

Method for manufacturing and the use of *Lignosus Rhinocerus* mycelia active substance for preventing and/or ameliorating lung injury

BACKGROUND

Technical Field

[0001] The present invention relates to a *Lignosus rhinocerus* mycelia active substance, a method for manufacturing said *Lignosus rhinocerus* mycelia active substance, and the use thereof. In particular, the present invention relates to a *Lignosus rhinocerus* mycelia active substance for ameliorating lung injury, a method for manufacturing said *Lignosus rhinocerus* mycelia active substance, and the use thereof in food or pharmaceuticals.

Description of Related Art

[0002] Among the ten leading causes of death in 2017 according to Taiwan's Ministry of Health and Welfare, four of them are closely associated with lung inflammation, namely, malignant neoplasms (ranked no. 1) including cancers of trachea, bronchus and lung, pneumonia (ranked no. 3), and chronic lower respiratory diseases (ranked no. 7).

[0003] Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (more severe than the former) are acute inflammatory lung diseases that are frequently seen clinically, both of which may cause respiratory failure and may be fatal. They are also associated with many respiratory diseases.

[0004] ALI-causing risk factors fall into two major groups: direct and indirect risk factors. Direct risk factors are those which originate in the lungs,

such as infective pneumonia caused by bacteria or viruses, aspiration of gastric acid or foreign objects, or lung contusion. On the other hand, indirect risk factors are those which do not originate in the lungs, such as sepsis, chronic use of alcohol and drugs, or transfusion of artificial blood plasma.

[0005] While common treatments of ALI include mechanical ventilation, beta-2 adrenergic receptor agonist, anticoagulation, thrombolysis, surfactant therapy, and surgery, there is still a lack of clinically effective drugs that can be used to manage ALI's case fatality rate due to the complex mechanism and high case fatality rate of ALI.

[0006] Chronic Obstructive Pulmonary Disease (COPD), the fourth leading cause of death in the world, is a common chronic respiratory disease with high disability rates and fatality rates. COPD affects over 300 million people worldwide.

[0007] The primary cause of COPD is smoking. Recently in Taiwan, however, particulate matter pollution (PM 2.5) has become a major cause of the disease due to emissions produced by scooters and cars, and to the increasing energy consumption, coal-burning and fuel-burning in the industries. Long-term exposure to such pollution tends to cause lung inflammation and airway narrowing, resulting eventually in lung tissue defects.

[0008] Treatments for COPD include smoking cessation, pulmonary rehabilitation, inhaled bronchodilators, corticosteroids, long-term oxygen therapy or lung transplantation. The latest new drug approved in 2015 is a combination of two muscle relaxants previously used for treating COPD. Nevertheless, no further breakthroughs have occurred ever since.

[0009] Therefore, research and development on effective treatments of ALI and COPD remain essential.

[0010] *Lignosus rhinocerus*, commonly known as tiger milk mushroom, belongs to family Polyporaceae in the division Basidiomycota. *Lignosus rhinocerus* has been used traditionally in Malaysia as a medication for treating diseases such as cough, asthma, bronchitis, vomit, fever and food poisoning. Recent pharmaceutical research has found that *Lignosus rhinocerus* has effects of immune response regulation, anti-inflammation, anti-oxidation, anti-microbial, anti-virus, anti-coagulation and anti-breast cancer. However, there has been no research done on ameliorating ALI and COPD using *Lignosus rhinocerus*.

SUMMARY

[0011] Provided herein is a *Lignosus rhinocerus* mycelia active substance and a method for manufacturing said *Lignosus rhinocerus* mycelia active substance, which can be used to ameliorate lung injuries. Compared to conventional drugs and treatments, the method for manufacturing the liquid fermentation of the *Lignosus rhinocerus* mycelia active substance disclosed herein is more natural, safer and simpler, and effective in ameliorating lung injuries such as ALI or COPD.

[0012] According to one embodiment of the present invention, a method is provided for manufacturing a *Lignosus rhinocerus* mycelia active substance for ameliorating lung injury. The method comprises the following steps:

[0013] (a) growing *Lignosus rhinocerus* mycelia on a PDA(potato dextrose agar) plate between 15 and 30°C for 1 to 2 weeks;

[0014] (b) inoculating the *Lignosus rhinocerus* mycelia of step (a) to a flask and culturing the mycelia between 15 and 30°C and at a pH of 2 to 6 for 3 to 14 days;

[0015] (c) inoculating the *Lignosus rhinocerus* mycelia of step (b) to a fermenter tank and culturing the mycelia by stirring between 15 and 30°C and at a pH of 2 to 6 for 3 to 21 days, so as to obtain a *Lignosus rhinocerus* mycelium fermentation liquid containing *Lignosus rhinocerus* mycelia active substance.

[0016] In one embodiment, the method for manufacturing a *Lignosus rhinocerus* mycelia active substance further includes steps of (d): freeze-drying the *Lignosus rhinocerus* mycelium fermentation liquid and grinding the freeze-dried product, so as to obtain a *Lignosus rhinocerus* mycelium powder containing *Lignosus rhinocerus* mycelia active substance.

