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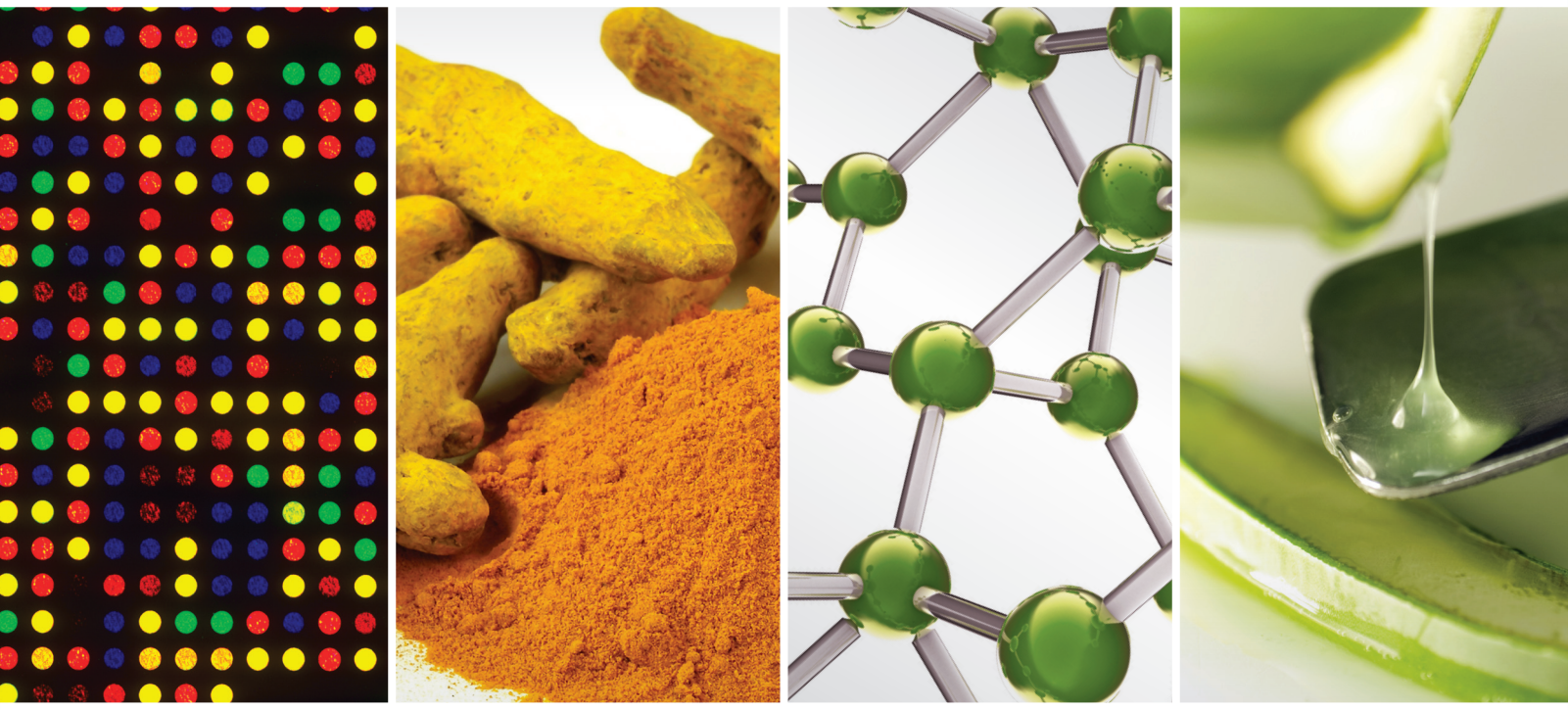
Medicinal Mushrooms

Guest Editors: Ulrike Lindequist, Ha Won Kim, Evelin Tiralongo, and Leo Van Griensven



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Editorial
Medicinal Mushrooms

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Since beginning of mankind nature is the most important source of medicines. Bioactive compounds produced by living organisms can be used directly as drugs or as lead compounds for drug development. Besides, the natural material can be used as crude drug for preparation of powder or extracts.

Plants have traditionally been used as a source of most medical systems and as such herbal medicines constitute an important part of traditional and evidence-based medicine worldwide. In contrast though, the broad medicinal use of mushrooms was for a very long time restricted to Asian countries. Nowadays, the medicinal use of mushrooms, so called, medicinal mushrooms', is increasing also in Western and other countries. This is underlined not only by growing sales of mushroom products but also by an increasing number of scientific papers and international conferences in this field.

Medicinal mushrooms can be defined as macroscopic fungi, mostly higher Basidiomycetes, which are used in the form of extracts or powder for prevention, alleviation, or healing of diseases and/or for nutritional reasons.

Presently medicinal mushrooms are mainly used as dietary supplements or functional food. Nevertheless they have the potential to become real drugs of traditional and/or evidence-based medicine. To explore this potential high quality products, preclinical and clinical trials according to the regulations, and legal authorization are necessary.

The most important mushroom species are *Ganoderma lucidum*, *Coriolus versicolor* (L.:Fr.)Quél. (syn. *Trametes versicolor* [L.:Fr.]Pilát), *Lentinula edodes* (Berk.)Pegler, *Agaricus brasiliensis* Wasser et al., *Cordyceps sinensis* (Berk.)Sacc. (*Ophiocordyceps sinensis* (Berk.)Sung & al.), *Grifola frondosa* (Dicks.:Fr.)Gray, *Hericium erinaceus* (Bull.:Fr.)Pers. and some others. Due to the extended traditional use of these mushrooms extensive knowledge about in vitro activities and mode of action and effects in animal assays is available. Some information about several chemical constituents responsible for the pharmacological effects is also available. Needless to say more research on the pharmacology and chemistry of these and others, so far less explored mushrooms, is urgently needed.

What's also missing, however, are investigations about structure-activity-relationships and possible toxicological risks of these mushrooms and their products, clinical trials and suitable quality critera for mushroom products and established methods for its control.

We received 13 research papers in this field which indicates that mushroom research is still very limited. However, this special issue includes 6 high-quality peer-reviewed papers demonstrating essential new findings about different pharmacological effects of several medicinal mushrooms and their components in vitro, in animal assays and in humans.

Chan et al. and Kawai et al. showed that *Amauroderma rugosum* and *Pleurotus eryngii* have promising antiinflammatory properties. *A. rugosum* is used by indigenous communities in Malaysia, *P. eryngii* is consumed as fresh cultivated mushroom worldwide. *Clitocybe nuda*, also known as *Lepista nuda*, is an edible mushroom in Europe. It is known for its antioxidative and antimicrobial properties. The study by

Shi et al. validated the antidiabetic and hypolipidemic effects of *Clitocybe nuda* in diabetic mice and gives detailed insight into its mode of action.

Beta-glucans (MBGS) constitute one of the most important groups of bioactive compounds in mushrooms. They have been reported as anticancer agents mainly by strengthening immune activities. The papers of Chen et al. and Wu et al. focus on investigations of beta-glucans from *Ganoderma lucidum*, the famous Reishi or Ling Zhi mushroom. The beta-glucans isolated from solid culture of *Ganoderma lucidum* inhibit, in combination with radiation, tumor metastasis in Lewis lung carcinoma bearing mice (Chen at el.), whereas the oral administration of MBG, also obtained from mycelium of *G. lucidum*, modulates immune responses in an allergy murine model (Wu et al.).

The study of Rossi et al. investigated the influence of a mixture of *Ganoderma lucidum* and *Ophiocordyceps sinensis* on the performance and stress resistance of cyclists by monitoring the testosterone/cortisol ratio in saliva, as well as oxidative stress. Although only a small number of participants were included in this study, the presented results confirm the potential clinical use of mushrooms in general and the protection of the athletes from overtraining syndrome in particular.

Acknowledgments

The editors would like to express their greatest appreciation to the authors for their fascinating and interesting contributions. The editors would like also to express their gratitude to the reviewers for their important time and valuable suggestions/comments to make the special issue a success. The papers published in this special issue not only extend our knowledge about medicinal mushrooms and confirm the great potential of mushrooms for development of new drugs, but hopefully also inspire the reader to get involved in medicinal mushroom research.

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Ha Won Kim
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Research Article

Pleurotus eryngii Ameliorates Lipopolysaccharide-Induced Lung Inflammation in Mice

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Pleurotus eryngii (*P. eryngii*) is consumed as a fresh cultivated mushroom worldwide and demonstrated to have multiple beneficial effects. We investigated the anti-inflammatory effect of *P. eryngii* in mice with acute lung injury (ALI). Intranasal instillation of lipopolysaccharide (LPS) (10 μg/site/mouse) induced marked lung inflammation (increase in the number of inflammatory cells, protein leakage, and production of nitric oxide in bronchoalveolar lavage fluid) as well as histopathological damage in the lung, 6 h after treatment. Mice administered heat-treated *P. eryngii* (0.3–1 g/kg, p.o. (HTPE)) 1 h before LPS challenge showed decreased pulmonary inflammation and ameliorated histopathological damage. These results suggest that HTPE has anti-inflammatory effects against ALI. Thus, *P. eryngii* itself may also have anti-inflammatory effects and could be a beneficial food for the prevention of ALI induced by bacterial infection.

1. Introduction

Pleurotus eryngii is an edible mushroom native to Europe. Its natural habitat is the dead roots of the weed *Eryngium campestre*. *P. eryngii* is cultivated widely, and its production has been increasing in Asia, including Japan [1]. *P. eryngii* is considered to be a health food because it is low in fat and calories but rich in amino acids, vitamins, and dietary fiber. *P. eryngii* is also bioactive, with hypolipidemic [2], antitumor [3], antioxidant [4–6], and antiallergic activities [1]. In particular, *in vitro* studies have demonstrated that the antiallergic activity of *P. eryngii* is caused by the down-regulation of allergy-related signaling proteins (including inflammation-related proteins) by inhibition of the nuclear factor of activated T cells, nuclear factor-kappa B (NF-κB), and high-affinity immunoglobulin E receptor (FcεRI) mediated signaling in antigen-stimulated mast cells. However, whether *P. eryngii* has clinical effectiveness remains to be determined.

Acute respiratory distress syndrome is a result of acute inflammation of the lung and noncardiogenic pulmonary edema that often leads to multiorgan system failure and death [7, 8]. Lipopolysaccharide (LPS) is present in the outer membrane of Gram-negative bacteria. LPS can cause acute inflammation of the lung because of neutrophil recruitment and pulmonary edema [9, 10]. Intranasal instillation of LPS in mice (as an animal model of acute lung injury (ALI)) has been shown to result in the release of proinflammatory cytokines, which cause aggregation of inflammatory cells and, consequently, injury to lung tissue [11, 12]. There have been reports with regard to these mechanisms and the features of this model. Such reports have shown that LPS activates alveolar macrophages directly and stimulates neutrophils to migrate into the lung and that the proinflammatory mediators released from these inflammatory cells recruit lymphocytes to the lung [7, 13]. The critical feature of LPS-induced ALI is the destruction of vascular integrity and the subsequent upregulated permeability results in protein leakage and pulmonary edema [9, 10, 14]. It has also been reported that nitric oxide (NO) has important roles in the pathogenesis of ALI because inhibitors of NO synthase inhibit LPS-induced damage [9, 15–17].

Figure 1: The fresh fruiting body of *Pleurotus eryngii* and freeze-dried powder of heat-treated *P. eryngii* (HTPE).

The aim of this study was to examine the anti-inflammatory effect of *P. eryngii in vivo* using an LPS-induced ALI model in mice. We used the whole *P. eryngii* to illustrate the functional utility of this mushroom as food.

2. Materials and Methods

2.1. Animals. Male BALB/c mice (6 weeks of age: Japan SLC, Ltd., Shizuoka, Japan) were used. They were kept under controlled temperature (21–23°C) and humidity (45–65%). The room was lit from 7:00 am to 7:00 pm and during the behavioral test. Food and water were available *ad libitum*. The study was approved by the Committee for Animal Experiments at the University of Toyama (Toyama, Japan).

2.2. Agents. LPS (*Escherichia coli 0111:B4*), dexamethasone, and pentobarbital sodium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other agents used in this study were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). LPS was dissolved in phosphate-buffered saline (PBS) and instilled intranasally. Dexamethasone, which was dissolved in saline with 10% ethanol, was given to mice in the dexamethasone group by intraperitoneal injection 1 h before LPS administration. Pentobarbital sodium was dissolved in saline containing 0.4% propylene glycol and 1.05% ethanol, and given by intraperitoneal injection.

2.3. Preparation of P. eryngii Intakes. The fruiting body of *P. eryngii* (Figure 1) was obtained from Hokuto Co. (Nagano, Japan). It was then cut into small pieces and boiled in an equal amount of distilled water for 10 min. Heat-treated *P. eryngii* (HTPE) was freeze-dried and powdered. HTPE was resuspended in tap water and administered orally 1 h before intranasal administration of LPS.

2.4. LPS-Induced ALI Model in Mice. BALB/c mice were challenged with intranasal instillation (i.n.) of LPS (10 μg in 50 μL PBS per mouse) to induce lung inflammation. Control mice were given PBS (i.n.) without LPS. After 6 h, collection of bronchoalveolar lavage fluid (BALF) was carried out following the method of Chu et al. under anesthesia (sodium pentobarbital, 80 mg/kg, i.p.) [14]. After centrifugation (25×g, 4°C, 5 min), cell pellets were resuspended in PBS for total cell counts using a hemacytometer. The supernatant was used for NO analyses and protein analyses.

2.5. Measurement for Protein Concentration. The protein concentration in BALF was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Measurement for NO Production. A metabolite of NO, nitrite (NO^{2-}), in BALF was measured using Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, 2.5% phosphoric acid). Briefly, 50 μL of BALF and 50 μL of Griess reagent were mixed in a 96-well plate. The azo dye formed was determined with a spectrophotometer (Multiskan FC: Thermo Fisher Scientific K.K., Yokohama, Japan) at 540 nm using sodium nitrite as the standard.

2.7. Hematoxylin and Eosin (H&E) Staining. Anesthesia (sodium pentobarbital, 80 mg/kg, i.p.) was induced in mice 6 h after LPS treatment. Mice were then decapitated. Lungs were removed and, after washing with PBS, placed in 10% formalin solution. Preparation of paraffin-embedded sections and staining with H&E were undertaken using standard procedures. Staining was observed under a light microscope (AX80: Olympus, Osaka, Japan) with a charge-coupled device camera (Axio Cam; Carl Zeiss, Jena, Germany).

2.8. Immunohistochemical Staining. Anesthesia (sodium pentobarbital, 80 mg/kg, i.p.) was induced in mice 6 h after LPS treatment. Mice were then decapitated. Lungs were removed and, after washing with PBS, placed in 10% formalin solution. Preparation of paraffin-embedded sections and deparaffinization were undertaken using standard procedures. Deparaffinized sections were treated with methanol containing 0.3% hydrogen peroxide and then with 0.2% Triton X-100 in PBS. After treatment with 0.3% fetal bovine serum in PBS to block immunoglobulin binding, sections were incubated with rabbit anti-myeloperoxidase (MPO) antibody (DAKO, Glostrup, Denmark) or rat anti-Mac-2 antibody (Cedarlane, Ontario, Canada) at 4°C overnight, followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (DAKO) or horseradish peroxidase-conjugated anti-rat IgG antibody (DAKO). Color was developed using DAKO liquid with a 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate chromogen system (DAKO) and counterstained with hematoxylin. These stained sections were observed under a light microscope (AX-80; Olympus) coupled to a CCD camera (Axio Cam; Carl Zeiss).

2.9. Statistical Analyses. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance between groups was assessed using one-way analysis of variance and *post hoc* Holm-Sidak multiple comparisons. $P < 0.05$ was considered significant. Statistical analyses were done using Sigmaplot v11 (Systat Software, Inc., Chicago, IL, USA).

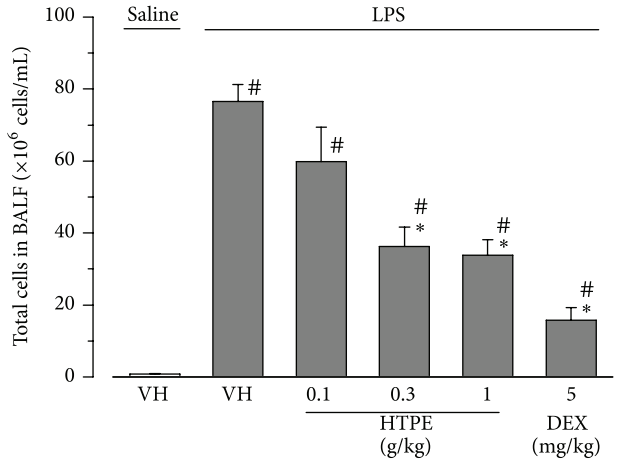

Figure 2: Effect of HTPE on the total number of cells in the BALF of LPS-induced ALI mice. Mice were administered, via the oral and intraperitoneal routes, HTPE (0.1–1 g/kg) and dexamethasone (DEX: 5 mg/kg, positive control group), respectively, 1 h before intranasal administration of LPS (10 μg/site). Vehicle (VH: tap water, vehicle for HTPE) was also administered orally 1 h before intranasal administration of LPS (LPS group) or saline (vehicle for LPS, negative control group). BALF was collected 6 h after LPS challenge to measure the total number of cells. Values are presented as the mean and SEM ($n = 6$ for VH or HTPE; $n = 4$ for DEX). $^{\#}P < 0.05$ versus negative control group (VH + saline), $^{*}P < 0.05$ versus LPS group (VH + LPS) (Holm-Sidak multiple comparisons).

3. Results

3.1. Effects of HTPE on the Aggregation of Inflammatory Cells Induced by LPS in the Lung. LPS-induced ALI resulted in an increase in the number of inflammatory cells, such as neutrophils and macrophages, in the lung [14]. Six hours after intranasal instillation of LPS, the number of inflammatory cells in BALF increased significantly compared with intranasal instillation of saline (vehicle for LPS) (Figure 2). Oral pretreatment with HTPE (0.1–1.0 g/kg) significantly inhibited the LPS-induced increase in the number of cells in BALF compared with that of the vehicle (for HTPE) pretreated group (Figure 2). Intraperitoneal pretreatment with dexamethasone (5 mg/kg) also significantly decreased the number of cells in the BALF of LPS-treated mice compared with that of the vehicle (for HTPE) pretreated group (Figure 2).

3.2. Effect of HTPE on LPS-Induced Vascular Permeability in the Lung. One of the major pathological changes observed in LPS-induced ALI is increased vascular permeability, which results in increased protein leakage in BALF [14]. Six hours after intranasal instillation of LPS, the concentration of protein in BALF increased significantly compared with the intranasal instillation of saline (vehicle for LPS) (Figure 3). Oral pretreatment with HTPE (0.1–1.0 g/kg) significantly inhibited LPS-induced protein leakage in BALF compared with that of the vehicle (for HTPE) pretreated group (Figure 3). Intraperitoneal pretreatment with dexamethasone (5 mg/kg) also significantly attenuated the concentration of protein in the BALF of LPS-treated mice compared with that of the vehicle (for HTPE) pretreated group (Figure 3).

3.3. Effect of HTPE on LPS-Induced NO Production in the Lung. It has been reported that NO plays an important part in the pathogenesis of ALI [8, 15–17]. Six hours after intranasal instillation of LPS, the concentration of nitrite and metabolites of NO in BALF increased significantly with intranasal instillation of saline (vehicle for LPS) (Figure 4). Oral pretreatment with HTPE (0.1–1.0 g/kg) significantly inhibited LPS-induced NO production in the lung compared with that of the vehicle (for HTPE) pretreated group (Figure 4). Intraperitoneal pretreatment with dexamethasone (5 mg/kg) showed a tendency toward inhibition of LPS-induced NO production, but not in a significant manner ($P = 0.051$) (Figure 4).

3.4. Effect of HTPE on LPS-Induced Pulmonary Histopathological Changes. To evaluate the histopathological changes in LPS-treated mice, lung sections 6 h after LPS treatment were stained with H&E. Normal pulmonary histology was observed in mice that underwent intranasal instillation of saline (vehicle for LPS) (Figure 5(a)). LPS-treated lungs exhibited an apparent increase in infiltration of inflammatory cells, interstitial edema, and hyperemic thickening of the alveolar wall (Figure 5(b)). In particular, the infiltrated inflammatory cells in LPS-treated lung were neutrophils (MPO-immunoreactive cells) (Figure 5(c)) and macrophages (Mac-2-immunoreactive cells) (Figure 5(d)). However, these histopathological changes in lungs treated with LPS were ameliorated by pretreatment with HTPE (1 g/kg) (Figure 5(e)) or dexamethasone (5 mg/kg) (Figure 5(f)).

4. Discussion

We evaluated the anti-inflammatory activities of HTPE usinga LPS-induced model of ALI in mice. Pretreatment with HTPE as well as dexamethasone decreased the number of total cells, protein leakage, and NO production in the BALF of LPS-induced ALI mice. Histopathological studies revealed infiltration of inflammatory cells (such as neutrophils and macrophages), interstitial edema, and thickening

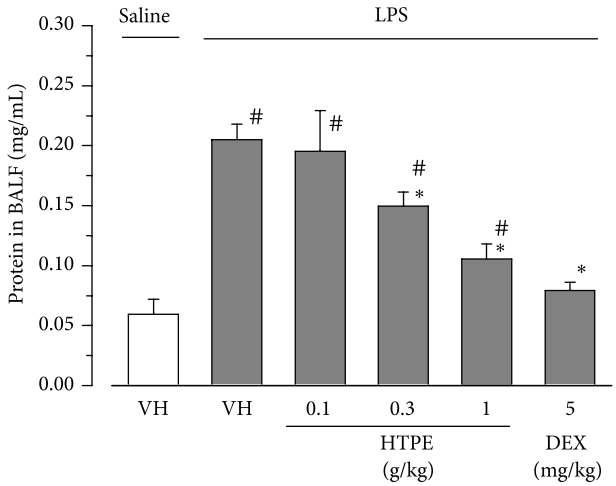

Figure 3: Effect of HTPE on total protein concentration in the BALF of LPS-induced ALI mice. Mice were administered, via the oral and intraperitoneal routes, HTPE (0.1–1 g/kg) and dexamethasone (DEX: 5 mg/kg, positive control group), respectively, 1 h before intranasal administration of LPS (10 μg/site). Vehicle (VH: tap water, vehicle for HTPE) was also administered orally 1 h before intranasal administration of LPS (LPS group) or saline (vehicle for LPS, negative control group). BALF was collected 6 h after LPS challenge to measure total protein concentration. Values are presented as the mean and SEM ($n = 6$ for VH or HTPE; $n = 4$ for DEX). $^{\#}P < 0.05$ versus negative control group (VH + saline), $^{*}P < 0.05$ versus LPS group (VH + LPS) (Holm-Sidak multiple comparisons).

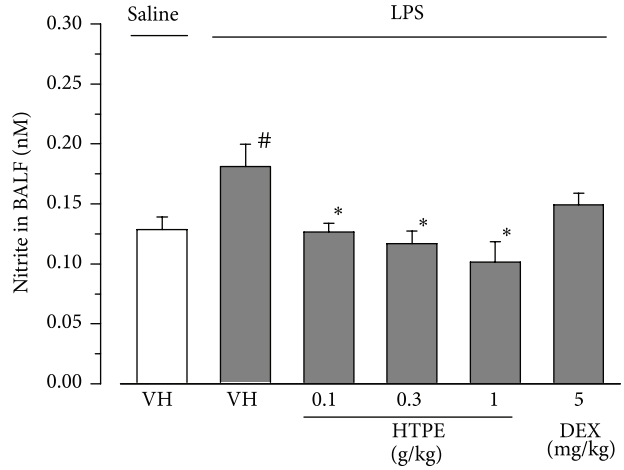

Figure 4: Effect of HTPE on production of nitric oxide in the BALF of LPS-induced ALI mice. Mice were administered, via the oral and intraperitoneal routes, HTPE (0.1–1 g/kg) and dexamethasone (DEX: 5 mg/kg, positive control group), respectively, 1 h before intranasal administration of LPS (10 μg/site). Vehicle (VH: tap water, vehicle for HTPE) was also administered orally 1 h before intranasal administration of LPS (LPS group) or saline (vehicle for LPS, negative control group). BALF was collected 6 h after LPS challenge to measure the nitrite (a metabolite of nitric oxide) concentration. Values are presented as the mean and SEM ($n = 6$ for VH or HTPE; $n = 4$ for DEX). $^{\#}P < 0.05$ versus negative control group (VH + saline), $^{*}P < 0.05$ versus LPS group (VH + LPS) (Holm-Sidak multiple comparisons).

of the alveolar walls in the lungs of LPS-induced ALI mice. These histopathological changes were also prevented in mice given HTPE and dexamethasone. These results suggest that HTPE improves the lung injury induced by LPS in mice through inhibition of the recruitment of inflammatory cells and overproduction of NO.

Mushrooms are low in calories, abundant in amino acids, vitamins, and dietary fiber, and are popular foods worldwide. It has been reported that some edible mushrooms have a wide range of pharmaceutical properties, including anti-inflammatory and antioxidant activities [4–6, 18–20].

It is well known that *P. eryngii* contains mainly β-glucans, including β-(1,3)-(1,6)-glucans [21, 22]. The *P. eryngii* (per 100 g of fresh fruiting body) used in the present study contained β-glucan (1.9 g). Polysaccharides such as β-glucans are known to be biologically active substances [23–25]. β-Glucans have been reported to possess immunomodulatory/immunostimulatory activities, and β-glucans usually have a β-(1,3)-linked main chain and β-(1,6)-linked branches [23, 24, 26, 27]. The frequency of branching varies, and immunomodulatory/immunostimulatory activities are dependent upon the structure of β-glucans [28, 29]. It has been reported that β-(1,6) branches could contribute to the stimulatory activity of β-glucans [28]. In the present study, we did not have information on the function and structure of β-glucans. However, *P. eryngii* contains β-(1,3)-(1,6)-glucans [21, 22]. Lentinan is a β-glucan from the fruiting bodies of *Lentinus edodes* and a β-(1,3)-glucan with β-(1,6) branching. Lentinan has been shown to suppress LPS-induced secretion of NO and tumor necrosis factor-α from RAW264.7 macrophages [25]. Thus, β-(1,3)-(1,6)-glucans in HTPE may have important roles in anti-inflammatory actions and NO production in lungs treated with LPS. In the present study, nitrite concentration was correlated with total cell numbers in BALF ($r = 0.716$, $P \leq 0.001$). However, the decrease in nitrite concentration by HTPE was not completely correlated with the decrease in total cell numbers. β-Glucans inhibit LPS-induced NO production [25]. Thus, it is suggested that the decrease in nitrite concentration by HTPE is associated with the decrease in total cell numbers and inhibition of NO production by HTPE in inflammatory cells. The *P. eryngii* (per 100 g of fresh fruiting body) used in the present study also contained ergosterol (45.5 mg) and vitamin D2 (1.9 μg). The fungal sterol ergosterol (known as provitamin D2) is abundant in mushrooms, as is its peroxide [30, 31]. Sterols suppress LPS-induced inflammatory responses in RAW264.7 macrophages through inhibition of the transcriptional activity of NF-κB and CCAAT-enhancer-binding protein (C/EBP) β as well as phosphorylation of mitogen-activated protein kinases (MAPKs) [31]. Supplementation with vitamin D results in reductions in the levels of proinflammatory cytokines such as interleukin (IL)-4, IL-5, and IL-13 in the BALF of mice challenged with ovalbumin [32, 33]. Moreover, it has been reported that *P. eryngii* has a significantly higher amount of total phenolic compounds and has a close relationship

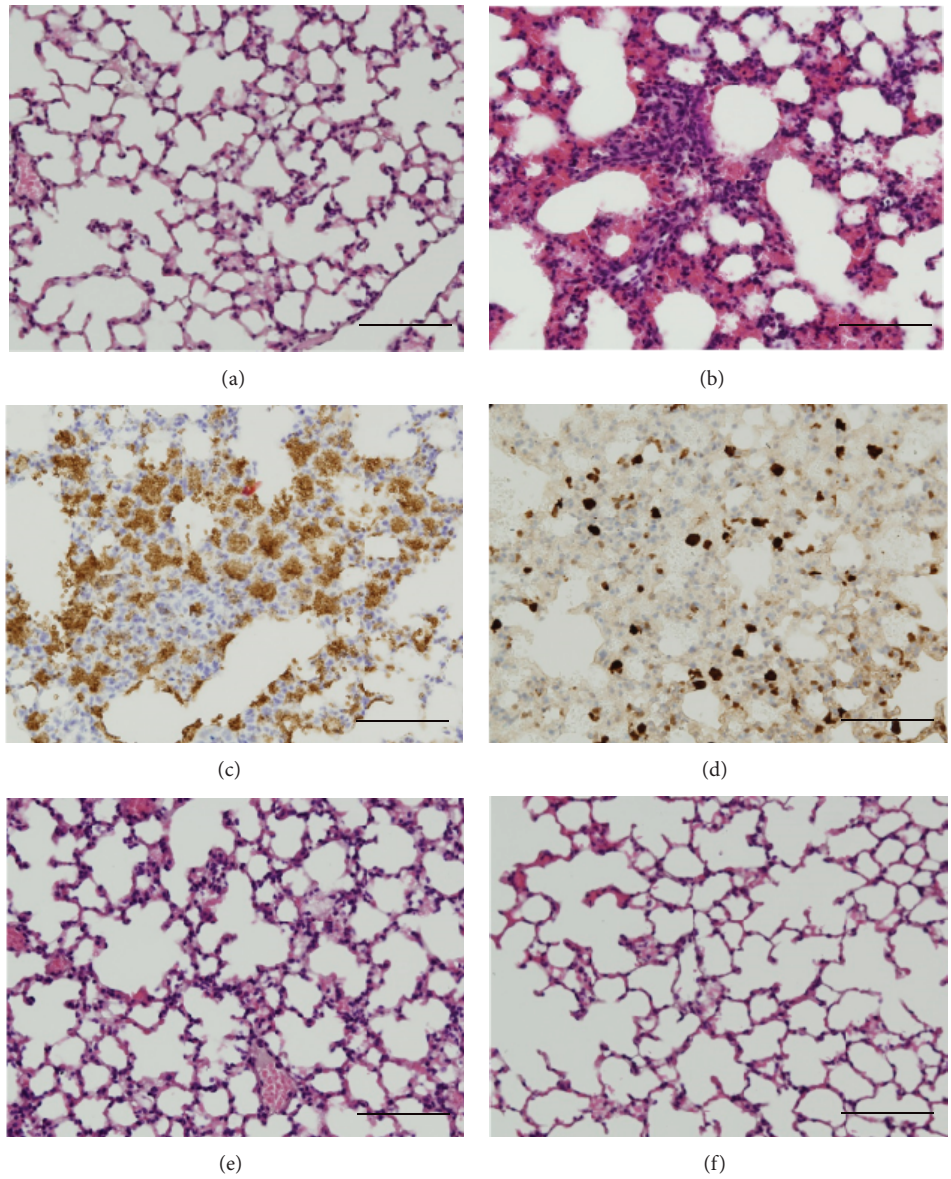

Figure 5: Effect of HTPE on histological changes in the lungs of LPS-induced ALI mice. Mice were administered, via the oral and intraperitoneal routes, HTPE (1 g/kg) and dexamethasone (DEX: 5 mg/kg, positive control group), respectively, 1 h before intranasal administration of LPS (10 μg/site). Vehicle (tap water, vehicle for HTPE) was also administered orally 1 h before intranasal administration of LPS (LPS group) or saline (vehicle for LPS, saline group (negative control group)). Lungs (n = 3-4) from each experimental group were processed for histological evaluation 6 h after LPS challenge. The result in each group was almost identical. Lung sections were stained with hematoxylin and eosin ((a), (b), (e), and (f)). Lung sections in (c) and (d) show the immunoreactivity of myeloperoxidase (a marker of neutrophils) and Mac-2 (a maker of macrophages). Scale bar: 100 μm. (a) Saline group (negative control group); ((b), (c), and (d))LPS group; (e) HTPE-treated LPS group; (f) DEX-treated LPS group (positive control group).

with antioxidant activity and 2,2-diphenylpicrylhydrazyl-scavenging activity [6]. Taken together, these findings suggest that the components of *P. eryngii* may contribute to the anti-inflammatory actions of HTPE *in vivo*.

