

## Idescarpin Isolated from the Fruits of *Idesia polycarpa* Inhibits Melanin Biosynthesis

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Received: January 26, 2005

Accepted: March 29, 2005

**Abstract** Tyrosinase is an enzyme that catalyzes the biosynthetic pathway of melanin pigments participating in the coloring of skin, hair, and eyes, and is widely distributed in nature. The inhibitory compounds of tyrosinase have been extensively used as a cosmetic agent with a skin-whitening effect. In this paper, several plant extracts were screened using Melan-a cells for the melanin biosynthesis inhibition activity, and *Idesia polycarpa* was selected. A melanin biosynthesis inhibitor was isolated from *I. polycarpa* fruits by activity-guided fractionation, and the inhibitor was identified as 6-hydroxy-2-[[[(1-hydroxy-6-oxo-2-cyclohexen-1-yl)carbonyl]oxy]methyl]phenyl  $\beta$ -D-glucopyranoside (idescarpin) by comparing it with reported spectral data. Idescarpin ( $IC_{50}=8 \mu\text{g/ml}$ ) reduced melanin content compared with the vehicle. In addition, the inhibitory activity of idescarpin for melanin synthesis is mediated by decreasing tyrosinase protein rather than directly inhibiting the tyrosinase activity. These results suggest that idescarpin isolated from *I. polycarpa* fruits may be used as a skin-whitening agent.

**Key words:** *Idesia polycarpa*, idescarpin, tyrosinase, Melan-a cell, melanin biosynthesis inhibitor, skin-whitening

Melanin is the pigment responsible for the color of human skin and hair. It is secreted in the basal layer of the dermis by melanocyte cells [1, 2]. Its roles are to protect the skin from ultraviolet (UV) damage and remove reactive oxygen species (ROS). Although melanin has an important role in protecting the skin against UV damage, an excessive or uneven melanin production can lead to the formation of freckles and age spots. Various dermatological disorders are concomitant with the accumulation of an excessive

level of epidermal pigmentation [3]. The type and amount of melanin synthesized by melanocyte and its distribution in the surrounding keratinocytes determine the actual color of the skin. Synthesis of melanin starts from the conversion of the amino acid L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), and the oxidation of L-DOPA yields dopaquinone by the enzyme tyrosinase (EC 1.14.18.1). The enzyme catalyzes two distinct reactions involving molecular oxygen. Quinones are highly reactive compounds and can be polymerized spontaneously to high-molecular-weight compounds or brown pigments (melanins), or quinones can react with amino acids and proteins that enhance the yellow color produced [4, 5]. Tyrosinase is a key enzyme in melanogenesis. However, it has recently been shown that melanocytes contain a second group of melanogenic enzymes, dopachrome tautomerase (Dct), and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase (Tyrp1) [6–8]. Melanin biosynthesis can be inhibited by avoiding UV exposure, by inhibiting tyrosinase, or by inhibiting melanocyte metabolism and proliferation [9, 10]. A number of melanogenesis inhibitors such as arbutin and kojic acid have been reported and are being used as cosmetic additives. However, there is no report on natural compounds that increase melanogenesis [11]. Recently, much attention has been focused on the application of natural products in cosmetics [12–21]. From this point of view, the effect of the extracts of plants on the inhibition of melanin biosynthesis was examined. Idescarpin was isolated from *Idesia polycarpa* fruits.

*Idesia polycarpa* is a deciduous tree of the Flacourtiaceae family and is distributed in forests at medium and, rarely, at low altitudes. A variety of compounds have been found in this plant; pyrocatechol, salirepin, benzyl alcohol, phenethyl alcohol, fatty acids, idesin, idesin hydrogen sulfate, and flavonoids [22, 23]. In the present study, the inhibitory effect of the compound obtained from *Idesia polycarpa* on melanin biosynthesis was investigated.

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## MATERIAL AND METHODS

### Plant Materials

The methanol extract of *Idesia polycarpa* was obtained from Plant Extract Bank in KRIBB (Daejeon, Korea).

