The Study on Application to Cosmetics of *Phellinus linteus*

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Key Words

phellinus linteus, B16 melanoma cell, 1,1-diphenyl-2-picrylhydrazyl(DPPH), whitening effect, melanogenesis, scavenger, antioxidative activity, immunological activity

Abstract

Phellinus linteus was artificially cultivated in kangwon province in Korea.

The air-dried *phellinus linteus* was frozen in liquid nitrogen tank and powdered in jar. 10g of the powder was extracted with each 200g of ethanol, methanol, distilled water and 1,3-burylene glycol/distilled water (6:4, w/w) 4 hours under refluxing and then the liquid extract was concentrated under reduced pressure. As a result of analysis by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC), many kinds of sugar and flavonoids were detected. Also we knew that *phellinus linteus*' extract had a strong UV-ray absorption.

In the efficacy test for applying to cosmetics, free radical scavenging effect was confirmed. As a result, 2% of sample was the most potent inhibitory effect and the free radical scavenging activity (SC_{50}), was 0.31%. This is more effective than any other material. In the test of antioxidative activity against lipid autoxidation, *phellinus linteus*' extract had a good effect by 46% while vitamine E was 42.3%.

The immunological activity of *phellinus linteus* was showed through the activation of macrophage cell. Actually, *Phellinus linteus* activated macrophage function of 1.1-1.8 times including nitrite (NO_2-) production compared to control.

The whitening effect of *phellinus linteus* was showed through the inhibition of tyrosinase activity, melanin biosynthesis of *S. bikiniensis* and B16 melanoma cells. *Phellinus linteus*' extract was showed strong mushroom tyrosinase inhibitory activity with IC_{50} value of 0.5% and inhibited melanin biosynthesis with 28mm inhibition zone at 0.005%/paper disc in *S. bikiniensis*, a bacterium used as an indicator organism in this work. Also it inhibited melanin biosynthesis in B16 melanoma cells with a minimum inhibitory concentration of 0.134%.

I. Introduction

Phellinus linteus, as it belongs to pine Hymenochaetceae, is a kind of mushroom produced at the bottom of old mulberry bark. Its shape looks like a yellow lump of clay. The shell, leaves and roots of mulberry bark have been applied to many kinds of aspects, medicine and cosmetics. For example, it is utilized anticancer agent and immunomodulatory agent.

Recently, the investigation of natural products has been increased expecting the effectiveness and safety. For example, components extracted or isolated from crude drugs are utilized in order to improve the blotches and freckle causing by suntan as a cosmetic ingredient (1). As regards synthesis, the development of practical cosmetic materials has been carried out by deriving noble compounds in order to improve on whitening effect or stability (2).

We found that *phellinus linteus*' extracts were effective on inhibition of tyrosinase activity.

In this report, we identified various kinds of active ingredients from *phellinus linteus* and researched efficacy for whitening agents, antioxidation effect and immunological activity in order to apply to cosmetics.

II. Materials and Methods

1. Experimental reagents and materials

Mulberry root extract, licorice PT 40, kojic acid and arbutin were obtained from Ichimaru Co., Maruzen Co., Tokyo Kasei Co. and Kaden Co. (Japan). Other experimental reagents were used by Sigma Co. (USA).

2. Collection and extraction of *phellinus linteus*

Phellinus linteus was artificially cultivated in kangwon province in Korea. The air-dried *phellinus linteus* was frozen in liquid nitrogen tank and powdered in jar. 10g of the powder was extracted with each 200g of ethanol, methanol, distilled water and 1,3-butylene glycol/distilled water (6:4, w/w) 4 hours under refluxing and then the liquid extracts were concentrated under reduced pressure.

3. Identification of ingredients

3-1. Thin layer chromatography (TLC) analysis: TLC was performed on precoated silicagel RP-18F_{254} S plates (Merck Co.) and spots were detected by aniline hydrogen phthalate or 10% sulfuric acid followed by heating. Spots were compared to sugar standard materials (Sigma Co.). The development solvent of TLC was 1-butanol: acetic acid: distilled water (4:1:2 (v/v)).

