



(51) International Patent Classification:
C07D 209/46 (2006.01) *A61K 8/49* (2006.01)
A61K 31/4035 (2006.01)

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(21) International Application Number:
PCT/KR2008/000804

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(22) International Filing Date:
12 February 2008 (12.02.2008)

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(25) Filing Language: Korean

(26) Publication Language: English

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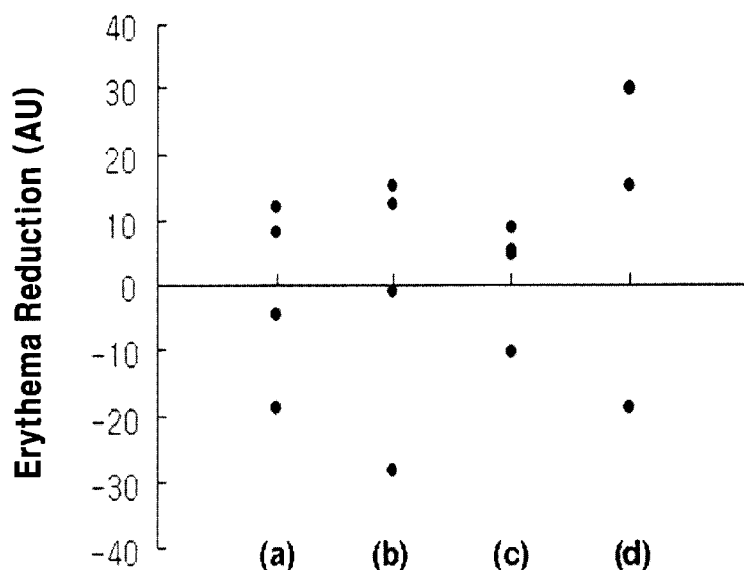
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(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: NOVEL CLITOCYBIN DERIVATIVES, PREPARATION METHOD THEREOF AND COMPOSITION CONTAINING THE EXTRACT OF CLITOCYBE AURANTIACA KCTC 11143BP OR THE NOVEL CLITOCYBIN DERIVATIVES FOR PREVENTION OF AGING AS AN ACTIVE INGREDIENT

FIGURE 9



(57) Abstract: Disclosed are novel clitocybin derivatives and methods for the preparation thereof. Also, anti-aging, pharmaceutical or cosmetic composition comprising the clitocybin derivatives as active ingredient are also provided. The extract from *Clitocybe aurantiaca* (KCTC 11143BP) or the novel clitocybin derivatives have excellent ability to scavenge oxygen radicals, to inhibit oxidative DNA damage and to reduce UV-induced erythema, so that they can be useful in the prophylaxis and treatment of diseases attributable to the accumulation of excess active oxygen and can be applied to the preparation of pharmaceutical or cosmetic compositions for the prevention of aging.



Published:

— *with international search report (Art. 21(3))*

[DESCRIPTION]**[Invention Title]**

NOVEL CLITOCYBIN DERIVATES, PREPARATION METHOD
THEREOF AND COMPOSITION CONTAINING THE EXTRACT OF CLITOCYBE
5 AURANTIACA KCTC 11143BP OR THE NOVEL CLITOCYBIN DERIVATES
FOR PREVENTION OF AGING AS AN ACTIVE INGREDIENT

[Technical Field]

The present invention relates to novel clitocybin
derivatives, preparation methods thereof, and anti-aging
10 compositions comprising *Clitocybe aurantiaca* extracts or
novel clitocybin derivatives as an active ingredients.

[Background Art]

Reactive oxygen species, including oxygen ions, free
radicals, and peroxides, such as superoxide (O_2^-), hydroxyl
15 radical ($\cdot OH$) and hydrogen peroxide (H_2O_2), which are
generated by various oxidative reactions, chemicals, foods,
diseases, radiation, and other factors, oxidize the lipids
in cell membranes, which are abundant in unsaturated fatty
acid, with the concomitant production of lipid peroxides.
20 Lipid peroxides accumulated in cell membranes lower the
fluidity and functionality of cell membrane, suppressing
overall cellular functionality and causing changes in cell
structure. Thus, tissues are regionally injured, resulting

in various diseases. For example, reactive oxygen and free radicals modify or destroy cellular constituents, such as nucleic acids, carbohydrates, etc., causing various diseases including cancer, liver diseases such as alcoholic hepatitis, cerebral apoplexy, myocardial infarction, diabetic vascular diseases, hyperlipidemia, acute inflammation, and rheumatoid diseases [Arthritis. Res. Ther. 6(6): 265-78 (2004), J. Neuropathol. Exp. Neurol. 65(7): 631-41 (2006), Trends. Mol. Med. 12(11): 521-8 (2006), Int. J. Biochem. Cell. Biol. 39(1); 44-84 (2007)]. In addition, reactive oxygen is a cause of food rancidity because it is likely to react with unsaturated fatty acids in food, thus compromising the safety and quality of food. Besides, problems caused by reactive oxygen species are found in various fields, including oxidative stress on animals and plants and a decrease in yield of microorganism fermentation.

Hence, non-toxic materials with potent antioxidative activity are very usefully applied to the development of therapeutics or as anti-aging cosmetic additives and food additives.

Thus far, synthetic anti-oxidative agents, such as BHT (butylated hydroxy toluene) and BHA (butylated hydroxy anisol) have been used as lipid peroxide inhibitors. In spite of their superior inhibitory activity against lipid peroxidation, BHT and BHA cannot be used for a long period

of time because they are very likely to cause cancer or deformations. There is a need for the development of natural anti-oxidant agents free of toxicity and side effects.

5 There are about 20 kinds of anti-oxidant materials that have been discovered from microorganism metabolites. Most of the anti-oxidant materials discovered from microorganisms thus far are derived from *Streptomyces*, with the most predominance of carbazole compounds. Typical of
10 the carbazole-type anti-oxidant materials is carquinostatin, which is an acetone extract from *Streptomyces exfoliates*. In addition, antiostatin, carazostatin, neocarozostatin, and carbazomycin have been reported. Also, phenazine-type anti-oxidant materials have
15 been reported, which include benthocyanin, benthophoenin and phenazoviridin. In addition, thiazolestatin?, naphterpin, and pyrrolostatin have been reported as natural thiazole-type anti-oxidant agents.

 In Korea, the study and research of lipid
20 peroxidation inhibitors have been performed to examine anti-oxidative activity mostly on food materials and thus have been limited to use as food preservatives. In contrast, there have been few studies on the extraction, purification and structural analysis of antioxidant
25 principle materials.

 In the early 1990s, KRIBB (the Korea Research

Institute of Bioscience and biotechnology) started to search for lipid peroxidation inhibitors and succeeded in extracting a lipid peroxidation inhibitor from actinomycetes cultures or products. In addition, KRIBB has
5 studied anti-oxidant materials from Korean wild mushrooms, with the achievement of excellent research results, including "New Indole Derivatives with Free Radical Scavenging Activity from *Agrocybe cylindracea*" (J. of Natural Products, 60 : 721- 723, 1997), "Novel
10 antioxidative compounds from *Daldinia concentrica* and antioxidative composition comprising the same as an active material" (Korean Patent No. 0466031, 2005), "Hispidin-series chemical compound capable of removing active oxygen and process for preparation thereof" (Korean Patent
15 0320787, 2002), "Method of extracting active compound from *Polyozellus multiplex* with ethyl alcohol and of refining the active compound" (Korean Patent No. 0362394, 2002), and "Antioxidative extract from a culture of *Cyathus stercoreus* fruit bodies and preparation method thereof "(Korean Patent
20 Application No. 2006-96391).

In the search for potent antioxidative materials in Korean wild mushrooms, intensive and thorough studies, conducted by the present inventors, resulted in the finding
25 that an extract from *Clitocybe aurantiaca* (KCTC 11143BP) or a novel clitocybin derivative, an iso-indole compound,

possesses potent anti-oxidative activity, leading to the present invention.

[Disclosure]

[Technical Problem]

5 Accordingly, the present invention has been made keeping in mind the above problems occurring in the prior art, and an object of the present invention is to provide a novel compound which has potent antioxidant activity.

10 It is another object of the present invention to provide a method for the preparation of the novel clitocybin derivative.

 It is a further object of the present invention to provide a potent antioxidant agent.

15 It is still a further object of the present invention to provide a UV screening agent which is highly protective of UV-induced erythema.

 It is still another object of the present invention to provide a pharmaceutical composition for the prophylaxis and treatment of diseases attributable to the accumulation
20 of excess active oxygen.

 It is yet another object of the present invention to provide a pharmaceutical composition for the prevention of aging.

25 It is yet a further object of the present invention to provide a cosmetic composition for the prevention of

aging.

It is yet still another object of the present invention to provide a method for the treatment of diseases attributable to the accumulation of active oxygen.

5 It is yet still a further object of the present invention to provide the use of the novel clitocybin derivative in the prophylaxis and treatment of diseases attributable to accumulation of excess active oxygen.

[Technical Solution]

10 In order to accomplish the above objects, the present invention provides provide a novel clitocybin derivative, represented by the following Chemical Formula 1.

 In another aspect of the present invention, there is provided a method for the preparation of the novel
15 clitocybin derivative.

 In a further aspect of the present invention, there is provided an antioxidant agent comprising an extract from *Clitocybe aurantiaca* (KCTC 11143BP) or a novel clitocybin derivative as an active ingredient.

20 In still a further aspect of the present invention, there is provided a UV screening agent comprising an extract from *Clitocybe aurantiaca* (KCTC 11143BP) or a novel clitocybin derivative as an active ingredient.

 In still another aspect of the present invention,
25 there is provided a pharmaceutical composition for the

prophylaxis and treatment of diseases attributable to the accumulation of excess active oxygen, comprising an extract from *Clitocybe aurantiaca* (KCTC 11143BP) or a novel clitocybin derivative as an active ingredient.

5 In yet another aspect of the present invention, there is provided a pharmaceutical composition for the prevention of aging, comprising an extract from *Clitocybe aurantiaca* (KCTC 11143BP) or a novel clitocybin derivative as an active ingredient.

10 In yet a further aspect of the present invention, there is provided a cosmetic composition for the prevention of aging, comprising an extract from *Clitocybe aurantiaca* (KCTC 11143BP) or a novel clitocybin derivative as an active ingredient.

15 In yet still another aspect of the present invention, there is provided a method for the treatment of diseases attributable to the accumulation of active oxygen, comprising the administration of the novel clitocybin derivative to patients suffering therefrom.

20 In yet still a further aspect of the present invention, there is provided a method for the treatment of diseases attributable to the accumulation of active oxygen, comprising the administration of an extract from *Clitocybe aurantiaca* in water, acetone or a mixture thereof to a
25 patient in need thereof.

 In yet another additional aspect of the present

invention, there is provided a method for the treatment of diseases attributable to the accumulation of active oxygen, comprising the administration of an ethyl acetate fraction from the extract from *Clitocybe aurantiaca* in water, acetone or a mixture thereof to a patient in need thereof.

In yet a further additional aspect of the present invention, there is provided the use of the novel clitocybin derivative in the prophylaxis and treatment of diseases attributable to accumulation of excess active oxygen.

In is yet still another additional aspect of the present invention, there is provided the use of an extract from *Clitocybe aurantiaca* in water, acetone or a mixture thereof in the prevention and treatment of a disease attributable to accumulation of excess active oxygen.

In is yet still a further additional aspect of the present invention, there is provided the use of an ethyl acetate extract from *Clitocybe aurantiaca* in the prevention and treatment of a disease attributable to accumulation of excess active oxygen, the ethyl acetate extract being prepared by fractioning an extract from *Clitocybe aurantiaca* in water, acetone or a mixture thereof into ethyl acetate.

[Advantageous Effects]

Having excellent ability to scavenge oxygen radicals,

to inhibit oxidative DNA damage and to reduce UV-induced erythema, the extract from *Clitocybe aurantiaca* (KCTC 11143BP) or the novel clitocybin derivative in accordance with the present invention can be useful in the prophylaxis and treatment of diseases attributable to the accumulation of excess active oxygen and can be applied to the preparation of pharmaceutical or cosmetic compositions for the prevention of aging.

[Description of Drawings]

10 FIG. 1 is a ^{13}C NMR spectrum of the novel Clitocybin A compound according to the present invention;

 FIG. 2 is a ^1H NMR spectrum of the novel Clitocybin A compound according to the present invention;

 FIG. 3 is an HMBC spectrum of the novel Clitocybin A compound according to the present invention;

 FIG. 4 is a ^{13}C NMR spectrum of the novel Clitocybin B compound according to the present invention;

 FIG. 5 is a ^1H NMR spectrum of the novel Clitocybin B compound according to the present invention;

20 FIG. 6 is a ^{13}C NMR spectrum of the novel Clitocybin C compound according to the present invention;

 FIG. 7 is a ^1H NMR spectrum of the novel Clitocybin C compound according to the present invention;

 FIG. 8 provides fluorescence microphotographs displaying images of lymphocytes without or with damaged

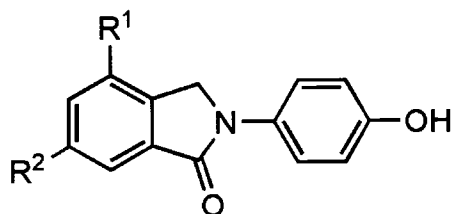
DNA after treatment with PBS only (a, control), novel
 clitocybin A (b); novel clitocybin B (c); novel clitocybin
 C (d); hydrogen peroxide (e); novel clitocybin A + hydrogen
 peroxide (f); novel clitocybin B + hydrogen peroxide (g)
 5 and novel clitocybin C + hydrogen peroxide (h); and

FIG. 9 is a graph in which erythema reduction is
 plotted for animals treated with novel clitocybin A (a),
 novel clitocybin B (b), novel clitocybin C (c) and control
 (d).