We used HTPE obtained from the whole fresh fruiting bodies of *P. eryngii*. HTPE (0.3–1.0 g/kg) was effective against ALI in mice. HTPE at 0.3–1.0 g/kg is equivalent to a fresh fruiting body at 3–10 g/kg. The effective dose in mice cannot be applied directly for humans, but a fresh fruiting body of *P. eryngii* (180–600 g per person (60 kg body weight)) should be eaten to protect against ALI. We consider that intake of *P. eryngii* (which has anti-inflammatory effects and which many people can obtain) on a daily basis could be a useful prevention strategy against ALI.

5. Conclusion

HTPE obtained from the heat-treated fresh fruiting body of *P. eryngii* prevented LPS-induced ALI through inhibition

of infiltration of inflammatory cells, destruction of vascular integrity, and overproduction of NO and led to alleviation of histopathological damage. Our study supports the hypothesis that *P. eryngii* could be a beneficial food for the prevention of ALI associated with bacterial infection.

Conflict of Interests

The authors state that there is no conflict of interests.

Authors' Contribution

Junya Kawai and Tsugunobu Andoh contributed equally to this work.

Acknowledgment

The authors thank Mr. K. Matsunaga of the University of Toyama for technical assistance with the preparation of lung sections.

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Research Article

Improving Training Condition Assessment in Endurance Cyclists: Effects of *Ganoderma lucidum* and *Ophiocordyceps sinensis* Dietary Supplementation

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The main reasons for taking daily dietary supplements are to maintain good health, to improve homeostasis, and to create conditions for reducing the risk of disease. Due to growing market demand, the search for effective, nontoxic, natural compounds with antioxidant and ergogenic properties has increasingly become a matter of interest. This paper describes how a specific combination of fungal supplements can help improve the performance of endurance athletes. We report the effects of a brief 3-month trial of two fungal supplements, *Ganoderma lucidum* and *Cordyceps sinensis* (3 capsules of *O. sinensis* and 2 capsules of *G. lucidum* per day), in 7 healthy male volunteers, aged between 30 and 40 years, who are all amateur cyclists that participate in "Gran Fondo" cycling races. This trial investigated the effects of fungal supplements on the level of physical fitness of the athletes by monitoring and comparing the following biomarkers just before and after physical exertion: the testosterone/cortisol ratio in the saliva and oxidative stress (DPPH free radical scavenging activity). A decrease of more than 30% in the testosterone/cortisol ratio after race compared to before race was considered as a risk factor for nonfunctional overreaching (NFO) or the overtraining syndrome (OTS). The results show that, after 3 months of supplementation, the testosterone/cortisol ratio changed in a statistically significant manner, thereby protecting the athletes from NFO and OTS. Antioxidant activity was measured by quantifying the scavenging ability of the human serum on the synthetic free radical DPPH. After 3 months of fungal supplementation, the data demonstrate an increased scavenger capacity of free radicals in the athletes' serum after the race, thereby protecting the athletes from oxidative stress.

1. Introduction

Fatigue and underperformance are common in athletes and can affect more than 65% at the peak of their career. The European College of Sport Science has defined these symptoms as nonfunctional overreaching (NFO) and the overtraining syndrome (OTS) [1–3]. Athletes increase their training load in order to improve their performance. However, a maladapted response to excessive exercise, without adequate rest, can lead to nonfunctional performance, such as a discrepancy between the stress incurred during training or competing and the athlete's ability to recover after exercise. The most commonly encountered effects are underperformance, reduced tolerance to training load, decreased coordination, and increased heart rate.

NFO is a performance decrement that requires 72 hours of recovery, while OTS is a performance decrement that requires days or weeks of recovery after maximal physical exercise [1–3]. It is clinically difficult to differentiate between NFO and OTS as the difference is based on the time it takes for athletes to recover and not necessarily on the degree or type of symptoms. Thus, a period of complete rest is often required before it is possible to diagnose one syndrome or the other. Many authors consider overreaching and overtraining as a continuum, while others suggest that NFO precedes OTS [3].

It would be very useful to understand the mechanisms underlying underperformance in order to assess, manage, and educate the athlete. The overtraining syndrome (OTS) disturbs many body systems (neurologic, endocrinologic, and immunologic systems). Many hypotheses have been presented to explain the pathogenesis of the OTS [2]. The hypothalamic hypothesis suggests that deregulation of the hypothalamus and hormonal axes activates the hypothalamic-pituitary-adrenal axis in endurance athletes, but data in the literature are contradictory. Furthermore, this hypothesis suggests that endurance athletes may experience changes in levels of cortisol, adrenocorticotropic hormone, testosterone, and other hormones. Unfortunately, literature data prove contradictory in terms of the pattern of hormonal changes involved [4–7]. One of the indices that is used to evaluate the stress induced by physical exercise is the ratio between the levels of testosterone and cortisol (T/C), both in basal conditions and after exercise. The T/C ratio is used to define the general metabolic trend in an organism: a decrease indicates a catabolic tendency, while an increase indicates an anabolic trend. In particular, data from the scientific literature show that a decrease of less than 30% in the T/C ratio indicates an effective workout, while a decrease of more than 30% from baseline in the T/C ratio is a risk factor for overtraining [7].

The oxidative stress hypothesis suggests that excessive oxidative stress causes muscle damage and fatigue. A small amount of data indicates that markers of oxidative stress are higher at rest in overtrained athletes and increase with exercise.

Many fungal species have recently been reported to display antioxidant and ergogenic properties, demonstrating what has already been claimed by Traditional Chinese Medicine (TCM) [8, 9]. One of the most popular fungi in this group is *Ganoderma lucidum* (Curtis) P. Karst., better known in western countries by the Japanese term "Reishi"; it is defined in Traditional Chinese Medicine (TCM) as the "mushroom of immortality." *Ganoderma lucidum* preferentially grows on broadleaved trees, such as oak or chestnut, mainly as a saprotroph and only rarely as a parasite. This species has been reported to have multiple beneficial values because it contains a vast number of bioactive compounds, the most pharmacologically active of which being triterpenes and polysaccharides [10–14]. Another two of these bioactive compounds are adenosine monophosphate, thought to help lower blood pressure and remove blood vessel blockage, and superoxide dismutase, an antiaging substance. The main medicinal properties of this fungus are immune response modulating, anticancer, antihypertensive, antioxidant, antiaging, anti-inflammatory, antiviral, and cardio- and nephroprotection [15, 16].

Another important medicinal fungal species is *Ophiocordyceps sinensis* (Berk.) G. H. Sung, J. M. Sung, Hywel-Jones & Spatafora, more commonly known as *Cordyceps sinensis* (Berk.) Sacc. According to the last taxonomic revision, it is an ascomycete, endemic to the Tibetan Plateau and surrounding Himalayas. The fungus is parasitic and colonizes the larvae of a moth, *Hepialus armoricanus*, forming a complex that includes the remains of the parasitic larva and the stroma of the fungus (sclerotia). In its growth phase, the fungus gradually turns into a fruiting body. *Ophiocordyceps sinensis* is used in Traditional Chinese Medicine (TCM) to promote health, longevity, and athletic power because of its tonic effect, reducing fatigue. Recent studies have demonstrated that the chemical constituents extracted from this species have various protective effects on the nervous system, cardiovascular diseases, proliferation of tumor masses, lipid metabolism, and infection [17]; moreover, it has antiaging properties [18]. One of the constituents isolated from *O. sinensis,* cordycepin (3′-deoxyadenosine), is a derivative of the nucleoside adenosine, differing only in the absence of an oxygen molecule in the 3′ position of the ribose.

Athletes who perform endurance training undergo oxidative stress and potentially suffer from the overtraining syndrome. The aim of the present study was to evaluate the effects of two fungal supplements, used in TCM, on the athletic performance of road cyclists. Considering the different properties of the two fungal species mentioned above, we suggested that the athletes in our study group take this specific combination of dietary supplements.

Table 1: Age, weight, height, body mass index (BMI), and related SD of amateur cyclists who participated in the trial.

	Age (years)	Weight (Kg)	Height (cm)	BMI (Kg/m^2)
Mean	39.7	73.8	176.7	23.7
SD	5.0	6.1	5.5	1.6
n	7	7	7	7

2. Materials and Methods

2.1. Subjects. We selected a sample of 7 healthy male volunteers, aged between 30 and 40 years, who were all amateur cyclists and duly informed them of the procedures to be followed in the study. The subject age, weight, height, and BMI are reported in Table 1. All the subjects had been cycling for more than 10 years and usually performed about 300 km per week and 12,000/15,000 km per year. Each procedure was drawn up in agreement with the Helsinki Declaration adopted at the Eighteenth General Assembly of the World Medical Association (WMA), held in 1964 on ethical principles for medical research involving human subjects, and with the permission of the Ethic Committee of the University of Pavia, Italy.

The subjects were instructed not to change their lifestyle during the trial, including exercise, diet, and other routine activities, and not to take any other medicinal herbs or drugs. They were also instructed to report any adverse events to the investigators during the trial: none occurred.

2.2. Study Design. To assess the training condition of the athletes, according to the hypothalamic hypothesis, we evaluated the T/C ratio before and after race [7]: an increase in the after race value of more than 30% compared to the before race value was considered a risk factor for overtraining. Using

saliva to calculate the levels of cortisol and testosterone is a valid alternative to using plasma: the concentration of cortisol and testosterone in saliva represents the hormone-free part and thus the biologically active component [19]. In order to test the oxidative stress hypothesis, we measured the free radical scavenging activity by DPPH.

We performed a double-blind clinical study. The volunteers took placebo supplements for the first month and then active supplements of *O. sinensis* and *G. lucidum* for the following 3 months. During the trial, the athletes performed daily workouts and took part in 2 "Gran Fondo" cycling races. During the placebo phase, we monitored the athletes before and after the first race, Gran Fondo Laigueglia, with a distance of 110 km, a change in elevation of 1651 m, a duration of about 3 hours and 40 minutes, and an average speed of 36 km/h. During the fungal supplementation phase, we monitored the athletes before and after the second race, Gran Fondo Aprica, with a distance of 85 km, a change in elevation of 1850 m, a duration of about 4 hours, and an average speed of 33 km/h. The latter course is particularly hard due to the slopes and technical difficulties.

2.3. Equipment. In order to monitor energy expenditure during races, we used a Sense Wear Pro Armband TM: a commercially available device used to estimate energy expenditure [20, 21]. The Sense Wear Armband (Sense Wear Body Monitoring System by BodyMedia Pittsburgh, PA, USA) is a single device that combines five different sensors. This device is worn on the right upper arm over the triceps muscle and monitors various physiological and movement parameters: heat flux, accelerometer, galvanic skin response, skin temperature, near body temperature, and demographic characteristics including gender, age, height, and weight. These parameters are used to estimate energy expenditure utilizing proprietary equations developed by the manufacturer. Computer software (Sense Wear Professional software 6.1 by BODYMEDIA) applies activity-specific algorithms to calculate energy expenditure based on the analysis of the pattern of signals from the sensors. Metabolic equivalents (METs expressed in Kcalorie/Kg/hour) represent the energy cost of physical activities as multiples of the metabolic rate at rest [20]. Data were collected and MET were measured during the Gran Fondo races, during both the placebo and fungal supplementation phases.

2.4. Chemicals. All chemicals and solvents used in this study were supplied by Sigma-Aldrich Co. and kits for the ELISA test were supplied by the company DiaMetra©, 20090 Segrate-Milano, Italy.

2.5. Supplementation Protocol. Subjects were provided with either two commercially available capsules, one of which contained a formulation of *Ophiocordyceps sinensis* and the other a formulation of *Ganoderma lucidum*, or with placebo capsules that were identical in shape and colour. The placebo capsules contained hydroxypropyl methylcellulose (HPMC) and 18% mannitol. Every day, during the placebo phase, athletes took 5 capsules: 1 with breakfast, 2 with lunch, and 2 with dinner. Every day, during the fungal supplementation phase, athletes took 3 capsules: 1 capsule of *O. sinensis* with breakfast, 1 capsule of *O. sinensis* and 1 capsule of *G. lucidum* with lunch, and 1 capsule of *O. sinensis* and 1 capsule of *G. lucidum* with dinner.

The first phase of the trial protocol consisted of a month of placebo capsule intake as specified above. At the end of this phase, the athletes participated in the first bicycle race.

Immediately following the placebo phase, there was a three-month fungal supplementation phase with fungal capsule intake as specified above, up to three days before the second race, which took place at the end of this phase. The dose was increased for the final three days of this three-month period before the second race (6 capsules of *O. sinensis* + 2 capsules of *G. lucidum*/day).

Ophiocordyceps sinensis (Cordyceps sinensis). Ingredients per capsule are 445 mg standardized extract obtained from mycelium. Nutritional information is as follows: energy per capsule 0.12 Kcal, 33.2% polysaccharides including 7.8% β-glucan, 25.3% protein, 18.3% mannitol, 3.6% fat, and 0.45% adenosine.

Ganoderma lucidum. Ingredients per capsule are 390 mg pure extract and 3.9 mg of silicon dioxide. Nutritional information is as follows: energy per capsule 0.12 Kcal, 33% polysaccharides of which 24.6% β-glucans, 23% protein, 2.1% fat, and 1.5% triterpenes.

Daily fungal supplementation consisted of 1335 mg of standardized *Ophiocordyceps sinensis* extract from mycelium containing 34.71 mg of β-glucans and 1170 mg of *Ganoderma lucidum* pure extract, containing 95.9 mg of β-glucans and 4.5 mg of triterpenes.

2.6. Collection and Processing of Saliva and Human Serum Samples. Saliva and blood were collected just before and just after physical exertion, for each race.

The subjects were instructed to deposit saliva into a collector (Saliva Collector Devices ce DKO063-DiaMetra© Italy). The saliva was frozen at −20°C and maintained at this temperature until it arrived in the laboratory. The samples were centrifuged for 15 minutes at 655 g to remove any remaining food or possible contaminants, after which the recovered supernatant was analyzed.

Health care workers obtained venous blood samples from the volunteers using serum calibrated vacuum tubes, containing gel polymers. The collected blood was immediately centrifuged at 218 g for 15 minutes to separate the serum from the cellular component. The serum obtained was stored at −80°C.

2.7. Determination of Testosterone and Cortisol in Saliva by Direct Enzyme Immunoassay. The calculation of the amount of cortisol [22] and testosterone [23] in saliva were performed by means of competitive ELISA immunoenzymatic colorimetric methods (DiaMetra©, Italy).

2.8. Evaluation of Human Serum Antioxidant Capacity. We assessed the antioxidant effect of the athletes' serum on the stable radical cation chromophore, DPPH (1,1′-diphenyl-2-picrylhydrazyl), one of the most stable and easy-to-use synthetic free radicals. We therefore assessed the ability of the molecules present in the serum to act as scavengers of the free radical reaction, which consists in donating a hydrogen molecule to DPPH. The antioxidant capacity was calculated using a spectrophotometric measurement (spectrophotometer Shimadzu UV-1800), by the decrease in absorbance at a wavelength (λ) of 517 nm, which was observed following the capture of the free radical. Antioxidant activity is expressed in FRSA (free radical scavenging activity) as DPPH reduced μ mol/mL sample [24]. Human serum samples were treated before being analyzed: the serum was boiled at 95°C for 10 minutes to deproteinize the sample (mineralization). Subsequently, the sample was centrifuged at 2000 g for 20 minutes and the supernatant used for analysis [24].

2.9. Data Analysis. Descriptive statistics were expressed as means and standard error of the mean (SEM). Statistical analysis for the difference between before and after race and intergroup differences was performed using a univariate Student's paired t test. The level of statistical significance was set at a P value of <0.05 ($^{*}P$ value <0.05; $^{**}P$ value <0.01). NS means not statistically significant. Student's t test was applied after evaluating data assumption of normality with Shapiro-Wilk test.

3. Results

Seven healthy male volunteers, aged between 30 and 40 years, who are all amateur cyclists that participate in "Gran Fondo" cycling races were included in the trial.

During both placebo and fungal supplementation we measured energy expenditure during exercise by means of a Sense Wear Pro Armband TM (see Section 2): MET values for all 7 athletes ranged between 4.5 and 5 METs. All subjects in both conditions (placebo and fungal supplements) have the same energy expenditure during exercise, suggesting similar exercise intensity during races.

3.1. Performance Assessment. Figure 1(a) reports salivary testosterone levels just before the race (before race) and just after the race (after race) in the placebo condition. In 4 out of the 7 athletes tested, the testosterone level decreased after the race (samples 1, 2, 3, and 6; mean value after race, 0.06 ± 0.01 ng/mL versus 0.025 ± 0.006 ng/mL value before race, mean reduction 66.7%, $n = 4$, $P < 0.049$), whereas in the other 3 athletes, the testosterone level increased after the race (samples 4, 5, and 7, mean value after race, 0.055 ± 0.006 ng/mL versus 0.034 ± 0.01 ng/mL, $n = 3$, mean increase 61.7%, $n = 3$, t test not significant).

Figure 1(b) shows salivary cortisol level just before and just after the race in the placebo condition. In 4 out of the 7 athletes tested, the cortisol level increased after the race (samples 2, 5, 6,and 7; mean value after race, 17.9 ± 2 ng/mL versus 6.34 ± 1.83 ng/ml value before race, mean increase, 182.3%, $n = 4$, $P < 0.01$), whereas in the other 3 athletes, the cortisol level decreased after the race (samples 1, 3, and 4; mean value after race, 7.55 ± 3.22 ng/mL versus 12.57 ± 1.49 ng/mL value before race, $n = 3$, mean decrease 39.9%, $n = 3$, t test not significant).

Figure 1(c) reports the testosterone/cortisol ratio before and after the race in the placebo condition (values multiplied by 100). In 5 out of the 7 athletes tested (subjects 2, 3, 5, 6, and 7), the testosterone/cortisol ratio decreased after the race compared to before the race by more than 30% (values multiplied by 100: 0.19 ± 0.04 mean value after race versus 0.7 ± 0.11 mean value before race, mean decrease 72.9%, $n = 5$, $P < 0.0006$). These 5 athletes are therefore at risk of overtraining. However, in the other 2 athletes (subjects 1 and 4) the testosterone/cortisol ratio increased after the race compared to before the race (values multiplied by 100: before race value 0.4 ± 0.11 versus 1.04 ± 0.003 after race value, increase ratio 160%, $n = 2$). More specifically, subject 1, who is a well-trained athlete, showed a slight decrease in testosterone level and a great decrease in cortisol level after race, whereas subject 4 showed a slight increase in testosterone level and a great decrease in cortisol level after race.

Therefore, we divided the athletes into two groups: well-trained (WT, subjects 1 and 4) and at risk of overtraining (OT, subjects 2, 3, 5, 6, and 7).

3.2. Effects of Fungal Supplements on Well-Trained Athletes. We studied the effects of two fungal supplements on the two well-trained athletes (Figure 2). The before race basal level of salivary testosterone increased after the fungal supplementation phase that lasted 3 months (0.09 ± 0.02 ng/mL after fungal supplementation versus 0.044 ± 0.005 ng/mL in the placebo condition, Figure 2(a)). The after race testosterone level after fungal supplementation increased even more compared to the after race level in the placebo condition (0.19 ± 0.016 ng/mL after fungal supplementation versus 0.045 ± 0.002 ng/mL in the placebo condition; see Figure 2(a)).

The before race basal level of salivary cortisol decreased after the fungal supplementation phase that lasted 3 months (7.5 ± 2.3 ng/mL after fungal supplementation versus 11.7 ± 2.12 ng/mL in the placebo condition, Figure 2(b)). The after race cortisol level after fungal supplementation increased compared to the after race level in the placebo condition (6.5 ± 1.9 ng/mL after fungal supplementation versus 4.33 ± 0.22 ng/mL in the placebo condition (Figure 2(b))).

The increase in the testosterone/cortisol ratio is shown in Figure 2(c). After 3 months of fungal supplementation, the testosterone/cortisol ratio of the two athletes increased both before race (1.2 versus 0.38) and after race (ratio 2.9 versus 1.04) compared to the ratio in the placebo condition.

3.3. Effects of Fungal Supplements on Athletes at Risk of Overreaching/Overtraining. Figure 3 shows the before race basal level of salivary testosterone in the placebo phase and after 3 months of fungal supplementation. The mean before race basal testosterone level increased after fungal supplementation compared to the level in the placebo condition (0.08 ± 0.02 ng/mL versus 0.052 ± 0.01 ng/mL, $n = 5$,

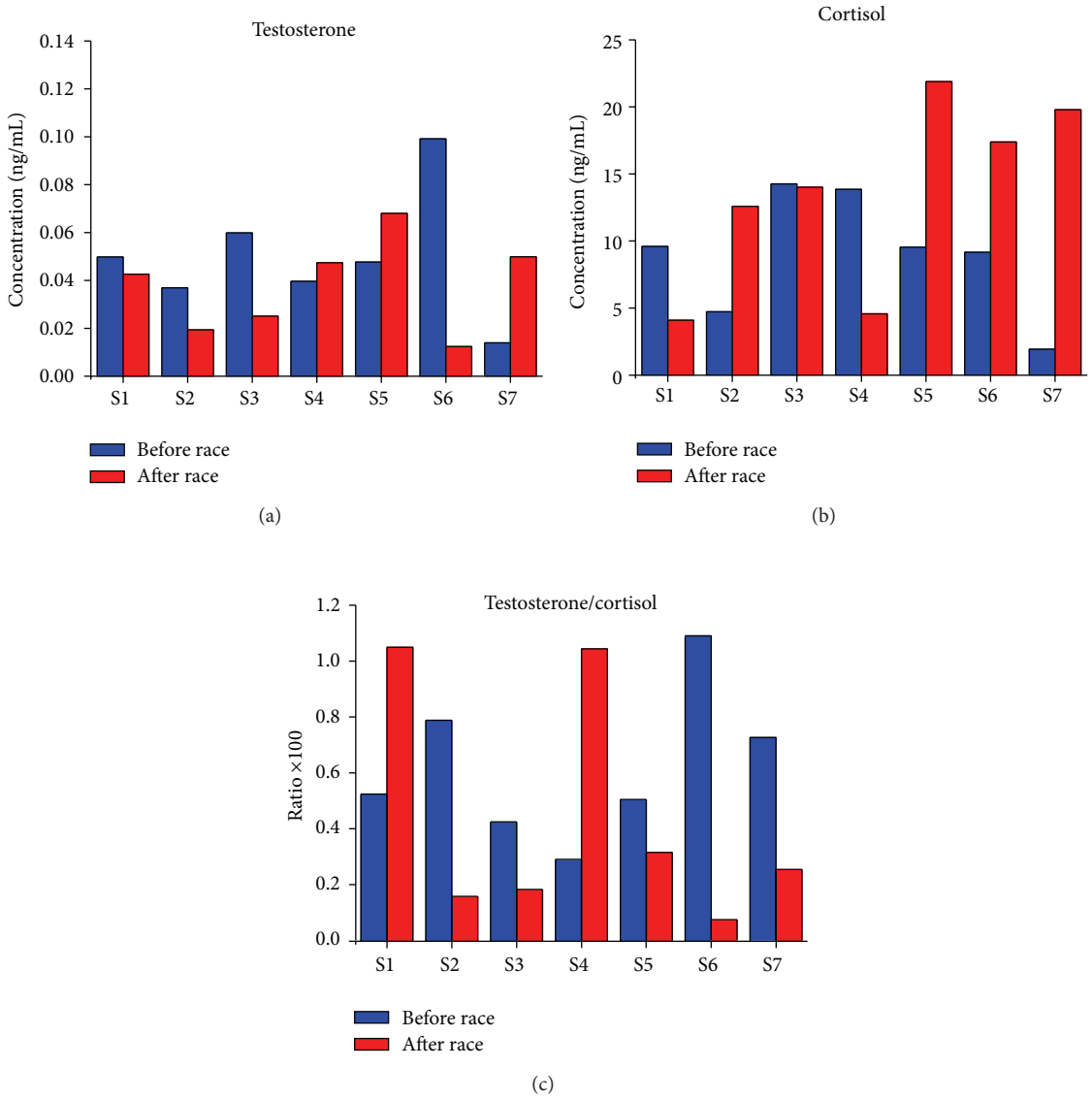

Figure 1: Placebo supplementation. Training condition assessment. Salivary testosterone (a), cortisol (b), and testosterone/cortisol ratio (c) in the placebo condition in 7 athletes (subjects S1–S7) just before the race (before race, blue) and just after the race (after race, red). The testosterone/cortisol ratio value is multiplied by 100.

$P < 0.027$). The after race testosterone level after fungal supplementation increased 3.4-fold compared to the after race level in the placebo condition (0.12 ± 0.04 ng/mL versus 0.035 ± 0.01 ng/mL, $P < 0.016$, Figure 3(a)).

The before race basal level of salivary cortisol after fungal supplementation is not statistically different compared to the level in the placebo condition (6.43 ± 1.75 ng/mL versus 7.92 ± 2.12 ng/mL). The after race cortisol level after fungal supplementation increased compared to the level in the placebo phase (10.58 ± 2.2 ng/mL versus 17.13 ± 1.74 ng/mL). The 2.2-fold increase of cortisol level in the placebo condition is statistically different (before race versus after race, $P < 0.03$), whereas the 1.6-fold increase after fungal supplementation is not statistically different.

We evaluated the testosterone/cortisol ratio in athletes at risk of overtraining, just before and just after the races in both the placebo phase and after 3 months of fungal supplementation (Figure 3(c)). After 3 months of fungal supplementation, the ratio values of the two athletes increased both before race (1.24 versus 0.66, values multiplied by 100) and after race (ratio 1.13 versus 0.2, values multiplied by 100).

In the placebo condition, the testosterone/cortisol ratio decreased by an average of −69.3%, suggesting that the athlete was at risk of overtraining [20], while after fungal supplementation it decreased by an average of −8.7%, so the athlete was no longer at risk of overtraining.

Four out of the 5 athletes who were shown to be at risk of overtraining in the placebo condition overcame

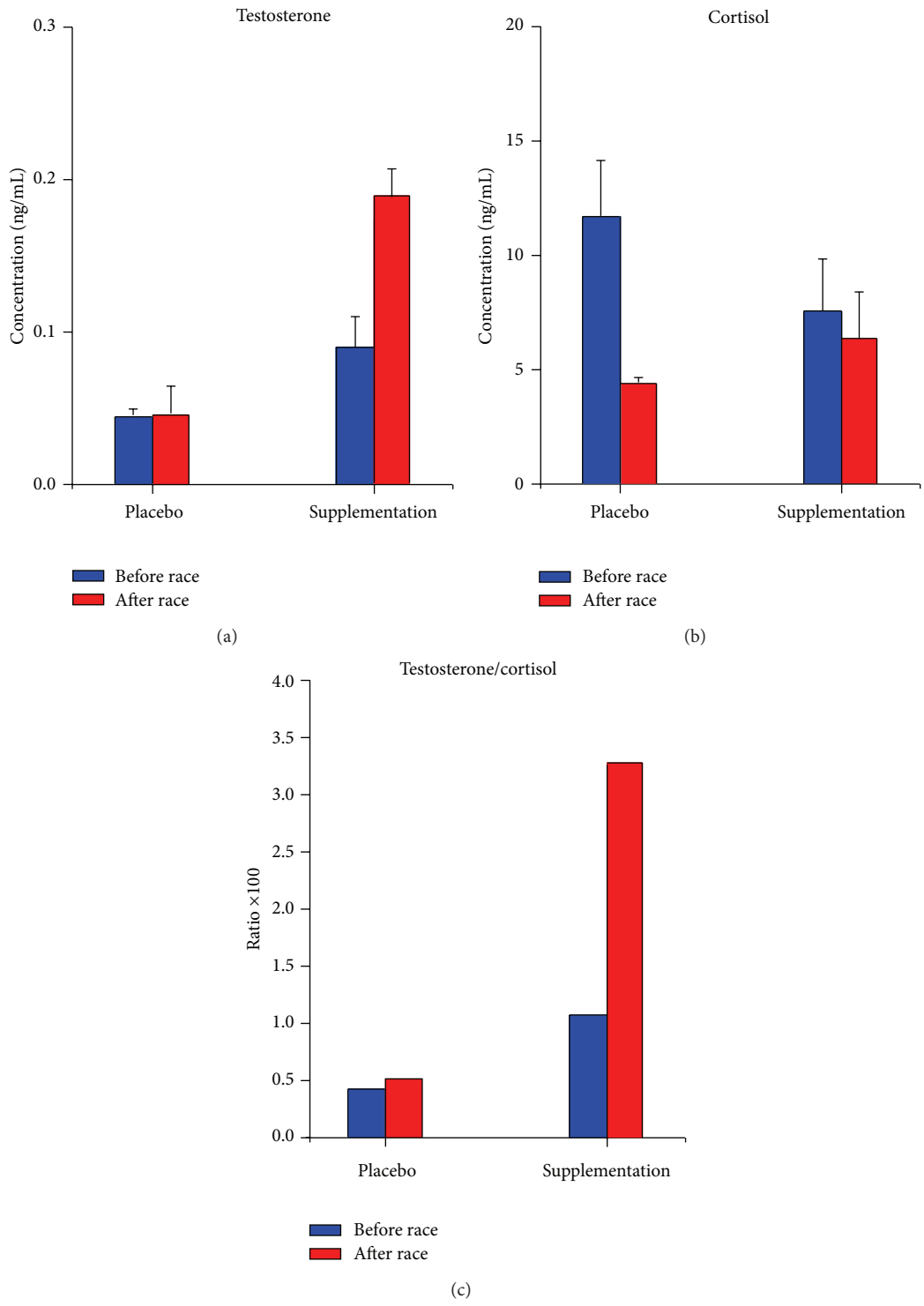

Figure 2: Well trained athletes. *Ophiocordyceps ganoderma* supplementation. Effects of fungal supplementation on well-trained athletes. Salivary testosterone (a), cortisol (b), and testosterone/cortisol ratio (c) after 3 months of *Ophiocordyceps ganoderma* fungal supplementation before the race (before race, blue) and after the race (after race, red). Data are reported as mean ± sd. The testosterone/cortisol ratio value is multiplied by 100.