3-2. High performance liquid chromatography (HPLC) analysis: The sugar components of the extract were quantitatively analyzed by using HPLC (Waters Co.) with 510 pump, M996 UV detector and 410 RI detector (analysis condition — column: sugar pak 1, eluent: water, column temperature: 90°C, flow rate: 0.5ml/min).

3-3. UV absorption: We measured ultraviolet spectrum by UV/VIS spectrophorometer (Cary, Varian). The concentration was 0.1/10 (ml/ml) in ethanol.

4. Free radical scavenging activity

Free radical scavenging activity was determined by the modified method of Fujita et al (3) using a moderately stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). 1.0 ml of ethanolic sample solution was added to 1.0 ml of 0.1 mM DPPH methanolic solution, and allowed to stand for 30 min at 37°C. The amount of free radicals in the mixture was measured by absorbance at 516nm. The free radical scavenging activity (SC_{50}) was defined as the concentration of the sample required for 50% of the free radicals to be scavenged. Free radical inhibition (FRI) was calculated as follows:

$$FRI\ (\%) = \{1 - (E - B)/C\} \times 100$$

B: blank group
C: control group
E: experimental group

5. Antioxidative activity against lipid autoxidation

The reaction mixture (1.0 ml) containing 2 mg/ml of linolenic acid, 10 mg/ml of Tween-20 and 0.2 M potassium phosphate (pH 7.4) in the presence of sample were incubated for 24 hours at 37°C. 0.1 ml of reaction mixture was added to 9.7 ml of 80% ethyl alcohol, then 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM ferrous ammonium sulfate-3.5% hydrochloric acid were added. After 3 min, the optical density was measured at 500 nm (4).

6. Immunological activity

Mouse peritoneal cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal calf serum (FCS). The cell suspension (5×10^4 - 1×10^5 cells/well) was poured into 96-well microtiter plates (Corning Co.) and added a different concentration of the test materials. Then, incubated in 5% CO_2 at 37°C for 48 hours. 200 µl of each supernatant was transferred to wells of a fresh 96-well microtiter plate and stirred at room temperature for 10 min. The absorbance of cultures, cell-free control samples and a water blank was measured in a microtiter plate reader (Bio-Tek, ELX800) at 550 nm. 90 µl of the Griess reagent was added into a well plate and stirred at room temperature for 20 min. The absorbance was measured in a microtiter plate reader at 550 nm. The amount of nitrite (NO_2^-) released was calculated (5).

7. Whitening effect

7-1. Inhibition of tyrosinase activity: This was a modification of the methods of Horowits and others (6). The reaction mixture consisted of 150 µl of 0.1 M phosphate buffer (pH 6.5), 1.5 mM L-tyrosine solution (25 µl) and 2,380 U/ml mushroom tyrosinase (10 µl) in 0.05 M phosphate buffer (pH 6.5). A sample solution (15 µl) was added to the reaction mixture in 96 well microtiter plates and incubated at 37°C for 10 min. The optical density at 490 nm was measured by a microtiter plate reader. The inhibitory activity of the sample was expressed as the concentration which inhibits 50% of enzyme activity (IC_{50}). The inhibitory percentage of tyrosinase was calculated as follows:

Tyrosinase inhibition (%) = {[(D-C) - (B-A)] / (D-C)} × 100

A/B: The absorbance before/after reaction with inhibitory agent
C/D: The absorbance before/after reaction without inhibitory agent

7-2. Inhibition of melanin production of S. bikiniensis: A preserved culture of *S. bikiniensis* NRRL B-1049 (KCTC-9172) was inoculated on a Papavizas VDYA agar slant which contained V-8 juice (Campbell Soup Co.) 200 ml, glucose 2 g, yeast extract (Difco Co.) 2 g, $CaCO_3$ 1 g, agar (Difco Co.) 20 g, and distilled water 800 ml, the pH being adjusted to 7.2 before autoclaving.

