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# Melanogenesis Inhibitory Effects of Methanolic Extracts of *Umbilicaria esculenta* and *Usnea longissima*

**Moo-Sung Kim[1] and Hong-Bum Cho[2]***

[1]*R&D Center, Macrocare Tech., Ltd, Ochang 363-883, Republic of Korea*
[2]*Department of Biological Engineering, Seokyeong University, Seoul 136-704, Republic of Korea*

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**The primary objective of this study was to assess the *in vitro* melanogenesis inhibitory effects of methanolic extracts of the edible and medicinal lichens, *Umbilicaria (Gyrophora) esculenta* and *Usnea longissima*. The quantities of the total phenolic compounds of methanolic extract of the two lichen extracts were determined to be 1.46% and 2.62%, respectively. In order to evaluate the antioxidative effects of the extracts, we also measured electron donating abilities (EDA) and lipid peroxidation rates. The EDA values measured by the reduction of 1.1'-diphenyl-2-picrylhydrazyl (DPPH) were 72.8% and 80.7% for the extracts, with $SC_{50}$ (median scavenging concentration) values of 1.29±0.05 mg/ml and 1.03±0.06 mg/ml, respectively. The rates of inhibition of lipid peroxidation using linoleic acid were 92.1% and 97.3% for the extracts, with $IC_{50}$ (median inhibitory concentration) values of 0.57±0.05 mg/ml and 0.53±0.06 mg/ml, respectively. The inhibitory rates of the extracts against tyrosinase were 67.4% and 84.8%, respectively. The extracts were shown to reduce melanin formation in human melanoma cells. Melanin contents in the samples treated with 0.01% and 0.1% *U. esculenta* were 47.1% and 31.2%, respectively, and those treated with 0.01% and 0.1% *Usnea longissima* were 51.1% and 34.9%, respectively, whereas a value of 54.0% was registered when ascorbic acid was utilized as a positive control. In addition to direct tyrosinase inhibition, it was determined that the lichen extracts affected the activity of tyrosinase via the inhibition of tyrosinase glycosylation. As a result, the methanolic extracts of *U. esculenta* and *Usnea longissima* evidenced melanogenesis inhibitory effects, which occurred via multiple routes.**

***Keywords***: anti-oxidation, tyrosinase, glycosylation, melanogenesis inhibition, *Umbilicaria (Gyrophora) esculenta*, *Usnea longissima*

Lichens are symbiotic microorganisms that consist of algae and fungi. They have been demonstrated to evidence a number of secondary metabolites, which may protect them against physical stresses or biological attacks during their slow growth (Crittenden and Porter, 1991; Herbert, 1992; Kahng *et al.*, 2004). Some lichen species or their compounds are utilized extensively for medicinal and industrial purposes (Richardson, 1988; Muller, 2001). Among the medicinal lichens, *Umbilicaria esculenta* and *Usnea longissima* have been utilized in the Far East. *Umbilicaria* (*Gyrophora*) *esculenta* (Miyoshi) Minks, a foliose-type macrolichen, is an edible mushroom which is utilized in the preparation of traditional foods and medicines in Korea, Japan, and China. Extracts or prescriptions of *U. esculenta* have proven effective in the treatment of many types of inflammation, bleeding, and poisoning (Lo, 1994). It has also been reported that *U. esculenta* evidenced a variety of effects, including cholesterol synthesis inhibition, anti-tumor, glucosidase inhibitory, and anti-thrombotic effects (Kim, 1982; Sone *et al.*, 1996; Lee and Kim, 2000; Kim and Lee, 2006). *Usnea longissima* Ach., a fructicose-type lichen, has been employed as a traditional medicine in both Eastern and Western countries. Extracts or prescriptions of *U. longissima* have been shown to exert anti-bacterial, anti-inflammatory, and detoxifying effects (Lo, 1994; Okuyama *et al.*, 1994; Lauterwein *et al.*, 1995). Many constituents of *U. longissima* have been previously identified (Mallavadhani *et al.*, 2004), but little information is currently available regarding the biological potency of the lichen. This study was conducted in an effort to determine the whitening effects and mechanisms of the edible and medicinal lichens, *U. esculenta* and *Usnea longissima.*

* To whom correspondence should be addressed.
(Tel) 82-2-940-7187 ; (Fax) 82-2-912-7932
(E-mail) hbcho@skuniv.ac.kr

## Materials and Methods

### *Lichen collection*

Fresh thalli of *U. esculenta* and *U. longissima* were collected in June 2003 from Mt. Jiri (35ºN, ~1,200 m altitude), in Korea. The collected samples were identified in accordance with their morphological characteristics (Yoshimura, 1994) and lyophilized for storage.