[0017] In one embodiment, the method for manufacturing *Lignosus rhinocerus* mycelia active substance further includes step (e): extracting the *Lignosus rhinocerus* mycelium freeze-dried powder with at least one solvent, so as to obtain a *Lignosus rhinocerus* mycelium fluid extract containing *Lignosus rhinocerus* mycelia active substance.

[0018] In one embodiment, the method for manufacturing *Lignosus rhinocerus* mycelia active substance further includes step (f): drying the *Lignosus rhinocerus* mycelium fluid extract, so as to obtain *Lignosus rhinocerus* mycelia active substance.

[0019] In one embodiment, the solvent of step (e) is selected from water or ethanol.

[0020] In one embodiment, the culturing of step (b) is a shake flask culture, and the shaking speed is between 110-130 rpm.

[0021] In one embodiment, a gas is further fed into the fermenter tank of step (c), and the gas comprises air, oxygen, carbon dioxide, helium or a combination thereof; the pressure of the fermenter tank is 0.5 to 1.0 kg/cm², and a gas flow rate is 0.01 to 1.5 VVM.

[0022] In another embodiment of the present invention, a *Lignosus rhinocerus* mycelia active substance is provided, which is manufactured using the said method.

[0023] In yet another embodiment of the present invention, a composition for ameliorating lung injury is provided, which comprises said *Lignosus rhinocerus* mycelia active substance having an effective concentration, and a pharmaceutically acceptable carrier, excipient, diluent or adjuvant.

[0024] In one embodiment, the *Lignosus rhinocerus* mycelia active substance has an effective concentration of greater than 1 mg/ml when the solvent used for extraction is water; the *Lignosus rhinocerus* mycelia active substance has an effective concentration of greater than 25 µg/ml when the solvent used for extraction is ethanol.

[0025] In yet another embodiment of the present invention, a use of said *Lignosus rhinocerus* mycelia active substance is provided, which is used to ameliorate lung injury.

[0026] In one embodiment, said lung injury includes chronic obstructive pulmonary disease and/or acute lung injury.

[0027] In one embodiment, said composition is a pharmaceutical composition or a food additive.

[0028] For the purpose of further illustrating the above and other aspects of the present invention, several exemplary embodiments will be described with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] Fig. 1 shows the test result after administering *Lignosus rhinocerus* mycelia active substance intra-peritoneally to acute lung injury

models.

[0030] Fig. 2 shows the protein leakage test result after *Lignosus rhinocerus* mycelia active substance was administered intra-peritoneally.

[0031] Fig. 3 shows the test results of (A) leukocytes, (B) phagocytes and (C) neutrophils after administering *Lignosus rhinocerus* mycelia active substance intra-peritoneally .

[0032] Fig. 4 shows the test result after administering *Lignosus rhinocerus* mycelia active substance orally to acute lung injury models.

[0033] Fig. 5 shows the protein leakage test result after *Lignosus rhinocerus* mycelia active substance was administered orally.

[0034] Fig. 6 shows the test results of (A) leukocytes, (B) phagocytes and (C) neutrophils after administering *Lignosus rhinocerus* mycelia active substance orally.

[0035] Fig. 7 shows the cell injury test result after inducing COPD in A549 cells (alveolar epithelial cells) using cigarette smoke extracts, and treating the cells with *Lignosus rhinocerus* mycelia water extracts, wherein LrH(L), LrH(M) and LrH(H) refer to *Lignosus rhinocerus* mycelia water extracts having a concentration of 0.25 mg/ml, 0.5 mg/ml and 1.0 mg/ml, respectively.

[0036] Fig. 8 shows the cell injury test result after inducing COPD in A549 cells (alveolar epithelial cells) using cigarette smoke extracts, and treating the cells with *Lignosus rhinocerus* mycelia alcohol extracts, wherein LrE(L), LrE(M) and LrE(H) refer to *Lignosus rhinocerus* mycelia ethanol extracts having a concentration of 6.25 μ g/ml, 12.5 μ g/ml and 25 μ g/ml, respectively.

DETAILED DESCRIPTION

Example 1: Culturing *Lignosus rhinocerus* mycelia

[0037] The *Lignosus rhinocerus* mycelia used in this example were purchased from the fruit bodies of wild type *Lignosus rhinocerus* in Malaysia. The fruit bodies were first surface sterilized, cut to make a notch, and then ripped so as to obtain an uncontaminated region. A small piece of fruit bodies was placed on an agar plate (PDA) by using tweezers for culture. Next, isolation of fungal species was performed according to different types of hyphae morphologies. Species identification was then performed when the types of hyphae were confirmed to be identical.

[0038] Mycelia were collected from the liquid-cultured species, and DNA was extracted therefrom by grinding in liquid nitrogen. ITS gene segments were then amplified using ITS-1 and ITS-4 primers (ITS-1: TCCGTAGGTGAACCTGCGG/ ITS-4: TCCTCCGCTTATTGATATGC).

