COPRINO

Antioxidant, Anti-photoageing, Anti-inflammatory

- **COPRINO POWDER**
  (Powder, Food Grade)
- **COPRINO POWDER C**
  (Powder, Cosmetic Grade)
- **COPRINO EXTRACT-PO.5**
  (Water-soluble powder, Food Grade)
- **COPRINO EXTRACT-PCO.5**
  (Water-soluble powder, Cosmetic Grade)
- **COPRINO EXTRACT-LC**
  (Water-soluble liquid, Cosmetic Grade)

ORYZA OIL&FAT CHEMICAL CO., LTD.

Ver. 1.5 YF

COPRINO

Beauty enhancement foods and cosmetic ingredients

1. Introduction

Coprino, or its scientific name *Coprinus comatus*, is a variety of edible white mushroom. *Coprinus comatus* is widely grown and distributed in temperate regions in Europe and North America starting from spring to autumn. Coprino, as illustrated below, it is white in colour with cylindrical body growing from the ground covered with umbrella-shaped cap. The natural beauty of Coprino does not last long as it will turn black and dissolve itself hours after being picked or depositing spores. In Italy and other European countries, the mushroom *Coprinus comatus* has been regarded and consumed as high quality food ingredient due to limited availability. Meanwhile, its delicious taste is similar to the taste of marshmallow and is believed to taste and blend well with oil.

In recent years, ergothioneine, a naturally-occurring amino acid with strong antioxidant activity has fascinated the attention of the industry. Ergothioneine cannot be synthesized in the human cells but can be acquired from the diet [1)]. It is commonly found in bacterial, plants and animals where it is loaded in bolete and oyster mushroom. Upon absorption, ergothioneine is concentrated in the erythrocytes, lens of eyes and in the skin. The antioxidant properties of ergothioneine has been reported to be stronger than L-cysteine and ascorbic acid. Consequently, it is widely used in topical formulation for cosmetic effect. *In vitro*, ergothioneine has been demonstrated to inhibit tyrosinase and elastase activity while other studies documented the anti-inflammatory and anti-stress effect of ergothioneine.

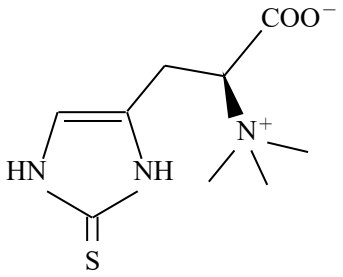

Fig.1 The structure of ergothioneine

Professor Toshihiko Osawa, honourable professor of Nagoya University, Assoc. Professor Yumi Itoh from Hokkaido University of Education and others evaluated the content of ergothioneine in a variety of food. It is clearly shown that Coprino is the richest source of ergothioneine and upon comparison with other fungi, content of ergothioneine is 5x higher in Coprino (Fig. 2).

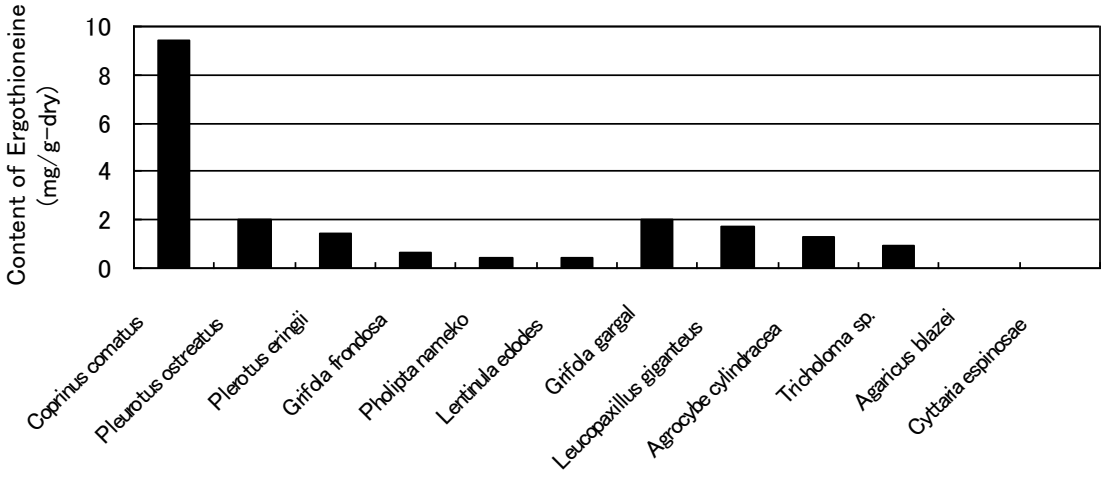

Fig.2 The comparison of ergothioneine content

In addition, Coprino can be regarded as tasty health food due to the presence of glutamic acid (>1%) and guanylic acid. Glutamic acid is the main contributor to the good taste of kelp.

The commercial cultivation of Coprinus comatus is limited by its predisposition to disintegrate into inky mess. Nevertheless, with the joint collaboration and cooperation from The Institute of Healthcare System Inc., Iwade Mycology Research Institute, Oryza Oil & Fat Chemical Co., Ltd., Nagoya University, Hokkaido University of Education and Meijo University have managed to commercialize large-scale cultivation and extraction of *Coprinus comatus*, Coprino with functional effect as an excellent anti-ageing and skin lightening agent.

The raw material of Coprino is grown and cultivated in private facility rich in natural fertilizers for maintenance of consistency and production of safe and excellent quality product. Coprino is produced as new generation functional food and cosmetics ingredients which benefits health.

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2. Physiological Antioxidant

In vitro and cell studies documented that ergothioneine as a superoxide scavenger. K. Obayashi [2] et al., has reported that ergothioneine trapped superoxide radicals in a concentration dependent manner at very low range of concentration. In addition, ergothioneine has been reported to prevent the occurrence of lipid peroxidation as compared to other relatively strong and popular antioxidants such as cysteine.

