Anti-melanogenesis effect of 2,5-dimethyl-4-hydroxy-3 [2H]-furanone

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Abstract

DMHF (2.5-dimethyl-4-hydroxy-3[2H]-furanone), an antioxidative compound from the reaction of L-cysteine/D-glucose scavenged efficiently 1,1-diphenyl-2-picryl hydrazyl free radicals. It exhibited an inhibitory effect on the autoxidation of linolenic acid, and the protective effect against UV cytotoxicity in cultured human fibroblast. In addition, DMHF appeared to prevent the cellular melanogenesis in the cultured murine melanoma cells more effectively than kojic acid, a well known inhibitor of melanogenesis, while the former was not so effective as the latter for the inhibition of the tyrosinase. Considering that cellular melanogenesis is a metabolic process triggered by oxidative stress, it òvas tentatively deduced that the antioxidative property of DMHF might afford the effect against cellular pigmentation by alleviating the causative stress. In toxicological tests such as irritation and sensitization, this compound turned out to be safe. The results of this study suggest that DMHF may be a novel inhibitor of melanogenesis, and that might be useful for application in cosmetics.

I. Introduction

In mammals, most visible pigmentation results from the synthesis and distribution of melanins, which are inert and heterogeneous biopolymer. Melanins play a critical role in the absorption of free radicals, and melanogenesis in the skin is a kind of process that produces photoprotective agents against damaging effect of UV. For the Orientals, white skin has been thought to be one of the criteria of beauty, and skin pigmentation such as freckles has been thought to be a nuisance. So. many cosmetic companies have tried to find inhibitor of melanogenesis that causes cellular pigmentation. Melanins are formed through the progressive oxidation of the amino acid, tyrosine. Melanogenesis starts with the action of tyrosinase that converts tyrosine to dopaquinone and proceeds with series of oxidation processes to form melanin. The regulation of cellular pigmentation can be controlled at many different stages of melanogenesis. Especially, tyrosinase inhibitor and antioxidant can be used for inhibition of cellular piomentation, because melanin producing process is enzymatic and nonenzvmatic oxidation reaction. We previously isolated HMF (4-hydroxy-5-methyl-3[2H]-furanone) from pine needles as

antioxidative ingredient, and reported that this compound reduced oxidative stress in skin cell and inhibited synthesis of melanin (I). In this study. we report stability and safety of DMHF (2, 5-dimethyl-4-hydroxy-3[2H]-furanone), a derivative of HMF, and its inhibitory effect on tyrosinase and melanogenesis.

II. Materials and Methods

1. Materials and Instruments

DMHF was prepared by the reaction of xylose and glycine through Millard reaction (2) and analyzed by HPLC with Ci91 4100 pump and SM 5000 detector of Thermo Separation Products Co. For UV spectra, CECIL CE5500 spectrometer was used.

2. Methods

(1) Free radical scavenging activity

To determine free radical scavenging activity of DMHF, 100 μM of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and various concentrations of samples were added to 2 ml of ethyl alcohol. After 30 min. of incubation at 37°C. the optical density was measured at 516 nm (3).

(2) Antioxidative activity against lipid autoxidation

The reaction mixtures (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).

(3) Inhibition of 3, 4-dihydroxyphenylalanine (DOPA) autoxidation

The reaction mixtures containing 500 μM 3, 4-dihidroxyphenylalanine and 50 mM potassium phosphate buffer (pH 6.8) in the presence of various concentrations of samples were incubated for 48 hours at 37°C, then UV absorbance was measured at 475 nm (5).

(4) Inhibition of tyrosinase

0.1 mg/ml of tyrosine. 70 units/ml of mushroom tyrosinase were added to 1.5 ml of 50 mM potassium phosphate buffer (pH 6.8) containing samples. The reaction mixture was incubated for 10 min. at 37°C and W absorbance was measured at 475 nm (6).

(5) Inhibition of cell pigmentation

Murine melanoma cells were cultured in Eagle's minimal essential medium containing 10 % fetal bovine serum. Murine melanoma cells were seeded in 75 T flask containing this medium at density of 1.0 x 10^8 cells/flask, and incubated for 2 days at 37°C in 5 % CO_2 after sample addition. Then trypsin was added and cells were collected by centrifugation. The degree of cell pigmentation was observed.