### Mushroom Tyrosinase Inhibitory Activities

The reaction mixture for the mushroom tyrosinase (E.C 1.14.18.1, Sigma) activity determination consisted of 150  $\mu$ l of 0.1 M phosphate buffer (pH 6.5), 3  $\mu$ l of sample solution, 8  $\mu$ l of mushroom tyrosinase (2,100 unit/ml, 0.05 M phosphate buffer, pH 6.5), and 36  $\mu$ l of 1.5 mM L-tyrosine in a 96-well microplate (SPL, Korea). The tyrosinase activity was determined by reading the optical density at 490 nm on a microplate reader (Bio-Rad 3550, CA, U.S.A.) after incubating at 37°C for 20 min. The inhibitory activity of the sample was expressed as the concentration that inhibits 50% of the enzyme activity ( $IC_{50}$ ).

### Inhibitory Effect of Melanin Production in *Streptomyces bikiniensis*

The melanin synthesis inhibitory activity was determined by the paper-disc agar diffusion method. A preserved culture of *S. bikiniensis* NRRL B-1049 was inoculated on a Papavizas' VDYA agar slant, which contained V-8 juice (Campbell Soup Co.) 200 ml, glucose 2 g, yeast extract (Difco) 2 g, CaCO<sub>3</sub> 1 g, agar (Difco) 20 g, and distilled water 800 ml. The pH was adjusted to 7.2. After incubation 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 mycelium was scraped with an inoculating loop. The spore suspension thus obtained was transferred to sterile microtubes. A 0.4-ml aliquot of the spores suspension of *S. bikiniensis* was added to the agar medium ISP No. 7 (40 ml) supplemented with Bacto-yeast extract (Difco) 0.2%, and was spread over the agar surface uniformly with a glass hockey bar. After the agar surface was dried, a paper disc (8-mm diameter) soaked with the sample solution was placed on the agar plate. The plate was incubated at 28°C for 48 h; the diameter zone (mm i.d.) of melanin formation was measured from the reverse side of the plate.

### Extraction and Isolation

The methanol (MeOH) solution was evaporated to dryness (1.92 g). The MeOH extract was suspended with H<sub>2</sub>O (500 ml) and then partitioned successively with hexane, ethyl acetate (EtOAc), and butanol (BuOH). Among the solvent fractions, the EtOAc fraction showed the inhibitory activity of melanin synthesis in Melan-a cells. Accordingly, the EtOAc fraction (0.4 g) was concentrated *in vacuo* and chromatographed on a C-18 reversed phase HPLC with an elution solvent system of 50% aqueous acetonitrile (YMC-ODS-AM 250 $\times$ 6 mm, 10  $\mu$ m; flow rate, 1.5 ml/min; detection, UV at 220 nm) to afford compound (40 mg).

### Cell Cultures

The melanocyte line, Melan-a, was kindly provided by D.C. Bennett (St George's Hospital Medical School, London, U.K.). The Melan-a line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), streptomycin-penicillin (100  $\mu$ g/ml each), and 200 nM tetradecanoyl phorbol acetate (TPA), a potent tumor promoter, at 37°C in 5% CO<sub>2</sub>. Cells were passed every 3 days until a maximal passage number of 40. Confluent monolayers of melanocytes were harvested with a mixture of 0.05% trypsin and 0.53 mM EDTA (Gibco BRL, Grand Island, NY, U.S.A.).

### Cell Viability Assay

Cell viability was determined using a crystal violet assay. After being incubated with the test substances for 24 h, the culture medium was removed and replaced with 0.1% crystal violet in 10% ethanol. Cells were stained for 5 min at room temperature and rinsed three times. The crystal violet retained by adherent cells was then extracted with 95% ethanol. Absorbance was determined at 540 nm.

### Melanization Inhibition Assay on Melan-a Cell

Cells were seeded into a 24-well plate (Falcon, U.S.A.) at a density of  $1 \times 10^5$  cells per well and allowed to attach overnight. The medium was replaced with fresh medium containing various concentrations of compounds. Cells were cultured for 72 h and further incubated for a day. After washing them with phosphate-buffered saline (PBS), the cells were lysed with 250  $\mu$ l of 0.85 N KOH and transferred to a 96-well plate. The melanin contents were estimated by measuring the absorbance at 405 nm. Phenylthiourea (PTU) was used as a positive control [24].