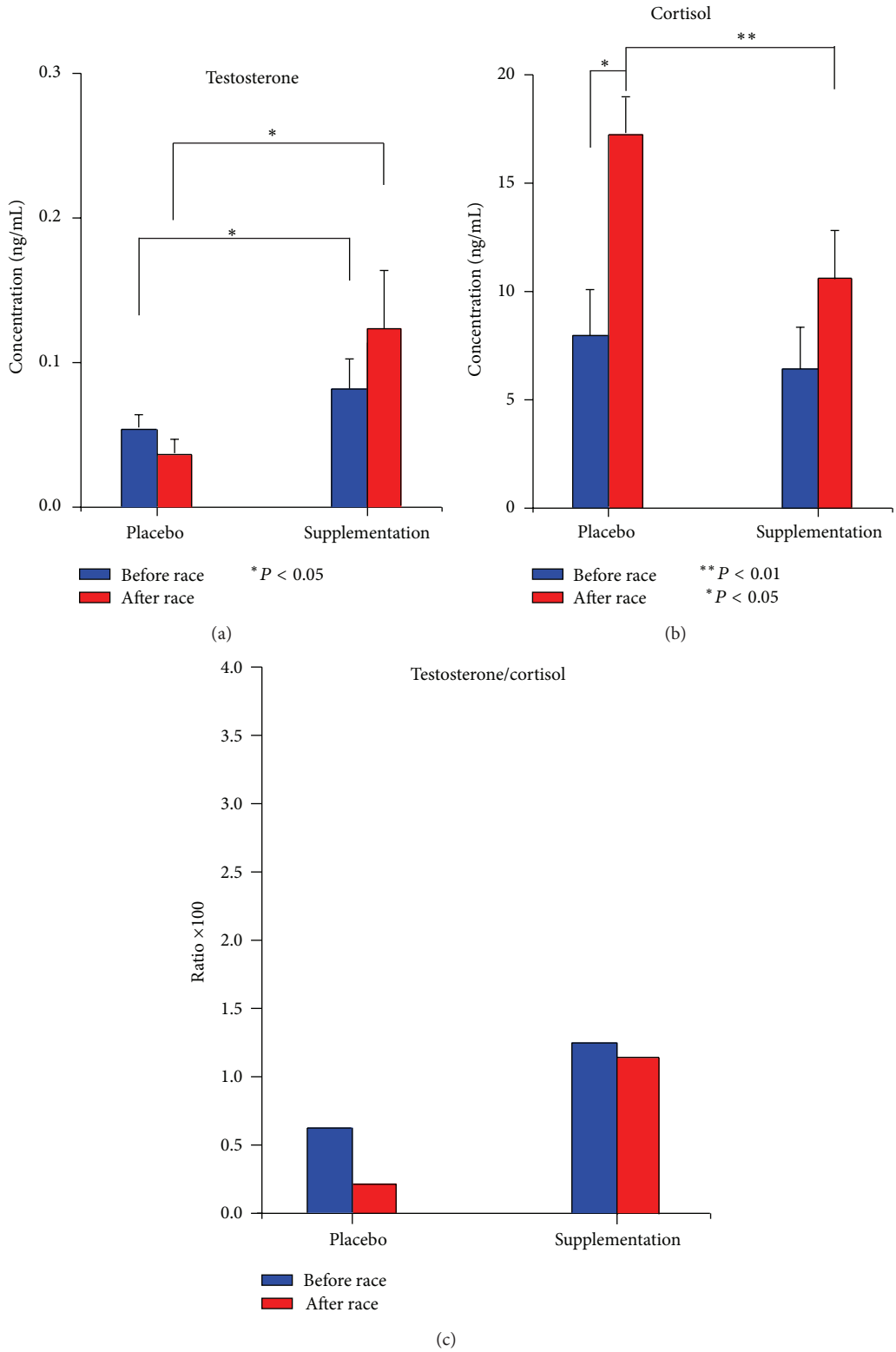

Figure 3: Inadequately trained athletes. *Ophiocordyceps ganoderma* supplementation. The effects of fungal supplementation on athletes at risk of overreaching/overtraining. Salivary testosterone (a), cortisol (b), and testosterone/cortisol ratio (c) after 3 months of *Ophiocordyceps ganoderma* fungal supplementation before the race (before race, blue) and after the race (after race, red). Data are reported as mean ± SEM. The testosterone/cortisol ratio value is multiplied by 100. The level of statistical significance was set at a P value of <0.05 ($^{*}P$ value <0.05; $^{**}P$ value <0.01). NS: not statistically significant.

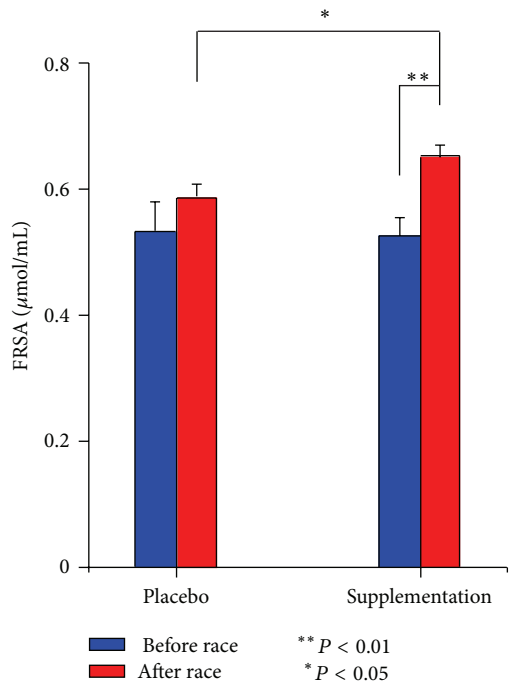

Figure 4: DPPH. *Ophiocordyceps ganoderma* supplementation. Human serum antioxidant capacity. Analysis of DPPH before and after the race. Measurements expressed in free radical scavenging activity (FRSA) were carried out in triplicate for each subject and are presented as mean ± mean standard error. Experimental conditions: placebo values just before the race (before race placebo) and just after the race (after race placebo); 3 months of fungal supplementation just before the race (before race *Ophiocordyceps ganoderma*) and just after the race (after race *Ophiocordyceps ganoderma*). The level of statistical significance was set at a P value of <0.05 ($^{*}P$ value <0.05; $^{**}P$ value <0.01). NS: not statistically significant.

these symptoms after fungal supplementation, whereas the testosterone/cortisol ratio in the remaining athlete in this group did not improve. After fungal supplementation, all athletes displayed an increase in the T/C ratio after race compared to before race.

3.4. Assessment of the Antioxidant Capacity of the Human Serum. The antioxidant capacity of the serum was assessed by its ability to scavenger DPPH (Figure 4) before and after race in the placebo phase and after 3 months of fungal supplementation. The antioxidant activity is expressed in FRSA (free radical scavenging activity) as DPPH reduced μmol/mL sample (see Section 2).

In the placebo condition, before and after race DPPH values were not statistically significant. After 3 months of fungal supplementation, before and after race average DPPH values were statistically different ($n = 7$, $P < 0.009$, paired t test). Furthermore, after 3 months of fungal supplementation, FRSA after race values increased in a statistically significant manner compared to after race values in the placebo condition ($n = 7$, $P < 0.027$, paired t test).

4. Discussion

This paper investigates the problem of underperformance in athletes by referring to two different hypotheses in the pathogenesis of nonfunctional overreaching and overtraining: the hypothalamic hypothesis and the oxidative stress hypothesis.

We tested the effect of the dietary fungal supplements *Ophiocordyceps sinensis* and *Ganoderma lucidum* both on the levels of testosterone and cortisol in the saliva of road cyclists and on the scavenger ability of free radicals in their serum.

The link between hormonal status and oxidative stress has not been considered in previous publications. Excessive oxidative stress is an important mediator of a decline in steroid hormone production mediated by activated p38 mitogen-activator protein kinase (p38MAPK) [25].

The "stress response" paradigm is comparable to adrenal hyperfunction, according to which psychophysical stress and sensible activation of the hypothalamic-hypophyseal-adrenal axis cause oxidative damage. In animal models of restraint stress, adrenal hyperfunction leads to a decrease in antioxidant enzymes (SOD, Cat, GSH transferase, and reductase) and a reduction of GSH and urate in serum. In the same animal model, lipid peroxidation and carbonyl contents significantly increase in the brain, liver, and heart suggesting a causal role of stress hormones in oxidative processes induced during the adaptive response [26, 27]. Oxidative stress and inflammation are associated with fatigue and bad recovery and are risk factors for overreaching in intense exercise. Antioxidant treatment may have a positive effect on markers of oxidative stress, inflammation, and cortisol response [28]. Furthermore, there is a correlation between sex hormones and plasmatic total antioxidant capacity (TAC), and TAC significantly correlated with total testosterone [29].

The generation of reactive oxygen and nitrogen species (RONS) in response to intense exercise can occur via several pathways, including mitochondrial respiration (electron leakage from electron transport chain and subsequent production of the superoxide radical), which is the main metabolic mechanism involved in the performance of cyclists. An alteration in the redox state in favor of the generation of RONS is necessary in order to initiate signaling pathways; however, when acute or chronic free radicals are produced, the antioxidant defense system can be overwhelmed, thereby disrupting normal redox-sensitive signaling and causing a permanent shift in "redox homeostasis" [30, 31]. The development of procedures to ameliorate the production of undesirable RONS may become one of the core research areas in this field.

It is common practice for athletes to use antioxidant dietary supplements as they are known to prevent the adverse effects of exercise-induced oxidative stress, hasten the recovery of muscle function, and improve performance [32, 33]. The most commonly used supplements include vitamin E, vitamin C, β-carotene, coenzyme Q10, lipoic acid, N-acetylcysteine, allopurinol, quercetin, resveratrol, and several other polyphenolic compounds or a combination of the above. However, free radicals not only cause damage but they also have a role in cell signaling. RONS produced during exercise act as signals that regulate molecular events that

are important in muscle cell adaptations to exercise [34]. The practical consequence is that antioxidant administration prevents such adaptations. Antioxidants, especially in high doses, have recently been shown to increase markers of exercise-induced oxidative stress or to inhibit the beneficial physiological or muscle adaptations induced by ROS [34, 35]. The practical implication is that by decreasing the effects of RONS using antioxidants, beneficial cell adaptations may be hindered during exercise.

Our data suggest that athletes may have a viable alternative to taking antioxidant dietary supplements, namely, micotherapy supplements containing nutriceuticals that do not greatly interfere with antioxidant exercise-induced adaptations, thereby improving systemic redox.

The fungal species *Ophiocordyceps sinensis* is mainly used in TCM as a tonic. It has been suggested that its antioxidant effects improve performance. The molecular mechanism responsible for the effects this fungus has on physical fitness has not yet been clarified. A pilot study [36] carried out using a fermented product of standardized *O. sinensis* on 20 healthy elderly subjects describes a 10% increase of the metabolic threshold (from 0.84 to 0.93 L/min) and of the ventilatory threshold (8.5%). In rats, oral administration of *O. sinensis* causes a statistically significant increase in the period of endurance (from 1.79 to 2.9 times) compared to the control group [36]. In the gastrocnemius muscle of the rat, higher levels of some molecules were measured, with metabolic functions at muscular level [37].

Ophiocordyceps sinensis is a powerful hydrogen donor, and the protection effect of oxidative damage is due to its free radical scavenger ability. The protective effects of *O. sinensis* against oxidative damage to lipids, proteins, and low-density lipoproteins (LDL) are not due to the presence of cordycepin and adenosine but due to the presence of polyphenols and flavonoids [38].

Antioxidant properties have also been reported in assays for lipid oxidation of the polysaccharide fraction from the mycelium of *O. sinensis* [39]. Sporophore water and ethanol extracts of *Ophiocordyceps sinensis* possess potent antioxidant properties [40].

The other fungal species in this study, *Ganoderma lucidum*, contains many bioactive compounds amongst which triterpenes have displayed antioxidant effects and exert a scavenging action on free radicals (superoxide, peroxyl, DPPH) *in vitro* [41, 42]. It is noteworthy that some differences between European or Chinese strains and sporophores have been registered [43, 44].

The administration of extracts of triterpenes for 1 month has been shown to enhance the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase, GPX in mice, *in vivo* [41, 42]. Even at low concentrations, triterpenes have been found to be effective in preventing DNA damage and in reducing apoptosis. They also reduce the formation of reactive oxygen species (ROS) and increase the activity of endogenous antioxidant enzymes in splenic lymphocytes subjected to irradiation. This suggests that triterpenes isolated from *G. lucidum* are able to protect cells from radiation-induced damage, indicating possible applications in the field of therapy [38, 39]. Furthermore, crude ethanol and water extracts from *Ganoderma* are a rich source of antioxidant compoundssuch as phenols, ascorbic acid, β-carotene, and lycopene and display antioxidant properties in some different assays, namely, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and the metal chelating activity against ferrous ions.

We divided the athletes who participated in this study into two groups, according to their testosterone/cortisol ratio before and after race: well-trained athletes (2 subjects) and athletes at risk of overtraining (5 subjects).

There was no difference between the before and after race testosterone levels in the two well-trained athletes during the placebo phase, whereas there was a 2-fold increase in the before race basal testosterone level after fungal supplementation compared to the placebo condition and a 4-fold increase in the after race level. Cortisol levels did not change in a statistically significant manner before and after fungal supplementation or before and after race. The T/C ratio and scavenger activity of free radicals improved in both athletes.

After 3 months of fungal supplementation, the T/C ratio and the scavenger activity of free radicals increased in a statistically significant way in the other 5 athletes, who were potentially at risk of OTS during the placebo phase (as a result of incorrect or insufficient training), thus protecting them from overreaching and/or overtraining. In the placebo condition, after race levels of testosterone decreased compared to before race, while after race levels of cortisol increased compared to before race. After fungal supplementation, before race basal testosterone levels increased compared to before race levels in the placebo condition, and before race levels increased even more compared to the placebo condition, reaching a 3-fold increase. Before race basal cortisol levels did not change after fungal supplementation, but after race levels increased after fungal supplementation compared to levels in the placebo condition, demonstrating that the fungal supplements protected the athletes at risk of overtraining. The statistically significant increase in FRSA after race after fungal supplementation and the statistically significant difference in after race values after fungal supplementation compared to the placebo condition suggest that the 7 athletes are protected from an increase in free radical production during physical exercise.

We cannot distinguish between the two states of underperformance, because the difference is based on time before recovery and not on the degree or type of symptoms [2].

We can therefore conclude that a 3-month period of *O. sinensis* and *G. lucidum* dietary supplementation may protect endurance athletes from nonfunctional overreaching/overtraining. An interesting future development of this research would be to analyze inflammatory parameters in order to understand the role fungal supplementation plays on the immune system. The study should continue to select standardized fungal dietary supplements, but it should be expanded to include a larger number of endurance athletes, due to the variability in their athletic condition [45].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

The Effect of Mushroom Beta-Glucans from Solid Culture of *Ganoderma lucidum* on Inhibition of the Primary Tumor Metastasis

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This study evaluates the effect of mushroom beta-glucans (MBGS) derived from solid culture of *Ganoderma lucidum* on tumor inhibition by examining size of the primary tumor and rate of metastasis in Lewis lung carcinoma (LLC) bearing mice (C57BL/6), given oral administration of MBGS with radiation therapy. A previous result showed that MBGS enhances NK cell-mediated cytotoxicity in mice without LLC bearing in advance. Furthermore, applications of MBGS in conjunction with radiation therapy were effective in controlling tumor growth, and rate of metastasis, life threatening, and can potentially serve as a protective factor for wounds and hair loss that resulted from the overgrowth of primary tumor in LLC bearing mice.

1. Introduction

Mushrooms have been valued for their health benefits and medicinal effects for centuries. One of the special components found from mushrooms is beta-glucan, which is predominantly composed in the fungal cell wall and is mostly composed of beta-D-glucose. In many researches, beta-glucan effectively stimulates the host immune response to defend against bacterial, viral, fungal, or parasitic infections [1]. Moreover, it is known as biological response modifier since it primarily achieves its disease protective activity through modulating the host immune system [2]. The stimulation of beta-glucan to macrophages, neutrophils, and natural killer (NK) cells is proved by binding to the receptor (dectin-1) of these cells and modulates the systems [3, 4]. In clinical applications, beta-glucan is usually used as an adjuvant to enhance the effectiveness of the medicine [5, 6]. To sum up the experimental and clinical results, the potential anticancer activity from beta-glucan has been proven, and thus beta-glucan has been gaining prominence in clinical research during the past few years [6, 7].

The rapidly grown cancer cells turn into tumors, which compete with other somatic cells for space and nutrient. The tumor oppresses normal tissues, affects the normal function of surrounding tissues, and invades adjacent blood vessels or the lymphatic system which leads to metastasis [8]. In fact, many cancer patients do not decease from the exacerbation of the primary tumors. Instead the most common cause of death is from the establishment of secondary tumors in other areas through metastasis. When these tumor cells successfully proliferate in the new host environment, a secondary tumor is formed, which completes the metastatic process and is a potential risk factor during current cancer therapy and patients life threaten [9].

Natural killer (NK) cells have the ability to distinguish self- versus non-self-cells through the MHC- (major histocompatibility complex-) class I molecules on the cell

surface [10]. The MHC-class I molecules on self-cells inhibit the NK cell-mediated cytotoxicity. Atypical cells or infected cells will try to evade being identified by the host immune system through reducing or eliminating the cell surface presentation of MHC-class I molecules. Since most cancer cells are derived from the abnormal proliferation of self-cells, a normal immune system will not necessarily distinguish and eradicate the cancer cells effectively. Therefore the addition of cytotoxic function, such as NK cell-mediated cytotoxicity in eliminating cancer cells, plays an important role in cancer therapy [11].

This study estimated the NK cell-mediated cytotoxicity of mice which is treated by MBGS and, furthermore, used the tumor-bearing murine model of inducing the metastasis from the primary tumor by radiation [12] and observes for the effectiveness of MBGS in conjunction with the radiation therapy to control cancer metastasis.

2. Materials and Methods

2.1. Mushroom Beta-Glucans (MBGS) Preparation and Cell Culture. Manufacturing process of MBGS was initiated by culturing of *G. lucidum* in a culture broth containing glucose, lactose, galactase, sucrose, mannose, and yeast extract using a shaker incubator in temperature that ranged from 21 to 25°C for 2 weeks. Subsequently, cultured mycelium of *G. lucidum* was then inoculated into a sterile solid medium containing brown rice, oats, and buckwheat in a temperature of 25°C for approximately 6 months. Following emergence of the fruit body, all materials in the culture flasks were then dried and grinded into a fine powder. The powder was then dissolved in distilled water at 1 : 5 ratio and stirred using a magnetic stirrer for 6 ~ 10 h at 20 ~ 30°C. Following centrifugation, 95% of alcohol was then added into the supernatant to give a final concentration of 60% alcohol. The precipitation was then collected and redissolved in approximately 3 times of the distilled water. The crude MBGS solution was then concentrated by a ceramic membrane. HPLC analysis showed that MBGS contained high molecular weight particles that ranged from 9.6 ~ 298 kDa, and GC-MS analysis showed that MBGS contained 2-; 4-; and 6-linked galactopyranosyl residues and 3-; 4-; 3,4-; 2,4-; 4,6-; and 3,4,6-linked glucopyranosyl residues. The crude MBGS solution was dried and grinded into the fine powder form. Beta-glucan concentration of MBGS determination by commercial "Megazyme (Ireland) mushroom and yeast beta-glucan kit" was demonstrated at approximately 70–75%.

Lewis lung carcinoma (LLC) cell line and YAC-1 cell lines were purchased from the Bioresource Collection and Research Center (Taiwan). The LLC cells were anchorage-dependent which were cultured in DEME medium (with 10% FBS). The YAC-1 were suspension cells and being cultured in PRMI-1640 medium (with 10% FBS). When the cells grew to the designated quantity, they were collected separately and used as tumor inducing cells for the mice and as target cells for the cytotoxicity analysis.

2.2. Dosage Determination. Since MBGS is a novel, highly purified beta-glucan that has been utilized in the present study, the dosages for mice were determined as 10 mg/kg/day based on a prior study conducted by Itoh et al., which utilized a similar polysaccharide isolated from *Agaricus blazei* with antitumor properties [13].

2.3. Experiment Animals and Tumor Bearing Procedures. C57BL/6 mice (6 weeks old) were purchased from the Laboratory Animal Center in National Taiwan University College of Medicine and housed in animal rooms in compliance with the institutional guidelines. For the present study, animals with the same gender and treatments were housed together by using polycarbonate cages with paddy husk bedding in the animal room. Rodent feed 5010, LabDiet, PMI, Nutrition International (Brentwood, MO) and drinking water, was provided ad libitum throughout the study period. The room temperature and relative humidity were maintained at 21 ± 2°C and 55 ± 20%, respectively, with a 12 h light/dark cycle. The animals were allowed to acclimatize for a minimum of 6 days before the initiation of experiments.

The LLC cells cultured with a preset length of time were treated with trypsin and then brought into suspension and collected by centrifugation. The cells collected were rinsed and resuspended with PBS to undergo further centrifugation for cell collections. This process was repeated for 3 times. Cells collected during the final round of centrifugation were brought into suspension in PBS, counted, and diluted to a cell density of $2 \times 10^5/\mu$L. Using a procedure modified from Camphausen et al. [12] and Gorelik et al. [14], 50 μL of LLC cell fluids were injected subcutaneously into the right hind leg of the mice and monitored for 3 to 5 days to ensure the implantation was successful.

2.4. The Effect of MBGS on NK Cell-Mediated Cytotoxicity in Mice. To observe the change in cytotoxicity associated with the different lengths in MBGS treatments, a total of 27 C57BL/6 mice (6 weeks old) were randomly divided into 9 groups. With the control group being fed standard diets, the other 8 groups received MBGS (gavage) treatment between 1 and 8 days. Mice were euthanized and the monocytes from the spleen were extracted for measurements of cytotoxicity. During the extraction procedure, spleens were removed and shredded with forceps, followed by separating the monocytes with centrifugation using Histopaque. The isolated monocytes were used as the effector cells after being washed twice with PBS buffer and had the cell density adjusted to 1×10^6/mL in RPMI 1640 medium.

YAC-1 cells, intended to be used as the target cells, were collected by centrifugation and had the cell density adjusted to 1×10^6/mL. The cells were then stained with DiOC-18 at 37°C, 5% CO_2 for 20 min, followed by a PBS rinse, and suspended to 1×10^6/mL in RPMI 1640 medium. For the assay of NK cell-mediated cytotoxicity, the effector and target cells were mixed in ratios of 10 : 1 followed by adding the propidium iodide (PI) staining solution to each mixture. Finally, the cell mixtures were incubated at 37°C, 5% CO_2 for 2 h, and analyzed with flow cytometer. Lysed

(PI^+ and DiOC-18^+) and viable (DiOC-18^+ and PI^-) YAC-1 cells were identified by their dual- or single-positive staining. Assessment of the NK cell-mediated cytotoxicity was defined by the percentage increase in cytotoxicity relative to the baseline level set by the control group (100%).

2.5. Tumor-Bearing Mice and Radiation Treatment Procedure. 40 tumor-bearing mice were randomly divided into 4 groups: the control group, the group assigned to MBGS treatments only, the group assigned to radiation treatments only, and the group assigned to both MBGS and radiation treatments. To set up for the radiation treatments, LLC tumor-bearing mice were immobilized in a customized harness that allowed the right hind leg to be exposed. The tumor-bearing sites were irradiated by Cobalt 60 unit with 10 Gy per day for 8 consecutive days. MBGS treatments were provided for 22 days throughout the intervention period, which started out from initiation of the radiation therapy and continued for another 14 days after that. At the 23rd day following the interventions, 5 mice from each group were euthanized immediately to measure the primary tumor sizes. Moreover, the numbers of tumor nodules in the lung were counted to analyze the extent of metastasis. Finally, the remainders of the mice were observed daily and euthanized when criteria from the guidelines for euthanasia [15] were met and the length of survival was recorded.

2.6. Statistical Analysis. One-way ANOVA followed by Turkey's HSD test was used to evaluate the statistical significances of differences amongst groups. A P value less than 0.05 ($P < 0.05$) was considered of a statistical significance. Results are presented as mean ± SD.

3. Results

3.1. The Effect of MBGS on NK Cell-Mediated Cytotoxicity in Mice. The NK cell-mediated cytotoxicity from healthy C57BL/6 mice receiving MBGS treatments was recorded for 8 consecutive days. The cytotoxicity from the control group that did not receive MBGS treatments was referenced as 100%. Results indicated that the cytotoxicity level increased significantly upon MBGS for 4 consecutive days, and with the level maintained above the 200% mark (Figure 1).

3.2. The Effects of Administering MBGS on Cancer Therapy. To estimate the tumor growth phenomenon, the primary tumor was calculated with an ellipsoid volume calculator and the volume average was recorded from each group. The volume average of the primary tumor was the greatest (11876 mm^2) in the control group (Figure 2 and Figure 3(a)). The group assigned to radiation therapy only had significant primary tumor growth inhibition rates when compared to the control group; however, the mice from this particular group also had significant hair loss and external wounds on the surface of the hind leg (Figure 2 and Figure 3(b)). Both groups that received MBGS treatments had a significant decrease in the primary tumor volume than control (Figure 2). In addition, the less hair loss and less severe wounds occurred in groups that received MBGS treatments (Figures 3(c) and 3(d)).

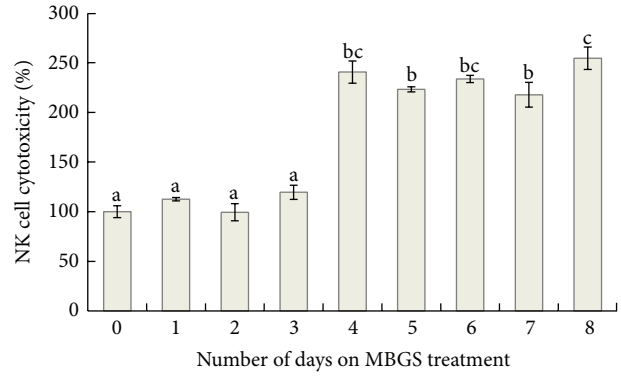


Figure 1: The effect of MBGS on NK cell-mediated cytotoxicity. Changes in cytotoxicity upon MBGS treatments were recorded daily. The cytotoxicity of the group that did not receive MBGS treatments (control) was assigned to 100%, and the relative cytotoxicity level after taking MBGS treatments was calculated. Each histogram represented the mean ± SD cytotoxicity measured from 3 mice. The lower case letters indicate significant difference between groups from each day ($P < 0.05$).

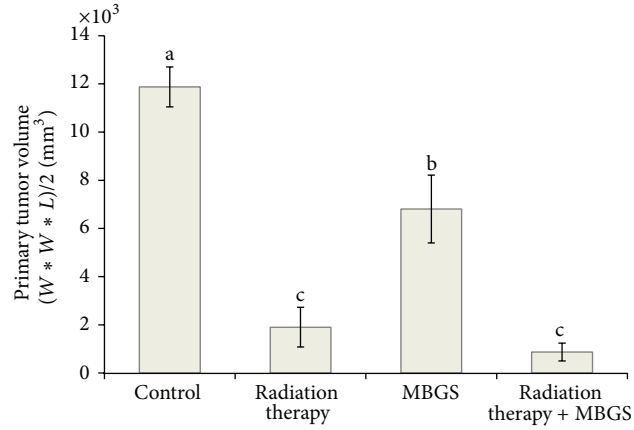
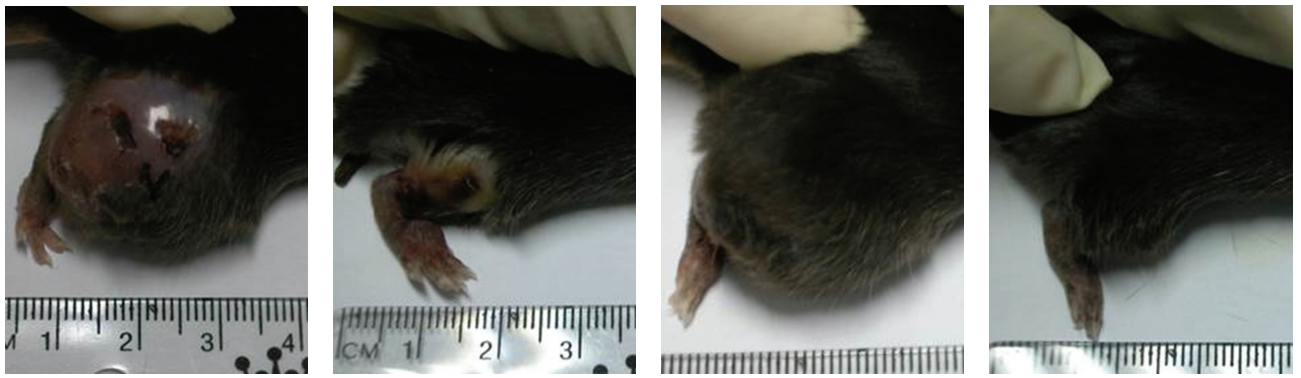


Figure 2: Different treatments options versus primary tumor volume. The primary tumor volume was the greatest in the control group as compared to the others. The group receiving radiation therapy only and the one receiving both radiation therapy and MBGS treatments showed statistically significant reductions in the primary tumor volume. Each histogram represents the mean ± SD of primary tumor volumes calculated from each group containing 5 mice. The lower case letters indicate significant difference between groups ($P < 0.05$).