### *Preparation of extracts*

Dried thalli of *U. esculenta* and *U. longissima* were ground in a mixer and extracted with absolute methanol at a ratio of 1:10 (w/v) for 2 h in a heating mantle at 70-80°C. The extract was filtered and concentrated *in vacuo* at 50°C. The quantity of total phenolic compounds was determined via the official method established by the AOAC (1985).

***Antioxidation activity by electron donating ability***
Electron donating ability (EDA) was determined in accordance with the method described by Blois (1958). That is, $4\times10^{-4}$ M DPPH (1.1-diphenyl-2-picrylhydrazyl) solution was added to 0.2 ml of each sample (solid concentration 6.0 mg/ml). After 10 min, optical density was measured at 525 nm using a UV/Vis Spectrophotometer (Jasco, Japan). EDA was expressed as a percentage of the differences between the extract and the control.

***Antioxidative activity measurement by inhibition of lipid peroxidation***
Osawa's method (1981) was used to measure antioxidative activity in accordance with the inhibition of lipid peroxidation. Linoleic acid (25 mg/ml in ethanol), ferrous chloride (2.45 mg/ml in 3.5% HCl), ammonium thiocyanate (0.3 g/ml in water), and 0.2 M phosphate buffer (pH 7.0) were utilized as stock solutions. 0.2 ml of each sample solution (solid concentration 6.0 mg/ml) was mixed with 0.2 ml of linoleic acid in a test tube, and subsequently 0.4 ml of phosphate buffer and 0.2 ml of distilled water was added. During incubation at 40°C in darkness, the linoleic acid peroxidation rate was measured at regular intervals. A 0.1 ml aliquot of the mixture was mixed with 3 ml of 70% ethanol, 0.1 ml of ammonium thiocyanate solution, and 0.1 ml of ferrous chloride solution in a test tube, and the optical density was assessed at 500 nm.

***Tyrosinase inhibition***
Tyrosinase inhibition activity was determined via the method described by Wong *et al.* (1971). Tyrosinase (mushroom tyrosinase, Sigma) dissolved in 50 mM sodium phosphate buffer solution (pH 7.0), and a 0.2 ml aliquot of the enzyme solution with or without 0.5 ml of extract solution was added to 2.8 ml of substrate solution (10 mM catechol). The changes in the optical density of the mixture were measured at 420 nm. One unit of enzyme activity was defined as the quantity of enzyme required for a 0.001 change in optical density per 1 min. Tyrosinase inhibition activity was expressed as a percentage of inhibition of the extract compared to the control.

***Effect on melanin formation in human melanoma cells***
Human melanoma cells (HM3KO cells) were cultivated in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) at 37°C and an atmosphere of 5% $CO_2$. Cells were inoculated in each flask at a cell count of $3\times10^5$ and were cultivated with 0.01% and 0.1% of the lichen extracts, respectively. Cells cultivated at the maximum were harvested, and their color and melanin contents were determined. Melanin content was determined by optical density measurements at 490 nm after treatment with 1 N NaOH.

***Effect on tyrosinase glycosylation of human melanoma cells***
HM3KO cells were cultivated with 0.01% and 0.1% of the lichen extracts. After cultivation, the cells were harvested and disrupted in cell lysis buffer (2% CHAPS in 50 mM HEPES and 200 mM NaCl, pH 7.5, protease inhibitors) with an ultrasonicator. The lysates were centrifuged for 10 min at 12,000×g and endoglycosidase H (100 units, Sigma) was added to the supernatant. After 1 h of reaction at 37°C, the proteins of the supernatant were separated via electrophoresis.

***Statistical analysis***
The experimental results were expressed as the Mean±S.D. Analysis of variance was performed by using SPSS (SPSS Inc., USA). Duncan's new multiple range test was used to determine the difference of means.