### Western Blot Analysis

Cells were grown in a 6-well plate and treated with test substances. They were washed with ice-cold PBS 3 times, and lysed in cold lysis buffer (0.1 M Tris-HCl, pH 7.2, 1% Nonidet P-40, 0.01% SDS, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin). An aliquot of lysate was measured by the Bradford assay (Bio-Rad, #500-0002) using BSA as a standard. Thirty  $\mu$ g of protein per lane was separated by 8% SDS-polyacrylamide gel electrophoresis. Resolved proteins were blotted onto PVDF membrane (Milipore, Bedford, MA, U.S.A.). The membrane was blocked with 5% non-fat skim milk in Tris-buffered saline containing 0.05% Tween 20. Blots were incubated with the appropriate primary antibodies. Tyrosinase, Tyrp1, Dct, and  $\beta$ -actin were detected using rabbit polyclonal anti- $\alpha$ PEP7 antibody, rabbit polyclonal anti- $\alpha$ PEP1 antibody, rabbit polyclonal anti- $\alpha$ PEP8 antibody (1:1,000, a gift from Dr. V. J. Hearing, National Institutes of Health, Bethesda, MD, U.S.A.) and mouse monoclonal anti- $\beta$ -actin antibody (1:5,000, Sigma, St. Louis, MO, U.S.A.). Then, they were further incubated with horseradish peroxidase-

conjugated secondary antibody. Bound antibodies were detected using the Amersham ECL system.

## RESULTS

### Structure Elucidation of Compound

Compound (Idescarpin): syrup; ESI-MS  $m/z$  441.1  $[M+H]^+$ ;  $^1H$ -NMR ( $CD_3OD$ , 300 MHz, ppm): 7.01 (t, H-4), 6.87 (dd,  $J=2.0$ , 7.8, H-5), 6.79 (dd,  $J=2.0$ , 7.8, H-3), 6.13 (m, H-3"), 5.74 (dt,  $J=1.5$ , 8.5, H-2"), 5.46 (d,  $J=12.5$ , H-7a), 5.29 (d,  $J=12.5$ , H-7b), 4.61 (d,  $J=6.7$ , H-1'), 3.50-3.45 (m, H-2',3',4'), 3.85 (dd,  $J=2.2$ , 12.5, H-6'), 3.73 (dd,  $J=4.8$ , 12.5, H-6'), 3.35 (m, H-5'), 2.93-2.85 (m, H-5"a), 2.65-2.41 (m, H-4, H-5"b);  $^{13}C$ -NMR ( $CD_3OD$ , 75 MHz, ppm): 207.3, 171.4, 151.0, 144.7, 133.3 (CH), 131.1, 129.2 (CH), 126.8 (CH), 121.0 (CH), 118.3 (CH), 107.2 (CH), 79.2, 78.3 (CH), 77.7 (CH), 75.3 (CH), 70.9 (CH), 64.9 ( $CH_2$ ), 62.3 ( $CH_2$ ), 36.8 ( $CH_2$ ), 27.1 ( $CH_2$ ).

From  $^1H$ -NMR,  $^{13}C$ -NMR, and ESI-MS spectra data, the compound was identified as idescarpin [6-hydroxy-2-[[[(1-hydroxy-6-oxo-2-cyclohexen-1-yl)carbonyl]oxy]methyl] phenyl  $\beta$ -D-glucopyranoside] [22] (Fig. 1).

### Effects of Idescarpin on Tyrosinase Activity and *S. bikiniensis* Melanin Biosynthesis

The use of L-tyrosine as substrate enabled us to measure the inhibition rate of *o*-hydroxylation of tyrosine. The inhibitory activity was monitored by spectrophotometric measurement of dopachrome formation. We determined the tyrosinase activity, and found that idescarpin was not inhibited by the oxidation of tyrosine (Table 1). Furthermore, idescarpin did not inhibit melanin biosynthesis of *S. bikiniensis* (data not shown).