[0039] The amplification was performed at 95°C for 5 minutes, followed by 35 cycles of amplification performed at 95°C for 30 seconds, at 52°C for 30 seconds, and at 72°C for 1 minute 10 seconds. Finally, polymerase chain reaction was performed at 72°C for 5 minutes. The amplified products were then sequenced and compared through BLAST searches on NCBI databases. The above sequences and those of *Lignosus rhinocerus* showed a similarity of 99%.

[0040] However, *Lignosus rhinocerus* used in the present invention is not limited to the particular species described herein. *Lignosus rhinocerus* from other sources may be used and are not limited by this invention.

[0041] (1) Plate culture: A *Lignosus rhinocerus* mycelium was inoculated onto an agar plate and cultured between 15 and 30°C for 1 to 2 weeks (at 25°C for 7 days in this example). The recipe for the agar may include Potato Dextrose Agar (PDA), carbon sources and nitrogen sources, and is not specifically limited.

[0042] (2) Culture in a flask: *Lignosus rhinocerus* mycelia were scraped from the plate of step (1) and inoculated to a flask. The culture was grown at 110 to 130 rpm in a shaker incubator between 15 to 30°C and at a pH of 2 to 6 for 3 to 14 days (at 120 rpm, 25°C and pH 5 for 7 days in this example). The recipe of the shaking culture is shown in Table 1 below:

[0043] Table 1: Recipe for the culture medium

Ingredient	Amount used in this example (weight %)	Preferred range of amount (weight %)
Mixed carbon and nitrogen sources	1	1-3
Carbohydrates	2	1-4
Yeast extract	0.6	0.5-1
Peptone	0.6	0.5-1
Inorganic salts	0.05	0.01-0.05

[0044] Among the above ingredients, mixed carbon and nitrogen sources can be cereals (such as wheat flour) or legumes (such as soya bean powder, mung bean powder, Glycine max powder or cinnamon powder); carbohydrates can be glucose, fructose, maltose, sucrose or the alike; and inorganic salts can be magnesium sulfate, dipotassium phosphate, potassium dihydrogen phosphate, ferric sulfate or the alike. It should be noted that the recipe as shown in Table 1 is only exemplary, and that the ingredients therein are not specifically limited and can be adjusted according to actual needs or used in combination with commercially available culture media.

[0045] (3) Culture in a fermenter tank: The culture in the flask of step (2) was further inoculated into a fermenter tank and was stirred between 50 to 150 rpm in temperature between 15 to 30°C, the tank pressure between 0.5 to 1.0 kg/cm² and the pH between 2 to 6. Meanwhile, gas was fed into the tank at a gas flow rate of 0.1 to 1.0 VVM. The resulting culture was incubated for 3 to 21 days to obtain a *Lignosus rhinocerus* mycelium fermentation liquid (in this example, the culture conditions were 25°C, 0.5 kg/cm², pH 5, 80 rpm and 1.0 VVM (air) for 14 days). The medium used in the fermenter tank may contain either the same recipe as the culture in a flask of step (2) or other appropriate media. This *Lignosus rhinocerus* mycelium fermentation liquid includes the *Lignosus rhinocerus* mycelia active substance of the present invention. The *Lignosus rhinocerus* mycelium fermentation liquid can be further freeze-dried to obtain a freeze-dried powder of *Lignosus rhinocerus* mycelia. In this example, 100 L of *Lignosus rhinocerus* mycelium fermentation liquid was freeze-dried into 2 kg of freeze-dried powder.

[0046] (4) Preparation of extracts: The freeze-dried powder of the *Lignosus rhinocerus* mycelium fermentation liquid was divided into two portions. RO water and ethanol weighing 20 times as much as each portion was added for extraction. The extract dissolved by water was heated at 100°C for 30 minutes, and centrifuged after cooling. The supernatant obtained after centrifugation was freeze-dried to obtain a water extract of *Lignosus rhinocerus* mycelia. The extract dissolved by ethanol was sonicated for 1 hour using ultrasonic and centrifuged. The supernatant obtained after centrifugation was treated with vacuum concentration to obtain an ethanol extract of *Lignosus rhinocerus* mycelia.

[0047] Water extracts and ethanol extracts containing *Lignosus rhinocerus* mycelia active substance at higher concentrations can be obtained at the extraction step. *Lignosus rhinocerus* mycelia active substance is

contained in various forms including *Lignosus rhinocerus* mycelium fermentation liquid (mycelia and liquid), a freeze-dried powder made from fermentation liquid, a water extract, an ethanol extract, a mixture of water extracts and ethanol extracts, a freeze-dried powder of water extracts and ethanol extracts, or in other formulations. In the following Example 2, *Lignosus rhinocerus* mycelia active substance is included in the form of a freeze-dried powder, and included in the forms of a water extract and an ethanol extract in Example 3.

