

USE OF *HERICIUM ERINACEUS* MYCELIA ACTIVE SUBSTANCE FOR PREVENTING OR CURING RETINOPATHY

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0001] The present invention relates to a use of a *Hericium erinaceus* mycelium active substance and, in particular, to preparation of a composition for preventing or treating retinopathy using the *Hericium erinaceus* mycelium.

DESCRIPTION OF THE PRIOR ART

[0002] Age-related macular degeneration (AMD) is a disease of progressive degeneration of the retina macula responsible for vision and color perception, mainly caused by the death of the central retinal photoreceptor. The retinal pigment epithelium (RPE) cells located between the photosensitive layer and the choroid are also involved in the pathogenesis of early AMD. As described in the name "age-related", the prevalence of AMD gradually increases with age, becoming the main cause of blindness in the elderly.

[0003] The pathogenesis of AMD is related to many factors, such as metabolic disorders, immunity, inflammation, reactive oxygen species (ROS) and so on. Recent studies have shown that the oxidative stress caused by ROS is the main pathogenic factor of AMD. In the past few decades, the methods used to treat AMD include: (1) laser photocoagulation, (2) transpupillary thermotherapy (TTT), (3) photodynamic therapy. (PDT), (4) antiangiogenic therapy, (5) nutritional supplement therapy, (6) gene therapy, (7) antioxidants and (8) a combination of the

above therapies, etc. However, surgical therapy has side effects and the post-blindness rate after surgery is still increasing. Therefore, the development of new therapeutic agents with lower toxicity is quite important for the prevention or treatment of AMD.

[0004] *Hericium erinaceus* (Bull.) Pers is a precious medicinal and edible fungus, mainly distributed in temperate regions with higher latitudes. The "Encyclopedia of Chinese Wild Vegetable Recipes" believes that it can cure indigestion, gastric ulcer, gastritis, stomach pain, stomach bloating and neurasthenia. It shows that *Hericium erinaceus* has a gastric protective function in addition to being delicious and can be used as a dual-purpose mushroom for food and medicine. Since 1994, Kawagishi has isolated and purified a variety of Erinacine (A, B, C, etc.) from *Hericium erinaceus* mycelia. Erinacine can stimulate mouse stellate cells and increase the secretion of nerve growth factor (NGF), which can treat diseases such as mental decline and neurasthenia. Therefore, *Hericium erinaceus* is also believed to promote nerve and brain health. However, there is currently no research showing that *Hericium erinaceus* has curative effect on retinopathy.

SUMMARY OF THE INVENTION

[0005] The present invention provides a use of a *Hericium erinaceus* mycelium active substance in manufacture of a composition for preventing or treating retinopathy. A preparation method of the *Hericium erinaceus* mycelium active substance includes the following steps:

[0006] (a) cultivating a *Hericium erinaceus* mycelium on a plate medium at 15-35°C for 7-15 days;

[0007] (b) inoculating the *Hericium erinaceus* mycelium in step (a) into a flask and culturing the mycelium at 15-35°C and pH 2-8 for 3-5 days; and

[0008] (c) inoculating the *Hericium erinaceus* mycelium cultured in step (b) into a fermentation tank and culturing the mycelium by stirring at 15-35°C and pH 4.5-5.5 for 7-15 days to obtain a *Hericium erinaceus* mycelium fermentation liquid containing the *Hericium erinaceus* mycelium active substance.

[0009] In one embodiment, the preparation method of the *Hericium erinaceus* mycelium active substance further includes a step (d): freeze-drying and then grinding the *Hericium erinaceus* mycelium fermentation liquid to obtain a *Hericium erinaceus* mycelium lyophilized powder containing the *Hericium erinaceus* mycelium active substance.

[0010] In one embodiment, the preparation method of the *Hericium erinaceus* mycelium active substance further includes a step (e): extracting the *Hericium erinaceus* mycelium lyophilized powder with a solvent to obtain a *Hericium erinaceus* mycelium extract containing the *Hericium erinaceus* mycelium active substance.

[0011] In one embodiment, the preparation method of the *Hericium erinaceus* mycelium active substance further includes a step (f): drying the *Hericium erinaceus* mycelium extract to obtain the *Hericium erinaceus* mycelium active substance.

[0012] In one embodiment, the solvent in the step (e) is ethanol, and the *Hericium erinaceus* mycelium lyophilized powder is extracted twice with ethanol.