Incidence of metastasis was calculated by recording the numbers of mice with metastasis versus the ones without at the end of the experiment. The metastatic tumor nodules visual examination was shown in Figure 4. Mice without metastasis were confirmed by visual confirmation and histopathological analysis. As indicated, the metastasis incidence was the greatest in the control and radiation therapy group (100%), followed by the group receiving MBGS treatments (80%) and the combination of MBGS treatments



(a) (b) (c) (d)

Figure 3: Observation on the tumor-bearing site of experimental mice. The mouse from the control group showed the greatest primary tumor volume and demonstrated external wounds and hair loss that resulted from the rapid growth of the primary tumor (a). The mouse from the radiation therapy group showed reduction in primary tumor size but with wounds eschar on the surface of the hind leg (b). Both groups receiving MBGS treatments had improved condition in hair loss and wounds regardless of whether the radiation therapy was provided ((c) and (d)).

and radiation therapy group (20%) (Figure 5). Finally, the number of metastatic tumor nodules from each treatments group was counted and calculated for the average (as shown in Figure 6). The results indicated that mice from the radiation therapy group had the highest numbers of metastatic tumor nodules (nodules = 17), followed by the control group (nodules = 14), MBGS treatments group (nodules = 2), and the MBGS and radiation therapy group (nodules = 1). In comparison, both groups that received MBGS treatments had significant lower findings in tumor metastatic nodules than the ones without ($P < 0.05$) (Figure 6).

The length of survival was recorded from each group and calculated for the average length of survival. Results showed a significant longer average length in group receiving both MBGS treatments and radiation therapy (35 days) ($P < 0.05$), followed by the MBGS only treatments group (29 days) ($P < 0.05$), and the radiation therapy group (28 days) ($P < 0.05$) compared to the control group (24 days), respectively (Figure 7).

4. Discussion

Beta-glucan was known to enhance and stimulate the immune system and express antitumoral activities in animal models [3, 4, 16]. Compared to the other beta-glucans extracted from the yeast or oats that promoted similar antimetastatic activities, beta-glucans from the mushroom were comprised of short β (1, 6) branches coming off a β (1, 3) backbone, thereby lacking the extra β (1, 3) branch extending from the β (1, 6) branch point [17, 18]. In addition to the structural difference, chemical viabilities including molecular weight and water solubility also made the beta-glucans from mushrooms more bioavailable and favorable for adjunct therapeutic applications [19–22].

NK cells are specialized large granular lymphocytes of the innate immune system responsible for eliminating virus-infected and tumor cells [23]. Also, these cells triggered spontaneous cytotoxicity that plays a critical role in immune surveillance and cancer therapy [24]. In 2000, research indicated that the cytotoxic anticancer agents have immunomodulating effects, which has been shown to enhance the activation of macrophages with associated increases of cytokines, can exert immunity-dependent curative effects in mouse tumor models [25]. In the present study, the potential adjunct therapeutic effects of MBGS treatment in a murine tumor-bearing model have been observed. The antimetastatic effect, as demonstrated in the present study, might be a result of an increase in NK-cell mediated cytotoxicity, as previously suggested by Di Luzio and Williams [26]; Ŝandula et al. [27]; and Chen et al., 2011 [17]. The NK cell-mediated cytotoxicity increases significantly after four days of MBGS treatment in mice. Additionally, a continuous daily MBGS treatment effectively maintains NK cell-mediated cytotoxicity. Despite the lack of statistical significant difference in primary tumor size between the group that received a combination of MBGS and radiation therapy and the group that received only the radiation therapy, the combination treatment group demonstrates a promising result for the overall treatment performance when factors such as damages inflicted on the outer epithelial layer, rate of tumor metastasis, and the average length of survival are compared in this study.

In 1981, effective results of single injection of 100 or 200 mg/kg or daily injections of 20 or 50 mg/kg of schizophyllan after removal of the primary tumor markedly inhibited pulmonary metastases [28]. As in the murine tumor-bearing model, the incidence of metastasis was 100% in both the control and radiation therapy group, 80% in the group receiving MBGS treatment and 20% in the group receiving both MBGS treatment and radiation therapy. These results suggest that the glucans, such as MBGS acts as an effective adjunct therapy to radiation therapy in reducing metastatic potential of the primary tumor. This is further confirmed during the necropsy by the large numbers of metastatic tumor nodules found in the lung tissues from both groups that did not receive MBGS

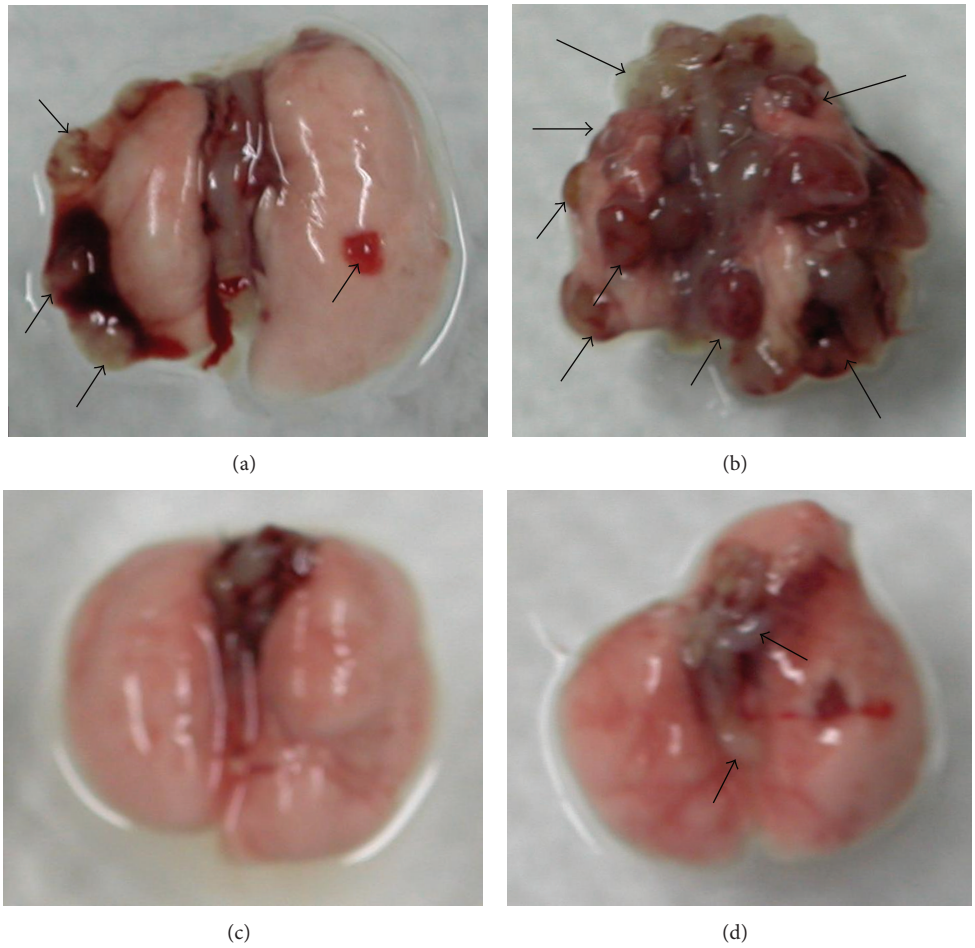


Figure 4: Necropsy examinations of mice lungs. Lungs from the tumor-bearing mice were removed and visually examined for the numbers of metastatic tumor nodules. As the results indicated (a) in the control group lungs with a few surface metastatic tumors nodules (arrows); (b) in the radiation therapy group lungs with a large number of surface metastatic tumors nodules (arrows); (c) in the MBGS treatments group lungs appeared to be free of metastatic tumor nodules; (d) in the combination of MBGS treatments and radiation therapy group lungs with a few surface metastatic tumors nodules (arrows).

treatments. We believe that the increase of NK cell-mediated cytotoxicity is highly associated with the protective effect in reducing the incidence of metastasis.

As another marker for the treatment efficacy, we observed an increasing trend in the length of survival in the treatment groups, with the control group being the shortest (24 days) and the group receiving a combination of MBGS treatment and radiation therapy being the longest (35 days). Moreover, the group that received the combination treatments survived 7 additional days than those who received radiation therapy only (28 days), suggesting an improved posttreatment prognosis using MBGS as an adjunct therapeutic agent with radiation therapy to increase the length of survival.

Furthermore, aside from lethargy, fatigue, and unintentional weight loss, which are common clinical manifestations for the cancer bearing mice, we have also observed open wounds and severe alopecia inflicted by the rapid growth of the primary tumor, from which the symptoms cannot be alleviated by the radiation therapy. However, within groups that received MBGS treatments, there are minimal hair loss and less severe wounds observed on the surface of the primary tumor. Findings from Kougias et al. indicate that in addition to macrophages, neutrophils and NK cells, membrane receptors for beta-glucan, are present in the human dermal fibroblasts [29], which promote wound repair and re-epithelialization of a full-thickness skin by stimulating human dermal fibroblast collagen biosynthesis through a nuclear factor-1 dependent mechanism [30, 31]. In addition, as suggested by prior researches, interferon-γ (IFN-γ), one of the primary cytokine produced by NK cells responsible for cancer immunosurveillance and immunoediting, can effectively active macrophage and dendritic cell to promote immunotherapeutic approaches to control and/or eliminate cancers [32]. As demonstrated from the result of this study, we suggested that the inhibition of the primary tumor metastasis was related to the expression of IFN-γ. This hypothesis was further confirmed by an unpublished data from a follow-up study which has shown a comparable outcome with respect to the current one.

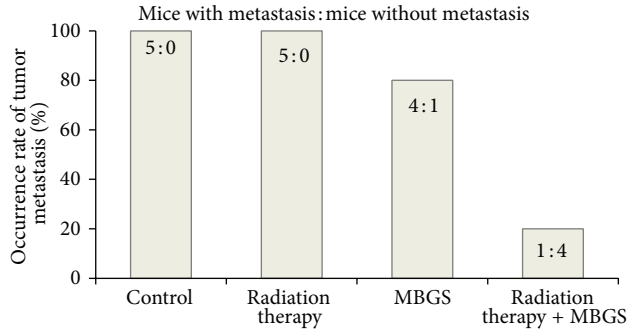


Figure 5: Different treatments options versus the incidence of metastasis. The incidence of metastasis was the greatest in the control and radiation therapy group (100%), followed by the group receiving MBGS treatments (80%) and the combination of MBGS treatment and radiation therapy group (20%).

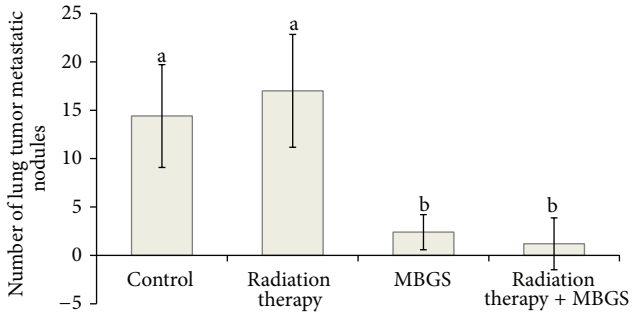


Figure 6: Comparisons in the number of metastatic nodules. At the 23rd day following the interventions, mice from each group were euthanized and the numbers of tumor nodules in the lung were counted to analyze the extent of metastasis. Both groups received MBGS treatments had significant lower findings in tumor metastatic nodules than the ones without ($P < 0.05$). Each histogram represented the mean ± SD nodules found in 5 mice. The lower case letters indicate significant difference between each group ($N = 5$) ($P < 0.05$).

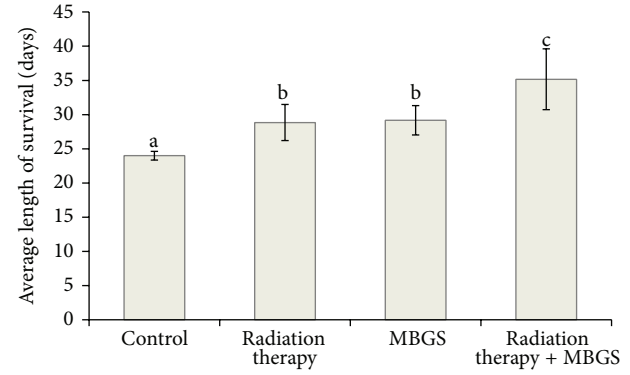


Figure 7: Comparison in the average length of survival. The average length of survival in group receiving both MBGS treatments and radiation therapy is 35 days, followed by the MBGS only treatments group (29 days), and the radiation only therapy group (28 days) compared to the control group (24 days), respectively. The lower case letters indicate significant difference between each group ($N = 5$) ($P < 0.05$).

Radiation therapy is one of the mainstays in cancer therapies to date. During the treatments process, high energy waves are projected to the cancerous growth, causing damage within the cells and ultimately cause the tumor to shrink. However, one of the disadvantages of radiation therapy is that the effectiveness is limited only to the localized tumor rather than a metastatic cancer, and a combination of immunotherapy and radiation therapy is recommended for the best posttreatments prognosis [33]. In this study, we look at the potential mechanism of MBGS treatments in reducing the rate of tumor growth and enhancing NK cell-mediated cytotoxicity against LLC tumor in mice. In addition, the results have further suggested that MBGS treatments reduce the rate of metastasis and improve the effectiveness of radiation therapy by providing additional length of survival for the tumor-bearing mice.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Oral Administration of MBG to Modulate Immune Responses and Suppress OVA-Sensitized Allergy in a Murine Model

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Recently studies performed on mushroom isolated polysaccharides demonstrated that β-(1,3)-glucan may affect the balance of Th1/Th2 cell response. Using ovalbumin (OVA) as a hypersensitivity inducer, we evaluated the ability of mushroom beta-glucan (MBG) in modulating Th1/Th2 cell responses in B6 mice. As compared to the control group, administration of MBG resulted in an increase of phagocytic activities, Th1 cytokine productions, immunoglobulins including IgG2A and IgA, and a significant expression of the splenic surface markers including CD3, CD4, CD8, and F4/80. In contrast, administration of MBG has significantly suppressed IgE and IgG1 levels and Th2 cytokines including IL-4, IL-5, and IL-6. Histopathological observation of MBG-treated followed by OVA-treated mice showed less filtration of eosinophil in pulmonary tissue sections. Our data suggested that administration of MBG treatments alters the natural course of the IgE-mediated hypersensitivities. In this investigation, we realize the mushroom beta glucan alter the Th2 response toward the Th1 in the allergic, resulting in a reduction in IgE productions which played a substantive role in reducing the severity of IgE-mediated hypersensitivity.

1. Introduction

A rapid increase in the prevalence of allergies and other chronic inflammatory diseases worldwide has highlighted the need to develop an effective intervention [1, 2]. Currently, the medical management of patients with allergy-related diseases includes allergen avoidance, pharmacotherapy, and immunotherapy, of which allergen-specific immunotherapy (SIT) is typically recommended for patients whose allergic symptoms cannot be ameliorated by environmental control and medications; however, this type of treatment involves an increased risk of anaphylaxis and physiological side effects [3–5].

Previous studies have shown that mushroom extract containing β-(1-3)-glucan, such as lentinan, protects against IgE-mediated allergy in a murine model [6, 7]. This protective effect is mediated through the stimulation of monocytes, Nature Killer cell (NK cell), and dendritic cells, resulting in the amelioration of a skewed Th1/Th2 balance and inflammation [8].

The Th1 and Th2 polarization is built on cytokine patterns, which begins when the antigen-presenting cells (APC) interact with the naive T cells and polarize into type 1 and type 2 cells in response to the type of antigen encountered [9]. While Th1 and Th2 cells secrete different cytokines, the Th1 cells are reliant on interlukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor (TNF), which are involved in cell-mediated immunity against pathogens, and the Th2 cells are mostly dependent on interleukin-4 (IL-4) and interleukin-5 (IL-5), which stimulate the production of IgE antibodies and eosinophil responses, resulting in the allergic diseases [10, 11]. While an imbalanced Th1/Th2 immune response is linked to certain hypersensitivity disorders such as allergy, asthma, and hay fever [12], studies have suggested that using

biological response modifier (BRM) to restore the balance between Th1 and Th2 immune response can be a potential treatment option for the IgE dependent hypersensitivity [13]. *Ganoderma lucidum (G. lucidum)* is a medicinal mushroom which has been widely used as a folk medicine in oriental countries such China and Japan for hundreds of years for the immunomodulating and antitumor effects. Many biological available substances, in particular polysaccharides, with immunity enhancement effects have been isolated from the extract of *G. lucidum* [14]. The present study was designed to evaluate the efficacy of orally administrating MBG, a polysaccharide isolated from the *G. lucidum* to suppress the onset of the OVA-sensitized allergy in a murine model.

2. Materials and Methods

2.1. MBG and Dosage Determination for Animal Study. In this experiment, mycelium of *G. lucidum*, subcultured and maintained in sterile YM agar (0.02%), was used for the production of MBG. The manufacturing process was initiated by preparing a culture medium containing glucose, lactose, galactose, sucrose, mannose, and yeast extract. Mycelium of *G. lucidum* was then introduced into the sterile medium and cultured using a shaker incubator at temperatures ranging from 27 to 32°C for 3–5 weeks to achieve full polymerization of MBG in the culture system. Subsequently, MBG from cultured mycelia was homogenized and disrupted using high speed homogenizer and ultrasonic vibration. The MBG solution was then filtered and concentrated using a ceramic membrane to strip most of the residual small molecules in the solution. The concentrated MBG was dried by lyophilization and then grinded into the powdered form. The sample was demonstrated to contain approximately 95% carbohydrate, 1% fat, 1% protein, 2% ash, and 0.8% water. Using Megazyme (Ireland) mushroom and yeast Beta-Glucan kit, the crude extract was demonstrated to contain approximately 60–65% of MBG (MBG). The molecular weight of MBG was analyzed by high pressure liquid chromatographic (HPLC) using Shodex SUGAR KS series containing KS-G, KS-804, and KS-805 columns and detected using RI 2000 detector. Molecular weight was determined by referring to the standard cure using standard molecules including STDP-800 (molecular weight M.W. 8×10^5), STDP-400 (M.W. 4×10^5), STDP-200 (M.W. 2×10^5), STDP-100 (M.W. 1×10^5), and STDP-20 (M.W. 2×10^4). MBG was also processed for analysis of its glycosyl linkage. The sample was premethylated, depolymerized, reduced, and acetylated. The resultant partially methylated alditol acetates (PMAAs) were then analyzed by gas chromatography-mass spectrometry (GC-MS) according to the procedures described by York et al. and Ciucanu and Kerek [15, 16].

Result from the HPLC analysis showed that MBG powder contained high molecular weight particles in the range of 9.6~298 kDa. The result of GC-MS analysis showed that MBG powder contained 2-; 4-; and 6-linked galactopyranosyl residues and 3-; 4-; 3,4-; 2,4-; 4,6-; and 3,4,6-linked glucopyranosyl residues. It is the first beta-glucan extracted from the edible mushroom that has been generally recognized as safe (GRAS) under the US Food and Drug Administration (USFDA) regulation. The dosages for MBG treatments are determined based on a predetermined positive control, which is the equivalence of 17 mg/kg/day (0.85 g/50 kg/day) in accordance with the dosage level established by United States Department of Agriculture (USDA), and low, medium, and high dosages used in the experiments were the equivalence of 0.5, 3, and 10 times of the positive control dosage. The MBG solution was prepared fresh daily by dissolving assigned dose levels with 100 mL of sterile water.

2.2. Mice. A total of 50 male C57 BL/6 (B6) mice (six weeks old) of approximately 25 g each were obtained from the Laboratory Animal Center, National Taiwan University College of Medicine, for the experiment. All study procedures were performed in accordance with protocol approved by the National Taiwan University Animal and Use Committee (NTUAUC). For the present study, animals were housed in the Animal Housing Facility of National Taiwan University, College of Life Science, using polycarbonate cages with paddy husk bedding in the animal room. The room temperature and relative humidity were maintained at 21 ± 2°C and 55 ± 20%, respectively, with a 12-hour light/dark cycle. The animals were allowed to acclimatize for a minimum of six days before the initiation of experiments.

2.3. Treatment. 50 B6 mice were divided into five groups based on stratified randomization by using body weights taken before the initiation of treatments. Mice were administered orally (gavage) once daily (SID) with MBG at the dose level of 0 mg/kg/day (control group), 17 mg/kg/day (positive control group; PC), 8.5 mg/kg/day (low dose group; low), 51 mg/kg/day (medium dose group; med.), and 170 mg/kg/day (high dose group; high) for 42 consecutive days. Ovalbumin (OVA) (Sigma, St. Louis, MO, USA) was prepared by mixing with complete adjuvant (Sigma, St. Louis, MO, USA) and each mouse was injected intraperitoneally (IP) with 20 μg of OVA and 4 mg complete adjuvant in a total volume of 1 mL in a tail base at the 36th, 40th, and 41st day during course of the MBG treatment. All treated animals were euthanized by vertebral dislocation at the 43rd day of experiment for analysis. The dosing solutions were prepared fresh daily while the control animals received sterile water.

2.4. Assessment of NK Cell-Mediated Cytotoxicity. Assessment of the NK cell-mediated cytotoxicity was determined by the live/dead cell ratio using cell-mediated cytotoxicity kit (Invitrogen). To prepare for the target cells used in this part of the experiment, YAC-1 cells acquired from the Bioresource Collection and Research Center in Hsinchu, Taiwan, were adjusted to 1×10^6/mL in cell density, followed by staining with DiOC-18 at 37°C in 5% CO_2 for 20 min and a PBS rinse, and suspended to 1×10^6/mL in RPMI 1640 medium. For the assay of NK cell-mediated cytotoxicity, the effector (NK cell) and target cells (YAC-1) were mixed in ratios (effector : target) of 5 : 1, 10 : 1, and 20 : 1, followed by adding the propidium iodide (PI) staining solution to each mixture. Finally, the cell mixtures were incubated at 37°C in 5% CO_2 for 2 h

and analyzed with flow cytometer (CyFlow Counter, Partec, USA). Lysed (PI+ and DiOC-18+) and viable (DiOC-18+ and PI−) YAC-1 cells were identified by their dual- or single-positive staining.

2.5. Assessment of Phagocytic Activity. On the 43rd day after the administration of MBG treatments, all mice were euthanized by cervical dislocation and the monocytes/phagocytes were obtained by washing the peritoneal cavity with RPMI-1640 medium. After centrifugation (2000 rpm, 20 min, 4°C), the precipitated cells were suspended in RPMI-1640 medium containing 10% FBS. After cultivation, adherent cells were collected. Monocytes/phagocytes were adjusted to 1×10^6 cells in the 200 μL of RPMI-1640 medium per well in a 96-well plate and incubated at 37°C for 2 h in 5% CO_2. Upon removal of the nonattached cells after incubation, 100 μL pHrodo *E. coli* BioParticles Conjugate in RPMI was added, followed by incubating at 37°C for 2 h in 5% CO_2. RAW 264.7 cell line (net positive control) purchased from Bioresource Collection and Research Center in Hsinchu, Taiwan, was used to establish a baseline phagocytic activity. The phagocytic activity was measured by pHrodo BioParticles Conjugates for Phagocytosis kit (Invitrogen) using the following formula:

$$\text{phagocytic activity (\%)} = \left(\frac{\text{net experimental phagocytosis}}{\text{net positive control (RAW 264.7) phagocytosis}}\right) \times 100\%. \quad (1)$$

2.6. Assays for Cell Surface Markers. Splenocytes were collected and stained with FITC-conjugated rat anti-mouse monoclonal antibodies including CD3, CD4, CD8, CD22, and F4/80 (eBioscience, San Diego, CA). Using a flow cytometry (CyFlow Counter, Partec, USA), fluorescence intensity (FI) was recorded and calculated for the mean from different MBG dosage groups.

2.7. Assays for Cytokine Productions. Blood samples collected were allowed 3–6 h to clot, centrifuged (4,000 rpm, 10 min), and stored at −20°C until the analysis. Splenocytes acquired from the mice were adjusted to 1×10^4 cells per well and placed into a 96-well plate, added 10 μg of OVA, and incubated for 24 h at 37°C in 5% CO_2. Levels of cytokines IFN-γ, IL-2, IL-4, TNF-α, and IL-5 were measured from splenic cell culture supernatant and mice blood samples using ADI system kit (Enzo Life Sciences Inc., USA) and Mouse IL-5 ELISA kit (Abfrontier System, South Korea) in Microplate Spectrophotometer (μQuant, BioTec).

2.8. Analysis of Serum Antibody Productions. Mice IgA, IgG2a, IgG1, and IgE antibodies were measured from serum using an ELISA kit (eBioscience, San Diego, CA, USA) according to the indication of the manufacturer, and the detection protocol of anti-OVA related antibodies was followed by Thumbikat et al. [17].

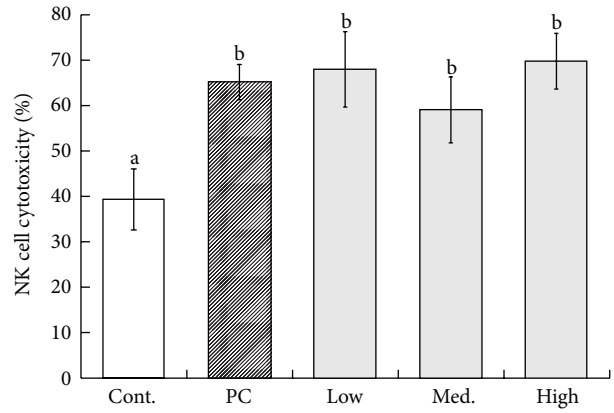

Figure 1: Detection of cytotoxic ability of splenic Nature Killer (NK) cells by measuring the percentage of dead Yac-1 cell line. Splenocyte samples were taken from experimental B6 mice. The mononuclear cell fraction was obtained from each sample. Each value represents the mean ± SD from ten independent experiments. Statistical significance was indicated by Duncan's test; the different letters represent a significant difference between the groups ($P < 0.05$). PC: positive control, low: low dose, med.: medium, high: high dose.

2.9. Histopathological Observations. A histopathological comparison was performed on the pulmonary tissues from the group which received MBG treatment after ovalbumin sensitization and the control group which received the MBG treatment only. Using Giemsa's staining. The pulmonary tissues were fixed in neutralized buffered formalin for observation under the microscope. The shamed mice were with MBG at the dose level of 0 mg/kg/day (control group).

2.10. Statistical Analysis. One-way ANOVA followed by Duncan's test was used to evaluate the statistical significance of differences amongst groups. A P value less than 0.05 ($P < 0.05$) was considered to be of statistical significance. Results are presented as mean ± SD. Different letters represent a statistically significant difference between the groups ($P < 0.05$); that is, a was different from b, b was different from c, and so forth, while the double-letter group (ab) shows that there was not statistically significant difference between this particular group (ab) and group a or b ($P > 0.05$).

3. Results

3.1. Effect of MBG on NK Cell-Mediated Cytotoxicity. A statistically significant difference has been observed in the NK cell-mediated cytotoxicity between the control group and the groups receiving MBG treatments ($P < 0.05$). The NK cell cytotoxic observed for the control group was 39% (Figure 1), while the mean cytotoxicity for the positive control group was 67% ($P < 0.05$), 67.8% for the low dose group ($P < 0.05$), 59.2% for the medium dose group ($P < 0.05$), and 69.6% for the high dose group ($P < 0.05$), respectively. However, during the experiment, we were unable to establish a statistically significant dose response relationship in cytotoxic activities due to the fact that the medium dose group presented a lower

Table 1: The expression levels of surface markers on mice splenocytes from B6 mice treated with MBG in different dosages.

Surface markers	Control	Treatment mice			
		PC	Low	Med.	High
CD3 (%)	24.96 ± 3.3	28.01 ± 0.9*	27.94 ± 2.1*	28.32 ± 2.5*	28.46 ± 0.8*
CD4 (%)	16.36 ± 2.4	21.27 ± 0.9*	24.85 ± 4.4*	20.03 ± 1.9*	20.12 ± 1.5*
CD8 (%)	7.73 ± 1.8	8.81 ± 0.5*	9.38 ± 1.5*	8.33 ± 1.0*	9.19 ± 1.5*
CD22 (%)	52.95 ± 6.5	51.18 ± 0.7	50.19 ± 4.6	52.12 ± 4.9	51.42 ± 1.1
F4/80 (%)	91.00 ± 4.0	99.61 ± 0.1*	90.92 ± 1.7	99.74 ± 0.2*	99.59 ± 0.1*

*Significant difference at $P < 0.05$ level compared with those of control. The values shown in the flow cytometry profiles are the mean fluorescence intensity (MFI) indices. Each value represents the mean ± SD from ten independent experiments. Statistical significance is indicated by P values (Duncan's test).

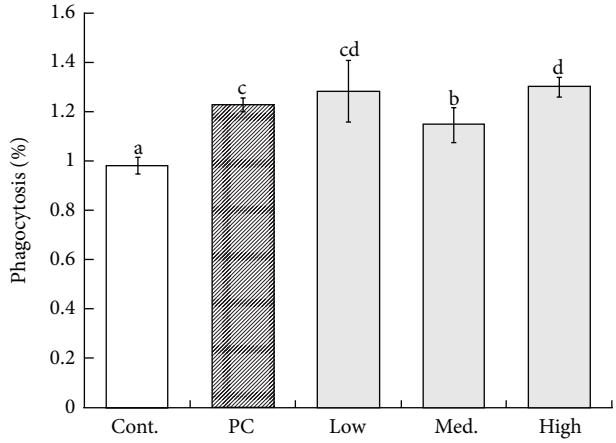

Figure 2: The comparison of the phagocytic activities performed in the splenocytes of the B6 mice administered with various MBG dosages. Each value represents the mean ± SD from ten independent experiments. Statistical significance was indicated by Duncan's test; the different letters represent a significant difference between the groups ($P < 0.05$). PC: positive control, low: low dose, med.: medium, high: high dose.

cytotoxic activity than those of the low dose and positive control group.

3.2. Effects of MBG on the Phagocytic Activity. Phagocytotic activities observed in the MBG treatment groups were higher than the control group ($P < 0.05$). As shown in Figure 2, the mean of phagocytic activity for MBG-treated groups presented 123% in phagocytic activity for the positive control group ($P < 0.05$), 128% for the lower dose group ($P < 0.05$), 114% for medium dose group ($P < 0.05$), and 130% for high dose treated group ($P < 0.05$), respectively. However, no statistically significant trend has been observed in phagocytic activities with respect to the increasing dosage between different groups.