Example 2: Analyzing the use of *Lignosus rhinocerus* mycelium freeze-dried powder to ameliorate ALI

[0048] One of the major risk factors leading to ALI is Gram-negative bacterial infection, whose envelope is mainly composed of endotoxins, also known as lipopolysaccharide (LPS). There have been experiments in which animal models of endotoxin-induced ALI are employed. See Yunhe Fu *et al.* (2017), Protective effect of TM6 on LPS-induced acute lung injury in mice, *SCIENTIFIC REPORTS*, 7:572. A mouse model of acute lung inflammation was established by LPS according to the above publication. LPS at 50 µg/ 20 µL was administered intranasally to induce acute lung injury. Analyses of changes in pulmonary pathology, total bronchoalveolar lavage fluid cell counts, protein concentration and various cytokines were then carried out, so as to assess the effect of synthesized peptides, such as cell-permeable TIR domain-derived decoy peptide, in animal model of ALI. Among various pathogens that caused ALI, LPS is widely considered as the most effective inducer of acute lung inflammation. Moreover, the model of this induction is the closest to the acute inflammation that occurs clinically. Therefore, suppressing LPS-caused lung inflammation as well as regulating immune functions and mechanisms shall achieve the effect of ameliorating ALI. In

this experiment, a mouse model of acute lung inflammation was also established using LPS. Similarly, analyses of changes in pulmonary pathology, total bronchoalveolar lavage fluid cell counts, protein concentration and various cytokines were then carried out to assess the effect of *Lignosus rhinocerus* mycelia active substance in animal model of ALI.

[0049] (1) BALB/c mice were used in this animal experiment and were divided into two major groups: intraperitoneal administration group and oral administration group. The two major groups were further divided into four groups: Control group, LPS group, LR group and Dexamethasone (DEX) group (dissolved in saline at a concentration of 5 µg/g, with 50 µL administered intra-peritoneally), making a total of four groups with six mice each.

[0050] (2) Endotoxins (LPS) were administered intranasally 30 minutes after the intraperitoneal administration of freeze-dried powder of *Lignosus rhinocerus* mycelia (the freeze-dried powder was dissolved in a water/alcohol solvent at a ratio of 1:1 with a concentration of 0.5 mg/g; 50 µL of the solution was administered intraperitoneally). Mice were sacrificed 24 hours after administration, and samples were collected to be analyzed as described below.

[0051] (3) Freeze-dried powder of *Lignosus rhinocerus* was administered orally once per day, for a total of three times (the freeze-dried powder was dissolved in a water/alcohol solvent at a ratio of 1:1 with a concentration of 0.5 mg/g; 200 µL of the solution was administered orally). Endotoxins (LPS) were administered intranasally 30 minutes after oral administration of *Lignosus rhinocerus* on day 3. Mice were sacrificed 24 hours after administration, and samples were collected to be analyzed as described below.

[0052] (4) Histopathological observation of lung tissues: one half of the right lung of the mice that was not treated with alveolar lavage was taken out and fixated immediately in formalin. The fixated product was made into paraffin sections and dyed with conventional hematoxylin and eosin stain (H&E stain). Histopathological changes were observed using light microscopy.

[0053] (5) Bronchoalveolar lavage (BAL): lung lavage was performed on the other half of the right lung with 1 mL of PBS three times using endotracheal intubation to wash out the contents in the alveolar cavity and to obtain BAL fluid (BALF). The BALF was centrifuged and the precipitated cells were measured using differential blood cell count. The protein concentration in the supernatant was determined using Bradford protein assay.

[0054] (6) Measurement of leukocytes count and type using flow cytometry: flow cytometry is a highly sensitive quantification method for determining different types of white blood cell in blood and BALF samples, based on specific antigens that are present on the surface of leukocytes. Targets of measurement include: (1) cell count in BALF; (2) leukocytes (CD45) count; (3) phagocytes (CD45+/CD11b) count; (4) neutrophils (CD45+/Ly6G+) count; and (5) macrophage [(CD45+/CD11b+) - (CD45+/Ly6G+)] count.

[0055] Statistical method: data are expressed as Mean $\pm$ standard deviation (SD) and analyzed using one-way ANOVA. Group differences are analyzed using an LSD post-hoc test. Results with a p value less than 0.05 are considered statistically significant.

[0056] Fig. 1 shows the test result of acute lung injury models after administering *Lignosus rhinocerus* mycelia active substance intra-peritoneally. In LR mycelia and Dexamethasone groups, LR mycelia (0.5

mg/g) and Dexamethasone (DEX, 5 μ g/g) were administered intraperitoneally to BALB/c mice. Endotoxins (LPS) were administered intranasally 30 minutes after intraperitoneal administration to induce acute lung inflammation. As shown in Fig. 1, no significant changes were observed in the Control group with most alveolar structure remaining intact. In the LPS only group (negative control), pathological changes in the lung were clearly observed with the alveolar structure being damaged and fused. On the other hand, the lung damages of the mice in the LR mycelia and DEX groups were clearly improved, similar to the results observed in the Control group. The above results show that *Lignosus rhinocerus* mycelia active substance proved to be effective in ameliorating inflammatory lung diseases.