(6) Stability test of DMHF

Stability test was carried out at 37°C and in the presence of light. 0.1 % solutions of sample in ethyl alcohol, 50 % ethyl alcohol and water were placed respectively in 37°C incubator and a window. The content change of sample was analyzed by HPLC with the passage of time. (Eluent: 5 % acetonitrile, flow rate: 1 ml/min. detection: UV 288 nm)

(7) Cytotoxicity test of DMHF

Human fibloblasts were seeded in 96 well plate at density of 10^4 cells/well, supplemented with 0.2 ml of EMEM medium containing 2 % FBS, and incubated for 24 hours. After sample addition. incubated for another 24 hours, the survival and proliferation of cells were evaluated by MTT assay (7). 0.1 ml of MTT solution was added to each wells and incubated for 3 hours. After removing of media, 0.5 ml of DMSO was added and formed formazan was measured by UV absorbance at 570 nm using ELISA reader.

(8) Acute oral toxicity of DMHF

According to SOP of National Health and Safety Research Institute. test groups were composed as Table-I. Eight-week-old ICR mice weighing 28 - 35 g were fed the samples solubilized in saline. Body weights and clinical signs were monitored for 2 weeks and LD^{50} were measured.

Table 1. Test of acute oral toxicity of HDMF

Dosage (g/kg)	Dose amount (ml/kg)	Sex	Number
0.5	20	M	5
		F	5
5.0	20	M	5
		F	5

9) Allergenicity test

Allergenicity test of DMHF was carried out by Guinea pig maximization test (8). Test materials in saline and FCA was injected subcutaneously for allergy induction. The challenge was carried out by closed patch test. For (-) control, ethyl alcohol and 1,3-butylene glycol were used and for (+) control, DNCB (2, 4-dinitro chlorobenzen) was used. Using the scoring system of Kilgman, the allergenic potential was evaluated.

III. Results and Discussions

DMHF is uncommonly present in some plant species (9) and can be synthesized by Maillard reaction of methylpentose and amino acids. It is very important as flavors of food. Antioxidative activity can be measured by reduction of DPPH to 1,1 diphenyl-2picrylhydrazine, which cause decrease in optical density at 516 nm. DPPH radical scavenging activity of DMHF (the concentration that reducing 50 % of 100 μM DPPH, SC^{50} = 24 μg/ml) was stronger than those of kojic acid and maltol that have similar structure with DMHF, but weaker than those of ascorbic acid and α-tochopherol (Table-2). DMHF also inhibited linolenic acid autoxidation. At concentration of 100 ppm, it inhibited lipid peroxidation stronger than α-tochopherol (Fig.l). The reason that DMHF had stronger inhibitory effect of lipid peroxidation in spite of having weaker DPPH free radical scavenging effect than a-tochopherol is that DMHF is more stable than α-tochopherol. In fact, DMHF was proved to be stable in stability test.

Table 2. DPPH free radical scavenging activity of various antioxidants

antioxidants	SC_{50} (ppm)
HDMF	24
kojic acid	250
maltol	500
L-ascorbic acid	3.3
α-tochopherol	9.5

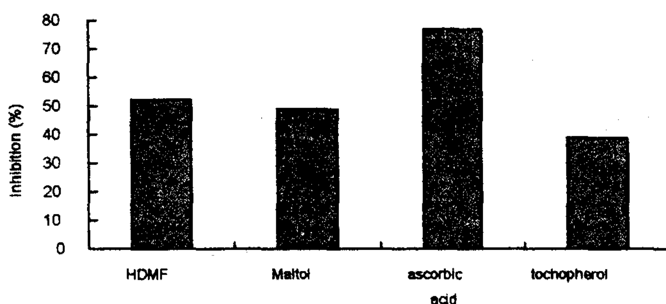


Fig. 1. Inhibitory effect of various antioxidants (100 g/ml) on the autoxidation of linolenic acid

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 oftyrosinase. In fact, kojic acid and arbutin which are known as tyrosinase inhibitors, have been used in cosmetics in expectation of skin whitening. In this study, the effect of DMHF on melanogenesis was examined using *in vitro* enzyme assay and cell culture method. At first, inhibition of tyrosinase-

catalyzed dopachrome formation was examined. DMHF (IC_{50}=120 ppm) inhibited stronger than arbutin (IC_{50}= 300 ppm) although weaker than kojic acid (IC_{50}= 7 ppm) (Fig. 2). In addition, DMHF inhibited dopa autoxidation stronger (IC_{50}= 110 ppm) than kojic acid while arbutin accelerated lipid autoxidation on the contrary (Fig. 3). DMHF also showed stronger effect on inhibition of pigmentation of cultured murine melanoma cell than kojic acid (Fig. 4). From this result, it is deduced that DMHF actually inhibited cellular pigmentation stronger than kojic acid by its antioxidative activity against oxidation of unstable intermediate of melanin as well as direct inhibition of tyrosinase. And its inhibitory activity against cellular pigmentation is thought to be largely due to its antioxidative property that can reduce oxidative stress and inhibit intermediate autoxidation, rather than tyrosinase inhibition.