[0013] In one embodiment, the cultivation in the flask in the step (b) is shake flask cultivation with a rotation speed of 10-250 rpm.

[0014] In one embodiment, the retinopathy is macular degeneration.

[0015] In one embodiment, a gas is further fed in the fermentation tank in the step (c), and the gas includes air, oxygen, carbon dioxide, helium or a combination thereof; pressure of the fermentation tank is 0.5-2.0 kg/cm², and a gas flow rate is 0.5-1 VVM.

[0016] In one embodiment, the composition is a pharmaceutical composition, and the pharmaceutical composition further comprises a pharmaceutically acceptable carrier, an excipient, a diluent or an adjuvant.

[0017] In one embodiment, the composition is a food additive.

[0018] In one embodiment, the composition is administrated orally.

[0019] In order to make the above-mentioned and other aspects of the present invention clearer, the following specific examples are given in conjunction with the accompanying drawings for description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] FIG. 1 shows the optical coherence tomography diagrams of mice in each group;

[0021] FIG. 2 shows the results of retinal thickness measurement in FIG. 1;

[0022] FIG. 3(A) shows H&E stain photographs of eyes of mice in each group, and FIG. 3(B) shows enlarged views of the dashed frames in FIG. 3(A); and

[0023] FIG. 4(A) shows the measurement results of the inner nuclear layer (INL) thickness of FIG. 3(A), and FIG. 4(B) shows the measurement results of the outer nuclear layer (ONL) thickness of FIG. 3(A).

DETAILED DESCRIPTION OF THE EMBODIMENTS

[0024] Source of *Hericium erinaceus* mycelia

[0025] The *Hericium erinaceus* strain used in the examples of the present invention was purchased from Taiwan Food Industry Development Research Institute, and the deposit number is BCRC 35669. The strain can be purchased directly from the official website of BCRC (<https://catalog.bcrc.firdi.org.tw/>) and is a strain that can be easily obtained by the public. However, the *Hericium erinaceus* active substance of the present invention is not limited to those obtained from this strain.

[0026] Liquid culture of mycelia

[0027] The *Hericium erinaceus* mycelia were inoculated on a plate medium and cultivated at an appropriate temperature of 15-35°C (preferably 25°C) for 7-15 days. The hyphae scraping was inoculated into a flask and cultivated under the conditions of 15-35°C (preferably 25°C), pH 2-8 (preferably pH 4-7, more preferably pH 5.5) and a shaking rate of 100-250 rpm for 3-5 days. Afterwards, the flask culture was inoculated into the fermentation tank culture medium (same as the flask culture medium) and cultivated at 15-35°C (preferably 25°C) under a tank pressure of 0.5-2.0 kg/cm², pH 4.5-5.5 and a stirring speed of 10-150 rpm or no stirring (air lift) with the introduction of air, oxygen, carbon dioxide, nitrogen or a mixture of the above gases (preferably air) at an aeration rate of 0.5-1 VVM for 7-15 days to obtain the *Hericium erinaceus* mycelium fermentation liquid. The fermentation liquid includes mycelia and supernatant. The aforementioned culture conditions are merely exemplary, and can be adjusted as needed.

[0028] The formulation of flask culture medium and fermentation tank culture medium used in the present invention can be as what follows:

Components	Content (wt%)
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Mixed carbon and nitrogen sources	0.01-10
Animal and plant-derived proteins and hydrolysates thereof	0.01-5
Yeast or malt extracts (powder, cream)	0.001-2
Inorganic salts	0.0001-2
Carbohydrates	0.01-20

[0029] The mixed carbon and nitrogen sources may be cereals (such as wheat flour) or beans (such as soybean flour, mung bean flour, soya bean flour, etc.). The inorganic salts can be magnesium sulfate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ferric sulfate and the like. The carbohydrates may be glucose, fructose, maltose, sucrose and the like. In particular, the medium used in the present invention is not limited to the components or formulation described above and can be adjusted according to the actual situation.

[0030] Drying of fermentation liquid

[0031] The *Hericium erinaceus* mycelium fermentation liquid can be further prepared into other dosage forms such as lyophilized powder through a drying step. The drying methods include, but are not limited to: spray drying, hot air drying, drum drying, freeze drying or other methods.

