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SHORT COMMUNICATION

Depigmenting effect of *Cinnamomum cassia* Presl in B16F10 melanoma cells

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Abstract–To find efficient depigmenting agents, we examined several Chinese herbs for melanogenesis inhibition and toxicity. *Cinnamomum cassia* Presl exhibited low cytotoxicity at even high concentration (200 μg/ml). The effects on melanogenesis of cultured B16 melanoma cells, mushroom tyrosinase activity, and free radical scavenging activity were further assessed. The methanol extracts of this plant showed the suppression of melanin synthesis. Melanin content was dose-dependently decreased by this herb extract as compared with control cells. It also showed good anti-oxidative activity (IC_{50}=3.7 μg/ml) but no inhibition of mushroom tyrosinase activity. This result showed that *Cinnamomum cassia* Presl extract might be useful and safe as a new whitening agent in cosmetics.

Key words: Melanogenesis, Depigmentation, Herb, *Cinnamomum cassia* Presl

INTRODUCTION

Melanocytes have specialized lysosome-like organelles, termed melanosomes, which contain several enzymes that mediate the production of melanin [1]. The copper-containing enzyme tyrosinase (a tyrosine hydroxylase; EC 1.14.18.1) oxidizes the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and DOPA to DOPA quinone, which are the first two rate-limiting steps in the melanin synthesis pathway [2]. At least two additional melanosomal enzymes are involved in the eumelanogenesis pathway that produces brown and black pigments, including TRP-1 (DHICA oxidase), and TRP-2 (DOPAchrome tautomerase). Depending on the incorporation of a sulfur-containing reactant (e.g., cysteine or glutathione) into the products, the melanogenesis pathway diverges, producing eumelanins or pheomelanins (amber and red pigments) or both [3].

Melanin plays an important role in preventing ultraviolet (UV) light-induced skin damage. However, increased levels of epidermal melanin synthesis can darken the skin. Various dermatologic disorders result in the accumulation of excessive levels of epidermal pigmentation. They include melasma, lentigines, and postinflammatory hyperpigmentation. Because of the visible nature of dermatologic diseases, they have a considerable psychological effect on affected patients [4]. Disfiguring facial lesions can significantly affect a person's overall emotional well-being and can contribute to decreases in social functioning, productivity at work or school, and self-esteem [5]. Therefore, a number of whitening compounds have been screened for their effectiveness in reducing melanogenesis [3,6-10]. Many of the traditionally used skin lightening products such as hydroquinone, corticosteroids and mercury-containing products are still used in many countries, in spite of serious health concerns, including irreversible cutaneous damage, ochronosis, and accumulating of mercury in the body [11-13]. These adverse effects have led to the search for safer, plant-based skin lightening products. The ideal skin lightening agent for cosmetic products is one that inhibits melanogenesis without cytotoxicity, reduces pigmentation in cells and is of "natural" or "plant" origin.

Cinnamomum cassia Presl, the stem of *Cinnamomum* spp., is a commonly used Chinese herbal drug that contains coumarin, cinnamyl alcohol, cinnamaldehyde, cinnamic acid, methoxycinnamaldehyde, and cinnamyl acetate as its bioactive constituents [14]. Cinnamon is high in antioxidant activity. The essential oil of cinnamon also has antimicrobial properties. In medicine it acts like other volatile oils and once had a reputation as a cure for colds. It has also been used to treat diarrhea and other problems of the digestive system. Cinnamon has been reported to have remarkable pharmacological effects in the treatment of type II diabetes [15].

In this study, we examined whether this herb could inhibit melanin synthesis. From the mechanism study, it was demonstrated that the methanol extract of that showed the suppression of melanin synthesis, good free radical scavenging activity, but did not affect cell viability. After fractionation by using solvent-solvent extraction, ethyl acetate fraction showed high inhibition in melanin synthesis without cell toxicity. From these results, we suggest that this extract might be useful as a new whitening agent in cosmetics.

MATERIALS AND METHODS

1. Materials and Reagents

Mushroom tyrosinase, L-DOPA (3,4-dihydroxy-L-phenylalanine), Arbutin, DMSO (dimethyl sulfoxide) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) were purchased from Sigma Chemical Co. (St. Louis, U.S.A). DMEM media, fetal calf serum, trypsin EDTA, Phosphate buffered saline (PBS), Penicillin/streptomycin were purchased from Invitrogen Corp. (CA, U.S.A).

