate the proposed biological phenomenon of *A. brasiliensis* extracts. Weight loss, SI value reduction, and spleen histological alterations were observed in mice treated with cyclophosphamide. However, *A. brasiliensis* aqueous extract did not reduce these alterations. Therefore, more studies on the supposed effects *in vivo* of *A. brasiliensis* extract are necessary before it can be used as an alternative or complementary therapy.

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# Antihyperglycemic and Antilipidperoxidative Effects of Polysaccharides Extracted from Medicinal Mushroom Chaga, *Inonotus obliquus* (Pers.: Fr.) Pilát (Aphyllophoromycetideae) on Alloxan-Diabetes Mice

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**ABSTRACT:** The optimum conditions for the extraction of crude polysaccharides from dry matter of culture broth (DMCB) of *Inonotus obliquus* were determined by using response surface methodology (RSM). In alloxan-induced diabetic mice, treatment with polysaccharide extract from DMCB of *I. obliquus* (150 and 300 mg/kg body weight for 21 days) showed a significant decrease in blood glucose level, with percentage reductions of 16.64% and 20.09% at the 7th day, and 29.71% and 36.36% at the 21st day, respectively. Furthermore, polysaccharide treatment significantly decreased serum contents of free fatty acid, total cholesterol, triglyceride, and low-density lipoprotein cholesterol, whereas it effectively increased high-density lipoprotein cholesterol, insulin levels, and hepatic glycogen contents in the liver of diabetic mice. In addition, the polysaccharides significantly increased catalase, superoxide dismutase, and glutathione peroxidase activities, whereas they decreased the maleic dialdehyde level in diabetic mice. A histological morphology examination showed that the polysaccharides restored the damage of pancreatic tissues in mice with diabetes mellitus. The results revealed that the *I. obliquus* polysaccharides possessed antihyperglycemic, antilipidperoxidative, and antioxidant effects in alloxan-induced diabetic mice.

**KEY WORDS**: medicinal mushrooms, polysaccharides, antihyperglycemic, antilipidperoxidative, antioxidant, diabetes, chaga mushroom, *Inonotus obliquus*

## I. INTRODUCTION

Diabetes mellitus (DM) is a chronic disorder of the metabolism caused by an absolute or relative lack of insulin. It is characterized by hyperglycemia in the postprandial and/or fasting state, and in its severe form is accompanied by ketosis and protein wasting.[1] In addition to hyperglycemia, several other symptoms, including hyperlipidemia, are involved in the development of microvascular and macrovascular complications of diabetes, which

## ABBREVIATIONS

**b.w.:** body weight; **CAT:** catalase; **DM:** diabetes mellitus; **DMCB:** dry matter of culture broth; **FFA:** free fatty acid; **GPx:** glutathione peroxidase; **H&E:** hematoxylin and eosin; **HDL-C:** high-density lipoprotein cholesterol; **LDL-C:** low-density lipoprotein cholesterol; **MDA:** maleic dialdehyde; **RSM:** response surface methodology; **SOD:** superoxide dismutase; **TC:** total cholesterol; **TG:** triglyceride

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are the major causes of morbidity and death.[2] The World Health Organization has predicted that 300 million people will have diabetes mellitus by the year 2025.[3]

DM is a pathologic condition, resulting in severe metabolic imbalances and nonphysiologic changes in many tissues, in which oxidative stress plays an important role in the etiology.[4] One of the reasons for injury related to hyperglycemia is the formation of glycated proteins, glucose oxidation, and increased free fatty acids.[5] Patients with diabetes and experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, thereby depleting the activity of the antioxidative defense system and thus promoting free-radical generation.[4] Moreover, some recent studies have suggested that reactive oxygen species (including free radicals) may also be involved in the initiation and development of vascular complications in patients with diabetes.[6] Oxidative stress combined with mitochondrial dysfunction leads to the activation of inflammatory signaling pathways, which may damage insulin-producing cells and further aggravate diabetes complications.[7]

The management of diabetes mellitus is considered as a global problem and successful treatment is yet to be discovered. Modern drugs, including insulin and other oral hypoglycemic agents such as biguanides, sulphonylureas, and α-glucosidase inhibitors, control the blood glucose level as long as they are regularly administered; however, they may also produce a number of undesirable side effects.[8,9] There is an increasing demand by patients for the use of natural products and other dietary modulators with antidiabetic activity. Recently, there has been a considerable interest in identifying natural antioxidants from fungus materials to replace synthetic ones. Data from both scientific reports and laboratory studies show that fungi contain a large variety of substances that possess antioxidant activity.[10]

*Inonotus obliquus* (Pers.: Fr.) Pilát [syn. *Fuscoporia obliqua* (Pers.: Fr.) Aoshima] is a white rot fungus that belongs to the Hymenochaetaceae family of higher Basidiomycetes and a typical tree disease fungus widely distributed over Europe, Asia, and North America. *I. obliquus* has traditionally been used for the treatment of gastrointestinal cancer, cardiovascular disease, and diabetes since the 16th century in Russia, Poland, and most of the Baltic countries.[11] In recent years, many extracts from this fungus have exhibited various biological activities, including hypoglycemic,[12,13] antiviral,[14] antifungal,[15] and anti-tumor activities.[16–19] Biochemical investigations of *I. obliquus* have resulted in the isolation of a series of polysaccharides, triterpenoids, steroids, small phenolics hydrolyzable tannins, flavonoids, polyphenols, and melanins.[20] Water-soluble polysaccharides from the sclerotia of *I. obliquus* have been shown to have strong antidiabetic activity and anti-tumor effects.[12] Ethanol extract containing triterpenoids and steroids has also presented a relatively strong antioxidant effect.[21]

Although research has focused on the therapeutic effects of *I. obliquus*, little information is available about the antihyperglycemic and antioxidant effects of this fungus. Our preliminary studies have shown the remarkable antihyperglycemic and antilipdiperoxidative effects of dry matter of culture broth (DMCB) of *I. obliquus* in submerged culture.[22] However, the major active component(s) responsible for the bioactivity of *I. obliquus* remains unknown. To further understand the antihyperglycemic, lipid-modulating, and antioxidative activity of *I. obliquus* in living systems and the possible mechanisms of this protection, we investigated the effect of polysaccharides of DMCB of *I. obliquus* against alloxan-diabetes mice.

## II. MATERIALS AND METHODS

### A. Materials

A voucher specimen *I. obliquus* (JNPF-IO01) is deposited in the Laboratory of Pharmaceutical Engineering, School of Medicine and Pharmaceutics, Jiangnan University (Wuxi, China). The DMCB of *I. obliquus* (mycelia and mycelial extracellular medium) was fermented in the laboratory.[22,23] The culture medium was composed of 3% glucose, 0.2% peptone, 2% bran extract, 0.2% $KH_2PO_4$, and 0.1% $MgSO_4$ in distilled water and adjusted to the initial pH of 5.0.

The kits of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C),

**TABLE 1. Box-Behnken Experimental Design Matrix with Experimental and Predicted Values of the Yield of Polysaccharides**

| | | $X_1$ | $X_2$ | $X_3$ | |
|---|---|---|---|---|---|
| Standard order[a] | Run order[b] | Temperature (°C) | Extraction time (h) | Liquid-solid ratio (v/w) | Yield of polysaccharides (%) |
| 1 | 10 | –1 (70) | –1 (1.5) | 0 (30) | 19.32 |
| 2 | 9 | –1 (70) | 0 (2) | –1 (25) | 19.88 |
| 3 | 13 | –1 (70) | 0 (2) | 1 (35) | 23.57 |
| 4 | 7 | –1 (70) | 1 (2.5) | 0 (30) | 22.83 |
| 5 | 8 | 0 (80) | –1 (70) | –1 (25) | 19.35 |
| 6 | 11 | 0 (80) | –1 (70) | 1 (35) | 21.27 |
| 7 | 16 | 0 (80) | 1 (2.5) | –1 (25) | 22.61 |
| 8 | 5 | 0 (80) | 1 (2.5) | 1 (35) | 23.84 |
| 9 | 6 | 1 (90) | –1 (50) | 0 (30) | 21.03 |
| 10 | 15 | 1 (90) | 0 (2) | –1 (25) | 22.35 |
| 11 | 12 | 1 (90) | 0 (2) | 1 (35) | 24.58 |
| 12 | 3 | 1 (90) | 1 (2.5) | 0 (30) | 23.83 |
| 13 | 1 | 0 (80) | 0 (2) | 0 (30) | 24.21 |
| 14 | 2 | 0 (80) | 0 (2) | 0 (30) | 24.45 |
| 15 | 4 | 0 (80) | 0 (2) | 0 (30) | 24.47 |

[a]Nonrandomized and [b]randomized.

high-density lipoprotein cholesterol (HDL-C), free fatty acid (FFA), catalase (CAT), maleic dialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glycogen were purchased from Nanjing Jianchen Bioengineering Institute (Jiangsu, China). Alloxan (Sigma-Aldrich Corporation, Shanghai, China), glibenclamide (Tianjin TIPR Pharmaceutical Responsible Company Ltd., Tianjin, China), a blood glucose kit (Shanghai Kexin Biotech Institute, Shanghai, China), and an insulin kit (Beijing Furui Biological Engineering Company, Beijing, China) were used in the study. The other chemicals used were reagent grade from commercial sources.

## B. Optimization of Extraction of Polysaccharides from DMCB

As shown in Table 1, the extraction of polysaccharides from DMCB of *I. obliquus* was optimized by a three-level Box-Behnken factorial design. Design Expert software (version 7.1.0, Stat-Ease Inc., Minneapolis, MN, USA) was performed for regression and graphical analysis of the obtained data.

Powdered DMCB (500 g) of *I. obliquus* was extracted with distilled water under the optimum process conditions. The combined aqueous phase was cooled, and 4 volumes of 95% ethanol were added and then allowed to precipitate overnight at 4ºC. By precipitation, a resulting precipitate (crude polysaccharides) was obtained following centrifugation and subsequent lyophilization.

## C. Determination of Content and Composition of Monosaccharides in Polysaccharides

The lyophilized polysaccharides were suspended in 1 M NaOH at 60°C for 1 h, and the sugar content in the supernatant (1 mL) was measured by using the phenol-sulfuric acid method.[24] To determine the monosaccharide composition, polysaccharides were hydrolyzed in 3 mL of 2 mol/L trifluoroacetic acid (TFA) (100°C, 8 h). The monosaccharide was acetylated[25] and determined by gas chromatography (GC; Shimadzu Corporation, Tokyo, Japan) as alditol acetates, using a capillary column (DB-1701, 30 m × 0.53 mm × 1.5 μm), 260°C temperature,

and a flame ionization detector. Inositol was used as an internal standard.

### D. Animals and Treatments

Male ICR mice (20 ± 2 g) were purchased from the Shanghai BK Experimental Animal Center (Shanghai, China) and acclimatized under 20 ± 2°C controlled temperature, 60% ± 5% humidity, and 12-h light/dark cycle conditions for 2 weeks before the start of the experiments. The mice were allowed to feed on standard laboratory diet and water. After randomization into various groups, all mice had free access to tap water and fasted 12 h before blood and tissue collection. The experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Mice were made diabetic by a single intraperitoneal injection of alloxan monohydrate (150 mg/kg b.w.) after overnight fast for 12 h. Alloxan was first weighed individually in an Eppendorf tube for each animal according to the weight and then solubilized with 0.2 mL saline (154 mmol/L NaCl) just prior to injection. Seventy-two hours after alloxan injection, mice with blood glucose levels above 11.1 mmol/L were included in the study and then treated with drugs 48 h later.[26] All of the animals were randomly divided into the five groups with 10 animals in each group: group I animals served as normal controls and received vehicle only; group II served as diabetic controls and received vehicle; group III animals received the standard drug glibenclamide (25 mg/kg b.w.); and groups IV and V received polysaccharides extract from DMCB of *I. obliquus* (150 and 300 mg/kg b.w, respectively). The vehicle or test drugs were administered orally (1% CMC-Na in distilled water). Blood samples were collected by tail nipping at weekly intervals until the end of study (3 weeks) and centrifuged (3000 rpm for 15 min at 4°C) to separate the serum. After that time, the serum was frozen at −70°C for the following assay. Finally, blood was collected from the eyes (venous pool) under ether anaesthesia, the animals were then sacrificed by cervical dislocation, and the liver and pancreas were removed promptly and weighed. The serum was stored at −70°C after separation for the following biochemical analysis. The tissues were also stored at −70°C until required.

### E. Measurement of Blood Glucose, Lipids, and Lipoprotein in Serum

Blood glucose was estimated by a commercially available glucose kit based on the glucose oxidase method.[27] FFA, TC, TG, HDL-C, and LDL-C levels were measured following the commercial kit's instructions.

### F. Measurement of CAT, SOD, and GPx Activities and MDA Level in Liver Homogenates

Livers were thawed, weighed, and homogenized with Tris-HCl (5 mmol/L containing 2 mmol/L EDTA, pH 7.4). Homogenates were centrifuged (1000 × *g*, 15 min, 4°C) and the supernatant was used immediately for the assays of CAT, SOD, GPx activities and MDA levels in liver homogenates according to the commercial kit's instructions.

### G. Measurement of Hepatic Glycogen Content in the Liver

Hepatic glycogen content was measured according to the anthrone-$H_2SO_4$ methods, with glucose as the standard.[28] Briefly, liver tissue (<100 mg) was homogenized in 5 volumes of an ice-cold 30% (w/v) KOH solution and dissolved in a boiling-water bath (100°C) for 20 min. The glycogen was then resolubilized in distilled water. The glycogen concentration was determined by treatment with an anthrone reagent (2 g anthrone/1 L of 95% (v/v) $H_2SO_4$) and the absorbance was measured at 620 nm.

### H. Measurement of Insulin Level in Serum

Serum insulin was measured by using the rat insulin RIA kit from Beijing Furui Biological Engineering Co. (Beijing, China).[29]

### I. Histopathological Examination

The pancreas was removed immediately from the animals after sacrifice and rinsed in ice-cold saline. The tissue samples were fixed with 10% formaldehyde, dehydrated in a graded series of ethanol, and embedded in paraffin wax before sectioning. The paraffin sections were then cut into sections (approximately 5-μm thickness), dewaxed, and rehydrated. The sections were stained with hematoxylin and eosin (H&E) and studied with a light microscope to determine histopathological changes, that is, the normal numbers and volume, as well as the deformation of the islet cells.[30] Histopathological damages were scored as follows: 0, normal; I, minor injury; II, moderate injury; III, obvious injury; and IV, severe injury. Each sample was observed at 400× magnification. The degree of injuries was expressed as the mean of 10 different fields in each slide.[31] According to the scale on the photo captured by the light microscope, diameters of islet cells were measured by a ruler. The volumes of islet cells were calculated according to the following formula:

$$V = \pi/6[(A+B)/2]^3,$$

where $V$ is the volume of the islet cell, $A$ is the longest diameter of the islet cell, and $B$ is the shortest diameter of the islet cell.

### J. Statistical Analysis

The data are expressed as means ± standard error of the mean (SEM). Statistical comparisons were conducted by using a one-way analysis of variance (ANOVA). The results were considered statistically significant if the $P$ values were .05 or less.

## III. RESULTS

### A. Optimization of Extraction Conditions of Polysaccharides and Their Monosaccharide Composition

The design arrangement and the experimental results of the optimization design are shown in Table 1.

The application of RSM yields the following regression equation, which is an empirical relationship between polysaccharide yield and the test variable in coded units:

$$Y = 24.38 + 0.78X_1 + 1.52X_2 + 1.13\ X_3 - 0.18X_1X_2 - 0.37X_1X_3 - 0.17X_2X_3 - 0.90X_1^2 - 1.72X_2^2 - 0.89X_3^2.$$

To validate the regression coefficient, an ANOVA for the polysaccharides was performed. The "Model Prob > $F$" value was 0.0012, and the "Lack of Fit Prob > $F$-value" was 0.0541, indicating that the model can adequately fit the experimental data. The optimal conditions obtained by RSM for the extraction of crude polysaccharides from DMCB included the following parameters: extraction temperature, 83°C; extraction time, 2.2 h; and liquid-solid ratio, 33:1.

After extraction under these optimal conditions, the yield of polysaccharides from DMCB was 25.08%. The sugar content in crude polysaccharides from DMCB of *I. obliquus* was 41.23 mg/g. In comparison with the monosaccharide standards, polysaccharides comprised Glc, Gal, Ara, and Man in a 1.00:0.23:0.05:0.04 ratio.

### B. Effect on Alloxan-induced Diabetic Mice

The effect of the polysaccharide extract from DMCB of *I. obliquus* on the fasting blood glucose levels of diabetic mice is shown in Table 2.

Intraperitoneal injection of alloxan monohydrate (150 mg/kg) led to an over 2.5-fold elevation of blood glucose levels ($P < .001$), which was maintained over a period of 4 weeks. Daily treatment with 150 and 300 mg/kg of the polysaccharide extract from DMCB for 3 weeks led to falls in blood glucose levels by 16.64% and 20.09% at the 7th day, 26.13% and 31.13% at the 14th day, and 29.71% and 36.36% at the 21st day, respectively. The percentages of the reduction of blood glucose levels in glibenclamide-treated mice were 21.87%, 27.75%, and 36.71%, respectively.

**TABLE 2. Effect of 3-Week Treatment with Polysaccharides from DMCB of *I. obliquus* on Glucose Levels in Alloxan-Induced Diabetic Mice**

| Group | Dose (mg/kg) | Blood glucose (mg/dL) | | | |
|---|---|---|---|---|---|
| | | Day 0 | Day 7 | Day 14 | Day 21 |
| Normal controls | – | 80.82 ± 2.18 | 79.92 ± 2.88 | 81.18 ± 2.88 | 80.64 ± 2.70 |
| Diabetic controls | – | 264.98 ± 22.42 | 268.71 ± 20.55* | 269.10 ± 21.78* | 282.26 ± 24.48* |
| Glibenclamide | 25 | 263.39 ± 21.93 | 205.96 ± 21.89*# (21.87) | 190.26 ± 2.32*# (27.75) | 166.68 ± 17.8*# (36.71) |
| Polysaccharides | 150 | 264.85 ± 17.53 | 220.77 ± 18.63*# (16.64) | 195.65 ± 19.66*# (26.13) | 186.18 ± 10.89*# (29.71) |
| Polysaccharides | 300 | 260.21 ± 18.83 | 207.92 ± 16.26*# (20.09) | 179.21 ± 14.96*# (31.13) | 165.61 ± 11.94*# (36.36) |

Values are means ± SEM for 10 mice. Value in parentheses indicate the percentage lowering of blood glucose in comparison to the reading at day 0 (72 h after alloxan injection). The diabetic control group was compared with the normal group, and experimental groups were compared with the corresponding values at day 0.
*$P$ < .01 compared with the normal control group.
#$P$ < .01 compared with the corresponding values at day 0.

### C. Effect on Lipids and Lipoprotein in Serum

Table 3 shows the levels of lipids and lipoproteins in the serum of the control and experimental animals in each group.

The FFA, TG, TC, and LDL-C levels of diabetic control mice were significantly increased, whereas the HDL-C levels were decreased in alloxan-induced diabetic mice as compared with normal mice. When diabetic mice were treated for 3 weeks, the polysaccharide extract from DMCB of *I. obliquus* (150 and 300 mg/kg) and glibenclamide (25 mg/kg) significantly decreased the levels of FFA, TG, TC, and LDL-C and simultaneously increased the HDL-C level.

### D. Effect on MDA Level and CAT, SOD, and GPx Activities in Liver Homogenates

Table 4 shows the MDA level and CAT, SOD, and GPx activities in liver homogenates of normal and experimental animals.

The MDA level was significantly increased in diabetic mice, whereas CAT, SOD, and GPx activities were significantly decreased in alloxan-induced diabetic mice compared with normal mice. The polysaccharide extract from DMCB of *I. obliquus* (150 and 300 mg/kg) and glibenclamide (25 mg/kg) treatment significantly decreased the MDA level and increased CAT, SOD, and GPx activities in the liver compared with alloxan-induced diabetic mice.

### E. Effect on Hepatic Glycogen Content in Liver and Insulin Level in Serum

As shown in Table 5, the levels of hepatic glycogen and insulin in serum were decreased significantly in diabetic mice.

The polysaccharides from DMCB of *I. obliquus* (150 and 300 mg/kg) and glibenclamide (25 mg/kg) increased the hepatic glycogen content in liver and the insulin level in serum in alloxan-induced diabetic mice.

### F. Histopathological Observations

Based on H&E-stained tissue sections, normal control animals showed no notable changes in pancreas histology throughout the 3-week study. In contrast, alloxan administration elicited severe injury of the pancreas, such as decreasing the numbers of islet

**TABLE 3. Effect of Polysaccharides from DMCB of *I. obliquus* on Lipids and Lipoprotein in Diabetic Mice**

| Group | Dose (mg/kg) | FFA (mmol/L) | TG (mmol/L) | TC (mmol/L) | LDL-C (mmol/L) | HDL-C (mmol/L) |
|---|---|---|---|---|---|---|
| Normal controls | – | 0.93 ± 0.13 | 0.98 ± 0.11 | 2.06 ± 0.18 | 0.91 ± 0.11 | 1.76 ± 0.15 |
| Diabetic controls | – | 2.01 ± 0.17* | 1.57 ± 0.17* | 2.91 ± 0.11* | 2.04 ± 0.22* | 1.29 ± 0.19* |
| Glibenclamide | 25 | 1.39 ± 0.22*# | 1.18 ± 0.18*# | 2.25 ± 0.15*# | 1.32 ± 0.21*# | 1.51 ± 0.17*# |
| Polysaccharides | 150 | 1.34 ± 0.18*# | 1.18 ± 0.18*# | 2.12 ± 0.14# | 1.20 ± 0.13*# | 1.84 ± 0.13*# |
| Polysaccharides | 300 | 1.23 ± 0.14*# | 1.05 ± 0.12# | 2.01 ± 0.11# | 1.11 ± 0.14*# | 1.87 ± 0.12*# |

Values are means ± SEM of 10 mice. The data were measured at the end of the experiment (day 21). FFA, free fatty acid; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.
*$P < .01$ compared with the normal control group.
#$P < .01$ compared with the diabetic control group.

cells and diminishing of the diameter of the pancreatic island. The islets were shrunken in diabetic mice when compared with normal mice. Administration of the polysaccharide extract from DMCB (150 and 300 mg/kg) and glibenclamide (25 mg/kg) showed moderate expansion of islet cells and significantly reduced injuries of the pancreas. The damage to pancreas tissues was mended in the polysaccharide-treated diabetic group and glibenclamide-treated diabetic group to some degree.

## IV. DISCUSSION

Previously, extracts of *I. obliquus* were reported as showing potent biological activities. Water-soluble polysaccharides from the sclerotia of *I. obliquus* have showed strong antidiabetic activity and anti-tumor effects.[12] Ethanol extract containing triterpenoids and steroids has also presented a relatively strong antioxidant effect.[21] Polysaccharides, steroids, and triterpenoids are considered as the main

**TABLE 4. Effect of Polysaccharides from DMCB of *I. obliquus* on MDA Level and CAT, SOD, and GPx Activities in Diabetic Mice**

| Group | Dose (mg/kg) | MDA (nmol/mgpr) | CAT (U/mgpr) | SOD (U/mgpr) | GPx (U/mgpr) |
|---|---|---|---|---|---|
| Normal controls | – | 7.71 ± 0.33 | 95.58 ± 2.06 | 302.55 ± 8.71 | 69.06 ± 2.88 |
| Diabetic controls | – | 11.56 ± 0.49* | 71.81 ± 4.15* | 258.36 ± 9.93* | 49.95 ± 2.65* |
| Glibenclamide | 25 | 5.15 ± 0.39*# | 88.44 ± 4.33*# | 294.57 ± 8.38*# | 62.73 ± 2.92*# |
| Polysaccharides | 150 | 6.38 ± 0.45*# | 83.09 ± 4.68*# | 302.32 ± 6.32# | 61.05 ± 2.68*# |
| Polysaccharides | 300 | 6.03 ± 0.53*# | 93.48 ± 4.42# | 308.81 ± 5.17*# | 63.51 ± 3.14# |

Values are means ± SEM of 10 mice. The data were measured at the end of the experiment (day 21). MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase.
*$P < .01$ compared with the normal control group.
#$P < .01$ compared with the diabetic control group.

**TABLE 5. Effect of Polysaccharides from DMCB of *I. obliquus* on Hepatic Glycogen Content in Liver and Insulin Level in Serum**

| Group | Dose (mg/kg) | Glycogen content (mg/kg liver tissue) | Insulin level (μIU/mL) |
|---|---|---|---|
| Normal controls | – | 3.31 ± 0.16 | 12.03 ± 0.49 |
| Diabetic controls | – | 2.01 ± 0.31* | 6.78 ± 0.33* |
| Glibenclamide | 25 | 2.78 ± 0.29*# | 11.28 ± 0.33# |
| Polysaccharides | 150 | 3.84 ± 0.56# | 10.87 ± 0.42*# |
| Polysaccharides | 300 | 4.34 ± 0.52# | 11.83 ± 0.39# |

Values are means ± SEM of 10 mice. The data were measured at the end of the experiment (day 21).
*$P < .01$ compared with the normal control group.
#$P < .01$ compared with the diabetic control group.

components in the *I. obliquus*.[32] In this study, crude polysaccharides were extracted from DMCB of *I. obliquus* and its monosaccharide composition was analyzed. Meanwhile, we reported the effect of the polysaccharide extract from DMCB on elements of diabetes mellitus including blood glucose, oxidative parameters, and hepatic glycogen content in the liver, insulin level in serum, and histopathological examination in mice with alloxan-induced diabetes.

Alloxan is one of the most commonly used substances to induce diabetes in experimental animals. In this study, there was a significant increase ($P < .01$) in fasting blood glucose in alloxan-induced diabetic mice. Treatment with polysaccharides from DMCB produced a significantly antihyperglycemic effect beginning from the first week of the study and progressing until the end of third week (Table 2). On the other hand, the increase in blood sugar was accompanied by marked increases in TC, LDL-C, and TG and a reduction in HDL-C in alloxan-induced diabetic rats (Table 3). The results revealed that regular administration of polysaccharides from DMCB for 3 weeks nearly normalized the lipid profile of diabetic animals. Polysaccharides (300 mg/kg) not only lowered FFA, TC, TG, and LDL-C, but also enhanced cardioprotective HDL-C.

Free radicals may play an important role in the causation and complications of DM.[33] Enzymatic antioxidants (SOD, CAT, and GPx) and MDA play a major role in scavenging toxic free radicals *in vivo*. Evidence suggests that the diabetogenic capacity of alloxan may depend on its ability to damage β-cells and induce oxidative stress. Lowered activities of enzymatic antioxidants, such as SOD, CAT, and GPx, have been well documented in alloxan-induced diabetic rats.[34,35] Our results corroborated these observations. A significant increase of SOD, CAT, and GPx activities in the polysaccharide-treated diabetic group was observed when compared with the diabetic group (Table 4). On the basis of these results, polysaccharides from DMCB of *I. obliquus* may exert their antioxidant effects by activating SOD, CAT, and GPx activities and decreasing MDA levels in the liver.

In this study, diabetic mice showed an obvious decrease in insulin level and the number of β-cells in the pancreas, whereas the pancreas of the polysaccharide-treated mice showed improved insulin levels and significant stimulation of β-cell replication or neogenesis. Chemicals with antioxidant effects may help to regenerate β-cells and protect pancreatic islets against cytotoxic effects of alloxan, which is evident from the histopathological examination of the pancreas. The present results also demonstrate that the serum insulin level and glycogen content were significantly higher in the group treated with polysaccharide extract compared with the diabetic mice.

In conclusion, polysaccharides from DMCB of *I. obliquus* (150 and 300 mg/kg) showed significant antihyperglycemic and antilipidperoxidative effects and potent antioxidant defense mechanisms in alloxan-induced diabetic mice. The mechanism of the antihyperglycemic effect may be mediated through the interaction between insulin and gluconeogenesis, which further affects glucose metabolism in the liver and other tissues in the body and introduces abnormal or disordered glucose

metabolism to normal or ordered states. Further pharmacological and biochemical investigations are in progress to elucidate the detailed mechanism of polysaccharides from DMCB of *I. obliquus* on glucose metabolism.

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# Influence of Culinary-Medicinal Maitake Mushroom, *Grifola frondosa* (Dicks.: Fr.) S.F. Gray (Aphyllophoromycetideae) Polysaccharides on Gene Expression in Jurkat T Lymphocytes

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**ABSTRACT:** The fruiting body of the culinary-medicinal Maitake mushroom *Grifola frondosa* contains β-glucans that have immunomodulatory effects and potential uses as adjuncts to cancer therapy. Numerous clinical trials are being conducted to assess the benefits of using commercial preparations containing *G. frondosa* extracts in cancer treatment. We tested a commercially available product containing polysaccharides from the *G. frondosa* (CGFP) and studied their effect on gene expression in Jurkat T cells. An mRNA differential display was performed to gain insight into the cell response to the substance. HAI1, hnRNP A2/B1, PKC iota, KLRC1, and genes of the ubiquitin system were identified as possible regulators in the mechanism of tumor-suppressor action of CGFP. Real-time polymerase chain reaction (qPCR) was used to investigate the effect of CGFP on cytokine expression in Jurkat T cells. The qPCR results showed that the expression of the gene for IL-5 is increased.

**KEY WORDS:** medicinal mushrooms, Maitake mushroom, *Grifola frondosa*, Jurkat T cells, differential display, gene expression

## I. INTRODUCTION

*Grifola frondosa* (Dicks.: Fr.) S.F. Gray (Maitake mushroom) is a higher Basidiomycetes belonging to the family Polyporaceae (Aphyllophoromycetideae).[1] Its fruiting body contains polysaccharides with a β-1,6-glucopyranoside main chain and branches of β-1,3-linked glucose.[2] The 1,3-branched-1,6-β-glucan, named D-Fraction, has been reported to have antitumor effects.[2-4] These polysaccharides are

## ABBREVIATIONS

**CGFP:** commercially available *Grifola frondosa* polysaccharides; **cDNA:** complementary deoxyribonucleic acid; $\mathbf{C_t}$: threshold cycle; **FBS:** fetal bovine serum; **HAI1:** hepatocyte growth factor activator inhibitor 1, isoform 1; **hnRNP A2/B1:** heterogeneous nuclear ribonucleoprotein A2/B1, isoform A2; **IL-1:** interleukin 1; **IL-5:** interleukin 5; **Itch:** itchy homolog E3 ubiquitin protein ligase; **KLRC1:** cell lectin-like receptor subfamily C, member 1; **mRNA:** messenger ribonucleic acid; **NK cells:** natural killer cells; **PBS:** phosphate buffered saline; **PCR:** polymerase chain reaction; **PKC:** protein kinase C; **qPCR:** real-time polymerase chain reaction; **TFA:** trifluoroacetic acid; **TLC**: thin-layer chromatography; **TNFα:** tumor necrosis factor-alpha; **Uba52:** ubiquitin and ribosomal protein L40 precursor; **UBR1:** ubiquitin protein ligase E3 component n-recognin 1

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considered to be biological response modifiers that can stimulate the immune response. They enhance the activity of cells such as macrophages, helper T cells, and cytotoxic T cells, which attack tumor cells and can be used as an adjunct to cancer therapy.[5] The remedial effects of β-glucans have led researchers to conclude that oral administration of β-glucans would be an effective treatment for patients with cancer. Orally administered D-Fraction was shown to reduce tumor size in mice without causing unwanted side effects.[4] Suzuki et al. examined the effects of the β-1,3-glucan obtained from liquid-cultured mycelium of *G. frondosa* (LELFD) on the growth of syngeneic tumors and immune responses in mice.[6] In solid tumor systems, LELFD administered intraperitoneally or intralesionally exhibited significant antitumor effects. However, the growth of L1210 and P388 leukemia was unaffected by the injection of LELFD. In the meantime, D-Fraction has been commercialized and its safety has been tested by the Consumer Product Testing Co. (Fairfield, NJ, USA).[7] These polysaccharides have also been used in a nonrandomized clinical trial in the treatment of patients with lung and breast cancer, and it was revealed that treatment with D-Fraction increased the activities of natural killer (NK) cells, cytotoxic T cells, macrophages, and delayed-type hypersensitivity T cells by 1.23 to 2.5 times.[8] Lin et al.[9] found that Maitake β-glucan induces granulocyte colony-stimulating factor production and has specific dose-related effects on human hematopoietic progenitor cells. In addition to their immunomodulating activity, β-glucans have also been studied for their direct cytotoxic activity on cancer cells.[10]

We studied the response of Jurkat T cells to *G. frondosa* polysaccharides on the transcriptional level by using the mRNA differential display technique. These experiments were based on commercially available polysaccharides from the mushroom *G. frondosa*. The data uncover a possible new mechanism of action of CGFP.