10 **【Best Mode】**

In accordance with an aspect thereof, the present
 invention provides a novel clitocybin derivative
 represented by the following Chemical Formula 1.

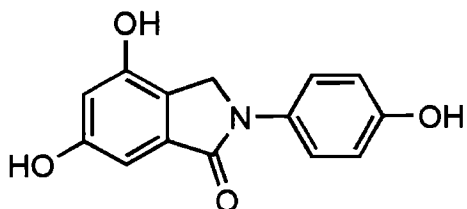
15 **【Chemical Formula 1】**



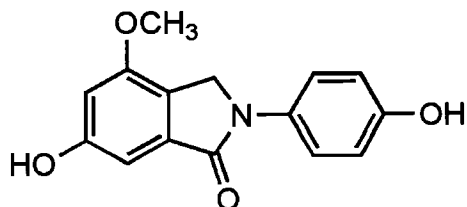
(wherein, R¹ and R² are independently OCH₃ or OH)

The clitocybin derivative represented by Chemical
 20 Formula 1 can be further illustrated by the following
 Chemical Formulas 11~13.

【Chemical Formula 11】

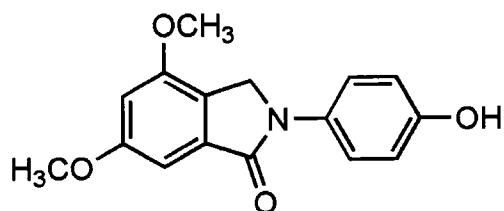


【Chemical Formula 12】



5

【Chemical Formula 13】



10 The compound of Chemical Formula 11 according to the present invention is prepared as a pale brown powder having the molecular formula $C_{14}H_{11}O_4N$ and named clitocybin A.

15 The compound of Chemical Formula 12 according to the present invention is prepared as a pale yellow powder having the molecular formula $C_{15}H_{13}O_4N$ and named clitocybin B.

Further, the compound of Chemical Formula 13 according to the present invention is prepared as a white powder having the molecular formula $C_{16}H_{15}O_4N$ and named

clitocybin C.

The compound of Chemical Formula 1 according to the present invention may be obtained preferably from *Clitocybe aurantiaca* using extraction and purification processes and
5 may be synthesized chemically.

The derivative represented by Chemical Formula 1 in accordance with the present invention may be in the form of pharmaceutically acceptable salts. Within the range of
10 these salts are included acid addition salts formed with pharmaceutically acceptable free acids. Useful as the free acids are inorganic acids such as hydrochloric acid, nitric acid, phosphoric acid, sulfuric acid, hydrobromic acid, hydroiodic acid, nitrous acid, and phosphorous acid, and
15 non-toxic organic acids such as aliphatic mono- and dicarboxylate, phenyl-substituted alkanooate, hydroxy alkanooate and alkanedioate, aromatic acids, aliphatic and aromatic sulfonic acids. Examples of such non-toxic bases include sulfate, pyrosulfate, bisulfate, sulfite,
20 bisulfite, nitrate, phosphate, monohydrogen phosphate, dihydrogen phosphate, metaphosphate, pyrophosphate chloride, bromide, iodide, fluoride, acetate, propionate, decanoate, caprilate, acrylate, formate, isobutyrate, caprate, heptanoate, propiolate, oxalate, malonate,
25 succinate, suberate, sebacate, fumarate, maleate, butyne-1,4-dioate, hexyne-1,6-dioate, benzoate, chlorobenzoate,

methylbenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, phthalate, terephthalate, benzene sulfonate, toluenesulfonate, chlorobenzenesulfonate, xylene benzoate, phenylacetate, phenylpropionate, phenylbutyrate, 5 citrate, lactate, β -hydroxybutyrate, glycolate, malate, tartrate, methanesulfonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate and mandelate.

The acid addition salts of the compound according to the present invention may be prepared using a conventional 10 method, for example, by dissolving the compound of Chemical Formula 1 in excess acid in water and precipitating the resulting salt in a water-miscible organic solvent, e.g., methanol, ethanol, acetone or acetonitrile.

Alternatively, the compound of Chemical formula 1 may 15 be heated along with the same amount of acid or alcohol in water, followed by evaporating the mixture and drying or filtering the precipitate to prepare acid addition salts thereof.

Also, metal salts formed with bases may fall within 20 the range of pharmaceutically acceptable salts of the compound of the present invention. Examples of the metal salts useful in the present invention include alkali metal salts and alkaline earth metal salts. For example, the compound of the present invention may be dissolved in 25 excess alkali metal hydroxide or alkaline earth metal hydroxide in water, and, after the filtration of the

solution to remove non-dissolved compound salts, the filtrate may be dried to afford the pharmaceutically acceptable salts of the compound of the present invention. Suitable for use in pharmaceuticals are sodium, potassium or calcium salts. Corresponding silver salts may be obtained by reacting the alkali metal or alkaline earth metal salts with suitable silver salt (e.g., silver nitrate).

In addition to the compound of Chemical Formula 1 and pharmaceutically acceptable salts thereof, solvates and hydrates that can be prepared therefrom are included within the scope of the present invention.

In accordance with another aspect thereof, the present invention provides a method for the preparation of the novel clitocybin derivative of the present invention.

As mentioned above, the novel clitocybin derivative according to the present invention may be prepared by extraction and purification from *Clitocybe aurantiaca* or through organic synthesis.

In an embodiment of the present invention, the method for the preparation of the novel clitocybin derivative comprises:

Culturing *Clitocybe aurantiaca* (KCTC 11143BP) (Step 1);

Forming an extract from the culture of Step 1 with acetone and ethyl acetate (Step 2);

Separating an active fraction from the extract of Step 2 through silica gel column chromatography eluting with a gradient of a mixture of chloroform and methanol (Step 3); and

5 Purifying the active fraction of Step 3 through Sephadex LH-20 column chromatography eluting with 100% methanol (Step 4).

10 In Step 1, *Clitocybe aurantiaca* (KCTC 11143BP) is cultured in a medium.

The medium for culturing *Clitocybe aurantiaca* (KCTC 11143BP) is preferably a yeast extract broth (YPS).

15 Step 2 is to extract active ingredients from the culture of Step 1 with acetone and ethyl acetate.

For this purpose, the culture obtained in Step 1 is treated with acetone, followed by re-extraction with ethyl acetone. A typical extraction process may be applied to this. The ethyl acetate layer after the re-extraction is
20 used for the next step.

Afterwards, the extract of Step 2 is subjected to silica gel column chromatography eluting with a gradient of a mixture of chloroform and methanol in Step 4.

25 The mixture of chloroform and methanol had a concentration gradient from 50:1 to 1:1, and elution at a

rate of 10 ~ 15 ml/min for 150 min affords an active fraction.

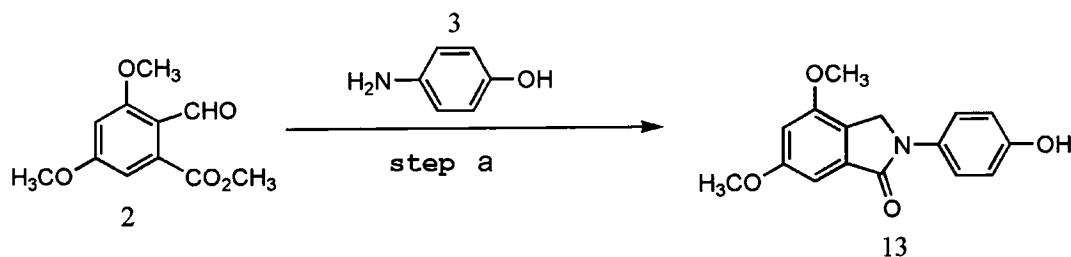
Subsequently, the active fraction of Step 3 is purified by Sephadex LH-20 column chromatography eluting with 100% ethanol to afford the clitocybin derivative of Chemical Formula 1 in Step 4.

Elution with the eluent (100% methanol) at a rate of 1 ~ 1.6 ml/min for 140 min in the Sephadex LH-20 column chromatography affords the novel clitocybin derivative as a light brownish fraction (Chemical Formula 11).

In another embodiment of the present invention, the method for the preparation of the novel clitocybin derivative, as illustrated in the following Reaction Scheme 1, comprises reacting the compound of Chemical Formula 2 with the compound of Chemical Formula 3 in an organic solvent to produce the compound of Chemical Formula 13 (Step a).

20

[Reaction Scheme 1]



(wherein the compound of Chemical Formula 13 falls

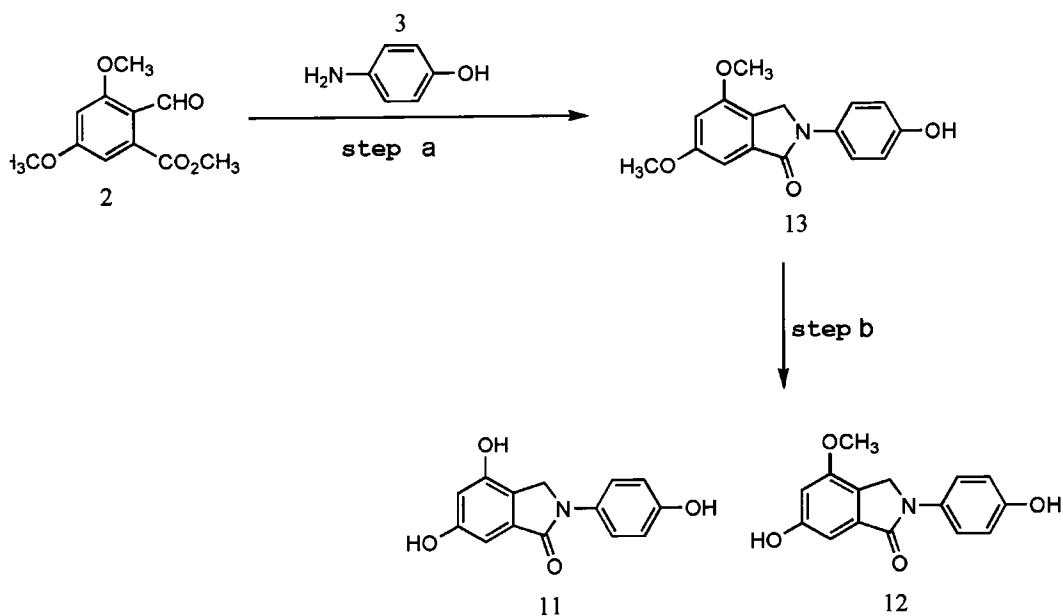
within the range of the compound of Chemical Formula 1)

In a further embodiment of the present invention, the method for the preparation of the novel clitocybin derivative, as illustrated in the following Reaction Scheme 2, comprises:

Reacting the compound of Chemical Formula 2 with the compound of Chemical Formula 3 in an organic solvent to give the compound of Chemical Formula 13 (Step a); and

Adding hydrobromic acid and acetic acid to the compound of Chemical Formula 4 of Step a, followed by extraction with ethyl acetate to afford the compound of Chemical Formula 1 (Chemical Formulas 11 and 12) (Step b).

[Reaction Scheme 2]



(wherein the compounds of Chemical Formulas 11 and 12 are included within the range of the compound of Chemical Formula 1)

5 In Step a, the compound of Chemical Formula 2 is reacted with the compound of Chemical Formula 3 in an organic solvent to produce the compound of Chemical Formula 13.

 Suitable for use as an organic solvent in this
10 reaction is methanol. The compounds of Chemical Formulas 2 and 3 are preferably mixed at an equivalent ratio of 1:1.

 In step b, the compound of Chemical Formula 13 is reacted with hydrobromic acid and acetic acid, followed by extraction with ethyl acetate to afford the compound of
15 Chemical Formula 1 (Chemical Formulas 11 and 12).

 In greater detail, the compound of Chemical Formula 13, obtained in Step a, is reacted with hydrobromic acid and acetic acid with heating reflux for 15~20 hrs. To this reaction is added ice water, followed by extraction with
20 ethyl acetate. The organic layer thus formed is washed with water, dried over anhydrous magnesium sulfate, and concentrated in a vacuum. Purification through silica gel column chromatography eluting with a mixture of 10:1 dichloromethane : methanol gives the clitocybin derivatives
25 of Chemical Formulas 11 and 12 at a yield of 80% or higher.

In accordance with a further aspect thereof, the present invention provides an antioxidative agent comprising an extract from *Clitocybe aurantiaca* or the novel clitocybin derivative as an active ingredient.

5 The extract from *Clitocybe aurantiaca* or the novel clitocybin derivative in accordance with the present invention was found to have superior radical-scavenging activity over trolox, BHA, ferulic acid and catechin, as measured using superoxide, DPPH and ABTS⁺ (FIG. 3).

10 Also, the increased oxidative DNA damage by hydrogen peroxide expressed by % tail DNA was found to be significantly inhibited by administering the novel clitocybin derivative of the present invention (FIG. 8 and Table 4).

15 Taking this into consideration, the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative in accordance with the present invention can be useful as an antioxidant agent.

20 In accordance with still another aspect thereof, the present invention provides a sun block agent comprising the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative as an active ingredient.

25 The extract from *Clitocybe aurantiaca* or the novel clitocybin derivative according to the present invention was found to have a sun block effect comparable to that of

a control, as determined by measuring erythema (FIG. 9). These results indicate that the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative can be used as a sun block agent.

5

In accordance with still a further aspect thereof, the present invention provides a pharmaceutical composition for the protection and treatment of diseases attributable to the accumulation of active oxygen, comprising the
10 extract from *Clitocybe aurantiaca* or the novel clitocybin derivative as an active ingredient.