## Results and Discussion

***Antioxidation activity measurements by electron donating ability***
Measurements of EDA using DPPH were conducted via the direct estimation method for antioxidation activity. Extracts of *U. esculenta* and *Usnea longissima* evidenced 72.8% and 80.7% electron-donation effects, respectively, at a concentration of 6 mg/ml (Fig. 1). The median scavenging concentration ($SC_{50}$), or the concentration resulting in 50% electron-donation ability, was identified as 1.29±0.05 mg/ml and 1.03±0.06 mg/ml, respectively, whereas the $SC_{50}$ of ascorbic acid as a positive control was 0.10±0.01 mg/ml.

As the majority of naturally existing antioxidation agents are phenolic compounds, we assessed the total phenolic contents in an effort to verify the correlation between the antioxidant activities of the lichen extract and the polyphenol content. The polyphenol content of the lichen extract was 1.46% in *U. esculenta* and 2.62% in *Usnea longissima*. The lichen extract evidenced potent antioxidation activity, but this effect was not proportional to the polyphenol content. It is assumed that certain compounds other than polyphenol might also function as an antioxidative agent.

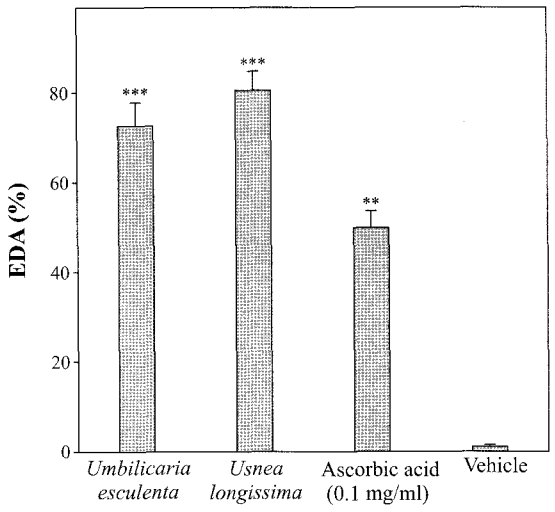


**Fig. 1.** Electron donating ability (EDA) in methanol extracts of *Umbilicaria esculenta* and *Usnea longissima*. DPPH ($4\times10^{-4}$ M) solution was added to 0.2 ml of each sample. Solid concentration of lichen extract was 6.0 mg/ml. Each value is expressed as Mean±SD in triplicate experiments. **Significantly different from the control ($P<0.01$), ***Significantly different from the control ($P<0.001$).

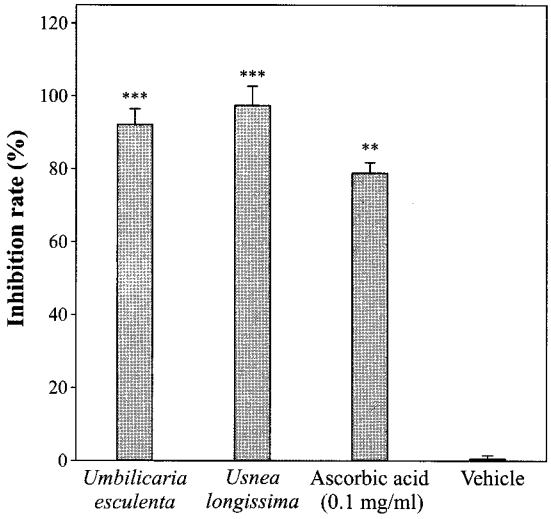


**Fig. 2.** Inhibition of lipid peroxidation in methanol extracts of *Umbilicaria esculenta* and *Usnea longissima*. Each sample solution (0.2 ml) was mixed with 0.2 ml of linoleic acid. Solid concentration of lichen extract was 6.0 mg/ml. Each value is expressed as Mean±SD in triplicate experiments. **Significantly different from the control ($P<0.01$), ***Significantly different from the control ($P<0.001$).