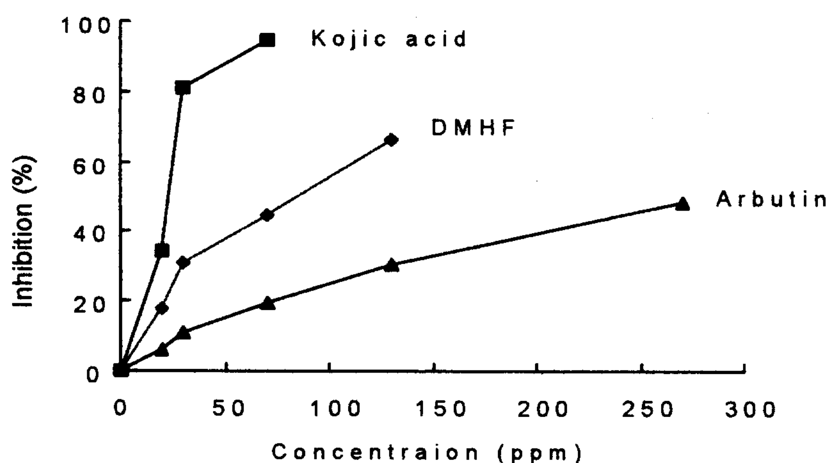


Fig 2. Effect against the tyrosinase-catalyzed dopachrome formation.

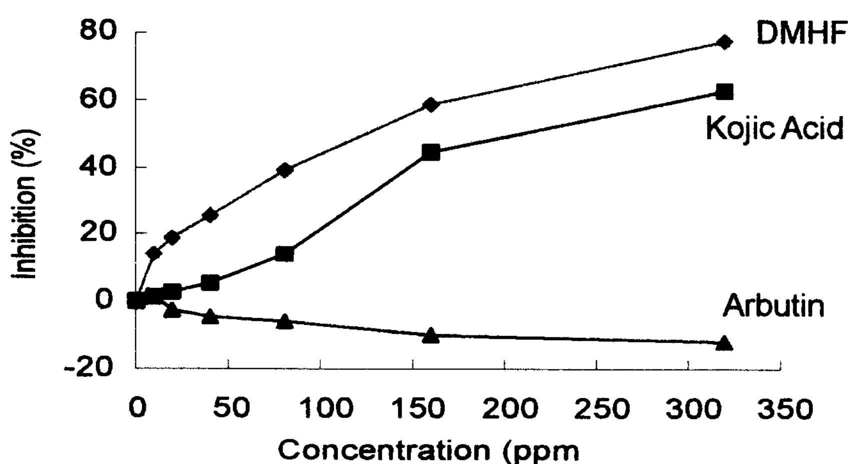
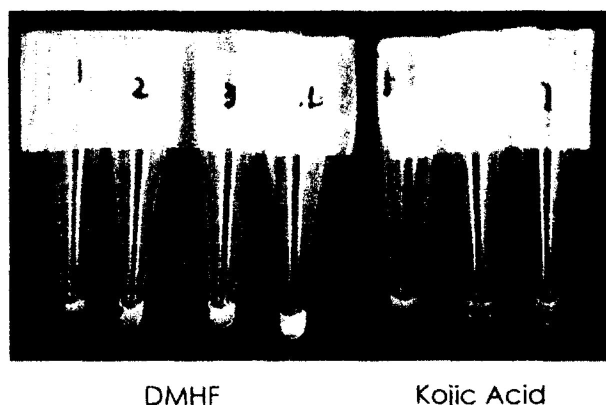


Fig 3. Antoxidative activity against DOPA autoxidation.



DMHF Kojic Acid

Fig. 4. Photograph of melanoma cells cultured in the absence and in the presence of DMHF or Kojic acid (1: control, 2: HDMF 20 μg/ml, 3: DMHF 50 μg/ml, 4: DMHF 100 μg/ml, 5: Kojic acid 20 μg/ml, 6: Kojic acid 50 μg/ml, 7: Kojic acid 100 μg/ml)

The safety of DMHF was evaluated by cytotoxicity on human fibroblasts, acute oral toxicity, and allegenicity tests. In cytotoxicity test on human fibroblasts, LD_{50} of DMHF was about 8000 ppm, while LD_{50} of L-ascorbic acid were 4 ppm, which showed that DMHF is much safer compound than ascorbic acid. In acute oral toxicity test, LD_{50} of DMHF was more than 5 g/kg, and in allegenecity test, DMHF was also proved to be safe. DMHF inhibited cellular pigmentation by reducing oxidative stress and inhibiting of tsrosinase and dopa autoxidation, and it is expected to have whitening effect in human skin. In addition. owing to its safety and stability, it can be applied to cosmetics.

IV. References

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