## II. MATERIALS AND METHODS

### A. Cell Culture and Treatment

The Jurkat T lymphocyte line, which is a human acute T-cell lymphoma cell line (TIB-152; ATCC, Manassas, VA, USA), was used. Jurkat cells are used for studying acute T-cell leukemia, T-cell signaling, and gene expression after exposure to various stimuli. The cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 2 mM glutamine (Sigma), 0.1% antibiotic/antimycotic (10,000 units/mL penicillin G, 10 mg/mL streptomycin sulfate) (Sigma), and 5% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) at 37°C in a 5% $CO_2$ incubator.

The polysaccharides used in this study are commercially available as an over-the-counter product (Shanghai Baixin Edible & Medicinal Fungi Co. Ltd., Shanghai, China) that is packed in capsules. For use in the experiments, the capsules were opened and the powder within was dissolved in phosphate buffered saline (PBS). To determine the appropriate dose of CGFP to be used in the experiments, the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS colorimetric assay; Promega, Madison, WI, USA) was performed according to the manufacturer's instructions. Cells ($2 \times 10^4$ cells/well) were incubated with 0.001, 0.01, 0.05, 0.1, and 5 mg/mL CGFP for 24 h. The CellTiter 96® AQueous One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)], which is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium.

For mRNA differential display experiments and real-time PCR analysis (qPCR), cells ($2.5 \times 10^6$ cells/well) were incubated with 0.1 mg/mL of polysaccharides in a 6-well plate and total RNA was isolated and purified after 24 h. In all experiments, the control untreated cells were treated with an equal volume of PBS.

### B. General Methods

CGFP (3 mg) was hydrolyzed in 1 mL of 2 M trifluoroacetic acid (TFA) for 12 h at 100°C. To recover the hydrolyzed polysaccharides, TFA was removed by vacuum evaporation. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 glass plates (Merck, Darmstadt, Germany) developed in a 4:2:2:1 mixture of ethyl acetate,

n-propanol, acetic acid, and water, with the sugars being detected with diphenylamine, aniline, 85% phosphoric acid solution in methanol.

Protein content was analyzed with the Pierce Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions using the standard curve for bovine serum albumin.

## C. Differential Display

The effect of CGFP on gene expression in Jurkat T cells was studied using the method described by Liang and Pardee.[11] Total RNA was isolated using the RNeasy Mini Kit combined with RNase-Free DNase Set (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The integrity of isolated total RNA was evaluated by formaldehyde gel electrophoresis, and RNA quantity was assessed by measuring absorbance at 280 nm on a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). RNA (250 ng) was reverse transcribed at 37°C for 2 h with OmniScript reverse transcriptase (Qiagen, Hilden, Germany), using one of the anchored primers H-$T_{11}$A, H-$T_{11}$C, H-$T_{11}$G (5′-HindIII-$T_{11}$A/C/G-3′). We used 2 µL of obtained cDNA as a template in a 25-µL PCR reaction containing 0.4 µM arbitrary 13-mer primer, 0.4 µM appropriate anchor primer, 400 µM dNTP, 0.625 U Taq polymerase (Promega, Madison, WI, USA), and 3 mM $MgCl_2$. PCR reactions were accomplished with combinations of 16 different random primers and H-$T_{11}$C, H-$T_{11}$G anchored primers (synthesized by Invitrogen). Reaction conditions were set to 30 sec at 94°C, 2 min at 42°C, 30 sec at 72°C for 40 cycles, and a termination step of 5 min at 72°C. RT-PCR reactions were all performed in duplicate. Subsequently, PCR products were loaded to GeneGel Clean sequencing gel (Amersham Pharmacia, Uppsala, Sweden) in a GenePhor electrophoresis unit (Amersham Pharmacia, Uppsala) and separated. A commercial kit (Amersham Pharmacia, Uppsala) was used to stain the gels with silver. Differentially stained cDNA bands were identified, excised from the gel using a sterile needle, and boiled in 20 µL of Tris-EDTA buffer for 5 min. We used 2 µL of solution to serve as a template for PCR-reamplification and the cloning into pGEM-T Easy vector (Promega, Madison, WI, USA). Plasmid DNA from three independent clones for each original DNA band was submitted to nonradioactive cycle sequencing (MWG-Biotech, Martinsried, Germany) with the SP-6 reverse primer. The nucleotide sequences obtained for the differentially expressed cDNA inserts were tested for homology with known sequences in the GenBank databases by a BLAST (basic local alignment search tool) search.

## D. Real-Time Polymerase Chain Reaction Analysis

The influence of CGFP on expression of cytokines in Jurkat T cells was investigated using the Human Common Cytokines RT² Profiler™ PCR Array (SABiosciences, San Jose, CA, USA), which profiles the expression of 84 important cytokine genes. The list of genes is given in Table 1. RNA (1 µg) was reverse-transcribed using the RT² First Strand Kit (SABiosciences) according to the manufacturer's protocol. Real-time PCR analysis (qPCR) was performed using the RT² SYBR® Green/ROX® qPCR Master Mix and the Human Common Cytokines RT² Profiler™ PCR Array (SABiosciences) on a ABI PRISM 7000 apparatus. The cycling program was as follows: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The threshold cycle ($C_t$) values obtained from the qPCR experiments were analyzed using the integrated RT² Profiler™ PCR Array Data Analysis software package. The software uses the comparative $C_t$ method to calculate fold-change from the threshold cycle data. In the comparative or the $\Delta\Delta C_t$ method of qPCR data analysis, the $C_t$ values obtained from two different RNA samples (experimental and the control samples) are normalized to a housekeeping gene and then compared. First, the difference between the $C_t$ values ($\Delta C_t$) of the gene of interest and the housekeeping gene (β2-microglobulin [B2M], hypoxanthine-guanine phosphoribosyl transferase [HPRT], glyceraldehyde-3-phosphate dehydrogenase [GAPD], β-actin [ACTB]) is calculated for each experimental sample. The difference in the $\Delta C_t$

**TABLE 1. List of Genes Included in the RT² Profiler™ Human Common Cytokines PCR Array**

| | |
|---|---|
| Interferons | IFNA1 (interferon α1), IFNA2, IFNA4, IFNA5, IFNA8, IFNB1, IFNG, IFNK |
| Interleukins | IL1A, IL1B, IL1F10, IL1F5, IL1F6, IL1F7, IL1F8, IL1F9, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL11, IL12A, IL12B, IL13, IL14, IL15, IL16, IL17A, IL17B, IL17C, IL18, IL19, IL20, IL21, IL22, IL24, IL25 (IL17E) |
| Bone morphogenic proteins (BMPs) and TGF-β family | BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8B, GDF2 (BMP9), GDF3, GDF5, GDF8, GDF9, GDF10 (BMP3B), GDF11 (BMP11), INHA, INHBA, NODAL, TGFA, TGFB1, TGFB2, TGFB3 |
| PDGF/VEGF family | FIGF (VEGFD), PDGFA |
| TNF superfamily | FASLG (TNFSF6), LTA, LTB, TNF, TNFRSF11B, TNFSF10, TNFSF11, TNFSF12, TNFSF13, TNFSF13B, TNFSF14, TNFSF4, CD70 (TNFSF7), TNFSF8 |
| Other growth factors/ cytokines | (MCSF), CSF2(GMCSF), FAM3B, LEFTY2 (EBAF) |

values between the experimental samples (CGFP-treated cells) and control samples (PBS-treated cells) $\Delta\Delta C_t$ is then calculated. The fold-change in expression of the gene of interest between the two samples is then equal to $2^{(-\Delta\Delta Ct)}$.

The response of the cells to the polysaccharides was confirmed with a subsequent qPCR analysis and a set of specific primers for interleukin-1 (IL-1) and IL-5 (10x QuantiTect Primer Assay, Qiagen, Hilden, Germany). Using oligo-dT primer mix, mRNA was transcribed with Omniscript reverse transcriptase (Qiagen, Hilden, Germany) from 1 µg of total RNA. qPCR was carried out on the ABI PRISM 7000 apparatus (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µL containing 5 µL of the RT mixture, 1X Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA, USA), 1 µL Rox reference dye, and 2.5 µL of the 10x QuantiTect Primer Assay specific primers for IL-1 and IL-5. The cycling program was as follows: 2 min at 50°C, and 2 min at 95°C followed by 45 cycles (15 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C). ACTB, B2M, HPRT, GAPD, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ), and ubiquitin C (UBC) (primer sequences obtained from the qPCR primer and probe database) housekeeping genes were checked for their stability by using geNorm normalization. Results obtained in qPCR experiments were normalized using the two most stable housekeeping genes: B2M and HPRT. A melting curve (60–95°C) and agarose gel electrophoresis of PCR products were performed to ensure the absence of artifacts. The qPCR results were analyzed by the comparative $C_t$ method.

## E. Flow Cytometry Assay of IL-5

The BD Cytometric Bead Array Human IL-5 Flex Set (BD Biosciences) was used according to manufacturer's protocol to assay the protein level of IL-5 in the cell culture supernatant. Briefly, Jurkat T lymphocytes ($10^6$ cells) were treated as previously described for the qPCR experiment. Cells were centrifuged and cell culture supernatant was used for further analysis. In an appropriate assay tube, 50 µL of the IL-5 capture beads, 50 µL of the human IL-5 PE detection reagent, and 50 µL of the sample were mixed and incubated for 3 h at room temperature protected from light. Samples were then washed with 1 mL of wash buffer and centrifuged at 3000 rpm for 5 min. Supernatant was carefully aspirated and discarded from each assay tube. The bead pellet was resuspended by adding 300 µL of wash buffer. A standard curve was prepared by serial dilutions of human IL-5 standard and used for determination of IL-5 concentrations in supernatants. Flow cytometry was performed using a FACSCalibur system (Becton Dickinson Inc., Franklin Lakes, NJ, USA).

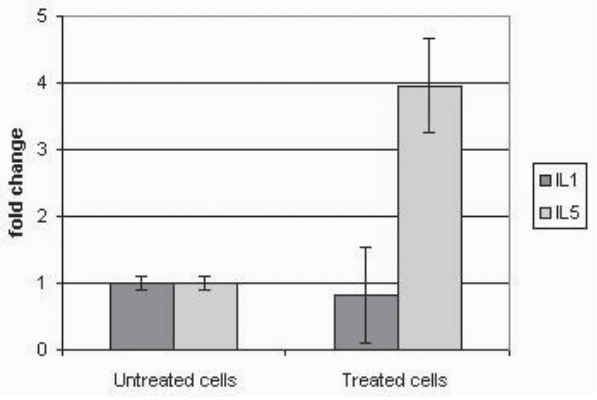


**FIGURE 1.** TLC chromatogram of the hydrolyzed and unhydrolyzed samples of CGFP. Plate: Merck, Silica gel 60 F254. Mobile phase: ethyl acetate–n-propanol–acetic acid–water = 4:2:2:1. Staining reagent: Diphenylamine, aniline, 85% phosphoric acid and methanol.

## III. RESULTS

### A. Analysis of CGFP

Hydrolyzed and unhydrolyzed samples of CGFP were tested by TLC. Glucose was detected in the hydrolyzed sample, whereas the unhydrolyzed tested sample was negative, indicating that no monosaccharides were present in the product (Fig. 1). No proteins were detected in the sample by the use of Coomassie Plus protein assay reagent, compared with the standard curve for bovine serum albumin.

### B. Effect of CGFP Treatment on the Gene Expression Profile of Jurkat T Cells

We used the differential display technique to examine the effects of CGFP on gene expression. Combinations of 32 different arbitrary and one-base anchored primers were used. Jurkat T cells were observed 24 h upon treatment and a total of 18 alterations were detected in the differential-display gel profile. Differentially stained cDNA bands were excised from the gel, reamplified, cloned, sequenced, and aligned using the GenBank database. The clone designation, identity, function, and change in expression of each of the differentially expressed genes are listed in Table 2. Identified genes have been previously connected to proliferation or immunomodulation, some of which are a part of the ubiquitin apparatus, code for kinases and enzymes involved in energy consumption and ion transport, or represent genes of unknown function.

### C. Impact of CGFP on Cytokine Expression

The influence of *G. frondosa* polysaccharides on cytokine expression in Jurkat T cells was tested after 24 h of treatment. A PCR array containing specific primers for 84 different cytokines was used. The only significant change in gene expression was found for IL-1 and IL-5, with increases of 2.5- and 2.3-fold, respectively.

To confirm these results, we repeated the qPCR analysis with another set of specific primers for IL-1 and IL-5, which confirmed the upregulation of the IL-5 mRNA expression (Fig. 2). When the production of the IL-5 protein was assayed by flow cytometry, we observed that although incubation of cells with CGFP significantly increased the expression of IL-5 mRNA, no increase in IL-5 protein level was detected in the medium after 24 h of treatment.

## IV. DISCUSSION

The antitumor activity of *G. frondosa* polysaccharides has been well documented and they have also been shown to affect various immune cells.[4,5,12] Due to the fact that *G. frondosa* polysaccharides are being proposed as a potential new drug in cancer therapy, we were interested in the changes that they cause on the transcriptional level in Jurkat T cells and possible new mechanisms of action involved in the antitumor effect of *G. frondosa* polysaccharides. For this purpose, we used the differential display technique and found 18 genes whose expression was up- or downregulated as a consequence of CGFP treatment. The obtained data show that CGFP modifies the transcription of genes that have previously been linked to pathological conditions, including cancer.

Of the 18 genes identified, itchy homolog E3 ubiquitin protein ligase (Itch), ubiquitin and ribo-

**TABLE 2. cDNA Fragments Isolated by Differential Display of mRNA from Jurkat T Cells Treated with CGFP**

| Clone | Accession | Encoded | Function | Gene map locus | Comment |
|---|---|---|---|---|---|
| 1 | NM_031483 gi:27477108 | ITCH, itchy homolog E3 ubiquitin protein ligase | Protein degradation | 20q11.22-q11.23 | Down |
| 2 | NM_002137 gi:156151373 | hnRNP A2/B1, heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2 | mRNA processing, metabolism and transport | 7p15 | Down |
| 3 | NM_001033930 gi:77539054 | Uba52, ubiquitin A-52-residue ribosomal fusion product or ubiquitin fused to ribosomal protein L40 | Protein degradation | 19p13.1-p12 | Up |
| 4 | NM_002259 gi:47717087 | KLRC1, killer cell lectin-like receptor subfamily C, member 1 | Recognition of the MHC class I HLA-E molecules | 12p13 | Up |
| 5 | NM_030809 gi:13540601 | TGF-β induced apoptosis protein 12 | Unknown | 12q13.11-q13.12 | Up |
| 6 | NM_004796 gi:41350303 | Neurexin 3 isoform α precursor | Cell adhesion | 14q31 | Down |
| 7 | NM_001018136 gi:66392202 | NME1-NME2 protein | Unknown | 17q21.3 | Down |
| 8 | NM_153375 gi:23503320 | Placenta-specific 2 | Unknown | 19p13.3 | Up |
| 9 | NM_004231 gi:20357546 | ATPase, H+ transporting, lysosomal 14kD, V1 subunit F | Acidification of eukaryotic intracellular organelles | 7q32 | Down |
| 10 | NM_001005241 gi:52546694 | Olfactory receptor, family 4, subfamily N, member 4 | G-protein-coupled receptors | 15q11.2 | Down |
| 11 | NM_023937 gi:22547132 | Mitochondrial ribosomal protein L34 | Protein synthesis | 19p13.1 | Down |
| 12 | NM_001040709 gi:105554420 | Mitsugumin 29 | Membrane ultrastructure and $Ca^{2+}$ signaling | 1p13.3 | Down |
| 13 | NM_004330 gi:4757855 | BCL2/adenovirus E1B 19kD interacting protein 2 | Proapoptotic protein | 15q22.2 | Down |
| 14 | NM_174916 gi:83656781 | UBR1, ubiquitin protein ligase E3 component n-recognin 1 | Protein degradation | 15q13 | Down |
| 15 | NM_001012339 gi:68077165 | DnaJ homology subfamily A member 5 isoform 2 | Protein importing, sorting, translocation, cell cycle regulation and exocytosis | 5p13.2 | Up |
| 16 | NW_927762 gi:89026611 | Hypothetical protein LOC650050 | Unknown | 7 | Up |
| 17 | NM_181642 gi:74027262 | HAI1, hepatocyte growth factor activator inhibitor 1 isoform 1 | Serine protease inhibitors | 15q15.1 | Down |
| 18 | NM_002740 gi:133908622 | PKC iota, protein kinase C, iota | Serine/threonine protein kinase in variety of processes | 3q26.3 | Down |


**FIGURE 2.** Influence of CGFP on IL-1 and IL-5 mRNA expression in Jurkat T cells. Jurkat cells were treated with PBS alone (untreated cells) and CGFP (0.1 mg/mL). After 24 h, total RNA was extracted and subjected to qPCR analysis for detection of IL-1 and IL-5 mRNA analysis.

somal protein L40 precursor (Uba52), and ubiquitin protein ligase E3 component n-recognin 1 (UBR1) are active in the ubiquitin system. The conjugation of ubiquitin to protein substrates has emerged as a fundamental mechanism for controlling turnover and abundance of proteins by targeting them for proteosomal or lysosomal degradation. Ubiquitin-mediated degradation of regulatory proteins is involved in a variety of cellular processes such as signal transduction, control of cell division, transcriptional regulation, and immune and inflammatory responses.[13]

Ubiquitin is a highly conserved protein that is composed of 76 amino acids and is synthesized in the cell as a precursor protein,[14] which consists either of polyubiquitin chains that are cleaved into individual ubiquitin moieties (Ubb or Ubc type) or single ubiquitin moieties fused to carboxyl extension proteins (CEPs) (Uba type). In humans, the two human ubiquitin-CEP genes are Uba80 and Uba52, which code for ubiquitin fused to ribosomal protein S27a and L40, respectively.[15] The function of these ubiquitin-ribosomal protein fusion genes has not yet been fully elucidated. The Uba52 gene may be important as a component of the respective ribosomal genes, as well as a significant source of ubiquitin within the cell.[15] In our experiment, cells treated with CGFP had upregulated expression of Uba52, which could be connected with the increase in protein degradation due to the induction of apoptosis.[16,17]

Protein ubiquitination requires the concerted action of ubiquitin-activating enzyme (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligases (E3).[18,19]

Itch and UBR1 are E3 ligases, and we found that expressions of both Itch and UBR1 were downregulated after treatment of Jurkat T cells with CGFP. Itch is an E3 ubiquitin ligase that was originally identified by genetic analysis of a mutant mouse with aberrant immunological phenotypes and constant scratching of the skin.[20] Itch mediates the transfer of the ubiquitin molecule from E2 ubiquitin-conjugating enzymes to substrates. One mechanism by which Itch regulates T-cell responses is the induction of T-cell anergy in which T cells become unresponsive after restimulation.[21] A number of Itch targets are proapoptotic molecules that display tumor-suppressive functions. Itch E3 activity is required for ubiquitination and proteosomal degradation of p73 and p63, two structural homologues of the tumor-suppressor transcription factor p53.[22,23] Several researchers have shown that, in response to DNA damage, Itch is rapidly downregulated; this response allows p73 levels to increase and become activated and stabilized, thus leading to cell cycle arrest and apoptosis.[22,24–27] Extracellular stimuli can also regulate protein turnover through inducible substrate phosphorylation, which confers recognition by E3 ligases.[28,29] The downregulation of Itch expression observed after treatment of Jurkat T cells with CGFP indicates that the degradation of proapoptotic mediators diminishes after treatment and the cells go to apoptosis. The second identified gene was UBR1, whose role is to mediate polyubiquitination and subsequent degradation of its substrates.[30] UBR1

is active in the N-end rule pathway, which is an ubiquitin-dependent system.[31]

Another gene whose expression was downregulated after treatment was PKC iota. Enzymes belonging to the protein kinase C (PKC) family represent one of the major mediators of signal transduction in cells. PKC iota participates in multiple aspects of the transformed phenotype of human cancer cells including transformed growth, invasion, and survival.[32] In melanocytes, PKC iota was the only isoform exclusively detected in tumor lysates in spontaneously transformed melanoma cells and melanoma cell lines, but not in normal melanocytes, and may therefore be associated with the transformed phenotype in human melanoma *in vitro* and *in vivo*.[33] Eder et al.[34] showed that an increased PKC iota transcription is associated with decreased progression-free survival in serous epithelial ovarian cancer. They suggested PKC iota as a potential oncogene and proposed it as a novel target for therapy. Regala et al.[35] demonstrated that PKC iota is a critical lung cancer gene that activates the Rac1→Pak→Mek1,2→Erk1,2 signaling pathway required for transformed growth. They claimed that PKC iota may be an attractive molecular target for mechanism-based therapies for treatment of lung cancer. Therefore, it is possible that *G. frondosa* polysaccharides can achieve tumor-inhibiting effects by reducing the transcription of PKC iota.

We also identified HAI1, which inhibits hepatocyte growth factor and was reported to have impact on cancer metastasis and tumor growth.[36] Heterogeneous nuclear ribonucleoprotein A2/B1, isoform A2 (hnRNP A2/B1), codes for the protein that is involved in cellular proliferation, differentiation, and protein synthesis and is up-regulated in non-small cell lung cancer. Zech et al.[37] considered hnRNP A2/B1 to be a valuable prognostic parameter in lung cancer. In our experiment, the expression level of hnRNP A2/B1 after treatment decreased, meaning that CGFP has a favorable antitumor effect on Jurkat T cells, as this is a leukemia cell line.

Apart from affecting proliferation and apoptosis connected genes, CGFP modified the expression of killer cell lectin-like receptor subfamily C, member 1 (KLRC1). It was shown that upregulation of KLRC1, an inhibitory NK cell receptor, downregulates antigen-specific cytotoxicity of cytotoxic T cells during both viral clearance and virus-induced oncogenesis**.** Upregulation of KLRC1 has been correlated with inefficient immune response against certain pathogens and cancer cells.[38] Increased expression of KLRC1 may mediate tumor escape from host immunity in human endometrial carcinoma.[39] Our results show that CGFP induces KLRC1, which does not coincide with the principle of anticancer activity. This discrepancy may be the consequence of the chosen cell model or the concentration of CGFP used in the experiment. Namely, KLRC1 may impair the function of T cells regarding their activation and promotion. On the other hand, the Jurkat T cells are a leukemia cell line and upregulation of this receptor may in fact reduce their activity.

The rest of the genes that we found to be differentially expressed in directional display experiments were either genes of unknown functions or could not be directly linked to processes involved in cancerogenesis. Further investigation may reveal their noteworthy part in the remedial effect of *G. frondosa*.

The effect of CGFP on the cytokine gene expression in Jurkat T cells was also a part of this study. Mushroom polysaccharides have mostly been studied for their immunomodulatory effects on peripheral blood mononuclear cells or spleen cells, or on lymph node cells obtained from treated experimental animals.[40–42] Surprisingly, treatment of Jurkat T cells with CGFP resulted only in IL-5 mRNA upregulation.

Relatively little is known about the regulatory mechanisms controlling the complex patterns of protein abundance and post-translational modification in tumors. Although positive correlations between translational and transcriptional products do exist, discordance between mRNA and proteins are not quite uncommon. Potential sources of discrepancy between RNA and protein levels include translational control and altered protein stability. To date, studies have mostly been conducted in yeast. These works have explored disparities between transcriptome and proteome data, and largely employed direct comparisons of mRNA and protein expression ratios between each sample pair.[43] In a study by Chen et al.,[44] mRNA and protein expression levels within the same tumor samples were

compared and 17% of the protein spots showed a statistically significant correlation between mRNA and protein. Le Naour et al.[45] profiled the changes in gene expression during differentiation and maturation of monocyte-derived dendritic cells using both oligonucleotide microarrays and proteomics. They demonstrated that a proteomics approach may provide information that could not be obtained at the RNA level, due either to poor correlation between mRNA and protein levels or due to post-translational modifications that may result in several isoforms generated from one mRNA.

After considering the results from DD experiments, a link between the down-regulation of Itch gene expression and up-regulation of IL-5 mRNA can be made. The same connection was studied by Fang et al.,[46] who assessed whether Itch deficiency affects T-cell development and function. They used heterozygous $Itch^{+/-}$ mice that were interbred to generate $Itch^{+/+}$, $Itch^{+/-}$, and $Itch^{-/-}$ littermates. T cells were isolated from the lymph nodes of $Itch^{-/-}$ mice. They found that $Itch^{-/-}$ T cells differentiate into T helper type 2 cells with an increased production of IL-5. This finding could explain the increase in the IL-5 mRNA levels in Jurkat T cells after treatment with CGFP.

Numerous studies have confirmed that polysaccharides extracted from *G. frondosa* have prominent beneficial effects on immune function, with the end result being the antitumor effect of the therapy. These polysaccharides promote the action of macrophages,[47–49] NK cells,[7,50,51] and T lymphocytes.[45] Fullerton et al.[10] have shown that the β-glucan from Maitake has a cytotoxic effect on prostatic cancer cells *in vitro* presumably through oxidative stress leading to apoptosis. Another possible mechanism was investigated by Matsui et al.,[52] who studied the effects of D-Fraction on carcinoma angiogenesis. They discovered that inhibition of tumor cell proliferation could also be a consequence of tumor angiogenesis induction provoked by the increased plasma level of vascular endothelial growth factor and direct damage of the tumor cells by the increased plasma TNF-α. We have found in this study that genes involved in the activity of the ubiquitin system are differentially expressed under stimulation with *G. frondosa* polysaccharides, adding this as a potential mechanism of action of CGFP. The ubiquitin system has emerged as the focus of molecular targeting in developing cancer therapeutics. In particular, alterations in ubiquitination are observed in most, if not all, cancer cells, and it is possible to target specific molecules involved in ubiquitination and proteosomal degradation to regulate many cellular processes such as signal transduction, proliferation, and apoptosis.[53] E3 ubiquitin ligases regulate a variety of biologic processes, including cell growth and apoptosis, through the timely ubiquitination and degradation of many cell cycle and apoptosis regulatory proteins. Abnormal regulation of E3 ligases has been convincingly shown to contribute to cancer development.[54] Thus, targeting E3 ubiquitin ligases for cancer therapy has gained increasing attention.

## V. CONCLUDING REMARKS

Genes important for the activity of the ubiquitin system and HAI1, hnRNP A2/B1, PKC iota, and KLRC1 were identified as being differentially expressed in Jurkat T cells, and regulation of their expression could be responsible for the activity of CGFP. These genes have implications in the development or treatment of cancer, and could be used as potential new targets in certain types of tumors. Surprisingly, the influence of CGFP on the expression of cytokines in the Jurkat T cells was limited to IL-5. This result, together with regulation of the expression of genes involved in the ubiquitin-mediated pathway, proposes a new potential mechanism in the effect of CGFP on the T-leukemia cell line.

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# Hepatoma Cell Growth Inhibition by Inducing Apoptosis with Polysaccharide Isolated from Turkey Tail Medicinal Mushroom, *Trametes versicolor* (L.: Fr.) Lloyd (Aphyllophoromycetideae)

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**ABSTRACT**: The *Trametes* (=*Coriolus*) *versicolor* polysaccharide (CVP) is well known as an anti-tumor drug in clinical applications. Although recent studies have demonstrated that CVPs can inhibit the proliferation of cancer cells *in vitro* and *in vivo*, different purity levels of CVPs have a different affect on various cancer cells. In this study, crude CVPs were extracted and purified from *T. versicolor* dry fruit bodies by hot-water extraction, ethanol precipitation, and phenol-vitriolic colorimetry. The *in vitro* cytotoxic activities of CVPs were examined on the human hepatoma cancer (QGY) cell lines by using an MTT assay. The cell cycle and cell death (apoptosis) of QGY cells were investigated by flow cytometry. The expression of genes involved in the apoptosis process, such as *p53*, *Bcl-2*, and *Fas*, was also studied using reverse transcriptase-polymerase chain reaction (RT-PCR) techniques. These results showed that CVPs inhibited the proliferation of QGY in low concentrations (<20 mg/L) and the $IC_{50}$ value was 4.25 mg/L. Changes that are characteristic of apoptosis, such as a found trapezoidal belt with DNA, were observed in the QGY cells treated with CVPs. There was a significant decrease in the expression of the cell cycle-related genes (*p53*, *Bcl-2*, and *Fas*) in these cells following treatment with CVPs. These results thus indicate that CVPs can be a potential candidate to ameliorate toxic effects when used in cancer therapy.

**KEY WORDS:** Turkey Tail mushroom, *Trametes* (=*Coriolus*) *versicolor,* medicinal mushrooms, polysaccharide, anti-tumor, apoptosis, human hepatoma cancer

## I. INTRODUCTION

Hepatoma is currently the fifth most common type of solid tumor worldwide and is the fourth leading cause of cancer-related death.[1] Therefore, finding new methods to treat this disease is becoming an increasingly important global issue. It is well known that cancer cells can acquire resistance to apoptosis by various mechanisms that involve regulators of various apoptosis signaling pathways. Apoptosis

## ABBREVIATIONS

**CV:** *Trametes* (=*Coriolus*) *versicolor*; **CVP:** *Trametes* (=*Coriolus*) *versicolor* polysaccharide; **DMSO:** dimethyl sulfoxide; **FBS:** fetal bovine serum; **FEW:** fungi water extract; **MTT:** 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide; **PI:** propidium iodide; **QGY:** human hepatoma cell line; **RT-PCR**: reverse transcriptase-polymerase chain reaction; **WRL:** human normal liver cell line

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is a physiological cell self-destruction program that has been implicated in multiple biological and pathological processes, including cancer. Analysis of the molecular mechanisms of apoptosis has led to a better understanding of many human diseases, notably cancer, in which the tumor-promoting genes will be inappropriately expressed and the tumor suppressor genes will be silenced in malignant tumor development. The induction and execution of apoptosis require the cooperation of a series of molecules, including signal molecules, receptors, enzymes, and gene-regulating proteins.[2–4] It has been shown that many genes, such as *p53*, *Fas*, or *Bcl-2*, should be able to shift the balance either to cell survival or cell death.[5–8]

Because of its medicinal value, the Turkey Tail medicinal mushroom, *Trametes* (=*Coriolus*) *versicolor* (L.: Fr.) Lloyd (Polyporaceae, Aphyllophoromycetideae) (CV), known as Yunzhi in China, was recorded in the *Compendium of Chinese Materia Medica* and *Shen Non Compendium Medica* thousands of years ago in China. CV has commonly been used in China for centuries to treat liver diseases. At the present time, the therapeutic potentials of CV have been gaining acceptance among patients worldwide.[9–11] Among various bioactive components derived from CV, polysaccharide (Krestin) is found to be the most prominent in anti-tumor and immunomodulatory activities. In recent years, many CV polysaccharides (CVPs) have been isolated from different strains of *T. versicolor* by using fermentation technology. Numerous *in vitro* studies have reported that CVPs can inhibit the proliferation of leukemia,[12,13] lymphoma,[14] hepatoma,[15,16] breast,[17] lung,[18] and prostate tumor cell lines.[19] However, CVPs can selectively suppress the proliferation of human hepatoma cancer. For example, *in vitro* CVPs can suppress the proliferation of QGY-7703 but can not suppress the proliferation of HepG2, 7721, PLC.[17] The anti-tumor activity of various CVPs seems to depend not only on the strains derived,[16] the habitat in which they grow,[17] and the source material and the method of recovery used,[20] but also on the prevention of tumor growth exerted through diverse mechanisms, including cell cycle arrest, induction of tumor cell death by apoptosis and secondary necrosis, together with stimulation of the anti-tumor activity of macrophages.[11] Previous studies have demonstrated that a standardized aqueous ethanol extract prepared from CV inhibited the proliferation of human leukemia cells and breast cancer via induction of apoptosis.[21] However, the mechanism implicated in apoptosis induced by CV extract and the effects on hepatoma growth *in vitro* are not yet fully understood. The present study aimed to examine the *in vitro* cytotoxic activities of hot-water extract from a culture grown of *T. versicolor* on human hepatoma cancer cell line (QGY), and to verify if crude CVPs can induce apoptosis of hepatoma cancer cells.