[0057] After intraperitoneal injection, protein leakage response caused by endotoxin-induced lung inflammation was analyzed. It was found that freeze-dried powder of *Lignosus rhinocerus* mycelia was effective in reducing protein leakage response, as shown in Fig. 2 and Table 2 below.

[0058] Table 2: Protein leakage response after intraperitoneal injection of *Lignosus rhinocerus* mycelia active substance

Group	BALF Protein leakage (mg/mL)
Control	2.87 $\pm$ 1.02
LPS only	9.93 $\pm$ 1.52#
LPS+LR mycelia freeze-dried powder 0.5 mg/g	4.26 $\pm$ 1.33*
LPS+DEX 5 μ g/g	3.83 $\pm$ 1.72*

(n=6)

Statistical significance compared to Control group (p<0.05)

* Statistical significance compared to negative control (LPS only) group (p<0.05)

[0059] After intraperitoneal injection, infiltration responses of (A) leukocytes (CD45) (B) phagocytes (CD45+/CD11b+) and (C) neutrophils (CD45+/Ly6G+) caused by endotoxin-induced lung inflammation were analyzed. It was found that freeze-dried powder of *Lignosus rhinocerus*

mycelia was effective in reducing infiltration response of leukocytes, phagocytes, and neutrophils, as shown in Fig. 3 (A to C) and Tables 3-5 below.

[0060] Table 3: leukocyte infiltration response after intraperitoneal injection of *Lignosus rhinocerus* mycelia active substance

Group	Leukocyte infiltration (10^5)
Control	0.14±0.06
LPS only	3.29±1.95#
LPS+LR mycelia freeze-dried powder 0.5 mg/g	0.46±0.18*
LPS+DEX 5 µg/g	0.56±0.29*

(n=6)

Statistical significance compared to Control group ($p<0.05$)

* Statistical significance compared to negative control (LPS) group ($p<0.05$)

[0061] Table 4: phagocyte infiltration response after intraperitoneal injection of *Lignosus rhinocerus* mycelia active substance

Group	Phagocyte infiltration (10^5)
Control	0.07±0.03
LPS only	1.62±0.67#
LPS+LR mycelia freeze-dried powder 0.5 mg/g	0.27±0.11*
LPS+DEX 5 µg/g	0.27±0.26*

(n=6)

Statistical significance compared to Control group ($p<0.05$)

* Statistical significance compared to negative control (LPS only) group ($p<0.05$)

[0062] Table 5: Neutrophil infiltration response after intraperitoneal injection of *Lignosus rhinocerus* mycelia active substance

Group	Neutrophil infiltration (10^5)
Control	0.04±0.02
LPS only	1.12±0.80#
LPS+LR mycelia freeze-dried powder 0.5 mg/g	0.18±0.12*
LPS+DEX 5 µg/g	0.16±0.16*

(n=6)

Statistical significance compared to Control group (p<0.05)

* Statistical significance compared to negative control (LPS only) group (p<0.05)

[0063] Similarly, freeze-dried powder of *Lignosus rhinocerus* mycelia (LR mycelia, 0.5 mg/g) was administered orally to BALB/c mice, and endotoxins (LPS) were administered intranasally 3 days after the oral administration to induce acute lung inflammation. As shown in Fig. 4, no significant changes were observed in the Control group with most alveolar structure remaining intact. In the LPS only group (negative control), pathological changes in the lung were clearly observed with the alveolar structure being damaged and fused. On the other hand, the lung damages of the mice in the LR mycelia and DEX groups were clearly improved. The above results show that freeze-dried powder of *Lignosus rhinocerus* mycelia, when administered orally, also proved to be effective in ameliorating inflammatory lung diseases.

[0064] After oral administration, protein leakage response caused by endotoxin-induced lung inflammation was analyzed. It was found that freeze-dried powder of *Lignosus rhinocerus* mycelia active substance was effective in reducing protein leakage response, as shown in Fig. 5 and Table 6 below.

[0065] Table 6: Protein leakage response after oral administration of *Lignosus rhinocerus* mycelia active substance

Group	BALF Protein leakage (mg/mL)
Control	2.60±0.24
LPS only	6.65±1.18#
LPS+LR mycelia freeze-dried powder 0.5 mg/g	2.65±0.43*
LPS+DEX 5 µg/g	3.11±0.64*

(n=6)

Statistical significance compared to Control group (p<0.05)

* Statistical significance compared to negative control (LPS only) group (p<0.05)

[0066] After oral administration, infiltration responses of (A) leukocytes (CD45), (B) phagocytes (CD45+/CD11b+) and (C) neutrophils (CD45+/Ly6G+) caused by endotoxin-induced lung inflammation were analyzed. It was found that freeze-dried powder of *Lignosus rhinocerus* mycelia active substance was effective in reducing infiltration responses of leukocytes, phagocytes and neutrophils, as shown in Fig. 6 (A to C) and Tables 7-9 below.