[0032] Extraction of lyophilized powder - ethanol extraction

[0033] Ethanol with a volume of 25 times the lyophilized powder of *Hericium erinaceus* mycelia was added into the lyophilized powder for the first extraction, which used ultrasonic vibration to extract for 1 hour, and then the suspension was

taken for centrifugation. After centrifugation, the supernatant was taken, the residue was subject to the above extraction step, and the second extraction was performed with ethanol. The supernatant obtained from the second extraction was mixed with the supernatant from the first extraction and concentrated under reduced pressure to obtain a cream-like ethanol extract of *Hericium erinaceus* mycelium, which contained the *Hericium erinaceus* mycelium active substance. The reason for repeating the extraction twice was to obtain a higher yield.

[0034] The *Hericium erinaceus* mycelium fermentation liquid, *Hericium erinaceus* mycelium lyophilized powder, and *Hericium erinaceus* mycelium ethanol extract all contain the *Hericium erinaceus* mycelium active substance of the present invention. In the following example, the *Hericium erinaceus* mycelium active substance was prepared according to the above-mentioned method, and biological experiments were carried out to evaluate its efficacy.

Example 1: Preparation of *Hericium erinaceus* mycelium active substance

[0035] Strain: The *Hericium erinaceus* strain used in the examples of the present invention is purchased from Taiwan Food Industry Development Research Institute, and the deposit number is BCRC 35669.

[0036] Plate culture: the mycelium was inoculated on the plate medium and cultured at 25°C for 7 days, and the medium was potato dextrose agar (PDA).

[0037] Flask culture: the hypha scraping from the plate was inoculated into the flask and cultivated using the following medium formulation at 25°C and pH 5.0 on a shaker with a rotation speed of 120 rpm for 5 days;

[0038] Medium formulation:

Ingredient	content (wt%)
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Glucose	3.0
Yeast extract	0.1
Soybean powder	0.1
Peptone	0.1
Magnesium sulfate	0.05
Water	Adding up to 100 wt%

[0039] Fermentation tank culture: The medium was the same as above, the flask culture was inoculated into the fermentation tank culture medium and cultivated at 25°C under a tank pressure of 1.0 kg/cm² and pH 5.0 with a stirring speed of 50 rpm and introduction of air at an aeration rate of 1.0 VVM for 12 days to obtain the fermentation liquid of *Hericium erinaceus* mycelia. The *Hericium erinaceus* mycelium fermentation liquid can be freeze-dried to obtain the lyophilized powder of *Hericium erinaceus* mycelia.

[0040] Preparation of extract: Ethanol, 95 % (v/v), with a volume of 25 times the lyophilized powder of *Hericium erinaceus* mycelia was added into the lyophilized powder for the first extraction, which used ultrasonic vibration at an oscillation rate of 120 rpm to extract for 1 hour, and then the suspension was taken for centrifugation. After centrifugation, the supernatant was taken, the residue from the first extraction was subject to the above extraction step, and the second extraction was performed with ethanol, 85 % (v/v). The supernatants obtained from both extractions were mixed and concentrated under reduced pressure to obtain a

cream-like ethanol extract of *Hericium erinaceus* mycelium (abbreviated as alcohol extract).

[0041] Results: The fermented liquid of *Hericium erinaceus* mycelia cultured in the 20 metric tons fermentation tank can be freeze-dried to obtain about 320 kg of lyophilized powder, which can be subject to the second extraction step to obtain about 20 kg of ethanol extract. The following biological experiments were carried out with the ethanol extract of the *Hericium erinaceus* mycelia.

Example 2 Animal model of retinopathy and analysis of related indexes

[0042] Establishment of mouse animal model of retinopathy

[0043] Sodium iodate (NaIO_3) is a stable oxidant and has been proven to be effective for inducing retinal degeneration. Retinal degeneration caused by sodium iodate is related to the regional loss of retinal pigment epithelial cells (RPE), and some morphological features of regional atrophy will also appear. Many studies have used different mammalian species to confirm that NaIO_3 is toxic to biological retinas, including sheep, rabbits, rats and mice. The above research pointed out that in the retina, the main target of NaIO_3 is RPE cells, which can be induced to death, such as necrosis, apoptosis or autophagy, and the secondary is choriocapillaris atrophy and panretinal degeneration.

[0044] In recent years, researchers have used low-dose NaIO_3 (15-35 mg/kg) to induce age-related retinopathy (AMD) models in mice and found that it is related to decreased visual function as well as localized RPE loss and external retinal damage, and is very similar to the common pathogenesis of dry AMD. Therefore, in recent years, such model has been mostly used to discuss RPE regeneration and the prevention of AMD.