## II. MATERIALS AND METHODS

### A. Chemicals

The RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY, USA). MTT (3-(4,5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide) and DMSO (dimethyl sulfoxide) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals used were reagent grade, and were purchased from a local chemical agent store.

### B. Cell Culture

The human hepatoma cell line (QGY) was obtained from Fudan University (China), and the human normal liver cell line (WRL) was provided by the Cancer Institute of Shanghai Jiao Tong University (China). Cells were cultured with RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 mg/L streptomycin, and 100 mg/L penicillin in 75-$cm^2$ tissue culture flasks in a humidified incubator at 37ºC with 5% $CO_2$.

### C. Preparation of *T. versicolor* Extract

The studied strain of *T. versicolor* is from the Agricultural Culture Collection of China (ACCC; strain no. 50435).

CVPs were obtained from the cultivated fruit bodies and crude CVPs were extracted according

to the method described by Zhou et al.[17] The fruiting bodies were selected, cleaned, quantified, and crushed. Based on a Soxhlet extraction method with petroleum ether as the extractive solvent, the materials were degreased and the residua were air-dried and then extracted with hot water at the proper proportion (1:30 [mass/volume]) and temperature (90°). After being filtrated and discolored, the solution was concentrated to 10% of its original volume using the reduced pressure method, followed by precipitation with 95% ethanol. The free proteins and pigment of the extracts were removed by the Sevage method and active carbon, and the extract was then refined. The experiment was replicated three times. The polysaccharide content was measured by the phenolsulphuric acid method, using glucose as standard.[19]

### D. MTT Assay

Cells were maintained as monolayer cultures in RPMI-1640 medium supplemented with 10% FBS and incubated at 37ºC for 48 h. CVPs (50 mg/L) in 0.2% DMSO were proportionally diluted with RPMI-1640 medium, and 50 µL of each solution (20, 10, 5, 2.5, 1.25, 0.625 mg/L) was added to triplicate wells, respectively. After 72 h, 0.15 mg (30 µL of 5 g/L) MTT was added to each well and incubated at 37ºC for an additional 4 h. The medium was removed and 100 µL DMSO was added into each well after the plate was shaken thoroughly for 10 min. The absorbance of the samples was measured at 570 nm with a Multiskan Spectrum Microplate Spectrophotometer (Thermo Labsystem, Germany).

### E. Cell Proliferation

QGY cells were cultured for 1 day in the basal medium. On the next day, the culture medium was replaced with medium containing desired concentrations of CVPs (6 mg/L), or the control medium. The cells were seeded on 12- or 24-well plates at a density of 1.0 to 2.0 × $10^5$ cells/well, and were cultured for 48 h and 72 h as specified in the medium with or without CVPs. The number of viable cells was identified and counted using flow cytometry.

### F. DNA Fragmentation

Cells were seeded in 75-$cm^2$ tissue culture flasks and incubated with desired concentrations of herbal extracts in a humidified incubator (37ºC in 5% $CO_2$) for 24 h. Cells were then harvested and washed once with 400 µL PBS. DNA of CVP (6 mg/L) treated and nontreated QGY cells were extracted as described in the protocol of the Apoptosis Cell DNA Ladder Isolation Kit (Biofuture, Beijing, China). We loaded 10 µL of each DNA sample on 1.2% TAE agarose gel to investigate whether any DNA ladders could be observed.

### G. RNA Isolation and RT-PCR

Total RNA of CVP (6 mg/L) treated and nontreated QGY cells were extracted following the protocol of Cell Total RNA Isolation Kit (Watson, Shanghai, China). The quality and concentration of RNA samples were examined by agarose gel electrophoresis and spectrophotometer analysis. After isolation, aliquots of 0.4 µg total RNA were employed to amplify the *p53* (NM_000546), *bcl-2* (NM_000633), and *Fas* (NM_152876) genes in the RT-PCR reaction. RT-PCR was performed at 50ºC for 30 min followed by 25 cycles of amplification (94ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 90 sec), using gene-specific primers P53F and P53R, Bcl-2F and Bcl-2R, FasF and FasR. Two primers, β-actin F and β-actin R, were used to amplify β-actin gene (NM_001101) as an internal control for RT-PCR (Table 1).

## III. RESULTS

### A. Effects of CVPs on the Cytotoxicity of QGY Cells

To estimate the anti-tumor effects of CVPs, QGY tumor cells and the human normal liver cell line (WRL) were incubated in the presence of various CVP concentrations (20, 10, 5, 2.5, 1.25, and 0.625

**TABLE 1. Primers for RT-PCR**

| Primer | Sequence |
|---|---|
| P53F | 5′-GCGCACAGAGGAAGAGAATCTCCGC-3′ |
| P53R | 5′-GGCCAACTTGTTCAGTGGAGCCCCGG-3′ |
| Bcl-2F | 5′-GCGTCAACCGGGAGATGTCGCCCCTG-3′ |
| Bcl-2R | 5′-TTTCTTAAACAGCCTGCAGCTTTGTTT-3′ |
| FasF | 5′-AGTACAGAAAACATGCAGAAAGCAC-3′ |
| FasR | 5′-CTCTGCAAGAGTACAAAGATTGGCT-3′ |
| β-actin F | 5′-GTGGGGCGCCCCAGGCACCAGGGC-3′ |
| β-actin R | 5′-CTCCTTAATGTCACGCACGATTTCCC-3′ |

mg/L) and tumor cell viability was determined after 48 h by MTT assay. CVPs reduced tumor cell viability in a dose-dependent manner, with calculated $IC_{50}$ values of 4.25 mg/L. CVPs did not inhibit the human normal liver cell line (WRL) (Fig. 1).

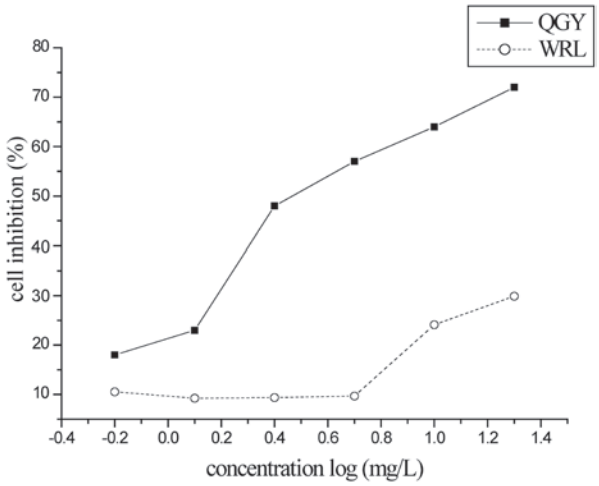


**FIGURE 1.** Anti-proliferative effects of CV extract on the human hepatoma cancer cell line (QGY) and the human normal liver cell line (WRL). Cells were incubated with increasing concentrations (0.625–20 mg/L with 2-fold increase) of the CV extract in culture medium for 72 h and the proliferative response was assessed by MTT assay.

### B. CVPs Induce G0/G1 Cell Cycle Arrest, Followed by Apoptosis of QGY Cells

We performed flow cytometric analysis of PI stained cells to further explore the type of cell death induced by CVPs. The proportion of apoptotic cells was significantly elevated after 48 h and 72 h of CVP (6 mg/L) treatment (Fig. 2). However, after 48 h of incubation with CVPs, 10.49% of the cells were apoptotic (Fig. 2A). After an additional 24 h of incubation, 27.39% were apoptotic (Fig. 2B). These results suggest that CVPs inhibit QGY cell proliferation in apoptotic tumor cell death.

### C. Analysis of QGY Cell DNA

DNA fragmentation occurred in apoptosis and was characterized by a ladder pattern indicating internucleosomal chromatin cleavage. A ladder of fragmented DNA was detected in QGY cells in 24-h culture treated with 6 mg/L of CVPs, whereas the 0.2% DMSO control (CON1) and medium control (CON2) did not show the same effect (Fig. 3). These results indicated that CVPs caused apoptosis.

### D. Analysis of Gene Expression

To examine the expression of apoptosis, cell cycle, and growth-related genes (*Bcl-2*, *p53*, and *Fas*) under CVP treatment using RT-PCR techniques, the treated and control QGY cells were used for RNA isolation after 24 h. In comparison with the 0.2% DMSO control (CON1) and medium control (CON2), there was a significant decrease in the expression of *p53* and *Fas* in the cells treated with CVPs (6 mg/L). However, there was a small decrease in the expression of *Bcl-2* in the cells treated with CVPs (6 mg/L) (Fig. 4).

## IV. DISCUSSION

### A. The Solubility of CVPs Is Relevant to Cytotoxic Effects

Previous studies demonstrated that a hot-water extract of *T. versicolor* possesses cytotoxic effects on human various tumor cells via apoptosis induction. The present results indicate that the CV extract significantly inhibited the proliferation of the human hepatoma cancer cell line (QGY-7703)

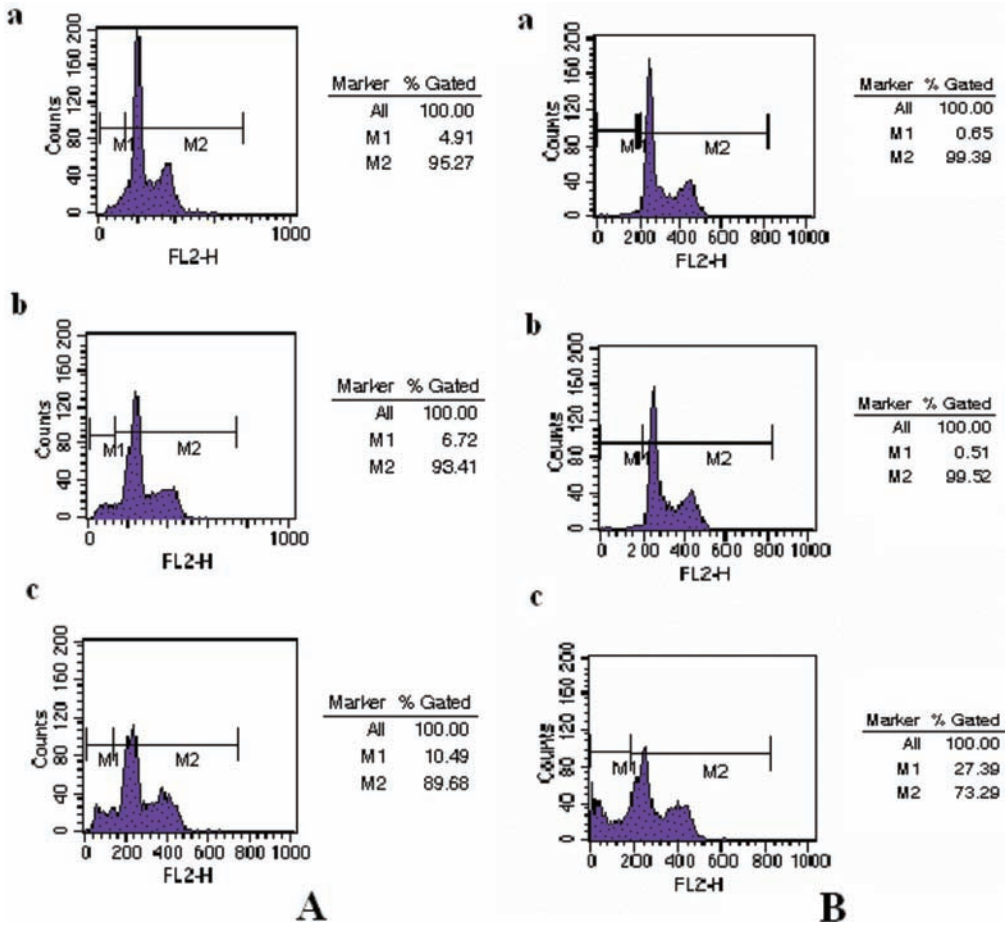


**FIGURE 2.** The effect of CVP on cell cycle, apoptosis, and necrosis of human hepatoma cancer cells line QGY. QGY cells (1.0 to 2.0 × $10^5$ cells/well) were cultured in the absence or presence of CVPs (6 mg/L). After the indicated 48 h (**A**) and 72 h (**B**), cells were stained with PI and analyzed by flow cytometry. Figure 2A shows cells that were stained with PI and analyzed by flow cytometry after the indicated 48 h. Figure 2B shows cells that were stained with PI and analyzed by flow cytometry after the indicated 72 h. a, control (medium); b, control (0.2% DMSO); c, CVPs (6 mg/L).

in dose- and time-dependent manners with the induction of apoptosis, which was confirmed by the internucleosomal DNA fragmentation (Fig. 3). Previous studies have shown that CVPs inhibited the proliferation of QGY-7703 in low concentrations, with $IC_{50}$ values of 18.37 mg/L.[17] Our results show that CVPs inhibited the proliferation of QGY-7703 in a lower concentration than shown in previous studies. The $IC_{50}$ value in this study was 4.25 mg/L. In our experiment, we found that the solubility of CVPs is different in various media. CVPs were solved in PBS solution in previous studies; however, we found that the solubility of CVPs is better in 0.2% DMSO solution than PBS solution (data not shown).

## B. CVPs Inhibit Hepatoma Cell Growth by an Untypical Pattern of Apoptosis

Apoptosis is known as an important type of cell death in response to cytotoxic treatment. The administration of many natural compounds with anticancer effects has been shown to be capable of inducing the apoptotic death of cancer cells. Two major apoptotic pathways have been identified thus far, the death receptor-mediated and mitochondria-mediated pathways.[21] Tumor suppressor gene *p53* is one of the pivotal molecules involved in apoptosis induction. *Fas* is a transmembrane receptor belonging to the tumor necrosis factor/nerve growth factor (TNF/NGF)-receptor superfamily that induces apoptosis

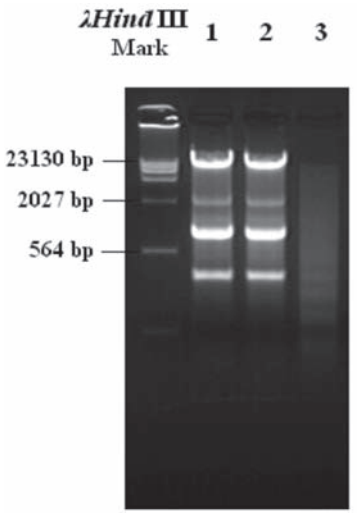


**FIGURE 3.** Analysis of QGY and cell DNA. Agarose gel electrophoresis of DNA was extracted from QGY cells that were incubated for 24 h with medium alone (lane 1), 0.2% DMSO (lane 2), or CVPs (lane 3). λ*Hind* III Mark shows the standard DNA markers.

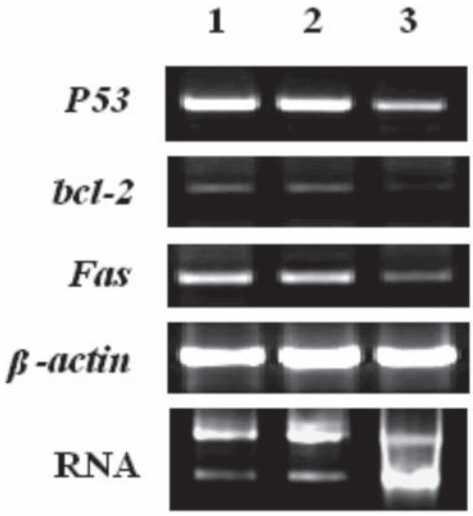


**FIGURE 4.** Analysis of gene expression. Total RNA was isolated from the QGY cells incubated for 24 h with medium alone (lane 1), 0.2% DMSO (lane 2), or CVPs (lane 3) and used in RT-PCR analysis for the expression of *P53*, *Bcl-2*, and *Fas*. β-actin was used as the internal control.

when triggered by agonistic antibodies or by its ligand, FasL.[22] *Bcl-2* is one of the major members of the Bcl-2 family, which works on mitochondria to prevent membrane permeabilization and thus the release of apoptogenic factors from the mitochondrial intermembrane space.[23] Previous studies demonstrated that CV aqueous ethanol extract dose-dependently suppressed the proliferation of HL-60 cells and induced apoptosis through the mitochondrial pathway (expression of anti-apoptotic protein *Bcl-2* was concomitantly down-regulated).[24] These results also indicated that it suppressed the proliferation of three breast tumor cell lines (T-47D, MCF-7, and MDA-MB-231) and induced apoptosis through the *p53*- and/or *Bcl-2*-dependent apoptotic pathways. Expression of *p53* protein was significantly up-regulated only in T-47D cells, and *Bcl-2* protein was down-regulated in MCF-7 and T-47D cells.[21] Regarding *Bcl-2* protein down-regulation expression, our results were consistent with the results of those for HL-60 cells and MCF-7 and T-47D cells. The tumor suppressor gene product *p53* is present in a wide variety of cells. Overexpression of *p53* has been reported to be associated with carcinogenesis, tumor progression, and poor prognoses in various types of cancer. However, our experiment revealed that CVPs could inhibit *p53* expression. Our data did not show whether the activation of *p53* may mediate cytotoxicity and cell cycle arrest in CVP-treated QGY because wt p53 was down-regulated by CVPs (QGY has a functional p53, wt p53). This result may be explained according to a study by Zhang et al.[25] Their results showed that medicinal fungi water extract (FEW), a concoction from a mixture that consists of equal amounts of *Cordyceps sinensis*, *Lentinus edodes*, *Agaricus brasiliensis*, and *Ganoderma lucidum*, could depress the levels of *bcl-2* and *p53* protein in the liver and lung cells. Zhang et al. posited that various polysaccharides from edible fungi, such as mushrooms, usually produce their anti-tumor effects by activating different immune responses in the host.[25] Interestingly, Youn et al. showed that aqueous extracts from *Inonotus obliquus* induced $G_0/G_1$ arrest with down-regulation of p53 expression in human hepatoma cells (HepG2).[26] They concluded that the down-regulation of p53 protein is closely associated with terminal differentiation or other signaling pathways levels. However, these questions unfortunately cannot be addressed at the present time.

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# Antioxidant and Free Radical Scavenging Activities of Culinary-Medicinal Mushrooms, Golden Chanterelle *Cantharellus cibarius* and Angel's Wings *Pleurotus porrigens*

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**ABSTRACT:** The antioxidant activity of ethyl acetate and methanol extracts of *Cantharellus cibarius* and *Pleurotus porrigens* were investigated by using six *in vitro* assay systems. Mushroom fruit bodies were obtained from the northern region of Iran. Ethyl acetate extracts had higher amounts of phenolic and flavonoids contents than their methanol extracts. Methanol extracts showed higher DPPH scavenging activity than ethyl acetate extracts. *C. cibarius* ethyl acetate extract showed better reducing powers than others, but was not comparable with vitamin C ($P < .001$). Methanol extracts exhibited better nitric oxide scavenging activity. The methanol extracts showed very powerful $Fe^{2+}$ chelating activity. The $IC_{50}$ values were 16.1 ± 2 for *C. cibarius* and 21.9 ± 0.9 μg $mL^{-1}$ for *P. porrigens*, respectively. There were no significant differences between methanol extracts and EDTA ($IC_{50}$ = 18 μg $mL^{-1}$, $P > .05$). These mushrooms can provide an optimal supply of antioxidant substances in the diet.

**KEY WORDS**: medicinal mushrooms, DPPH, flavonoids, iron chelator, phenols, thalassemia, *Cantharellus cibarius*, *Pleurotus porrigens*

## I. INTRODUCTION

Free radicals have been found to play an important role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer, and cardiovascular disease.[1,2] Recent studies have demonstrated the potential of naturally originated antioxidants against various diseases induced by free radicals.[3,4] However, synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have restricted use in foods because they are suspected to be carcinogenic.[5] Therefore, naturally originated compounds with antioxidative properties are being sought. In recent years, an increase in the consumption of wild edible mushrooms has been observed, even in the developed world. In fact, wild-growing mushrooms have been a popular

## ABBREVIATIONS

**AD:** Alzheimer's disease; **BHA:** butylated hydroxyanisole; **BHT:** butylated hydroxytoluene; **DPPH:** 1,1-Diphenyl-2-picryl hydrazyl; **EDTA:** ethylenediaminetetraacetic acid; **FTC:** ferric thiocyanate; **NO:** nitric oxide; **TCA:** trichloroacetic acid

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delicacy in many countries, mainly in central and eastern Europe[6] and northern Iran, because they add flavor and texture to foods. The golden chanterelle (*Cantharellus cibarius* Fr., Cantharellaceae, Aphyllophoromycetideae) is an edible mushroom with a worldwide distribution, fruiting in fall. It is highly appreciated for its wonderful fruity, apricot-like aroma, and is particularly prized for cooking throughout Europe.[7] In addition to its flavor and smell, the *C. cibarius* also has a pleasant texture. The *C. cibarius* is of great economical interest for the northeastern region of Iran apart from becoming an important commercial source. Previous studies on the chanterelle concerned the content levels of its amino acids,[8] vitamin D,[9] protein, fat, fiber, ash, minerals, ascorbic acid,[10] and trace elements[6]; the characterization of basidiolipids,[11] a ubiquitin-like peptide and homodimeric laccase; the use of different nitrogen sources; the production of bioactive secondary metabolites; and the biological activities of 2 fatty-acid derivatives formed as a response to injury[7]; its mutagenic[12] and insecticidal properties[13]; and its phenolic and organic acid profiles.[7] To the best of our knowledge, there are no available data on the antioxidant activity of ethyl acetate and its consecutive methanol extract of *C. cibarius.*

Lovastatin, a cholesterol-lowering agent derived from microscopic fungi present in the *Pleurotus* species, and its analogues are reported to be the best therapeutic agents for correcting hypercholesterolemia.[14] Ethyl acetate and methanol extracts of *P. ostreatus* var. *florida* have been found to exhibit potent scavenging of hydroxyl radicals and inhibition of lipid peroxidation activities.[15] Good antioxidant activity from *P. ostreatus* has recently been reported.[5] However, there has been no report on the antioxidant activity of ethyl acetate and methanol extract of *Pleurotus porrigens (*Pers.) P. Kumm. (Pleurotaceae, Agaricomycetideae).

The objective of this study was to examine the antioxidant activity of ethyl acetate and methanol extracts of the two above-mentioned mushrooms by employing six various *in vitro* assay systems (i.e., DPPH and nitric oxide radical scavenging, reducing power, scavenging of hydrogen peroxide, and its linoleic acid and iron ion chelating power) in order to understand the usefulness of this mushroom as a foodstuff and in medicine.

## II. MATERIALS AND METHODS

### A. Chemicals

Ferrozine, linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), and potassium ferricyanide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BHA, ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, ethylenediaminetetraacetic acid (EDTA), and ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

### B. Samples

*C. cibarius* and *P. porrigens*, the selected species of edible wild mushrooms, were obtained throughout the fall of 2008 from indigenous people who collect edible mushrooms from forests of Sari, Iran. The samples were authenticated by Dr. B. Eslami and the voucher specimens were deposited in the Sari School of Pharmacy herbarium. The macroscopic descriptions, including size, shape, color, texture, and odor, were noted. The color of the carpophore, shape of the cap and stipe, color of the flesh and latex, and its smell and habitat were also noted. The mushroom samples were transported to the Sari School of Pharmacy Laboratory and kept at <4°C within 24 h prior to sample preparation. Each mushroom species was replicated four times with 500 g of each species per time period.

### C. Preparation of Mushroom Extracts

Each mushroom (100 g) was extracted by the percolation method using ethyl acetate (400 mL) for 24 h at room temperature. The extract was then separated from the sample residue by filtration through Whatman No. 1 filter paper. This procedure was thrice repeated. The remaining sample residue was consecutively extracted with methanol (400 mL). Extracts were filtered and concentrated under reduced pressure at 40°C using a rotary evaporator.

### D. Determination of Total Phenolic Compounds and Flavonoid Contents

Total phenolic compound contents were determined by using the Folin-Ciocalteau method.[4,16] The extract samples (0.5 mL) were mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent for 5 min, and 2.0 mL of 75 g $L^{-1}$ sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. The results were expressed as gallic acid equivalents. Total flavonoids were estimated using the methods described in our recently published works.[17,18] Briefly, 0.5 mL solution of each extract in methanol was separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double-beam spectrophotometer (UV-Visible EZ201, Perkin Elmer, Norwalk, CA, USA). Total flavonoid contents were calculated as quercetin from a calibration curve.

### E. DPPH Radical Scavenging Activity

The stable DPPH radical was used to determine free radical scavenging activity of the extracts.[17,19,20] Different concentrations of each extract (50, 100, 200, 400, 800, and 1600 µg $mL^{-1}$) were added, at an equal volume, to a methanolic solution of DPPH (100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated three times. Vitamin C, BHA, and quercetin (Fluka, Germany) were used as standard controls. $IC_{50}$ values denote the sample concentration, which is required to scavenge 50% of DPPH free radicals.

### F. Reducing Power

The $Fe^{3+}$ reducing power of the extract was determined according to the method of Yen and Chen with a slight modification.[21,22] Different concentrations (50–800 µg $mL^{-1}$) of each extract (1 mL) were mixed with 1 mL phosphate buffer (0.2 M, pH 6.6) and 1 mL potassium hexacyanoferrate (1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 1 mL of TCA (10%) was added to terminate the reaction. The upper portion of the solution (1 mL) was mixed with 1 mL distilled water, and 0.2 mL $FeCl_3$ solution (0.1%) was added. The absorbance was measured at 700 nm against an appropriate blank solution. Vitamin C was used as the control. All tests were performed in triplicate.

### G. Determination of Metal Chelating Activity

We estimated the ability of the extracts to chelate ferrous ions in our recently published work.[23,24] Briefly, different concentrations of extracts (6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg $mL^{-1}$) were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), and the mixture was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage inhibition of the ferrozine-$Fe^{2+}$ complex formation was calculated as:

$$[(A_0 - A_1)/A_0] \times 100,$$

where $A_0$ was the absorbance of the control, and $A_1$ of the mixture containing the extract or the absorbance of a standard solution. EDTA was used as a standard.

### H. Assay of Nitric Oxide Scavenging Activity

This procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using the Griess reagent (1% sulfanilamide, 2% $H_3PO_4$, and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride). Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For this experiment, sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with differ-

ent concentrations of extracts dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extracts but with an equivalent amount of water, served as a control. After the incubation period, 0.5 mL of the Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as a positive control.[25]

### I. Scavenging of Hydrogen Peroxide

The ability of the extracts to scavenge hydrogen peroxide was determined according to the methods used in our recently published works.[16,20] A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extracts (0.1–3.2 mg $mL^{-1}$) in distilled water were added to a hydrogen-peroxide solution (0.6 mL, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows:

$$\% \text{ scavenged } [H_2O_2] = [(A_o - A_1)/A_o] \times 100,$$

where $A_o$ was the absorbance of the control, and A1 was the absorbance in the presence of the sample of extract and standard.

### J. Determination of Antioxidant Activity

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation.[26] The inhibitory capacity of extracts was tested against oxidation of linoleic acid by the FTC (ferric thiocyanate) method, which was adopted from Osawa and Namiki.[2,21] Twenty mg $mL^{-1}$ of samples dissolved in 4 mL of 95% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL), and distilled water (3.9 mL) and kept in screw-cap containers at 40°C in the dark. We then added 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate to 0.1 mL of this solution. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured. The absorbance was measured again every 24 h until the day the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as:

(%) inhibition = 100 – [(absorbance increase of the sample/absorbance increase of the control) × 100].

All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged. Vitamin C and BHA were used as positive controls.

### K. Statistical Analysis

Experimental results are expressed as means ± standard deviations (SD). All measurements were replicated three times. The data were analyzed by one-way analysis of variance (ANOVA), and Tukey multiple comparisons were carried out to detect significant differences ($P < .05$) between the mean values that had more than two groups. The $IC_{50}$ values were calculated from linear regression analysis.

## III. RESULTS AND DISCUSSION

### A. Recovery Percentage and Flavonoid and Phenolic Contents of Extracts

The yield and total phenolic and flavonoid contents of ethyl acetate and methanol extracts from mushrooms are shown in Table 1. Total phenol compounds were reported as gallic acid equivalents by reference to standard curves ($y = 0.0063x$; $r^2 = 0.987$). The total flavonoid contents were reported as milligrams of quercetin equivalent to grams of extract powder, by reference to the standard curve ($y = 0.0067x + 0.0132$; $r^2 = 0.999$). Ethyl acetate

**TABLE 1. Phenol and Flavonoid Contents and Antioxidant Activities of *Cantharellus cibarius* and *Pleurotus porrigens***

| $Fe^{2+}$ chelating ability (%)[a] or $IC_{50}$ (μg $mL^{-1}$) | $H_2O_2$ scavenging activity, $IC_{50}$ (mg $mL^{-1}$)[b] | Nitric oxide scavenging, $IC_{50}$ (μg $mL^{-1}$)[c] | DPPH free radical scavenging, $IC_{50}$ (μg $mL^{-1}$)[d] | Total flavonoid contents (mg $g^{-1}$) | Total phenol contents (mg $g^{-1}$) | Yield (%) | Sample name |
|---|---|---|---|---|---|---|---|
| 579.3 ± 18 | 1.2 ± 0.4 | 929.8 ± 36 | 548.5 ± 22 | 56.4 ± 1.2 | 20.4 ± 0.9 | 4 | CE |
| 16.1 ± 2 | 0.64 ± 0.02 | 603.1 ± 21 | 263.6 ± 11 | 5.7 ± 2.1 | 10.5 ± 0.3 | 13 | CM |
| 394.5 ± 15 | 0.97 ± 0.04 | 1025 ± 37 | 509.5 ± 19 | 73.9 ± 3.6 | 28.8 ± 1.1 | 1.6 | PE |
| 21.9 ± 0.9 | 0.59 ± 0.02 | 611 ± 19 | 246.7 ± 12 | 9.8 ± 1.1 | 15.9 ± 0.4 | 17 | PM |

Data are means ± SD. All measurements were replicated three times. Data were analyzed by ANOVA and Tukey multiple comparisons. The $IC_{50}$ values were calculated from linear regression analysis. CE, *C. cibarius* ethyl acetate extract; CM, *C. cibarius* methanol extract; PE, *P. porrigens* ethyl acetate extract; PM, *P. porrigens* methanol extract.
[a]Inhibition at 800 mg/$mL^{-1}$. EDTA was used as control ($IC_{50}$ = 18 ± 1.5 μg $mL^{-1}$).
[b]$IC_{50}$ values for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6 mg $mL^{-1}$, respectively.
[c]$IC_{50}$ value of quercetin was 5.28 ± 0.2 μg $mL^{-1}$.
[d]$IC_{50}$ values of BHA were 53.96 ± 3.1, vitamin C was 5.05 ± 0.1, and quercetin was 5.28 ± 0.2 μg $mL^{-1}$, respectively.

extracts had higher amounts of phenolic and flavonoid contents than their methanol extracts. The maximum extractable polyphenolic and flavonoid contents were recorded in *P. porrigens* ethyl acetate. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from natural sources and have been shown to possess significant antioxidant activities.[4]

## B. DPPH Radical Scavenging Activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples.[25] DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. Substances that are able to perform this reaction can be considered as antioxidants and thus as radical scavengers.[20] It was found that the radical scavenging activities of all of the extracts increased with increasing concentrations. The $IC_{50}$ values for DPPH radical scavenging activity are reported in Table 1. In general, high total phenol and flavonoid contents lead to better DPPH-scavenging activity.[2,19,27] However, *C. cibarius* methanol extract with the lowest amount of phenols and flavonoids showed the highest DPPH-scavenging activity in our study.