In accordance with yet another aspect thereof, the present invention provides an anti-aging pharmaceutical composition comprising the extract from *Clitocybe*
15 *aurantiaca* or the novel clitocybin derivative as an active ingredient.

In accordance with yet a further aspect thereof, the present invention provides a method for the treatment of diseases attributable to the accumulation of active oxygen,
20 comprising administering the novel clitocybin derivative to the patient.

In accordance with yet still another aspect thereof, the present invention provides a method for the treatment of diseases attributable to the accumulation of active
25 oxygen, comprising administering the patient with an extract from *Clitocybe aurantiaca* in water, acetone, or a

mixture thereof.

In accordance with yet still a further aspect thereof, the present invention provides a method for the treatment of diseases attributable to the accumulation of active oxygen, comprising administering the patient with an ethyl acetate fraction of an extract from *Clitocybe aurantiaca* in water, acetone, or a mixture thereof.

In accordance with still another additional aspect thereof, the present invention provides the use of the novel clitocybin derivative in the prevention and treatment of diseases attributable to the accumulation of excess active oxygen.

In accordance with still a further additional aspect thereof, the present invention provides the use of an extract from *Clitocybe aurantiaca* in water, acetone or a mixture thereof in the prevention and treatment of diseases attributable to the accumulation of excess active oxygen.

In accordance with yet another additional aspect thereof, the present invention provides the use of an acetone fraction of an extract from *Clitocybe aurantiaca* in water, acetone or a mixture thereof in the prevention and treatment of diseases attributable to the accumulation of excess active oxygen.

As demonstrated in an assay for radical scavenging activity against superoxides, DPPH and ABTS+, the extract from *Clitocybe aurantiaca* or the novel clitocybin

derivative in accordance with the present invention showed higher activity than did controls, such as trolox, BHA, ferulic acid and catechin (Table 3). In addition, the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative according to the present invention was found to have an effect of protecting DNA from active oxygen, as the increased oxidative DNA damage by hydrogen peroxide, expressed by % tail DNA, was significantly inhibited by administering the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative (FIG. 8 and Table 4). Active oxygen, such as hydrogen peroxide and free radicals, damages DNA, thus promoting aging (*Free Radic. Biol. Med.* 43: 477-503 (2007)). Thus, both the radical scavenging activity and the DNA protecting activity reflect the ability to reduce the generation of active oxygen.

Suppressing the generation of active oxygen, therefore, the pharmaceutical composition of the present invention is useful in the prevention and treatment of diseases attributable to the accumulation of excess active oxygen, including liver diseases such as alcoholic hepatitis, vascular diseases such as cerebral apoplexy, myocardial infarction, diabetic vascular diseases, hyperlipidemia, acute inflammation, rheumatoid diseases and cancer [*Arthritis. Res. Ther.* 6(6): 265-78 (2004), *J. Neuropathol. Exp. Neurol.* 65(7): 631-41 (2006), *Trends.*

Mol. Med. 12(11): 521-8 (2006), *Int. J. Biochem. Cell. Biol.* 39(1); 44-84 (2007)] as well as in the prevention of aging.

Optionally, the composition of the present invention
5 may further comprise one or more ingredients identical or similar in function to that of the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative.

For clinical practice, the composition in accordance with the present invention may be used in oral or non-oral
10 forms. It is usually formulated in combination with a diluent or an excipient, such as a filler, a thickening agent, a binder, a wetting agent, a disintegrant, a surfactant, etc. Solid agents intended for oral administration of the composition of the present invention
15 may be in the form of tablets, pills, powders, granules, capsules, and the like. In these solid agents, the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative is formulated in combination with at least one excipient, such as starch, calcium carbonate, gelatine,
20 etc. Liquid agents intended for oral administration include suspensions, internal use solutions, emulsion, syrups, and the like. In addition to a simple diluent, such as water or liquid paraffin, various excipients, such as wetting agents, sweetening agents, aromatics, preservatives, and
25 the like, may be contained in the liquid agents for the oral administration of the extract or the novel derivative

of the present invention. Also, non-oral dosage forms of the extract or the novel derivative of the present invention include sterile aqueous solutions, non-aqueous solutions, suspensions, emulsions, and freeze-dried agents.

5 Non-aqueous solutions and suspensions made from propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable esters such as ethyl oleate may be used.

The effective dosage of the extract or the derivative in accordance with the present invention depends on various factors, including the patient's weight, age, gender, state of health, diet, the time of administration, route of administration, excretion rate, severity of diseases, etc.

10 In general, the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative in accordance with the present invention may be administered in a single dose, and preferably in multiple doses per day at a daily dose ranging from 0.1 to 100 mg/day, and preferably from 5 to 50 mg/kg.

20 For application to the prevention and treatment of diseases attributable to the accumulation of excess active oxygen and the prevention of aging, the composition according to the present invention may be used alone or in combination with other therapies, including surgery, hormonal therapy, chemical therapy and/or biological reaction regulators.

25

In accordance with an additionally yet a further aspect thereof, the present invention provides an anti-aging cosmetic composition comprising the extract from
5 *Clitocybe aurantiaca* or the novel clitocybin derivative as an active ingredient.

The form of the cosmetic produced as the anti-aging cosmetic composition according to the present invention
10 includes lotions, ointments, gel, creams, patches and sprays, but is not limited thereto. In a cosmetic preparation for external application to the skin, the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative in accordance with the present invention may be
15 contained in an amount from 1 to 10 parts by weight, and preferably in an amount from 2 to 10 parts by weight, based on 100 parts by weight of the preparation. In addition to the novel clitocybin derivative of the present invention, the cosmetic preparation for external application to the
20 skin may comprise lipids, organic solvents, dissolving agents, thickening agents, gelling agents, softeners, anti-oxidants, suspending agents, stabilizers, foaming agents, aromatics, surfactants, water, ionic or non-ionic emulsifiers, fillers, sequestering agents, chelating
25 agents, preservatives, vitamins, UV blocking agents, wetting agents, essential oils, dyes, pigments, hydrophilic

or lipophilic activators, liposomes, and/or other general supplements used in the skin science field. These ingredients may be used in amounts that are generally accepted in the skin science field.

5 **[Mode for Invention]**

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

10

EXAMPLE 1: Preparation of Extract from *Clitocybe aurantiaca*

15 **<1-1> Preparation of Acetone Extract from *Clitocybe aurantiaca***

Clitocybe aurantiaca (KCTC 11143BP) was inoculated into 100 ml of yeast-malt extract (YPS) broth in a 500 ml Erlenmeyer flask and cultured at 27°C for 5 days in an incubator with rotational agitation at 140 rpm. Into a 5 L jar fermentor (Korea Fermentor Co. Ltd) containing 3 liters of sterilized yeast-malt extract (YPS) broth were transferred 100 ml of the culture. After incubation for 7 days under the same condition, the culture was mixed with 3 liters of 70% acetone and then subjected to reflux condensation at 100°C for 2 hrs. The resulting extract was

20

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filtered through a filter paper (Advantec No. 2) and the filtrate was concentrated in a vacuum using an evaporator (EYELA, N-1000, Japan) to afford an acetone extract from *Clitocybe aurantiaca* (48.3 g).

5

<1-2> Preparation of Water Extract from *Clitocybe aurantiaca*

The same procedure as in Example 1-1 was repeated, with the exception that water was used instead of acetone, to afford a water extract from *Clitocybe aurantiaca* (42.5 g).

10

EXAMPLE 2: Preparation of Ethyl Acetate Fraction of *Clitocybe aurantiaca*

15

The acetone extract (48.3 g) from *Clitocybe aurantiaca* prepared in Example 1-1 was dispersed in water and added with ethyl acetate. The ethyl acetate layer was concentrated in a vacuum to give an ethyl acetate fraction of *Clitocybe aurantiaca* (15.4 g).

20

EXAMPLE 3: Preparation of Active Fraction from Ethyl Acetate Fraction of *Clitocybe aurantiaca*

The fraction (15 g) obtained in Example 2 was subjected to silica gel column chromatography eluting with

25

a gradient of a mixture of chloroform and methanol (50:1 ~ 1:1) to give an active fraction (8.3 g).

**EXAMPLE 4: Preparation of Clitocybin A through
Isolation and Purification from Extract from *Clitocybe
aurantiaca***

The active fraction obtained in Example 3 was purified using Sephadex LH-20 column chromatography eluting with 100 % methanol to produce a pure compound as a light brown powder (6.5 mg). This was identified to be pure as analyzed by reverse-phase column (ODS) chromatography eluting with 35% methanol. The compound thus obtained was named clitocybin A.

15

**EXAMPLE 5: Preparation of Clitocybin Derivative
(Organic Synthesis)**

(1) Preparation of Clitocybin C

In methanol (40 ml) was dissolved methyl 2-formyl-3,5-dimethoxybenzoate (2.24 g, 10.0 mmol), followed by 4-aminophenol (1.1 g, 10.0 mmol). After being stirred for 1 hr, the resulting solution was added with NaBH₄ (0.75 g, 20.0 mmol) at 0°C and allowed to react therewith at room temperature for 18 hrs with stirring. The reaction was concentrated in a vacuum before the addition of water (50

25

ml) and a small amount of acetic acid. The precipitate thus formed was filtered through a glass filter, washed with water, and dried to give the object compound, named Clitocybin C (Chemical Formula 13; 2.6 g, 91%).

5 ¹H NMR (300 MHz, DMSO-d₆) δ 9.38 (s, 1H), 7.65 (s, 1H), 7.62 (s, 1H), 6.82-6.76 (m, 4H), 4.73 (s, 2H), 3.87 (s, 3H);

¹³C NMR (75 MHz, DMSO-d₆) δ 165.9, 161.4, 154.9, 154.2, 134.7, 131.1, 121.5, 115.2, 102.5, 97.8, 55.7, 55.6,
10 48.3

(2) Preparation of Clitocybin A

A 48% aqueous bromic acid solution (10 ml) and acetic acid (20 ml) were added to the Clitocybin C (2.0 g, 7.0 mmol) obtained in Example 5-(1), and were heated for 18 hrs with reflux. The resulting reaction was mixed with iced water, followed by ethyl acetate. The organic layer thus formed was washed with water, dried over anhydrous magnesium sulfate, concentrated in a vacuum and purified
20 through silica gel column chromatography (dichloromethane : methanol = 10 : 1) to produce the object compound Clitocybin A (1.5 g, 85%).

¹H NMR (300 MHz, DMSO-d₆) δ 9.98 (s, 1H), 9.61 (s, 1H), 9.36 (s, 1H), 7.61 (dd, J = 6.6, 1.8 Hz, 2H), 6.79
25 (dd, J = 6.6, 1.8 Hz, 2H), 6.56 (dd, J = 1.8 Hz, 1H), 6.49 (dd, J = 1.8 Hz, 1H), 4.65 (s, 2H);

^{13}C NMR (75 MHz, DMSO- d_6) δ 166.4, 161.4, 158.9, 154.1, 153.0, 134.9, 131.3, 121.5, 117.8, 115.2, 105.9, 100.1, 48.5

5 (3) Preparation of Clitocybin B

A 48% aqueous bromic acid solution (10 ml) and acetic acid (20 ml) were added to Clitocybin C (2.0 g, 7.0 mmol), obtained in Example 5-(1), and were heated for 18 hrs with reflux. The reaction was mixed with iced water, followed by ethyl acetate. The organic layer thus formed was washed with water, dried over anhydrous magnesium sulfate, concentrated in a vacuum and purified through silica gel column chromatography (dichloromethane : methanol = 10 : 1) to produce the object compound Clitocybin B (0.76 g, 40%), along with Clitocybin A.

^1H NMR (300MHz, DMSO- d_6) δ 9.85 (s, 1H), 9.36 (s, 1H), 7.62 (d, J = 9.3 Hz, 2H), 6.78 (d, J = 9.3 Hz, 2H), 6.67 (d, J = 1.8 Hz, 1H), 6.63 (d, J = 1.8 Hz, 1H), 4.70 (s, 2H), 3.84 (s, 3H);

20 ^{13}C NMR (75MHz, DMSO- d_6) δ 166.0, 159.3, 154.9, 154.1, 134.7, 131.2, 121.5, 119.0, 115.2, 102.5, 100.5, 55.4, 48.3.

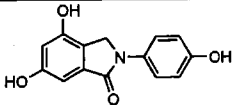
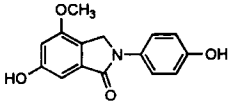
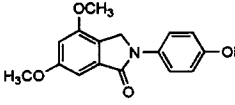
<Structural Analysis>

25 The physicochemical properties of the compounds prepared in Examples 4 and 5 were analyzed as follows.

Rotary power was determined using a polarimeter (JASCO DIP-370 polarimeter). The compounds were examined with regard to optical properties with a UV spectrophotometer (Shimadzu UV-260 spectrophotometer, Japan) for UV absorption spectra, with an IR spectrophotometer (FT-IR Equinox 55 spectrometer, Burker, USA) for IR absorption spectra, and a mass spectrometer (JEOL JMS HX-110 mass spectrometer, JEOL, Japan) for HR-ESI-MS spectra, so as to determine the molecular structures thereof.

Measurements are summarized in Table 1, below.