3.3. Expression of the Splenic Cell Surface Marker on MBG-Treated Animals. Results were presented in Table 1. The data indicated that groups which received MBG treatments in different dosages for consecutive 42 days have statistically significant increases in CD3, CD4, and CD8 expressions than those of the control group. However, no statistically significant difference has been observed in the CD22 expression in all groups. Moreover, we have observed a significantly higher F4/80 expression in the medium and high dosage groups when compared to the control group ($P < 0.05$). This suggested that MBG treatments at the medium or higher dosage could potentially induce F4/80 expression.

3.4. Effects on Cytokines and Serum Antibodies on MBG-Treated Animals. As illustrated in Figure 3, B6 mice that received the positive control, medium, and high dosage of MBG treatments showed statistically significant reductions in Th2 cytokines including IL-4, IL-5, and TNF-α in both OVA- and non-OVA-induced groups relative to the control group ($P < 0.05$) (Figures 3(a), 3(b), and 3(c)). In contrast, measurements of Th1 cytokines including IFN-γ and IL-2 from the same dosage groups showed statistically significant increments as compared to the control group ($P < 0.05$) (Figures 3(d) and 3(e)). Analysis from the serum immunoglobulins also indicated that IgA and IgG2a productions have increased significantly after administering MBG treatment for consecutive 42 days in both non-OVA- and OVA-induced groups ($P < 0.05$) (Figures 4(a) and 4(b)). In contrast, measurements of IgG1 and IgE have decreased significantly in both groups ($P < 0.05$) (Figures 4(c) and 4(d)).

3.5. Histopathological Observations. Histopathological findings confirmed that significant infiltration of the inflammation cells was observed when B6 mice were induced with OVA (Figure 5). Compared to the OVA-sensitized mice which received MBG treatments, a significant finding in eosinophil infiltration was observed in the group which received no MBG treatment. The result suggested that MBG treatment effectively suppresses the pulmonary inflammation by averting eosinophil infiltration in the pulmonary alveolus.

4. Discussion

An increase in the prevalence of allergic diseases triggered by environmental allergens has been reported [2]. The pathogenesis of an allergic disease was initiated by the cross-linking of IgE molecules on the surface of the mast cells/basophils, resulting in the release of a host of mediators which ultimately cause hypersensitivity reactions [18, 19]. Polysaccharides isolated from mushrooms reveal a number

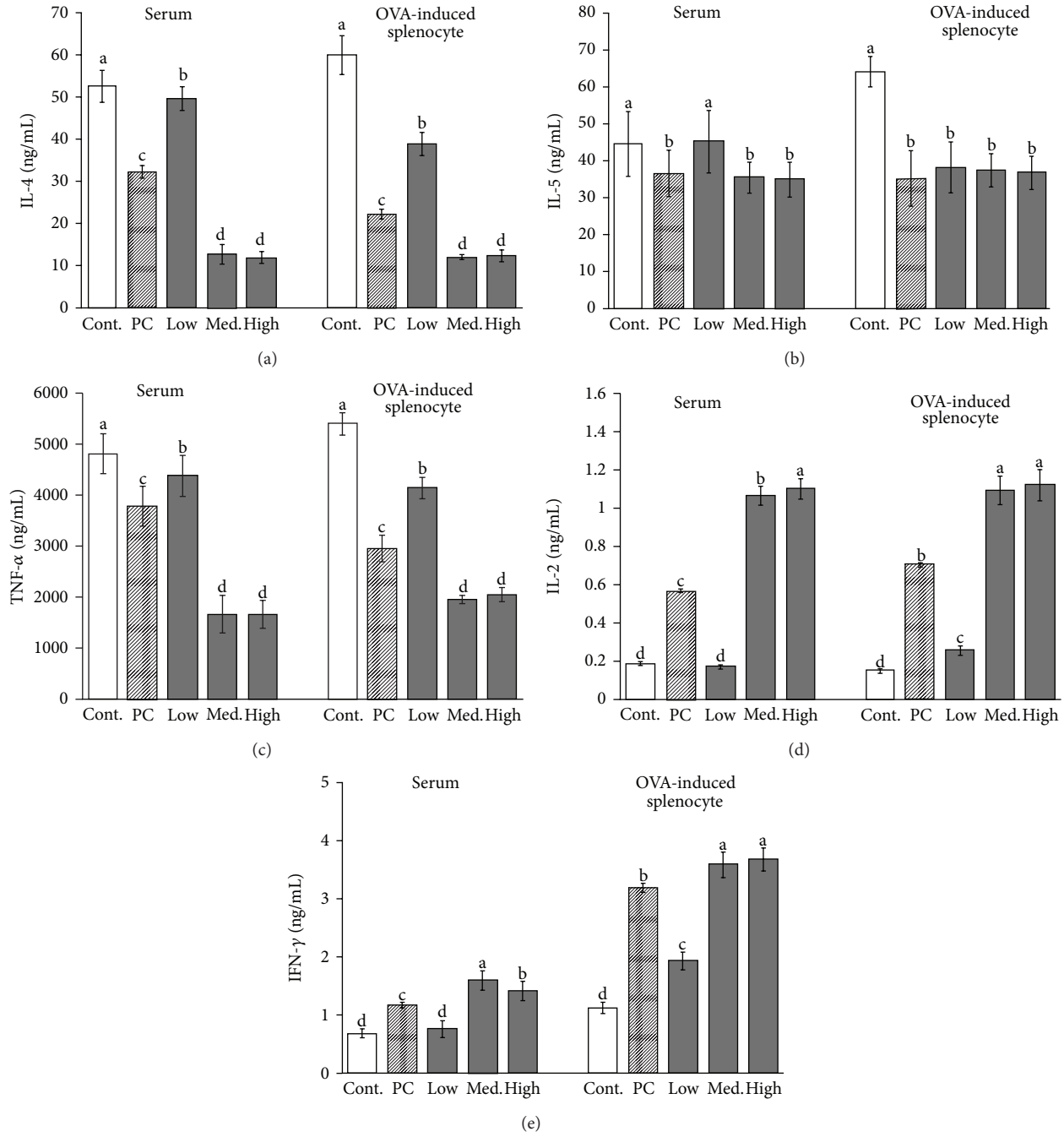

Figure 3: The comparison of cytokine concentrations (IL-4, IL-5, TNF-α, IL-2, and IFN-γ) in serum and splenocyte cultured fluid from the B6 mice administered with MBG in different dosages for 6 weeks. As shown in (a) and (c), concentrations of IL-4 and TNF-α were significantly decreased among MBG treatment groups. In contrary, concentrations of IL-2 and IFN-γ were significantly increased among MBG treatment groups ((d) and (e)). Each value represents the mean ± SD from ten independent experiments. Statistical significance was indicated by Duncan's test; the different letters represent a significant difference between the groups ($P < 0.05$). PC: positive control, low: low dose, med.: medium, high: high dose.

of therapeutic [20] properties including immunomodulation [21] and anti-inflammation [22], which were mediated through the stimulation of immune cells such as NK cells, monocytes, dendritic cells, and T-lymphocytes [23–25]. Several studies also indicated that these bioactive compounds from the mushrooms prevent the progression of allergic diseases by promoting the cellular immunity and skewed the immunological function toward Th1 activity [26–28]. Findings from the previous studies suggested that a pretreatment of *G. lucidum* extracts increased the level of cytokine secretion in mice, as well as enhancing the activities of the cultured human blood-derived primary macrophages and NK cell-mediated cytotoxicity in vitro [29]. As demonstrated in the present study, we have observed a similar outcome

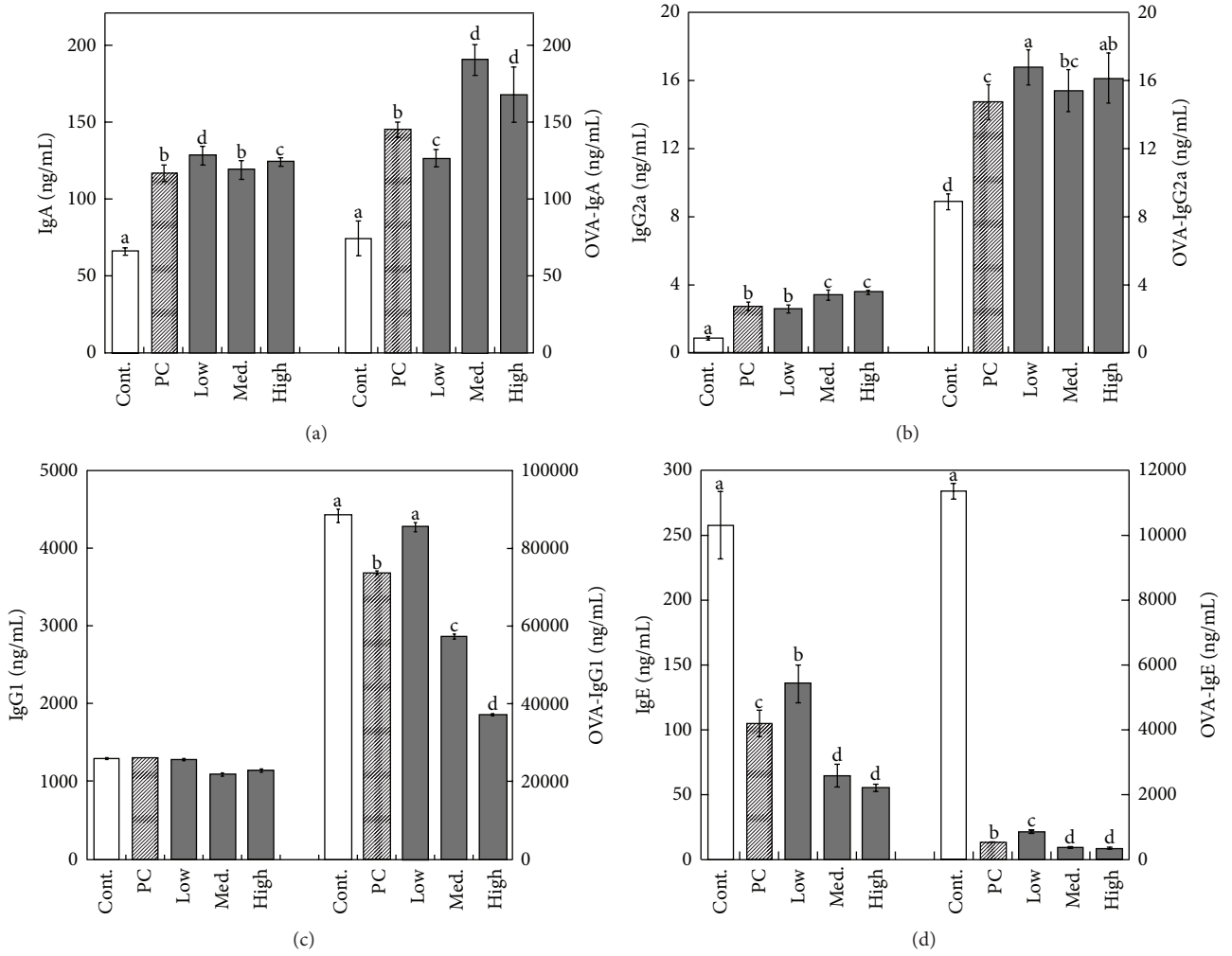

Figure 4: The comparison of IgA, IgG2a, IgG1, and IgE concentration in OVA-stimulated mice administered with MBG of different dosages. The productions of serum IgA and IgG2a were significantly elevated compared to the control group ((a) and (b)). The levels of IgG1 and IgE in OVA-stimulated group were significantly reduced in experimental groups receiving MBG when compared with the measurement from the control group ((c) and (d)). Each value represents the mean ± SD from ten independent experiments. Statistical significance was indicated by Duncan's test; the different letters represent a significant difference between the groups ($P < 0.05$). PC: positive control, low: low dose, med.: medium, high: high dose.

in our experiment using MBG extracted from *G. lucidum*, where it enhanced the NK cell-mediated cytotoxicity of the treatment group by at least 30% at the effector/target cell ratio (E/T) of 20 : 1 as compared to the previous findings [30]. In the presented data, we also observed a similar enhanced percentage of the MBG-treated group compared to control group. Regarding the level of cytokine productions, as demonstrated from the results, the productions of Th2 cytokines such as IL-4 and IL-5 were significantly higher than those of the Th1 cytokines, including IL-2 and IFN-γ during an episode of IgE-mediated hypersensitivity. Additionally, an increase expression of cell surface markers including CD3, CD4, CD8, and F4/80 in mice treated with MBG, indicating the activation or proliferations of T helper cells, cytotoxic T cells (TC), macrophages, and monocytes prompted by MBG treatment. With the absence of CD22 expression, the result strongly suggested the variation in immunoglobulin profiles associated with cytokines produced from T cells instead of B cells. Furthermore, Levels of IL-4, IL-5, and TNF-α were significantly reduced after administering MBG treatments for 42 consecutive days. In contrast, measurements of IL-2 and IFN-γ were significantly increased. Therefore, we suggested that with daily MBG treatments drove the differentiation of T cell toward the Th1 cells rather than Th2 type. A similar result was reproduced from the groups receiving both OVA sensitization and MBG treatments, where the increased levels of Th1 immunoglobulins (IgA and IgG2a) and decreased levels of Th2 immunoglobulin (IgE and IgG1) have been observed. These findings strongly suggested that treatments of MBG modulate the immunoglobulin production by directing the naive T cells to differentiate towards the Th1 type. The results confirmed our hypothesis that MBG reduces the production of Th2 cells and increases the production of Th1 cells, which could potentially reduce the onset of hypersensitivity

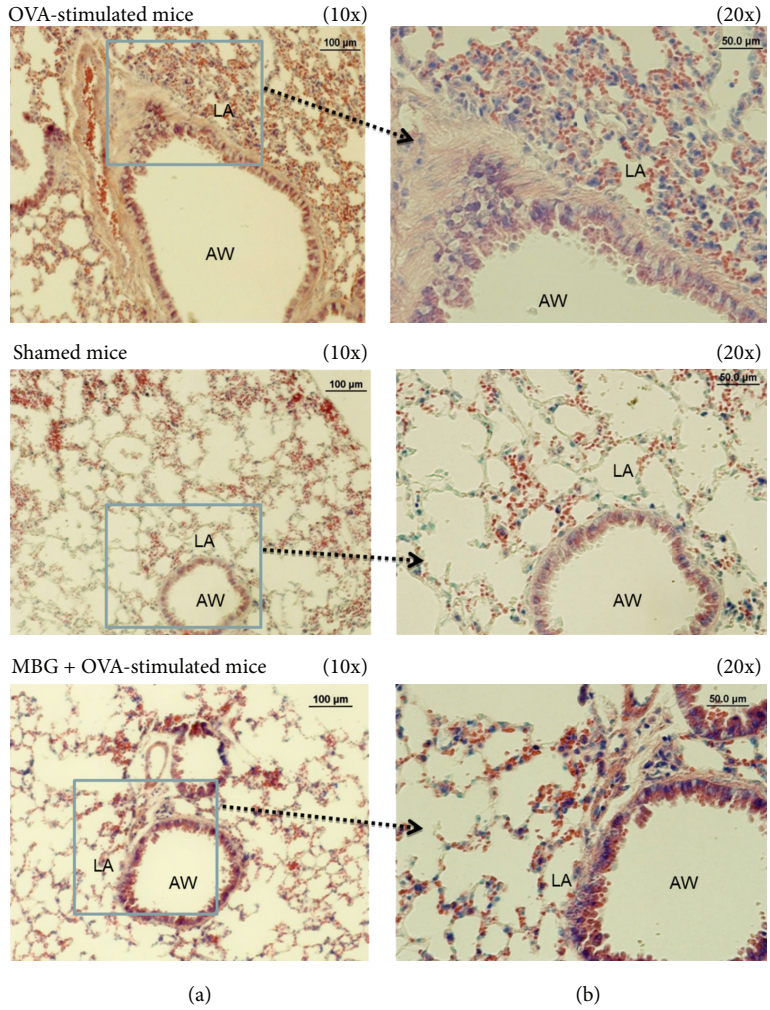

Figure 5: Histopathological observations of the pulmonary tissues obtained from three different experimental groups receiving OVA stimulation. Tissue sections at thickness of 5 μm were made and stained with Giemsa for observation. Each figure was a representative from ten independent sections ((a) low-power magnification ×10 and (b) high-power magnification ×20). LA: lung alveolus. AW: airway. Shamed mice: the control animals receiving sterile water.

reactions. The Th2 cytokines secreted by bronchial epithelial cells, tissue mast cells, alveolar macrophages, and inflammatory cells were recognized as strong promoters for airway hyperresponsiveness [31, 32]. Eosinophils are responsible for the pathogenesis of hypersensitivity related inflammation [33]. The transmigration of eosinophils across the vascular and into the pulmonary tissues is a complex process triggered by Th2 cytokines such as IL-4, IL-5, and chemokines [34, 35]. As a result, prior to the histopathological analysis, we suggest that with a reduction in Th2 cytokine and immunoglobulin production levels results in a significant reduction of inflammations observed in the pulmonary tissue. B6 mice administered with MBG treatments showed significant reductions of eosinophil infiltration found in the pulmonary tissue. This result suggested a potential therapeutic approach by reducing the Th2 cells types to manage respiratory related hypersensitivities. Our results suggested that MBG could suppress IgE-mediated hypersensitivities by downregulating Th2 cytokine and immunoglobulin productions and upregulating those of Th1. The featured parameters of the Th1 immune response were identified as the reductions in serum IL-4, IL-5, IgG1, and IgE and the induction of IL-2, IgG2a, and IgA. Moreover, as only a few filtrations of eosinophil were identified from the pulmonary tissue sections during the histopathological observation, we have further identified a potential protective effect by MBG against type I hypersensitivity by shifting the Th1/Th2 balance toward Th1, and such pathways may play a substantive role in preventing and relieving symptoms associated with respiratory hypersensitivities.

Conflict of Interests

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this paper.

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Research Article

Validation of the Antidiabetic and Hypolipidemic Effects of *Clitocybe nuda* by Assessment of Glucose Transporter 4 and Gluconeogenesis and AMPK Phosphorylation in Streptozotocin-Induced Mice

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The study was designed to investigate the effects of extract of *Clitocybe nuda* (CNE) on type 1 diabetes mellitus and dyslipidemia in streptozotocin- (STZ-) induced diabetic mice. Diabetes was induced by injection of STZ. Diabetic mice were randomly divided into five groups and given orally CNE (C1: 0.2, C2: 0.5, and C3: 1.0 g/kg body weight) or metformin (Metf) or vehicle for 4 weeks. STZ induction decreased in the levels of insulin, body weight, and the weight of skeletal muscle, whereas the levels of blood glucose, hemoglobin nonenzymatically (percent HbA1c), and circulating triglyceride ($P < 0.001$, $P < 0.001$, and $P < 0.01$, resp.) were increased. CNE decreased the levels of blood glucose, HbA1c, and triglyceride levels, whereas it increased the levels of insulin and leptin compared with the vehicle-treated STZ group. STZ induction caused a decrease in the protein contents of skeletal muscular and hepatic phosphorylation of AMP-activated protein kinase (phospho-AMPK) and muscular glucose transporter 4 (GLUT4). Muscular phospho-AMPK contents were increased in C2-, C3-, and Metf-treated groups. CNE and Metf significantly increased the muscular proteins of GLUT4. Liver phospho-AMPK showed an increase in all CNE- and Metf-treated groups combined with the decreased hepatic glucose production by decreasing phosphenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), and 11beta hydroxysteroid dehydroxygenase (11β-HSD1) gene, which contributed to attenuating diabetic state. The study indicated that the hypoglycemic properties of CNE were related to both the increased muscular glucose uptake and the reduction in hepatic gluconeogenesis. CNE exerts hypolipidemic effect by increasing gene expressions of peroxisome proliferator-activated receptor α (PPARα) and decreasing expressions of fatty acid synthesis, including acyl-coenzyme A: diacylglycerol acyltransferase (DGAT) 2. Therefore, amelioration of diabetic and dyslipidemic state by CNE in STZ-induced diabetic mice occurred by regulation of GLUT4, PEPCK, DGAT2, and AMPK phosphorylation.

1. Introduction

Clitocybe nuda (Fr.) Bigelow & Smith (*Lepista nuda,* commonly known as wood blewit or blue stalk mushroom) is an edible woodland mushroom found in Europe, North American, Asia, and Australia [1]. Due to its special fragrance and delicate texture, it has been cultivated in France, Holland, Britain, and Taiwan. Several bioactive extracts from *C. nuda* have been found to exhibit antioxidant and antimicrobial properties [2–4], but few reports have described medicinal

activities and health benefit of human disorders. A new drimane sesquiterpenoid including 3-keto-drimenol, 3beta-hydroxydrimenol, and 3beta, 11, 12-trihydroxydrimene had been shown to exert inhibitory activities against two isozymes of 11beta-hydroxysteroid dehydrogenases (11β-HSD1), which catalyze the interconversion of active cortisol and inactive cortisone [5]. Inhibitors of 11β-HSD1 are known to have a potential treatment for the metabolic syndrome [6].

The prevalence of diabetes mellitus (DM) represents a chronic metabolic disorder and growing global health problem. The prevalence of diabetes is increasing globally and is predicted to raise by twofold from 150 million in the year 2000 to 300 million by the year 2030 [7]. Streptozotocin (STZ), a nitrosurea derivative, is one of the most universally used diabetogenic agents to induce diabetes in experimental animals [8]. It is prominent for its selective pancreatic β-cell cytotoxicity and has been extensively used to induce insulin-dependent diabetes mellitus or type I diabetes [9]. STZ is taken up by pancreatic β cells and subsequently induces their death. Collectively, the potent alkylating properties of STZ are the main reason for its toxicity. STZ is a nitric oxide donor, nitric oxide was found to bring about the destruction of pancreatic islet cells, and STZ by itself was found to generate reactive oxygen species, which contributed to DNA fragmentation and evoked other deleterious changes in the cells. Therefore, the synergistic action of both nitric oxide and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes [10, 11].

Glucose transporter 4 (GLUT4) is the major insulin-regulated glucose transporter expressed mainly in skeletal muscle and adipose tissue [12, 13]. Insulin stimulates glucose uptake in these cells primarily by inducing net translocation of GLUT4 from the intracellular storage sites to the plasma membrane [14]. Impairment of GLUT4 expression, GLUT4 translocation, and/or insulin signaling may affect insulin-stimulated glucose uptake, and that would result in insulin resistance and hyperglycemia [15, 16]. Therefore, the improvements of GLUT4 contents and/or translocation to the plasma membrane have long been regarded as a potential target in the treatment of diabetes mellitus. GLUT4 translocation is mainly regulated by two pathways: the insulin signaling pathway and AMP-activated protein kinase (AMPK) pathway [17].

The stress kinase, AMPK, has also been shown to regulate GLUT4 translocation [17], and therefore we investigated whether CNE activated AMPK in liver tissue and skeletal muscle. However, the antidiabetic activity of CNE is not well defined in streptozotocin-induced diabetic mice. AMPK is considered as a therapeutic target for treatment of diabetes and dyslipidemia [18, 19]. Activation of the AMPK results in increased lipid and glucose catabolism [20]. Phosphorylation of Thr 172 of α subunits is essential for AMPK activity [21]. As one of the possible mechanisms of action, this study also examined the effect of *Clitocybe nuda* (CNE) on the expression of genes involved in antidiabetes, lipogenesis, and triglyceride lipase in the liver tissue, including phosphoenolpyruvate caboxykinase (PEPCK), glucose 6-phosphatase (G-6Pase), 11beta-HSD1, and diacylglycerol acyltransferase 2 (DGAT2).

2. Materials and Methods

2.1. Materials and Preparation of Extract of Clitocybe nuda (CNE). The mushroom (or fungal) strain of *Clitocybe nuda* (stain Tainung number 1) was cultured under compost extract agar medium. The preparation of grain spawn was as follows: wheat grains were washed with distilled water, boiled for 20 min, and removed from water by filtration. Then, they were added to 1% $CaCO_3$ and mixed well, transferred to the flask, and were sterilized at 121°C and 1.2 kg/cm^3 for 1 h. After one day, the hyphal chunk of the described above *C. nuda* was implanted to the flask at 24°C for 15 days for mycelia to cover grains, called grain spawn. The fruiting of *C. nuda* is as follows: the grain spawn of *C. nuda* was mixed with the fermented rice straw compost, incubated at 24°C for 21–28 days for spawn running, and covered with 1-2 cm peat and the condition was 13°C, 90–95% relative humidity, 1000 ppm CO_2 concentration, and at daylight for 8 h. During the period, they were periodically supplied with water until fruiting, and then the mushrooms were harvested. After lyophilizing, 30 g of the dried mushroom samples was homogenized and extracted with 40 times volumes of hot water under reflux at 100°C for 40 min. The aqueous extract was filtered over Whatman number 1 paper and the filtrate was evaporated to a small volume. The filtrate was lyophilized and designated the hot-water soluble fraction (CNE) and was stored frozen at −20°C until required. The total phenolic contents were determined by the Folin-Ciocalteau method [22]. The total phenolic contents of CNE were 1.29%. The polysaccharides of CNE were 12.62% by phenol-sulfuric acid method [23]. The total anthocyanin contents of CNE were 0.045%. The CNE was diluted adjusted, and then administrated orally to mice in a volume of 0.2, 0.5, and 1.0 g/kg bodyweight (C1: 0.2, C2: 0.5, and C3: 1.0 g/kg bodyweight), respectively. Distilled water was administered in a similar volume to control mice.

2.2. Acute Effects of CNE. The acute effects of CNE on fasting glucose were studied in diabetic mice. Animals were fasted for 15–18 h but were allowed access to water. Initial blood samples were obtained for determination of basal glucose levels from retroorbital sinus using heparinized capillary tubes. Blood samples were then taken at 1, 3, 5, and 7 h following dosing with 0.5 and 1.0 g/kg CNE or with an equivalent volume of normal saline.

2.3. Experimental Chronic Induction of Diabetic Mice. Male C57BL/6J mice, aged 5 weeks, were used. Diabetes was induced by intraperitoneal injection of streptozotocin (Sigma Chemical, St. Louis, MO, USA) for five consecutive days. The dosage of STZ is 55 mg/kg (dissolved in 0.05 M cold sodium citrate buffer, pH 4.5). The normal control mice received only citrate buffer of the same volume. After 2 weeks, the mice with severe diabetes exerting hyperglycemia (fasting blood glucose range of above 250 mg/dL) were considered as diabetic and selected for the experiment. Diabetic mice were

randomly divided into five groups and were either treated with vehicle (distilled water), CNE (0.2, 0.5, or 1.0 g/kg), or metformin (300 mg/kg) in a similar volume. The vehicle, CNE, or metformin was administered orally to mice once daily and thereafter for 28 days. During the experiment, all mice were fasted overnight and blood was collected from retroorbital sinus under ether anaesthesia. At the end of the experiment, mice were sacrificed by carbon dioxide inhalation. Liver, adipose tissue, and skeletal muscle were removed and immediately frozen in liquid nitrogen and stored at −80°C for various assays. Blood sample was allowed to clot at 25°C for 5 min. Plasma samples were collected by centrifugation at 1600 ×g for 15 min at 4°C. The separation of the plasma was finished within 30 min. Aliquots of the supernatant were obtained for total cholesterol (TC) and triglyceride (TG) assay and immediately frozen at −80°C until use.

2.4. Analysis of Fasting Blood Glucose and Biochemical Parameters. Blood samples were collected from the retroorbital sinus of fasting mice and the glucose level was measured by the glucose oxidase method (Model 1500; Sidekick Glucose Analyzer; YSI Incorporated, Yellow Springs, USA). The concentrations of TG and TC were measured using commercial assay kits according to the manufacturer's directions (Triglycerides-E test and Cholesterol-E test, Wako Pure Chemical, Osaka, Japan).

2.5. Analysis of Adipocytokine Levels. The levels of insulin and leptin were measured by ELISA using a commercial assay kit according to the manufacturer's directions (mouse insulin ELISA kit, Sibayagi, Gunma, Japan, and mouse leptin ELISA kit, Morinaga, Yokohama, Japan).

2.6. Histology Analysis of Epididymal WAT and Liver Tissue. Small pieces of epididymal WAT and liver were fixed with formalin (200 g/kg) neutral buffered solution and embedded in paraffin. Sections (8 μm) were cut and stained with hematoxylin and eosin. For microscopic examination, a microscope (Leica, DM2500) was used, and the images were taken using a Leica Digital camera (DFC-425-C) at 10 (ocular) × 20 (object lens) magnification.

2.7. Isolation of RNA and Relative Quantization of mRNA Indicating Gene Expression. Total RNA from the epididymal WAT and liver was isolated with a Trizol Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's directions. The integrity of the extracted total RNA was examined by 2% agarose gel electrophoresis, and the RNA concentration was determined by the ultraviolet (UV) light absorbency at 260 nm and 280 nm (Spectrophotometer U-2800A, Hitachi). The quality of the RNA was confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA after electrophoresis on 2% agarose gel containing 6% formaldehyde. Total RNA (1 μg) was reverse transcribed to cDNA in a reaction mixture containing buffer, 2.5 mM dNTP (Gibco-BRL, Grand Island, NY, USA), 1 mM of the oligo (dT) primer, 50 mM dithiothreitol, 40 U Rnase inhibitor (Gibco-BRL, Grand Island, NY, USA), and 5 μL Moloney murine leukemia virus reverse transcriptase (Epicentre, USA) at 37°C for 1 h and then heated at 90°C for 5 min to terminate the reaction. The polymerase chain reaction (PCR) was performed in a final 25 μL containing 1 U Blend Taq-Plus (TOYOBO, Osaka, Japan), 1 μL of the RT first-strand cDNA product, 10 μM of each forward (F) and reverse (R) primer, and 75 mM Tris-HCl (pH 8.3) containing 1 mg/L Tween 20, 2.5 mM dNTP, and 2 mM $MgCl_2$. Preliminary experiments were carried out with various cycles to determine the nonsaturating conditions of the PCR amplification for all the genes studied. The primers are shown in Tables 1 and 2. The products were run on 2% agarose gels and stained with ethidium bromide. The relative density of the band was evaluated using AlphaDigiDoc 1201 software (Alpha Innotech, Co., San Leandro, CA, USA). All the measured PCR products were normalized to the amount of cDNA of GAPDH in each sample.