2. Cell Culture

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B16F10 murine melanoma cells were purchased from ATCC (American Type Culture Collection). B16 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% (v/v) fetal bovine serum, 100 units/ml of penicillin and 100 units/ml of streptomycin at 37 °C in a humidified, CO_2-controlled (5%) incubator. The cells were sub-cultured every three days until a maximal passage number of 30 were achieved.

3. Extraction and Isolation

The dried bark was minced by grinder (Mill Powder Tech Solution, Taiwan). The sample powder was extracted three times with four volumes of a Methanol (MeOH) 95% for 24 hrs. The resulting mixtures were filtrated and concentrated to dryness at 40 °C under vacuum to produce the MeOH extract. The MeOH extract was partitioned successively with Hexane, Chloroform ($CHCl_3$), Ethylacetate (EtOAc), and distilled water.

4. Assays

4-1. Melanin

B16 cells were cultured at 6×10^4 cells in 6-well plates. After 24 hrs, the cells were treated with various concentrations of samples for 48 hrs. The cells were then harvested by trypsination. After washing twice with PBS, samples were dissolved in 200 ml of 1 N NaOH containing 10% DMSO. The samples were then heated at 80 °C for 1 hr and cooled. The amount of melanin was determined spectrophotometrically based on absorbance at 405 nm.

4-2. Cell Viability

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) based colorimetric assay for cell proliferation was used. B16F10 melanoma cells were cultured at 2.5×10^3 cells in 96-well plates. After 24 hrs, the cells were treated with various concentrations of sample for 48 hrs. After 48hrs incubation with test compounds, 100 ml of MTT (5 mg/ml in phosphate-buffered saline) solution was added to the wells. After 4 hrs of incubation, the medium was removed, and 100 ml of DMSO was added to dissolve the formazan produced in the cells. The absorbance of each well was then read at 540 nm by using an ELISA microplate reader. The optical density of formazan formed by control cells was used as a reference (assumed to be 100%) [16].

4-3. Cellular Tyrosinase

B16 cells were cultured at 6×10^4 cells in 6-well plates. After 24 hrs, the cells were treated with various concentrations of sample. The cells were harvested by trypsinization and washed three times with ice-cold phosphate-buffered saline (PBS) by centrifugation at 5,000 rpm for 5 min. The cells were lysed in a 0.1 M sodium phosphate buffer (pH 7.0) containing 1% Triton ×100). The cells were then disrupted by sonicating for one hour at 4 °C, and the lysates were then clarified by centrifugation at 13,000 rpm for 20 minutes. After protein content was quantified by using a protein assay kit (Bio-Rad, U.S.A.), the cell lysates were adjusted to the same amount of protein with a lysis buffer. The reaction mixtures, consisting of 40 μg of protein, 40 μl of 5 mM L-DOPA and 0.1 M PBS (pH 6.8), were assayed on a 96-well plate at 37 °C. Absorbance was measured at 475 nm with an ELISA reader at 1 hr [6].

4-4. Mushroom Tyrosinase

Reaction mixtures consisting of 100 μl of sample, 22 U mushroom tyrosinase, 40 μl of 5 mM L-DOPA, and 0.1 M PBS (pH 6.8) were assayed on a 96-well plate at 37 °C. After 20 minutes, absorbance was measured as described above. Each sample was measured in triplicate. Kojic acid was used as positive control.

4-5. Free Radical Scavenging Assay

The radical scavenging activity of a sample was determined by the DPPH method. A sample was dissolved in a DMSO with different concentrations. Reaction mixtures consisting of 100 μl of sample and 100 μl of DPPH solution were assayed on a 96-well plate. The absorbance at 517 nm of the solution was measured after 30 minutes. The radical scavenging activity was calculated by using the following equation: % scavenging activity=[A control−A sample]/A control×100. Each sample was measured in triplicate. The 50% inhibitory concentration (IC_{50}; concentration of sample required to scavenge 50% of DPPH radicals) values were determined by the method of probit-graphic interpolation for eight concentration levels (1→25 μg/ml) [17].

4-6. Statistical Analysis

The values are expressed as mean standard deviation (S.D.). Differences between results were assessed for significance using the Student's t-test and a P value of <0.05 was accepted as significant.

RESULTS

1. Effect of *Cinnamomum cassia* on Melanin Synthesis

To investigate the effect *Cinnamomum cassia* on melanin synthesis, B16F10 melanoma cells were exposed to this plant extract from 12.5 μg/ml to 200 μg/ml for two days and then melanin contents were measured. As shown in the Fig. 1, the melanin synthesis was effectively inhibited in a dose-dependent manner. At a concentration of 100 μg/ml, this sample can inhibit 35% of melanin synthesis (compared with Arbutin which inhibits 20% of melanin content at 200 μg/ml).