TABLE 1

	Clitocybin A	Clitocybin B	Clitocybin C
Structural Formulas			
Properties	Pale brownish powder	Pale yellow powder	White powder
Rotary Power	$[\alpha]_D^{20} - 1.40^\circ$ ($c=0.3$)	$[\alpha]_D^{20} - 1.40^\circ$ ($c=0.9$)	$[\alpha]_D^{20} - 1.40^\circ$ ($c=1.0$)
Chemical Formulas	$C_{14}H_{11}O_4N$	$C_{15}H_{13}O_4N$	$C_{16}H_{15}O_4N$
Mw	257	271	285
HR-ESI-MS m/z	Found: 258.07571 ($M+H^+$) Cal.: 258.07608	Found: 272.0923 ($M+H^+$) Cal.: 272.0923	Found: 286.1079 ($M+H^+$) Cal.: 286.1079
UV Spectra [UV nm(in	Abs. λ_{max} (0.4) cm^{-1}	Abs. λ_{max} (0.8) cm^{-1}	Abs. λ_{max} (0.8) cm^{-1}

methanol)]			
IR Abs. Spectra[IR (cm ⁻¹)]	3399, 2925, 1616, 1516, 1453, 1256, 1144, 1097	3399, 2925, 1616, 1516, 1453, 1256, 1144, 1097	3399, 2925, 1616, 1516, 1453, 1256, 1144, 1097
Solubility	MeOH, DMSO	EtOAc, DMSO	EtOAc, DMSO
Insolubility	hexane, water	hexane, water	hexane, water

As seen in Table 1, Clitocybin A according to the present invention was produced as a pale brownish powder and was found to have (M+H⁺) at m/z 258.07571, which is almost the same as the calculated value m/z 258.07608, as analyzed by high resolution electrospray ionization mass spectrometry (HR-ESI-MS). Therefore, the molecular formula was determined to be C₁₄H₁₁O₄N. As for UV spectra, maximum absorption was read at 211 nm and 286 nm while a band corresponding to a hydroxy group was observed at 3399 cm⁻¹ on the IR spectra. Taking these results into consideration, Clitocybin A was identified as a novel iso-indolone-based compound.

Clitocybin B according to the present invention was produced as a pale yellowish powder and was found to have (M+H⁺) at m/z 272.0923, which is almost the same as the calculated value m/z 272.0923, as analyzed by high resolution electrospray ionization mass spectrometry (HR-ESI-MS). Therefore, the compound was determined to have a molecular formula of C₁₅H₁₃O₄N. As for UV spectra, maximum absorption was read at 211 nm and 286 nm while a band

corresponding to a hydroxy group was observed at 3399 cm^{-1} on the IR spectra. Taking these results into consideration, Clitocybin B was identified as a novel iso-indolone-based compound.

5 Clitocybin C according to the present invention was produced as a pale yellowish powder and was found to have $(M+H^+)$ at m/z 286.1079, which is almost the same as the calculated value m/z 286.1079, as analyzed by high resolution electrospray ionization mass spectrometry (HR-ESI-MS). Therefore, the compound was determined to have a
10 molecular formula of $C_{16}H_{15}O_4N$. As for UV spectra, maximum absorption was read at 211 nm and 286 nm while a band corresponding to a hydroxy group was observed at 3399 cm^{-1} on the IR spectra. Taking these results into consideration,
15 Clitocybin B was identified as a novel iso-indolone-based compound.

Also, Clitocybin A ~ C were NMR analyzed for ^1H and ^{13}C NMR, HMQC (^1H -detected Heteronuclear Multiple-Quantum
20 Coherence) and HMBC (Heteronuclear Multiple-Bond Coherence) spectra using an NMR apparatus (Burker AMX 300, Burker, USA). These are shown in FIGS. 1 ~ 7 and Table 2.

TABLE 2

Carbon Nos. (position)	Clitocybin A		Clitocybin B		Clitocybin C	
	δC (ppm)	δH (ppm)	δC (ppm)	δH (ppm)	δC (ppm)	δH (ppm)

1	155		154.1		154.2	
2	108	6.50 (d, J=1.8 Hz)	102.5	6.63 (d, J=1.8 Hz)	102.5	6.63 (d, J=1.8Hz)
3	162		159.3		161.4	
4	102	6.71 (d, J=1.8 Hz)	102.5	6.67 (d, J=1.8 Hz)	97.8	6.99 (d, J=1.8Hz)
5	120		119.0		121.1	
6	136		134.7		134.7	
7	172		166.0		165.9	
8	50	4.71 (s)	48.3	4.70 (s)	48.3	4.68 (s)
9	133		131.2		131.1	
10, 14	124	7.54 (d, J=9.0 Hz)	121.5	7.62 (d, J=9.3 Hz)	121.5	7.68 (d, J=9.0 Hz)
11, 13	117	6.84 (d, J=9.0 Hz)	119.0	6.78 (d, J=9.3 Hz)	115.2	6.89 (d, J=9.0 Hz)
12	157		154.9		154.9	
15			55.4	3.84 (s)	55.6	3.83 (s)
16					55.7	3.87 (s)
Standard material: TMS						
¹ H NMR : 600 MHz						
¹³ C NMR : 150 MHz						

Optical analyses of Clitocybin A according to the present invention are given in FIG. 1 for ¹³C NMR spectrum, FIG. 2 for ¹H NMR spectrum and FIG. 3 for an HMBC spectrum.

5 Optical analyses of Clitocybin B according to the present invention are given in FIG. 4 for ¹³C NMR and FIG. 5 for ¹H NMR spectra.

Optical analyses of Clitocybin C according to the present invention are given in FIG. 6 for the ¹³C NMR spectrum and FIG. 7 for the ¹H NMR spectrum.

10

As seen in FIGS. 1 ~ 7 and Table 2, proton peaks of

the hydrogen atoms bonded to the aromatic rings are observed at δ 6.50(1H), δ 6.71(1H), δ 6.84(2H), and δ 7.54(2H) in ^1H -NMR spectra. The chemical shift of δ 4.71(2H) indicates a bond with nitrogen. Also, the ^{13}C -NMR and HMQC spectra show the presence of hydroxy carbons at δ 155(C1), δ 162(C3) and δ 157(C12), a carbonyl carbon at δ 172(C7) and a carbon at δ 50(C8). Also, three tertiary carbons were analyzed to be located at δ 120(C5), δ 136(C6) and δ 133(C9) with the existence of four methyne carbons at δ 124(C10, C14) and δ 117(C11, C13). Long range correlation was observed at δ 4.71, δ 6.50, δ 6.71, δ 6.84 and δ 7.54 from the HSBC spectra.

EXPERIMENTAL EXAMPLE 1: Assay for Radical-Scavenging Activity of Extract from *Clitocybe aurantiaca* or Novel Clitocybin Derivative

The following experiments were carried out to examine the radical-scavenging activity of the extract from *Clitocybe aurantiaca* or the novel Clitocybin derivative according to the present invention.

(1) Radical-Scavenging Activity Against 2,2-Azino-bis-3-ethylbenzothiazoline sulfonate Cation Radical (ABTS⁺)

A 7 mM aqueous ABTS solution was mixed with 2.45 mM potassium persulfate to produce ABTS⁺, which was then

diluted to an aqueous ABTS⁺ radical solution having an OD of about 0.7 at 734 nm. To 190 µl of the dilution of ABTS⁺ was added 10 µl of a solution of the extract from *Clitocybe aurantiaca*, the ethyl acetate fraction thereof, or the novel clitocybin derivative (clitocybin A, B, or C) in DMSO, after which 7 min incubation was followed by the measurement of absorbance at 734 nm. A solution of trolox or BHA in DMSO was used as a positive control, while DMSO alone was used as a negative control. The absorbance measurements were used to calculate ABTS⁺ radical scavenging activity according to the following Mathematical Formula 1.

(2) Radical-Scavenging Activity Against 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical

90 µl of 150 µM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in ethanol was mixed with 10 µl of a solution of the extract from *Clitocybe aurantiaca*, the ethyl acetate fraction, or the novel clitocybin derivative (clitocybin A, B, or C) in DMSO, and was allowed to stand for 30 min, followed by the measurement of absorbance at 517 nm. For comparison, trolox or BHA was used as a positive control while DMSO alone was used as a negative control. The measurements of absorbance were used to calculate DPPH radical scavenging activity according to the following Mathematical Formula 2.

(3) Radical Scavenging Activity Against Superoxide

To each well of 96-well plate were added 50 mM potassium phosphate buffer, pH 7.8, 1 mM EDTA, 0.04 mM nitroblue tetrazolium (NBT), 0.18 mM xanthine and 250 mU/ml xanthine oxidase, along with a solution of the extract *Clitocybe aurantiaca*, the ethyl acetate fraction thereof, or the novel clitocybin derivative (clitocybin A, B, or C) in DMSO, after which incubation was performed for 30 min at 37°C in a darkroom. Xanthine oxidase catalyzes the oxidation of xanthine into uric acid and superoxide. Superoxide reduces NBT, with the concomitant formation of blue formazan, which allows ELISA to measure absorbance at 560 nm. For comparison, DMSO only was used as a negative control, while ferulic acid or catechin was used as a positive control. Radical scavenging activity was calculated on the basis of the difference in absorbance between the test group and the control group in accordance with the following Mathematical Formula 2.

Each experiment was conducted in triplicate and the absorbance changes were means of three measurements.

[Mathematical Formula 1]

$$\text{Radical Scavenging Activity (\%)} = \left(\frac{\Delta A_{\text{Control}}}{\Delta A_{\text{Sample}}} - 1 \right) \times 100$$

[Mathematical Formula 2]

$$\text{Radical Scavenging Activity (\%)} = \left(1 - \frac{\Delta A_{\text{Control}}}{\Delta A_{\text{Sample}}}\right) \times 100$$

(wherein,

ΔA_{Sample} : a change of the absorbance measured in well containing sample compound.

5 $\Delta A_{\text{Control}}$: A change of the absorbance measured in well containing DMSO free of any sample)

The measurement results of the radical scavenging activity are summarized in Table 3, below. The results are expressed as concentrations (EC_{50}) of the compound of interest at which 50% of the used radicals are removed.

TABLE 3

Samples	Radical Scavenging Activity ($EC_{50} \pm SD$ (μM))		
	ABTS ⁺	DPPH	Superoxide
Acetone Extract of <i>Clitocybe aurantiaca</i>	10.26 $\pm$ 0.23	>100	15.42 $\pm$ 3.22
Ethyl Acetate Fraction of <i>Clitocybe aurantiaca</i>	8.65 $\pm$ 0.13	>100	12.04 $\pm$ 1.12
Clitocybin A	6.45 $\pm$ 0.32	>100	10.3 $\pm$ 0.43
Clitocybin B	9.63 $\pm$ 0.22	>100	5.98 $\pm$ 0.33
Clitocybin C	94.91 $\pm$ 5.52	>100	3.61 $\pm$ 0.38
Trolox	14.54 $\pm$ 0.08	37.61 $\pm$ 1.68	
BHA	11.70 $\pm$ 0.26	97.09 $\pm$ 05.52	
Ferulic Acid			150.22 $\pm$ 6.55
Catechin			47.61 $\pm$ 1.68

15 As understood from the data of Table 3, the extracts

from *Clitocybe aurantiaca* and the novel clitocybin derivatives in accordance with the present invention were measured to have an EC50 ranging from 6.45 ± 0.32 to 10.26 ± 0.23 μM against an ABTS⁺ radical, and from 3.61 ± 0.38 to 15.42 ± 3.22 μM against a superoxide radical, indicating far superiority in radical scavenging activity over the positive control group (trolox, BHA, ferulic acid, catechin), which are known as excellent radical scavengers. As for the DPPH radical, the compounds of the present invention showed radical scavenging activity comparable with that of BHA.

Accordingly, the excellent radical scavenging activity enables the extracts from *Clitocybe aurantiaca* or the novel clitocybin derivatives according to the present invention to be effectively applied not only to the prevention and treatment of diseases, attributable to the accumulation of excess active oxygen, but also to the prevention of aging.

EXPERIMENTAL EXAMPLE 2: Protection Effect of Extract from *Clitocybe aurantiaca* or Novel Clitocybin Derivative on DNA

The extracts from *Clitocybe aurantiaca* or the novel clitocybin derivatives in accordance with the present invention were examined as to whether they can reduce

cellular DNA damage, using the comet assay reported by Jeon, et al. (The Korean Journal of Nutrition, 37(6): 440-447, 2004).

For this purpose, first, lymphocytes isolated from the blood sample from healthy adult women were incubated in media with PBS alone as a negative control, or with 100 μ l of the extract from *Clitocybe aurantiaca*, the ethyl acetate fraction thereof, the novel clitocybin derivative, or H_2O_2 , or with a combination of the extract or the novel compound according to the present invention and H_2O_2 . The amount of the tail DNA produced was measured to quantitatively analyze cellular DNA damage while the cells were subjected to image analysis using a fluorescence microscope (Leica DMLB, Germany).

The bigger the tail DNA was, the more serious the DNA damage was.

Measurement results are given in FIG. 8 and Table 4.

In the fluorescence microphotographs of FIG. 8, the lymphocytes which were treated with PBS only, as a negative control, and with hydrogen peroxide only, as a positive control, are shown in panels (a) and (e), respectively. The lymphocytes which were incubated with the novel clitocybin compounds A, B and C alone are shown in panels (b), (c) and (d), respectively. The lymphocytes were incubated with the novel clitocybin compounds A, B and C in accordance with the present invention after exposure to hydrogen peroxide,

or with the novel compounds and hydrogen peroxide simultaneously in panels (f), (g) and (h), respectively.