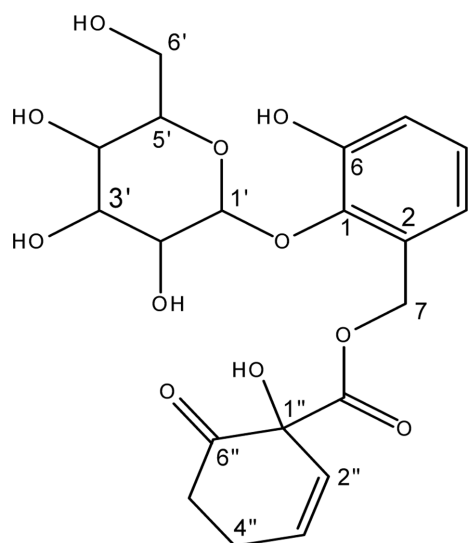


Fig. 1. Chemical structure of idescarpin.

Table 1. Effects of idescarpin against mushroom tyrosinase.

Sample	Concentration ( $\mu g/ml$ )	Inhibition <sup>a</sup> (%)	IC <sub>50</sub> <sup>b</sup> ( $\mu g/ml$ )
Idescarpin	100	10.3 $\pm$ 0.8	>200
	10	11.1 $\pm$ 2.2	
	1	5.6 $\pm$ 0.3	
Kojic acid	100	67.7 $\pm$ 0.8	50
	10	34.3 $\pm$ 0.9	
	1	11.6 $\pm$ 1.5	

<sup>a</sup>Tyrosinase was preincubated with test substances at 25°C for 5 min prior to incubation with tyrosinase for 30 min, and the absorbance was read at 490 nm. Each value represents the mean S.D. of three experiments.

<sup>b</sup>50% inhibitory concentration.

### Effects of Idescarpin on Melanin Synthesis of the Melan-a Cell Line

Desirable skin-whitening agents should inhibit the synthesis of melanin in melanosomes by acting specifically to reduce the synthesis or activity of tyrosinase, exhibit low cytotoxicity, and be nonmutagenic [25]. Therefore, idescarpin, PTU, and kojic acid were examined for melanin biosynthesis of Melan-a cells. Reduced pigmentation was seen in the cells treated both idescarpin and PTU. The 50% inhibitory concentration of melanin biosynthesis was about 8  $\mu g/ml$  by idescarpin and 1  $\mu g/ml$  by PTU. However, kojic acid had no effect on the melanin production of Melan-a cells (Fig. 2) (Table 2).

### Idescarpin Reduces Tyrosinase Synthesis in Melan-a Cell

Three melanocyte-specific enzymes [tyrosinase, tyrosinase-related protein 1 (Tyrp1), and tyrosinase-related protein 2 (Dct)] are involved in catalytic processes that convert tyrosine to melanin [26]. To characterize the amount of those enzymes during the decreased pigmentation by

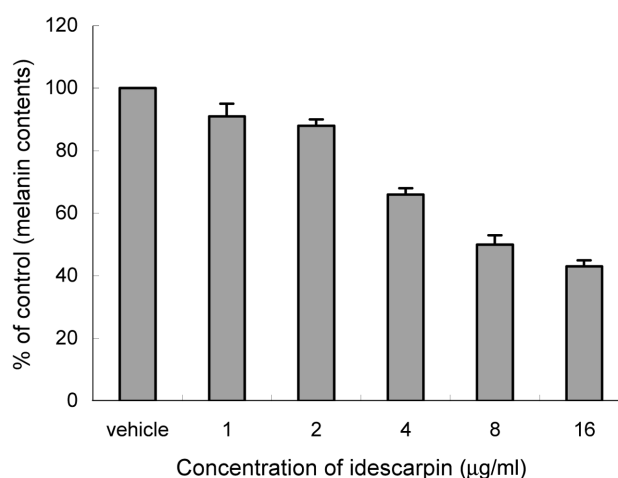


Fig. 2. Effect of idescarpin on melanogenesis in Melan-a cells. Melan-a cells were treated with idescarpin at various concentrations for 60 h. Data are expressed as % of control and each column represents the mean $\pm$ S.E. of three determinations.

**Table 2.** Effects of idescarpin, PTU, and kojic acid on cell growth and melanin production of Melan-a cells.

Sample	Melanin synthesis IC <sub>50</sub> (µg/ml)	Cytotoxicities LD <sub>50</sub> (µg/ml)
Idescarpin	8	30
PTU	1	100
Kojic acid	150	>150

Melan-a cells were grown to confluence in 24-well culture plates overnight. Test samples were added to the plates and incubated for 3 d. The medium containing compound was renewed every day and further incubated for a day. After 5 days of incubation, ELISA determined viability and melanin content in Melan-a cells. Phenylthiourea (PTU) was used as a positive control. The results were reproduced with three different cultures.