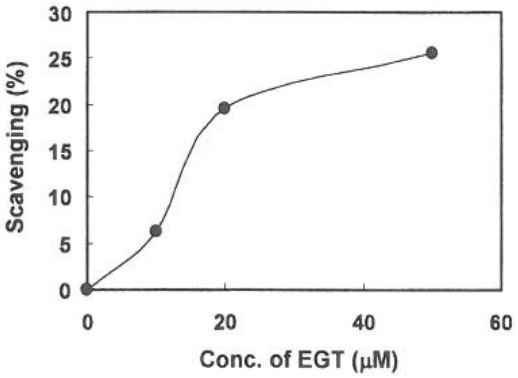


Fig. 3. The scavenging effect of ergothioneine on superoxide radicals.

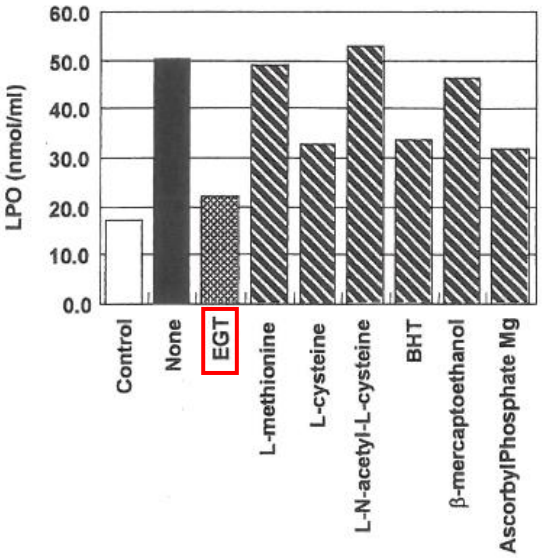


Fig. 4. The effect of various antioxidants on lipid peroxidation.

Furthermore hydrogen peroxide induced oxidative stress increased cells and mitochondrial DNA damage which consequently reduce cells' survival rate. Meanwhile BD Paul [3] et al., reported that addition of ergothioneine significantly revived cells from oxidative damage. In the same studies, it was demonstrated that lipid peroxidation was prevented in cell line treated with ergothioineine. Interference of RNAi depletes

ergothioneine transporter, increasing cells susceptibility to lipid peroxidation clarifying the physiologic cytoprotection of ergothioneine. As a result, this sulphur-containing amino acid, ergothioneine plays an important role as physiologic antioxidant.

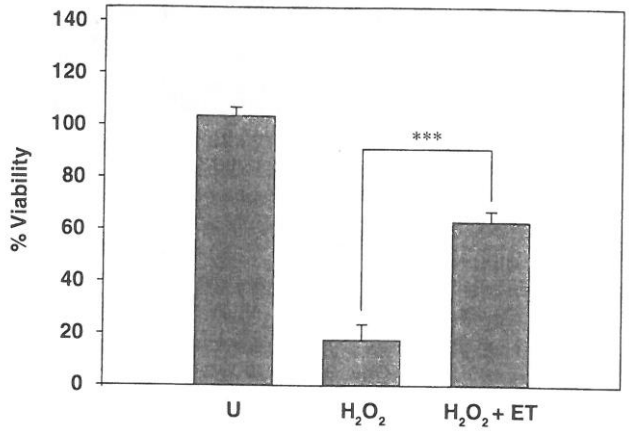


Fig. 5. The effect of ergothioneine on hydrogen peroxide-induced cell damage[3)]

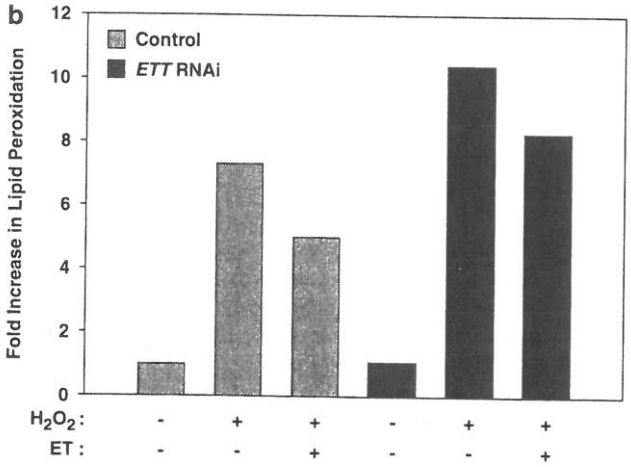


Fig. 6. The effect of ergothioneine on RNAi-mediated cell depletion of ergothioneine and lipid peroxidation.

3. Anti-photo-ageing

Photo-ageing refers to damages of the skin, the epidermis and the dermis, by intense and chronic UV exposure resulting in the appearance of fine lines, wrinkles and age spots.

(1) Anti-photo-ageing *in vitro*

The effect of Coprino on UV-induced photoageing on fibroblasts cells was examined. Normal human diploid fibroblasts (TIG-108, derived from female aged 40 of Japanese

origin) was pre-treated 24 hours prior to the addition of test samples followed by UV irradiation. Photoageing was induced with UV-B irradiation at 755mJ/cm^2, 14.4mW/cm^2. Next, MTT cytotoxic assay was carried out to determine intensity of cell damage while PCR was carried out to evaluate the expression of matrix metalloproteinases (MMP-1) and mRNA expression of TNF-α respectively.

As illustrated in Fig. 7, UV-B irradition decreased cell proliferation, similarly, growth of fibroblasts is inhibited. However, in cells treated with Coprino® Extract and ergothioneine, recovery of cells proliferation is observed with increasing concentration. Particularly, significant recovery of cells proliferation observed in cells treated with Coprino® Extract 300 μg/mL and ergothioneine 100 μg/mL.
Consequently, it is suggestive that Coprino® Extract with ergothioneine is preventive against photoageing.