TABLE 4

	Tail DNA (%)		
	Mean	SD	Error
Negative Control	5.72	5.10	0.72
Acetone Extract from <i>Clitocybe aurantiaca</i>	7.55	5.32	0.78
Ethyl acetate Fraction from <i>Clitocybe aurantiaca</i>	7.36	5.30	0.76
Clitocybin A	7.01	5.29	0.75
Clitocybin B	6.49	5.68	0.80
Clitocybin C	5.09	4.18	0.59
H ₂ O ₂	61.1 1	17.2 6	2.44
Acetone Extract from <i>Clitocybe aurantiaca</i> + H ₂ O ₂	38.2 6	16.0 3	2.38
Ethyl acetate Extract from <i>Clitocybe aurantiaca</i> + H ₂ O ₂	36.8 5	15.9 5	2.30
Clitocybin A + H ₂ O ₂	35.3 9	15.9 2	2.25
Clitocybin B + H ₂ O ₂	38.3 4	18.0 0	2.55
Clitocybin C + H ₂ O ₂	32.3 2	15.5 9	2.25

5

As seen in FIG. 4, exposure to hydrogen peroxide caused 61.11% tail DNA on average whereas treatment with the extract from *Clitocybe aurantiaca*, the ethyl acetate

fraction thereof, or the novel clitocybin derivative in accordance with the present invention allowed tail DNA at 32.32~38.34% on average, reducing the oxidative DNA damage by as much as about 40%.

5

Fluorescence microscopy, as shown in FIG. 8, displays lymphocytes with damaged DNA, evidenced by a comet upon exposure to H_2O_2 (e), and lymphocytes with a small number of tail DNA, visible as significantly shortened comets, upon treatment with the clitocybin derivative according to the present invention (f ~ h).

The extract from *Clitocybe aurantiaca* or the novel clitocybin derivatives in accordance with the present invention can reduce the oxidative DNA damage induced by potent reactive oxygen species, such as hydrogen peroxide, which leads to aging, by about 40%, and thus can be used as an active ingredient of an anti-aging cosmetic composition.

EXPERIMENTAL EXAMPLE 3: UV Screen Effect of Extract from *Clitocybe aurantiaca* or Novel Clitocybin Derivative

The following experiment was carried out with guinea pig erythema models in order to examine the ability of the extract from *Clitocybe aurantiaca* or the novel clitocybin derivative according to the present invention to afford protection from UV light.

For this purpose, albino guinea pigs were shaved and the extract from *Clitocybe aurantiaca* or the novel clitocybin derivatives (clitocybin A, B, and C) in accordance with the present invention were applied to shaved regions 4 days, 2 days and 2 hrs before UV exposure, or immediately after UV exposure. For erythema induction, UV light was radiated at a dosage of 2 MED - 400 MJ/cm². A mixture of propyl gallate, known as an antioxidant, ethyl alcohol and water (PG : EtOH : DIW = 5 : 3 : 2) was used as a control.

Erythema was observed with the naked eye, photography, a chromameter and histochemical staining, and erythema reduction was calculated according to the following Mathematical Formula 3.

15

[Mathematical Formula 3]

$$\text{Erythema Reduction (AU)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}$$

wherein AU is arbitrary unit, and A is a chromameter value

20

The measurement results are depicted in FIG. 9.

In FIG. 9, erythema reduction obtained after treatment with the extract from *Clitocybe aurantiaca* or the novel clitocybin derivatives in accordance with the present invention (a~c) and with the control, known as an

25

antioxidant, are plotted.

As seen in FIG. 9, the extract from *Clitocybe aurantiaca* or the novel clitocybin derivatives in accordance with the present invention have erythema
5 reduction effects comparable with those of the control, which is known to have excellent erythema reduction effects. Also, histochemical staining analysis with H&E showed no significant differences in erythema reduction between the control and the extract from *Clitocybe*
10 *aurantiaca* or the novel clitocybin derivatives in accordance with the present invention (not shown).

Having excellent UV screen effects, the extract from *Clitocybe aurantiaca* or the novel clitocybin derivatives in accordance with the present invention are therefore useful
15 as an ingredient for functional cosmetic compositions.

EXPERIMENTAL EXAMPLE 4: Assay of Novel Clitocybin Derivatives for Acute Toxicity

20 The novel clitocybin derivatives according to the present invention were assayed for acute toxicity as follows.

Six-week-old specific pathogen-free (SPF) SD rats were divided into groups, each consisting of two rats.
25 After being suspended in 0.5% methyl cellulose, each of the novel clitocybin derivatives prepared in the above Examples

was orally administered once at a dose of 1 g/kg/15 ml to the rat groups.

Afterwards, observations were made of the death, clinical symptoms, and weight changes of the animals, and serological and serobiochemical assays were conducted. Also, an autopsy was carried out to check for abnormalities in the abdominal and thoracic organs with the naked eye.

None of the animals to which the compounds of interest were administered exhibited noticeable clinical symptoms or died. Cytotoxicity was not observed in the weight change, serological assay, serobiochemical assay, or autopsy observations for the animals administered with the compounds of the present invention.

Having no toxic effects up to a dose of 1,000 mg/kg, all of the compounds according to the present invention were determined to have an oral lethal dose (LD₅₀) of 1,000 mg/kg, and were thus regarded as safe.

The novel clitocybin derivative in accordance with the present invention was formulated in combination with auxiliary agents, such as excipients, binders, lubricants, disintegrants, diluents, etc., into pharmaceutical preparations as follows.

FORMULATION EXAMPLE 1: Preparation of Pharmaceutical Compositions

1. Tablet Preparation

A tablet comprising the novel clitocybin derivative or a pharmaceutically acceptable salt thereof in accordance with the present invention as an active ingredient was prepared as follows.

The compound according to the present invention was sieved, mixed with lactose, starch and pre-gelatinized corn starch and granulized with a suitable volume of distilled water. After being dried, the granules thus obtained were mixed with magnesium stearate and pressurized into a tablet.

The tablet comprised the following ingredients.

	Novel Clitocybin derivative	5.0 mg
15	Lactose BP	150.0 mg
	Starch BP	30.0 mg
	Pre-gelatinized Corn Starch BP	15.0 mg
	Mg Stearate	1.0 mg

2. Capsule Preparation

A capsule comprising the novel clitocybin derivative or a pharmaceutically acceptable salt thereof in accordance with the present invention as an active ingredient was prepared as follows.

The compound of the present invention was mixed with a predetermined amount of excipients and magnesium

stearate, and the resulting mixture was loaded into a gelatin capsule to afford a capsule medicine.

The capsule comprises the following ingredients.

	Novel Clitocybin derivative	5.0 mg
5	Starch 1500	100.0 mg
	Mg Stearate BP	1.0 mg

3. Injection Preparation

10 An injection comprising the novel clitocybin derivative or a pharmaceutically acceptable salt thereof in accordance with the present invention as an active ingredient was prepared as follows.

The novel clitocybin derivative was dissolved in a suitable volume of injectable sodium chloride BP, and was
15 adjusted to a pH of 3.5 using dilute hydrochloric acid BP. Injectable sodium chloride BP was further added to achieve a desired volume, and the solution was sufficiently mixed. The mixture solution was then put into a 5-ml type I ampule made of transparent glass. The glass was melted to seal the
20 ampule, which was subsequently autoclaved at 120°C for 15 min, thereby giving an injectable solution.

The injection comprises the following ingredients.

	Novel Clitocybin derivative	100 µg/ml
	Dilute Hydrochloric acid BP	to pH 3.5
25	Injectable NaCl BP	Up to 1 ml

**FORMULATION EXAMPLE 2: Preparation of External
Application to the Skin**

1. Cream Preparation

5	Cetostearyl alcohol	2.8 wt. parts
	Bees wax	2.6 wt. parts
	Stearic acid	1.4 wt. parts
	Lipophilic glyceryl monostearate	2 wt. parts
	PEG-100 Stearate	1 wt. part
10	Sorbitan sesquioleate	1.4 wt. parts
	Jojoba oil	4 wt. parts
	Squalane	3.8 wt. parts
	Polysorbate 60	1.1 wt. parts
	Macadamia oil	2 wt. parts
15	Tocopherolyl acetate	0.2 wt. parts
	Methylpolysiloxane	0.4 wt. parts
	Ethyl paraben	0.1 wt. parts
	Propyl paraben	0.1 wt. parts
	Euxyl K-400	0.1 wt. parts
20	1,3-Butylene glycol	7 wt. parts
	Methyl paraben	0.05 wt. parts
	Glycerin	6 wt. parts
	d-Panthenol	0.2 wt. parts
	Novel Clitocybin derivative	4.6 wt. parts
25	Triethanolamine	0.2 wt. parts
	pt 41891	0.2 wt. parts

p-H₂O 46.05 wt. parts

2. Lotion preparation

	Cetostearyl alcohol	1.6 wt. parts
5	Stearic acid	1.4 wt. parts
	Lipophilic glyceryl monostearate	1.8 wt. parts
	PEG-100 stearate	2.6 wt. parts
	Sorbitan sesquioleate	0.6 wt. parts
	Squalene	4.8 wt. parts
10	Macadamia oil	2 wt. parts
	Jojoba oil	2 wt. parts
	Tocopheryl acetate	0.4 wt. parts
	Methylpolysiloxane	0.2 wt. parts
	Ethyl paraben	0.1 wt. parts
15	Propyl paraben	0.1 wt. parts
	1,3-Butylene glycol	4 wt. parts
	Methyl paraben	0.1 wt. parts
	Xanthan gum	0.1 wt. parts
	Glycerin	4 wt. parts
20	d-Panthenol	0.15 wt. parts
	Allantoin	0.1 wt. parts
	Novel Clitocybin derivative	3.5 wt. parts
	Carbonate (2% aq. Sol)	4 wt. parts
	Triethanolamine	0.15 wt. parts
25	Ethanol	3 wt. parts
	pt 41891	0.1 wt. parts

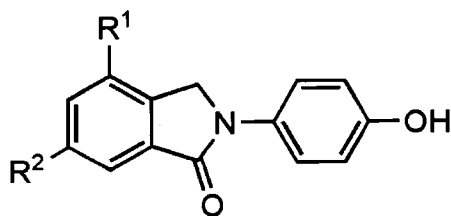
p-H₂O

48.3 wt. parts

[CLAIMS]**[Claim 1]**

A clitocybin derivative, represented by the following
 5 Chemical formula 1:

[Chemical Formula 1]



(wherein, R¹ and R² are independently OCH₃ or OH.)

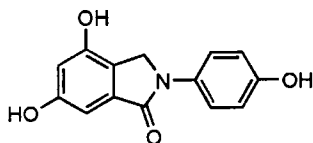
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[Claim 2]

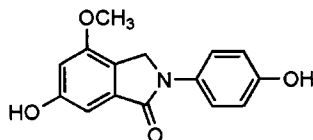
The Clitocybin derivative according to claim 1,
 wherein the clitocybin derivative is one of compounds
 represented by the following Chemical Formulas 11 to 13:

15

[Chemical Formula 11]

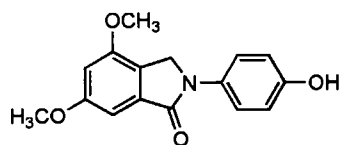


[Chemical Formula 12]



20

[Chemical Formula 13]



【Claim 3】

5 A method for preparing the clitocybin derivative of claim 1, comprising:

 culturing *Clitocybe aurantiaca* (KCTC 11143BP) (Step 1);

 forming an extract from a culture of Step 1 with
10 acetone and ethyl acetate (Step 2);

 separating an active fraction from the extract of Step 2 through silica gel column chromatography eluting with a gradient of a mixture of chloroform and methanol (Step 3); and

15 purifying the active fraction of Step 3 through Sephadex LH-20 column chromatography eluting with 100% methanol (Step 4).

【Claim 4】

20 The method according to claim 3, wherein *Clitocybe aurantiaca* (KCTC 11143BP) of Step 1 is cultured in a yeast extract broth (YPS).

【Claim 5】

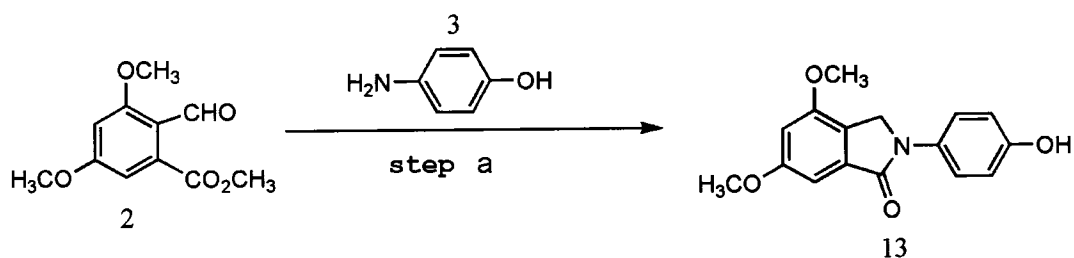
The method according to claim 3, wherein the mixture of chloroform and methanol of Step 3 has a concentration gradient from 50:1 to 1:1.

5 **【Claim 6】**

A method for preparing the clitocybin derivative of claim 1, as illustrated in the following Reaction Scheme 1, comprising:

reacting a compound of Chemical Formula 2 with a
10 compound of Chemical Formula 3 in an organic solvent to produce the compound of Chemical Formula 13 (Step a).