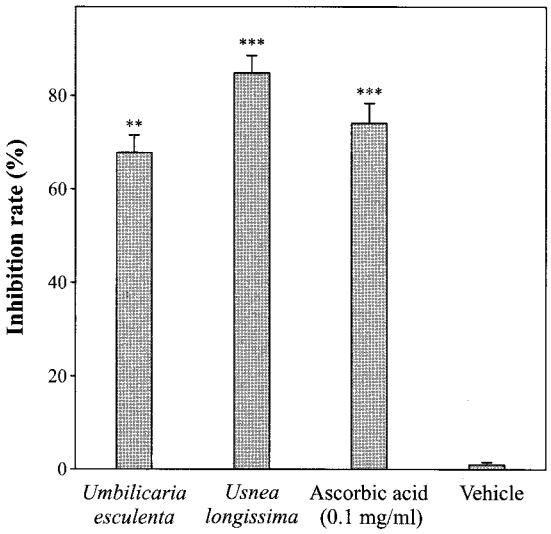


**Fig. 3.** Inhibition of tyrosinase in methanol extracts of *Umbilicaria esculenta* and *Usnea longissima*. An aliquot of tyrosinase solution (0.2 ml) with or without 0.5 ml of extract solution was added to 2.8 ml of substrate solution (10 mM catechol). Each value is expressed as Mean±SD in triplicate experiments. **Significantly different from the control ($P<0.01$), ***Significantly different from the control ($P<0.001$).

### *Antioxidation activity measurement by inhibition of lipid peroxidation*

The inhibition of linoleic acid peroxidation was measured as the other method for the estimation of antioxidation activity. As is shown in Fig. 2, the inhibition effects were measured at 92.1% in *U. esculenta* and 97.3% in *Usnea longissima* at a concentration of 6 mg/ml, whereas it was 78.8% in ascorbic acid (0.1 mg/ml), which was utilized as a positive control. The median inhibitory concentration ($IC_{50}$), the concentration at which the inhibition level was 50%, was identified as 0.57±0.05 mg/ml in *U. esculenta* and 0.53±0.06 mg/ml in *Usnea longissima*, while it was 0.05±0.01 mg/ml in ascorbic acid.

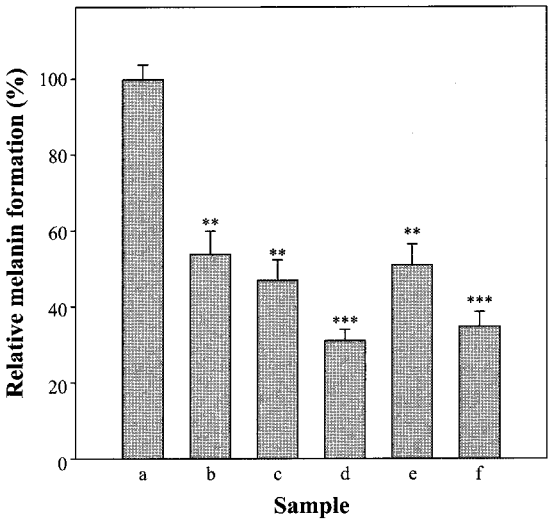


**Fig. 4.** Effect of lichen extracts for melanin formation in human melanoma cells. a, vehicle (water:propylene glycol=7:3); b, incubated with 0.01% ascorbic acid; c, incubated with 0.01% *Umbilicaria esculenta* extract; d, incubated with 0.1% *Umbilicaria esculenta* extract; e, incubated with 0.01% *Usnea logissima* extract; f, incubated with 0.1% *Usnea logissima* extract. HM3KO cells grown in minimum essential medium were inoculated in each flask at a cell count of $3\times10^5$ and were cultivated with 0.01% and 0.1% of the lichen extracts, respectively. Each value is expressed as Mean±SD in triplicate experiments. **Significantly different from the control ($P<0.01$), ***Significantly different from the control ($P<0.001$).