## C. Reducing Power

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action.[16] In this assay, the presence of antioxidants in the sample would result in the reduction of $Fe^{3+}$ to $Fe^{2+}$. The amount of $Fe^{2+}$ complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm.[19,28,29] Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1 shows the dose response curves for the reducing powers of the extracts. It was found that the reducing powers of all of the extracts also increased with the increase of their concentrations. There were significant differences ($P < .05$) among the extracts. *C. cibarius* ethyl acetate extract showed better activity than the others, although none of them was comparable with vitamin C ($P < .001$). Polyphenol contents of all the sample extracts seem to function as good electron and hydrogen atom donors, and thus should be able to terminate radi-

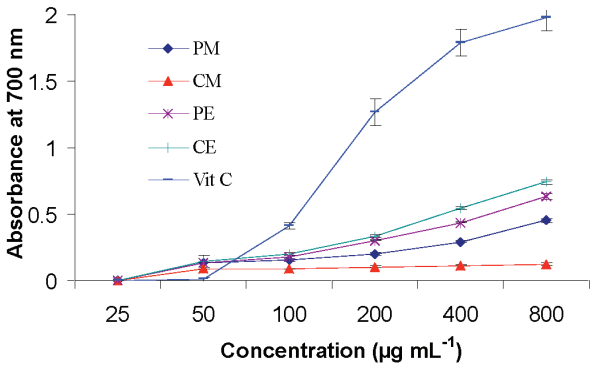


**FIGURE 1**. Reducing power of *C. cibarius* and *P. porrigens*. CE, *C. cibarius* ethyl acetate; CM, *C. cibarius* methanol; PE, *P. porrigens*. ethyl acetate; PM, *P. porrigens* methanol extract. Vitamin C was used as the control.

cal chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observations between the polyphenolic constituents in terms of dose-dependent and reducing power activity have been reported for several extracts.[19,21] Ethyl acetate extracts with higher amounts of phenols and flavonoids showed the highest reducing power activity compared to methanol extracts. It was evident that ethyl acetate extracts could serve as electron donors for terminating the radical chain reaction.

## D. Nitric Oxide Scavenging Activity

This procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated by using a Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. The percentage of inhibitions was increased with increasing concentration of the extracts, the results of which are shown in Table 1. Methanol extracts exhibited better activity than ethyl acetate extract. However, the activity of quercetin was much more pronounced than that of our extracts (17 ± 1.5 µg $mL^{-1}$). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer, and other pathological conditions.[30] The plant and plant products may have the property to counteract the effect of nitric oxide formation, and in turn may be of considerable interest in preventing the ill effects of excessive nitric oxide generation in the human body. Furthermore, the scavenging activity may also help to arrest the chain reactions initiated by excess generation of nitric oxide that are detrimental to human health.

## E. $Fe^{2+}$ Chelating Ability

Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in humans, thereby improving quality of life and overall survival in patients with diseases such as thalassemia major.[31] In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in the pathology of Alzheimer's disease (AD); thus, iron chelation could be considered as a rational therapeutic strategy for patients with AD.[27] Foods are often contaminated with transition metal ions that may be introduced by processing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry.[1] These processes can be delayed by iron chelation and deactivation. The transition metal iron is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease.[30] Because $Fe^{2+}$ also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing $Fe^{2+}$ concentration in Fenton reactions affords protection against oxidative damage. We estimated the chelating of ferrous ions by the extracts in our recently published works.[19,22–24] Ferrozine can quantitatively form complexes with $Fe^{2+}$. In the presence of other chelating agents, the complex formation is disrupted, with a resulting decrease in the red color of the complexes. In this assay, both the extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of the $Fe^{2+}$-ferrozine complex was decreased in a dose-dependent manner. For

example, the activity increased when the concentration was increased from 6.25 to 800 μg $mL^{-1}$. Metal chelating capacity was significant because the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation.[25] It has been reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion[32] (Table 1). The *C. cibarius* and *P. porrigens* methanol extracts showed very powerful $Fe^{2+}$ chelating activity (Figure 2). The $IC_{50}$ values were 16.1 ± 2 μg $mL^{-1}$ for *C. cibarius* and 21.9 ± 0.9 μg $mL^{-1}$ for *P. porrigens*, respectively. EDTA showed similar activity ($IC_{50}$ = 18 μg $mL^{-1}$). In spite of the higher potency of *C. cibarius*, there were no significant differences between the methanol extracts and EDTA ($P > .05$).

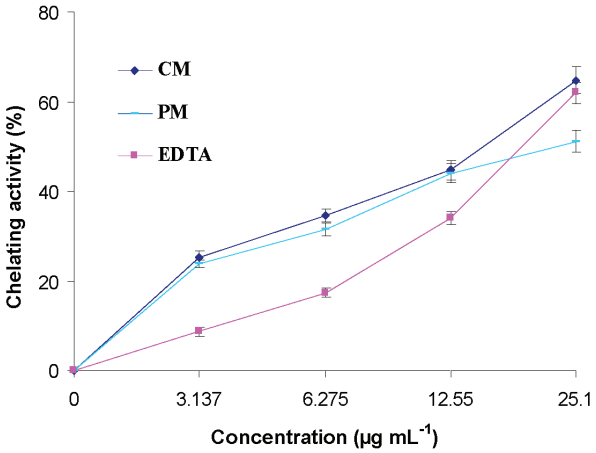


**FIGURE 2**. $Fe^{2+}$ chelating activity of *C. cibarius* methanol (CM) and *P. porrigens* methanol extract (PM). EDTA was used as the control. Ethyl acetate extracts showed lower activity that was not comparable with the control.

### F. Hydrogen Peroxide Scavenging

Scavenging of $H_2O_2$ by extracts may be attributed to their phenolics, which can donate electrons to $H_2O_2$ and thus neutralize it to water.[16,20] The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner (Table 1). Methanol extracts showed weak activity that was not comparable with vitamin C and quercetin ($P < .001$). Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing $H_2O_2$ is very important throughout food systems.

### G. FTC Method

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specifically, linoleic acid and arachidonic acid are targets of lipid peroxidation.[26] The inhibition of lipid peroxidation by antioxidants may be due to their free radical scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical.[21] Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Plant extracts did not show any activity in peroxidation inhibition in the FTC method. Vitamin C and BHA were used as controls and showed 91% to 98% inhibition at different incubation times (24–96 h).

## IV. CONCLUSIONS

The extracts examined in this study exhibited different levels of antioxidant activity in the tested methods. The results indicate that the extracts have strong iron chelatory activities. Further investigation of the individual compounds, their *in vivo* antioxidant activities, and use in different antioxidant mechanisms is needed. Such identified potential and natural constituents could be exploited as cost-effective food/feed additives for human and animal health.

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# Antinociceptive Activity of Agaricoglycerides Extracted from Mycelium of Ling Zhi or Reishi Medicinal Mushroom, *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Aphyllophoromycetideae)

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**ABSTRACT:** The antinociceptive activity of agaricoglycerides extracted from mycelium of Ling Zhi or Reishi medicinal mushroom, *Ganoderma lucidum* (AMG) was studied. The writhing test, formalin test, and hot-plate test, as well as the *in vitro* neurolysin inhibition assay, were respectively used to evaluate the antinociceptive activity of agaricoglycerides. The extracted agaricoglycerides inhibited acetic acid-induced abdominal constrictions in mice in a dose-dependent manner ($P < .05$). Both phases of response to the formalin test were also significantly inhibited ($P < .05$). The results from the hot-plate test showed that the extract significantly ($P < .05$) inhibited the reaction time to thermal stimuli at 30, 60, and 90 min after application. In the neurolysin inhibition assay, AMG showed strong activities against neurolysin ($IC_{50}$ = 100 nM). Our results indicate that if the same effects in humans or higher animals are shown, AMG may have potential applications as a component of analgesic food/feed and possibly even as a medicine.

**KEY WORDS:** medicinal mushrooms, agaricoglycerides, antinociceptive activity, *Ganoderma lucidum*, Ling Zhi or Reishi mushroom

## I. INTRODUCTION

The medicinal use of mushrooms has a very long tradition in Asian countries. Some mushroom extracts and compounds have been found to have special central effects that could be of neurological interest. Benzyl-alcohol derivatives from *Hericium erinaceus* induce the synthesis of nerve growth factors and might have an ameliorative effect in Alzheimer's dementia.[1] Erinacine E from *Hericium coralloides* fermentation broth is a highly selective agonist at the κ-opioid receptor.[2] Such compounds may exhibit antinociceptive activity without the side effects observed with μ-receptor agonists such as morphine.[3] Agaricoglycerides are a new class of fungal secondary metabolites that constitute esters of chlorinated 4-hydroxy benzoic acid and glycerol (Fig. 1). They are produced in mycelial cultures of several mushrooms, some of which are edible. The main active principle, agaricoglyceride A, has shown strong activities against neurolysin, a protease involved in the regulation of dynorphin

## ABBREVIATIONS

**AMG:** agaricoglycerides extracted from mycelium of *Ganoderma lucidum*; **i.p.**: intraperitoneal; **DMSO**: dimethyl sulfoxide; **p.o.**: oral administration; **YHZT:** Yuanhuzhitong

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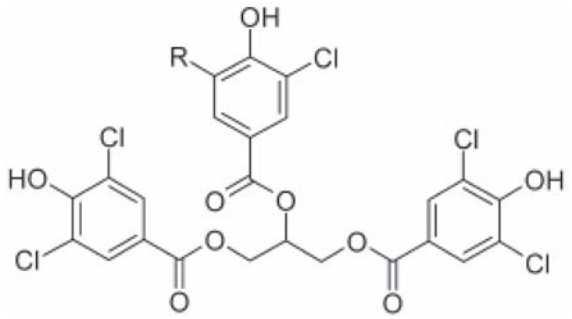


**FIGURE 1.** Chemical structures of agaricoglyceride.

and neurotensin metabolism, and has even exhibited moderate analgesic *in vivo* activities in an *in vivo* model.[4]

*Ganoderma lucidum* has been popularly used for its health-promoting properties.[5] The effects of *G. lucidum* extract on cancer, hypertension, hypercholesterolemia, and hepatitis have been demonstrated by several researchers.[6–8] In addition, *G. lucidum* has also been reported to show antinociceptive activity. Administration of hot water-soluble extracts of *G. lucidum* decreased pain dramatically in two patients with post-herpetic neuralgia recalcitrant to standard therapy and in two other patients with severe pain due to herpes zoster infection.[9] An earlier study[10] performed with a dichloromethane extract of *G. lucidum* demonstrated that these extracts possess antinociceptive activity. However, to our knowledge, there has been no report of the antinociceptive activity of agaricoglycerides extracted from *G. lucidum.*

The objective of the present study was to demonstrate whether agaricoglycerides extracted from *G. lucidum* also present antinociceptive properties.

## II. MATERIALS AND METHODS

### A. Chemicals

All chemicals were provided by Sigma Aldrich (Deisenhofen, Germany), whereas media ingredients were purchased from Tianjin Xing Yu Chemical Co., Ltd. (Tianjin, China). Yuanhuzhitong capsules (YHZT) were purchased from Jinan Limeng Pharmaceutic Factory (China). YHZT is a type of Chinese medicine that is used to treat pain, and it is composed of two traditional Chinese herbs, including *Rhizoma corydalis* and *Radix angelicae*.

### B. Mycelium of *G. lucidum*

A strain of *G. lucidum,* GL09101, purchased from the Agricultural Culture Collection of China (Beijing, China) was used in this study. First, the culture was grown at 28°C for 5 days on PDA slants (1000 mL of 20% potato extract liquid + 20.0 g of dextrose + 20.0 g of agar) and then maintained at 4°C in a refrigerator. Five to six pieces of the mycelia of *G. lucidum* were transferred from a slant into 250-mL Erlenmeyer flasks containing 100 mL of liquid medium (20% potato extract liquid + 2.0% dextrose + 0.1% $KH_2PO_4$ + 0.05% $MgSO_2$). The culture was incubated at 27°C on a rotary shaker at 180 rpm for 3 days.

A 72-h-old liquid culture was homogenized using a sterilized blender and then inoculated to 500-mL Erlenmeyer flasks containing 300 mL of culture medium (20% potato extract liquid + 2.0% dextrose + 0.1% $KH_2PO_4$ + 0.05% $MgSO_2$). The volume of inoculum was 15 mL, which was then cultivated under the same conditions. The 72-h-old culture medium was used for extraction.

### C. Agaricoglycerides Extraction from Mycelium of *G. lucidum*

The methodology for agaricoglyceride extraction was described in detail by Stadler et al.[4] Briefly, mycelia were separated from the culture fluid by filtration and extracted twice with acetone in an ultrasonic bath. The extract was filtered and the acetone was removed *in vacuo* to yield an aqueous residue. This residue was diluted with tap water and subsequently extracted three times with EtOAc. The combined organic phases were dried over $Na_2SO_4$ and evaporated *in vacuo* to yield an oily residue. This crude extract was separated by preparative high performance liquid chromatography at a retention time of 60 to 64 min, detected as single peak at 210 nm.

## D. Animals

Kunming outbreed mice weighing 20 g to 22 g were purchased from the Experimental Animal Center of Shandong University (Shandong, China). The mice were maintained at room temperature under an alternating natural light/dark photoperiod, and had access to standard laboratory food and fresh water *ad libitum*. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals. Care was taken to minimize discomfort, distress, and pain to the animals.

## E. Evaluation of Antinociceptive Activity

### *1. Neurolysin Inhibition Assay In Vitro*

To determine *in vitro* activities against neurolysin (Endopeptidase EC 3.4.24.16), AMG were dissolved in DMSO (final concentration: 10 to 0.078 μM). The final concentration of neurolysin solution in dilution buffer was 1.5 nM. Following the manufacturer's instructions, 2-mL samples were incubated with 50 mL of a neurolysin solution in each well of OptiPlate 96-well microtiter plates. At the same time, the peptidic substrate, DAB-CYLGlu-Arg-Nle-Phe-Leu-Ser-Phe-Pro-EDANS, was diluted in 50 mM Tris/HCl buffer (pH 7.5) to a final concentration of 5 mM in each well. The samples were then incubated for 90 min at room temperature. Thereafter, the $IC_{50}$ values were determined by fluorometric methodology (λex = 320 nm, λex = 520 nm).

### *2. Writhing Test*

The writhing test was used to evaluate analgesic activity. Mice were treated with AMG (1, 5, and 10 mg/kg, orally) 60 min before receiving a 0.6%-acetic acid injection (10 mL/kg, i.p.). The number of contractions or writhings, determined by abdominal muscle contractions and hind paw extension, was recorded for 20 min, starting 10 min after the administration of acetic acid.[13] YHZT capsules (5 mg/kg, p.o.) were used as standard.

### *3. Formalin Test*

The formalin test, which causes a local tissue injury to the paw, was used as a model for tonic pain and localized inflammatory pain. Twenty microliters of a 1% formalin solution were injected into the right hind paw of mice, and the licking time was recorded after the first 5 min (first phase, corresponding to a direct chemical stimulation of nociceptors) and after 20 min (second phase, involving inflammation) for 5 min each time. Animals were pretreated with AMG 60 min (p.o.) before intraplantar formalin injection.[14,15] YHZT capsules were used as standard.

### *4. Hot-plate Test*

In the hot-plate test, mice were preselected according to their reactions to a thermal stimulus (jumping or licking of hind limbs when placed on a hot plate at 55ºC). Latency times were recorded immediately before and 30, 60, and 90 min after drug administration, up to a maximum time of 40 sec to avoid paw lesions.[16]

### *5. Acute Toxicity*

The acute toxicity of AMG was investigated using a single oral administration of the substance in mice. In this assay, increasing doses of the test substance were orally administered to animals (up to 150 mg/kg). The animals were observed for 14 days. At the end of this period, the number of survivors was counted and body weight and spleen weight were recorded.

## F. Statistical Analysis

All data were analyzed by using a one-way analysis of variance, and the differences between means were established by Duncan's multiple-range test.[18] The data represent the means and standard deviations. A 5% significance level ($P < .05$) was used as the minimum acceptable probability for the difference between the means.

## III. RESULTS AND DISCUSSION

Neurolysin (EC 3.4.24.16) is a zinc metalloprotease that inactivates particular biologically active peptides, such as neurotensin and dynorphin A, by specific cleavage.[19] Whereas the κ-opioid receptor agonist dynorphin A is a well-known and obvious endogenous pain-relieving peptide, neurotensin has been reported to have analgesic properties when applied centrally in animal models.[20] Therefore, neurolysin inhibitors are likely to be useful substitutions for morphine and other opiates in the treatment of severe pain.[21–23] However, only few examples of nonpeptidic inhibitors of this enzyme have been found. In a neurolysin inhibition assay, AMG showed strong activity against neurolysin ($IC_{50}$ = 100 nM). Moreover, AMG even exhibited moderate analgesic activities in the next *in vivo* model.

In the writhing test, AMG (1, 5, and 10 mg/kg) inhibited the acetic acid-induced abdominal constrictions in mice in a dose-dependent manner after oral administration (41%, 61%, and 83%) (Table 1). The acetic acid-induced writhing reaction in mice, described as a typical model for inflammatory pain, has long been used as a screening tool for the assessment of analgesic properties of new agents.[24] The constrictions induced by acetic acid in mice result from an acute inflammatory reaction related to the increase in the peritoneal fluid levels of $PGE_2$ and $PGF_2\alpha$.[25] The fact that AMG was able to inhibit constrictions shows that this fraction has a peripheral antinociceptive effect.

**TABLE 1. Inhibitory Effects of AMG in Mice Submitted to the Writhing Test (N = 10)**

| Dose, route of administration | Contractions (20 min), n | Inhibition, % |
|---|---|---|
| Control: distilled water, 1 mL/kg, p.o. | 23.5 ± 1.6 | — |
| AMG, 1 mg/kg, p.o. | 13.9 ± 1.8 | 41* |
| AMG, 5 mg/kg, p.o. | 9.0 ± 1.9 | 62* |
| AMG, 10 mg/kg, p.o. | 4.1 ± 1.7 | 83* |
| YHZT, 5 mg/kg, p.o. | 3.9 ± 1.7 | 83* |

The asterisks in the last column indicate a statistical difference ($P < .05$).

The formalin test is different from most models of pain in that it is possible to assess the manner in which an animal responds to moderate, continuous pain generated by injured tissue. This model is constituted by two distinct phases: the first phase represents the irritating effects of formalin at the sensorial C fibers,[26] and the second is an inflammatory pain response. Therefore, it is possible to appraise the animal's answer to moderate and continuous pain caused by the tissue lesion, as well as the role of pain regulatory endogenous systems. Although both phases of the response were significantly inhibited in the formalin test in the present study (Table 2), the AMG effect was predominant in phase two and caused 61% and 48% inhibition of licking time at the orally administered doses of 5 and 10 mg/kg, respectively, similarly to YHZT capsules. These results indicate that both peripheral

**TABLE 2. Antinociceptive Effects of AMG in Mice Submitted to the Formalin Test (N = 10)**

| | Licking time, sec | | Inhibition, % | |
|---|---|---|---|---|
| Dose, route of administration | 1st phase | 2nd phase | 1st phase | 2nd phase |
| Control: distilled water, 1 mL/kg, p.o. | 61.2 ± 5.5 | 31.4 ± 3.0 | — | — |
| AMG, 1 mg/kg, p.o. | 64.7 ± 3.6 | 46.5 ± 3.9 | — | — |
| AMG, 5 mg/kg, p.o. | 71.09 ± 4.7 | 13.4 ± 3.7 | — | 57* |
| AMG, 10 mg/kg, p.o. | 53.2 ± 2.9 | 16.7 ± 3.3 | 13 | 47* |
| YHZT, 5 mg/kg, p.o. | 50.2 ± 3.0 | 12.9 ± 3.6 | 18 | 59* |

The asterisks in the last column indicate a statistical difference ($P < .05$).

**TABLE 3. Effects of AMG in Mice Submitted to the Hot-plate Test (N = 10)**

| | Reaction time to the thermal stimulus, sec | | | |
|---|---|---|---|---|
| **Dose, route of administration** | **0 min** | **30 min** | **60 min** | **90 min** |
| Control: distilled water, 1 mL/kg, p.o. | 11.8 ± 0.8 | 10.6 ± 0.9 | 8.3 ± 0.80 | 9.5 ± 0.6 |
| AMG, 1 mg/kg, p.o. | 13.9 ± 1.1 | 8.2 ± 0.9 | 10.4 ± 1.7 | 7.5 ± 0.9 |
| AMG, 5 mg/kg, p.o. | 7.9 ± 0.9 | 10.5 ± 1.1 | 16.4 ± 0.8* | 15.8 ± 1.2* |
| AMG, 10 mg/kg, p.o. | 10.9 ± 1.2 | 14.3 ± 0.8* | 15.8 ± 1.1* | 16.4 ± 1.1* |
| YHZT, 5 mg/kg, p.o. | 9.9 ± 1.0 | 13.5 ± 1.1* | 16.4 ± 0.8* | 16.4 ± 1.1* |

The asterisks indicate a statistical difference ($P < .05$).

**TABLE 4. Acute Toxicity Trial of the Effects of AMG on Mice (N = 10)**

| Outcome measure | 0 days | 14 days |
|---|---|---|
| Deaths, n | — | 0 |
| Body weight, g | 20.2 ± 1.1 | 23.1 ± 0.9 |
| Spleen weight, mg/g | 4.29 ± 0.9 | 4.3 ± 0.6 |

No significant changes in body weight or spleen weight were observed at the dose level of 150 mg/kg.

analgesic properties and central analgesic effects are the antinociception mechanisms of AMG.

Hot-plate tests were also conducted to corroborate that AMG also has analgesic actions. The hot-plate test is commonly used to assess narcotic analgesics or other centrally acting drugs.[27] In this study, this test was performed to assess the central antinociceptive effect of AMG (Table 3). Our results showed that AMG significantly inhibited the reaction time to thermal stimuli at 30, 60, and 90 min after oral administration of 1, 5, and 10 mg/kg compared with controls, similarly to the YHZT capsules.

In preliminary toxicologic studies, deaths were not observed even at the 150-mg/kg level. In addition, AMG did not cause significant changes in body weight or spleen weight of the animals, demonstrating its safety (Table 4).

In the present study, we report a potent antinociceptive activity of agaricoglycerides extracted from fermented medicinal mushroom *G. lucidum*. Our results show that AMG is a potent analgesic compound. To our knowledge, this is the first study to show the antinociceptive properties of agaricoglycerides of *G. lucidum* and our results support the folk medicinal use of this mushroom.

## ACKNOWLEDGMENT

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# Anti-Human Papillomavirus (HPV) 16 E6 Activity of Ling Zhi or Reishi Medicinal Mushroom, *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Aphyllophoromycetideae) Extracts

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**ABSTRACT:** Cancer of the cervix ranks second in the demographics of cancers affecting women worldwide. Human papillomavirus (HPV) type 16 is one of the major causative agents for cervical cancer. The E6 region of the HPV 16 genome encodes for an oncoprotein responsible for the pathogenesis of the disease. The E6 oncoprotein is abundant in cervical cancer and cervical cancer-derived cell lines. Mushrooms of the genus *Ganoderma* have long been used traditionally as an immunomodulating and antitumor agent, with recent studies validating its use in chemotherapeutics. Due to the importance of the E6 oncoprotein in the carcinogenesis of cervical cancer, this study was undertaken to screen the crude dichloromethane, ethanol, water, and polysaccharide extracts of *Ganoderma lucidum* for the ability to suppress the expression of HPV 16 E6 and to evaluate secondary metabolite classes present in the active crude extract. CaSki cells were treated with the crude extracts and were then subjected to immunocytochemistry protocols. All crude extracts of *G. lucidum* presented HPV 16 E6 suppression, with the dichloromethane extract being the most active compared to the other crude extracts. The crude dichloromethane extract was then subjected to thin-layer chromatography to evaluate the secondary metabolite classes present. The present study shows that the crude dichloromethane extract of *G. lucidum* possesses flavonoids, terpenoids, phenolics, and alkaloids. Further isolation work on the dichloromethane extract of *G. lucidum* could direct us to lead compounds with promising anti-HPV 16 E6 activity.

**KEY WORDS:** medicinal mushrooms, Ling Zhi or Reishi, *Ganoderma lucidum*, cervical cancer, EnVision™+, CaSki, terpenoids

## I. INTRODUCTION

Cervical cancer is the second most common cancer affecting women worldwide and ranks as the number one cancer in women from developing countries.[1] High-risk human papillomavirus (HPV), such as subtype HPV 16, has been found to contribute directly to the development of cervical cancer.[2] HPV 16 produces E6 and E7 oncoproteins directly involved in cellular transformations of the cervical epithelia leading to neoplasm, whereby E6 strongly contributes to malignant tumor formation and E7 strongly contributes to benign tumor formation.[3] E6 oncoprotein plays a major role in malignant cervical tumor due to the following mechanisms. First, it has been shown that E6 induces gross chromosomal alterations; second, it inhibits cellular differentiation; and third, it may induce telomerase

## ABBREVIATIONS

**DMSO:** dimethyl sulfoxide; **FBS:** foetal bovine serum; **HPV:** human papillomavirus; **LLC:** Lewis lung carcinoma; **NR:** neutral red; **OD:** optical density; **PBS:** phosphate buffered saline; **TLC:** thin-layer chromatography

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activation—all of which are responses in cases of malignancy.[3] The E6 protein is abundant in cervical cancer and cervical cancer-derived cell lines such as CaSki. A CaSki cell harbors 500 to 600 copies of the HPV 16 genome.[4]

Therapies for cervical cancer are readily available via hysterectomy,[5] radiotherapy,[6] and chemotherapy,[7] all of which could present complications and/or side effects. A target-specific treatment via the direct suppression of E6 oncoprotein could thus serve as a novel HPV 16-induced cervical cancer treatment.

Mushrooms of the genus *Ganoderma* have long been used traditionally as an immunomodulating and antitumor agent.[8] The fruit bodies of Ling Zhi or Reishi medicinal mushrooms, *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Ganodermataceae, Aphyllophoromycetideae), have been widely prescribed in Chinese medicine as treatment for health problems such as insomnia, hypertension, and cancer.[9,10] Studies have validated the use of *G. lucidum* as a cancer therapeutic agent. This species has demonstrated antitumor effects on Lewis lung carcinoma (LLC)-implanted mice,[11] suppression of invasive breast cancer cells MDA-MB-231,[12] as well as antiangiogenic properties in prostate cancer cells[13] and human lung cancer cells.[14]

As yet, no research has been established on the effects of *G. lucidum* extracts against HPV 16 E6 activities. This study aims to address whether *G. lucidum* extracts are able to suppress E6 activity manifested by HPV 16 in CaSki cell lines, and if suppression does occur, to determine which of the crude *G. lucidum* extracts are responsible for the activity. This study also addresses the possible secondary metabolites of the active crude *G. lucidum* extract involved in the suppression of HPV 16 E6 oncoprotein.

## II. MATERIALS AND METHODS

### A. Mushroom Extraction and Preparation of Stock Solution

Sliced and dried fruit bodies of *G. lucidum* were obtained from a mushroom farm (Vita Agrotech, Malaysia). The mushroom slices (100 g) were cut, blended, and subjected to extraction with ethanol (99.8%) by reflux (3 h) and concentrated *in vacuo* to yield ethanol extract (2.6249 ± 0.0001 g). The residue from the extraction was boiled (4 h) in distilled water (2 L) and filtered. Ethanol (99.8%) was added to the filtrate at a 1:1 ratio to form precipitates. The precipitates were collected and centrifuged (4000 rpm, 10 min, 4ºC) to obtain pellets. The pellets were combined and dialyzed in a dialysis tube against distilled water (72 h, 4ºC) to obtain the polysaccharide extract (0.0790 ± 0.0001 g). Cut and blended mushroom slices (100 g) were extracted with dichloromethane (72 h, 25ºC) and concentrated *in vacuo* to yield dichloromethane extract (1.4567 ± 0.0002 g). Cut and blended mushroom slices (100 g) were boiled (4 h) in distilled water (2 L) and lyophilized to yield water extract (3.192 ± 0.0001 g).

Preparation of extract stock solutions was as follows. The dichloromethane, ethanol, and polysaccharide extracts of *G. lucidum* were weighed (0.02 g) and diluted in 1 mL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) as stock solution. The water extract was weighed (0.02 g) and diluted in 1 mL distilled water. It was observed that the dichloromethane extract did not fully dissolve in DMSO.

### B. Cell Culture

Carcinoma of cervical squamous cells with representations of HPV 16 genome (i.e., CaSki; ATCC, Manassas, VA, USA) were maintained in RPMI-1640 culture media (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS) (PAA, Austria), and incubated (37ºC, 5% $CO_2$, and humidified air).

### C. Cell Treatment and Immunocytochemistry Protocol for HPV 16 E6 Activity

The extract stock solutions were diluted and added to the cells in tissue culture flasks (Thermo Fisher Scientific, Waltham, MA, USA) to give final treatment concentrations of 1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL.

Cells not treated with any extract served as the negative control. Incubation was carried out (72 h, 37ºC, 5% $CO_2$, and humidified air). The cells were then harvested and fixed in four wells on 12-well Teflon-coated slides (Thermo Fisher Scientific) with acetone.

The treated CaSki cells were gradually hydrated using a serial hydration protocol prior to staining. PBS with pH 7.6 was used as the washing solution. Hydrogen peroxidase was used as an endogenous peroxidase blocking solution. The cells in four wells were incubated with anti-HPV 16 E6 antibody (Chemicon, Billerica, MA, USA). A polymer-based immunocytochemistry protocol, EnVision™+ (Dako, Glostrup, Denmark), was performed on the cells to evaluate expression of HPV 16 E6 still present in the CaSki cells. Qualitative observations of four wells were photographed to determine the most active crude extracts of *G. lucidum*. The test was performed in three individual experiments.

## D. Neutral Red Cytotoxicity Assay Against Cell Lines

The neutral red (NR) cytotoxicity assay was conducted based on the method developed by Borenfruend and Puerner.[15] CaSki cells were seeded ($3 \times 10^4$ cells per well, 24 h, 37ºC, 5% $CO_2$, and humidified air) in 96-well microtiter plates (Thermo Fisher Scientific). Extracts were then added to the cells to give final treatment concentrations of 1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL. Cells not treated with any extract served as the negative control. Incubation was carried out (72 h, 37ºC, 5% $CO_2$, and humidified air). After the incubation period, the culture media were replaced with NR dye (ICN Biomedicals, Solon, OH, USA) (200 µL per well) and further incubated (3 h, 37 ºC, 5% $CO_2$, and humidified air) to allow maximum uptake of the dye by viable cells. Subsequently, the cells were washed with NR washing solution. The NR dye was extracted from viable cells with NR extraction solution (200 µL per well) and the microtiter plate was placed in gentle agitation (30 min, 25ºC). Optical density (OD) was determined spectrophotometrically at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The OD obtained is the mean between three wells. The average data were expressed in terms of the percentage of killing relative to negative controls. The test was performed in three individual experiments.

The value of the inhibition concentration that results in 50% cell death ($IC_{50}$) was obtained by developing a scatter plot based on each individual experiment and generating a logarithmic equation. The $IC_{50}$ value of each experiment was obtained by using "50" as the "y" value to the generated equation. The mean $IC_{50}$ values of the three individual experiments (N = 3) were reported as the $IC_{50}$ value of the extract ± standard error of the mean (SEM). These measurements were conducted using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA) and SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software.

## E. Secondary Metabolites Profile of the Active Crude Extract

The *G. lucidum* extract exhibiting the most active anti-HPV 16 E6 activity was subjected to thin-layer chromatography (TLC) to establish its secondary metabolites' profile. The crude extract was spotted on the TLC plate (silica gel 60, aluminum-backed) (Merck, Germany) and developed using the chloroform:ethanol (93:7) solvent system.

