

USE OF CORDYCEPS CICADAЕ MYCELIUM ACTIVE SUBSTANCE FOR PREVENTING OR TREATING MACULAR DEGENERATION

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0001] The present invention relates to a use of a *cordyceps cicadae* mycelium active substance and, in particular, to preparation of a composition for preventing or treating macular degeneration using the *cordyceps cicadae* 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] Cordyceps cicadae is a kind of entomogenous fungus, also known as soil cordyceps cicadae, insect flower, cicada grass, cordyceps sobolifera, cicada fungus, cicada pupa grass, golden cordyceps cicadae, cicada velvet or silkworm velvet, etc. Cordyceps cicadae belong to *Ascomycotina*, *Clavicipitales*, *Clavicipitaceae*, *Cordyceps* fungi and is formed through the death of larvae of infect cicada pupa, *cicada flammata*, *platyleura kaempferi*, *cryptotympana pustulata* and *platylomia pili*, etc. and formation of the flower bud-like seat at the tip of the cicada pupa or on the head of the worm, so it is named cordyceps cicadae. Natural wild cordyceps cicadae are mostly produced in tropical and subtropical areas south of the Yangtze River. There are also traces of fruit bodies of wild cordyceps cicadae in some mountainous areas in Taiwan.

[0005] Cordyceps cicadae is a valuable traditional Chinese medicinal material, which has been used as medicine for more than a thousand years. Modern pharmacological experiments show that cordyceps cicadae and their artificial cultures can significantly regulate immunity, regulate the nervous system, resist fatigue, calm, relieve pain and fever, improve kidney function, lower blood sugar, lower blood pressure, slow down heart rate, and inhibit atherosclerosis formation, resist tumor, resist radiation and nourish and strengthen the body. However, there is currently no research showing that cordyceps cicadae have curative effects on macular degeneration.

SUMMARY OF THE INVENTION

[0006] The present invention provides a use of a *cordyceps cicadae* mycelium active substance in manufacture of a composition for preventing or treating macular degeneration. A preparation method of the *cordyceps cicadae* mycelium active substance includes the following steps:

[0007] (a) cultivating a *cordyceps cicadae* mycelium on a plate medium at 15-35°C for 2-10 days;

[0008] (b) inoculating the *cordyceps cicadae* mycelium in step (a) into a flask and culturing the mycelium at 15-35°C and pH 2-8 for 3-7 days; and

[0009] (c) inoculating the *cordyceps cicadae* mycelium cultured in step (b) into a fermentation tank and culturing the mycelium by stirring at 15-35°C and pH 2-8 for 3-5 days to form a *cordyceps cicadae* mycelium fermentation liquid containing the *cordyceps cicadae* mycelium active substance.

[0010] In one embodiment, the preparation method of the *cordyceps cicadae* mycelium active substance further includes a step (d): freeze-drying and then grinding the *cordyceps cicadae* mycelium fermentation liquid to obtain a *cordyceps cicadae* mycelium lyophilized powder containing the *cordyceps cicadae* mycelium active substance.

[0011] In one embodiment, the preparation method of the *cordyceps cicadae* mycelium active substance further includes a step (e): extracting the *cordyceps cicadae* mycelium lyophilized powder with a solvent to obtain a *cordyceps cicadae* mycelium extract containing the *cordyceps cicadae* mycelium active substance.

[0012] In one embodiment, the preparation method of the *cordyceps cicadae* mycelium active substance further includes a step (f): drying the *cordyceps cicadae* mycelium extract to obtain the *cordyceps cicadae* mycelium active substance.

[0013] In one embodiment, the solvent in the step (e) is water.

[0014] In one embodiment, the cultivation in the flask in the step (b) is shaking flask cultivation with a rotation speed of 10-250 rpm.

[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 0.01-1.5 kg/cm², and a gas flow rate is 0.5-1.0 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(A) shows photographs of ocular fundus of mice in each group, and FIG. 1(B) shows the optical coherence tomography diagrams at the positions corresponding to the red/vertical line marks in the photographs;

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

[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 *cordyceps cicadae* mycelia

[0025] The *cordyceps cicadae* mycelia used in this embodiment are collected from Taiwan wild *cordyceps cicadae* fruit bodies, not fruits. The fruit bodies are separated to obtain their hyphae, and then subcultured on the plate medium. This strain has been identified as *cordyceps cicadae* by Taiwan Food Industry Development Research Institute, and it has been deposited with the Bioresources Research Center (BCRC) of the Food Industry Development Research Institute under the registration number MU30106. This strain is also deposited in the China General Microorganism Collection Management Center, and the preservation label is CGMCC No.10486. However, the *cordyceps cicadae* mycelium active substance described in the present invention is not limited to that obtained from this strain, and other types of *cordyceps cicadae* strains can also be used.