2.8. Western Immunoblotting Analysis of Phospho-AMPK (Thr172) Proteins. Protein extractions and immunoblots for the determination of AMPK phosphorylation were carried out on frozen liver tissue and skeletal muscle from mice according to a previous report [24]. Briefly, liver samples (0.1 g) were powdered under liquid nitrogen and homogenized for 20 s in 500 μL buffer containing 20 mM Tris-HCl (pH 7.4 at 4°C), 2% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 100 mM NaF, 2 mM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μL/mL pepstatin [25]. 40 μg of each homogenate was mixed with an equal amount of 2 × standard SDS sample loading buffer containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.25% bromophenol blue and boiled for 10 min before electrophoresis. Proteins were separated by 12% SDS-PAGE according to the method of Laemmli [26] and transferred by electroblotting onto PolyScreen PVDF transfer membrane (NEN) using semidry transfer cell (Bio-Rad) according to the manufacturer's manual. The membrane was then treated sequentially with blocking solution (phosphate-buffered saline (PBS) containing 5% nonfat skim milk), with appropriate dilution of anti-phospho-AMPKα (Thr 172) antibody (Abcam Inc., Cambridge, MA, USA) and with anti- G6PD (G6PD) (glucose 6 phosphate dehydrogenase antibody; Abcam Inc, USA) conjugated to peroxidase (Zymed Inc., South San Francisco, CA, USA). Finally, the membrane was soaked in a chromogen/substrate solution (TMB single solution; Zymed) for color development.

2.9. Statistical Analysis. Data were expressed as mean ± S.E. values. Whenever possible, data were subjected to analysis of variance, followed by Dunnett's multiple range test, using SPSS software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

Table 1: Primers used in this study.

Gene	Accession numbers	Forward primer and reverse primer	PCR product (bp)	Annealing temperature (°C)
Liver				
DGAT2	NM_026384.3	F: AGTGGCAATGCTATCATCATCGT R: AAGGAATAAGTGGGAACCAGATCA	149	50
G6Pase	NM_008061.3	F: GAACAACTAAAGCCTCTGAAAC R: TTGCTCGATACATAAAACACTC	350	50
11β-HSD1	NM_008288.2	F: AAGCAGAGCAATGGCAGCAT R: GAGCAATCATAGGCTGGGTCA	300	50
PPARα	NM_011144	F: ACCTCTGTTCATGTCAGACC R: ATAACCACAGACCAACCAAG	352	55
PEPCK	NM_011044.2	F: CTACAACTTCGGCAAATACC R: TCCAGATACCTGTCGATCTC	330	52
ATGL	AY894805	F: AGG ACA GCT CCA CCA ACA TC R: TGG TTC AGT AGG CCA TTC CT	165	50
GAPDH	NM_031144	F: TGTGTCCGTCGTGGATCTGA R: CCTGCTTCACCACCTTCTTGA	99	55

Table 2: Absolute tissue weight, weight gain over 4-week treatment (g), and blood profiles.

Parameter	CON	STZ	STZ + C1 0.2[a]	STZ + C2 0.5[a]	STZ + C3 1.0[a]	STZ + Metf 0.3[a]
Absolute tissue weight (g)						
EWAT	0.235 ± 0.009	0.075 ± 0.007$^{\#\#\#}$	0.058 ± 0.010	0.061 ± 0.005	0.067 ± 0.011	0.090 ± 0.010
MWAT	0.305 ± 0.014	0.216 ± 0.011$^{\#\#\#}$	0.197 ± 0.018	0.237 ± 0.017	0.247 ± 0.015	0.250 ± 0.014
RWAT	0.024 ± 0.002	0.016 ± 0.009	0.007 ± 0.002	0.007 ± 0.003	0.005 ± 0.001	0.012 ± 0.003
Visceral fat	0.259 ± 0.010	0.091 ± 0.012$^{\#\#\#}$	0.065 ± 0.011	0.070 ± 0.007	0.072 ± 0.012	0.102 ± 0.012
BAT	0.081 ± 0.010	0.100 ± 0.049	0.056 ± 0.004	0.044 ± 0.006	0.052 ± 0.003	0.048 ± 0.003
Liver (g)	0.992 ± 0.032	1.031 ± 0.048	1.048 ± 0.031	1.063 ± 0.064	1.121 ± 0.049	1.074 ± 0.020
Spleen	0.073 ± 0.004	0.055 ± 0.003$^{\#}$	0.069 ± 0.009	0.061 ± 0.006	0.056 ± 0.002	0.055 ± 0.003
Skeletal muscle	0.270 ± 0.021	0.175 ± 0.012$^{\#\#\#}$	0.155 ± 0.014	0.147 ± 0.016	0.175 ± 0.012	0.183 ± 0.005
Weight gain (g)	0.10 ± 0.32	−0.19 ± 0.73	−0.92 ± 0.20	−1.24 ± 0.35	−0.58 ± 0.70	−0.79 ± 0.67
Blood profiles						
TC (mg/dL)	95.0 ± 5.9	140.7 ± 7.3$^{\#\#\#}$	160.0 ± 10.4	157.5 ± 6.2	153.3 ± 6.8	114.3 ± 7.8*
Insulin (μU/mL)	10.27 ± 0.59	4.07 ± 0.38$^{\#\#\#}$	8.88 ± 0.81**	11.08 ± 1.87**	13.29 ± 0.79***	11.25 ± 1.67**

All values are means ± S.E. ($n = 9$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the control (CON) group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the streptozotocin (STZ) + vehicle (distilled water) (STZ) group. C1, C2, and C3: extracts of *Clitocybe nuda*. BAT: brown adipose tissue; EWAT: epididymal white adipose tissue; RWAT: retroperitoneal white adipose tissue; MWAT: mesenteric white adipose tissue; EWAT + RWAT: visceral fat; TC: total cholesterol; TG: triglyceride.
[a]Dose (g/kg/day).

3. Results

3.1. Acute STZ Induced Diabetic Mice. Following treatment with 0.5 g/kg CNE, blood glucose levels were decreased after 7 h. In the treatment with 1.0 g/kg CNE, blood glucose levels were decreased after 5 h and the hypoglycemic effect continued to 7 h (Figure 1(a)).

3.2. Chronic Effects

3.2.1. Body Weight and Tissue Weight. STZ induction caused a decrease in the weight of white adipose tissue (WAT) (including epididymal, mesenteric WAT, and visceral fat) ($P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.) and skeletal muscle ($P < 0.001$). There is no significant difference in the weights of retroperitoneal WAT, visceral fat, and skeletal muscle between the CNE-treated STZ group and vehicle-treated STZ group (Figure 1(c) and Table 2).

3.2.2. Plasma Glucose Levels and Blood Glycated Hemoglobin (HbA1c). All group mice started with similar mean body weights at the beginning of the study (19.6 ± 0.4 g). At the end of the experiment, the STZ induced mice caused a significant decrease in body weight and skeletal muscle weight compared with the CON group ($P < 0.001$, $P < 0.001$, resp.). There is no significant difference in the body weight between the CNE- and Metf-treated STZ group and vehicle-treated STZ group (Figure 1(b)).

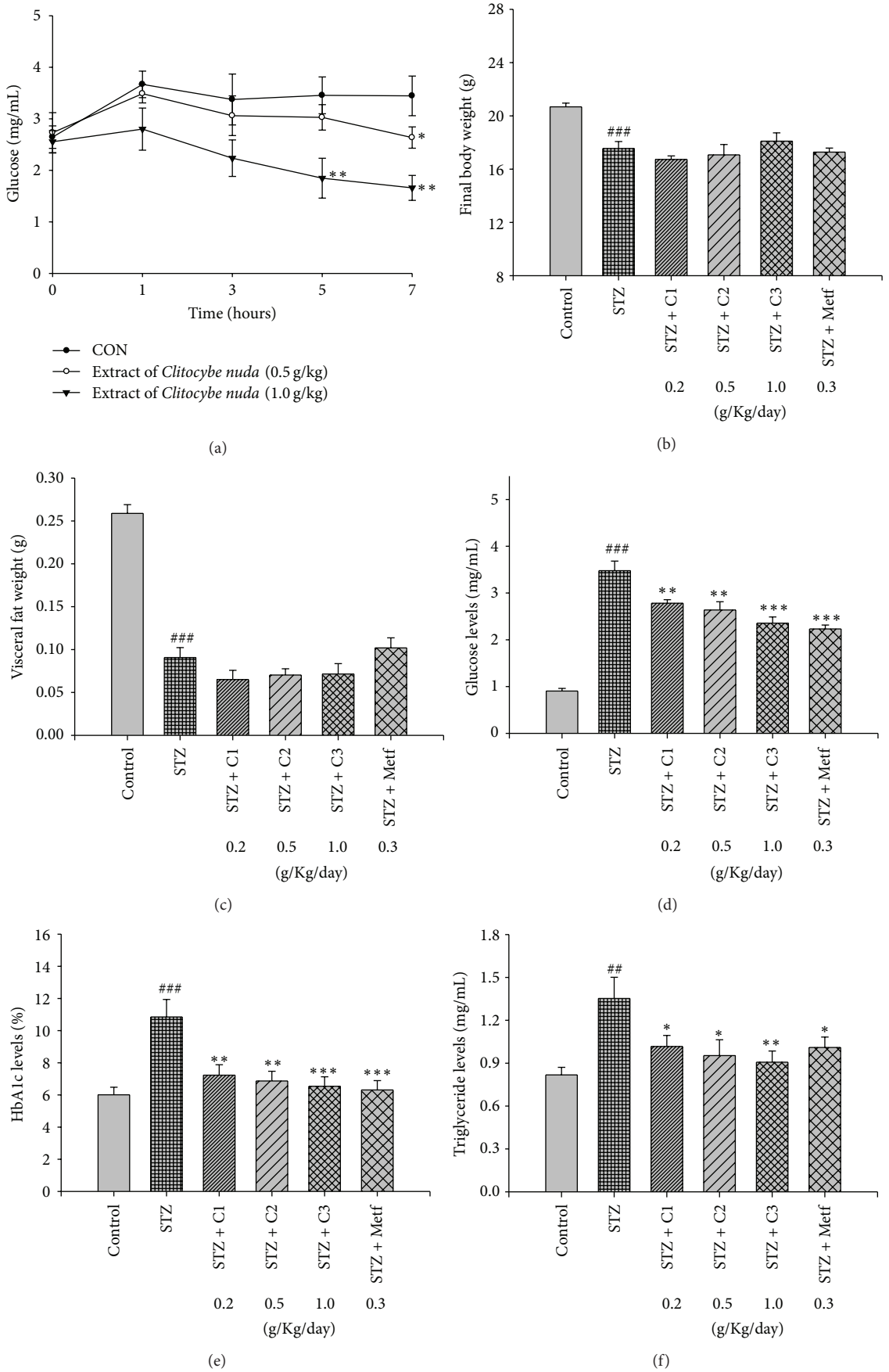


Figure 1: Continued.

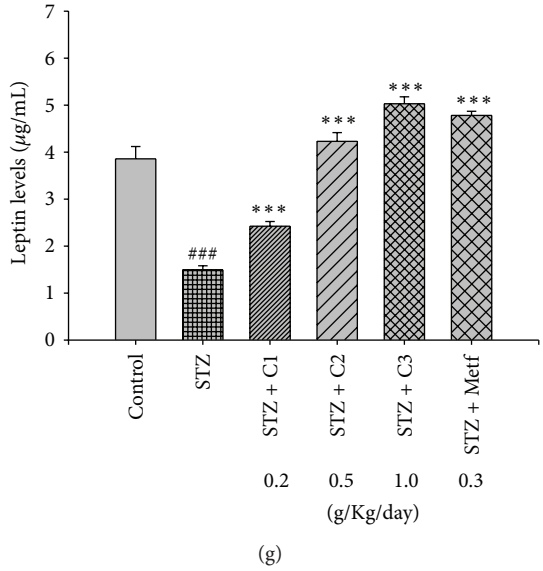


Figure 1: Effects of extract of *Clitocybe nuda* on (a) the levels of blood glucose in STZ induced acute test, (b) final body weight, (c) visceral fat weight, (d) blood glucose levels, (e) the glycosylated hemoglobin (HbA1c) levels, (f) circulating triglyceride levels, and (g) leptin levels at the end of experiment in STZ induced chronic test. All values are means ± S.E. ($n = 9$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the control (CON) group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the streptozotocin + vehicle (distilled water) (STZ) group. C1, C2, and C3: extracts of *Clitocybe nuda* (C1: 0.2, C2: 0.5, and C3: 1.0 g/kg bodyweight); Metf: metformin (0.3 g/kg body weight). The HbA1c is represented by % hemoglobin.

At the beginning of the study, all mice started with similar levels. At the end of the experiment, the glucose levels of the STZ group were significantly greater than the CON group ($P < 0.001$). Treatment with C1, C2, C3, and Metf showed a significant reduction in plasma glucose compared with the vehicle-treated STZ group ($P < 0.01$, $P < 0.01$, $P < 0.001$, and $P < 0.001$, resp.) (Figure 1(d)). The percent of hemoglobin was evaluated nonenzymatically (percent HbA1c), as an integrated measure of long-term blood glucose regulation. STZ induction caused an increase in HbA1c in the STZ group compared to the CON group ($P < 0.001$). After treatment, all CNE- and Metf-treated groups showed a significant reduction in HbA1c as compared with the STZ group (Figure 1(e)).

3.2.3. Plasma Lipid. At the end of the experiment, the levels of TC and TG were 48.1% and 65.4% greater in the STZ group than in the CON group ($P < 0.001$, $P < 0.01$, resp.). Treatment with C1, C2, C3, and Metf suppressed the STZ diet-induced increases in the concentrations of TG ($P < 0.05$, $P < 0.05$, $P < 0.05$, and $P < 0.05$, resp.). There is no significant difference in the concentrations of TC between the CNE-treated STZ group and vehicle-treated STZ group (Table 2 and Figure 1(f)).

3.2.4. Insulin and Leptin Concentration. As shown in Table 2 and Figure 1(g), at the end of the experiment, the concentrations of insulin and leptin were lower in the STZ group than in the CON group ($P < 0.001$, $P < 0.001$, resp.). C1, C2-, C3,- and Metf-treated groups significantly increased the levels of insulin compared with the vehicle-treated STZ group ($P < 0.01$, $P < 0.01$, $P < 0.001$, and $P < 0.01$, resp.). C1-, C2-, C3-, and Metf-treated groups significantly increased leptin levels compared with the vehicle-treated STZ group ($P < 0.01$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.).

3.2.5. Histology of Epididymal WAT and Liver Tissue. STZ induced the adipocytes smaller than the CON group in epididymal WAT. Following treatment with C1 and C2 caused the atrophy compared with the vehicle-treated STZ group. The results obtained from the other mice are similar to those shown in Figure 2. STZ induction does not cause ballooning of hepatocyte compared with the CON group. Afterwards, treatment with C1, C2, C3, and Metf exerts no ballooning phenomenon. These morphological results strongly suggest that neither STZ induction nor CNE caused the hepatic TG accumulation. The results obtained from the other mice are similar to those shown in Figure 3.

3.2.6. Expressions of PEPCK, 11β-HSD1, Glucose-6-Phosphatase (G-6Pase), Peroxisome Proliferator-Activated Receptor α (PPARα), Adipose Triglyceride Lipase (ATGL), and DGAT2 in Liver Tissue. As shown in Figure 4, at the end of the experiment, the mRNA levels of PEPCK, 11β-HSD1, glucose-6-phosphatase (G6Pase), and DGAT2 were higher in the STZ group than in the CON group, whereas there was no significant difference in PPARα and ATGL expression of mRNA in the STZ group compared with the CON group. Following treatment, the C1-, C2-, C3-, and Metf-treated groups significantly decreased the mRNA level of PEPCK ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.), 11β-HSD1 ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.), G6Pase

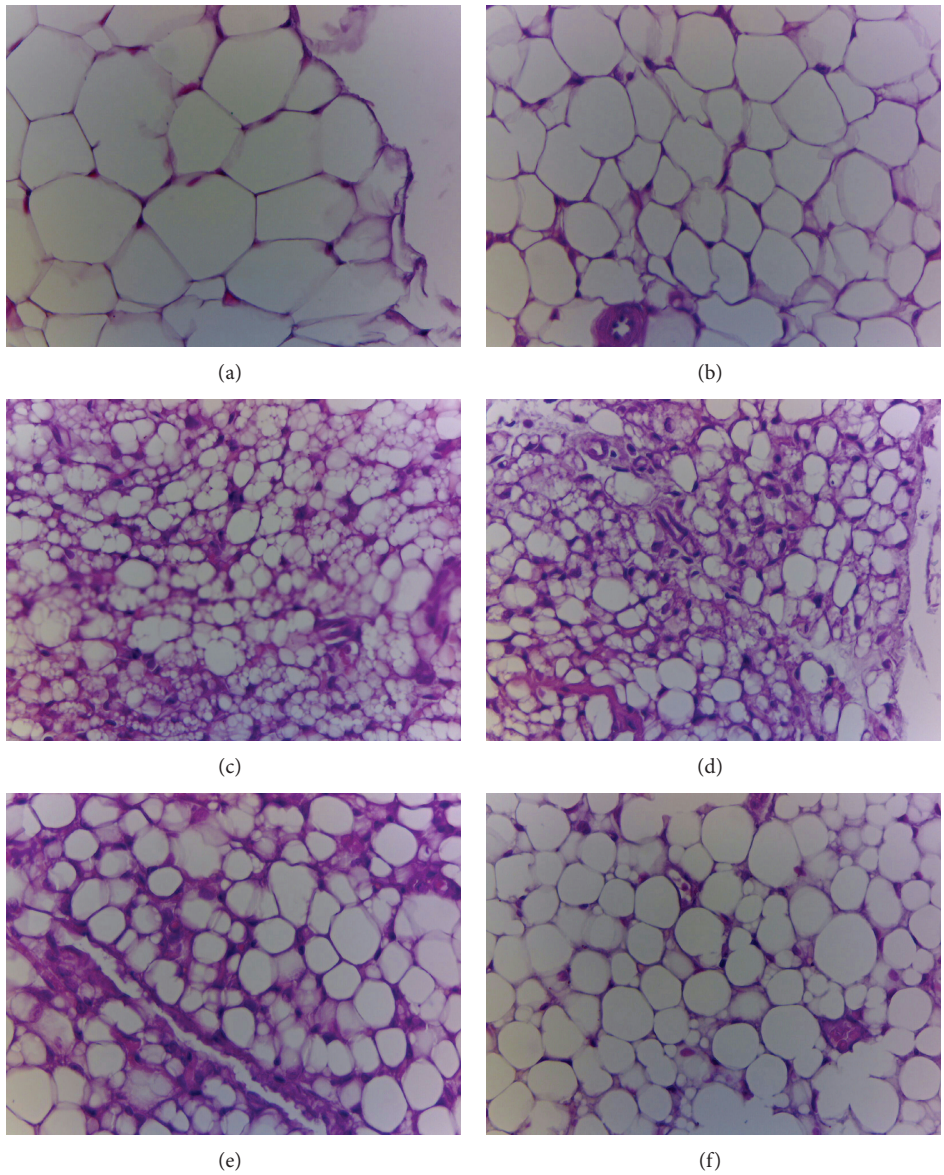


Figure 2: Histology of epididymal white adipose tissue (WAT) of mice in the (a) control (CON), (b) streptozotocin + vehicle (distilled water) (STZ), (c) STZ + C1, (d) STZ + C2, (e) STZ + C3, or (f) STZ + metformin (Metf) groups by hematoxylin and eosinstain. Magnification: 10 (ocular) × 20 (object lens). C1, C2, and C3: extracts of *Clitocybe nuda* (C1: 0.2, C2: 0.5, and C3: 1.0 g/kg bodyweight); Metf: metformin (0.3 g/kg body weight).

($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.), and DGAT2 ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.). Following treatment, the C1-, C2-, C3-, and Metf-treated groups significantly increased the mRNA level of PPARα ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.) and ATGL ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.01$, resp.) as compared with vehicle-treated STZ group (Figure 4).

3.2.7. The Phospho-AMPK (Thr172) Protein Contents in Liver Tissue and Skeletal Muscle. At the end of the experiment, the protein contents of phospho-AMPK protein were lower in the STZ group than in the CON group in liver and skeletal muscle ($P < 0.001$, $P < 0.001$, resp.). After treatment, the protein contents of hepatic phospho-AMPK were increased in the C1-, C2-, C3-, and Metf-treated groups compared with the STZ group ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.) (Figure 5(a)). Following treatment, the muscular protein contents of phospho-AMPK were increased in the C2, C3, and Metf-treated groups compared with the STZ group ($P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.) (Figure 5(b)).

3.2.8. The GLUT4 Protein Contents in Skeletal Muscle. At the end of the experiment, the protein contents of GLUT4 protein were lower in the STZ group than in the CON group in skeletal muscle ($P < 0.001$). After treatment, the

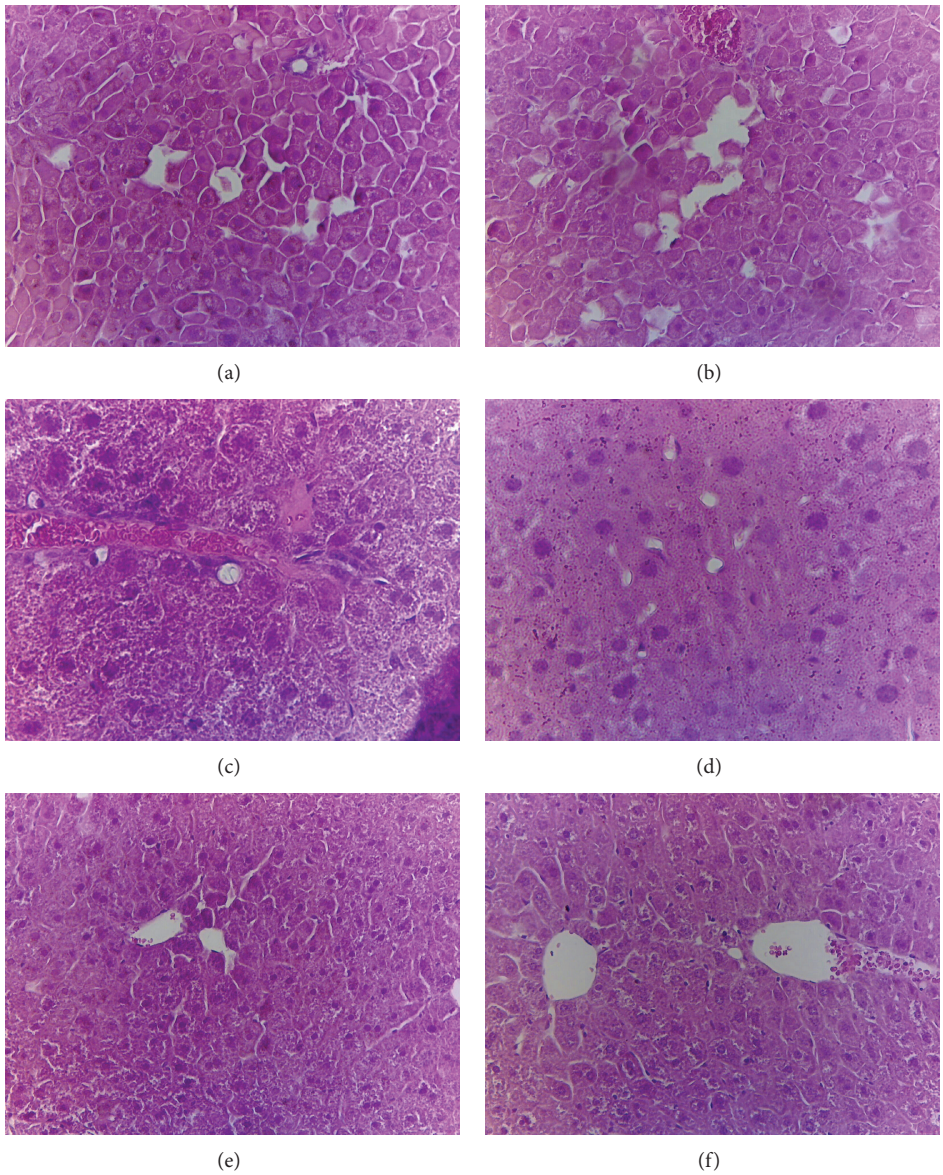


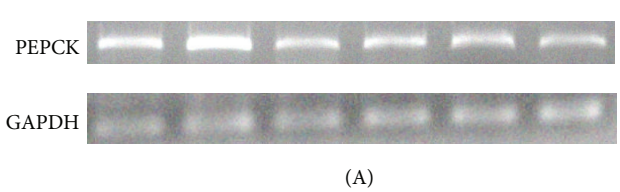
Figure 3: Histology of liver tissue of mice in the (a) control (CON), (b) streptozotocin + vehicle (distilled water) (STZ), (c) STZ + C1, (d) STZ + C2, (e) STZ + C3, or (f) STZ + metformin (Metf) groups by hematoxylin and eosinstain. Magnification: 10 (ocular) × 20 (object lens). Mice treated with the streptozotocin + vehicle (distilled water) (STZ) group. C1, C2, and C3: extracts of *Clitocybe nuda* (C1: 0.2, C2: 0.5, and C3: 1.0 g/kg bodyweight); Metf: metformin (0.3 g/kg body weight).

skeletal muscular protein contents of GLUT4 were greater in C1, C2, C3-, and Metf-treated groups than in the STZ group ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.) (Figure 5(c)).

4. Discussion

The primary aim of the present study was to examine the effects and mechanism of CNE-mediated glucose lowering effect in a diabetic model, STZ induced diabetic mice, and to compare with metformin. STZ induced diabetic models have been used as type 1 diabetic models [27]. STZ is pancreatic β-cell toxin that induces rapid and irreversible necrosis of β cells. STZ induced model had also elevated levels of triglycerides. Therefore, STZ mouse model was chosen to address antidiabetic and hypolipidemic properties of CNE.

The present study demonstrated that the CNE exerted antihyperglycemic effect both in acute and chronic STZ induced diabetic mice. Our result that metformin reduced blood glucose in diabetes is consistent with results from a previous study [28]. To examine the antidiabetic properties of CNE, we chose GLUT4 translocation in skeletal muscle as the target. This value measures the movement of the insulin responsive glucose transporter GLUT4 to the cell surface, an essential step for insulin-responsive glucose in skeletal



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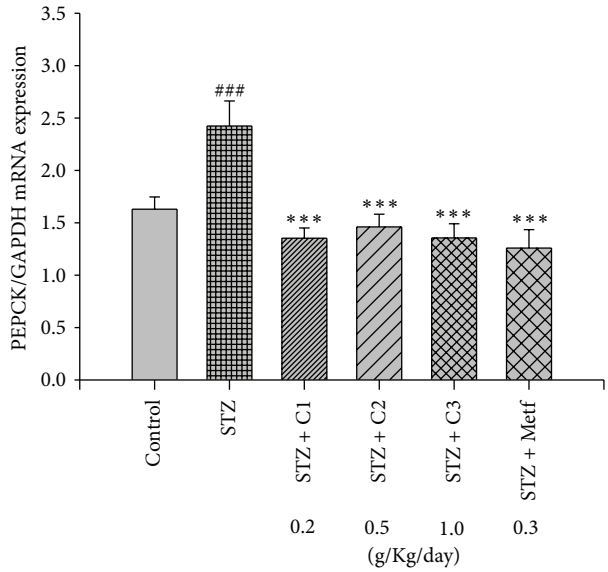
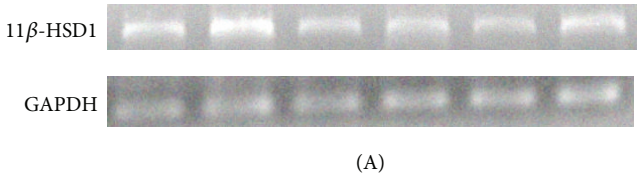


(B)

(a)



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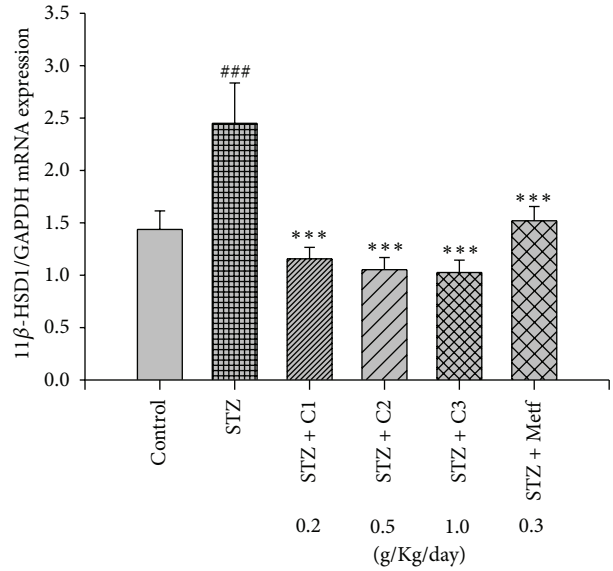

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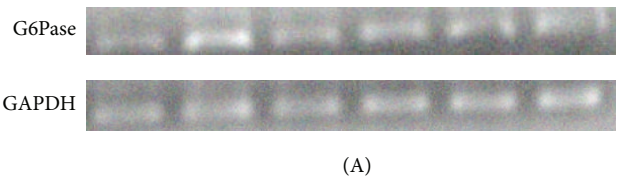

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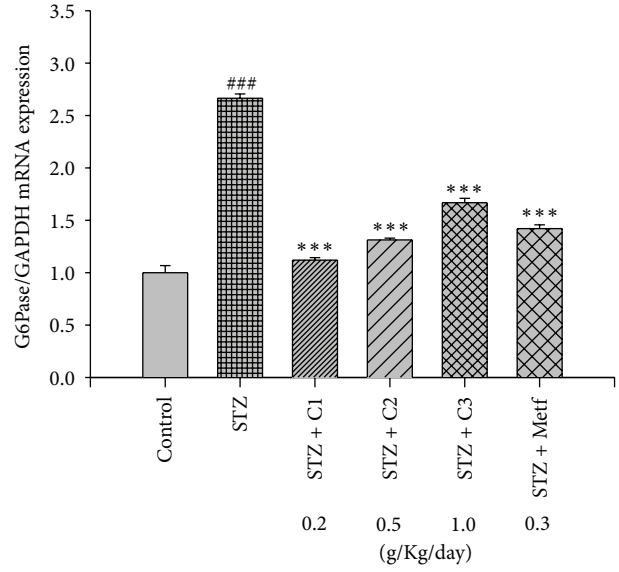

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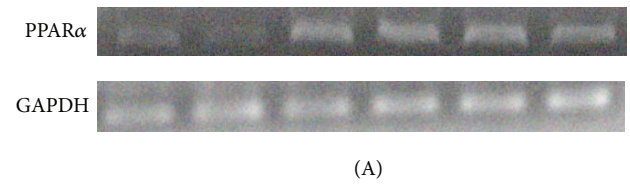
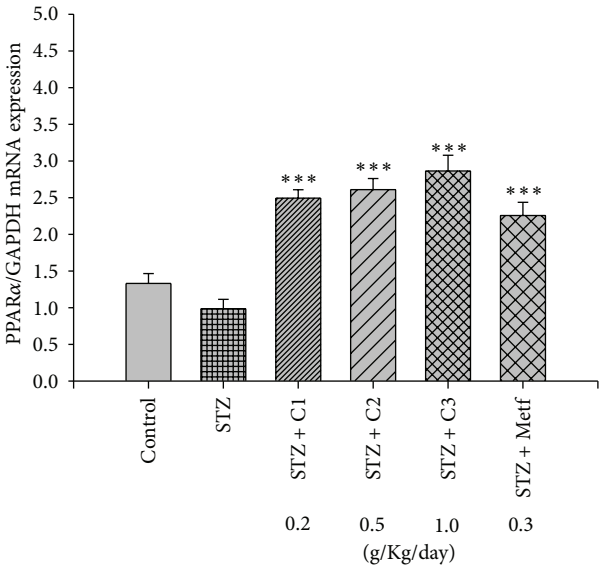


(A)



(B)

(d)

Figure 4: Continued.