[0045] In this example, a 40 mg/kg dose of NaIO₃ was used to induce AMD in mice, so as to evaluate the preventive and therapeutic effects of *Hericium erinaceus* mycelium active substances on retinopathy (in particular macular degeneration).

[0046] Experimental animals: Male mice of strain Balb/c aged about 6-8 weeks with a weight of about 26.21±1.76 g grams were purchased from Taiwan National Laboratory Animal Center. The mice were raised in the Experimental Animal Center of Chung Shan Medical University, provided with normal clean feed and drinking water. The breeding environment was under cycles of 12 hours of light and 12 hours of darkness. The temperature was controlled at 20±2°C, and the humidity was controlled at 50±5%.

[0047] Feeding dosage and experimental procedures

[0048] The experiment was carried out for 21 days. Before the experiment, the mice were divided into 4 groups, each of which has 6 mice:

[0049] (1) Blank control group (Mock): 100 µL of phosphate buffered saline (PBS) was injected intravenously (i.v.), and 200 µL of PBS was given orally and daily.

[0050] (2) Negative control group: 40 mg/kg of sodium iodate NaIO₃ was given intravenously (i.v.) to induce AMD, and 200 µL of PBS was given orally and daily.

[0051] (3) *Hericium erinaceus* ethanol extract group (experimental group): First, 100 mg/kg of the *Hericium erinaceus* ethanol extract prepared in Example 1 was given orally and daily for 14 days, then 40 mg/kg of iodine sodium NaIO₃ was given intravenously to induce AMD, and then 100 mg/kg of the *Hericium erinaceus* ethanol extract was fed daily for 7 days.

[0052] Mice in each group were sacrificed 21 days after the experiment for retinal damage detection and related index analysis.

[0053] Retinal damage detection and related index analysis

[0054] 1. Image detection of ocular fundus

[0055] Phoenix-Micro IV fundus image detection system was used, in which the optical fundus mirrors, fluorescent fundus angiography and retinal tomography could be utilized to evaluate the integrity of mouse retinal tissues and changes in smoothness. FIG. 1 shows the optical coherence tomography diagrams of mice in each group on the 7th day after the injection of sodium iodate.

[0056] In FIG. 1, the optic nerve of mice was taken as the center, the retina thickness (red/vertical line range) was measured for 6 times in the range of 300 μm to 600 μm on the nasal and temporal sides (shown by the yellow/horizontal line), and the average value was calculated to obtain the retinal thickness of mice. The result is shown in FIG. 2.

[0057] Please refer to the optical coherence tomography diagrams of FIG. 1. It can be seen from the portion marked with red line of the Mock group that the retina structure is complete. As to the mouse group administered with sodium iodate (NaIO_3) alone, the retina arrangement is relatively irregular and loose, the ordering between tissues is less smooth, and the boundary line between tissues is more blurred with the occurrence of obvious retinal distortion. The group of mice pre-treated with *Hericium erinaceus* extract (HE) has significantly less irregularities and blurring of the retina arrangement than the group of mice administered with sodium iodate (NaIO_3) alone.

[0058] The results of retinal thickness measurement in FIG. 2 shows that (where the * mark indicates $p < 0.05$ compared with the NaIO_3 alone group, and there is a significant difference), compared with the blank control group (Mock), the retina thickness of the group of mice administered with sodium iodate (NaIO_3) alone became significantly thinner 7 days after receiving sodium iodate injection. As to

the group of mice pre-treated with the *Hericium erinaceus* extract (HE), though the thickness became thinner, there was still a significant increase, compared with the group of mice administered with sodium iodate (NaIO_3) alone.

[0059] Accordingly, it can be known that the *Hericium erinaceus* extract of the embodiment of the present invention can reduce distortion and thinning of retina of mice induced by sodium iodate NaIO_3 , thereby improving the condition of AMD.

[0060] 2. Histopathological analysis (H&E stainine)

[0061] On the 7th day after sodium iodate injection, mice in each group were sacrificed and blood was collected. The eye tissue sections were stained with hematoxylin and eosin to distinguish the structure and type of RPE. The photographs are shown in FIG. 3 (A). FIG. 3(B) shows enlarged views of the dashed frames in FIG. 3(A) for observing the thickness of the inner nuclear layer (INL) and the outer nuclear layer (ONL) and the number of inflammatory cells.