After incubating at 28°C for 2 weeks, 2 ml of sterile water was added onto the slant culture and the spore mass formed on the aerial mycellium was scraped with an inoculating loop. The spore suspension thus obtained was transferred to a sterile test tube. Agar medium ISP No. 7 supplemented with 0.2% Bacto-yeast extract (Difco Co.) was poured into petri dishes (90 mm i.d.). After solidification, 0.4 ml of the spore suspension of *S. bikiniensis* was added to the agar plate and spread over the agar surface uniformly with a glass hockey bar.

After drying of the agar surface, a paper disc (8 mm diameter) soaked with sample solution was placed on the agar plate. The plate was incubated at 28°C for 48 hr; the resulting zone (mm i.d.) of inhibition of melanin formation was measured from the reverse side of the plate. 4-Hydroxyanisole was used as a reference standard (7).

7-3. Inhibition of melanin syntbesis in B16 melanoma cells: B16 melanoma cells (ATCC CRL No. 6323) were suspended in DMEM containing 10% FCS at 5×10^4 cells/ml. The cell suspension (5 ml) was poured into a tissue culture flask (Corning Co.) and added a different concentration of the test materials. Then, incubated in 5% CO_2/95% air atmosphere at 37°C for 4 days. After 4 days of incubation, the adherent cells were washed with phosphate-buffered saline (PBS) and trypsinizated. The cell suspension was centrifugated at 1,500 rpm for 10 min. The color and volume of resulting cells were compared with those of controls(7).

III. Results and Discussions

1. Identification of ingredients

As the result of TLC analysis, *phellinus linteus*' extracts were separated into 6 spots (Fig. 1). As compared with the Rf (rate of flow) values of standard sugar materials (spot no. 3: rhamnose, spot no. 5: ribose, glucose, fructose, spot no. 6: maltose, spot no. 7: lactose), *phellinus linteus*' ethanol extract contained rhamnose, maltose and lactose. There were 2 more spots (Rf value: 0.77, 0.83) in addition to standard sugar in methanol extract. Therefore, we think that methanol extract contains other ingredients except sugar component.

The result of HPLC analysis of *phellinus linteus*' methanol extracts was shown in Fig. 2.

As compared with the chromatogram of standard sugar materials (maltose, lactose, glucose, xylose, rhamnose, galactose, manose, fructose, arabinose, ribose), *phellinus linteus*' extracts contained maltose, lactose, glucose, rhamnose, manose, fructose, arabinose and ribose. We think that these sugar materials are concerned in immunological activity and hygroscopicity in skin.

Phellinus linteus' extracts showed UV absorption peak at 270-280 nm (Fig. 3) and stronger UV absorbing ability than any other whitening agent (arbutin, kojic acid).

2. Free radical scavenging activity

An active oxygen was continuously created by normal cellular metabolic process(8), drug metabolic process (9), inflammation (10) and ultraviolet (11) in cells. Then, an bioorganism was always exposed to harmful effects of the free radical reaction occurring by them.

Especially, as cellular aging, these harmful effects were gradually accumulated and then all kinds of aging diseases were produced (12, 13, 14).

Free radical scavenging activity can be measured by reduction of DPPH to 1,1-diphenyl-2-picrylhydrazine, which causes decrease in optical density at 516nm. DPPH radical scavenging activity of *phellinus linteus*' extract (the concentration reducing 50% of 100 μM DPPH, SC_{50} = 0.31%) was stronger than that of Ginkgo biloba, but weaker than that of Green-tea (Fig. 4).

3. Antioxidative activity against lipid autoxidation

Linolenic acid is colorless, unsaturated fatty acid oil which is easily oxidized by air. When this fatty acid oxidized and reacted with iron is put into ammonium thiocyanate solution, the mixed solution is changed into red color. We could know antioxidative effect by measuring color change of the mixed solution with sample or without sample. Antioxidative activity against lipid autoxidation was showed in Fig. 5.

In cosmetics, vitamine E is well known for its antioxidative activity. In this experiment, *phellinus linteus* ethanol extract had a good effect by 46% while vitamin E was 42.3%.

4. Immunological activity

The macrophage cell activating properties are possibly the most interesting aspect of their immunomodulating

properties. So, we measured the amount of nitrite produced by activated macrophage cell for immunological properties. The immunological activity of *phellinus linteus* showed through the activation of macrophage cell. Actually, *phellinus linteus* activated macrophage function of 1.1-1.8 times including nitrite production compared to control (Fig. 6).