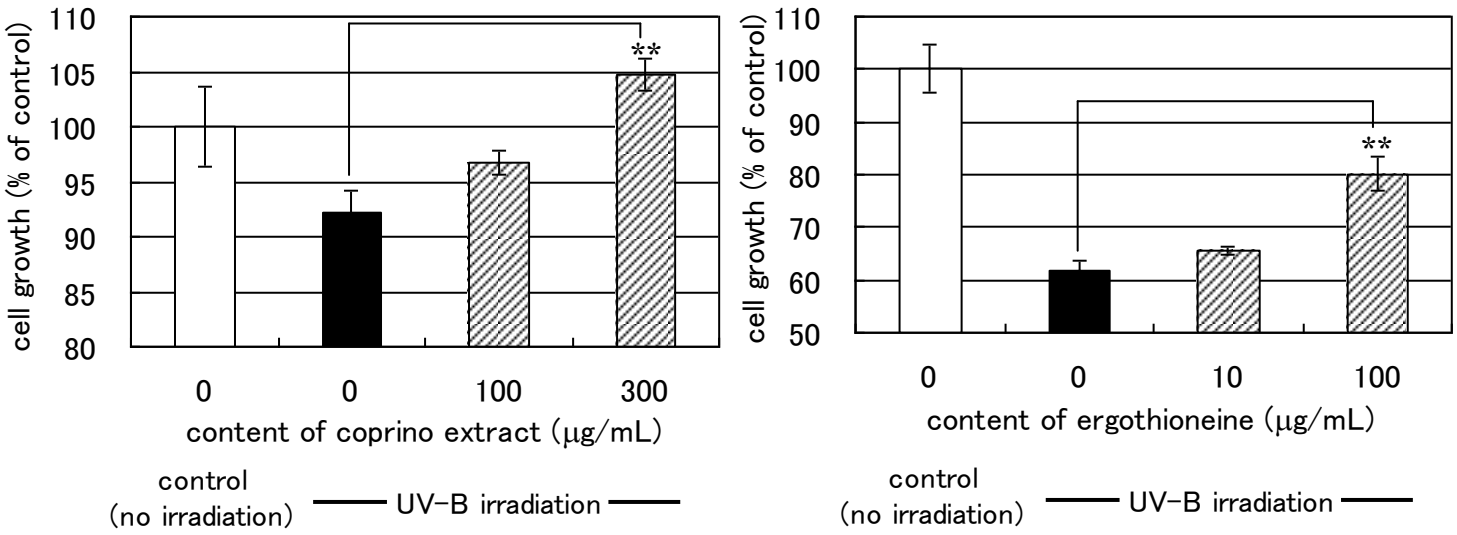
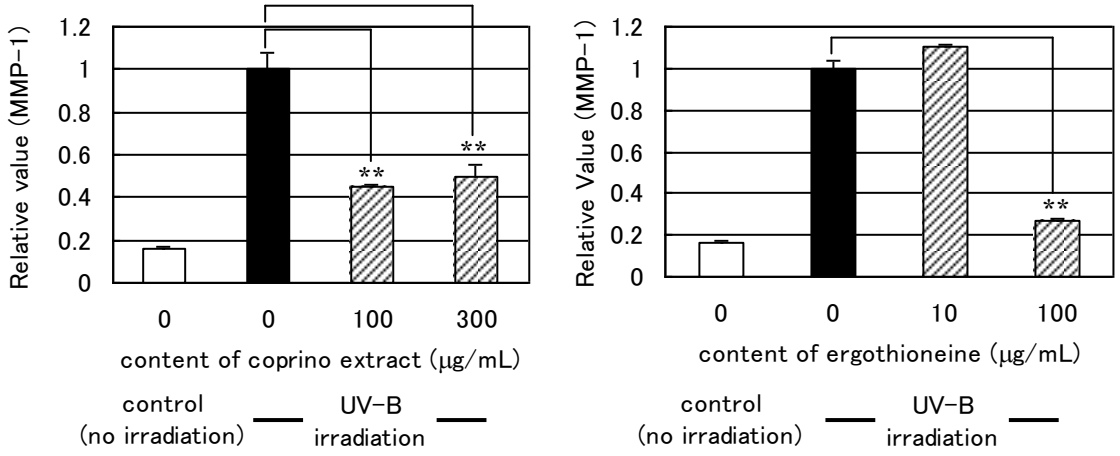


Fig.7 Cell proliferation effect of Coprino® Extract and ergothioneine

In addition, upon irradiation of UV-B, MMP-1(matrix metalloproteinases), the marker of fibroblast collagenase and TNF-α, the inflammatory marker, were activated to initiate the cleavage of collagen and induce inflammation respectively. In the experiment, cells treated with Coprino® Extract and ergothioneine demonstrated a down-regulation on the expression of MMP-1 and TNF-α which once again suggested the prevention of photoageing by Coprino® Extract.

The above findings confirm the protective effect of Coprino® Extract against UV-induced photoageing, preventing the breakdown of collagen and inflammation of the skin.



Relative value : β-actin as correction factor for gene expression
** : $p < 0.01$, N=4, average±S.E.