[0067] Table 7: leukocyte infiltration response after oral administration of *Lignosus rhinocerus* mycelia active substance

Group	Leukocytes infiltration (10 ⁵)
Control	0.38±0.22
LPS only	3.17±0.82#
LPS+LR mycelia freeze-dried powder 0.5 mg/g	0.68±0.31*
LPS+DEX 5 µg/g	0.88±0.25*

(n=6)

Statistical significance compared to Control group (p<0.05)

* Statistical significance compared to negative control (LPS only) group (p<0.05)

[0068] Table 8: phagocyte infiltration response after oral administration of *Lignosus rhinocerus* mycelia active substance

Group	Phagocyte infiltration (10 ⁵)
Control	0.06±0.04
LPS only	0.62±0.35#
LPS+LR mycelia freeze-dried powder 0.5 mg/g	0.09±0.06*
LPS+DEX 5 µg/g	0.08±0.05*

(n=6)

Statistical significance compared to Control group (p<0.05)

* Statistical significance compared to LPS only group (p<0.05)

[0069] Table 9: Neutrophil infiltration response after oral administration of *Lignosus rhinocerus* mycelia active substance

Group	Neutrophils infiltration (10^5)
Control	0.02±0.01
LPS	0.29±0.09#
LPS+LR mycelia freeze-dried powder 0.5 mg/g	0.03±0.01*
LPS+DEX 5 µg/g	0.04±0.02*

(n=6)

Statistical significance compared to Control group ($p < 0.05$)

* Statistical significance compared to negative control (LPS) group ($p < 0.05$)

[0070] The *Lignosus rhinocerus* mycelia active substance of the present invention has been proved through the above experiment to be effective in ameliorating ALI. The active substance can be combined with a pharmaceutically acceptable carrier, excipient, diluent or adjuvant to manufacture a pharmaceutical composition, and can also be used as a food additive.

Example 3: Analysis the use of *Lignosus rhinocerus* mycelium water and alcohol extracts for ameliorating COPD

[0071] COPD models have been established through experiments using cigarette smoke extracts (CSE) and human lung cancer epithelial cells (A549 cells). See Miaomiao Chen et al. (2015), Azithromycin attenuates cigarette smoke extract-induced oxidative stress injury in human alveolar epithelial cells, *MOLECULAR MEDICINE REPORTS*, 11: 3414-3422. As described in the above publication, an MTT assay was conducted after A549 cells were incubated with CSE and Azithromycin to assess the therapeutical effectiveness of Azithromycin on COPD. Injury of alveolar epithelial cells by CSE is a key feature of COPD mechanisms. As such, COPD may be

improved if the injury of alveolar epithelial cells by CSE can be suppressed. In this experiment, an MTT assay was examined after A549 cells were incubated with CSE and *Lignosus rhinocerus* mycelia active substance to assess the therapeutical effectiveness of *Lignosus rhinocerus* mycelia active substance on COPD.

[0072] (1) Human lung cancer epithelial cells (A549 cells) were collected. The concentration of the cell suspension was adjusted to 50,000-100,000/mL. The suspension was then inoculated onto a 96-well plate.

[0073] (2) The 96-well plate of step (1) was incubated at 37°C with 5 % CO₂ in an incubator for 16 to 24 hours, and then treated with different doses of the water extracts (0, 0.25, 0.5, 1 mg/ml) and ethanol extracts (0, 6.25, 12.5, 25 µg/ml) of *Lignosus rhinocerus* mycelia obtained in Example 1. The extracts of *Lignosus rhinocerus* mycelia were then dissolved in DMSO, the concentration of which did not exceed 0.1 % during the assay in order to prevent its cytotoxicity from impeding cell growth. In the Control group, 0.1% DMSO was used and extracts at each concentration level were tested in triplicates. The resulting mixtures were incubated at 37°C with 5 % CO₂ in an incubator for 24 hours;

[0074] (3) The resulting mixtures of step (2) were first centrifuged at 400xg for 10 minutes, and the supernatant was removed. 12.5% CSE was then added to induce the cytotoxicity of A549 cells, while no treatment was performed in the Control group. The resulting mixture was incubated at 37°C with 5% CO₂ in an incubator for 6 hours;

[0075] (4) The supernatant of the resulting mixtures of step (3) was removed. 100 µl of MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added into each well. After 2 hours of reaction at 37°C, the incubation was terminated and the MTT solution in each well was carefully removed;

[0076] (5) 100 µl DMSO was added into each well of step (4). The resulting mixtures were shaken for 2 minutes and the absorbance was measured at 570 nm. This absorbance can be seen as cellular capability of reducing MTT (the amount of formazan produced), that is, mitochondrial activity (number of living cells). The greater the absorbance at 570 nm, the higher the cell viability.