5. Whitening effect

Melanogenesis is a series of oxidative polymerization reaction starting from tyrosine and activated by oxidative stress caused by UV. Tyrosinase plays an important role in melanogenesis. Dopaquinone, an intermediate of melanogenesis, is unstable and converted to dopachrome by tyrosinase or autoxidation, and melanin can be formed through subsequent polymerization reaction. So inhibition of melanogenesis can be achieved by antioxidation and inhibition of tyrosinase. In fact, kojic acid and arbutin known as tyrosinase inhibitors, have been used in cosmetics in expectation of skin whitening. In this study, the effect of *phellinus linteus*' extract on melanogenesis was examined by using *in vitro* enzyme assay, cell culture and microorganism culture method. At first, inhibition of tyrosinase catalyzed dopachrome formation was examined. *Phellinus linteus*' ethanol extract, kojic acid, arbutin and licorice PT 40 had both the effect of tyrosinase inhibition and depigmenting effect in melanoma cells (Table 1, Fig. 7). Kojic acid and arbutin had the effect of tyrosinase inhibition but didn't inhibit melanin formation of *S. bikiniensis* (Fig. 8).

The values of IC_{40} of 0.037%, 0.4%, 0.5% were obtained from kojic acid, arbutin and *phellinus linteus*' extract. Also *phellinus linteus*' extract inhibited melanin biosynthesis in B16 melanoma cells with a minimum inhibited concentration of 0.134%. Mulberry root extract didn't inhibit melanin formation of B16 melanoma cells but inhibited melanin formation of *S. bikiniensis* (Fig. 8). From this result, we deduced that *phellinus linteus*' extract actually inhibited cellular pigmentation by its antioxidative activity against oxidation of unstable intermediate of melanin as well as direct inhibition of tyrosinase.

Therefore, in order to estimate whitening effect, it is necessary that depigmenting activity in normal cells as well as tyrosinase inhibition should be examined.

Owing to these effects of *phellinus linteus*, we can apply its extracts to cosmetics.

IV. References

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Table 1. Inhibitory effects on mushroom tyrosinase and melanin formation of *S. bikiniensis* and B16 melanoma cells.

Compound	S. bikiniensis NRRL B-1049	B16 Melanoma	Mushroom tyrosinase
	Inhibition zone(㎜)	MIC(%)/(+, -)	IC_{50}
4-Hydroxyanisole	24㎜	ND**	ND**
Kojic acid	NF	0.034/+	0.037%
Hydroquinone	25㎜	0.00026/+	0.007%
Arbutin	NF*	0.034/+	0.4%
Phellinus linteus' MeOH ext.	28㎜	ND**	below 0.5%
Phellinus linteus' EtOH ext.	16㎜	0.134/+	below 0.5%
Phellinus linteus' 1,3-BG/DW(6:4) ext.	6㎜	ND**	-
Phellinus linteus' DW ext.	NF*	0.668/-	-
Mulberry root ext.	12㎜	0.334/+	below 10%
Licorice PT 40	16㎜	0.0002/+	0.0014%
P-methoxyphenol	30㎜	ND**	-

* : not formed, ** : not determined
+ : inhibition of melanin formation, - : noninhibition of melanin formation

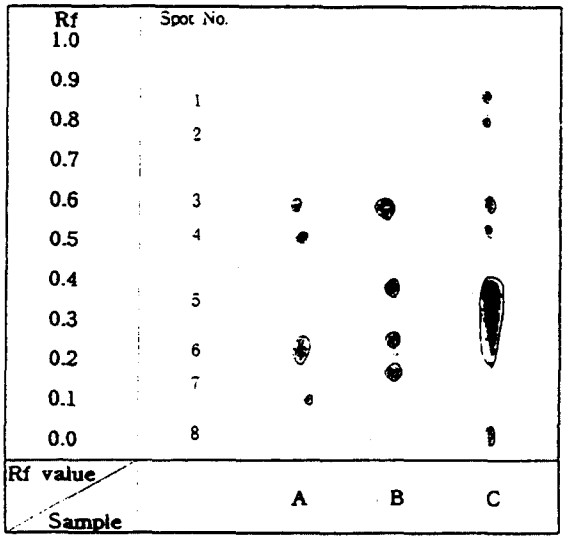

Fig. 1. TLC Chromatogram of Sugar.
A : Ethanol extract of *Phellinus linteus*
B : Sugar Standard Materials
C : Methanol extract of *Phellinus linteus*