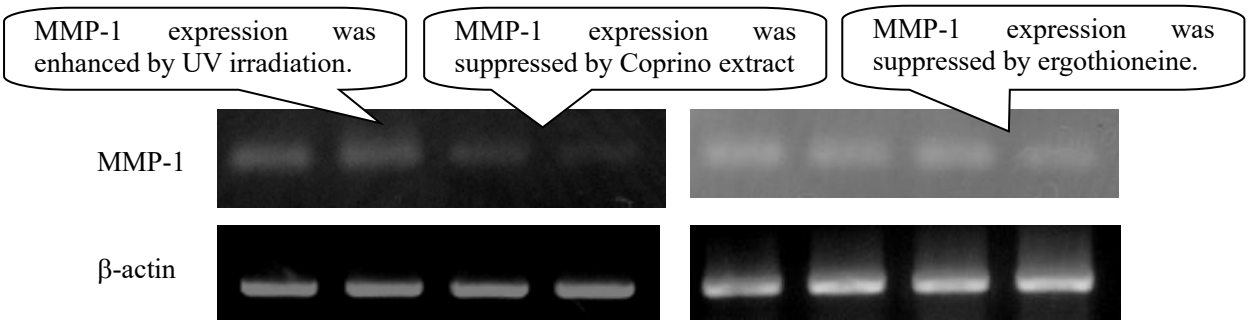
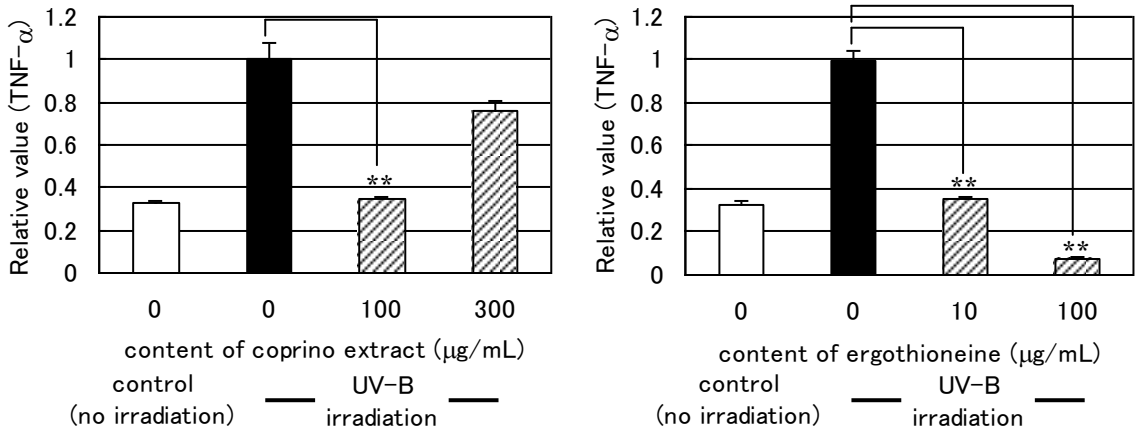


Fig. 8.The effect of Coprino® Extract and ergothioneine on MMP-1 mRNA expression



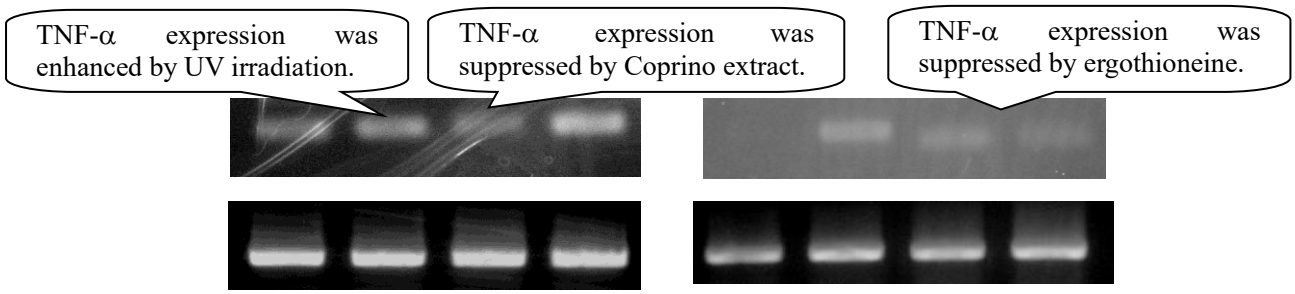


Fig. 9. The effect of Coprino® Extract and ergothioneine on TNF-α mRNA expression

(2) Anti-photo-ageing effect (*in vivo*)

Coprino® Extract (ergothioneine content 1%) was given to hairless mice to evaluate whether Coprino® Extract suppress the wrinkle formation caused by UV irradiation.

[Methods]

Hairless mise (Hos; HR-1, ♂ ,6 weeks old) were preliminary bred for 3 days. Coprino® Extract (10, 100 and 200 mg/kg) were orally administration to mice for 90 days. UVB (120 mJ/cm^2) was irradiated to the back median line using solar simulator within 3hours after oral administration. The day 91, skin replica and specimen of irradiation area were collected. Wrinkle area/25 mm^2 of skin replica was measured using software (NIH image, Adobe Photoshop 7.0,WinRoof6.1 and lumen formation analysis software). The mRNA of skin specimen was extracted and applied for RT-PCR to determine the mRNA expression levels of each enzyme. Furthermore, immunostaining was performed to evaluate the expression of hyarulonan-binding protein in skin.

[Results]

Measurement of skin replica revealed that Coprino® Extract tended to change the skin surface smoothly (Fig. 10). As a result of image analysis, Coprino® Extract decreased the wrinkle area and its ratio (Table 1).

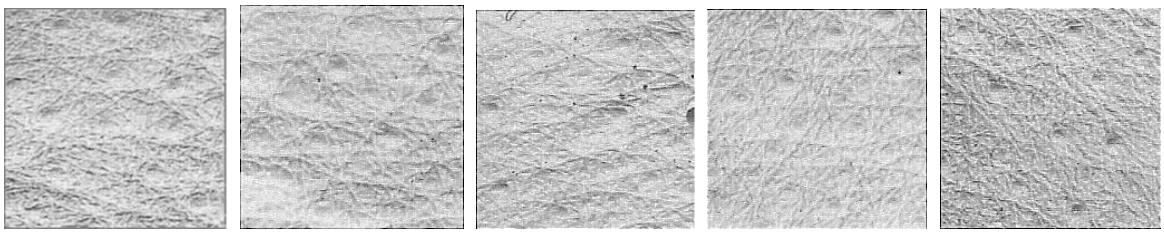


Figure 10 mouse skin replica image

Table 1. Analysis value of skin replica of UV-irradiated mice administered Coprino

	dose (mg/kg)	UV-B	wrinkle area [a)] (mm^2)	Ratio of wrinkle area [a)] (%)
Normal	-	-	4.42±0.37	17.7±1.5
Control	-	+	**5.04±0.41**	**20.2±1.6**
Coprino®Extract	10	+	**4.39±0.74**	**17.6±3.0**
	100	+	**4.00±0.52**	**16.0±2.1**
	200	+	**3.89±0.62**	**15.5±2.5**

Mean±SE (n=5), [a)] NIH image

Oral administration of Coprino® Extract increased the expression of hyaluronan synthase (HAS2, HAS3) in the skin of UV-irradiated mice.(Table 2).