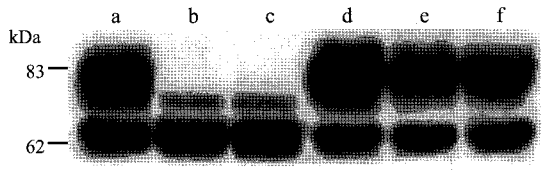


**Fig. 5.** Effect of lichen extracts for tyrosinase glycosylation. Large, upper bands represent 80 kD glycosylated tyrosinase and small, lower bands represent 60 kD non-glycosylated tyrosinase. Lane description : a, control, treated with endoglycosidase H; b, incubated with 0.01% *Umbilicaria esculenta* extract, treated with endoglycosidase H; c, incubated with 0.01% *Usnea logissima* extract, treated with endoglycosidase H; d, control, not treated with endoglycosidase H; e, incubated with 0.01% *Umbilicaria esculenta* extract, not treated with endoglycosidase H; f, incubated with 0.01% *Usnea logissima* extract, not treated with endoglycosidase H. HM3KO cells were cultivated with 0.01% and 0.1% of the lichen extracts. Cells were harvested and disrupted in cell lysis buffer (2% CHAPS in 50 mM HEPES and 200 mM NaCl, pH 7.5, protease inhibitors). The lysates were treated with endoglycosidase H (100 units) and the proteins of the supernatant were separated via SDS- PAGE.

***Tyrosinase inhibition***

Tyrosinase is a rate-limiting enzyme in the melanogenesis of a living body. The lichen extracts utilized in this study evidenced potent inhibitiory activity against tyrosinase. The *U. esculenta* and *Usnea longissima* extracts employed in this study evidenced tyrosinase inhibition rates of 67.8% and 84.8%, respectively (Fig. 3), whereas ascorbic acid, which was widely utilized as an effective tyrosinase inhibitor, inhibited tyrosinase activity by 73.4% at a concentration of 0.1 mg/ml. Considering the crude state of the lichen extract, those tyrosinase inhibition rates reflect a high degree of efficacy.

***Effects on melanin formation in human melanoma cells***

In order to evaluate the effects of lichen extracts on melanin formation in living cells, human melanoma cells were cultivated with the extracts. Ascorbic acid, which was used as a positive control at a concentration of 0.01% reduced melanin formation to 54.0%, whereas *U. esculenta* extracts of 0.01% and 0.1% reduced melanin formation to 47.1% and 31.2%, respectively, and the *Usnea longissima* extract reduced melanin formation to 51.1% and 34.9%, respectively (Fig. 4). Considering the crude state of the lichen extract, as in the case of direct tyrosinase inhibition, those melanin reduction rates are reflective of a high degree of efficacy. We confirmed that the color of the cells treated with lichen extract was less dark than was observed in the untreated cells.

***Effects on tyrosinase glycosylation in human melanoma cell***

Changes in glycosylation may affect protein structure and function (Na and Song, 2000; Lee *et al.*, 2002). Tyrosinase is a glycoprotein which completes its maturation via a glycosylation process, in which many types of glucosidase participate (Toyofuku, 2001). If tyrosinase matures abnormally due to any problem in this glycosylation process in the carbohydrate portion, it cannot migrate and function properly in melanosomes, in which melanin is synthesized.

Tyrosinase, in which the carbohydrate chain is normally formed, evidenced approximately an 80 kD matured glycoprotein because it resists hydrolysis by endoglycosidase. However, tyrosinase, in which the carbohydrate chain is not formed normally by the inhibition of glucosidase participating in the glycosylation process, evidenced only approximately a 60 kD protein due to the hydrolysis of carbohydrate chain by endoglycosidase. As is shown in Fig. 5, the tyrosinase of comparative group (a) was larger in size (about 80 kD) because the carbohydrate chain was not broken by endoglycosidase, but the tyrosinase treated with 0.01% of *U. esculenta* and *Usnea longissima* extract was small in size (about 60 kD) because the carbohydrate portion was completely hydrolyzed by endoglycosidase (b, c). Without endoglycosidase treatment, all of the comparative group (d) and the lichen extract-treated group (e, f) evidenced large-sized (about 80 kD) normal tyrosinase. This result indicated that lichen extract could influence the melanogenesis activity of tyrosinase, by causing problems in the formation of mature glycoproteins via glucosidase inhibitory activity.

In conclusion, the formation of melanin in the human body could be influenced or reduced by several mechanisms, including anti-oxidation, direct tyrosinase inhibition, melanin inhibition of migration from cell-to-cell and hormonal activities, etc. (Prota and Thomson, 1976; Pawelek and Korner, 1982).

In this study, we determined that the methanolic extracts of *U. esculenta* and *Usnea longissima* affected melanin formation not only via mechanisms of antioxidative action and direct tyrosinase inhibition, but also via the inhibition of tyrosinase maturation. It can be assumed that lichen extracts might be utilized as an effective whitening material operating via multiple mechanisms.

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