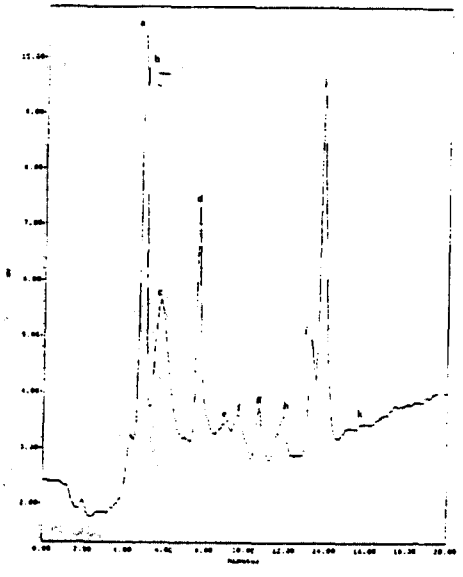

Fig. 2. HPLC Chromatogram of *Phellinus linteus'* Extract.
(b : maltose, c : lactose, d : glucose, e : xylose, f : rhamnose, g : galactose, h : manose, i : fructose, j : arabinose, k : ribose)

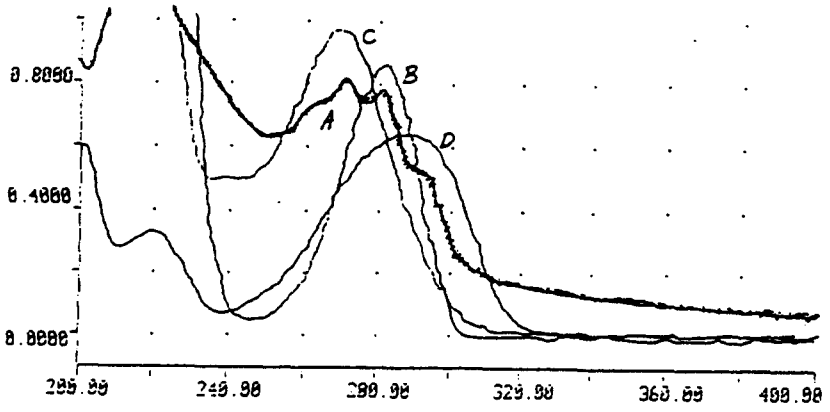

Fig. 3. UV Absorption Spectrum of *Phellinus linteus*' Extract.
(A : *Phellinus linteus*' EtOH extract, B : 10% Arbutin in DW
C : 5% Kojic acid in DW, D : Songyi EtOH extract)

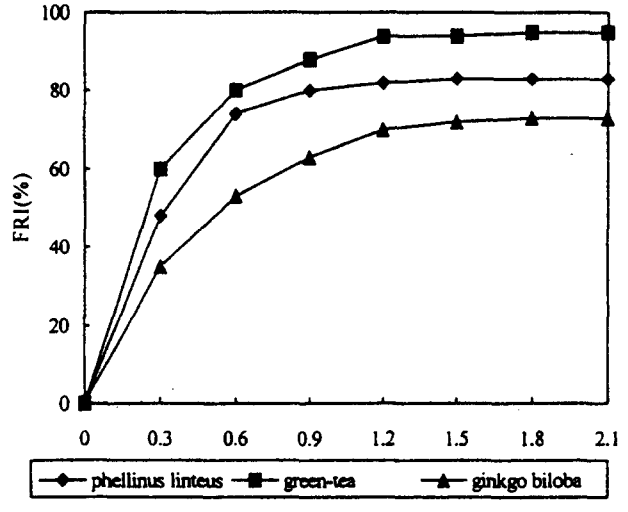

Fig. 4. Scavenging effect of *phellinus linteus*' extract against DPPH free radicals.