## F. Thin-Layer Chromatography Viewing Agents and Staining Reagents

A UV cabinet (UVP LLC, Upland, CA, USA) was used to emit $UV_{254}$ and $UV_{365}$ on developed TLC plates. The fluorescence spots were marked with a pencil. Iodine crystals (Sigma-Aldrich) were placed in an enclosed chamber and developed TLC plates were exposed to the iodine vapor. The brown spots were marked with a pencil. Vanillin-sulfuric acid reagent was prepared as described by Wall.[16] The developed TLC plate was heated (10 min, 110ºC). The spots, which were blue, purple, pink, or green in color, were marked with a pencil. Ferric (III) chloride reagent was prepared as described by Sharma et al.[17] The developed TLC plate was heated (10 min,

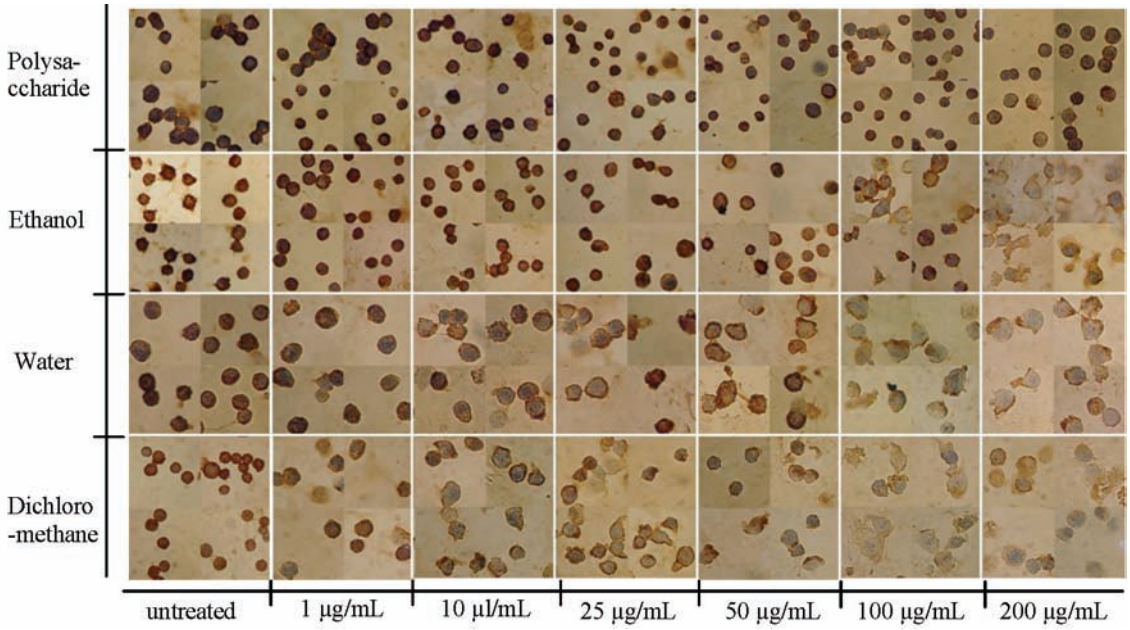


**FIGURE 1.** Comparison of untreated CaSki cells and CaSki cells treated with four crude extracts of *Ganoderma lucidum* at different concentrations.

110ºC). The spots, which were blue in color, were marked with a pencil. Dragendorff's reagent was prepared as described by Tsai et al.[18] The orange spots were marked with a pencil.

## III. RESULTS

### A. Anti-HPV 16 E6 Activity in Crude Extracts of *G. lucidum*

The CaSki cells, treated separately with various concentrations of crude dichloromethane, ethanol, water, and polysaccharide extracts of *G. lucidum* were analyzed for the expression of HPV 16 E6 oncoprotein. The untreated CaSki cells were stained and used as negative controls. The antibody against HPV 16 E6 and subsequent binding with the EnVision™+ system were used to detect the distribution and quantity of E6 oncoprotein *in situ*.[19] The E6 oncoprotein was recorded as present when a reddish-brown stain was clearly observed in the nuclear and/or cytoplasmic regions. The appearance of treated and untreated CaSki cells is shown in Figure 1.

A high intensity of the reddish-brown stain corresponded to a high quantity of E6, whereas lowered or weaker intensity (relative comparison to the untreated) corresponded to lower or suppressed expression of the E6 oncoprotein. Figure 1 shows the highest intensity of brown products in the untreated CaSki cells, which corresponded to abundant expression of HPV 16 E6. In general, a reduction in the intensity of the reddish brown stain was observed in cells treated with *G. lucidum* extracts, suggesting the suppression of the HPV 16 E6 oncoprotein by the extracts. The suppression of the E6 oncoprotein seemed to increase with increased concentrations of the extracts, which suggests that the anti-HPV 16 E6 activity by *G. lucidum* was dose-dependent. When compared, CaSki cells treated with the dichloromethane extract showed the lowest intensity of brown products, suggesting the best HPV 16 E6 suppression activity by this extract. This was followed by water, ethanol, and polysaccharide extracts of *G. lucidum*. It was also observed that cells treated with ethanol, water, and dichloromethane extracts of *G. lucidum* showed enlargement of cellular morphology with increased concentration of the extracts. These observations hold true for all three replicates for the bioassay.

### B. Cytotoxic Effects of Dichloromethane *G. lucidum* Extract

The $IC_{50}$ of dichloromethane *G. lucidum* extract was determined to be 40.02 ± 8.73 µg/mL. Crude extracts that express $IC_{50}$ values of ≤20 µg/mL were deemed as actively cytotoxic.[20] This extract is thus regarded as not actively cytotoxic toward CaSki cell lines, even though there is an increasing trend of inhibition percentage with the increase of treatment concentration with the dichloromethane *G. lucidum* extract (Fig. 2).

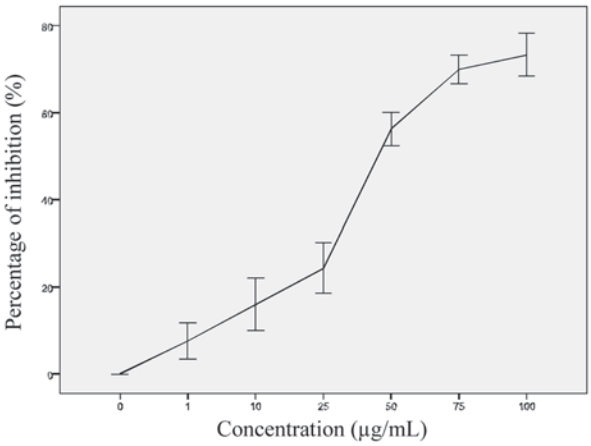


**FIGURE 2.** Concentration of the dichloromethane *Ganoderma lucidum* extract against the percentage of inhibition of CaSki cells. The values were expressed as mean percentage of inhibition ± SEM (N = 3).

### C. Secondary Metabolite Profile of Dichloromethane *G. lucidum* Extract

Having been demonstrated as the most active *G. lucidum* crude extract for anti-HPV 16 E6 activity, the dichloromethane extract was subjected to TLC. TLC profiling was carried out to analyze the group of secondary metabolites present in the extract. A sheet of TLC plate was used to spot the crude dichloromethane extract in six longitudinal lanes (marked with a pencil). Having developed the TLC plate, the plate was cut according to the lanes into six pieces. This process was conducted to avoid ambiguity in corresponding the spots to one another should the six plates be developed separately. Each of the cut longitudinal lanes was then subjected to viewing agents as described in Table 1.

A total of 25 spots were detected from the viewing agents and reagents (Fig. 3). The marked bands showed positive reaction toward all of the TLC viewing agents used. The chemical classes of compounds in the dichloromethane extract of *G. lucidum* were identified as flavonoids, terpenoids, phenolics, and alkaloids (Table 1, Fig. 3).

## IV. DISCUSSION

When it functions normally, the p53 tumor-suppressor protein negatively regulates cell growth. The cell cycle regulation by p53 is disrupted with continuous expression of HPV 16 E6. The E6 protein of high-risk HPV, such as HPV 16, causes p53 to lose its apoptotic function and the infected cervical cells to have increased survivability even if they are genetically unstable.[24] Therefore, protection of p53 is vital, and suppression of the E6 oncoprotein is also important. The current findings show that dichloromethane extract of *G. lucidum* has the ability to suppress the expression of HPV 16 E6 oncoprotein in CaSki cells. However, the mode of action of HPV 16 E6 suppression was not investigated in this study.

It was also observed that as E6 expression decreased, the cellular morphology of CaSki became abnormal (Fig. 1). This finding gave rise as to whether the decrease of E6 expression was caused by the seemingly cytotoxic effect of CaSki cells induced by the *G. lucidum* extracts. CaSki cells were treated with active dichloromethane *G. lucidum* extract to determine if this extract was cytotoxic toward the cells. An $IC_{50}$ value of 40.02 ± 8.73 µg/mL was obtained for treatment of dichloromethane *G. lucidum* extract against CaSki cells. This result showed that the extract was not actively cytotoxic, although there was a potential decrease of E6 expression activity. In addition, previous research has also shown that HPV E6 oncoprotein has anti-apoptotic activity in HPV-positive tumor cells (in which SiHa cells were used), and that the E6 oncoprotein is required for the survival of these tumor cells. Apoptosis occurred when E6 was sup-

**TABLE 1. TLC Spot-viewing Agents with Corresponding Detected Secondary Metabolites**

| Spot-viewing agent | Characteristics of detected compounds |
|---|---|
| $UV_{254}$ | Conjugated double bonds, flavonoids[21] |
| $UV_{365}$ | Fluorescent compounds, flavonoids[21] |
| Iodine | Double bonds[22] |
| Vanillin-sulfuric acid | Terpenoids[23] |
| Ferric (III) chloride | Phenols[17] |
| Dragendorff's reagent | Alkaloids[21] |

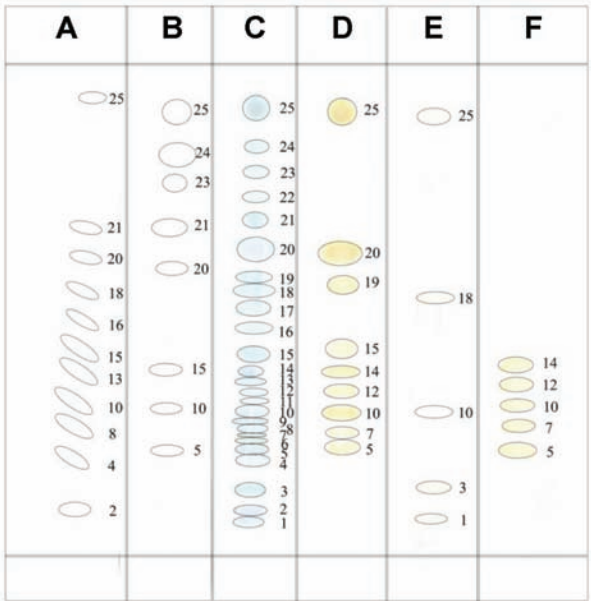


**FIGURE 3.** Depiction of a TLC profile on the dichloromethane extract of *Ganoderma lucidum*. The developed TLC plate was cut into six longitudinal lanes and subjected to $UV_{254}$ exposure (**A**), subjected to $UV_{365}$ exposure (**B**), sprayed with vanillin-sulfuric acid (**C**), exposed to iodine vapor (**D**), sprayed with ferric (III) chloride (**E**), and sprayed with Dragendorff's reagent (**F**). The bands were marked and labeled as shown.

pressed, whereas the SiHa cells showed no apoptotic activity when E6 was not suppressed.[25] In another study by Cho et al.,[26] HPV 16 E6 antisense was used to target E6 suppression. The authors used CaSki cells, which contain 500 to 600 copies of the HPV 16 E6 genome, in an effort to maximize specificity of the E6 suppression effect. Cho et al. found that the suppression of HPV 16 E6 in CaSki cells induced apoptosis.[26] These findings suggest that the E6 decrease is not a result of cytotoxicity, as seen in the abnormal cellular morphology phenomenon (Fig. 1). Findings of a study by Sima et al.[27] indicated that therapies directed against HPV oncoproteins may be effective in the search for therapy against cervical cancer.

Polysaccharides extracted from *G. lucidum* were shown to have anticancer bioactivities, and thus became a group of interest in the screening of anti-HPV 16 E6 activities in the present study. Dichloromethane, ethanol, and water were used as agents to extract compounds from *G. lucidum* to possibly cover a wider spectrum of chemical classes.

TLC performed in this study showed that the dichloromethane *G. lucidum* extract contained flavonoids, terpenoids, phenolics, and alkaloids. This finding corresponds with the review by Gao et al.[28] that states that chemical classes of compounds in *G. lucidum* include terpenoids and other compounds such as polysaccharides, nucleosides, ergosterols, fatty acids, proteins, and trace elements. In a review by Paterson,[29] it was summarized that metabolites of the genus *Ganoderma* consist mainly of terpenoids and polysaccharides. Houghton and Raman[21] stated that the extraction of an organism using dichloromethane could yield alkaloids, aglycones, phenols, and terpenoids.

In their review, Huie and Di[30] stated that pharmacological activities of triterpenoids (a class of the terpenoid group) isolated from *G. lucidum* have been well established. Novel triterpenes of *G. lucidum* exhibited cytotoxic activities in several tumor cell lines *in vitro*, namely, LLC cells and mice tumor cells Sarcoma 180.[31,32] Hajjaj et al.[33] isolated four triterpenoids from *G. lucidum* and found that all four compounds possessed *in vitro* cholesterol-lowering activities. Other studies have shown that triterpenoids of *G. lucidum* possess *in vitro* apoptotic activities in human lung cancer cells 95-D,[34] as well as anti-androgenic activities.[35] Further work must be carried out to determine which of the compounds in the dichloromethane extract of *G. lucidum* were responsible for the

suppression of HPV 16 E6 oncoprotein. To date, no phenolic and alkaloid groups extracted from *G. lucidum* have been reported to be biologically or pharmacologically active.

The dichloromethane extract of *G. lucidum* contains flavonoids, terpenoids, phenolics, and alkaloids, and has shown the highest anti-HPV 16 E6 activity among the crude extracts of the mushroom evaluated in this study. Additional research is needed to investigate the mode of action of HPV 16 E6 suppression. Further isolation of this extract and screening for HPV 16 E6 suppression bioactivity could establish a lead compound to be used in the prevention or treatment of HPV 16-induced cervical cancer.

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# Comparison of Some Metabolites Among Cultured Mycelia of Medicinal Fungus, *Ophiocordyceps sinensis* (Ascomycetes) from Different Geographical Regions

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**ABSTRACT:** *Ophiocordyceps sinensis*, also named Dong Chong Xia Cao, is one of the best-known Chinese medical fungus, with great benefits to human health and huge economical values. In the present study, cultured mycelia were obtained from submerged cultures with 18 authentic and representative strains from different origins on the Tibetan plateau. The contents of the main metabolites, including adenosine, uridine, guanosine, cordycepin, and D-mannitol, were then determined. There was no obvious trend in the amount of nucleosides between the cultured *O. sinensis* mycelia and natural products. Among the tested nucleosides, uridine showed the highest concentration. Cordycepin has been confirmed in submerged cultured mycelia. Natural products have a significantly higher content of D-mannitol compared with the submerged cultured mycelia. Based on the content of nucleosides and D-mannitol, hierarchical clustering analysis was performed. The tested samples were divided into two main clusters for cultured mycelia and natural products, respectively. The cultured mycelia of strains from different regions did not group together, suggesting that sample diversity had a greater effect on metabolite content than did the geographical origin. The results also suggested that strain selection is important when *O. sinensis* is used to manufacture health foods.

**KEY WORDS:** medicinal fungi, medicinal mushrooms, *Ophiocordyceps sinensis*, submerged culture, metabolites, hierarchical clustering, geographical origin

## I. INTRODUCTION

*Ophiocordyceps sinensis* (Berk.) G.H. Sung et al. (=*Cordyceps sinensis* (Berk.) Sacc.), Ophiocordycipitaceae (Ascomycetes) is also known as Chinese Caterpillar Fungus or "Dong Chong Xia Cao" (winter worm, summer grass). *O. sinensis* is an entomogenous fungus that parasitizes larvae of moths (Lepidoptera), especially *Thitarodes armoricanus* (=*Hepialus armoricanus*), and converts each larva into a sclerotium from which the stroma and fruit body grow.[1] The complex used for medicine includes the fungal stroma and the sclerotium.

As one of the most valued traditional Chinese medicines (TCMs), *O. sinensis* is compared with ginseng and deer velvet and is considered to have similar medicinal effects. It has been used in China for hundreds of years from at least the 17th century,[2,3] and has been officially regarded as a recognized drug in the Chinese Pharmacopeia since

## ABBREVIATIONS

**HPLC:** high-performance liquid chromatography; **nrDNA**: nuclear ribosomal DNA; **PCR**: polymerase chain reaction; **PDA**: potato dextrose agar; **RSD**: relative standard deviation; **TCM**: traditional Chinese medicine

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1963.[4] *O. sinensis*, reputed in TCM to replenish the kidney and sooth the lungs, is prescribed for the treatment of a wide range of conditions, including respiratory, liver, and cardiovascular diseases; hyposexuality; and hyperlipidemia (see reviews[5,6]).

*O. sinensis* is endemic to alpine habitats (elevation of 3000–5000 m) on the Tibetan plateau.[7,8] Natural *O. sinensis* is limited and expensive because of its confined geographic distribution and overexploitation. Due to the fact that cultivation of fruiting bodies of *O. sinensis* has not been successfully developed, the submerged fermentation mycelium of this fungus has been proven as a promising way to meet the needs of human consumption and to reduce the ecological pressure on the species, which is in danger.[8]

Many strains have been isolated from natural *O. sinensis*. Twenty-two names in 13 anamorph genera have previously been associated with *O. sinensis*.[7] Some of them have been manufactured in large quantities by fermentation technology such as *Paecilomyces hepiali* Chen et Dai (Jinshuibao capsule produced by Jiangxi Jinshuibao Pharmaceutical Factory), *Mortierella hepiali* Chen et Liu (Zhiling capsule produced by Shanxi Datong Liqun Pharmaceutical Factory), and *Cephalosporium sinensis* Chen (Ningxinbao capsule produced by Shenyang Dongxin Pharmaceutical Factory). However, molecular evidence[9,10] supports only *Hirsutella sinensis* Liu et al. as being the correct anamorph of *O. sinensis*.

Many health care products containing so-called fermented mycelia of *O. sinensis* are available and popular in the market. However, prices of the fermented products differ from natural products by over 100-fold. Whether these products have similar components and pharmacological effects is a question that concerns the consumer.

Much research has been devoted to analyzing the chemical composition and bioactive ingredients of *Ophiocordyceps* species, but no attention has been devoted to comparing the metabolites among cultured mycelia of *O. sinensis* from different regions. Hsu et al.[11] has compared the amino acid profiles, proximate compositions, and contents of adenosine and cordycepin among *O. sinensis*, its counterfeit and mimic forms, and fermented mycelia of *O. sinensis.* The results suggested that adenosine and cordycepin might be used as indexing ingredients for differentiating *Ophiocordyceps* from the counterfeit and mimic forms. More than 10 nucleosides and related compounds have been isolated from *Ophiocordyceps* including adenine, adenosine, uracil, uridine, guanidine, guanosine, hypoxanthine, inosine, thymine, thymidine, and deoxyuridine.[12-17] Among them, adenosine has been used as marker for quality control of *O. sinensis*.[18] Much research has been conducted to determine the content of nucleosides, and the results suggested that cultured *Ophiocordyceps* mycelia contain a much higher concentration than natural *O. sinensis*.[19,20] Hierarchical cluster analysis was performed based on 32 peaks in capillary electrophoresis profiles of natural and cultured *Ophiocordyceps*,[13] and the results showed that natural and cultured samples were in different clusters.

However, the cultured *O. sinensis* mycelia used in these studies were obtained from Jiangxi Jinshuibao Pharmaceutical Factory, Wanfeng Pharmaceutical Factory (Wanfeng), Shanxi Datong Liqun Pharmaceutical Factory, Baoding Pharmaceutical Factory, Yuxi Pharmaceutical Co. Ltd., Yunnan, Wanji Pharmaceutical Co. Ltd., and Shenzhen. Most of the products were apparently not products of a strain of *O. sinensis* (see Jiang and Yao[21] for a review).

Cordycepin, a nucleoside analogue 3′-deoxyadenosine with a broad spectrum of biological activity, was first extracted from *Cordyceps militaris* (L.) Link.[22,23] Many bioactivities of cordycepin have been reported such as anti-inflammation, anti-tumor activity, and inhibition human platelet aggregation.[23–25] However, the existence of cordycepin in natural or cultured mycelium of *O. sinensis* has been controversial in past decades. It was not detected in natural *O. sinensis*[26] nor in cultured mycelia of *O. sinensis*.[17] Li et al.[27] confirmed the existence of cordycepin in natural and cultured *O. sinensis* but the content is beyond the lower limit of linear range, whereas Wang et al. reported that cordycepin is the main active component of *O. sinensis* and has a relatively higher content.[28] Guo et al.[14] reported that the concentrations of cordycepin in *Cordyceps* mycelia are much higher than in natural *O. sinensis*. Therefore, the existence and content of cordycepin, both in natural and cultured mycelia

of *O. sinensis*, need to be ascertained by using authentic materials.

D-mannitol, also called cordycepic acid, was isolated from *O. sinensis* in 1957,[29] and has been shown to have diuretic, antitussive, and anti-free-radical activities.[30] D-mannitol has been used as one of the markers for quality control of many cultured mycelia of *O. sinensis*.[18] However, the study of D-mannitol content has been limited in natural mycelia from different origins,[31] and to date there has been no comparison between cultured mycelia from different origins.

Most of the commercial products manufactured by fermentation technology on the market claim to be derived from natural *O. sinensis* and are sold as products of the fungus. Substitutes such as *C. militaris* have been used, and adulterants also confuse the market. Therefore, quality control of *O. sinensis* and its products is very important to ensure its safety and efficacy. At present, single compounds such as adenosine and D-mannitol are being used as the markers. Unfortunately, these markers are not unique to *O. sinensis* and cannot be used to distinguish authentic *O. sinensis* from other species.[32] Extensive work is still needed to look for rational markers to be used for authentication.

In the present study, cultured mycelia were obtained from submerged culture with 18 authentic and representative strains from different origins on the Tibetan plateau. The contents of the main metabolites, including adenosine, uridine, guanosine, cordycepin, and D-mannitol, were then determined and compared. Based on the results, hierarchical clustering analysis was performed to reveal the correlation between metabolite content and geographical origin.

## II. MATERIALS AND METHODS

### A. Chemicals

Adenosine, cordycepin, cytidine, guanosine, thymidine, uridine, and uracil were purchased from Sigma (St. Louis, MO, USA). Methanol in high-performance liquid chromatography (HPLC) grade was purchased from Tianjin Shield Company (Tianjin, China). Sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate dodecahydrate from Beijing Chemical Reagents Company (Beijing, China); mannitol from Shanghai Zhengxiang Science and Technology Company (Shanghai, China); and sodium periodate from Guangdong Shantou Xilong Chemical Factory (Shantou, China). All reagents were of analytical grade. Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

### B. Natural and Cultured Mycelia of *O. sinensis*

Natural *O. sinensis* was collected from the provinces of Qinghai, Sichuan, Tibet, and Yunnan on the Tibetan plateau. After the soil and plant debris on the fresh specimens were removed using a toothbrush, specimens were dried with silica gel in the field. The identity of these natural *O. sinensis* specimens was determined by careful morphological examination, and the voucher specimens have been deposited at the Herbarium Mycologicum, Academia Sinica (HMAS), Beijing, China. The sample from Xiaojin County, Sichuan Province was also divided into two parts (i.e., sclerotium and stroma). Dried samples were ground to fine powder. The details of the natural specimens used in this study are listed in Table 1.

Cultured mycelia of *O. sinensis* were obtained by submerged culture using strains originally isolated from fresh specimens collected from the major production areas on the Tibetan plateau (Table 2). Soil and plant debris on the fresh specimens were removed by using a toothbrush, and the surface of the specimens was sterilized with 70% ethanol before isolation. The exoskeleton of host larva was peeled off using a scalpel, and small pieces of the inner tissue of sclerotium of *O. sinensis* were inoculated on potato dextrose agar (PDA) supplemented with 5% wheat bran and 0.5% peptone.[33] Pure cultures were obtained by subculturing hyphal tips of primary isolates. The details of strains used in this study are listed in Table 2.

The identity of the strain was confirmed by means of both morphological and molecular methods. The internal transcribed spacer (ITS1-5.8S-ITS2) of nuclear ribosomal DNA (nrDNA) was

**TABLE 1. Natural *Ophiocordyceps sinensis* Used in this Study**

| Location | Average weight per specimen (g) | Elevation (m) | Voucher |
|---|---|---|---|
| Hainan County, Qinghai | 0.236 | 3800 | HMAS 173820 |
| Xiaojin County, Sichuan | 0.273 | 4200 | HMAS 132152 |
| Linzhi County, Tibet | 0.227 | 4600 | HMAS 173829 |
| Baima Snow Mountain, Yunnan | 0.196 | 4050 | HMAS 173808 |

amplified and sequenced from the culture. All of the strains were subjected to DNA extraction, PCR amplification, and sequencing, following the methods of Jiang and Yao.[34] Sequencing was performed using the ABI PRISM Big Dye Terminator Cycle sequencing Ready Reaction Kit (Version 2.0) on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Each fragment was sequenced in both directions for confirmation, and the two strands of sequences were assembled with the SeqScape program (ABI Prism® SeqScape Software™, Version 1.1; Applied Biosystems) and by careful manual examination to eliminate base-calling errors. The sequence of the DNA fragment was compared with a data set generated in this laboratory containing ITS sequences from dried specimens and living strains of *O. sinensis* obtained from various regions of the Tibetan plateau.

The strains were first incubated in the same medium as for the isolation at 18°C for 60 d in Petri dishes. Seed cultures were grown in 500-mL Erlenmeyer flasks containing 100 mL of liquid medium (the same as above, but without agar) inoculated with a 5-mm agar disc from the 60-d culture. The flasks were rotated at 100 rpm at 18°C for 15 d, and 100 mL medium in a 500-mL Erlenmeyer flask was then inoculated with 10 mL of seed culture mycelium and incubated on a rotary shaker at 100 rpm and 18°C for 25 d. All of the strains were incubated under the same conditions in the same medium.

The mycelia were harvested by centrifugation for 15 min at 8000 *g* to separate them from the liquid medium. After repeated washing with distilled water, the mycelial pellets were lyophilized using a VirTis freeze dryer (VirTis Co., Gardiner, NY, USA) for later experiments.

## C. Determination of Nucleosides by HPLC

Accurate amounts of adenosine, uridine, guanosine, and cordycepin were dissolved in 90% methanol solution at 2 mg/mL as a stock solution. A certain amount of the stock solution was transferred to a 10-mL volumetric flask and made up to volume with the same solvent so as to obtain the desired concentration. All solutions were stored at 4°C for 2 weeks.

Samples were extracted by 90% methanol solution using the reflux extraction method at 60°C for 2 h, and filtered through a 0.45-mm filter membrane prior to injection into the HPLC system.

The analysis was performed by reverse-phase HPLC using a Waters system (Waters, Milford, MA, USA) equipped with a 1525 Binary HPLC pump, a 717 autosampler, 2487 Dual Wavelength Absorbance Detector and Waters Breeze System. A pre-packed column Puritex C18 (4.6 × 200 mm, 5-µm particle size; Dalian Elite Analytical Instruments Co. Ltd., Dalian, China) was used. The mobile phase was a mixture of methanol/0.01 M phosphate buffer at pH 6.5 (15: 85). The flow rate was 1 mL/min and the injection volume was 10 µL. The system was operated at 25°C. Nucleosides and their bases were monitored and quantified at 260 nm.

The recovery was performed by adding 120.00, 160.00, and 680.00 µg of adenosine, guanosine, and uridine into an accurately weighed (0.5 g) 762 cultured *Ophiocordyceps* mycelia and 150.00 µg cordycepin into an accurately weighed (0.5 g) 1016 cultured mycelia, respectively. The mixture was extracted and analyzed using the above-mentioned methods. The reproducibility

**TABLE 2. Strains of *Ophiocordyceps sinensis* Used in this Study**

| No. | Origin | Isolation source | Isolation date |
|---|---|---|---|
| 977 | Menyuan County, Qinghai | Sclerotium | 2004-5-28 |
| 992 | Maqin County, Qinghai | Sclerotium | 2004-6-1 |
| 986 | Zaduo County, Qinghai | Sclerotium | 2004-5-29 |
| 974 | Haulong County, Qinghai | Sclerotium | 2004-5-27 |
| 762 | Kangding County, Qinghai | Sclerotium | 2000-6-9 |
| 239 | Xiaojing County, Xichuan | Stroma | 2000-6-1 |
| 880 | Xiaojin County, Sichuan | Sclerotium | 2004-4-30 |
| 882 | Xiaojin County, Sichuan | Sclerotium | 2004-4-30 |
| 246 | Xiaojin County, Sichuan | Sclerotium | 2000-6-2 |
| 291 | Xiangcheng County, Sichuan | Sclerotium | 2001-6-7 |
| 266 | Yulong mountains, Lijiang, Yunnan | Sclerotium | 2001-5-3 |
| 285 | Baima snow-mountains, Yunnan | Sclerotium | 2001-6-4 |
| 286 | Baima snow-mountains, Yunnan | Stroma | 2001-6-4 |
| 295 | Zhongdian County, Yunnan | Sclerotium | 2001-6-8 |
| 961 | Biru County, Tibet | Sclerotium | 2004-6-10 |
| 952 | Linzhi County, Tibet | Sclerotium | 2004-6-2 |
| 958 | Gongbujiangda County, Tibet | Sclerotium | 2004-6-3 |
| 964 | Jiacha County, Tibet | Sclerotium | 2004-6-10 |

(n = 3) was also determined by using 762 or 1016 cultured mycelia.

### D. Determination of D-mannitol Content

The contents of D-mannitol of mycelia of *O. sinensis* were determined using the colorimetric method.[35] In brief, mycelia of *O. sinensis* were extracted with boiled water three times and the filter was combined and condensed for the determination of D-mannitol. One milliliter of the solution containing 0.2 mg of extract and 1 mL of 0.015 M sodium periodate were mixed. After 10 min, 2 mL of 0.1% rhamnose and 4 mL of fresh Nash regent (2 M ammonium acetate mixed with 2 mL acetic acid and 2 mL acetyl acetone) were added to the mixture, which was placed in a water bath at 53°C for 15 min. The absorbance was measured at a wavelength of 412 nm on a Unico-2100 spectrophotometer (Unico, Shanghai, China). A blank test was prepared by substituting distilled water for the extract solution. A standard curve was prepared using a D-mannitol standard. One milliliter of solution containing up to 50 $\mu g/mL^{-1}$ of D-mannitol was determined by the above method, and the D-mannitol content of samples was calculated by the linear regression equation from the standard curve.

### E. Hierarchical Cluster Analysis Based on the Content of D-mannitol and Nucleosides

Before the cluster analysis, the data were autoscaled to mean zero and variance 1, with the aim of assuring that all variables contributed equally to the model.[36]

$$Z_{ij} = \frac{X_{ij} - \overline{X_j}}{S_j}$$

where the $X_{ij}$ is the value of variable J of sample I, $\overline{X_j}$ is the mean value of variable J, and $S_j$ is the standard deviation of variable J.

The hierarchical cluster analysis was carried out using SPSS 11.5 (SPSS Inc., Cary, NC, USA). The between-groups linkage method was applied and the squared Euclidean distance was chosen to measure the distances between clusters.

## III. RESULTS

### A. Strain identification

In addition to the morphological characters in culture, the identity of the strains was confirmed by a molecular method through which the internal transcribed spacer (ITS1-5.8S-ITS2 of nuclear ribosomal DNA) was amplified from the cultures. The sequences of the complete ITS region of the strains were 563 or 555 bp in length, and were compared with the data set containing over 100 ITS sequences of dried specimens and living strains of *O. sinensis* from almost all of the major production areas of the species. The ITS sequences were almost identical and only 0–9 bases were different from that of the CS18 (*O. sinensis*, submitted by this laboratory to GenBank and accessed as AY608925).