[0062] Both INL and ONL layer thickness tests were centered on the optic nerve, and after 6 thickness (marked by the line in FIG. 3(B)) measurements on the nasal and temporal sides around $300\text{ }\mu\text{m} \sim 600\text{ }\mu\text{m}$, the average value was calculated. The results are shown in FIG. 4(A) and FIG. 4(B) respectively, where the * mark indicates $p < 0.05$ compared with the NaIO_3 alone group, so there is a significant difference.

[0063] Compared with the blank control group (Mock), for the mouse group administered with sodium iodate (NaIO_3) alone, the thickness of both the ONL and INL layers of the retina became thinner on the 7th day after the injection of sodium iodate. However, the group of mice pre-treated with the *Hericium erinaceus* extract (HE) could significantly slow down the thinning of ONL and INL layer of mouse

retina induced by sodium iodate, compared with the group of mice administered with sodium iodate (NaIO_3) alone.

[0064] Accordingly, it can be known that the *Hericium erinaceus* extract of the embodiment of the present invention can slow down the thinning of the inner and outer nuclear layers of the mouse retina induced by sodium iodate NaIO_3 , thereby improving the condition of AMD.

Example 3 Preparation of composition

[0065] If the *Hericium erinaceus* mycelium active substance of the present embodiment is applied to medicinal purposes, the following Composition 1 is taken as an illustrative example.

[0066] Composition 1: The lyophilized powder or ethanol extract (20 wt%) of the *Hericium erinaceus* mycelium active substance of Example 1 was mixed thoroughly with magnesium stearate (8 wt%) as a lubricant and silicon dioxide (7wt%) as a preservative and then dissolved in pure water (65wt%), followed by storage at 4°C for later use. The aforementioned wt% refers to the proportion of each component to the total weight of the composition.

[0067] However, although the *Hericium erinaceus* mycelium active substance was orally fed to mice in Example 2, other dosage forms such as drops, suppositories, etc. could also be used in practical applications.

[0068] If the strain of the present disclosure is applied to food use in a liquid form, the following composition 2 is taken as an illustrative example.

[0069] Composition 2: The lyophilized powder or ethanol extract (20 wt%) of the *Hericium erinaceus* mycelium active substance of Example 1 was mixed thoroughly with benzyl alcohol (8 wt%) as a preservative, glycerin (7 wt%) as a diluent and sucrose (10 wt%) as a diluent and then dissolved in pure water (55wt%), followed

by storage at 4°C for later use. The aforementioned wt% refers to the proportion of each component to the total weight of the composition.

CLAIMS

What is claimed is:

1. Use of a *Hericium erinaceus* mycelium active substance in the manufacture of a medicament for preventing or treating age-related macular degeneration, wherein a preparation method of the *Hericium erinaceus* mycelium active substance includes the following steps:

(a) cultivating a *Hericium erinaceus* mycelium on a plate medium at 15-35°C for 7-15 days;

(b) inoculating the *Hericium erinaceus* mycelium in step (a) into a flask and culturing the mycelium at 15-35°C and pH 2-8 for 3-5 days;

(c) inoculating the *Hericium erinaceus* mycelium in step (b) into a fermentation tank and culturing the mycelium by stirring at 15-35°C and pH 4.5-5.5 for 7-15 days to obtain a *Hericium erinaceus* mycelium fermentation liquid containing the *Hericium erinaceus* mycelium active substance;

(d) freeze-drying and then grinding the *Hericium erinaceus* mycelium fermentation liquid to obtain a *Hericium erinaceus* mycelium lyophilized powder containing the *Hericium erinaceus* mycelium active substance;

(e) extracting the *Hericium erinaceus* mycelium lyophilized powder with ethanol twice to obtain a *Hericium erinaceus* mycelium extract containing the *Hericium erinaceus* mycelium active substance; and

(f) drying the *Hericium erinaceus* mycelium extract to obtain the *Hericium erinaceus* mycelium active substance.

2. The use of claim 1, wherein the cultivation in the flask in the step (b) is shake flask cultivation with a rotation speed of 10-250 rpm.

3. The use of claim 1, wherein a gas is further fed in the fermentation tank in the step (c), and the gas includes air, oxygen, carbon dioxide, helium or a combination thereof; pressure of the fermentation tank is 0.5-2.0 kg/cm², and a gas flow rate is 0.5-1 VVM.
4. The use of claim 1, wherein the medicament is a pharmaceutical composition, and the pharmaceutical composition further comprises a pharmaceutically acceptable carrier, an excipient, a diluent or an adjuvant.
5. The use of claim 1, wherein the medicament is to be administered orally or as a drop or a suppository.