Table 2. Expression of skin mRNA in UV-irradiated mice treated with Coprino®Extract

	dose (mg/kg)	UV-B	Hyaluronan synthase	
			HAS2	HAS3
Normal	-	-	4.84±1.83	4.76±2.14
Control	-	+	1.00±0.31	1.00±0.39
Coprino®Extract	10	+	6.77±6.23	5.05±5.03
	100	+	6.66±6.62	—
	200	+	6.36±3.35	5.53±4.28

Mean±SE (n=5)

As a result of immunostaining, Coprino® Extract increased hyaluronan-binding protein in mouse skin (Fig. 11). Hyaluronan-binding protein (brown staining site) in the upper dermis of mouse skin was greatly reduced by UV irradiation (Fig. 11a, b red line). Administration of Coprino® Extract increased hyaluronan-binding protein (brown staining site) and recovered to the same or more level as normal after UV irradiation.

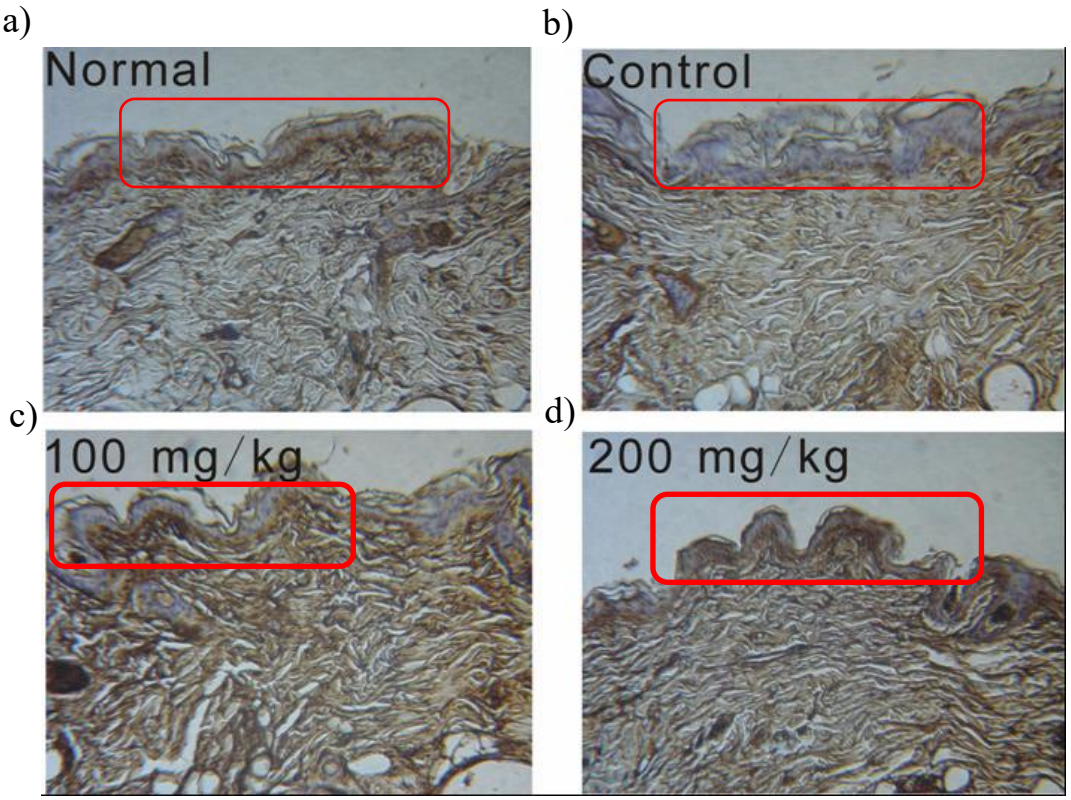


Figure 11 Immunostaining image of mouse hyaluronan-binding protein
a) Normal : No UV irradiation group, b) Control: UV irradiation group,
c), d) 100 (200) mg/kg: UV irradiation + Coprino® Extract administration groups
The hyaluronic acid-binding protein (brown staining area) in the upper dermis of mouse skin

4. Skin Whitening

Skin pigmentation is a result of excessive production of melanin leaving skin with freckles and spots. The synthesis of melanin is catalyzed by the enzyme tyrosinase, hence inhibition of tyrosinase is believed to prevent skin pigmentation.
In vitro experiments showed that Coprino® Extract inhibit melanin synthesis and tyrosinase activity respective. Therefore, it is suggestive that Coprino® Extract posses skin whitening properties.

(1) Inhibition of Melanin Production

In collaboration with Meijo University, the effect of Coprino® Extract on melanin synthesis was examined *in vitro* using B16 melanoma cells. Results revealed that melanin synthesis was significantly suppressed in samples treated with Coprino® Extract.

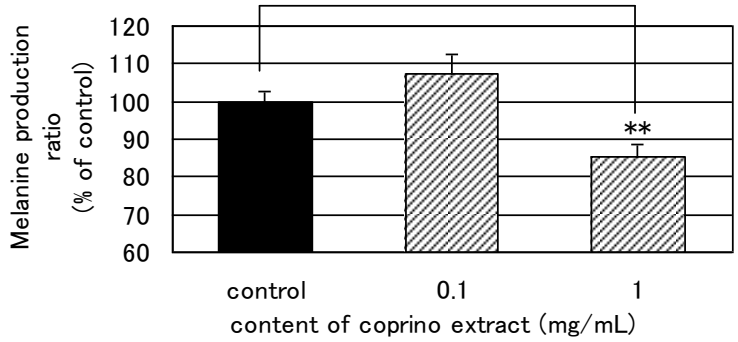


Fig. 10. The Effect of Coprino® Extract on the inhibition of melanin synthesis

(2) Inhibition of Tyrosinase Activity

Further experiment was prompted to investigate the skin whitening effect of Coprino. *In vitro* experiment showed that Coprino® Extract and its bioactive component, ergothioneine, demonstrated concentration-dependent inhibition on tyrosinase activity.