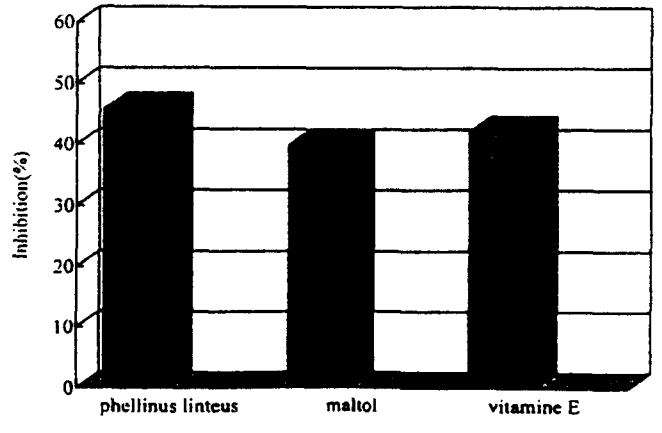

Fig. 5. Inhibitory effect of *phellinus linteus*' extract on the autoxidation of linolenic acid.

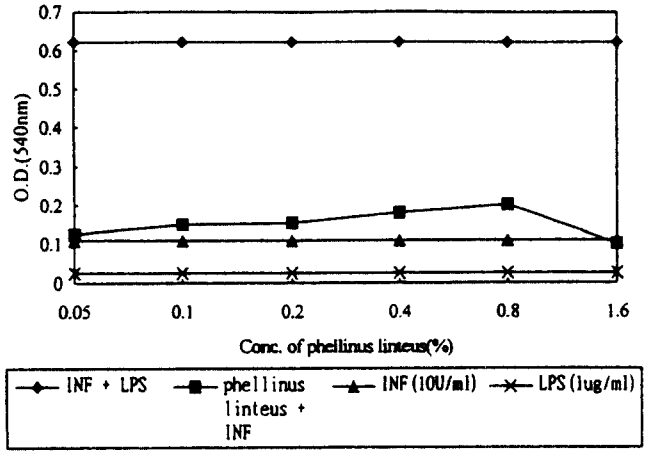

Fig. 6. Immunological activating effect of *phellinus linteus*' extract.

Fig. 7. Photograph of B16 melanoma cells cultured in the absence and in the presence of samples.
(A: control, B: licorice PT40, C: *phellinus linteus*' ethanol extract, D: kojic acid, E: arbutin, F: mulberry root extract)

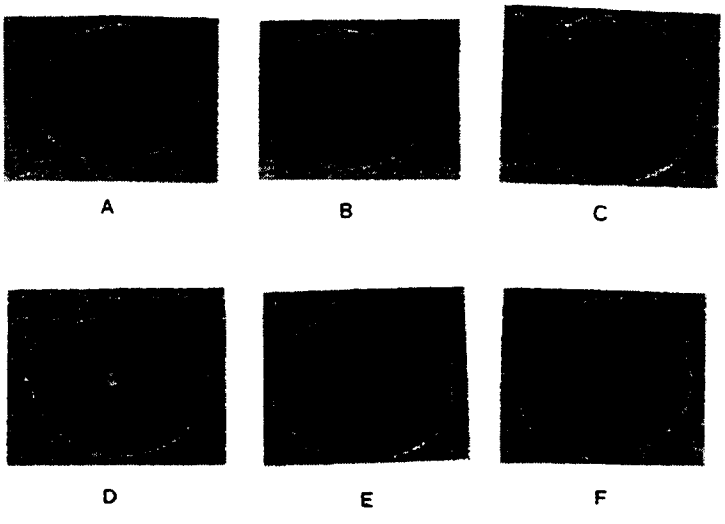

Fig. 8. Photograph of melanin formation of *S. bikiniensis* in the absence and in the presence of samples.
(A: control, B: kojic acid, C: mulberry root extract, D: *phellinus linteus*' methanol extract, E: arbutin, F: 4-hydroyanisole)