[0077] Statistical method: data are expressed as Mean $\pm$ standard deviation (SD). Group differences are analyzed using Student's t-test. Results with a p value less than 0.05 are considered statistically significant.

[0078] Results of the experiment using water extracts are shown in Fig. 7 and Table 10 below. While CSE may induce A549 cell cytotoxicity and reduce cell viability, adding water extracts of *Lignosus rhinocerus* mycelia active substance may suppress cell injury significantly and thus improve cell viability. The *Lignosus rhinocerus* mycelium water extracts obtained in Example 1 of the invention are effective in ameliorating COPD when administered at a concentration of greater than 1 mg/ml.

[0079] Table 10: Results of experiment using *Lignosus rhinocerus* mycelium water extracts

Group	Cell viability (%)
Control	100 $\pm$ 3.61
CSE	84.54 $\pm$ 1.49#
CSE + LR mycelium water extract 0.25 mg/ml	84.64 $\pm$ 4.95
CSE + LR mycelium water extract 0.5 mg/ml	90.28 $\pm$ 6.23
CSE + LR mycelium water extract 1 mg/ml	96.48 $\pm$ 5.66*
LR mycelium water extract 1 mg/ml	104.91 $\pm$ 2.26

(n=3)

Statistical significance compared to Control group (p<0.05)

* Statistical significance compared to negative control (CSE) group ($p < 0.05$)

[0080] Results of experiment using ethanol extracts are shown in Fig. 8 and Table 11 below. Similarly, the *Lignosus rhinocerus* mycelium ethanol extracts obtained in Example 1 of the invention are effective in ameliorating COPD when administered at a concentration of greater than 25 $\mu\text{g/ml}$.

[0081] Table 11: Results of experiment using *Lignosus rhinocerus* mycelium ethanol extracts

Group	Cell viability (%)
Control	100 $\pm$ 5.92
CSE	85.12 $\pm$ 2.67#
CSE + LR mycelium alcohol extract 6.25 $\mu\text{g/ml}$	86.46 $\pm$ 4.90
CSE + LR mycelium alcohol extract 12.5 $\mu\text{g/ml}$	89.24 $\pm$ 4.80
CSE + LR mycelium alcohol extract 25 $\mu\text{g/ml}$	94.57 $\pm$ 3.51*
LR mycelium alcohol extract 25 $\mu\text{g/ml}$	103.07 $\pm$ 3.32

(n=3)

Statistical significance compared to Control group ($p < 0.05$)

* Statistical significance compared to negative control (CSE) group ($p < 0.05$)

[0082] It should be noted that the effective concentrations of *Lignosus rhinocerus* mycelium water and ethanol extracts in the above experiment are merely illustrative, and may vary among subjects during actual administration based on individual differences.

[0083] The *Lignosus rhinocerus* mycelia active substance of the present invention has been proved through the above experiment to be effective in ameliorating COPD. The active substance can be combined with a pharmaceutically acceptable carrier, excipient, diluent or adjuvant to manufacture a pharmaceutical composition, and can also be used as a food additive.

Example 4: Manufacturing composition

[0084] The present invention provides a composition comprising a *Lignosus rhinocerus* mycelia active substance to be manufactured into a pharmaceutical composition as well as a health food.

[0085] The composition further includes an additive. In a preferred embodiment, the additive can be an excipient, preservative, diluent, filler, absorption enhancer, sweetener, lubricant, thickener or a combination thereof. The excipient can be selected from sodium citrate, calcium carbonate, calcium phosphate, sucrose or a combination thereof. The preservative, such as benzyl alcohol, parabens, silica or a combination thereof, can prolong the shelf life of pharmaceutical compositions. The diluent can be selected from water, alcohol, propylene glycol, glycerol or a combination thereof. The filler can be selected from lactose, high molecular weight polyethylene glycol or a combination thereof. The absorption enhancer can be selected from dimethyl sulfoxide (DMSO), laurocapram, propylene glycol, glycerol, polyethylene glycol or a combination thereof. The sweetener can be selected from Acesulfame K, aspartame, saccharin, sucralose, neotame or a combination thereof. The lubricant can be selected from magnesium stearate or gum Arabic. The thickener can be cornstarch. In addition to the additives listed above, other ones may be selected according to actual needs provided that the pharmaceutical effects of the composition are not affected.

[0086] The composition can be developed into various products in the pharmaceutical industry. In a preferred embodiment, the composition is a drug, feed, drink, nutritional supplement, dairy product or health food.

[0087] The composition can be various forms to meet the subject's needs. In a preferred embodiment, the composition can be in powder, tablet,

granule, suppository, microcapsule, ampoule/ampule, liquid spray or suppository form.

[0088] The composition of the present invention can be administered to an animal or a human. Provided that the effects of the composition are not affected, it can be made into any dosage forms and administered via an appropriate route to the animal or human depending on the dosage form.