[Reaction Scheme 1]



15 (wherein the compound of Chemical Formula 13 falls within the range of the compound of Chemical Formula 1)

【Claim 7】

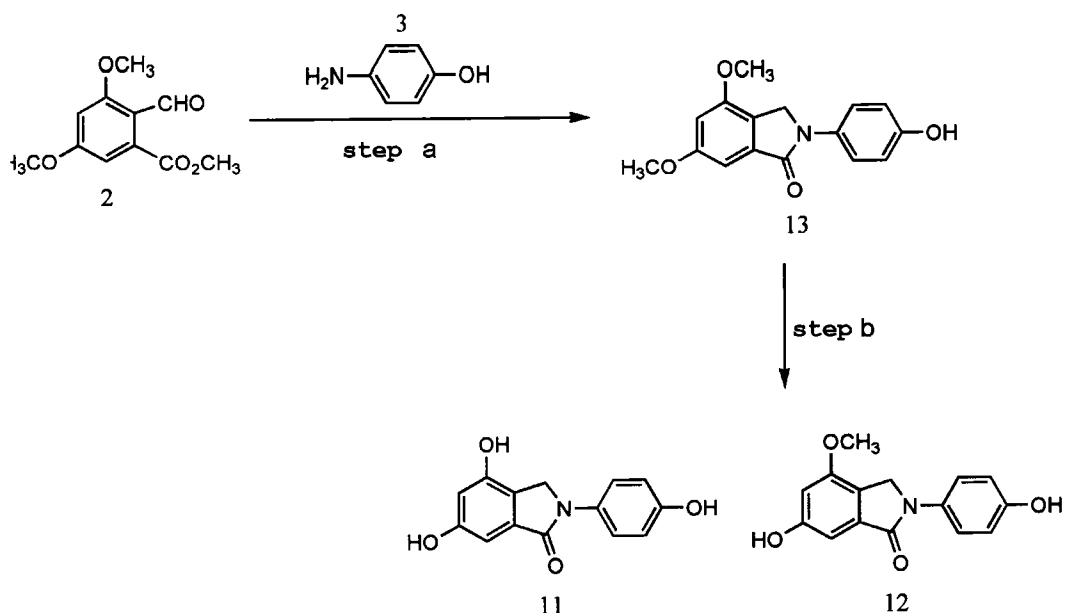
A method for preparing the clitocybin derivative of claim 1, as illustrated in the following Reaction Scheme 2, comprising:

reacting a compound of Chemical Formula 2 with a
20 compound of Chemical Formula 3 in an organic solvent to

give a compound of Chemical Formula 13 (Step a); and

adding hydrobromic acid and acetic acid to the compound of Chemical Formula 4 of Step a and extracting the clitocybin derivative with ethyl acetate to afford the compound of Chemical Formula 1 (Step b).

[Reaction Scheme 2]



(wherein the compounds of Chemical Formulas 11 and 12 are included within the range of the compound of Chemical Formula 1)

【Claim 8】

The method according to claim 7, wherein the solvent of Step a is methanol.

【Claim 9】

An antioxidant agent, comprising the clitocybin derivative of claim 1 as an active ingredient.

5 **【Claim 10】**

A UV screening agent, comprising the clitocybin derivative of claim 1 as an active ingredient.

【Claim 11】

10 A pharmaceutical composition for prevention and treatment of a disease attributable to accumulation of excess active oxygen, comprising the clitocybin derivative of claim 1 as an active ingredient.

15 **【Claim 12】**

The pharmaceutical composition according to claim 11, wherein the disease attributable to the accumulation of excess active oxygen is liver disease, cerebral apoplexy, myocardial infarction, diabetic vascular disorder,
20 hyperlipidemia, acute inflammation, rheumatoid disease or cancer.

【Claim 13】

An anti-aging pharmaceutical composition, comprising
25 the clitocybin derivative of claim 1 as an active ingredient.

【Claim 14】

An anti-aging cosmetic composition, comprising the clitocybin derivative of claim 1 as an active ingredient.

5

【Claim 15】

An antioxidant agent, comprising a *Clitocybe aurantiaca* extract as an active ingredient, said extract being prepared using water, acetone or a mixture thereof as a solvent.

10

【Claim 16】

The antioxidant agent according to claim 15, wherein the *Clitocybe aurantiaca* extract is further fractioned into ethyl acetate to form an ethyl acetate fraction, and the ethyl acetate fraction is used as the active ingredient.

15

【Claim 17】

The antioxidant agent according to claim 16, wherein the ethyl acetate fraction is purified through silica gel column chromatography eluting with a gradient of a mixture of chloroform and methanol (50:1 ~ 1:1) to give an active fraction, and the active fraction is used as the active ingredient.

20

25

【Claim 18】

A UV block agent, comprising a *Clitocybe aurantiaca* extract as an active ingredient, said extract being prepared using water, acetone or a mixture thereof as a solvent.

5

【Claim 19】

The UV block agent according to claim 18, wherein the *Clitocybe aurantiaca* extract is further fractioned into ethyl acetate to form an ethyl acetate fraction, and the ethyl acetate fraction is used as the active ingredient.

10

【Claim 20】

The antioxidant agent according to claim 19, wherein the ethyl acetate fraction is purified through silica gel column chromatography eluting with a gradient of a mixture of chloroform and methanol (50:1 ~ 1:1) to give an active fraction, and the active fraction is used as the active ingredient.

15

20

【Claim 21】

A pharmaceutical composition for prevention and treatment of a disease attributable to accumulation of excess active oxygen, comprising a *Clitocybe aurantiaca* extract as an active ingredient, said extract being prepared using water, acetone or a mixture thereof as a solvent.

25

【Claim 22】

The pharmaceutical composition according to claim 21,
wherein the *Clitocybe aurantiaca* extract is further
5 fractioned into ethyl acetate to form an ethyl acetate
fraction, and the ethyl acetate fraction is used as the
active ingredient.

【Claim 23】

10 The pharmaceutical composition according to claim 22,
wherein the ethyl acetate fraction is purified through
silica gel column chromatography eluting with a gradient of
a mixture of chloroform and methanol (50:1 ~ 1:1) to give
an active fraction, and the active fraction is used as the
15 active ingredient.

【Claim 24】

The pharmaceutical composition according to claim 21,
wherein the disease attributable to the accumulation of
20 excess active oxygen is liver disease, cerebral apoplexy,
myocardial infarction, diabetic vascular disorder,
hyperlipidemia, acute inflammation, rheumatoid disease or
cancer.

【Claim 25】

25 An anti-aging pharmaceutical composition, comprising

a *Clitocybe aurantiaca* extract as an active ingredient, said extract being prepared using water, acetone or a mixture thereof as a solvent.

5 **【Claim 26】**

The anti-aging pharmaceutical composition according to claim 25, wherein the *Clitocybe aurantiaca* extract is further fractioned into ethyl acetate to form an ethyl acetate fraction, and the ethyl acetate fraction is used as
10 the active ingredient.

【Claim 27】

The anti-aging pharmaceutical composition according to claim 26, wherein the ethyl acetate fraction is purified
15 through silica gel column chromatography eluting with a gradient of a mixture of chloroform and methanol (50:1 ~ 1:1) to give an active fraction, and the active fraction is used as the active ingredient.

20 **【Claim 28】**

An anti-aging cosmetic composition, comprising a *Clitocybe aurantiaca* extract as an active ingredient, said extract being prepared using water, acetone or a mixture thereof as a solvent.

25

【Claim 29】

The anti-aging cosmetic composition according to claim 28, wherein the *Clitocybe aurantiaca* extract is further fractioned into ethyl acetate to form an ethyl acetate fraction, and the ethyl acetate fraction is used as the active ingredient.

【Claim 30】

The anti-aging cosmetic composition according to claim 29, wherein the ethyl acetate fraction is purified through silica gel column chromatography eluting with a gradient of a mixture of chloroform and methanol (50:1 ~ 1:1) to give an active fraction, and the active fraction is used as the active ingredient.

【Claim 31】

A method for treatment of a disease attributable to accumulation of excess active oxygen, comprising administering the clitocybin derivative of claim 1 to a patient in need thereof.

【Claim 32】

A method for treatment of a disease attributable to accumulation of excess active oxygen, comprising administering a *Clitocybe aurantiaca* extract to a patient in need thereof, said extract being prepared using water, acetone or a mixture thereof as a solvent.

【Claim 33】

The method according to claim 32, wherein the *Clitocybe aurantiaca* extract is further fractioned into ethyl acetate to form an ethyl acetate fraction, and the ethyl acetate fraction is administered to the patient.

【Claim 34】

Use of the clitocybin derivative of claim 1 in prevention and treatment of a disease attributable to accumulation of excess active oxygen.

【Claim 35】

Use of a *Clitocybe aurantiaca* extract in prevention and treatment of a disease attributable to accumulation of excess active oxygen, said extract being prepared using water, acetone or a mixture thereof as a solvent.

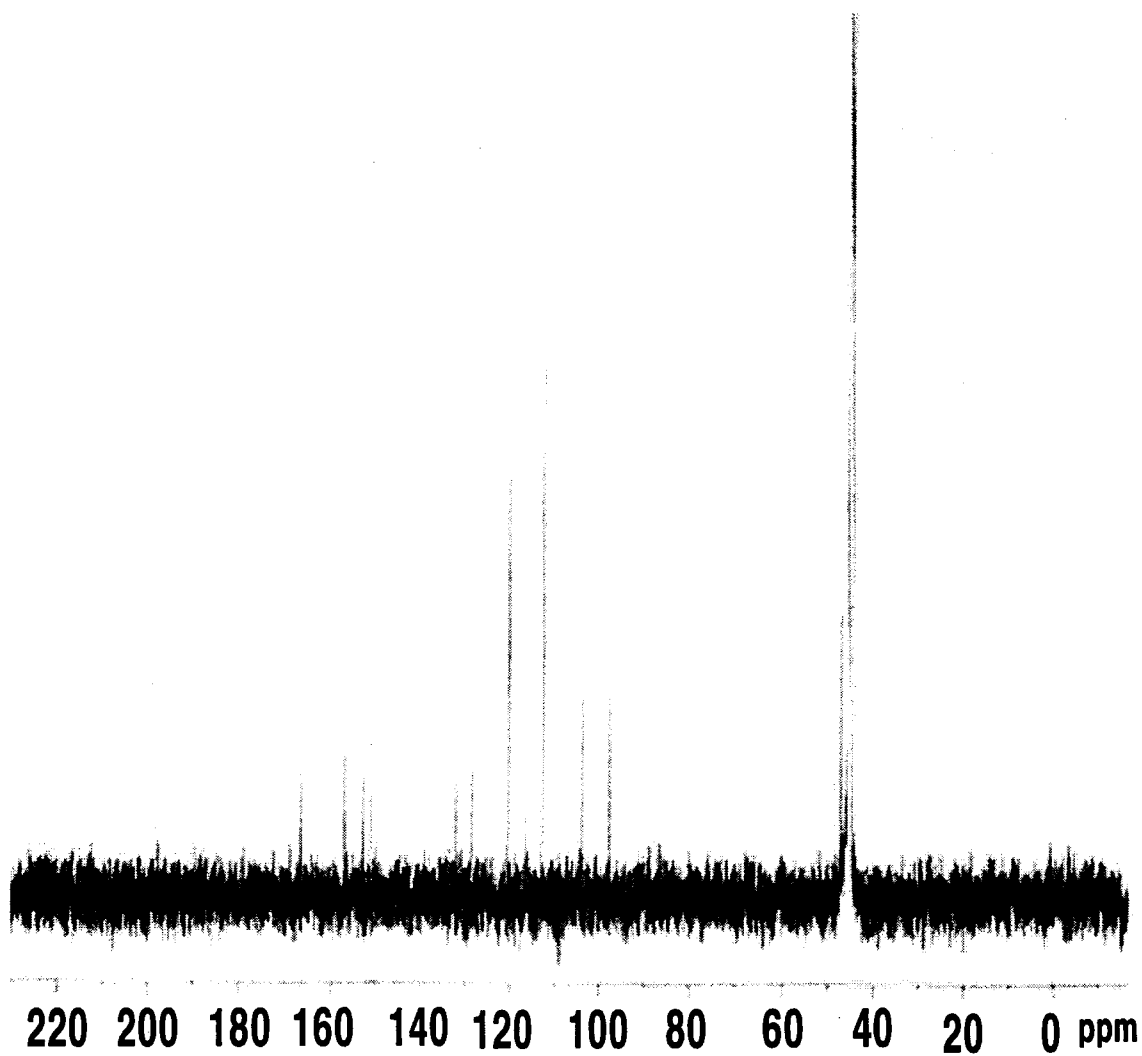
【Claim 36】

Use of an ethyl acetate extract from *Clitocybe aurantiaca* in prevention and treatment of a disease attributable to accumulation of excess active oxygen, said ethyl acetate extract being prepared by fractioning an extract from *Clitocybe aurantiaca* in water, acetone or a mixture thereof into ethyl acetate.