### B. Quantitation of Nucleosides

In the HPLC system, four nucleosides (adenosine, cordycepin, guanosine, and uridine) can be separated completely (Fig. 1). The linearity ranges, recovery, and reproducibility for the investigated compounds were reported in Table 3. The correlation coefficient ($r^2 > 0.99$) values indicated good correlations between investigated compound concentrations and their peak areas within the test range. The recoveries and reproducibilities showed that the method used was satisfactory.

The peaks were indentified by comparing their retention times and UV spectra with those obtained on injecting standards under the same conditions, and by spiking *Ophiocordyceps* samples with stock standard solutions.

By using the calibrated curve of each investigated compound, the nucleoside content in natural *O. sinensis* and cultured mycelia was determined (Table 4). There was no obvious trend in the amount of nucleosides when the cultured *O. sinensis* mycelia were compared to natural products. Among the tested nucleosides, uridine showed the highest concentration in both natural and cultured products. Cordycepin was not detected in natural *O. sinensis;* however, it was contained in cultured *O. sinensis*, although the content was beyond the lower limit of linear range in this report. The cultivated fruit body of *C. militaris* showed a much higher concentration of cordycepin. The amount of adenosine in natural *O. sinensis* was greater than 0.01%; however, among cultured mycelia of *O. sinensis* from different regions, the content varied from 0.0051% to 0.0236%.

### C. D-mannitol Content

As for the content of D-mannitol, there was a significant difference among the tested samples ($P < .05$, Table 4). Natural mycelia have significantly higher content when compared with the submerged cultured mycelia. Among cultured mycelia of *O. sinensis* from different regions, the content varied from 1.47% to 10.69%. There was no significant difference among sclerotium and stroma and natural samples from Sichuan, but a significant difference was found between strains 285 and 286, which separated from the sclerotium and stroma of the same specimen ($P < .05$), respectively.

### D. Hierarchical Cluster Analysis

Hierarchical cluster analysis was performed based on the content of nucleosides and D-mannitol of natural and cultured *O. sinensis*. The average-linkage-between-groups method was applied, and the squared Euclidean distance was selected as the form of measurement. Figure 2 shows the results of the tested 24 samples of *O. sinensis*, which are divided into two main clusters. Cultured mycelia of *O. sinensis* are in cluster one, and natural products are in cluster two. However, in cluster one, the

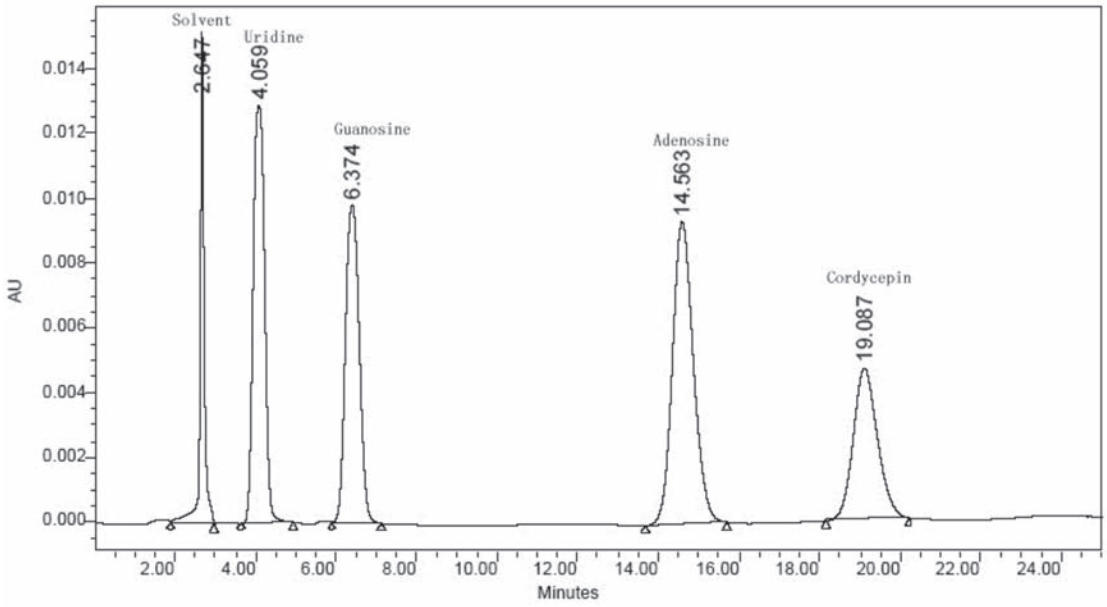


**FIGURE 1.** HPLC chromatographs of mixed standard substances.

**TABLE 3. Linear Regression Equation and Recoveries of Investigated Compounds from *Ophiocordyceps sinensis***

| | Regression data | | | | |
|---|---|---|---|---|---|
| Nucleotide | Linear range ($\mu g/mL^{-1}$) | Regression equation | Correlation coefficient | Recovery (%) | RSD (%)[1] |
| Adenosine | 5–80 | Y = 33259X – 40042 | 0.9985 | 101.80 | 1.06 |
| Cordycepin | 2.5–40 | Y = 40750X – 13730 | 0.9994 | 101.77 | 1.05 |
| Guanosine | 5–80 | Y = 24095X – 4420.2 | 0.9995 | 101.51 | 1.82 |
| Uridine | 5–80 | Y = 25532X – 27056 | 0.9979 | 100.59 | 1.51 |

[1]Relative standard deviation of three replicates.

cultured mycelia of strains from different regions have not grouped together according to their geographic origin.

## IV. DISCUSSION

The use of *O. sinensis* as a medicine and tonic food has been appreciated for hundreds of years in China. Recently, fermented mycelia have been promoted as health foods, functional foods, or nutraceuticals. Much research has been devoted to analyzing the chemical composition and bioactive ingredients of *Ophiocordyceps* species; however, there has been no report, to our knowledge, on the comparison of metabolites among cultured mycelia of *O. sinensis* from different regions. In the present study, the submerged cultured mycelia were obtained from authentic strains from different origins on the Tibetan plateau. The contents of adenosine, uridine, guanosine, cordycepin, and D-mannitol, both in natural and cultured mycelia of *O. sinensis* from different regions, were then determined and compared. The results showed that natural and cultured mycelia of *O. sinensis* could be distinguished on the basis of their nucleosides and D-mannitol content, whereas there was no correlation between the content of metabolites and the geographic origin for the cultured mycelia from different origins.

**TABLE 4. Contents of Nucleotides and D-mannitol of Natural and Cultured Mycelia of *Ophiocordyceps sinensis***

| Samples | | | Adenosine (%) | Uridine (%) | Guanosine (%) | Total content (%) | Cordycepin (%) | Content of D-mannitol (%) |
|---|---|---|---|---|---|---|---|---|
| Natural | Tibet | | 0.0127 | 0.0193 | 0.0037 | 0.0357 | 0 | 15.09 ± 0.32 a |
| | Qinghai | | 0.0201 | 0.0373 | 0.0075 | 0.0469 | 0 | 12.51 ± 0.42 c |
| | Sichuan | | 0.0264 | 0.0567 | 0.0069 | 0.0900 | 0 | 13.52 ± 0.22 b |
| | Sclerotium[2] | | 0.0183 | 0.0495 | 0.0033 | 0.0711 | 0 | 13.36 ± 0.44 b |
| | Stroma | | 0.0599 | 0.0947 | 0.0150 | 0.1696 | 0 | 13.17 ± 0.24 b |
| | Yunnan | | 0.0205 | 0.0228 | 0.0014 | 0.0447 | 0 | 12.72 ± 0.27 c |
| Cultured mycelium | Sichuan | 239 | 0.0162 | 0.1430 | 0.0297 | 0.1889 | Nd[1] | 5.93 ± 0.13 i |
| | | 246 | 0.0093 | 0.0632 | 0.0051 | 0.0776 | Nd | 3.05 ± 0.16 m |
| | | 291 | 0.0196 | 0.0663 | 0.0051 | 0.091 | Nd | 8.78 ± 0.09 e |
| | | 762 | 0.0236 | 0.1359 | 0.0313 | 0.1908 | Nd | 8.71 ± 0.16 e |
| | | 880 | 0.0051 | 0.0956 | 0.0120 | 0.1127 | Nd | 1.78 ± 0.07 n |
| | | 882 | 0.0179 | 0.0413 | 0.0127 | 0.0719 | Nd | 4.81 ± 0.06 jk |
| | Yunnan | 266 | 0.0222 | 0.1664 | 0.0302 | 0.2188 | Nd | 7.83 ± 0.10 fg |
| | | 285 | 0.0110 | 0.1548 | 0.0145 | 0.1803 | Nd | 10.69 ± 0.39 d |
| | | 286 | 0.0074 | 0.0391 | 0.0074 | 0.0539 | Nd | 8.22 ± 0.34 f |
| | | 295 | 0.0199 | 0.1089 | 0.0375 | 0.1663 | Nd | 1.47 ± 0.03 no |
| | Tibet | 958 | 0.0063 | 0.0062 | 0.0051 | 0.0176 | Nd | 6.73 ± 0.23 h |
| | | 952 | 0.0114 | 0.0252 | 0.0067 | 0.0433 | Nd | 7.64 ± 0.28 g |
| | | 961 | 0.0057 | 0.0742 | 0.0021 | 0.082 | Nd | 4.81 ± 0.06 jk |
| | | 964 | 0.0160 | 0.0246 | 0.0019 | 0.0425 | Nd | 6.97 ± 0.28 h |
| | Qinghai | 974 | 0.0128 | 0.0408 | 0.0073 | 0.0609 | Nd | 8.62 ± 0.08 e |
| | | 977 | 0.0121 | 0.0888 | 0.0120 | 0.1129 | Nd | 1.09 ± 0.03 o |
| | | 986 | 0.0202 | 0.0511 | 0.0153 | 0.0866 | Nd | 5.95 ± 0.18 i |
| | | 992 | 0.0055 | 0.0727 | 0.0064 | 0.0846 | Nd | 2.73 ± 0.08 m |
| *C. militaris* | Fruitbody | | 0.0110 | 0.0424 | 0.0119 | 0.0653 | 0.5009 | 4.72 ± 0.09 k |
| | Cultured mycelia of 1016 | | 0.0214 | 0.0346 | 0.0118 | 0.0678 | 0.0299 | 3.83 ± 0.42 l |

[1]Nd indicates that the value is beyond the test limit and cannot be determined accurately.
[2]The samples of sclerotium and stroma are from Xiaojin County, Sichuan Province.

*O. sinensis* is endemic to the Tibetan plateau, including the Tibet, Qinghai, Sichuan, Yunnan, and Gansu provinces in China[7,8] (see Fig. 3A). Much research has been devoted to the separation of strains. In fact, 22 fungal anamorphic (asexual) names, involving 13 genera, were found to be associated with the isolates from *O. sinensis* since the 1980s. At times, they all have been called *O. sinensis*, although many of them have no close link to the species. Extensive fieldwork in vari-

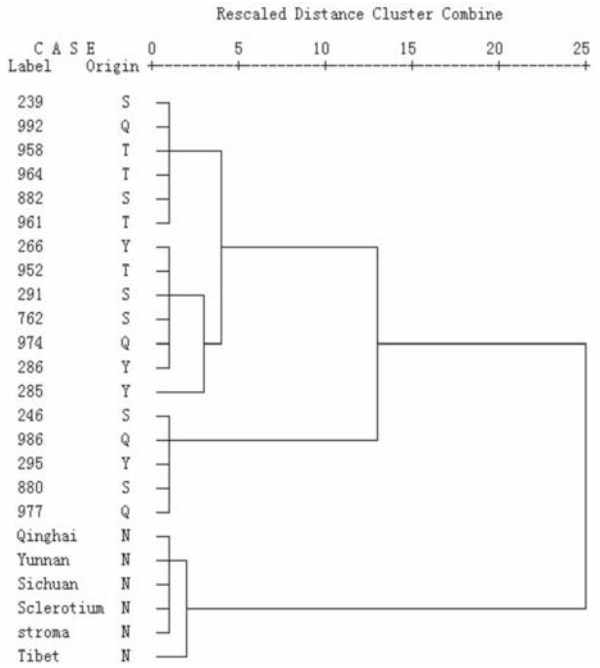


**FIGURE 2.** Cluster dendrogram of cultured and natural *O. sinensis* from different origins based on the content of nucleosides and D-mannitol. The hierarchical cluster analysis was done by SPSS software. A method known as average linkage between groups was applied and the squared Euclidean distance were chosen for the measurement of the distances between clusters. Yunnan, Sichuan, Qinghai, Tibet represent the natural *O. sinensis* from Province of Yunnan, Sichuan, Qinghai, Tibet separately; others are the cultured mycelium. Y, Q, S, T represent the strain from Yunnan, Sichuan, Qinghai, Tibet separately. Sclerotium and stroma are from natural *O. sinensis* of Sichuan. N represents natural *O. sinensis*.

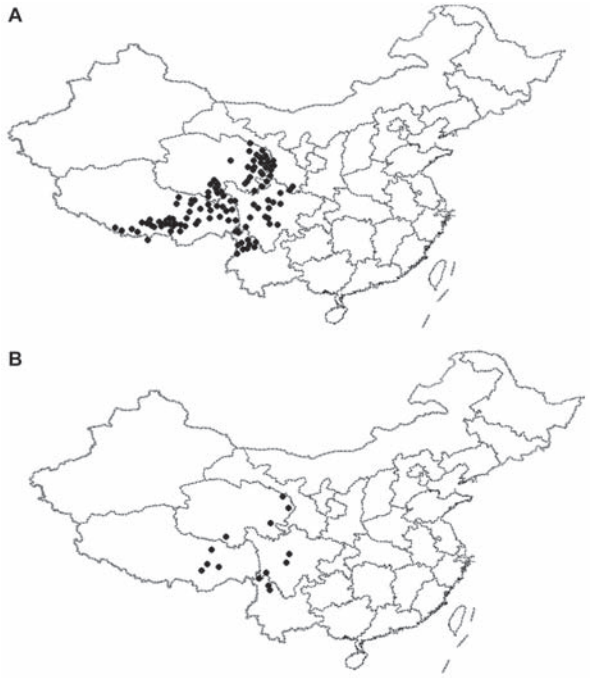


**FIGURE 3.** Distribution of *Ophiocordyceps sinensis* on the Tibet plateau and the origin of the strains used in this study. (A) Distribution of *Ophiocordyceps sinensis* on the Tibetan plateau; (B) the origin of the strains of *O. sinensis* used in this study (the strains from the same county were labeled with one marker).

ous regions on the Tibetan plateau carried out by this laboratory has resulted in a large number of collections of *O. sinensis*, including many living strains. The identity of these strains was confirmed by morphological, physiological, and molecular characteristics, especially the DNA sequence of ITS in nrDNA. A wide selection of strains with their identities confirmed by molecular methods has made the study of metabolites comparison possible.

Eighteen strains used in the study were separated from different geographical origins in the Tibetan plateau (see Fig. 3B). The reliability of geographical origin of the strains used in the study can be guaranteed because all of the species were collected or bought at the location and all of the strains were separated by this laboratory. The identity of the strains was confirmed by both morphological and molecular methods. Most of the strains were separated from the sclerotium. However, there are also strains from sclerotium and stroma, strain numbers 285 and 286, from one specimen. Some of the strains were from different specimens at the same geographical origin. Thus, there is sufficient authenticity and representation for the strains used in this study.

Cultured *Ophiocordyceps* mycelia have been reported to contain a much higher concentration of nucleosides than natural *O. sinensis*.[19,20] In the present study, the same trend was found when the natural mycelia were compared with the commercial submerged mycelia such as the Jinshuibao capsule (produced by the Chinese Medicine Factory of Jiangxi with the strain *Paecilomyces hepiali*) and the Ningxinbao capsule (produced by the Shenyang Dongxin Pharmaceutical Factory with the strain *Cephalosporium sinensis*, data not shown.) However, there was no obvious trend in the amount of nucleosides when the cultured authentic *O. sinensis*

mycelia were compared to natural products in this study. Adenosine is believed to be one of the key active components in *Ophiocordyceps* and content of over 0.01% was the quality-control marker for the natural *O. sinensis* in the Pharmacopoeia of the People's Republic of China. In this study, the content of adenosine was over the marker for all natural samples. However, some of the cultured mycelia had a lower content of adenosine. Perhaps the content of the nucleosides could be increased by optimizing the medium. Cordycepin has been confirmed in submerged cultured mycelia, although the amount was beyond the lower limit of linear range in this report. Cordycepin was not detected in natural *O. sinensis*. The material used in earlier tests that showed cordycepin as the main active ingredient in cultured *O. sinensis*, occurring in concentrations greater than the natural *O. sinensis*, is questionable.

D-mannitol has also been used as a quality control at levels of 7.0% for the Bailing capsule and 6.0% for the Jinshuibao tablet, which are the recommended standards under the auspices of the Ministry of Health, People's Republic of China. In this study, the D-mannitol content in natural *O. sinensis* was much higher than this criterion, but the variability was great among the cultured mycelia of *O. sinensis*. Because a significant difference has been found between the strains isolated from the sclerotium and stroma of the same sample, the effect of sample diversity on D-mannitol content was much greater than the geographical origin. The same trend has been reported as that for the D-mannitol content in natural *O. sinensis* in that the effects of sample diversity and micro-ecological environment might exceed that of district and climate difference.[31]

To reveal the correlation of the natural and cultured *O. sinensis* from different origins, a hierarchical cluster analysis was performed based on the content of nucleosides and D-mannitol. The tested samples of *O. sinensis* were divided into two main clusters and cultured mycelia and natural products were in different clusters, respectively. The fact that cultured mycelia did not group according to the geographical origin has suggested that the effect of sample diversity on the content of metabolites was much greater than the geographical origin. This result also suggests that in the same culture condition, the content of metabolites may be different when strains from different origins are used. Strain selection is important when *O. sinensis* is used in the manufacture of health foods.

## ACKNOWLEDGMENTS

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# Free Radical Scavenging Activity of Culinary-Medicinal Morel Mushrooms, *Morchella* Dill. ex Pers. (Ascomycetes): Relation to Color and Phenol Contents

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**ABSTRACT:** Morels (*Morchella* spp.) are sought-after edible mushrooms encompassing several different species that differ in head color and ecological characteristics. High phenotypic variability, including differences in color, is also found within the species. In the present report, the relationships between antioxidant content as radical-scavenging activity (RSA), phenol content, and color within populations of two *Morchella* species, *M. vulgaris* and *M. esculenta*, were studied. Total RSA measured using the radical DPPH (1,1-diphenyl-2-picrylhydrazyl), total phenols measured after methanolic extraction, and ascocarp darkness measured by chromatometer were correlated. All three parameters exhibited high levels of variability among individual ascocarps within each species population. For both species, RSA correlated well with phenol content ($R^2$ = 0.85 and 0.82 for *M. vulgaris* and *M. esculenta*, respectively). In addition, darker mushrooms within populations of each species had higher RSA and phenol contents than light-colored mushrooms.

**KEY WORDS**: medicinal mushrooms, antioxidant activity, color, DPPH, *Morchella*, phenol, radical-scavenging activity

## I. INTRODUCTION

Epidemiological studies suggest that fruits and vegetables have a protective effect against various diseases,[1,2] and provide recommendations for consumption to promote health.[3] One of the main groups with beneficial activity is the antioxidants. Antioxidants are produced in living organisms to protect against oxidative damage, and include small molecules such as ascorbic acid and tocopherol (vitamins C and E) and enzymes (superoxide dismutase and catalase). Oxidation processes are important in living organisms; however, when unbalanced oxidation occurs (i.e., when high levels of reactive oxygen species [ROS] are produced) these processes may cause damage, termed oxidative stress.[4] Oxidative damage is related to various processes of physiological deterioration in the body and causes a wide range of diseases, as well as aging.[5] In several fruits, color intensity is correlated with antioxidant content: the darker the fruit, the higher the antioxidant level.[6–8] Among antioxidant compounds, the group of phenols has been well documented.[9]

Medicinal mushrooms have also been shown to have high antioxidant content.[10,11] They have

## ABBREVIATIONS

**BHA:** butylated hydroxyanisole; **DPPH:** 1,1-diphenyl-2-picrylhydrazyl; **ROS:** reactive oxygen species; **RSA:** radical-scavenging activity; **TEAC:** trolox-equivalent antioxidant capacity

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long been consumed as an important food source, and they are also used in traditional medicine in Asian countries.[12] Mushrooms are now considered functional foods, and an increasing number of studies have emerged investigating their content of beneficial biologically active compounds. Various medicinal mushrooms have been shown to exhibit anticarcinogenic, anti-inflammatory, immunosuppressor, and antibiotic activities.[13,14]

Morel mushrooms (*Morchella* spp., family Morchellaceae, Operculate Discomycetes, Ascomycota) are known as one of the top culinary species. They are in high demand for their aroma and delicate taste, and have been subjected to determinations of antioxidant content. Antimicrobial, anticancer, and anti-inflammatory activities have been found in different species of *Morchella*.[15–17] In general, there are three main groups of morels, based on head color: black morels, gray morels, and yellow morels, with several different species in each group.[18] However, ascocarps may be highly diverse in appearance (i.e., color, size, and morphology) within populations of a particular species due to environmental and physiological conditions of aging.

From a culinary point of view, no one species dominates the others. There are no reports on potential within-species relationships between the mushroom's nutritional quality and its head color. Such information could be important for both applied and fundamental aspects of the significance of color variation in morel biology.

In the present study, were compared and correlated ascocarp color with antioxidant content measured as radical-scavenging activity (RSA) and phenol content in mushrooms of a *M. vulgaris* (Pers.) Boud. (=*M. conica* Pers.) (black morel) and *M. esculenta* (L.) Pers. (yellow morel).

## II. MATERIALS AND METHODS

### A. Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl), quercetin, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and Folin-Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical-grade methanol was obtained from Gadot (Haifa, Israel), and hydrochloric acid from Bio-Lab (Jerusalem, Israel).

### B. Fungal Material

Ascocarps of the *Morchella* species *M. conica* (black morel) and *M. esculenta* (yellow morel) were collected from their natural habitat in northern Israel. Thirty fruit bodies of *M. vulgaris* and of *M. esculenta* were collected from only one site for each species, representing one population. *M. vulgaris* mushrooms appeared in the spring after a forest fire and the ascocarps were collected during the course of 1 day. *M. esculenta* ascocarps were collected when spotted, throughout the season. *M. vulgaris* ascocarps were usually smaller than *M. esculenta* ascocarps. Each ascocarp was analyzed separately. The heads and stalks of each mushroom were separated and the stalks were discarded. The heads were freeze-dried and pulverized using a mortar and pestle. The powder was kept in dark, dry conditions until analysis.

### C. Ascocarp Color Analysis

The color of each freeze-dried ground ascocarp was measured using a chromatometer (Chroma Meter CR-400, Minolta Co., Osaka, Japan). Color parameters were expressed as tri-stimulus colorimetric measurements (i.e., L*, a*, and b*). A lower L* value indicates darkness and a higher L* value indicates lightness, whereas a positive a* value indicates brown color and a highly positive b* value indicates a more yellow color.[19] The chroma C value, calculated as $C = (a^{*2} + b^{*2})$, indicates color intensity or saturation.[8,20]

### D. Radical-Scavenging Activity

Antioxidant activity was determined as RSA using a modified version of the assay reported by Miller et al.[21] with DPPH as the added free radical. The assay is based on reduction of the purple color of fresh-made DPPH in 50% aqueous methanol solution in the presence of a radical-scavenging

compound. Freeze-dried powdered tissue (5 mg) from each ascocarp was added directly into 40 mL 0.1 mM DPPH in 50% aqueous methanol for RSA determination. The reaction system was incubated in a rotating incubator for 4 h at 30ºC. The reduction in DPPH color in the reaction system was determined by a Jasco (UV-VIS 7800, Tokyo, Japan) spectrophotometer at 517 nm. Absorption values were also measured in DPPH blank solutions before incubation and after 4 h at 30ºC. All tests for each fruit body were carried out in duplicate. A calibration curve was used with Trolox as the standard, in which Trolox (stock solution of 1 mg/mL) was added to the DPPH solution to a final concentration of 2 to 10 ppm.

Mushroom RSA (antioxidant content) was determined as the activity responsible for reducing the active free radical DPPH, and calculated as follows:

$$\frac{Blank_{trolox} - OD_{trolox}}{C_{trolox}} = K_{trolox} \quad (1)$$

In Equation (1), $K_{trolox}$ is the calibration constant in OD/ppm Trolox units. $Blank_{trolox}$ is the absorbance of the solution containing all of the compounds except Trolox in the calibration curve. $OD_{trolox}$ is the absorbance of the reaction solution with different Trolox concentrations. ($C_{trolox}$) served as the standard.

$$\frac{Blank_{trolox} - OD_{trolox}}{K_{trolox}} = M_{antioxidant} \quad (2)$$

In Equation (2), $K_{trolox}$ is the calibration constant in OD/ppm Trolox units, retrieved from Equation (1). $M_{antioxidant}$ is the antioxidant content in the mushroom. $Blank_{trolox}$ is the absorbance of the solution containing all the components without added antioxidant after 4 h incubation at 30ºC. $OD_{trolox}$ is the absorbance of the reaction solution containing powdered mushroom tissue after 4 h of incubation at 30ºC. $M_{antioxidant}$ presented mushroom antioxidant activity in terms of Trolox units (ppm), and then data were transformed to milligram Trolox equivalents per gram of dry mushroom weight (mg TE/g DW).

### E. Phenol Content

The content of total phenols was determined in the supernatant fraction of freeze-dried ground ascocarp tissue extracted with acidified methanol (HCl:methanol:water 1:80:10, v/v) for 2 h at room temperature by vigorous shaking (stirrer), using the Folin-Ciocalteu method.[22] The phenol content was determined for each mushroom separately. All tests were carried out in duplicate. Quercetin was used for the standard calibration curve, and total phenol content was determined as milligram quercetin equivalents per gram of dry mushroom weight (mg QE/g DW).

### F. Statistical Analyses

All analyses were performed in 30 independent repetitions, each with two replicates. Each repetition is the results of two average replicates with relative standard deviation (RSD) > 10. The mean and standard deviation (SD) values of the color parameters, antioxidants, and phenols (in the two distinct morel populations, *M. vulgaris* and *M. esculenta*) were calculated from the measurements of all repetitions for ascocarps in each population. Means were compared for each pair of data using the Student's *t* test. Variance analysis was obtained by JMP 5 statistical discovery software (SAS Institute Inc., Cary, NC, USA).

## III. RESULTS

### A. Color Properties

Matured fruit bodies of *M. vulgaris* and *M. esculenta* differ in head color, the former surface being basically black and the latter being basically yellow. However, both species exhibit a range of colors within each population. In general, *M. vulagris* ascocarps had a dark outer tissue and a white inner tissue with a white stalk, but outer color could range from black to gray to white (Fig. 1). Similar to *M. vulgaris* ascocarps, in *M. esculenta* ascocarps the outer tissue was darker than the inner tissue. Ascocarps of the *M. esculenta* population were found in a range of colors as well: from dark brown and gray to dark and bright yellow and beige, with the colored stalk being either white or similar to its head color. The different colors of the ascocarps

**FIGURE 1.** Color variations between *M. vulgaris* ascocarps picked on the same day at the same site.

**FIGURE 2.** Color variations in freeze-dried, ground ascocarps within the *M. esculenta* population.

were stable after freeze-drying and grinding, as illustrated in Figure 2, which shows the high variability in color of the individual ascocarps within each of the two populations.

The color characteristics were measured by a chromometer. Color parameters a*, b*, and C differed significantly between the studied *M. esculenta* and *M vulgaris* ascocarps, that is, mushrooms were more reddish-brown and yellow and had a stronger color intensity in the *M. esculenta* population than in *M. vulgaris*, as noted by visual observation. A large range of color values was documented for ascocarps within each of the populations for all color parameters (Table 1). Dark- and light-colored mushrooms from both populations were subjected to molecular study to avoid errors in species identification, and were shown to have the same internal transcribed spacer (ITS) rDNA region sequence (data not shown)

## B. Phenol and RSA

The average phenol and antioxidant contents in the analyzed ascocarps were higher in *M. esculenta* than in *M. vulgaris* (Fig. 3, Table 2), but not significantly. Phenols reached an average level of 14.4 mg and 12.0 QE/g DW in *M. esculenta* and *M. vulgaris*, respectively. The average antioxidant content was 27.9 mg and 24.1 TE/g DW for *M. esculenta* and *M. vulgaris*, respectively. However, as with the color measurements, phenol and antioxidant levels exhibited a wide range of values among mushrooms within each species population (antioxidants: 13.5–55.7 and 11–48 mg TE/g DW for *M. vulgaris* and *M. esculenta*, respectively; phenols: 6.3–28.5 and 7.3–23.7 mg QE/g DW for *M. vulgaris* and *M. esculenta*, respectively) (Figs. 3 and 4). For both species, a positive correlation was found between phenol and antioxidant contents ($R^2$ = 0.85 and 0.82 for *M. vulgaris* and *M. esculenta*, respectively) (Fig. 4, Table 2).

## C. Relationships Between RSA, Phenol Contents, and Color

The relationships between RSA or phenol contents and color were examined in the *M. esculenta* and

**TABLE 1. Average Values and Range of Color Parameters L*, a*, b*, and C for the Two Different Populations of *M. vulgaris* and *M. esculenta.***

| Species | Color parameters (Mean ± SD) | | | |
|---|---|---|---|---|
| | L* | a* | b* | C |
| *M. vulgaris* | 60.33 ± 8.8A | 2.46 ± 1.08A | 13.15 ± 2.86A | 13.44 ± 2.75A |
| *M. esculenta* | 58.23 ± 9.37A | 5.14 ± 2.10B | 15.78 ± 4.58B | 18.58 ± 4.39B |

Values for the same parameters followed by different uppercase letters indicate significant differences ($P < .05$). Data expressed as mean ± standard deviation (SD). Data were analyzed by JMP 5 statistical discovery software. Means were compared for each pair of data using the Student's *t* test.

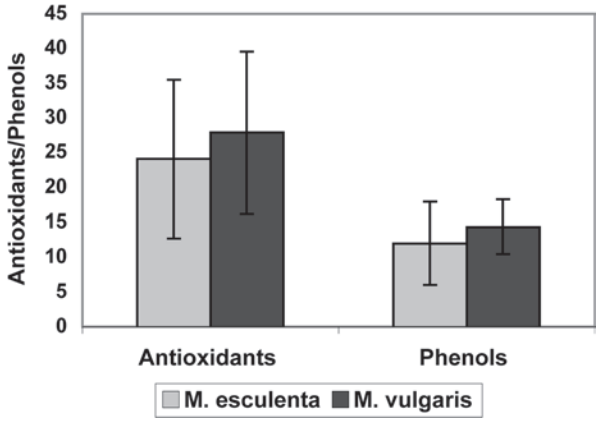


**FIGURE 3.** Average values of total antioxidants and total phenols for the two different populations (*M. vulgaris* and *M. esculenta*). Values are mean ± SD. Similar uppercase letters for the same parameters indicate no significant differences ($P < .05$). Data expressed as mean ± SD. TE, Trolox equivalents; QE, quercetin equivalents.

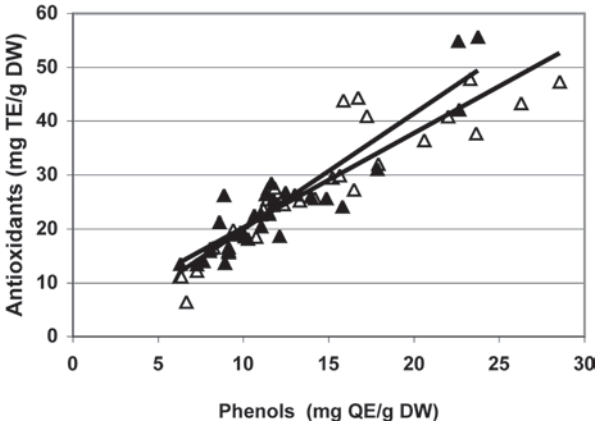


**FIGURE 4.** Correlation between antioxidant and phenol contents in mushrooms belonging to *M. esculenta* (▲) and *M. vulgaris* (Δ) populations. Data represent individual ascocarp heads. TE, Trolox equivalents; QE, quercetin equivalents.