[0089] **Manufacturing of composition**

[0090] When the *Lignosus rhinocerus* mycelia active substance of the present invention is of dietary use, the form of the Composition 1 as described below shall be an illustrative example of the *Lignosus rhinocerus* mycelia active substance.

[0091] Composition 1: Freeze-dried powder of the *Lignosus rhinocerus* mycelia active substance (20 wt%) was well-mixed with magnesium stearate used as a lubricant (8 wt%) and silica used as a preservative (7 wt%). The resulting mixture was dissolved in pure water (65 wt%) and stored at 4°C for future use. The notation “wt%” refers to the proportion of the weight of each ingredient relative to the total weight of the composition.

[0092] When the *Lignosus rhinocerus* mycelia active substance of the present invention is of medical use in a liquid form, the form of the Composition 2 as described below shall be an illustrative example of the *Lignosus rhinocerus* mycelia active substance.

[0093] Composition 2: Freeze-dried powder of the *Lignosus rhinocerus* mycelia active substance (20 wt%) was well-mixed with sucralose used as a sweetener (8 wt%), gum Arabic used as a lubricant (7 wt%) and sucrose used as an excipient (10 wt%). The resulting mixture was dissolved in pure water (55 wt%) and stored at 4°C for future use. The

notation “wt%” refers to the proportion of the weight of each ingredient relative to the total weight of the composition.

[0094] These examples, though disclosed in the above description, do not intend to limit the present invention. A person ordinarily skilled in the art should be able to make appropriate changes to the features described therein with reference to the above teachings and still achieve the effects as claimed by the present application. Therefore, the scope of claims protecting the present invention shall be determined by the appended claims.

WHAT IS CLAIMED IS:

1. A method of manufacturing *Lignosus rhinocerus* mycelia active substance for preventing and/or ameliorating lung injury, the method comprising the following steps:
 - (a) growing a *Lignosus rhinocerus* mycelia on an agar plate between 15 and 30°C for 1 to 2 weeks;
 - (b) inoculating the *Lignosus rhinocerus* mycelia of step (a) to a flask and culturing the mycelia between 15 and 30°C and at a pH of 2 to 6 for 3 to 14 days; and
 - (c) inoculating the *Lignosus rhinocerus* mycelia of step (b) to a fermenter tank and culturing the mycelia by stirring between 15 and 30°C at a pH of 2 to 6 for 3 to 21 days, so as to obtain a *Lignosus rhinocerus* mycelium fermentation liquid containing *Lignosus rhinocerus* mycelia active substance.
2. The method of claim 1, wherein the method further comprises steps of (d): freeze-drying the *Lignosus rhinocerus* mycelium fermentation liquid and grinding the freeze-dried product, so as to obtain a *Lignosus rhinocerus* mycelium powder containing *Lignosus rhinocerus* mycelia active substance.
3. The method of claim 2, wherein the method further comprises steps of (e): extracting the freeze-dried powder of *Lignosus rhinocerus* mycelium using at least one solvent, so as to obtain a *Lignosus rhinocerus* mycelium extract containing *Lignosus rhinocerus* mycelia active substance.
4. The method of claim 3, wherein the method further comprises steps of (f): drying the *Lignosus rhinocerus* mycelium extract, so as to obtain the *Lignosus rhinocerus* mycelia active substance.
5. The method of claim 3 or 4, wherein at step (e), the solvent is selected from water or ethanol.
6. The method of claim 1, wherein the culturing of step (b) is a shake flask

culture, and the shaking speed is between 110-130 rpm.

7. The method of claim 1, wherein a gas is further fed into the fermenter tank of step (c), and the gas comprises air, oxygen, carbon dioxide, helium or a combination thereof; pressure of the fermenter tank is 0.5 to 1.0 kg/cm², and a gas flow rate is 0.01 to 1.5 VVM.
8. The method of claim 1, wherein the lung injury includes chronic obstructive pulmonary disease and/or acute lung injury.
9. A *Lignosus rhinocerus* mycelia active substance, which is manufactured using the method of any one of claims 1-8.
10. A composition for preventing and/or ameliorating lung injury, which comprises the *Lignosus rhinocerus* mycelia active substance of claim 9 having an effective concentration, and a pharmaceutically acceptable carrier, excipient, diluent or adjuvant.
11. The composition of claim 10, wherein the *Lignosus rhinocerus* mycelia active substance has an effective concentration of greater than 1 mg/ml when the solvent is water.
12. The composition of claim 10, wherein the *Lignosus rhinocerus* mycelia active substance has an effective concentration of greater than 25 µg/ml when the solvent is ethanol.
13. The composition of claim 10, wherein said lung injury includes chronic obstructive pulmonary disease and/or acute lung injury
14. A use of *Lignosus rhinocerus* mycelia active substance of claim 9, which is used for manufacturing a composition for preventing and/or ameliorating lung injury.
15. The use of claim 14, wherein said lung injury includes chronic obstructive pulmonary disease and/or acute lung injury.
16. The use of claim 14, wherein the composition is a pharmaceutical composition or food additive.