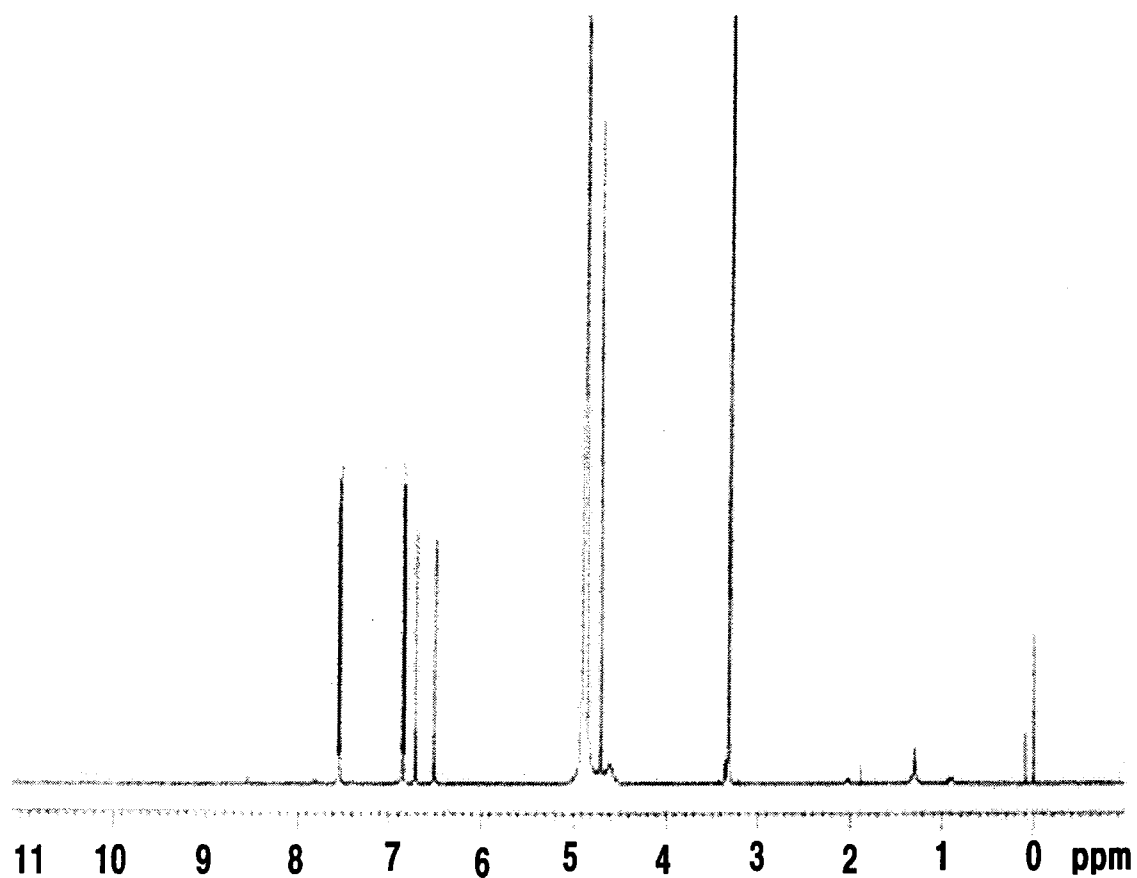
DRAWINGS

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FIGURE 1

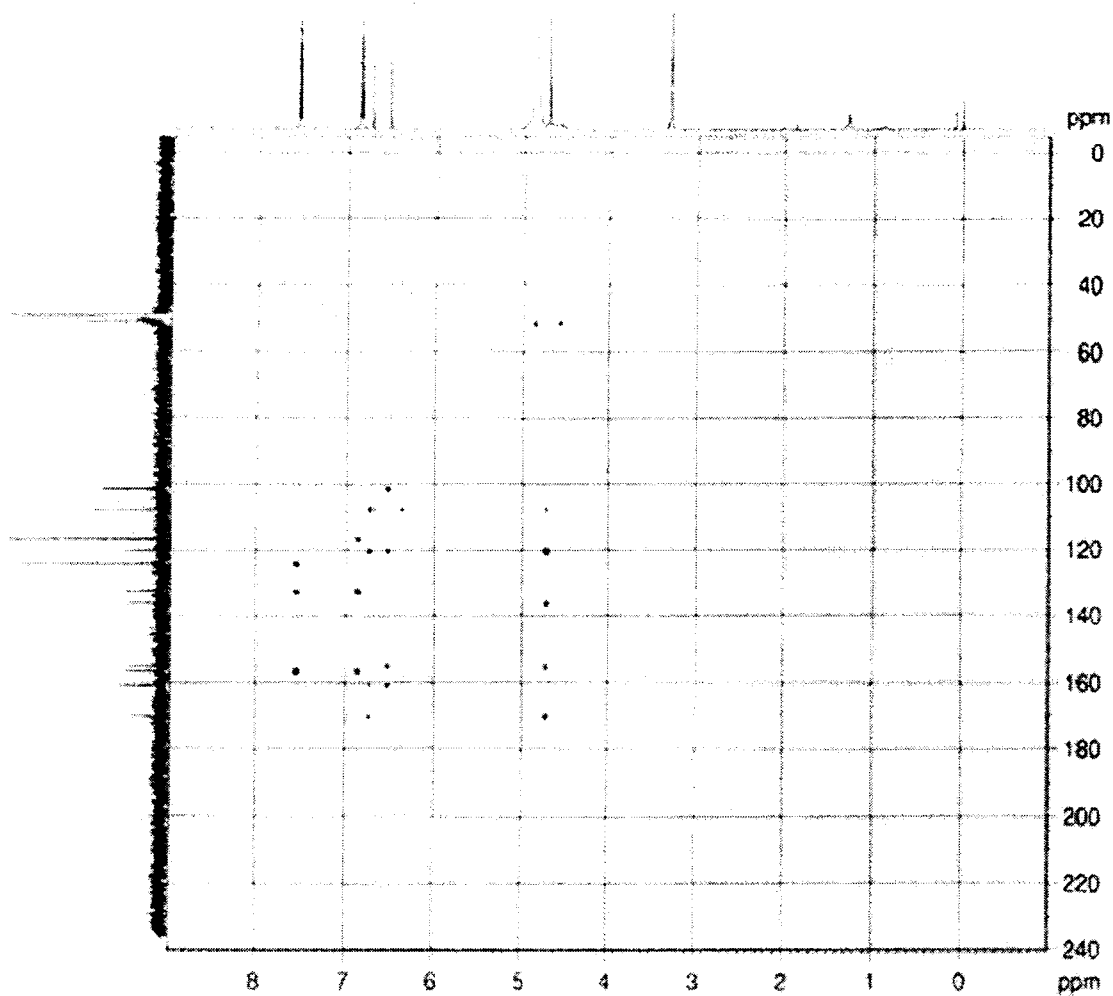


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FIGURE 2



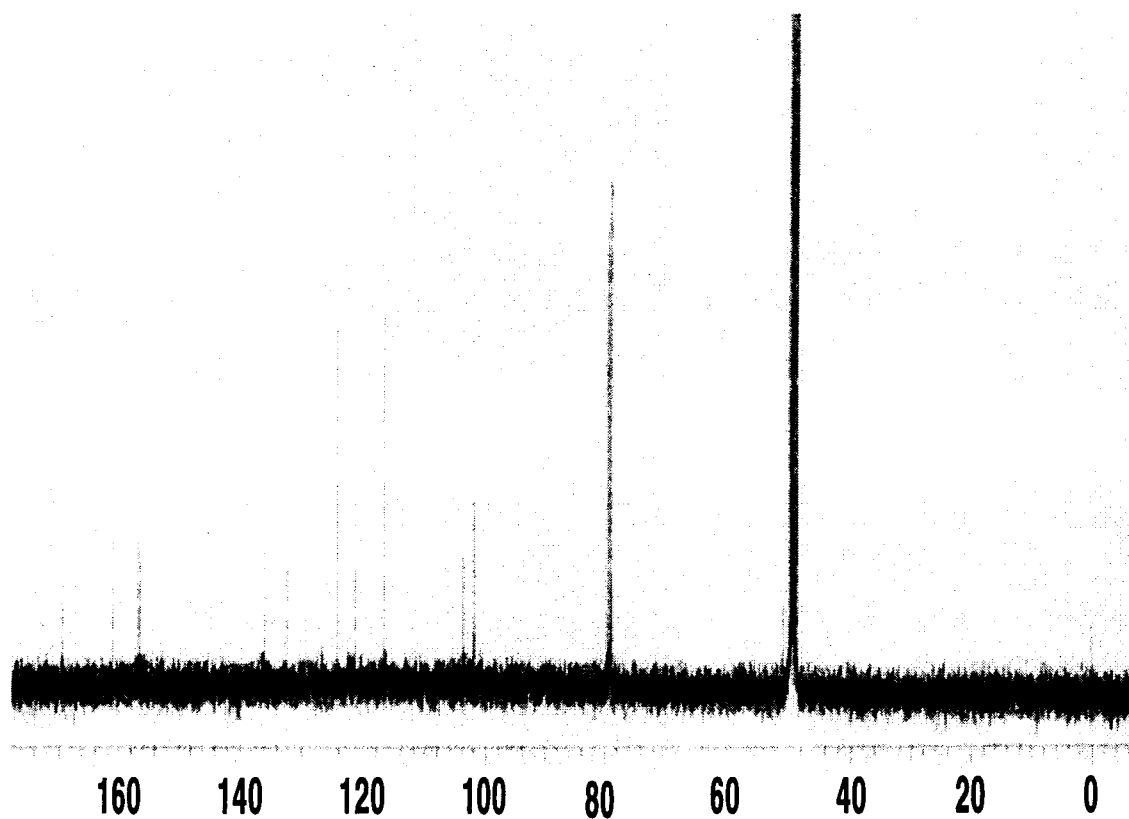
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FIGURE 3