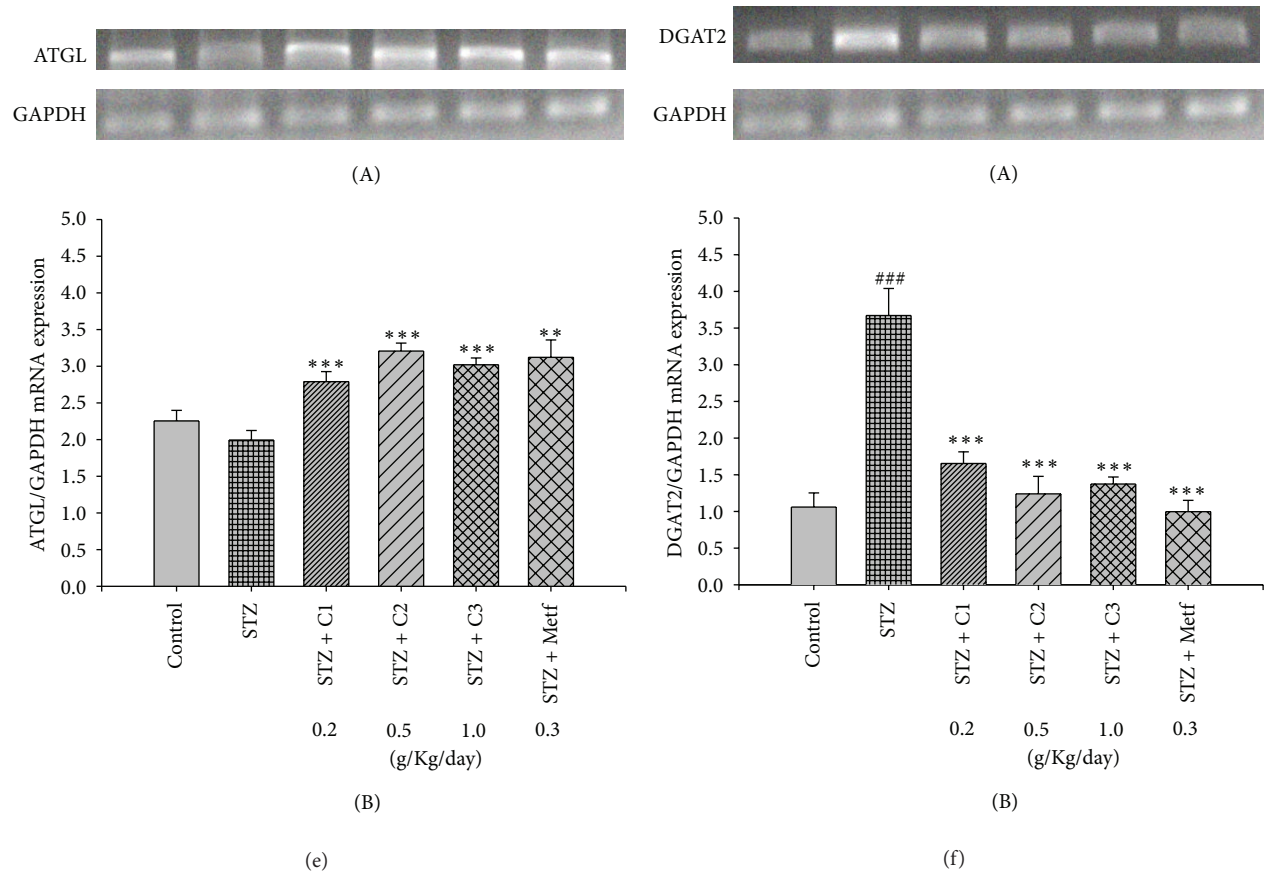


Figure 4: Semiquantitative RT-PCR analysis on (a) PEPCK, (b) 11β-HSD1, (c) G6Pase, (d) PPARα, (e) ATGL, and (f) DGAT2 mRNA expression in liver tissue of the mice by oral gavage extracts of *Clitocybe nuda* (C1: 0.2, C2: 0.5, and C3: 1.0 g/kg bodyweight); Metf: metformin (0.3 g/kg body weight). Total RNA (1 μg) isolated from tissue was reverse transcripted by MMLV-RT; 10 μL RT products were used as templates for PCR. The expression levels of PEPCK, 11β-HSD1, G6Pase, PPARα, ATGL, and DGAT2 mRNA were measured and quantified by image analysis. Values were normalized to GAPDH mRNA expression. All values are means ± S.E. ($n = 9$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the control (CON) group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the streptozotocin + vehicle (distilled water) (STZ) group.

muscle. GLUT4 translocation of skeletal muscle was reduced in STZ induced diabetic rats [29]. Treatment with CNE exerted a significant increase in GLUT4 protein contents and exhibits a strong effect to stimulate GLUT4 translocation by severalfold in skeletal muscle to a level that was comparable to metformin stimulation, suggesting that the hypoglycemic effect of CNE was related to glucose uptake by skeletal muscle.

Hypertriglyceridaemia and hypercholesterolaemia have been reported to occur in STZ diabetic animals [30, 31]. Our findings of STZ induction may elevate levels of triglycerides and total cholesterol is in agreement with the results of Choi et al. [30] and Sharma et al. [31]. The data presented show that repeated treatment with CNE decreased plasma levels of triglycerides in diabetic mice. Thus, it was clearly shown that CNE had an improving effect on the hypertriglycemia induced by STZ. Nevertheless, treatment with CNE did not decrease the levels of total cholesterol in STZ induced mice.

Glycosylated hemoglobin is a biochemical marker that strongly correlates with the level of ambient glycemia during a 2- to 3-month period and is a more accurate and reliable measure than fasting blood glucose level [32]. Glycosylated hemoglobin level is known as a key target for the prognosis of diabetes-related complications [33]. The observed increase in the level of glycosylated hemoglobin in the experimental diabetic mice implies the oxidation of sugars and extensive damage to both sugars and proteins in some tissues, continuing and reinforcing the cycle of oxidative stress and damage [34]. Treatment with CNE significantly decreased the concentrations of glycosylated hemoglobin, suggesting that it may prevent oxidative damage caused by the glycation reaction in diabetic states. The present results of levels of glucose and glycosylated hemoglobin demonstrate that CNE exerts the beneficial effects in preventing the pathogenesis of diabetic complications caused by impaired glucose metabolism.

Streptozotocin-induced diabetes is characterized by a severe loss in body weight [35]. The decrease in body weight is due to both the loss and degradation of structural

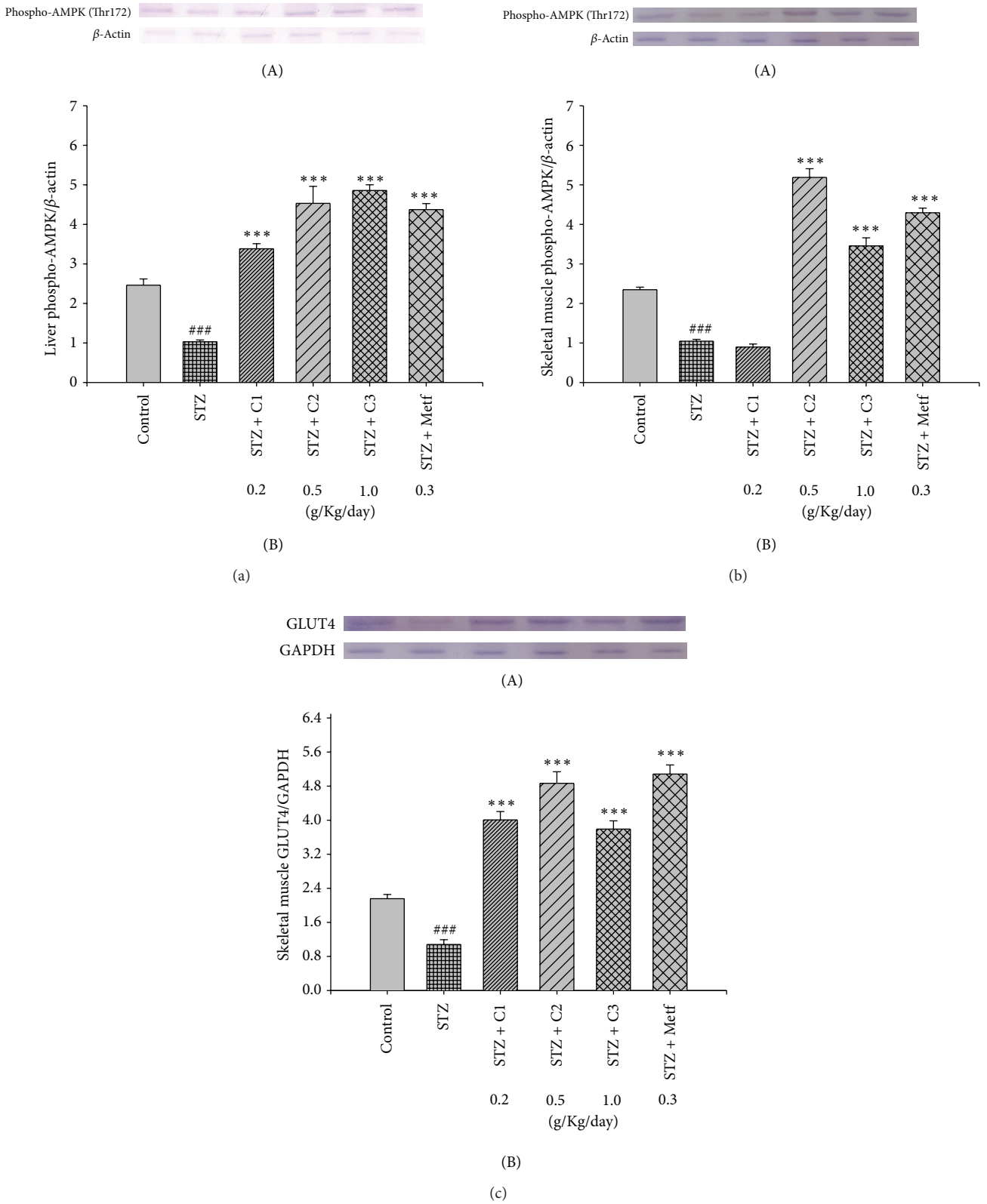


FIGURE 5: The phospho-AMPK (Thr172) protein contents in (a) liver and (b) skeletal muscle and (c) GLUT4 protein contents inskeletal muscle of the mice by oral gavage extract of *Clitocybe nuda*. Protein was separated by 12% SDS-PAGE detected by western blot. All values are means ± S.E. ($n = 9$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the control (CON) group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the streptozotocin + vehicle (distilled water) (STZ) group. C1, C2, and C3: extracts of *Clitocybe nuda* (C1: 0.2, C2: 0.5, and C3: 1.0 g/kg bodyweight); Metf: metformin (0.3 g/kg body weight).

proteins [36] and the altered carbohydrate metabolism [37]. Insulin is an important anabolic hormone. Due to decreased production of ATP and absolute or relative deficiency of insulin, protein synthesis was decreased in all tissues [38]. In the present study, the results of STZ induction decreased body weight and insulin levels were in accordance with a previous study. Following treatment with CNE, there was no difference in body weights. CNE caused a significant increase in insulin levels and decrease in blood glucose levels. Our study demonstrated that CNE exerts antidiabetic activity. The ability of CNE to reduce blood glucose levels in diabetic mice is due to its potential of secreting insulin from the existing islet β cells.

GLUT4 is the rate-limiting step for glucose uptake in skeletal muscle. Skeletal muscle is the major tissue responsible for insulin-mediated glucose utilization [39]. Insulin stimulates glucose uptake in skeletal muscle by promoting the translocation of the GLUT4 to the plasma membrane [40]. GLUT4 proteins of skeletal muscle were reduced in STZ induced diabetic animals [41]. In this study, there was a significant increase in the protein contents of GLUT4 in the CNE-treated diabetic groups. Increased protein contents of GLUT4 demonstrated that CNE improved glucose utilization in skeletal muscle by restoring translocation of GLUT4 to the plasma membrane.

The liver plays a vital role in carbohydrate metabolism. Hepatic glucose overproduction is a crucial factor in diabetic hyperglycemia. Hepatic gluconeogenesis accounts for approximately 60%~97% of the hepatic glucose production [42]. PEPCK is a key rate-limiting enzyme of gluconeogenesis. The activities of glucose 6-phosphatase (G-6Pase) increased significantly in the liver of diabetic rats [43]. G-6Pase plays a role in glucose homeostasis [44]. Insulin integrates hepatic carbohydrate metabolism by increasing the biosynthesis of enzymes of glycolysis and glycogenesis and by inhibiting gluconeogenesis [45]. Treatment with CNE reduced the expressions of these enzymes including PEPCCK and G-6Pase. Furthermore, selective inhibition of 11β-HSD1 has been shown to improve hepatic insulin sensitivity in hyperglycemic KKAy mice [46]. Thus, compounds that decrease 11β-HSD1 may impart antidiabetic effects and promote insulin sensitivity. In this study, 11β-HSD1 mRNA was decreased in all CNE-treated groups in liver tissue. Therefore, in addition to downregulation of PEPCK and G-6Pase, a decrease of 11β-HSD1 also contributes to the antidiabetic effect of CNE. The increased insulin and decreased glucose in STZ-diabetic mice might be the results of restoration of these carbohydrate metabolism enzymes.

The phosphorylation of AMPK pathway is another major regulator of GLUT4 translocation during exercise or in response to some antidiabetic agents such as AICAR and metformin [17]. We found that C2 and C3 were able to increase the phosphorylation of AMPK in muscle to a level comparable to metformin. Whether the phosphorylation of AMPK is responsible for the stimulation of GLUT4 translocation by the CNE remains to be further studied.

Metformin acts by increasing the phosphorylation and activation of AMPK [47]. Activation of AMPK is known to decrease hepatic glucose production, and the overall effect is to decrease glucose levels [47]. Liver phospho-AMPK proteins were increased in CNE- and Metf-treated groups. This might also indicate that CNE has the ability to improve hyperglycemia through AMPK activities in gluconeogenesis. Therefore, it is possible that CNE caused glucose lowering both by activation of AMPK, and inhibiting hepatic glucose production via PEPCK and G-6Pase downregulation.

The second objective of this study was to look into the antihyperlipidemic effect of CNE. PPARα is highly expressed in liver and controls β-oxidation [48]. To evaluate whether the effects of CNE on lipid profiles were mediated by alterations in PPARα target gene expression in liver, we measured the mRNA levels of various targets, including DGAT2. PPARα ligands are used widely to lower triglyceride levels in dyslipidemia and coronary heart disease [48]. Adipose triglyceride lipase (ATGL) is responsible for triacylglycerol hydrolase activity in cells that control the rate-limiting step of lipolysis in many insulin sensitive tissues. ATGL has been considered as a possible therapeutic target for dyslipidemia and fatty liver [49]. In this study, STZ induced diabetic mice exert lower PPARα mRNA levels than the CON mice. Our findings show that STZ induced mice fed with CNE were found to have significantly higher ATGL and PPARα, and lower DGAT2 mRNA levels than vehicle-treated STZ mice. However, CNE administration substantially enhanced hepatic PPARα functions in STZ induced mice. In this study, following treatment with CNE, triglycerides lowering occurred as a result of downregulation of the enzyme, DGAT2, which catalyzes the final step in the synthesis of triglycerides [50]. Therefore, the downregulation of DGAT2 appears to be responsible for the hepatic triglyceride output, which, in turn, contributed to the lowering of circulating triglycerides. In this study, CNE caused decrease in serum levels of triglycerides and these further confirm CNE's lipid-lowering effects via downregulation of genes involved in lipid synthesis.

Another finding of this study showed that the treatment of mice with CNE significantly increased leptin concentrations. Minokoshi et al. [51] demonstrated that leptin activated AMPK. The activation is strongly associated with the induction of gene expression, such as PPARα, the enhancement of fatty acid oxidation, and suppression of hepatic lipid accumulation. It is a noteworthy finding of the present study that the treatment with CNE markedly increased the phosphorylation of AMPK. Based on the reports of Minokoshi et al. [51], the AMPK phosphorylation by CNE may be linked to leptin secretion. There were two possibilities that CNE could directly activate AMPK or increase the secretion of leptin by inducing AMPK activation. The target molecule for CNE should be identified.

Analysis of WAT histology showed that C1 and C2 treatment decreased the number of large adipocytes and increased the number of small adipocytes (Figure 2). Since lipids that accumulate in adipose tissue are largely derived from circulating TG [52] and liver is a major target tissue for lipid and lipoprotein metabolism, CNE may be able to mobilize fat from adipose tissue by increasing lipid catabolism in liver. Based on our results, the increased fatty acid oxidation and

possibly decreased TG synthesis in liver effectively regulated morphometric adipocytes.

Recently, the researchers have shown that leptin could substitute for insulin to control blood sugar fluctuations in patients with type 1 diabetes [53]. Furthermore, the researchers showed that, in a mouse model of type 1 diabetes, leptin was as effective as insulin in controlling blood sugar; nevertheless the mechanism of action is far from clear. The study demonstrates that CNE had favorable effect on leptin levels, suggesting that CNE plays a role in glucose metabolism.

Metformin has been approved since 1994 by the US Food and Drug Administration for the treatment of diabetes. Metformin decreases hepatic glucose output and increases glucose uptake in the skeletal muscle [54]. Metformin exerts an insulin-sensitizing effect through AMPK pathway [55]. In this study, Metf decreases glucose production by downregulation of PEPCK and increases glucose uptake by increasing muscular GLUT4 proteins. These results were in accordance with the results of Woods et al. [54]. Moreover, our results demonstrated that Metf increases phospho-AMPK both in liver tissue and skeletal muscle. AMPK is known to play a role in glucose and lipid metabolism. Our results indicate that Metf exerts the lipid-lowering effect through regulation of lipolysis and lipogenesis via altering the expressions of hepatic ATGL and DGAT2 in STZ induced mice. Our findings indicate that Metf not only improves lipid metabolism but also has beneficial glucose metabolism both in liver and skeletal muscle.

In conclusion, CNE increased the phosphorylation of AMPK both in skeletal muscle and liver tissue. This activation of hepatic AMPK leads to a reduction gluconeogenesis (downregulated PEPCK and G-6Pase expression), thus resulting in reduced glucose level. Moreover, CNE also increased the protein contents of muscular GLUT4 to elevate glucose uptake, thus resulting in lowering blood glucose. This activation of hepatic AMPK caused a decrease in hepatic triglyceride synthesis (downregulated DGAT2 expression), whereas an increase in fatty acid oxidation, which, in turn, contributed to the lowering of circulating triglycerides. Theoretically, activation of AMPK provides an explanation for many of the pleiotropic beneficial effects of CNE. Our findings demonstrated that CNE exerted antidiabetic and hypolipidemic properties in STZ induced diabetic mice.

Abbreviations

AMPK: AMP-activated protein kinase
ATGL: Adipose triglyceride lipase
BAT: Brown adipose tissue
11β-HSD1: 11beta hydroxysteroid dehydrogenase
CON: Control
DGAT: Acyl-coenzyme A:diacylglycerol acyltransferase
FFA: Free fatty acid
GLUT4: Glucose transporter 4
G-6Pase: Glucose-6-phosphatase
HF: High-fat control
Metf: Metformin
PEPCK: Phosphoenolpyruvate carboxykinase
PPARα: Peroxisome proliferator-activated receptor α
PPARs: Peroxisomal proliferator-activated receptors
STZ: Streptozotocin
TC: Total cholesterol
TG: Triglyceride
WATs: White adipose tissues.

Conflict of Interests

The authors have declared that there is no conflict of interests.

Acknowledgments

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Research Article

Amauroderma rugosum (Blume & T. Nees) Torrend: Nutritional Composition and Antioxidant and Potential Anti-Inflammatory Properties

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Amauroderma rugosum is a wild mushroom that is worn as a necklace by the indigenous communities in Malaysia to prevent fits and incessant crying by babies. The aim of this study was to investigate the nutritive composition and antioxidant potential and anti-inflammatory effects of *A. rugosum* extracts on LPS-stimulated RAW264.7 cells. Nutritional analysis of freeze-dried mycelia of *A. rugosum* (KUM 61131) from submerged culture indicated a predominant presence of carbohydrates, proteins, dietary fibre, phosphorus, potassium, and sodium. The ethanol crude extract (EE), its hexane (HF), ethyl acetate (EAF), and aqueous (AF) fractions of mycelia of *A. rugosum* grown in submerged culture were evaluated for antioxidant potential and anti-inflammatory effects. EAF exhibited the highest total phenolic content and the strongest antioxidant activity based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. HF showed dose-dependent inhibition of NO production in LPS-stimulated RAW264.7 cells and NO radical scavenging activity. Gas chromatographic analysis of HF revealed the presence of ethyl linoleate and ergosterol, compounds with known anti-inflammatory properties. In conclusion, the nutritive compositions and significant antioxidant potential and anti-inflammatory effects of mycelia extracts of *A. rugosum* have the potential to serve as a therapeutic agent or adjuvant in the management of inflammatory disorders.

1. Introduction

Oxidative stress is caused by the imbalance between the production of reactive oxygen species (ROS) and the ability of the biological systems to detoxify reactive intermediates. This imbalance causes damage to important biomolecules and organs with potential impacts on the entire organism [1]. Most ROS are generated in cells by the mitochondrial respiratory chain, which is largely modulated by the rate of electron flow through the respiratory chain complexes. The biological reduction of molecular oxygen in aerobic cells produces ROS such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and organic peroxides, and the excess production of these radicals can oxidise and damage proteins, nucleic acids, and lipids [2]. Hence, ROS are often associated with chronic inflammation and a wide variety of cancers [3].

Inflammation is the reaction of tissue to irritation, injury, or infection, characterised by pain, redness, and swelling [4]. It is a protective process of the body that functions to destroy invading organisms or repair tissues after injury [5]. However, sustained or excessive inflammation may predispose the host to various chronic inflammatory diseases such as arthritis, asthma, multiple sclerosis, and atherosclerosis [3–5]. During inflammation, activated macrophages secrete several inflammatory mediators, including nitric oxide (NO) [6–8]. NO is synthesised by nitric oxide synthase (NOS) via oxidative deamination of L-arginine. In particular, inducible nitric oxide synthase (iNOS) catalyses the formation of a large amount of NO, which contributes to the pathogenesis of inflammatory diseases [8, 9].

Considering the undesirable side effects of anti-inflammatory drugs available in the market, natural

products/herbal medicines have gained significant interest as a source of new effective therapeutic agents. Mushrooms have been consumed by humans as a component of their normal diet since ancient times. The increased interest in scientific studies on mushrooms is attributed to the significant amounts of bioactive compounds produced by the fruiting body and the mycelium liquid culture [10]. In Malaysia, selected mushrooms are used by the locals and indigenous people as a home remedy [11]. *Amauroderma rugosum* is a basidiomycete with stipe that is black with white pore surfaces that bruise to a blood red colour when touched [12]. *Amauroderma* sp., which is also known as the "epileptic child mushroom" or "cendawan budak sawan" in the Malay language, is worn around the neck by the indigenous people in Malaysia to prevent fits and incessant crying by babies [13, 14]. Fits or epilepsy has been linked with inflammation and its development is termed epileptogenesis [15]. The study of the antioxidant potential and anti-inflammatory effects of *A. rugosum* was initiated based on the traditional aboriginal belief that this wild mushroom can reduce or prevent fit episodes. To the best of our knowledge, there are no scientific reports available on the nutritional composition or antioxidant and anti-inflammatory properties of *A. rugosum*. The aim of this study was to assess the nutritive composition, antioxidant activities, and total phenolic content of mycelia extracts of *A. rugosum* grown in submerged culture and to investigate the NO radical scavenging ability and inhibitory activity of the extracts on LPS-stimulated NO production in RAW264.7 cells.

2. Methods

2.1. Chemicals. Potato dextrose broth (PDB) and potato dextrose agar (PDA) were purchased from Difco (BD, USA), ethanol was purchased from System (Selangor, Malaysia), and hexane, ethyl acetate, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific Inc. (New Hampshire, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, trolox, butylated hydroxytoluene (BHT), gallic acid, Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), *Escherichia coli* (O55:B5) lipopolysaccharide (LPS), N_{ω}-nitro-l-arginine-methyl ester (L-NAME), sulphanilamide, N-(1-naphty) ethylenediamine, phosphoric acid (H_3PO_4), quercetin, and sodium nitroprusside were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Calbiochem, Merck Millipore (Darmstadt, Germany), and potassium persulphate, Folin-Ciocalteau phenol reagent, and sodium carbonate (Na_2CO_3) were purchased from Merck & Co. (New Jersey, USA). Penicillin-streptomycin and fungizone were obtained from Biowest (MO, USA) and phosphate buffer saline (PBS) was purchased from Oxoid Ltd, Thermo Scientific (Hampshire, UK).

2.2. Mushroom Mycelia. Mycelia of *A. rugosum* (KUM 61131) were obtained from the Mycology Laboratory, Institute of Biological Sciences, University of Malaya (Kuala Lumpur, Malaysia), and maintained on PDA medium. Seven-day old mycelia grown on PDA were used as inoculum. Ten plugs from the periphery of the colony were transferred into 500 mL baffled Erlenmeyer flasks containing 100 mL of PDB medium and incubated in a shaker at 100 rpm and 27°C for 14 days. The whole broth was then freeze dried and stored at 4°C for further use.

2.3. Preparation of A. rugosum Extract. The freeze-dried mycelia broth (24.43 ± 2.12 g) was extracted with ethanol at a ratio of 1 : 10 (w/v) for two days at room temperature. The ethanolic extract was decanted and filtered using Whatman No. 4 filter paper. The extraction process was repeated five times with ethanol at a ratio of 1 : 10 (w/v), the filtrates were combined, and the excess solvent was evaporated using a rotary evaporator. The ethanol crude extract (EE; 7.74 ± 0.78 g) was subjected to further extraction with hexane at a ratio of 1 : 10 (w/v) to yield the hexane-soluble fraction (HF; 0.21 ± 0.08 g) and hexane-insoluble residue. The hexane insoluble residue was further partitioned between ethyl acetate and water (1 : 2; v/v) to yield the ethyl acetate fraction (EAF; 0.30 ± 0.08 g) and aqueous fraction (AF; 6.72 ± 1.52 g). All the extracts were stored at 4°C prior to bioassay.

2.4. Nutritional Composition of Freeze-Dried Mycelia of A. rugosum Grown in Submerged Culture. Two hundred grams of freeze-dried mycelia of *A. rugosum* grown in submerged culture was sent to the Consolidated Laboratory (M) Sdn. Bhd. (Kuala Lumpur, Malaysia) for nutritional analysis. All tests performed were in compliance with the standards recommended by the Association of Analytical Communities/Association of Official Agricultural Chemist (AOAC) and American Association of Cereal Chemists (AACC). The cholesterol level was measured using high-performance liquid chromatography (HPLC) and the mineral contents were assessed using inductively coupled plasma optical emission spectrometry (ICP-OES).

2.5. Antioxidant Activity and Total Phenolic Content

2.5.1. DPPH Scavenging Activity. The antioxidant activity was evaluated using DPPH, according to a modified method described by Brand-Williams et al. [16]. The DPPH was dissolved in ethanol and 195 μL of this solution was added to 5 μL of *A. rugosum* extract at different concentrations (0.025, 0.25, 2.5, 25, or 250 μg/mL). The mixture was incubated for 3 hrs in the dark and the absorbance was measured at 515 nm using a spectrophotometer (Biotek, USA). Ascorbic acid, trolox, and BHT were used as positive controls. The values were expressed as EC_{50}. EC_{50} is defined as the amount of antioxidant required to scavenge 50% of the DPPH radicals.

2.5.2. ABTS Radical Scavenging Activity. The ABTS assay was used to analyse the antioxidant capacity of the *A. rugosum* mycelia extract based on the method proposed by Re et al. [17]. Briefly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS with 2.45 mM potassium persulphate in the dark at room temperature for 12–16 hrs. The $ABTS^{\bullet+}$ was further diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. One hundred microliters

of $ABTS^{\bullet+}$ solution was added to 10 μL of extract and the absorbance reading was measured after 1 min. Ascorbic acid, trolox and BHT were used as positive controls. The values were expressed as EC_{50}.

2.5.3. Total Phenolic Content (TPC). Total phenolic content (TPC) was assessed based on a method reported by Cheung et al. [18] with slight modifications. Fifty microliters of 10% Folin-Ciocalteau phenol reagent was added to 50 μL of extract and incubated in the dark at room temperature for 3 mins. Next, 100 μL of 10% Na_2CO_3 was added to the mixture and the mixture was incubated in the dark at room temperature for 1 hr. The absorbance was measured at 750 nm using a spectrophotometer. Gallic acid was used as a standard phenolic compound. All determinations were carried out in triplicate and were expressed as gallic acid equivalents (GAEs).

2.6. Anti-Inflammatory Potential of Extract

2.6.1. Cell Culture. The murine macrophage cell line (RAW264.7 cells) from American Type Culture Collection (ATCC, CAT number TIB-71) was cultured in DMEM containing 10% FBS, 0.1% penicillin-streptomycin, 0.1% L-glutamine, and 0.1% fungizone at 37°C in a humidified atmosphere containing 5% CO_2. When RAW264.7 cells had reached 80–90% confluency, the cells were scraped to remove them from the cell culture flask and then centrifuged at 1000 ×g at room temperature for 5 mins. The cell viability was determined by trypan blue dye exclusion method and direct counting with a hemocytometer.