[0026] Liquid culture of mycelia

[0027] The *cordyceps cicadae* mycelia were inoculated on a plate medium and cultivated at an appropriate temperature of 15-35°C (preferably 25°C) for 2-10 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 10-250 rpm for 3-7 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 2-8 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 0.01-1.5 VVM for 2-10 days to obtain the *cordyceps cicadae* 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%)
Mixed carbon and nitrogen sources	0.01-5
Animal and plant-derived proteins and hydrolysates thereof	0.01-2
Yeast or malt extracts (powder, cream)	0.001-2
Inorganic salts	0.0001-0.05
Carbohydrates	0.01-10

[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 *cordyceps cicadae* 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 - water extraction

[0033] The distilled water with a volume of 20 times the lyophilized powder of *cordyceps cicadae* mycelia was added into the lyophilized powder for dissolution, followed by heating at a temperature of 100°C for 30 minutes and then freeze-drying after cooling to obtain a water extract of the *cordyceps cicadae* mycelia.

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

Example 1: Preparation of active substance of *cordyceps cicadae* mycelium

[0035] Strain: deposited in the Bioresources Research Center (BCRC) of the Food Industry Development Research Institute with a deposition number of MU30106 *cordyceps cicadae* mycelium. This strain can be purchased on the official website of the depository BCRC, and is available for people having ordinary skill in the art.

[0036] Plate culture: the mycelium was inoculated on the plate medium and cultured at 25°C for 5 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 5.5 on a shaker with a rotation speed of 120 rpm for 3 days;

[0038] Medium formulation:

Ingredient	content (wt%)
Sucrose	3.0
Yeast extract	0.5
Soybean powder	1.0
Peptone	1.0
Magnesium sulfate	0.05
Amino acetic acid	0.1

[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 5.5 with a stirring speed of 10 rpm and introduction of air at an aeration rate of 1.0 VVM for 5 days to obtain the fermentation liquid of *cordyceps cicadae* mycelia. The *cordyceps cicadae* mycelium fermentation liquid can be freeze-dried to obtain the lyophilized powder of *cordyceps cicadae* mycelium.

[0040] Preparation of extract: The distilled water with a volume of 20 times the lyophilized powder of *cordyceps cicadae* mycelia was added into the lyophilized powder for dissolution, followed by heating at a temperature of 100°C for 30 minutes and then freeze-drying after cooling to obtain a water extract of the *cordyceps cicadae* mycelia.

[0041] Results: The fermented liquid of *cordyceps cicadae* mycelia cultured in the 20 metric tons fermentation tank can be freeze-dried to obtain about 320 kg of lyophilized powder, which can be extracted to obtain about 20-30 kg of water extract. The following biological experiments were carried out with the water extract of the *cordyceps cicadae* mycelia.

Example 2 Animal model of macular degeneration and analysis of related indexes

[0042] Establishment of mouse animal model of macular degeneration

[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 macular degeneration (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 *cordyceps cicadae* mycelium active substances on 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) *Cordyceps cicadae* water extract group (experimental group): First, 100 mg/kg of the *cordyceps cicadae* water extract prepared in Example 1 was given 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 *cordyceps cicadae* water extract was fed daily for 7 days.

[0052] (4) Positive control group: Onion extract is known to be able to improve macular degeneration. As a positive control group, 1500 mg/kg of onion extract was given orally and daily for 14 days, then 40 mg/kg of sodium iodate (NaIO₃) was

given intravenously to induce AMD, and then 1500 mg/kg of onion extract was fed daily for 7 days.

[0053] Mice in each group were sacrificed 21 days after the experiment for retinal damage detection and related index analysis.

[0054] Retinal damage detection and related index analysis

[0055] 1. Image detection of ocular fundus

[0056] 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(A) shows photographs of ocular fundus of mice in each group on the 7th day after the injection of sodium iodate to induce macular degeneration (the 21st day of the experiment), and FIG. 1(B) shows the optical coherence tomography diagrams at the positions corresponding to the red/vertical line marks in the photographs.

[0057] In FIG. 1(B), the optic nerve of mice was taken as the center, the 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.