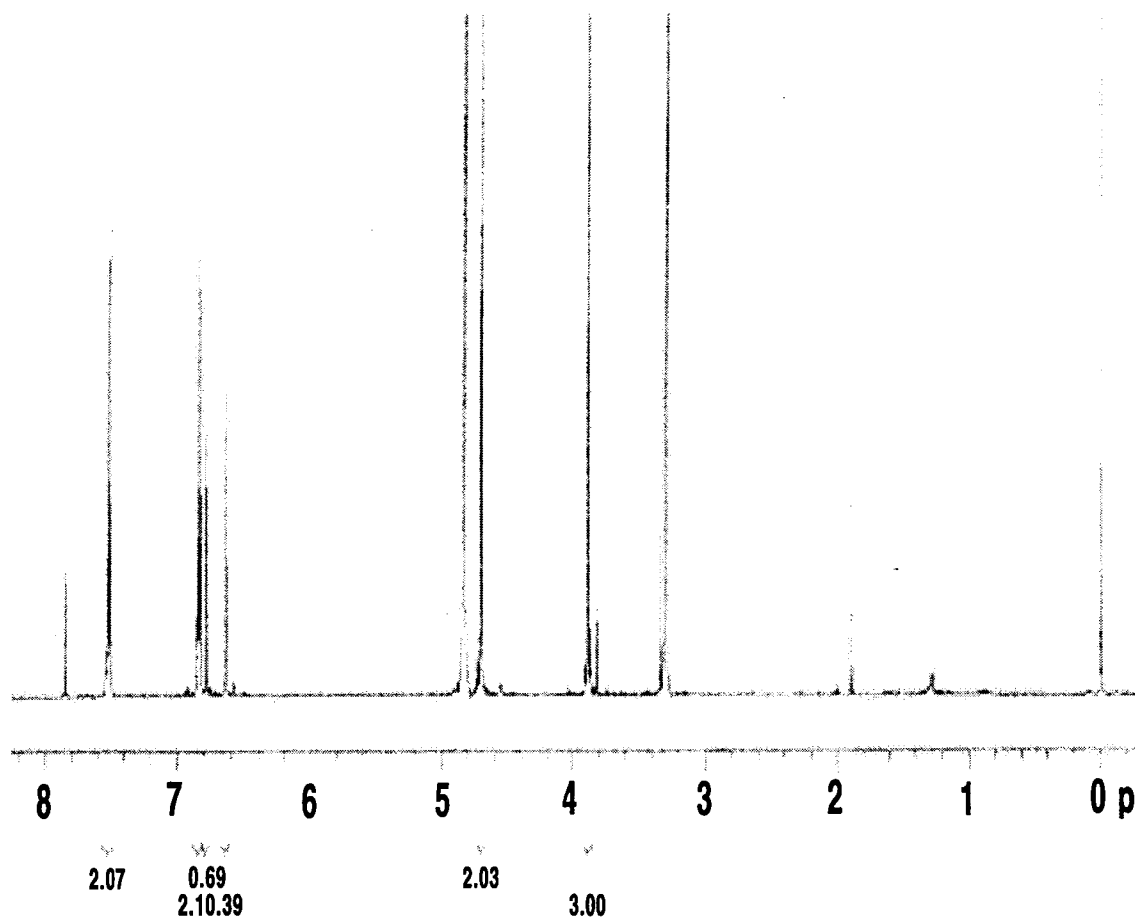


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FIGURE 4

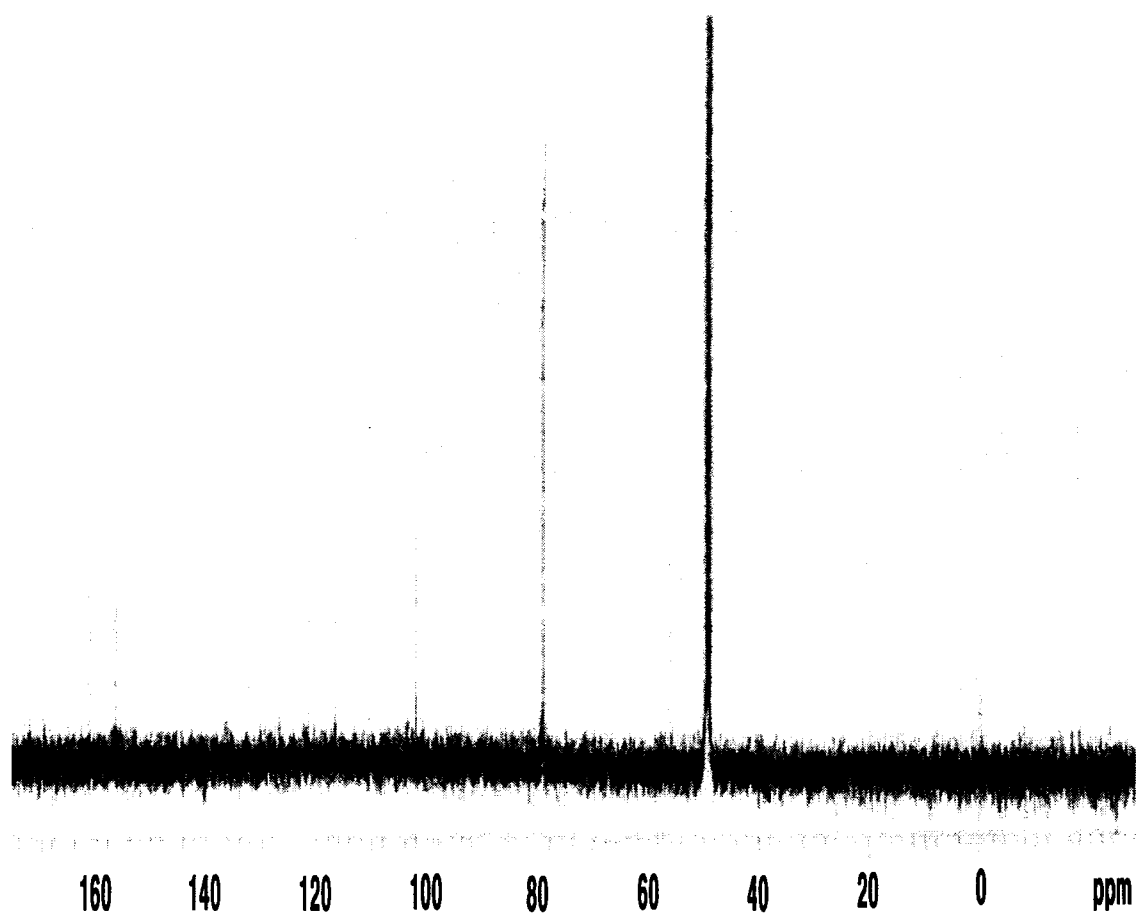


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FIGURE 5

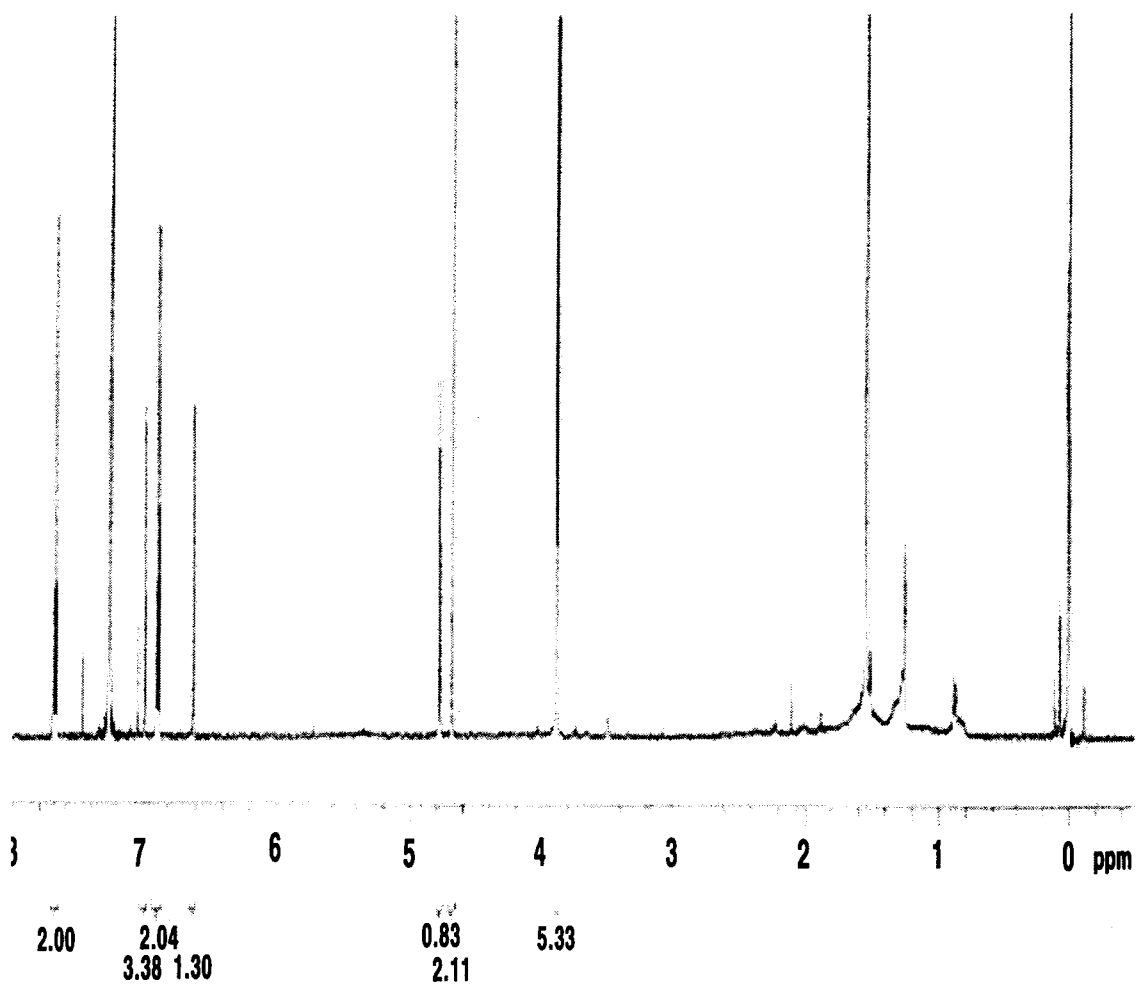


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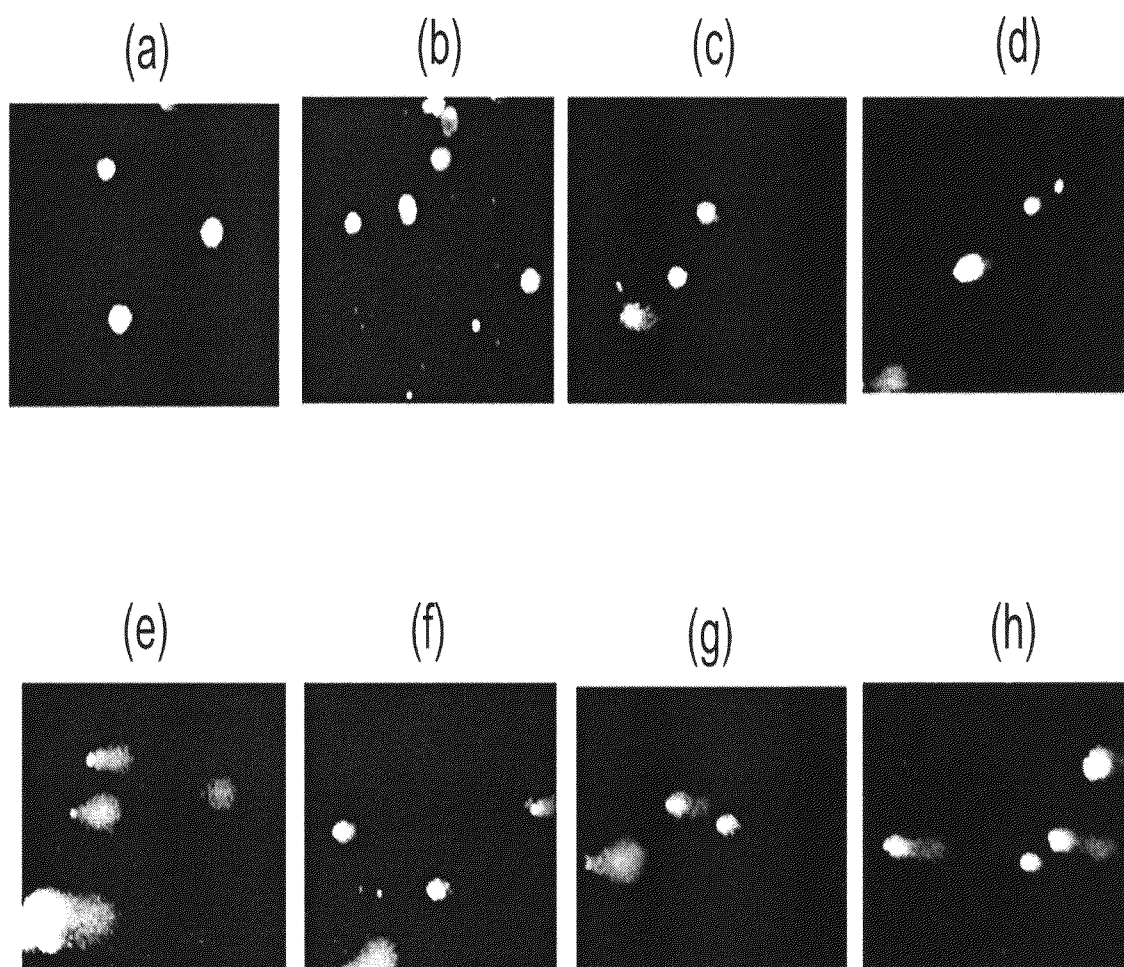
FIGURE 6



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FIGURE 7

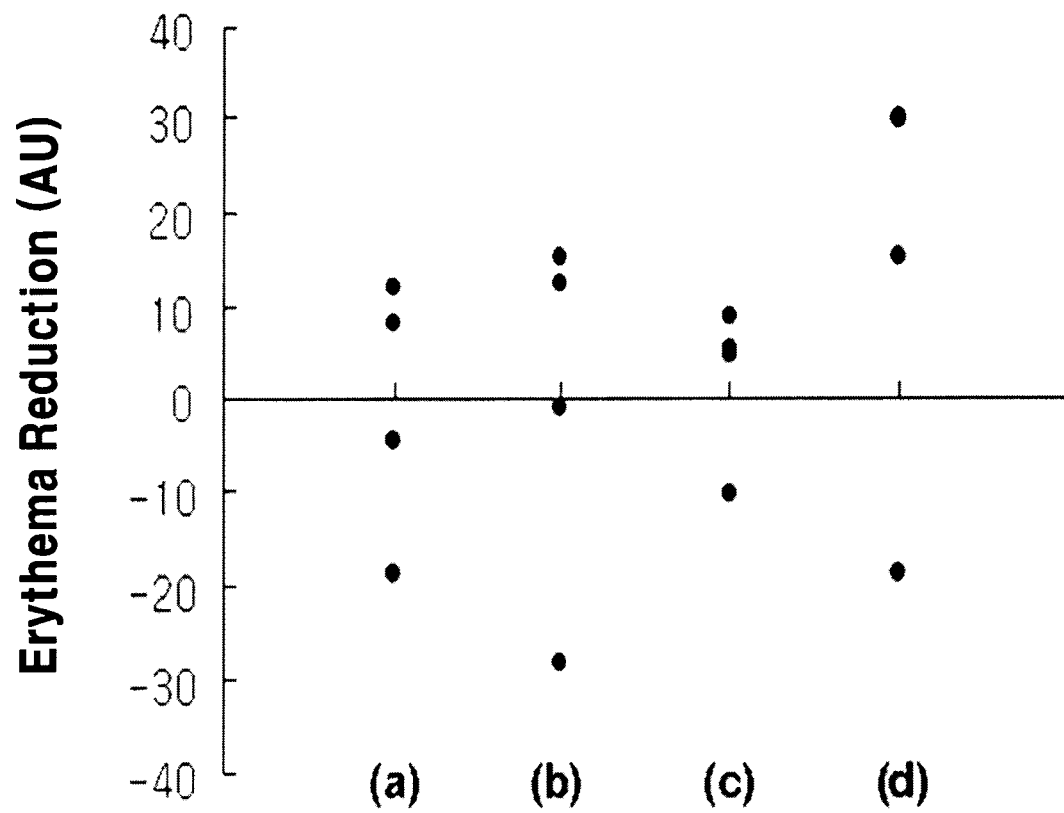


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FIGURE 8



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FIGURE 9



A. CLASSIFICATION OF SUBJECT MATTER*C07D 209/46(2006.01)i, A61K 31/4035(2006.01)i, A61K 8/49(2006.01)i*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 : A61K 31/47

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS(KIPO internal), PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	US 6429212 B1 (Yuichi Hashimoto) 06.08.2002. see column 2, 9, 13-15	1,2,6,7,8 9-14,34 3-5,15-30,35
Y A	US 20070092577 A1 (Micheal B. Zemel. et al) 26.04.2007. see paragraph 54-96 and claim 1	9-14,34 1-8,15-30,35
A	YooJin KIM. Studies on Constituents of the Higher Fungi of Korea(XLI) Antitumor components Extracted from the Carpophores of Clitocybe infundibuliformis. Korean Journal of Pharmacognosy. 1982, Vol.13, No.4, pp179.	1-30,34,35
A	KR 1020050087556 A (Korea Research Institute of Bioscience and Biotechnology) 31.08.2005. see claim 4-9	1-30,34,35
A	KR 1020010008772 A (Korea Research Institute of Bioscience and Biotechnology) 05.02.2001. see claim 3	1-30,34,35



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

29 OCTOBER 2008 (29.10.2008)

Date of mailing of the international search report

29 OCTOBER 2008 (29.10.2008)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-
gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

Na, Young Min

Telephone No. 82-42-481-8394



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2008/000804**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 31-33
because they relate to subject matter not required to be searched by this Authority, namely:
The subject matter of claim 31-33 relate to a method of treatment of the human body. Concerning the assessment of the industrial applicability of the subject matter relating to a treatment or diagnosis method, no unified criteria exist in the PCT. (Rule 43 bis.1(b), Rule 67.1(iv)).
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2008/000804

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6429212 B1	2002.08.06.	JP 10059938 A JP 10072346 A JP 10081666 A JP 10109975 US 6429212 B1 US 6515129 B1 WO 9807421 A1	1998.03.03. 1998.03.17. 1998.03.31. 1998.04.28. 2002.08.06. 2003.02.04. 1998.02.26.
US 20070092577 A1	2007.04.26.	none	
KR 1020050087556 A	2005.08.31.	none	
KR 1020010008772 A	2001.02.05.	none	