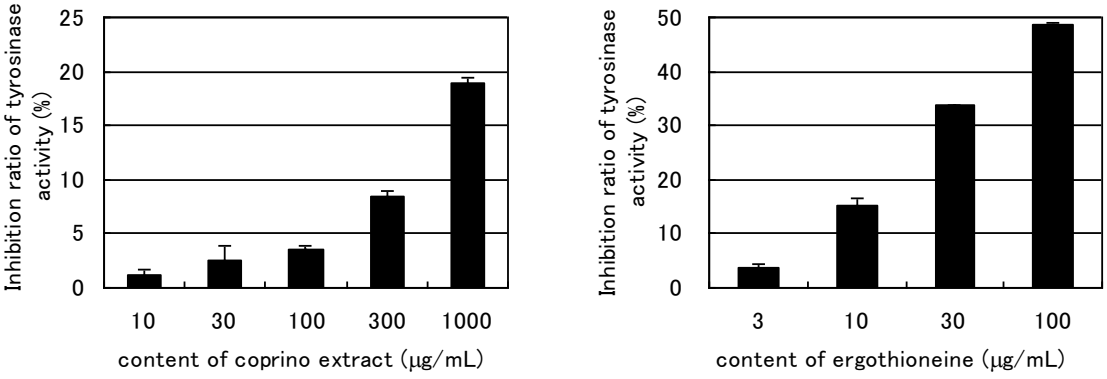


Fig. 11. The effect of Coprino® Extract and ergothioneine on the inhibition of tyrosinase activity.

5. Anti-inflammatory Effect

Further studies conducted with Nagoya University and Hokkaido University of Education revealed the anti-inflammatory effect of Coprino® Extract in mouse adipocytes (3T3-L1) using interleukin-6 (IL-6) as an inflammatory marker.

Addition of TNF-α after differentiation of mouse adipose precursor cells markedly increased the IL-6 concentration in the medium. The addition of Coprino® Extract significantly reduced the IL-6 concentration in the medium. This result suggests that Coprino® Extract has an inhibitory effect on IL-6 production.

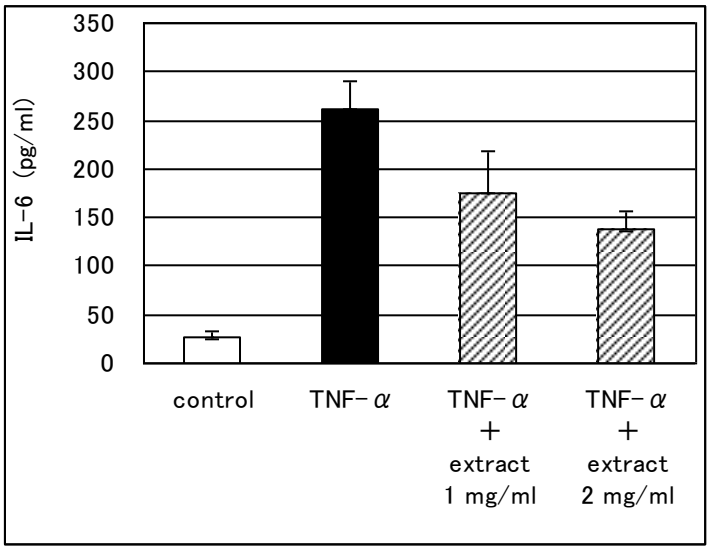


Fig. 12. The effect of Coprino® Extract on the inhibition of IL-6 production

Recently, it have been reported that Coprino inhibits UVB-induced inflammatory responses and DNA halogenation by Asahi et al[4]. In addition, Coprino and ergothioneine (main ingredient) showed anti-inflammatory effect by inhibiting myeloperoxidase which promotes DNA halogenation.[4]

6. Reference Journals

1) Ey J *et. al.*, *J. Agric. Food Chem.*, **55**, 6466-6474, 2007
2) Kei Obayashi *et. al.*, *J. Cosmet. Sci.*, **56**, 17-27, 2005
3) BD Paul and SH Snyder, *Cell Death Differ.*, **17**, 1134-1140, 2010
4) Asahi T. et al., Biosci Biotechnol Biochem. **80**, 313-7, 2016.

7. Stability Profile

(1) Heat Stability

As illustrated in Fig. 13, there is no significant decrease in the content of ergothioneine in Coprino powder and Coprino® Extract at normal temperature for sterilization.

Therefore, Coprino powder and Coprino® Extract are ingredients of high stability at normal food processing temperature.

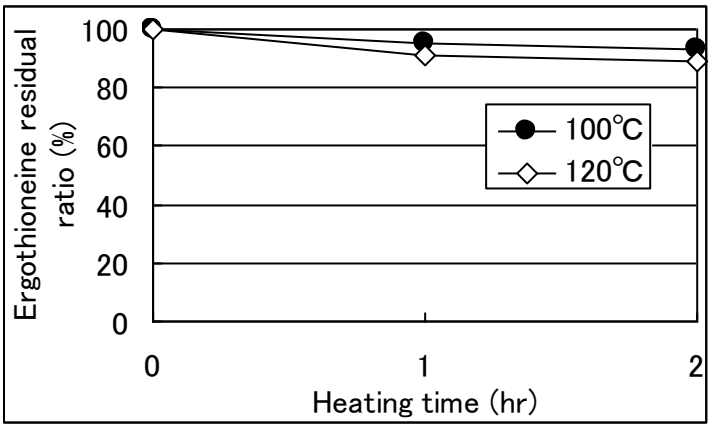


Fig. 13. The heat stability of Coprino® Extract

(2) pH Stability

As illustrated in Fig. 14, the content of ergothioneine in Coprino® Extract solution remained stable at pH ranges from 3 to 9 while significant reduction is observed at pH 10.