[0058] It can be seen from the fundus photographs of FIG. 1(A) that, compared with the blank control group (Mock), the mouse group administered with sodium iodate (NaIO_3) alone has relatively irregular retina arrangement and less smooth ordering and more blurred boundary line between the tissues on the 7th day after the injection of sodium iodate with the occurrence of obvious retinal distortion. The group of mice pre-treated with onion extract and *cordyceps cicadae* extract has

significantly less irregularities and blurring of the retina arrangement than the group of mice administered with sodium iodate (NaIO_3) alone.

[0059] 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. Compared with the group of mice administered with sodium iodate (NaIO_3) alone, the group of mice pre-treated with onion extract could significantly slow down the thinning of retina of mice induced by sodium iodate. Although there was no significant difference for *cordyceps cicadae* extracts, the thickness still picked up. However, the concentration of the onion extract used in the positive control group to slow down the thinning of retina needed to be as high as 1500 mg/kg, while the concentration of the *cordyceps cicadae* extract used in this example was only 100 mg/kg (1/15).

[0060] Accordingly, it can be known that the *cordyceps cicadae* 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, and the effect can be achieved at a lower concentration compared with the onion extract.

[0061] 2. Histopathological analysis (H&E stainine)

[0062] 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.

[0063] Both INL and ONL layer thickness tests were centered on the optic nerve, and after 6 thickness (marked by the yellow line in FIG. 3(B)) measurements on the nasal and temporal sides around 300 μm ~ 600 μm , 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.

[0064] 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 onion extract and *cordyceps cicadae* extract 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.

[0065] Accordingly, it can be known that the *cordyceps cicadae* 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

[0066] If the *cordyceps cicadae* mycelium active substance of the present embodiment is applied to medicinal purposes, the following Composition 1 is taken as an illustrative example.

[0067] Composition 1: The lyophilized powder or water extract (20 wt%) of the *cordyceps cicadae* 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.

[0068] However, although the *cordyceps cicadae* 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.

[0069] If the *cordyceps cicadae* mycelium active substance of the present disclosure is applied to food use in a liquid form, the following composition 2 is taken as an illustrative example.

[0070] Composition 2: The lyophilized powder or water extract (20 wt%) of the *cordyceps cicadae* 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 *cordyceps cicadae* mycelium active substance in the manufacture of a composition for preventing or treating macular degeneration, wherein a preparation method of the *cordyceps cicadae* mycelium active substance includes the following steps:

(a) cultivating a *cordyceps cicadae* mycelium on a plate medium at 15-35°C for 2-10 days;

(b) inoculating the *cordyceps cicadae* mycelium in step (a) into a flask and culturing the mycelium at 15-35°C and pH 2-8 for 3-7 days; and

(c) inoculating the *cordyceps cicadae* mycelium in step (b) into a fermentation tank and culturing the mycelium by stirring at 15-35°C and pH 2-8 for 3-5 days to obtain a *cordyceps cicadae* mycelium fermentation liquid containing the *cordyceps cicadae* mycelium active substance.

2. The use of claim 1, wherein the preparation method of the *cordyceps cicadae* mycelium active substance further includes a step (d): freeze-drying and then grinding the *cordyceps cicadae* mycelium fermentation liquid to obtain a *cordyceps cicadae* mycelium lyophilized powder containing the *cordyceps cicadae* mycelium active substance.

3. The use of claim 2, wherein the preparation method of the *cordyceps cicadae* mycelium active substance further includes a step (e): extracting the *cordyceps cicadae* mycelium lyophilized powder with a solvent to obtain a *cordyceps cicadae* mycelium extract containing the *cordyceps cicadae* mycelium active substance.

4. The use of claim 3, wherein the preparation method of the *cordyceps cicadae* mycelium active substance further includes a step (f): drying the *cordyceps cicadae* mycelium extract to obtain the *cordyceps cicadae* mycelium active substance.
5. The use of claim 3 or 4, wherein the solvent in the step (e) is water.
6. 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.
7. The use of claim 1, wherein the macular degeneration is age-related macular degeneration.
8. 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 0.5-1.0 kg/cm², and a gas flow rate is 0.01-1.5 VVM.
9. The use of claim 1, wherein the composition is a pharmaceutical composition, and the pharmaceutical composition further comprises a pharmaceutically acceptable carrier, an excipient, a diluent or an adjuvant.
10. The use of claim 1, wherein the composition is a food additive.
11. The use of claim 1, wherein the composition is administrated orally or as a drop or a suppository.