**TABLE 2. Statistic Parameters Including Variance Analysis of the Correlation Studies**

| Species | Dependent parameter | Independent parameter | $R^2$ | Prob>F | Mean of response | Equation |
|---|---|---|---|---|---|---|
| *M. vulgaris* | Antioxidants | Phenols | 0.85 | .0001 | 24.1 | Y = –1.56 + 2.14X |
| *M. esculenta* | Antioxidants | Phenols | 0.82 | .0001 | 27.9 | Y = 2.82 + 1.74X |
| *M. vulgaris* | Antioxidants | Color L | 0.58 | .0001 | 24.1 | Y = 77.60 – 0.88X |
| *M. esculenta* | Antioxidants | Color L | 0.67 | .0001 | 27.9 | Y = 86.55 – 1.01X |
| *M. vulgaris* | Phenols | Color L | 0.73 | .0001 | 12.0 | Y = 37.96 – 0.42X |
| *M. esculenta* | Phenols | Color L | 0.57 | .0001 | 14.4 | Y = 42.45 – 0.48X |
| *M. vulgaris* | Antioxidants | Color C | 0.45 | .0001 | 24.1 | Y = 56.98 – 2.45X |
| *M. esculenta* | Antioxidants | Color C | 0.45 | .0001 | 27.9 | Y = 60.78 – 1.77X |
| *M. vulgaris* | Phenols | Color C | 0.46 | .0001 | 12.0 | Y = 26.43 – 1.08X |
| *M. esculenta* | Phenols | Color C | 0.56 | .0001 | 14.4 | Y = 33.43 – 1.02X |

Linear fit analyses were obtained by JMP 5 Statistical Discovery Software.

*M. conica* populations. A moderate opposite correlation was found between lightness (L parameter) and antioxidant and phenol contents in both species (Figs. 5A and 5B, Table 2). The *M. conica* population exhibited a better correlation than the *M. esculenta* population for antioxidants ($R^2$ = 0.058 and 0.67 for *M. esculenta* and *M. vulgaris*, respectively), whereas *M. esculenta* exhibited better correlation for phenols ($R^2$ = 0.73 and 0.57 for *M. esculenta* and *M. vulgaris*, respectively). Moderate correlations were also obtained for total antioxidant and phenol contents in relation to the C parameter (indicating intensity), with similar correlation for both species for antioxidants ($R^2$ = 0.4512 and 0.4476 for *M. esculenta* and *M. vulgaris*, respectively), with *M. esculenta* exhibiting better correlation for phenols ($R^2$ = 0.56 and 0.46 for *M. esculenta* and *M. vulgaris*, respectively) (Fig. 6, Table 2).

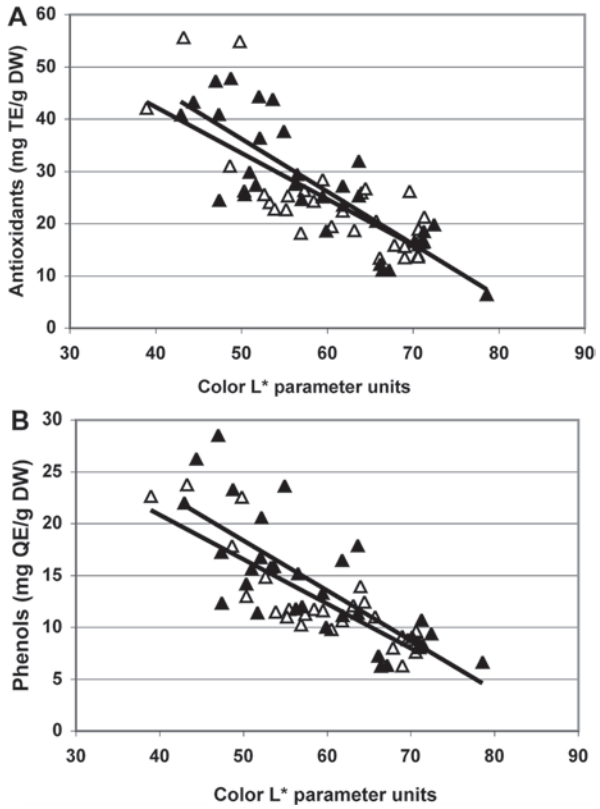


**FIGURE 5**: Relationships between total antioxidants (**A**) or total phenols (**B**) and L* (color parameter indicating darkness) in mushrooms belonging to *M. esculenta* (▲) and *M. vulgaris* (∆) populations. Lower L* parameter value indicates a darker mushroom. Data represent individual ascocarp heads. TE, Trolox equivalents; QE, quercetin equivalents.

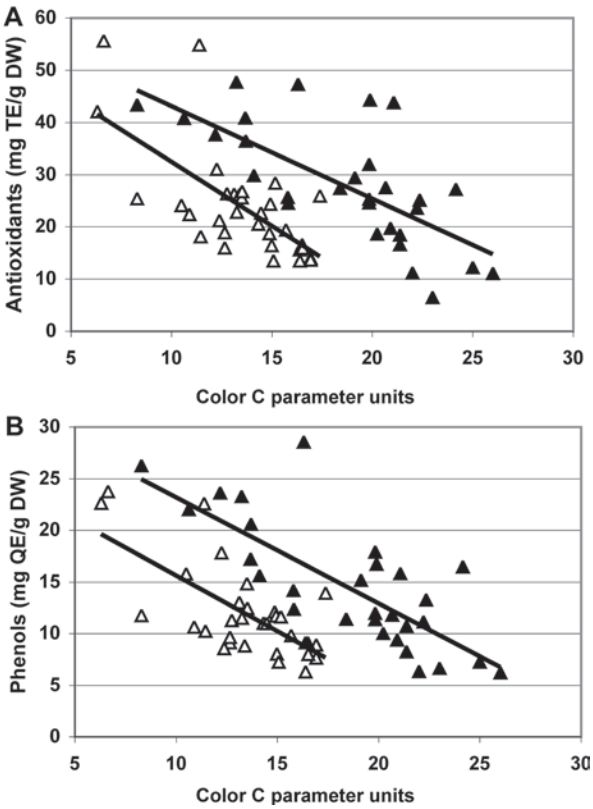


**FIGURE 6**: Relationships between total antioxidants (**A**) or total phenols (**B**) and C values (color parameter indicating color intensity) in mushrooms belonging to *M. esculenta* (▲) and *M. vulgaris* (∆) populations. Data represent individual ascocarp heads. TE, Trolox equivalents; QE, quercetin equivalents.

## IV. DISCUSSION

In the present study, antioxidant (as represented by RSA) and phenol contents were found to correlate with color in morel populations from two different species, *M. vulgaris* and *M. esculenta*: the darker the mushroom head, the higher the antioxidant and phenol contents, especially in *M. esculenta*.

Morels exhibit high color variation, both between and within species. The differences within a species are related to various environmental (soil, temperature) and physiological conditions. In fruits and vegetables, color appearance is also an important feature that indicates their physiological condition and determines their attractiveness.[8] Moreover, in some cases, antioxidant content is correlated to color darkness in fruits and vegetables. For example, berries (especially dark blue or red-colored berries) are known to exhibit high antioxidant activity; the same has been found for the darker fig varieties,[6,8,23,24] as well as red beans.[7] In plant material, pigmentation is the outcome of anthocyanin, chlorophyll, and carotenoid contents.[8]

In mushrooms, especially in most edible ones, earthy colors dominate, running the gamut from white to black, with yellow, reddish-brown, and gray. These colors are accompanied by different oxidation levels of phenolic compounds, from simple soluble phenol to dark insoluble melanin. The phenols change color mainly due to activation by oxidizing enzymes such as tyrosinase.[25–28] Color also serves to distinguish between species. In the *Morchella* genus, head color is a very important characteristic for distinguishing between the three main morel groups: black, yellow,

and gray. In the yellow group, the dominant species is *M. esculenta*, whereas the major black morel species is *M. vulgaris.* Yet, within these three groups, species distinction and identification are not at all clear-cut due to the high phenotypic variation within species, including a high variability in color. Each species (and each ecotype) and each population within a species can exhibit high phenotypic variation (the color range may vary widely, from white to black, in the same population). The mean darkness level of freeze-dried ground head tissues of both species was similar. Nevertheless, the other measured color parameters (a*, b*, and C) were higher in *M. esculenta* than in *M. vulgaris*, indicating stronger color intensity, and more brown and yellow. Measuring color parameters by the chromatometer is a widely used method to analyze the color appearance of fruits, vegetables, and powders, mainly for quality analyses, including the detection of fruit tissue browning.[19,29] In morels, the head tissue is honeycomb-like, making it difficult to measure its color in the whole fresh fruit body. Freeze-drying and grinding the tissue and measuring the powder color enabled a color determination of the whole head tissue.

Different methods can be found in the literature to determine the antioxidant content of fruits and vegetables. The different methods generate different data on these levels in different food sources, including mushrooms.[10] To determine total antioxidant content in morel mushrooms, we used the DPPH method, which analyzes the RSA of the whole tissue (antioxidant activity) without the need for its prior extraction. The use of ground whole tissue facilitated the interaction between DPPH and the different antioxidant molecules, as has been noted in a study by Miller et al.[21] in which the authors applied the grinding technique to examine antioxidant content in whole breakfast cereal grains using 50% methanolic DPPH solution. Using this method, both soluble and non-soluble compounds were in contact with the DPPH, and both contributed to the reduction in DPPH color due to electron-scavenging activity. Antioxidant compounds may be water-soluble, lipid-soluble, insoluble, or bound to the cell wall; in some studies, including those on mushrooms and particularly on *Morchella*, the antioxidant content of the mushrooms is determined by water or alcohol extraction.[17] Such extractions can only measure part of the antioxidant activity in the mushroom, that is, only that of the extracted compounds.

No significant difference was found between *M. vulgaris* and *M. esculenta* populations with respect to mean values of antioxidants and phenols when antioxidant activity was determined in the whole freeze-dried head tissue and phenol content in acidic methanolic extract. No difference in antioxidants and phenols content in other *Morchella* species, *M. vulgaris* and *M. esculenta*, was reported in an earlier work by Elmastas et al.[15] In their work, the antioxidant content was measured in the ethanolic extracts (extracted from air-dried mushrooms), resulting in 16.9 µg and 18 µg in 1 mg of ethanolic extract for *M. esculenta* and *M. vulgaris*, respectively. They did not report on the recovery of the ethanolic extracts and therefore their results cannot be compared to ours. In another study, Turkoglu et al.[17] determined antioxidant levels in an ethanolic extract of *M. vulgaris* to be 160 µg/mL. The ethanolic extract exhibited an antioxidant activity similar to that of 80 µg/mL butylated hydroxyanisole (BHA) measured by DPPH-degradation assay. No data on the extraction recovery were provided.

In addition to the positive correlation between ascocarp darkness and antioxidant content, a positive correlation was found in the current study between total antioxidants and total acidic methanol-extracted phenols ($R^2$ values of 0.82 and 0.85 for *M. vulgaris* and *M. esculenta*, respectively). These values are lower than the correlation found between total antioxidants (measured as TEAC [Trolox-equivalent antioxidant capacity]) and polyphenols measured in alcoholic extracts of fig, which reached 0.99.[8] The results thus indicate that other non-extracted or non-phenolic antioxidants are present in mushroom tissue, reacting with DPPH in the reaction system. This should be considered when determining the mushroom's total antioxidant activity for consumption as a food source.

Mushrooms, including species of genus *Morchella*, have high antioxidant contents. We suggest that within each *Morchella* population, color will be correlated with the level of whole-tissue antioxidant and extractable phenol contents. It should be noted that our study examined morel

tissue before cooking. *Morchella* mushrooms must be cooked before consumption and changes in antioxidant content may occur. A study will be carried out to address this aspect, as well as to further understand the role of the changes in antioxidant levels in the *Morchella* species life cycle and their effect on human health after consumption.

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# Evaluation of Alternate Substrate Pasteurization Techniques for Culinary-Medicinal White Oyster Mushroom, *Pleurotus ostreatus* var. *florida* (Agaricomycetideae) Cultivation

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**ABSTRACT:** Three traditional substrate pasteurization methods (chemical, steam, and hot water) and seven alternate methods (solarization, botanicals, and sanitizers) were evaluated for the cultivation of culinary-medicinal white oyster mushroom *Pleurotus ostreatus* var. *florida*. The investigation was undertaken to scientifically evaluate current methods and to educate farmers to adopt appropriate methods that will not only prevent contamination and give optimal yields, but that will also be eco-friendly and pose no health hazards to the workers. Steam pasteurization (80°C, 2 h) was found to be the most efficient method and showed the highest biological efficiency (BE) at 82.8%, followed by the hot water method (80°C, 60 min) with BE at 77.6%. The chemical sterilization technique (500 ppm formaldehyde + 75 ppm carbendazim-Bavistin) showed 49.6% BE and solarization 34.7% BE. All other treatments showed low BE. The hot-water, chemical sterilization, and solarization techniques were the most effective in preventing competitor molds.

**KEY WORDS:** culinary-medicinal mushrooms, white oyster mushroom, *Pleurotus ostreatus* var. *florida,* cultivation

## I. INTRODUCTION

Oyster mushrooms (*Pleurotus* spp.) are one of the most popular and suitable cultivated culinary-medicinal mushroom varieties grown in India. These mushrooms have carved a global niche due to their high nutritional value, pleasant taste, and medicinal value. Cultivation technology is relatively simple and the materials used as substrate are cost-effective. Oyster mushrooms can be cultivated on a wide range of lignocellulosic materials. Substrate pasteurization is one of the most critical steps during cultivation to diminish the damage caused by different pathogens (bacteria, molds, or pests) on mushroom development and yield. Different methods of pasteurization have been recommended over time.[1-3] The most common method of substrate pasteurization in India is hot water treatment. However, the energy input required and the method's labor-intensive nature have motivated growers to adopt the chemical sterilization technique as evolved by Vijay and Sohi.[4] Although it is easy to use, this

## ABBREVIATIONS

**ANOVA:** analysis of variance; **BE:** biological efficiency; **CD:** critical difference; **CRD:** complete random design; **CV%:** percent co-efficient of variance; **GBAG:** garlic barrier treatment; **SEd:** standard error of the difference

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technique has numerous drawbacks, some of which include: 1) the indiscriminate use of carcinogenic chemicals, such as formaldehyde, that can have ill effects on the health of workers who will be exposed to its fumes on daily basis; 2) the development of resistance among the competitor molds due to repeated carbendazim use; 3) the residue problem in mushrooms and its ill effect on consumer health; and 4) environmental pollution.

The current investigation was undertaken to evaluate the performance of three conventional methods of pasteurization (hot water, chemical, and steam), and to evolve some alternate methods of pasteurization that would be easy, cost-effective, safe, and eco-friendly. Investigations were undertaken incorporating 10 different treatments that were composed of botanicals, solar energy, and sanitizers. A case study was also undertaken on an oyster mushroom farm in the Mysore district in Karnataka state (M/s Mayaleya Mushroom, 2951 Temple Road, V. V. Mohalla, Mysore, 570002) to comparatively study chemical versus hot water pasteurization methods.

## II. MATERIALS AND METHODS

### A. Mushroom Materials

The experiments were conducted by using the white oyster mushroom *Pleurotus ostreatus* var. *florida* Eger (Strain PAU-05 from the culture collection of Department of Microbiology, Punjab Agricultural University, Ludhiana, India) as the cultivated species and paddy straw as the substrate.

### B. Substrate Preparation

Chopped paddy straw (30–60 mm in length, 1 kg of wet straw per bag) was used as substrate. A spawn dose (grain spawn prepared on sorghum) of 4% (by the straw wet weight) was used. The incubation temperature for spawn running was 25 ± 2°C. The environmental conditions during cropping were 25 ± 2°C temperature, 75% ± 5% humidity, and 8 h fluorescent light. The number of bags in each treatment varied from 9 to 20 (Table 1). The moisture content of the substrate varied from 65% to 78% in various treatments. The various pasteurization techniques used were as follows.

1. *Hot water pasteurization:* The wet straw was soaked in hot water (80 ± 2°C) for 60 min. After cooling and removal of excess water, the straw was filled in polypropylene (PP) bags (150-gauze thick, 300 × 355 mm) and spawned in a clean, closed bag filling and spawning room.
2. *Steam pasteurization*: Wet straw was pasteurized by steam at 80 ± 2°C for 2 h in an autoclave.
3. *Chemical sterilization technique*: Paddy straw was soaked for 18 h in an aqueous solution containing 75 ppm bavistin and 500 ppm formaldehyde.
4. *Bleaching powder treatment:* Paddy straw was soaked for 12 h in solution containing bleaching powder (commercial bleaching powder containing 33% chlorine) at 2%, 4%, 6%, 8%, and 10% (w/v) concentration.
5. *Hydrogen peroxide treatment:* Hydrogen peroxide solution at 1.5%, 3.0%, 6.0% and 8.0% (v/v) was used for soaking straw for 12 h.
6. *Neem cake:* Straw was soaked in 2%, 4%, and 8 % (w/v) neem-cake suspension.
7. *Onion-garlic treatment:* To yield a solution, 1 kg of onion and 0.5 kg of garlic were ground as a paste and soaked in 10 L of water. This solution was used for soaking straw at concentrations of 1%, 3%, and 5% (v/v).
8. *Garlic barrier treatment:* Garlic barrier treatment (GBAG) is a commercial insect repellent with a composition of 77% garlic oil and 22% neem oil (Garlic Research Labs Inc., Glendale, CA, USA; supplied in India by Veera Exim and Sales, Bangalore, India), and was used at a concentration of 0.33% for soaking straw.
9. *Protasan-DS treatment:* Protasan-DS treatment is a disinfectant composed of 11.5% benzalkonium chloride (Qualigens Fine Chemicals Division, GlaxoSmithKline Pharmaceuticals Limited, Mumbai, India), and is known to posses bactericidal, spori-

**TABLE 1. Incidence of Contamination in Various Pasteurization Techniques**

| Treatment | No. of inoculated bags | No. of contaminated bags | Extent of contamination | Contaminating agent |
|---|---|---|---|---|
| Hot water | 12 | NIL | NIL | NIL |
| Steam | 20 | 5 | 3 bags: 5%; 2 bags: 15% | *Trichoderma* and *Coprinus* |
| Chemical | 14 | NIL | NIL | NIL |
| Solarization | 14 | NIL | NIL | NIL |
| Bleaching powder | | | | |
| 2% | 11 | 11 | 2 bags: 15%; 9 bags: >50%* | *Trichoderma* |
| 4% | 11 | 11 | 8 bags: 5%; 1 bag: 25%; 2 bags: >50%* | *Trichoderma* and *Chaetomium* |
| 6% | 12 | 10 | 1 bag: 5%; 4 bags: 15%; 2 bags: 25%; 3 bags: 50% | *Trichoderma* and *Chaetomium* |
| 8% | 9 | 9 | 7 bags: 5%; 1 bag: 15%; 1 bag: 25% | *Trichoderma* and *Chaetomium* |
| 10% | 15 | 13 | 8 bags: 5%; 2 bags: 15%; 2 bags: 25%; 1 bag: >50%* | *Trichoderma* and *Chaetomium* |
| Hydrogen peroxide | | | | |
| 1.5% | 15 | 15 | 15 bags: >50 %* | *Trichoderma* |
| 3% | 15 | 7 | 1 bag: 5%; 1 bag: 15%; 2 bags: 25%; 3 bags: >50%* | *Trichoderma* |
| 6.0% | 15 | 7 | 4 bags: 5%; 1 bag: 50%; 2 bags: >50%* | *Trichoderma* |
| 8% | 15 | 4 | 3 bags: 5%; 1 bag: 50% | *Trichoderma* and *Chaetomium* |
| Neem cake | | | | |
| 2% | 15 | 12 | 3 bags: 5%; 3 bags: 15%; 4 bags: 25%; 2 bags: >50%* | *Trichoderma* |
| 4% | 15 | 3 | 3 bags: 5% | *Trichoderma* |
| 8% | 15 | 13 | 1 bag: 5%; 1 bag: 15%; 2 bags: 25%; 9 bags: >50%* | *Trichoderma* and *Chaetomium* |
| Onion-garlic solution | | | | |
| 2.5% | 15 | 8 | 3 bags: 5%; 1 bag: 15%; 4 bags: >50%* | *Trichoderma* |
| 7.5% | 12 | 6 | 3 bags: 5%; 1 bag: 25%; 2 bags: >50%* | *Trichoderma* |
| 12.5% | 11 | 10 | 2 bags: 5%; 1 bag: 15%; 7 bags: >50%* | *Trichoderma* |
| BGAG | | | | |
| 0.33% | 12 | 10 | 1 bag: 5%; 9 bags: >50%* | *Trichoderma* |
| Protasan-DS | | | | |
| 1.25% | 14 | 14 | 14 bags: >50%* | *Coprinus* |

cidal, and fungicidal properties. Protasan-DC was used at a concentration of 1.25% (v/v) for soaking straw.

10. *Solarization:* Wet paddy straw (25 kg) was spread on black polythene to a depth of 10 cm, and the top was covered with another transparent polythene sheet under sun exposure. The straw temperature was monitored for 1 day at hourly intervals from 9 A.M. to 4 P.M. The next day, the transparent polythene was removed and the straw was dried to the requisite moisture level, bagged, and spawned.

The major data recorded from all of these treatments included spawn running, contamination incidence, and yield.

### C. Scale of Measuring Contamination

The scale of contamination was measured by using the following formula:

$$\frac{\pi d^2}{2} + \pi d h$$

where d is equal to the diameter and h is equal to height.

The spawn run blocks were cylindrical. The formula of total surface area of cylinder was used with the following data:

- Diameter of the cylinder = 16.5 cm
- Height of the cylinder = 11 cm

Hence, the total surface area of the spawn run block was 997.3 $cm^2$.

The percentages of contaminated patches were scaled as follows:

- 1%–5% = 9.97-$cm^2$ to 49.85-$cm^2$ patch
- 6%–15% = 59.82-$cm^2$ to 149.55-$cm^2$ patch
- 16%–25% = 159.52-$cm^2$ to 249.22-$cm^2$ patch
- 26%–50% = 259.25-$cm^2$ to 498.50-$cm^2$ patch
- >50% = >498.50-$cm^2$ patch

## III. RESULTS AND DISCUSSION

As shown in Table 1, the hot water, solarization, and chemical sterilization techniques performed better in terms of prevention of competitors (0% contamination) as compared with other treatments. Protasan-DS and hydrogen peroxide (1.5%) resulted in complete contamination of all of the bags. The other treatments showed variable degrees of contaminated patches in the spawn run blocks. The chemical sterilization technique, which involves the use of formaldehyde and bavistin, could control competitors such as *Trichoderma harzianum* but was ineffective against *Coprinus comatus*, the spores of which are commonly found on paddy straw. In fact, contamination due to *C. comatus* increased many fold, sometimes leading to 100% losses in many of the farms that practiced the chemical sterilization technique. It is well known that most of the common fungal spores and bacteria do not survive temperatures greater than 50°C to 60°C. During hot water pasteurization, water at 80°C remains in direct contact with the substrate for 40 to 60 min. This results in killing all contaminants on the substrate as effectively as techniques that involve selective action by chemicals.

As shown in Table 2, the yields in the steam and hot water methods were significantly higher (233.00, 189.50 g/kg wet substrate, respectively) compared with the chemical sterilization technique (129.50 g/kg wet substrate). The yield in the chemical sterilization technique decreased by 44.42% compared with the steam pasteurization method and by 31.66% compared with the hot water method. Hot water and steam pasteurization aid in softening the straw, which helps in easier colonization and leads to better yield. In the steam pasteurization method, 25% of the bags showed contamination to an extent of 5% to 15% of the patch. However, the yield of contaminated bags in which the steam pasteurization technique was used (Table 3) was at par (157.0 g/kg wet substrate) compared with the yield of uncontaminated bags in which the chemical sterilization, hot water, or solarization techniques (129.50, 189.50, and 128.50 g/kg wet substrate, respectively) were used.

The chemical sterilization technique was first standardized by the National Research Center of Mushrooms in Solan, India,[4] with the objective of simplifying substrate pasteurization techniques for oyster mushroom cultivation. In the current study, the solarization, hot water, and chemical sterilization methods could effectively control contaminants. Various methods such as acid and alkali hydrolysis,

**TABLE 2. Effect of Different Pasteurization Methods on Yield of *Pleurotus ostreatus var. florida***

| Treatment | Concentration | No. of days for complete spawn run | Average yield (g/1 kg wet substrate) |
|---|---|---|---|
| Hot water | - | 8 | 189.50** |
| Steam | - | 9 | 233.00** |
| Chemical sterlization | - | 9 | 129.50* |
| Solarization | - | 13 | 128.50* |
| Bleaching powder | 2.0 | 21 | 55.00 |
| | 4.0 | 20 | 13.50 |
| | 6.0 | 20 | 19.00 |
| | 8.0 | 15 | 23.00 |
| | 10.0 | 15 | 20.00 |
| Hydrogen peroxide | 1.5 | Bags discarded due to high contamination | NIL |
| | 3.0 | 16 | 74.44 |
| | 6.0 | 16 | 125.50* |
| | 8.0 | 16 | 103.00* |
| Neem cake | 2.0 | 15 | 64.50 |
| | 4.0 | 15 | 91.50 |
| | 8.0 | 15 | 14.20 |
| Onion-garlic solution | 2.5 | 14 | 113.00* |
| | 7.5 | 14 | 99.00* |
| | 12.5 | 14 | 71.10 |
| BGAG | 0.33 | 20 | 21.50 |
| Protasan-DS | 1.25 | Bags discarded due to high contamination | NIL |

Single-factor ANOVA used for statistical analysis. CD 1% = 30.6627; CD 5% = 23.2348; SEd = 11.7708; CV% = 31.47. *At par. **Significantly higher.

steam, and microbes have been used for improving the digestibility of straw. The steam and hot water methods are very effective because they aid in swelling the fibers by hydrating cellulose molecules, thereby opening the fine structure, increasing enzyme processes, and providing a diffusion medium for enzymes, which are added to cellulose during hydrolytic cleavage of the glycosidic links of each molecule. Steam brings about very high levels of converting cell-wall polysaccharides into sugars, which aids in the fast colonization of the fungus.[5] This was also evident during the present investigation wherein the yield on steam and hot water pasteurized straw was significantly higher as compared with the chemical sterilization or solarization techniques. The chemical sterilization technique is not able to bring about any digestion of the straw. Therefore, the yield on the substrate treated with chemicals is low. Muhammad et al.[6] compared hot water, steam, and chemical (formaldehyde) pasteurization techniques of cotton waste for *P. ostreatus* var. *florida* cultivation. The authors reported a longer spawn running period and lower yield on chemically pasteurized substrate as compared with steam and hot water techniques. In the present study, we reported that steam is the best pasteurization method for oyster mushroom cultivation. Ficior et al.[7] concluded that the hot water method and chemical pasteurization with 0.01% Derosal were equally effective when corn cobs were used as the substrate. However, the effect of using such chemicals on a daily basis on the health of the workers and the environment is a

**TABLE 3. Comparative Yield of Contaminated Bags from Use of the Steam Pasteurization Method and Noncontaminated Bags from the Chemical Sterilization, Hot Water, and Solar Pasteurization Techniques**

***Data analyzed for comparative yield***

| Sl. no. | Treatment | Contamination incidence | Average Yield (g/1 kg wet paddy straw) |
|---|---|---|---|
| 1 | Hot water | NIL | 189.50 |
| 2 | Chemical | NIL | 129.50 |
| 3 | Solarization | NIL | 128.50 |
| 4 | Contaminated bags from steam pasteurization treatment | 5%–15% contaminated patch of *Trichoderma harzianum* | 157 |

***Data analyzed for unequal CRD***

| Treatment No. | Treatment No. | CD (5%) | CD (1%) |
|---|---|---|---|
| 1 | 4 | 62.424 | 91.198 |
| 2 | 4 | 62.424 | 91.198 |
| 3 | 4 | 62.424 | 91.198 |

matter of concern, and this is an issue that requires additional research.

To evaluate the chemical sterilization technique and hot water method, a case study was undertaken on a commercial farm in the Mysore district in Karnataka. As shown in Table 4, the contamination incidence was very high when the chemical sterilization technique was used (39.33%) as compared with the hot water treatment (11.72%). *C. comatus* was the dominant competitor mold in the chemical sterilization technique. The average yield obtained by the chemical sterilization technique decreased by 51.35% compared with the hot water treatment.

Among the alternate methods, solarization, hydrogen peroxide use (6% and 8%), and neem cake use (4%) showed similar yields of 128.50, 125.50, 103.00, and 91.50 g. (Table 2). It was interesting to note that competitor molds did not occur when the solarization technique was used. However, the yield in this technique was on par with the chemical sterilization technique but was low compared to the steam and hot water methods. These results can likely be attributed to the nonsoftening of straw, which prevents efficient substrate colonization. Table 5 shows the temperature regime during solarization. It was observed that merely covering wet straw with transparent polythene could raise the temperature of straw in the upper 5-cm layer to 59°C. However, heat conductance in the lower layers of straw was low because paddy straw is a poor conductor of heat. Milstein et al.[1] deduced that the solarization of straw in solar digesters (temperature of straw reaching 70°C for 10 h and 80°C for 6 h) reduced microbial levels and increased cellulose levels by 20% to 40%. The solarization of straw could be an alternate method because it

**TABLE 4. Yield Data from a Commercial Mushroom Farm at Mysore**

| Pasteurization method | Total no. of bags made | No. of contaminated bags | Average yield of noncontaminated bags (g/4 kg wet substrate) |
|---|---|---|---|
| Hot water | 128 | 15 | 1081.66* |
| Chemical | 267 | 105 | 735.00 |

Source of spawn: IIHR, Bangalore. Single-factor ANOVA used for statistical analysis. CD 1% = 112.0717; CD 5% = 83.0687; SEd = 40.5518; CV% = 12.23.
*Significantly higher yield.

**TABLE 5. Temperature of Paddy Straw at Hourly Intervals During Solarization**

| | Temperature (°C) of wet paddy straw during solarization at different depths | | | |
|---|---|---|---|---|
| Time | On surface | 5 cm from surface | 10 cm from surface | Floor temperature (°C) |
| 10:00 A.M. | 38.5 | 38.3 | 25.5 | 34.8 |
| 11:00 A.M. | 53.8 | 46.9 | 28.7 | 44.2 |
| 12:00 A.M. | 60.6 | 51.4 | 32.7 | 44.2 |
| 01:00 P.M. | 67.0 | 54.8 | 36.1 | 48.4 |
| 02:00 P.M. | 69.6 | 55.9 | 38.8 | 49.6 |
| 03:00 P.M. | 64.6 | 53.5 | 40.3 | 47.3 |
| 04:00 P.M. | 57.5 | 48.5 | 40.3 | 44.8 |

is eco-friendly, reduces production costs, and is suitable for rural areas. However, there is a need to further study and refine this technique.

Many botanicals and common sanitizers are known to possess antifungal and antibacterial properties. Hence, common botanicals such as neem cake, onion and garlic extract, and GBAG, as well as some commonly used sanitizers, such as bleaching powder, protasan, and hydrogen peroxide, were also used in our experiment. In experiments with the neem cake, the lower concentration (2%) was ineffective in pasteurization and resulted in 80% contamination (Table 1). At 4% neem-cake concentration, 20% of the bags showed contamination and a comparatively better yield of 91.50 g (Table 2). The higher concentration of 8% neem cake resulted in 86.66% contamination, with 73.33% of the bags showing an extent of more than 25% of contamination and a very low yield of 14.2 g (Tables 1 and 2). The likely reason for these results was the neem cake's inhibition of the mushroom mycelium coupled with its ineffectiveness toward contaminants. The other botanicals, such as onion and garlic solution and BGAG, were ineffective in killing contaminants and resulted in high contamination and lower yields (Tables 1 and 2).