Therefore, Coprino Powder and Coprino® Extract are recommended to be used at stable pH zone between pH 3 to pH 9.

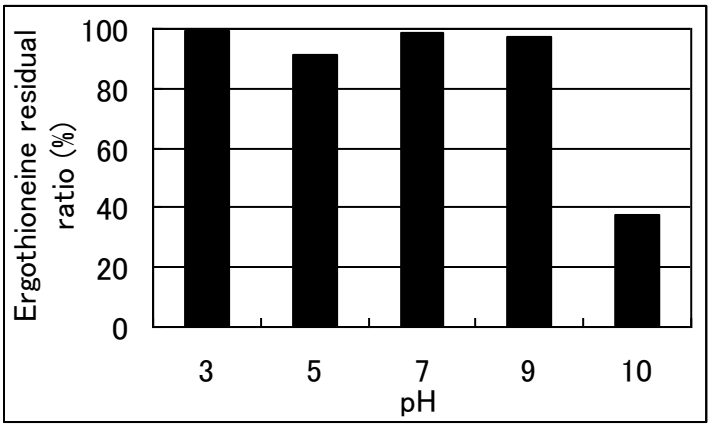


Fig. 14 The pH stability of Coprino® Extract solution

8. Nutritional Profile

Description	Amount per 100g		Analytical Method
	Coprino Powder	Coprino® Extract-P0.5	
Energy	177 kcal	347.6 kcal	Atwater Method (Revised) *1
Protein	23.3 g	15.3 g	Combustion Method *2
Fat	2.7 g	0.2 g	Acid degradation
Carbohydrate	41.3 g	70.6 g	100 g – (water + protein + fat + ash) *3
Natrium	— mg	64 mg	Atomic absorption spectrophotometory
Sodium	— g	0.1 g	Sodium Equiv. value
Moisture	5.7 g	3.2 g	Heat-drying at atmospheric pressure
Ash	9.0 g	10.7 g	Direct Incineration
Dietary fiber	18.0 g	1.1 g	Prosky Method

*1 Energy expression standard (Ministry of Health and Welfare's announcement No. 176)
Conversion factor: Protein 4, fat 9, sugar 4; dietary fiber 2
*2 Nitrogen, protein conversion factor: 6.25
*3 Carbohydrate expression standard (Ministry of Health and Welfare's announcement No. 176)

Coprino Powder
Test trustee Japan Food Research Laboratories / Date of analysis: December 25, 2009
Test No.: 09029156001-01

Coprino® Extract-P0.5
Test trustee: SRL, Inc / Date of analysis: September 21, 2010
Test No.:201009070021

9. Safety Profile

(1) Residual Agricultural Chemicals

Agricultural chemical inspection was conducted on Coprino as an requirement for importation of mushroom. Results showed that all tested items below the allowed reference range.

(2) Acute Toxicity (LD_{50})

Acute Toxicity test was conducted according to the Guidelines for Single-Dose Toxicity Tests for Pharmaceutical Products where Coprino® Extract 2000 mg/kg was orally given to starved mice (male & female ddy, 5 weeks old, weight ~30 g) for 14 days. No abnormalities and fatal event observed at 2000 mg/kg. No abnormalities of organs observed under macroscopic examination upon autopsy. Thus, LD_{50} of Coprino® Extract is deduced to be >2000 mg/kg.

(3)Mutagenicity

Ames test was conducted to evaluate the mutagenicity of Coprino® Extract (without binder) using *Salmonella typhimurium* TA98 and TA100 in S9mix. As a result, no

mutagenicity observed at concentration range of 19.5 – 5000 μg/plate.

10. Recommended Daily Dosage

Product Description	Functional Effect	Recommended dosage
Coprino Powder	Antioxidant Anti-photoageing Skin Whitening Anti-inflammatory	20-50 mg/day
Coprino® Extract –P0.5		10-30 mg/day

11. Applications

	Applications	Claims	Examples
Foods	Nutritional Food Diet Food Beauty Food	1) Antioxidant 2) Anti-photoageing 3) Skin Whitening 4) Anti-inflammatory	Beverages (soft drinks etc), hard and soft capsules, tablets, candies, chewing gum, cookies, chocolate wafers, jelly, *etc*.
Cosmetics	Beauty		Sunscreen, toner, lotion, body gel, shampoo, conditioner and bath salts, *etc*.

12. Packaging

Product Description	Packaging	Weight
Coprino Powder (powder, Food grade)	Interior Packaging: Aluminium bag Exterior Packaging: Cardboard	1kg 5kg
Coprino® Extract-P0.5 (Water soluble powder, food grade)		
Coprino Powder –C (powder, cosmetics grade) Coprino® Extract-PC0.5 (water soluble powder, cosmetics grade)	Interior Packaging: Aluminium bag Exterior Packaging: Cardboard	1kg 5kg
Coprino® Extract-LC (water soluble liquid, cosmetics grade)	Interior Packaging: Cubic polyethylene container Exterior Packaging: Cardboard	1kg

13. Storage

It is recommended to store in a cool, dry, dark place and avoid heat and humidity.
In particular, dessicant bag is recommended for storage of Coprino® Extract-P0.5 & -PC0.5 as once open it is highly hygroscopic.

14. Expression

＜Food＞

Coprino Powder
Expression：dried mushroom powder

Coprino® Extract-P0.5
Expression: dried processed mushroom powder

＜Cosmetic＞

Coprino Powder –C
INCI: Coprinus Comatus (Mushroom) Powder

Coprino® Extract-PC0.5
INCI: Coprinus Comatus (Mushroom) Extract (and) Dextrin

Coprino® Extract-LC
INCI: Water (and) Butylene Glycol (and) Coprinus Comatus (Mushroom) Extract

PRODUCT STANDARD
PRODUCT NAME

COPRINO POWDER

FOOD

This product is powder of dried *Coprinus Comatus*.
It guarantees minimum of 0.3 % ergothioneine.