2.6.2. Cell Viability. The cytotoxicity of *A. rugosum* extracts on RAW264.7 cells was determined using the MTT assay as described by Weyermann et al. [19]. RAW264.7 cells (4000 cells/well) were seeded in 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hrs. The attached cells were treated with *A. rugosum* extracts at different concentrations (0.01, 0.1, 1, 10, or 100 μg/mL). After 24 hrs incubation, 5 mg/mL of MTT reagent was added to each well. The supernatant from each of the 96 wells containing cells was removed after 4 hrs incubation and 100% DMSO was added to dissolve the formazan salts. The viable cells reduced the pale yellow substrate to a purple formazan product. The absorbance was measured at 560 nm and the percentage of viable cells was determined relative to the control group (untreated cells).

2.6.3. Nitric Oxide Determination. The nitric oxide (NO) was determined according to the method reported by Lee et al. [20]. RAW264.7 cells (4×10^5 cells/well) were seeded into 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hrs. The attached cells were coincubated with *A. rugosum* extracts (0.01, 0.1, 1, 10, or 100 μg/mL) and 1 μg/mL *Escherichia coli* (O55:B5) lipopolysaccharide (LPS) at 37°C in a humidified atmosphere containing 5% CO_2 for another 24 hrs. The production of NO was determined by measuring the nitrite levels in the culture supernatant using Griess reagent (1% sulphanilamide and 0.1% N-(1-naphty)ethylenediamine in 2.5% H_3PO_4) at 540 nm. The cell viability was determined using MTT assay. N-nitro-l-arginine-methyl ester (L-NAME) at a concentration of 250 μM was used as an iNOS inhibitor (positive control). A standard curve generated with sodium nitrite (0-100 μM) was used to calculate the levels of nitrite produced.

2.7. Nitric Oxide Radical Scavenging Assay. The nitric oxide radical scavenging assay was performed according to the method described by Lee et al. [20] with slight modifications. Briefly, 10 μL of *A. rugosum* extracts (0.05, 0.5, 5, 50, or 500 μg/mL) was added into 96-well plates. Then, 90 μL of sodium nitroprusside (5 mM dissolved in PBS) solution was added to each well and the plates were incubated for 90 mins with light exposure. Next, the Griess reagent was added to the wells and the resulting colour complex was measured at 540 nm. Quercetin was used as a positive control.

2.8. GC-MS Analysis. The GC-MS analysis of HF was performed with an Agilent Technologies 6890 N (United States) gas chromatography equipped with a 5975 inert mass selective detector (70 eV direct inlet) and an HP-5 ms (5% phenylmethylpolysiloxane) capillary column (30 m × 250 μm × 0.25 μm film thickness). The GC using helium as the carrier gas at a flow rate of 1 mL/min was initially set at 100°C, then programmed to increase to 300°C at a ramp rate of 5°C min^{-1}, and was put on hold for 10 minutes at 300°C. The total ion chromatogram obtained was autointegrated by Chemstation and the constituents were identified by comparison to the mass spectral database (NIST 05 Mass Spectral Library, USA).

2.9. Statistical Analysis. All values are expressed as means ± standard deviation (SD) of triplicate values. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Comparison Test using Statistical Product and Service Solutions, SPSS Statistics for Windows, Version 17.0, and $P < 0.05$ was denoted as being statistically significant. Effective concentrations (EC_{50}) were calculated using GraphPad Prism software version 5.0. The scatter plot and regression line for the correlation between TPC and antioxidant activities were plotted using GraphPad.

3. Results

3.1. Nutritional Composition of Freeze-Dried Mycelia of A. rugosum Grown in Submerged Culture. The nutritional composition of freeze-dried mycelia of *A. rugosum* grown in submerged culture is depicted in Table 1. The freeze-dried mycelia grown in submerged culture contain 0.3% of the recommended daily allowance (RDA) of total fat, a nondetectable level of cholesterol, 25.5% of the RDA of carbohydrate, 16.6% of the RDA of protein, and 38.4% of the RDA of dietary fibre and are rich in minerals such as phosphorus (14.4% of the RDA), potassium (11.6% of the RDA), and sodium (25.4% of the RDA).

3.2. Antioxidant Activity and Total Phenolic Content. The total phenolic content (TPC) of mycelia extracts of *A. rugosum* grown in submerged culture was quantified, and their antioxidant activities were evaluated based on DPPH

Table 1: Nutritional analysis of freeze-dried mycelia of *A. rugosum* grown in submerged culture.

Component	Method	Composition/100 g	Recommended daily allowance (RDA)
Energy	AOAC	341 kcal	—
Total fat	AOAC 989.05	0.2 g	65 g
Carbohydrate	AOAC	76.5 g	300 g
Protein	AACC 46-12	8.3 g	50 g
Cholesterol	HPLC	ND*	300 mg
Dietary fibre	AOAC 985.29	9.6 g	25 g
Magnesium	ICP-OES	5.69 mg	400 mg
Iron	ICP-OES	1.00 mg	18 mg
Zinc	ICP-OES	0.77 mg	15 mg
Phosphorus	ICP-OES	144.43 mg	1000 mg
Potassium	ICP-OES	404.86 mg	3500 mg
Copper	ICP-OES	0.01 mg***	2.0 mg
Manganese	ICP-OES	0.03 mg	2.0 mg
Selenium	ICP-OES	ND**	70 μg
Sodium	ICP-OES	609.90 mg	2400 mg
Calcium	ICP-OES	4.66 mg	1000 mg

ND: not detectable; AOAC: Association of Analytical Communities/Association of Official Agricultural Chemist; AACC: American Association of Cereal Chemists; HPLC: high-performance liquid chromatography; ICP-OES: inductively coupled plasma optical emission spectrometry. *Level of cholesterol <0.001 mg/100 g; **level of selenium <0.02 mg/kg; ***per kg.

Table 2: The antioxidant activities and total phenolic content of *A. rugosum* extracts.

	Total phenolic content (mg of GAEs/g of extract)	DPPH (EC_{50} μg/mL)	ABTS (EC_{50} μg/mL)
Ascorbic acid	—	2.84	6.43
BHT	—	6.84	18.99
Trolox	—	4.23	17.87
EE	93.62 ± 0.01	17.14	110.00
HF	154.11 ± 0.01	8.18	51.63
EAF	555.42 ± 0.01	2.30	18.34
AF	114.14 ± 0.00	10.05	52.59

DPPH and ABTS are expressed as half maximal effective concentrations (EC_{50}); TPC results are expressed as mean ± standard deviation ($n = 3$).

and ABTS assays; the results are shown in Table 2. The DPPH scavenging activity of the extracts in descending order of potency was EAF > HF > AF > EE, as shown in Table 2. The descending order of the ABTS scavenging potency of the extracts was EAF > HF > AF > EE (Table 2). Besides, EAF had the highest amount of phenolic compounds, followed by HF, AF, and EE. Overall, EAF had the highest antioxidant activities and total phenolic content. The relationship between EC_{50} values of antioxidant activities and TPC is shown in Figures 1 and 2. It was found that TPC and EC_{50} values for DPPH and ABTS had a weak correlation ($R^2 = 0.695$ and $R^2 = 0.571$, $P > 0.05$, resp.).

3.3. The Effects of A. rugosum Extracts on RAW264.7 Cell Viability. The MTT assay was used to determine the effect of *A. rugosum* extracts on RAW264.7 cell viability. The cell viability of the positive control (cells without any treatment) was denoted as 100%. All the extracts tested, except for AF, had no cytotoxic effects on RAW264.7 cells (Figure 3). AF treatment at 1 μg/mL caused a significant ($P < 0.05$) decrease in the number of viable cells. EE and HF promoted the proliferation of cells at concentrations greater than 1 μg/mL.

3.4. Inhibitory Effect of A. rugosum Extracts on Nitric Oxide (NO) Level in LPS-Stimulated RAW264.7 Cells. Murine macrophage RAW264.7 cells were challenged with LPS to produce NO and the effect of *A. rugosum* extracts on NO inhibition was assessed. All *A. rugosum* extracts, except for AF, inhibited NO production in a dose-dependent manner (Figure 4). The unstimulated cells secreted NO at the basal level of 1.39 ± 0.01 μM, while the nontreated LPS-stimulated cells showed an increase in NO production (15.20 ± 0.01 μM; 0% inhibition). L-NAME, a standard NOS inhibitor, was used as the positive control and it significantly ($P < 0.05$) inhibited NO (5.91 ± 0.01 μM; 61.1%) at 250 μM. Among the *A. rugosum* extracts tested, HF significantly ($P < 0.05$) inhibited NO production at all concentrations tested and inhibited it completely at 100 μg/mL (Figure 4); the cell viability of HF-treated cells was 104.7% (Figure 5).

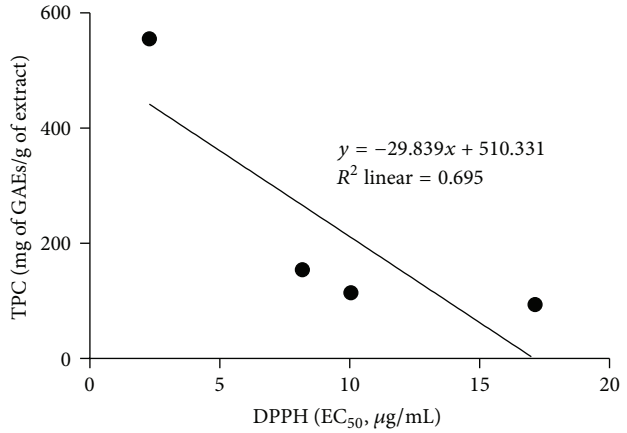

FIGURE 1: Correlation between EC_{50} value of DPPH and TPC.

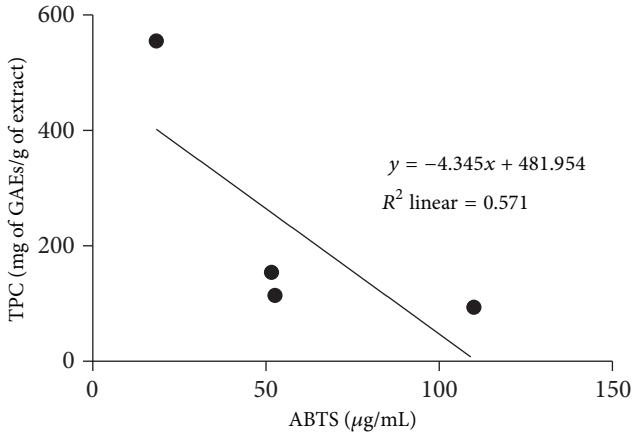

FIGURE 2: Correlation between EC_{50} value of ABTS and TPC.

3.5. Nitric Oxide (NO) Radical Scavenging Activity. The ability of *A. rugosum* extracts to scavenge NO radicals was evaluated. All *A. rugosum* extracts were able to scavenge NO radicals in a dose-dependent manner (Figure 6). At a concentration of 500 μg/mL, EAF scavenged 46.7% of the NO radicals and this was comparable to the NO scavenging of quercetin, which was 39.6%. Quercetin is a flavonoid widely found in plants that is well known for its antioxidant and radical scavenging properties.

3.6. Identification of Compounds in HF. The chemical investigation of HF led to the identification of two major components, ethyl linoleate and ergosterol (Table 3, Figure 7).

4. Discussion

Amauroderma rugosum belongs to the family of Ganodermataceae and its white pore surface bruises red when touched. The sliced hollow stipe is commonly worn as a necklace around the neck by the indigenous Temuan tribe in Peninsular Malaysia to prevent fits [13]. The Temuans also believe that babies wearing the fruiting bodies as a necklace will not cry at night [14]. Epilepsy, commonly known as fits,

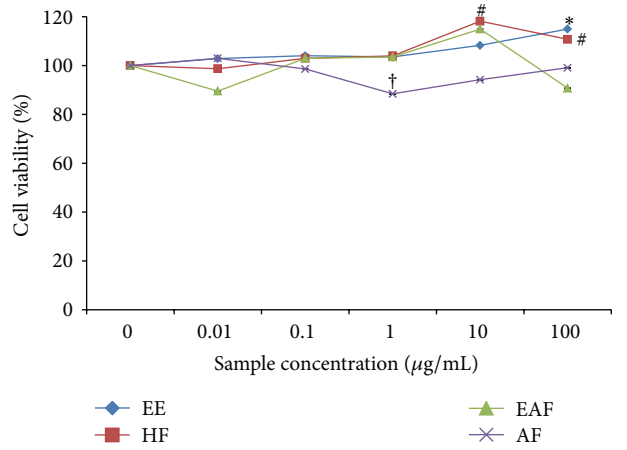

FIGURE 3: The effects of *A. rugosum* extracts on RAW264.7 cell viability. RAW264.7 cells were treated with *A. rugosum* extracts and cells without any treatment were expressed as 100%. Data were means ± S.D., $n = 3$, and $^{*,\#,\dagger}P < 0.05$ compared to control 100%.

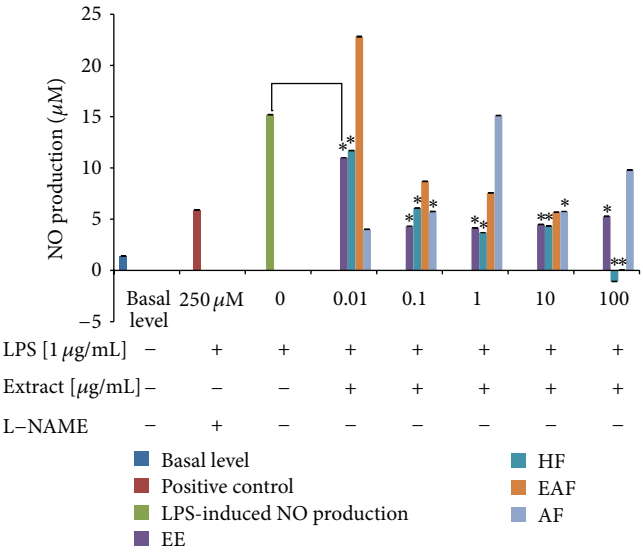

FIGURE 4: Effects of *A. rugosum* extracts on LPS-induced NO production by RAW264.7 cells. RAW264.7 cells were coincubated with various concentrations of *A. rugosum* extracts and 1 μg/mL LPS for 24 hrs. L-NAME (250 μM) served as positive control. Results shown represent the mean ± S.D., $n = 3$, and $^{*}P < 0.05$ versus LPS-induced NO level alone.

TABLE 3: Chemical constituents of HF.

Chemical constituents	RT (min)	MW	MF	Peak area (%)
Ethyl linoleate	24.811	308.27	$C_{20}H_{36}O_2$	4.30
Ergosterol	40.250	396.65	$C_{28}H_{44}O$	83.79

RT: retention time; MW: molecular weight (g mol^{-1}); MF: molecular formula.

is linked with inflammation and its development is termed epileptogenesis [15]. It has been reported that patients with refractory focal epilepsy display the hallmarks of chronic

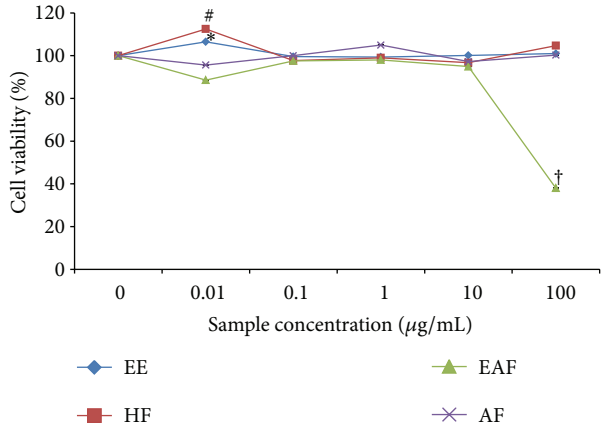

Figure 5: Effects of *A. rugosum* extracts on LPS-stimulated RAW264.7 cell viability. Cell viability of LPS-stimulated murine macrophage RAW264.7 cells was assessed using MTT method. Data shown were mean ± S.D., $n = 3$, and $^{*,\#,\dagger}P < 0.05$ compared to control 100%.

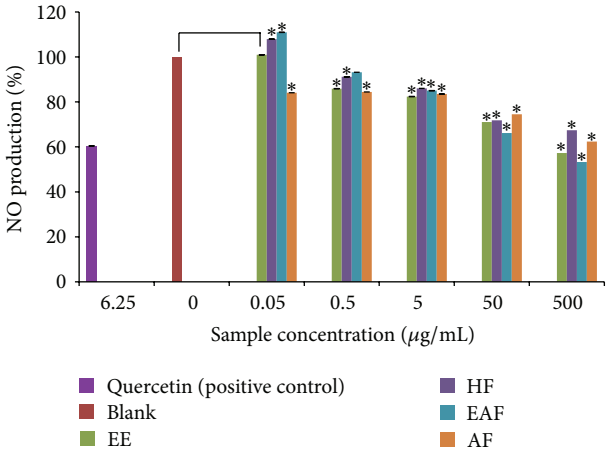

Figure 6: Effects of *A. rugosum* extracts on NO production by sodium nitroprusside (SNP). *A. rugosum* extracts were coincubated with SNP (5 mM dissolved in PBS) solution for 90 minutes. Quercetin (6.25 μg/mL) was served as positive control. Results shown represent the mean ± S.D., $n = 3$, and $^{*}P < 0.05$ versus SNP-produced NO alone.

inflammation such as infiltration of leukocytes and overexpression of cytokines and targeted proteins [21]. The traditional aboriginal belief that the use of *A. rugosum* to prevent fits led to the study of the antioxidant and anti-inflammatory effects of this wild mushroom. To date, the pharmacological and biochemical activities of *A. rugosum* have not been elucidated. Hence, to the best of our knowledge, this report is the first to describe the nutritional content and medicinal properties of mycelia of *A. rugosum* grown in submerged culture.

It has been reported that mushrooms are good sources of nutritional components such as proteins, minerals, and vitamins. However, there is no existing data in the literature on the nutritional components of *A. rugosum*. The knowledge of the composition and nutritional value of wild mushrooms is limited compared to vegetables or culinary and medicinal mushrooms [22]. Thus, it is important to examine the medicinal properties and biologically active components of wild mushrooms that may benefit humankind [23]. Moreover, increasing the understanding of the ethnomedicinal use of wild mushrooms is necessary for successful bioprospecting. According to the US Food and Drug Administration (FDA), it is important to disclose the nutrient content of a dietary supplement to ensure its safety and effectiveness before and after a product is marketed. Generally, fruiting bodies of mushrooms have low fat content and high protein content (including essential amino acids) that range from 19 to 35% of the RDA and large amounts of carbohydrate and fibre, ranging from 51 to 88% and from 4 to 20% of the RDA, respectively [24]. Mhd Omar et al. [25] reported that *Lentinus squarrosulus* mycelia extract contained 4.1% carbohydrate, 0.8% total fat, and <0.1 g of the 100 g RDA of crude fibre. The present study showed that the mycelia of *A. rugosum* grown in submerged culture contained 6.2 times more carbohydrate and 96 times more fibre content than the *L. squarrosulus* mycelia extract. Furthermore, the mycelia of *A. rugosum* grown in submerged culture have lower total fat than the *L. squarrosulus* mycelia and a nondetectable level of cholesterol and are rich in minerals, such as phosphorus, potassium, and sodium. Low total fat and cholesterol content are recommended as nutritional supplements for heart patients. Also, high levels of potassium help the body process sodium, which lowers blood pressure. Furthermore, phosphorus is one of the important minerals that combines with calcium to form calcium phosphate, which gives strength and rigidity to bones and teeth.

Reactive oxygen species (ROS), the most common form of free radicals, are produced during the normal metabolism of aerobic cells. Most of the free radicals produced are neutralised by cellular antioxidant defences (enzymatic and nonenzymatic systems), and the maintenance of equilibrium between the ROS generation and neutralisation systems is essential for the normal functions of an organism [10]. Thus, disequilibrium of this physiological process causes deleterious effects in living systems and eventually causes oxidative stress. Many studies have been carried out to determine effective ways of avoiding the onset of diseases that are caused by oxidative stress. The most effective way to combat oxidative stress is by supplying the body with a greater amount of natural antioxidants. Natural antioxidants are safe, cheap, and bioactive [26]. For this reason, extensive research has been conducted to identify natural products with antioxidant aptitude that may be used for human consumption or purified into drugs. In the current study, we have found that EAF had the highest total phenolic content and antioxidant activity of all the extracts tested. This is similar to the study reported by Öztürk et al. [27], which showed that the ethyl acetate extract of *Agaricus bitorquis* had the highest total phenolic content of all the extracts of that species. The significantly higher phenolic content of EAF than crude EE is most likely due to the concentration of the phenolic compounds in the fractionation process. Moreover, the high phenolic content in EAF may contribute to its antioxidant activity [28]. Mhd Omar et al. [25] reported that the aqueous extract of *L. squarrosulus* mycelia exhibited DPPH scavenging activity with an IC_{50} value of 14.29 mg/mL. Furthermore, the DPPH scavenging

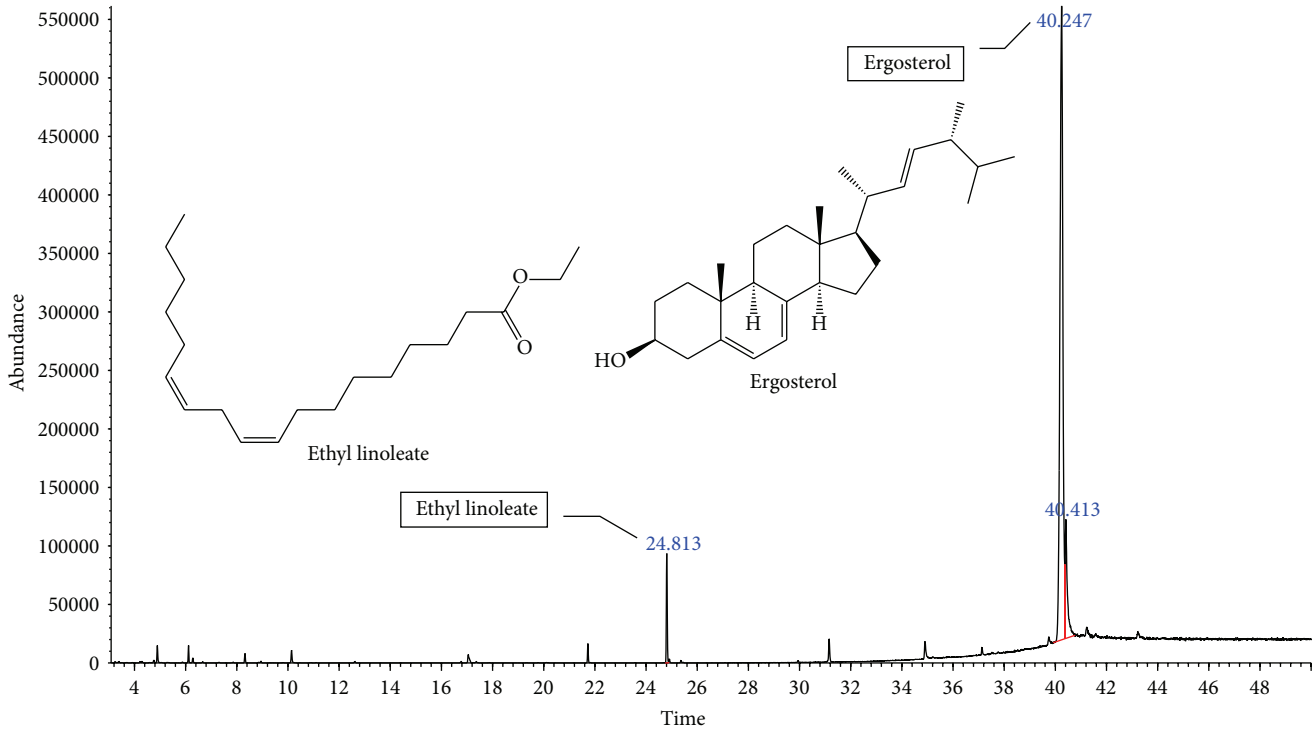

Figure 7: Chromatogram of HF of *A. rugosum*.

activity of the ethanol extract and hot aqueous extract of *Pleurotus ferulae* mycelium showed EC_{50} values of 12.0 mg/mL and 4.3 mg/mL, respectively [29]. According to Carvajal et al. [30], the DPPH scavenging activity of the *Agaricus brasiliensis* fruiting bodies was better than the *A. brasiliensis* mycelium, but the ABTS cation radical scavenging ability analysis indicated that the mycelia had greater antioxidant activity than the fruiting bodies. Carvajal et al. [30] suggested that the discrepancy of these results may be attributed to the difference in the types of polyphenols and nonphenolic components with antioxidant activity that are present in the various extracts.

Analysis of the relationship between the TPC and antioxidant activities of *A. rugosum* extracts revealed that there was no significant correlation between them. According to Prior et al. [31], the Folin-Ciocalteau assay is used to estimate the TPC present in the extract, but free radical scavenging assays are not specific for polyphenols. Various phenolic compounds respond differently to the DPPH and ABTS assays, and the results of these assays depend on the number of phenolic groups present in the extracts [32]. Therefore, the insignificant correlation between the TPC and antioxidant activity may be attributed to the presence of nonphenolic compounds with antioxidant activities [33].

In the present study, the MTT assay was used to evaluate the cytotoxic effect of *A. rugosum* extracts on RAW264.7 murine macrophage cells. It was found that *A. rugosum* extracts had no cytotoxic effects on RAW264.7 cells, except for the AF at a concentration of 1 μg/mL, which showed a significant ($P < 0.05$) but mild toxic effect. Moreover, the EE and HF significantly ($P < 0.05$) promoted proliferation of RAW264.7 cells at a higher concentration. During inflammation, numerous inflammatory mediators such as NO, cytokines, and prostaglandin E_2 are produced [20]. However, excessive production of NO is harmful to living organisms. In the present study, the abilities of *A. rugosum* extracts to scavenge NO radicals and inhibit NO production in RAW264.7 murine macrophage cells *in vitro* were evaluated. It was found that all the *A. rugosum* extracts were able to scavenge NO radicals and reduce LPS-induced NO production in macrophages. Among the *A. rugosum* extracts tested, the EAF and HF completely inhibited NO production at a concentration of 100 μg/mL. To determine whether the inhibition of NO was due to cell death or the downregulation of iNOS expression, the MTT assay was performed after challenging the cells with LPS. It was found that the reduced NO level caused by EAF at 100 μg/mL was due to cell death. On the other hand, when treated with HF at 100 μg/mL, RAW264.7 cells remained viable, and their NO production was inhibited. Additionally, the HF was able to scavenge NO radicals in a dose-dependent manner. Jedinak et al. [34] showed that oyster mushroom concentrate (*Pleurotus ostreatus*) markedly suppressed NO production in LPS-stimulated RAW264.7 cells at the highest concentration tested, which was 100 μg/mL. Lee et al. [20] also reported that the curcumin derivative, 2,6-bis(2,5-dimethoxybenzylidene)-cyclohexanone, significantly inhibited NO production in LPS-stimulated RAW264.7 cells with an IC_{50} of 13.66 $\pm$ 0.61 μM. However, the same report indicated that the curcumin derivative did not scavenge NO radicals at any concentrations tested. The HF was able to scavenge NO radicals and inhibit NO production effectively which may be an added value of this extract as a

potential anti-inflammatory agent. Therefore, HF was chosen for further identification of potential compounds that may be responsible for its anti-inflammatory activities.

Two major components were identified in HF, namely, ethyl linoleate and ergosterol. Ethyl linoleate has been reported to alleviate inflammation and its combination with antioxidants such as α-1-histidine, α-tocopherol, and tertiary butyl hydroquinone (TBHQ) can be used as a nonsteroidal topical agent [35]. Ergosterol is abundant in many mushrooms and is known to be provitamin D_2 [36]. The conversion of ergosterol to vitamin D_2 via UV radiation has a long history of commercial use for vitamin D_2 production for dietary supplements, pharmaceutical grade vitamin D preparations, and food fortification [37]. A few studies have reported the successful conversion of ergosterol to vitamin D_2 in mushrooms via UV irradiation [38, 39]. According to Ma et al. [40], ergosterol isolated from *Inonotus obliquus* was found to possess anti-inflammatory activities. Also, ergosterol purified from *Sarcodon aspratus* showed significant inhibition of LPS-induced inflammatory responses through nuclear factor-kappa B (NF-κB) and CAAT/enhancer binding protein-beta (C/EBPβ) and prevented the phosphorylation of p39, JNK, and ERK MAPKs [36].

Many studies have demonstrated that the NO inhibitory effect in RAW264.7 cells is due to the downregulation of iNOS. Inducible nitric oxide synthase (iNOS) is a soluble enzyme that catalyses the production of NO at not only the transcriptional level but also the posttranscriptional, translational, and posttranslational levels. The regulation of this pathway is important to ensure the maintenance of NO level at a constant level in living organisms. However, NO can be overproduced due to the overprotective effect of iNOS on microbial and viral pathogens. The generation of high concentrations of NO through the activation of iNOS by immunostimulating cytokines or bacterial pathogens and the activation of inducible nuclear factors such as NF-κB may predispose an individual to inflammatory diseases, such as atherosclerosis, rheumatoid arthritis, diabetes, septic shock, and multiple sclerosis [41]. Thus, the inhibition of iNOS exerts a beneficial anti-inflammatory effect on inflammatory disorders. This study demonstrated that the HF significantly ($P < 0.05$) inhibited NO production in LPS-stimulated murine macrophage cells and scavenged NO radicals effectively. Although the HF did not exhibit the highest antioxidant activity compared to the other extracts tested, it was able to scavenge the DPPH radical and inhibit the $ABTS^{\bullet+}$ radical cation with EC_{50} values of 8.18 μg/mL and 51.63 μg/mL, respectively. These results suggest that EAF has strong antioxidant activity, and HF has possible roles as an antioxidant with anti-inflammatory properties.

5. Conclusions

In conclusion, the mycelia of *Amauroderma rugosum* are a good source of nutrients and possessed significant antioxidant and anti-inflammatory activities. Hexane fraction (HF) showed antioxidant activity and this concurred with the scavenging of NO and inhibition of NO production in LPS-stimulated macrophages (RAW264.7 cells). Ethyl linoleate and ergosterol, the two major components detected in the HF, may contribute to the activities reported. In summary, this wild mushroom, traditionally used to control epileptic episodes in children, may have potential in the mitigation of inflammatory disorders leading to epilepsy. However, further analysis of mechanisms of activity including gene expression analysis is warranted.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Pui-Mun Chan carried out the experiment, drafted the paper, and is engaged in data acquisition and data interpretation. Gowri Kanagasabapathy is involved in data interpretation of GC-MS analysis. Yee-Shin Tan and Vikineswary Sabaratnam participated in acquisition of funding mushroom sampling and editing of the paper. Umah Rani Kuppusamy is involved in design of the study, coordinating and monitoring of research, data interpretation and paper editing.

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