2. Effect of *Cinnamomum cassia* on Cell Viability

To examine whether *Cinnamomum cassia* has cytotoxic effects, we treated B16 melanoma cells with this herb at various concen-

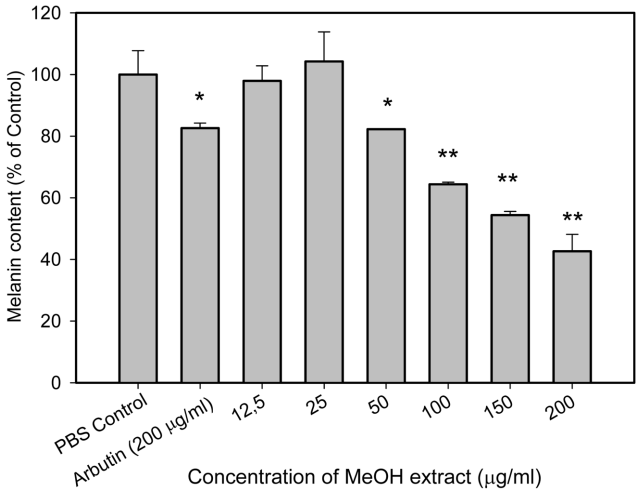

Fig. 1. Effect of crude extract of *Cinnamomum cassia* on melanin synthesis in cultured B16 melanoma cells. Data are expressed as percent change of the melanin content level relative to untreated control. Each determination was made in triplicate and data shown are means±S.D. *p<0.05, **p< 0.01: statistically significant vs. the value of the control group.

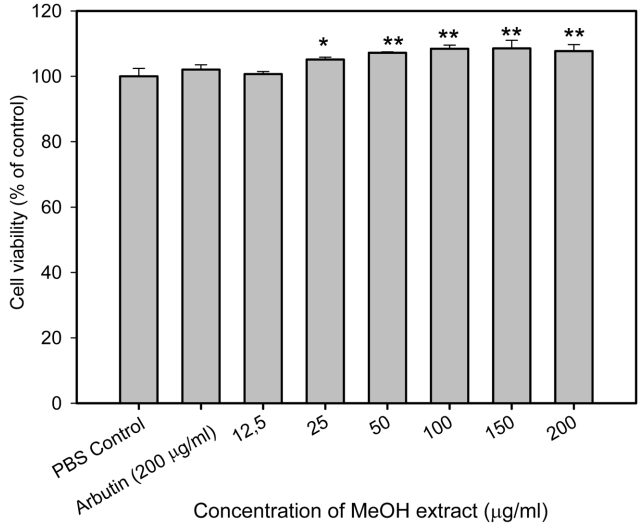

Fig. 2. Effect of crude extract of *Cinnamomum cassia* on cell viability in cultured B16 melanoma cells. Data are expressed as percent change of the cell viability level relative to untreated control. Each determination was made in triplicate and data shown are means±S.D. *p<0.05, **p<0.01: statistically significant vs. the value of the control group.

Table 1. Effect of crude extract of *Cinnamomum cassia* on tyrosinase activity

Concentration (μg/ml)	Tyrosinase activity (% of Control)	
	Cellular tyrosinase	Mushroom tyrosinase
100	120±3.48	102±0.45
200	123±4.25	100±1.42

Each determination was made in triplicate and data shown are means ±S.D.

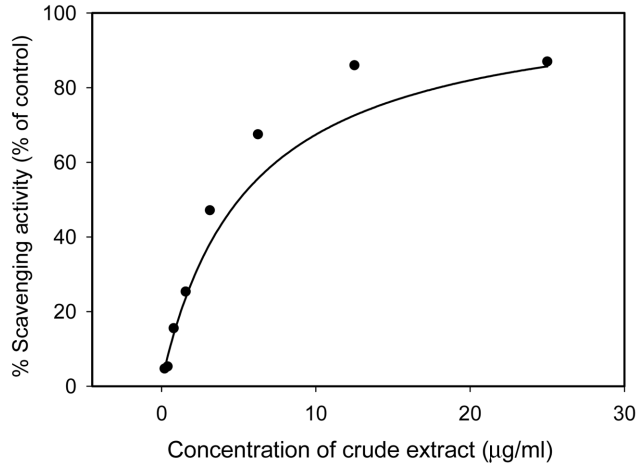

Fig. 3. Effect of crude extract of *Cinnamomum cassia* on free radical scavenging activity.

trations; cell viability was determined by using MTT assay. This plant did not show any effect on cell viability even at high concentration (200 μg/ml) (Fig. 2).