1. Appearance	Brown powder. Light unique smell.	
2. Ergothioneine	Min. 0.3 %	(HPLC)
3. Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1g, 105℃, 2h)
4. Purity Test		
(1)Heavy Metals (as Pb)	Max. 20 ppm	(Sodium Sulfide Colorimetric Method)
(2)Arsenic (as As_2O_3)	Max. 2 ppm	(Standard Methods of Analysis in Food Safety Regulation, The Third Method, Apparatus B)
5. Standard Plate Counts	Max. 3×10^3 cfu/g	(Analysis for Hygienic Chemists)
6. Moulds and Yeasts	Max. 1×10^2 cfu/g	(Analysis for Hygienic Chemists)
7. Coliforms	Negative	(Analysis for Hygienic Chemists)

8. Composition

Ingredient	Content
Dry *Coprinus Comatus*	100 %

PRODUCT STANDARD
PRODUCT NAME

COPRINO® EXTRACT-P0.5

FOOD

This product is extracted from *Coprinus Comatus* with water.
It guarantees minimum of 0.5 % ergothioneine. This product is water-soluble.

1. Appearance	Light yellow or light brown powder. Light unique smell.	
2. Ergothioneine	Min. 0.5 %	(HPLC)
3. Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1g, 105℃, 2h)
4. Purity Test		
(1)Heavy Metals (as Pb)	Max. 20 ppm	(Sodium Sulfide Colorimetric Method)
(2)Arsenic (as As_2O_3)	Max. 2 ppm	(Standard Methods of Analysis in Food Safety Regulation, The Third Method, Apparatus B)
5. Standard Plate Counts	Max. 3 $\times 10^3$ cfu/g	(Analysis for Hygienic Chemists)
6. Moulds and Yeasts	Max. 1 $\times 10^2$ cfu/g	(Analysis for Hygienic Chemists)
7. Coliforms	Negative	(Analysis for Hygienic Chemists)

8. Composition

Ingredient	Content
Coprinus Comatus Extarct	70 %
Dextrin	30 %

PRODUCT STANDARD
PRODUCT NAME

COPRINO POWDER C

COSMETIC

This product is powder of dried *Coprinus Comatus*.
It guarantees minimum of 0.3 % ergothioneine.

1. Appearance	Brown powder. Light unique smell.	
2. Ergothioneine	Min. 0.3 %	(HPLC)
3. Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1g, 105℃, 2h)
4. Purity Test		
(1)Heavy Metals	Max. 20 ppm	(The Second method of The Japanese Standards of Quasi-Drug Ingredients)
(2)Arsenic	Max. 2 ppm	(The Third method of The Japanese Standards of Quasi-Drug Ingredients)
5. Standard Plate Counts	Max. 1×10^2 cfu/g	(Analysis for Hygienic Chemists)
6. Moulds and Yeasts	Max. 1×10^2 cfu/g	(Analysis for Hygienic Chemists)
7. Coliforms	Negative	(Analysis for Hygienic Chemists)

8. Composition

Ingredient	Content
Coprinus Comatus (Mushroom) Powder	100 %

PRODUCT STANDARD
PRODUCT NAME

COPRINO® EXTRACT-PC0.5

COSMETIC

This product is extracted from *Coprinus Comatus* with water.
It guarantees minimum of 0.5 % ergothioneine. This product is water-soluble.

1. Appearance	Light yellow or light brown powder. Light unique smell.	
2. Ergothioneine	Min. 0.5 %	(HPLC)
3. Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1g, 105℃, 2h)
4. Purity Test		
(1)Heavy Metals	Max. 20 ppm	(The Second method of The Japanese Standards of Quasi-Drug Ingredients)
(2)Arsenic	Max. 2 ppm	(The Third method of The Japanese Standards of Quasi-Drug Ingredients)
5. Standard Plate Counts	Max. 1 $\times 10^2$ cfu/g	(Analysis for Hygienic Chemists)
6. Moulds and Yeasts	Max. 1 $\times 10^2$ cfu/g	(Analysis for Hygienic Chemists)
7. Coliforms	Negative	(Analysis for Hygienic Chemists)

8. Composition

Ingredient	Content
Coprinus Comatus (Mushroom) Extarct	70 %
Dextrin	30 %

PRODUCT STANDARD
PRODUCT NAME

COPRINO® EXTRACT-LC

COSMETIC

This product is extracted from *Coprinus Comatus* with water and is dissolved in aqueous 1,3-butyleneglycol.

1. Appearance — Light brown or brown liquid. Light unique smell.

2. Certification Test
Polyphenols — Dissolve 30 μl of this product in 3.5 ml water. Add 0.2 ml Folin-Denis reagent into the solution followed by 0.4 ml saturated Na_2CO_3.
The solution will turn into blue color.

3. Purity Test

(1)Heavy Metals (as Pb)	Max. 20 ppm	(The Second method of The Japanese Standards of Quasi-Drug Ingredients)
(2)Arsenic (as As_2O_3)	Max. 2 ppm	(The Third method of The Japanese Standards of Quasi-Drug Ingredients)

5. Standard Plate Counts — Max. 1×10^2 cfu/g (Analysis for Hygienic Chemists)

6. Moulds and Yeasts — Max. 1×10^2 cfu/g (Analysis for Hygienic Chemists)

7. Coliforms — Negative (Analysis for Hygienic Chemists)

8. Composition

Ingredient	Content
Water	69 %
Butylene Glycol	30 %
Coprinus Comatus (Mushroom) Extarct	1 %

ORYZA OIL & FAT CHEMICAL CO., LTD. striving for the development of the new functional food materials to promote health and general well-being.

From product planning to OEM - For any additional information or assistance, please contact：

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