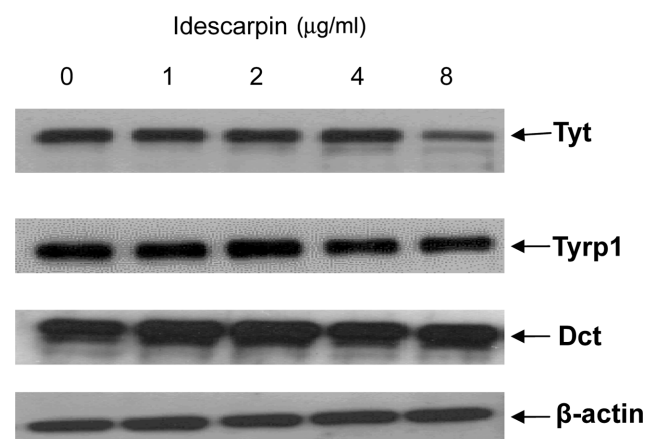
idescarpin, their expression levels were examined using Western blot analysis. This study showed that idescarpin decreased the expression of tyrosinase protein in Melan-a cells. However, it did not change the amount of TRP1 and Dct protein as much as the vehicle (Fig. 3).

## DISCUSSION

Recently, many efforts have been made in understanding the mechanism of melanogenesis to develop new therapeutic agents against skin pigmentation abnormalities [27–40]. The results of the present study revealed that the inhibitory effects of the crude extract of *Idesia polycarpa* fruits are attributable to idescarpin. Idescarpin is regarded as a promising skin-lightening agent because it inhibits melanin synthesis much stronger than other available skin-lightening agents, such as kojic acid. A compound with a concentration of 8 µg/ml exhibited 50% inhibition on the melanin

contents of Melan-a cells without cytotoxicity. In addition, the inhibitory activity of idescarpin for melanin synthesis is mediated not by direct inhibition of tyrosinase activity, like other drugs, but by decreasing tyrosinase protein.

In a Western blot analysis, idescarpin significantly decreased the amount of tyrosinase protein when compared with the extent level of expression in untreated control. Recently, it has been reported that tyrosinase, which is not matured in the endoplasmic reticulum (ER), was targeted for degradation and could not be transferred to melanosomes. In amelanotic melanoma cell lines, tyrosinase appeared mostly in a 70 kDa core-glycosylated, endoglycosidase H (endoH)-sensitive, immature form. This tyrosinase was retained in the ER and then degraded, but failed to reach the melanosomes [41–43]. In oculocutaneous albinism type 1, mutant tyrosinase, which is sensitive to endoH, is retained in the ER, and then degraded [44, 45]. According to another report, proper folding of tyrosinase is accomplished by direct interaction with calnexin for a definite time at passage through the ER [46]. However, if the dissociation from calnexin is inhibited, folding is prevented, thus the protein does not go through the normal secretory pathway and becomes targeted for degradation [47]. It has also been reported that DDB (2,2'-dihydroxy-5,5'-dipropyl-biphenyl) downregulates melanin synthesis by inhibiting the maturation of tyrosinase, leading to an acceleration of tyrosinase degradation. Furthermore, linoleic acid inhibited melanin synthesis by acceleration of tyrosinase degradation, in a manner similar to DDB [48–50]. From these results, immature tyrosinase, including misfolded tyrosinase, is retained in the ER and then degraded. Therefore, it is speculated that idescarpin may inhibit the formation of mature tyrosinase. However, more studies are needed to further clarify the mechanism in detail. The skin-whitening effect of idescarpin is currently under investigation.

**Fig. 3.** Effect of idescarpin on the expression of tyrosinase protein.

Idescarpin decreased the amount of tyrosinase protein in Melan-a cells, but no effect was found on Tyrp1 and Dct protein levels. Melanocyte cultures were treated with 0, 1, 2, 4, and 8 µg/ml of idescarpin for 60 h and analyzed using Western blotting, as described in Materials and Methods.

## Acknowledgments

This research was supported by a grant (PF0321205-00) from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology of the Korean Government.

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