The sanitizer bleaching powder contains chlorine, which has been used to kill harmful fungi and bacteria. In these studies, however, it was ineffective as a pasteurizing agent at all of the concentrations tested (Table 1). Hydrogen peroxide is another sanitizer that has been commonly used. In the present study, the contamination incidence decreased from 100% at 1.5% concentration to 26.66% at 8% concentration (Table 1). Hydrogen peroxide at 3% concentration showed 53.33% of the bags to have a greater than 50% contamination patch. As shown in Table 2, the yield increased from 0 (1.5% concentration) to 103.00 g (8% concentration). Wayne[8] standardized a method for cultivating oyster mushrooms using the hydrogen peroxide pasteurization method, and this method has shown promise but needs to be investigated in depth.

It can be concluded from the above-mentioned studies that hot water and steam pasteurization are better methods of substrate pasteurization for oyster mushroom cultivation. Although it is easy to use, the chemical sterilization technique may affect worker and environmental health and also appreciably reduces the yield. Other alternate methods, such as the solarization of wet straw and use of hydrogen peroxide, could be further studied for integration and refinement to form a low-cost and effective viable technology.

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# History of the Study of Thrombolytic and Fibrinolytic Enzymes of Higher Basidiomycetes Mushrooms at the V.L. Komarov Botanical Institute in Saint Petersburg, Russia

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**ABSTRACT:** The thrombolytic, fibrinolytic, milk-clotting, and general proteolytic activities of fruiting bodies of 400 species of Basidiomycetes belonging to various taxonomical and ecological groups were studied. A sufficient distinction between saprotrophic and symbiotrophic groups of Basidiomycetes in proteinase biosynthesis was established. It was found that the most pronounced proteolytic activity in combination with wide-range substrate specificity was characteristic for a group of saprotrophic mushrooms, whereas the symbiotrophic fungi were characterized by a low level or even complete absence of the aforementioned types of activity. The most pronounced fibrinolytic and thrombolytic activities were noted for lignotrophic saprotrophs and litter saprotrophs associated with lignin-cellulose complexes (rotten wood, compost, substrata, etc.). The list of species of Basidiomycetes that synthesize enzymes possessing high thrombolytic activity and low general proteolytic activity is presented.

**KEY WORDS:** Basidiomycetes, fruiting bodies, thrombolytic, fibrinolytic, milk-clotting, general proteolytic activities, list of active species, medicinal mushrooms

## I. INTRODUCTION

It is well known that proteolytic enzymes represent the molecular basis of an overwhelming majority of vital functions in organisms at any level of organization. The proteinases of bacteria, plants, and animals have been investigated rather intensively, whereas the knowledge on proteolytic systems of fungi is still far from complete.

In the last century, the mainstream of theoretical studies of mushroom proteinases was mainly connected with the molecular bases of ontogenesis in various groups of fungi and also with various mechanisms of pathogenesis, including phytopathogenesis. The main applied investigations focused on intensification of the metabolic processes in fungi (mainly in micromycetes), resulting in various biotechnological products such as feed protein, new antibiotics, diverse biological active compounds, enzymes of various nomenclature classes, and so forth.

The deficit of animal raw material that had arisen at the end of the 20th century led to active searches for alternative sources of proteolytic

## ABBREVIATIONS

**FA:** fibrinolytic activity; **HL**: hemolysis; **MCA**: milk-clotting activity; **PA**: general proteolytic activity; **TA**: thrombolytic activity

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enzymes with various substrate specificities, and became a "trigger" for intensive investigation of higher Basidiomycetes proteinases. The investigation predominately concerned the proteinases with caseinase and milk-clotting activities. These enzymes were studied most widely by Japanese researchers,[1–3] and patents were published.[4–8] At the same time, analogous work had begun in Russia.[9–12]

At the end of the 20th century, the number of applied investigations using cultures and fruiting bodies of higher fungi greatly increased. At the beginning of the 21st century, investigations of this group of fungi included unique applied niches, which differentiated this group significantly from filamentous fungi in experimental mycology and biotechnology.[13–16]

New knowledge of these mushrooms, accumulated in the fields of physiology, biochemistry, and molecular biology, as well as the experience in their medicinal application, opened new perspectives for the practical use of higher Basidiomycetes as a potential source of various biologically active compounds with medicinal value.[17–27]

The producers of fungal thrombolytic enzymes, as well as thrombolytic preparations for direct medicinal use, were studied in detail and characterized for different groups of micromycetes.[28,29] On the basis of the soil micromycete *Aspergillus terricola,* the fibrinolytic preparation "Terrilitine" was developed and proposed for use in Russia.[30,31] However, the limited number of thrombolytic preparations of microbial origin, and the serious side effects caused mainly by high levels of general nonspecific proteolysis, still prevent the wide use of these preparations in medicinal practice.

Biosynthesis of proteinases with fibrino- and thrombolytic actions in higher Basidiomycetes mushrooms is poorly studied. The lack of information on fibrinolytic and thrombolytic activities (FA and TA, respectively), as well as on the enzymes involved, has created the need to research different representatives of higher mushrooms. Unfortunately, the search for and study of new producers of thrombolytic enzymes still remain problematic for their use in practical medicine in many countries.

The first task-oriented investigations of FA and TA in basidial macromycetes were started in Russia at the V.L. Komarov Botanical Institute of Russian Academy of Sciences (Saint Petersburg, formerly known as Leningrad). Some of the pioneer work in this field was done by Falina[32] for FA and TA in cultures *Flammulina velutipes* (Curt.: Fr.) P. Karst. Later, investigations were continued on a wider basis of mycological material, including both cultures and fruiting bodies of higher Basidiomycetes from various taxonomical and ecological groups.[33–39]

In this article, we attempted to summarize our original material that was accumulated during 1980 to 2000 in Russia (part of the former USSR). On the basis of these data, we examined the general pattern of distribution of fibrinolytic and thrombolytic enzymes within various taxonomic groups of Basidiomycetes mushrooms.

## II. RESULTS AND DISCUSSION

The results of the analysis of FA and TA in fruiting bodies of basidial macromycetes from various taxonomic groups are presented in Tables 1 to 3. Table 1 contains a set of families in which active species were either not found or occurred only rarely. Tables 2 and 3 contain the families in which a considerable number of species with strongly pronounced activities of TA and FA occured.

On the basis of the data in Table 1, it can be seen that the pattern of active species populations in the families Strobilomycetaceae, Paxillaceae, Gomphidiaceae, Amanitaceae, and Cortinariaceae is similar. Fruiting bodies of all of the investigated species from these families are characterized by the complete absence of enzymes that lyzed blood thrombi and fibrin; all of these species belonged to the same ecological group, that is, symbiothrophic Basidiomycetes.

It should be noted that the species with strongly pronounced activities with respect to fibrin substrates only occurred much more frequently than the species possessing thrombo- and fibrinolytic effects (column TA in the Table 1).

The presence among higher Basidiomycetes species that develop both fibrino- and thrombolytic effects and of species active only against fibrin substrates (despite the fact that blood thrombi are actually fibrin-protein complexes) allows us

**TABLE 1. Families of Agaricoid Basidiomycetes with Rare Occurrence of Active Species**

| Taxon | No. of species (studied/active) | Active Species | | Concomitant activities (PA, MCA) and other comments |
|---|---|---|---|---|
| | | TA | FA | |
| **Boletales** | **48/3** | | | |
| Strobilomycetaceae | 2/0 | Absence | Absence | |
| Boletaceae | 38/3 | Absence | *Boletinus asiaticus,* Leccinum variicolor*, *Psiloboletinus lariceti* | *Boletus satanas* [PA*] |
| Paxillaceae | 5/0 | Absence | Absence | *Paxillus involutus* [PA*, HL] |
| Gomphidiaceae | 3/0 | Absence | Absence | |
| **Agaricales** | **85/5** | | | |
| Hygrophoraceae | 19/3 | Absence | *Pseudohygrocybe punicea, Hygrophorus agathosmus, H. chrysodon* | *H. chrysodon* [PA*] |
| Entolomataceae | 14/2 | *Entoloma abortivum** | *Clitopilus prunulus** | *C. prunulus* [PA*], *E. abortivum* [MCA] |
| Amanitaceae | 20/0 | Absence | Absence | |
| Cortinariaceae | 32/0 | Absence | Absence | |
| **Russulales** | **49/2** | | | |
| Russulaceae | 49/2 | Lactarius scrobiculatus* | *Lactarius blumii* | *L. scrobiculatus* [MCA*] |
| Total | 183/10 (5%) | 2 (1%) | 8 (4%) | |

*Indicates a high level of activity. HL, hemolysis.

to make several suggestions. First, the inhibitor blocking activity of the thrombolytic proteinases can be contained in the protein extracts of these mushrooms, which can be called "fibrinolytical" species. Another variant is possible—the enzymes in these species can be highly sensitive to inhibitors from blood serum. It is also possible that the blood thrombi are much more complicated protein substrates than the fibrin polymer, and this can explain the difference between enzyme sets of proteinases of "thrombolytical" and "fibrinolytical" species. Furthermore, we noticed that the fibrin polymer, used as a substrate, does not contain plazminogen. The absence of plazminogen allowed us to see the direct fibrinolytic effect of the studied enzymes in contradistinction to the "blood thrombi" substrate, in which it was possible to observe both direct and activator effects of thrombolysis. Therefore, we characterized the fruiting bodies of the investigated species as either species possessing predominantly thrombolytic activity or species possessing fibrinolytic activity.

Of all 183 studied species, only two possessed high levels of thrombolytic activity, namely, *Entoloma abortivum* (Berk. et Curt.) Donk and *Lactarius scrobiculatus* (Scop.: Fr.) Fr. At the same time, the high levels of thrombolytic activity in these species were accompanied by strongly pronounced milk-clotting activity (MCA), which was especially high in *L. scrobiculatus*. The presence of high levels of specific activities (TA and MCA) in the enzyme complex of obligate symbiothroph *L. scrobiculatus* (mycorrhizal with the *Pinus sylvestris*) at a low level of general proteolysis seems to be a significant feature, and this distinguishes it from other species of symbiothrophic Basidiomycetes.

As can be seen in Table 1, pronounced activity only against the fibrin substrate was found in eight species. In the family Boletaceae, pronounced activity was observed in fruiting bodies of *Lec-*

**TABLE 2. Families of Agaricoid Basidiomycetes with Frequent Occurrence of Active Species**

| Taxon | No. of species (studied/ active) | Active Species | | Concomitant activities (PA, MCA) and other comments |
|---|---|---|---|---|
| | | TA | FA | |
| **Agaricales** | | | | |
| Pleurotaceae | 11/5 | *Panus rudis,* Pleurotus ostreatus,* P. pulmonarius** | *Pleurotus cornucopiae, Panus tigrinus* | *P. tigrinus* PA*, HL; *P. ostreatus* PA*, MCA; *P. cornucopiae* PA*, MCA |
| Pluteaceae | 3/1 | *Pluteus atricapillus** [Δ] | | *P. atricapillus* PA* |
| Agaricaceae | 27/5 | *Chlorophyllum molybdites** | *Agaricus silvicola, A. abruptibulbus, A. placomyces*, Macrolepiota mastoidea** | *Ch. molybdites* (Singapore) |
| Coprinaceae | 16/8 | *Psathyrella candolleana**, *P. marcescibilis**, *P. microrhiza**, *P. spadiceo-grisea** | *Coprinus disseminatus,* Panaeolus* sp., *P. sphinctrinus*, *Panaeolina foenisecii* | |
| Bolbitiaceae | 13/6 | *Bolbitius vitellinus*, Agrocybe erebia** | *Conocybe tenera, C. hujsmanii*, Pholiotina arrhenii*, Agrocybe sphaleromorpha* | |
| Total** | 70/25 (36%) | 11 (16%) | 14 (20%) | |

*Indicates a high level of activity **except family Tricholomataceae. HL, hemolysis; [Δ], large spread of values.

*cinum variicolor* Watl. and *Psiloboletinus lariceti* (Singer) Singer. A strongly pronounced level of FA was detected in *Boletinus asiaticus* Singer. In this family, we observed a very high level of the general nonspecific PA in fruiting bodies of *Boletus satanas* Lenz.

In the family Hygrophoraceae, the enzymes with fibrinolytic action were found in litter saprotroph *Pseudohygrocibe punicea* (Fr.) Kovalenko, and in facultative symbiotrophs *Hygrophorus agathosmus* (Fr.) Fr. and *H. chrysodon* (Batsch.: Fr.) Fr. At the same time, FA in the species *H. chrysodon* was accompanied by a high level of general PA.

In the family Entolomataceae, *Clitopilus prunulus* (Scop.: Fr.) P. Kumm. (litter saprotroph and facultative symbiotroph) possessed high FA together with a high level of general PA.

A pronounced level of FA in the family Russulaceae was found only for *Lactarius blumii* Bon.

Therefore, proteinases with high thrombolytic activity were found in the fruiting bodies of only two (1%) of 183 studied species of agaricoid Basidiomycetes, and enzymes predominately affected the fibrin substrate in the fruiting bodies of eight species (4%). The overwhelming majority of studied species belonged to the group of mycorrhizal Basidiomycetes.

The character of distribution of active species in the other families of the order Agaricales s.l. is presented in Table 2. All of the studied species from the mentioned families (except the family Tricholomataceae) belonged to various groups of saprotrophic Basidiomycetes (xylotrophs, litter saprotrophs, ground litter saprotrophs, humus saprotrophs, coprotrophs, etc.).

Because the family Tricholomataceae is the most "multi-faced" in taxonomic and ecologotrophical aspects and has been studied on a large number of taxons, we have separated it in Table 3 for convenience of discussion.

As can be seen from the data in Table 2, in the family Pleurotaceae a pronounced level of biosynthesis of the thrombolytic enzymes was found in the fruiting bodies of *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm., *P. pulmonarius* (Fr.) Quél., *P. cornucopiae*

**TABLE 3. Distribution of the Active Species within Various Genera of the Family Tricholomataceae**

| Taxon | No. of species (studied/active) | Active species | |
|---|---|---|---|
| | | TA | FA |
| *Tricholoma* | 30/19 | *T. imbricatum* [Δ], *T. populinum* [Δ], *T. album*[Δ], *T. auratum*, *T. sudum*, *T. flavovirens* [PA*], *T. portentosum*, *T. sejunctum* [Δ], *T. saponaceum* [PA*], *T. scalpturatum* [Δ], *T. myomyces* [Δ], *T. mongolica* | *T. flavobrunneum*, *T. terreum*, *T. vaccinum*, *T. pessundatum* [HL, Δ], *T. triste*, *T. columbetta* [PA*], *T. terreum* [Δ] |
| *Lepista* | 9/7 | *L. gilva* [HL, Δ], *L. glaucocana* [PA*], *L. irina* [PA*], *L. personata* [PA*] | *L. nebularis*, *L. sordida* [PA*], *L. nuda* [PA*] |
| *Lyophyllum* | 4/2 | *L. decastes* [PA*, Δ], *L. ulmarium* | |
| *Clitocybe* | 11/5 | *C. maxima*, *C. odora* | *C. candicans*, *C. gibba*, *C. sinopica* |
| *Marasmius* | 6/3 | *M. wynnei* | *M. siccus*, *M. oreades* [PA*] |
| *Leucopaxillus* | 4/3 | | *L. gentianeus*, [HL], *L. tricolor*, *L. nauseosodulcis* |
| *Collybia* | 11/2 | *C. marasmioides* | *C. acervata*, |
| *Armillaria* | 2/1 | *A. mellea* [Δ] | |
| *Oudemansiella* | 4/1 | | *O. mucida* |
| *Flammulina* | 1/1 | *F. velutipes* | |
| *Tephrocybe* | 1/1 | | *T. palustris* |
| *Calocybe* | 1/1 | *C. gambosum* | |
| *Clitocybula* | 1/1 | *C. lacerate* | |
| *Myxomphalia* | 1/1 | | *M. maura* |
| *Rhodotus* | 1/1 | *R. palmatus* | |
| *Panellus, Laccaria, Mycena, Strobilurus, Melanoleuca, Tricholomopsis, Catathelasma, Xeromphalina* | 30/0 | Absence | Absence |
| Total | 117/49 (42%) | 27 (23%) | 22 (~19%) |

*Indicates a high level of activity. HL, hemolysis; [Δ], large spread of values.

(Paul.) Roll., and *Panus tigrinus* (Bull.: Fr.) Singer. We found that these species also have a high level of the general PA. Furthermore, we have seen that enzyme complexes from the fruiting bodies of species *P. tigrinus* caused blood hemolysis. Between species, only fruiting bodies of *Panus rudis* Fr. possessed an optimal combination of a high level of TA and low level of nonspecific proteolysis for medicinal use.

The species from the genus *Lentinus: L. edodes* (Berk.) Singer (commercial fruiting bodies from Japan), *L. fulvidus* (Bres.) Pilat., *L. lepideus* (Fr.: Fr.) Fr., and *L. lepideus* f. *rufescens* A. Petrov never possessed PA against the investigated protein substrates.

Many samples of fruiting bodies of the species from this family were collected in various geographic zones (Armenia, Azerbaijan, Belorussia, Japan,

North-West and Siberian regions of Russia, and other areas). A comparative analysis showed that the geographic region of the habitat of species from this family did not influence the level of enzymatic activity of the fruiting bodies.

In the family Pluteaceae, the enzymatic complex from xylotroph *Pluteus atricapillus* (Secr.) Singer is characterized by a high level of TA. However, no broad variability in levels of enzymatic activities were noted for this species dependent on the geographic region of habitat.

In the family Agaricaceae, pronounced TA were found in one species, *Chlorophyllum molybdites* (Meyer: Fr.) Mass., from a total of 27 species studied (from genera *Agaricus, Lepiota, Macrolepiota, Cystoderma, Chlorophyllum,* and *Phaeolepiota*). PA only against the fibrin substrate was found in all of the other species. The highest levels of FA were noted in the species *Agaricus placomyces* Peck and *Macrolepiota mastoidea* (Fr.) Singer.

In the family Coprinaceae (coprotrophs, humus saprotrophs, and xylotrophs), the species from genus *Psathyrella* (all of the samples of fruiting bodies were collected in Latvia) possessed high levels of TA – *P. candolleana* (Fr.) Maire, *P. marcescibilis* (Britz.) Singer, *P. microrhiza* (Lasch) Singer, and *P. spadiceo-grisea* (Fr.) Maire. The MCA, which is often accompanied by high TA, is completely absent in these species. Pronounced activity only against the fibrin substrate was found in *Coprinus disseminatus* (Pers.: Fr.) S.F. Gray, *Panaeolus sphinctrinus* (Fr.) Quél., *P. speciosus* P.D. Orton, and *Panaeolina foenisecii* (Pers.: Fr.) Maire.

In the family Bolbitiaceae, active biosynthesis of thrombolytic enzymes was found in the species *Bolbitius vitellinus* (Pers.: Fr.) Fr. and *Agrocybe erebia* (Fr.) Kühner. High activity only against the fibrin substrate was found in *Conocybe tenera* (Schaeff.: Fr.) Kühner, *C. hujsmanii* Knudsen et Watl., *Pholiotina arrhenii* (Fr.) Singer, and *Agrocybe sphaleromorpha* (Bull.: Fr.) Fayod.

Therefore, 25 active species of 70 species of various saprotrophs were found in the order Agaricales s.l. (except the family Tricholomataceae). A high level of thrombolytic enzymes was marked in the fruiting bodies of 11 species (16%). The proteolytic enzymes that attacked only a fibrin substrate were found in the fruiting bodies of 14 species of macromycetes (20%). The family Tricholomataceae stood out against the background of other more compact taxa of agaricoid mushrooms as a family with complicated taxonomic structure and a broad ecologo-trophic spectrum (symbiotrophs, litter and humus saprotrophs, xylotrophs, etc.). More than 100 species of 25 genera from this family were studied.

It can be seen from the data in Table 3 that the main part of active species (80%) of this family belonged to genera *Tricholoma, Lepista, Lyophyllum, Clitocybe, Marasmius,* and *Leucopaxillus*. The triad *Tricholoma-Lepista-Lyophyllum* invites attention due to the similarity of patterns in enzymatic activity (a high level of TA often connected with a high level of the general PA). Proteinases with high levels of TA were detected in the fruiting bodies of 28 species of this triad. Some species from the genera *Tricholoma* and *Lepista* showed a wide dispersion of values in enzymatic activities (without reference to the geographic zone of habitat of the species). However, high and stable values of TA were found for species *Tricholoma auratum* (Paul.: Fr.) Gill., *T. flavovirens* (Pers.: Fr.) S. Lundell [syn. *T. equestre* (L.: Fr.) P. Kumm.], *T. portentosum* (Fr.) Quél., *T. saponaceum* (Fr.: Fr.) P. Kumm., and *T. sudum* (Fr.) Quél. It is appropriate to mention that the borders between the genera of this triad are rather unclear. Some species combine characteristics in various combinations in such a manner that their generic attribution is different within various classification schemes.

The highest values of TA among obligate symbiotrophic species were noted in species from the sect. *Tricholoma* of genus *Tricholoma.*

High and stable levels of these enzymes among saprotrophs were found in the lignotrophic species *Flammulina velutipes* and *Rhodotus palmatus* (Bull.: Fr.) Maire; the rest of the active species belonged to other groups of saprotrophs, inhabiting mixed wood-humus substrates generally enriched with various organic components.

Of the 117 species studied, 49 active species were found in the family Tricholomataceae. High levels of thrombolytic TA were found in 27 species (23%). Enzymes with only fibrinolytic action were found in the fruiting bodies of 22 species (19%). The family Tricholomataceae had the greatest number of

active species (42%) of all of the studied families of agaricoid Basidiomycetes.

Altogether, PA against investigated protein substrates was studied in the fruiting bodies of more than 400 species of Basidiomycetes; TA, FA, PA, and MCA were determined for 370 of these species (as noted in Tables 1–3). Active biosynthesis of the enzymes of thrombolytic and fibrinolytic actions was detected in 84 species of various systematical and ecological groups.

Based on the analysis of the data in Tables 1 to 3, we have composed a generalized summary list of Basidiomycetes fruiting bodies that contain enzyme complexes providing the lysis of blood thrombi and fibrin films. Proteolytic enzymes of the mentioned species possess high TA and FA and do not possess high PA with respect to the other protein substrates. Species with the complete absence of PA and/or MCA are also included on the list. The species presented in the list of Basidiomycetes with narrow substrate specificity are, in our judgment, interesting and, in some instances, suitable objects for further medical-biological investigation.

Species of Basidiomycetes that synthesize thrombolytic enzymes are as follows:

- *Agrocybe erebia* (Fr.) Kühner—humus saprotroph
- *Bolbitius vitellinus* (Pers.: Fr.) Fr.—humus saprotroph, facultative xylotroph
- *Chlorophyllum molybdites* (Meyer: Fr.) Mass.—humus saprotroph
- *Clitocybe maxima* (Fl.Wett. : Fr.) P. Kumm.—humus saprotroph [absence of MCA, PA]
- *Clitocybula lacerata* (Lasch.) Métrod—humus saprotroph [absence of MCA]
- *Collybia marasmioides* (Britzelm.) Bresinsky et Stangl—[absence of MCA, PA]
- *Entoloma abortivum* (Berk. et Curt.) Donk—leaf litter saprotroph, xylotroph
- *Flammulina velutipes*—xylotroph
- *Lactarius scrobiculatus* (Scop.: Fr.) Fr.—symbiotroph [MCA*, absence of PA]
- *Lyophyllum ulmarium* (Bull.: Fr.) Kühner—xylotroph
- *Marasmius wynnei* Berk. et Bres.—litter saprotroph
- *Panus rudis* Fr.—xylotrophic [absence of MCA, PA]
- *Psathyrella candolleana* (Fr.) Maire—humus saprotroph, facultative xylotroph
- *P. marcescibilis* (Britz.) Singer—humus saprotroph, facultative xylotroph
- *P. microrhiza* (Lasch : Fr.) Konrad et Maubl.
- *P. spadiceo-grisea* (Fr.) Maire—humus saprotroph, xylotroph
- *Rhodotus palmatus* (Bull.: Fr.) Maire—xylotroph
- *Tricholoma auratum* (Paul.: Fr.) Gill.—symbiotroph [absence of MCA]
- *T. sudum* (Fr.) Quél.—symbiotroph [absence of MCA]

The presence of TA and FA in the fruiting bodies and cultures of higher Basidiomycetes (including some species not mentioned in this article) was checked and confirmed in *in vitro, ex vivo*, and *in vivo* experiments (in rats, rabbits, and dogs).

The method of obtaining enzyme preparations was developed with high TA and FA from fruiting bodies of *Tricholoma flavovirens* and *T. portentosum*.[40]

The enzyme preparations from cultures of *Flammulina velutipes, Coprinus domesticus* (Bolt.: Fr.) S.F. Gray, *C. cinereus* (Schaeff.: Fr.) S.F. Gray, *Cerrena unicolor* (Bull.: Fr.) Murrill, and others were obtained, and their influence on the processes of fibrinolysis and experimental thrombosis in blood vessels of experimental animals was studied. It was shown (Department of Pathological Physiology, Acad. I.P. Pavlov Saint Petersburg State Medical University) that enzyme complexes isolated from the aforementioned cultures possess a large affinity to fibrin and cause fibrin lyses. In addition, the enzyme complexes have a direct and stable thrombolytic effect that is more significant on fresh thrombosis.[36-38,41]

As a final note, *ex vivo* and *in vivo* experiments on dogs (Laboratory of Chemistry and Technology of Materials for Cardio-Vascular Surgery, Research Center of Cardio-Vascular Surgery Russian Academy of Medical Sciences, Moscow) have also shown that enzyme preparations from cultures of *Flammulina velutipes* and *Coprinus domesticus* can be used for developing new types of polymer materials with thromboresistant features.[39,42]

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# The 6th International Medicinal Mushroom Conference

## September 25 - 29, 2011
## Zagreb, Croatia

ORGANIZED BY: Dr Myko San – Health from Mushrooms Co. (Croatia)
Conference Organizing Committee
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## FIRST ANNOUNCEMENT: CALL FOR ABSTRACTS AND PRELIMINARY REGISTRATION

We are pleased to announce the 6th International Medicinal Mushroom Conference, which will be held in Zagreb, Croatia. We invite scientists, professionals, students and practitioners (mycologists, biologists, ecologists, biochemists, biotechnologists, pharmacologists, nutritionists, medical doctors, immunologists, oncologists, infection diseases specialists, cardiologists, neurologists, specialists in integrative, complementary and alternative medicine, bioremediators - especially in agriculture and forestry, etc), people working with mushrooms and in other food industries, nutraceutical, pharmaceutical and other related industries and entrepreneurial and commercial initiatives, as well as all those who are interested in studying, discussing and exploring practical applications of the most current research on fungi and their medicinal properties.

The Conference will be organized with parallel sessions and poster exhibitions. An International Industrial Exhibition of Medicinal Mushrooms and Mushroom Products is also planned.

The official language of the conference is English, including lectures and printed materials.

## Conference Topics

1. Systematics, taxonomy, distribution, ecology and fungal culture collections
2. Medicinal and nutritional value of mushrooms and pharmacology of active compounds
3. Mushroom nutraceuticals in theory and practice – production, regulation, promotion and marketing worldwide
4. Fungal physiology, biochemistry and genetics
5. Mycotechnology and cultivation in research and industry
6. Mycoremediation in forestry and agriculture
7. Ethnomycology, traditional and alternative medicines using medicinal mushrooms
8. Special panel discussion:
   a. Medicinal mushrooms and cancer, and/or
   b. Medicinal mushrooms and viral infections

## Conference Structure

The conference program will be based on the main topics and will also include plenary keynote lectures, symposia, special panel discussion, poster sessions and a trade exhibition.

## Call For Papers

We would be pleased to receive contributions from interested authors that follow the conference themes. Abstracts should focus on current issues relevant to science progress and/or to industry, and should contain scientific and/or practical content. Abstracts should clearly define the objectives of the presentation, topics covered, key conclusions reached, and potential benefits for science development and/or industry, including options for oral or poster presentation. Abstracts should be at least 500 words in length.

Full papers elaborating on abstracts will be published in forthcoming issues of the International Journal of Medicinal Mushrooms.

Abstracts should be sent to Solomon P. Wasser, Head of the Publishing Committee, Editor-in-Chief, International Journal of Medicinal Mushrooms, before January 15, 2011 in word or rich text format. Send to Solomon P. Wasser, Institute of Evolution, Haifa University, Mt. Carmel 31905, Israel, or e-mail them to spwasser@research.haifa.ac.il . Additionally, abstracts should be sent as soon as possible to immc6.loc@gmail.com , which will enable the Conference Organizing Committee to be more effective in the long-term shaping of the conference program.

## Main Organizers

Dr Myko San – Health from Mushrooms Co (Croatia)

## Co-organizers

Faculty of Agriculture (University of Zagreb, Croatia)

Faculty of Food Technology and Biotechnology (University of Zagreb)

Faculty of Forestry (University of Zagreb)

Croatian Society of Food Technologists, Biotechnologists and Nutritionists (Croatia)

## Conference Fees

€ 300 for regular attendees

€ 100 for students (with student card)

€ 370 for late registrants (after June 1, 2011)

On-line registration will soon be available at the Conference website, which will be launched during May 2010.

Conference fees include: admission to all scientific sessions, free entrance to the conference exhibition, abstracts book, scientific excursions, welcome reception, conference dinner party, and coffee breaks.

## Draft Program

The Conference's scientific program will extend from September 26 – 29, 2011. September 25 is registration day and September 28 is a touring day.

## Deadlines

| | |
|---|---|
| December 1, 2010 | Return the preliminary registration form |
| January 15, 2011 | Submission of abstracts |
| April 1, 2011 | Notification of authors on abstracts acceptance |
| June 1, 2011 | Early registration fee payment |
| June 1, 2011 | Hotel reservations |

## Accommodations

There will be several hotels of different categories available. More information on available hotels will be added to the conference website.

## About Croatia and Zagreb

Republic of Croatia, the country candidate for the membership in the European Union, is a Central European and Mediterranean country. With its very rich cultural heritage (7 world heritage sites) and magnificent natural beauty (8 national parks), Croatia is the 18th most popular tourist country in the world. Consisting of very different geographical and climate regions - plains, lakes and rolling hills in the continental north and northeast Pannonian plain, densely wooded mountains in Lika and Gorski Kotar and rocky coastlines on the Adriatic Sea with over one thousand islands. Croatia has great biodiversity, including mushrooms.

Zagreb, the capital of Croatia, is its political, economic, cultural and scientific center, with more than 800 000 inhabitants. Sometimes marked as „a little Vienna" regarding its architectural scenery, Zagreb has a great cultural heritage and the oldest continuously operating University in the region (founded in 1669). From an old medieval core to the modern areas, Zagreb is a modern European city and one of the leading centers of this region of Europe, very well connected and integrated into contemporary European transportation.

## Conference Organizing Committee

**President**: Dr. Ivan Jakopovich

**Vice president**: Prof. Mislav Jurin

**Members**: Prof. Romano Bozac, Prof. Milan Glavas, Boris Jakopovich, Prof. Drazenka Komes, Prof. Ibrahim Mujic

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## Publishing Committee

Prof. S.P. Wasser

Prof. Omon S. Isikhuemhen

Prof. Mislav Jurin

# The 6th International Medicinal Mushroom Conference (IMMC6)

## Preliminary Registration Form

Please send completed form to:
IMMC6 Conference Organizing Committee (Dr Myko San)
Miramarska 109, 10000 Zagreb
CROATIA, NA385

E-mail: immc6.loc@gmail.com
Fax: +385-1-4660095

Conference Fees

□ Regular (300 €) □ Student (100 €)

PLEASE USE LATIN BLOCK LETTERS. USE TOPIC NUMBERS AS DEFINED ABOVE.

TITLE _____
NAME (First, Last) _____
INSTITUTION _____
ADDRESS _____
ZIP CODE _____ CITY _____ COUNTRY _____
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ATTENDANCE: □ Passive □ Active
I PREFER: □ Oral presentation □ Poster presentation □ Trade Exhibit
TOPIC OF MY PRESENTATION: 1 2 3 4 5 6 7
I AM INTERESTED IN THE FOLLOWING TOPICS: 1 2 3 4 5 6 7 8a 8b
□ I WISH TO HELP SPONSOR THE CONFERENCE