3. Effect of *Cinnamomum cassia* on Tyrosinase Activity

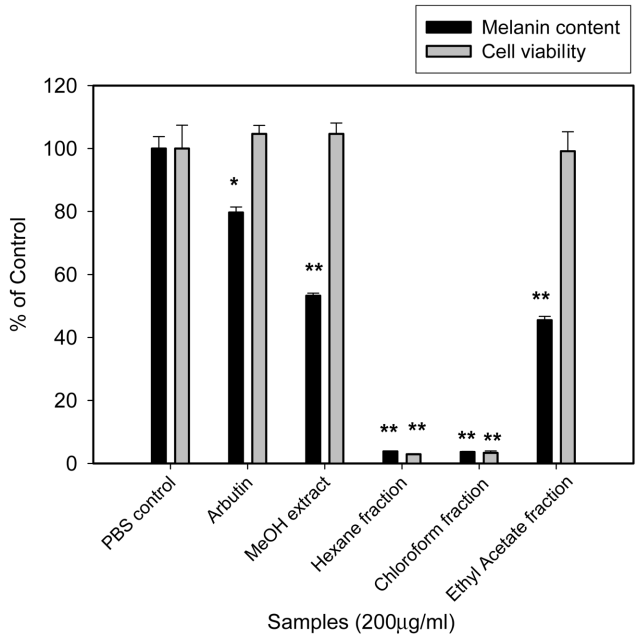

Fig. 4. Effect of solvent fraction of *Cinnamomum cassia* on melanin synthesis and cell viability in cultured B16 melanoma cells. Data are expressed as percent change of the content level relative to untreated control. Each determination was made in triplicate and data shown are means±S.D. *p< 0.05, **p<0.01: statistically significant vs. the value of the control group.

The direct effects of *Cinnamomum cassia* on tyrosinase activity were investigated by using mushroom tyrosinase. This herb did not show any effect on inhibition of mushroom tyrosinase activity. It also did not inhibit cellular tyrosinase activity (Table 1).

4. Effect of *Cinnamomum cassia* on Antioxidant Activity

To examine whether *Cinnamomum cassia* has antioxidant activity, DPPH assay was used. As shown in Fig. 3, *Cinnamomum cassia* has a good antioxidant activity with IC_{50}=3.7 μg/ml.

5. Effect of Solvent Extracts of *Cinnamomum cassia* on Melanin Synthesis and Cell Viability

In order to find the active constituents from this plant, the methanol extract was chromatographed by using solvent-solvent partition. Hexane, chloroform, and ethyl acetate were used. As shown in Fig. 4, ethyl acetate fraction showed high inhibition in melanin synthesis without any cytotoxicity.

DISCUSSION

A number of melanogenesis inhibitors have been reported and are being used as cosmetic additives for the treatment of hyperpigmented skin disorders. However, many of them are of limited effectiveness, cause reactions or side effects after long-term use and are difficult to formulate. Therefore, many efforts have been devoted to screening putative depigmenting agents from natural products. In this study, with an attempt to find and develop new depigmenting agents, *Cinnamomum cassia* Presl showed good inhibition in melanin synthesis and did not show cell toxicity even at high concentration (200 μg/ml).

Oxidative stress may be induced by increasing generation of re-

active oxygen species (ROS) and other free radicals. UV radiation can induce formation of ROS in skin such as singlet oxygen and superoxide anion, promoting biological damage in exposed tissues via iron-catalyzed oxidative reactions. These ROS enhance melanin biosynthesis, damage DNA, and may induce proliferation of melanocytes. Presl showed good antioxidant activity (IC_{50}=3.7 μg/ml) but did not inhibit tyrosinase activity. These results suggest that the depigmenting effect of this sample works through removing ROS but does not inhibit tyrosinase activity directly.

After fractionation by using solvent-solvent extraction, ethyl acetate fraction showed high inhibition in melanin synthesis without cell toxicity. Further study on the isolation of the exactly effective pure compound and the mechanism of melanin inhibition should be continued.

CONCLUSION

In this study, we showed that *Cinnamomum cassia* Presl exhibited low cytotoxicity and high depigmenting activity. Possible mechanism of depigmenting is removal of radicals due to its antioxidant property. Considering that this herb is traditionally used as an edible food additive, *Cinnamomum cassia* Presl extract can be useful and safe as a new whitening agent in cosmetics.

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