Inhibitory Effects of Active Compounds Isolated from Safflower (*Carthamus tinctorius* L.) Seeds for Melanogenesis

Jung Seop ROH,[a] Ji Young HAN,[a] Jung Han KIM,[b] and Jae Kwan HWANG*,[b]

[a]*HAITAI Confectionary R & D Center; Seoul, 140–190, Korea:* [b]*Department of Biotechnology, Yonsei University; Seoul, 120–749, Korea.* Received August 20, 2004; accepted September 1, 2004

In order to develop a new skin whitening agent, safflower (*Carthamus tinctorius* L.) seeds were evaluated for melanogenesis inhibitory activity and its active principles were identified following activity-guided isolation. The 80% aqueous methanol extract and ethyl acetate fraction from safflower seeds showed a significant inhibition for mushroom tyrosinase. Three active compounds, *N*-feruloylserotonin, *N*-(*p*-coumaroyl)serotonin, and acacetin, were isolated from the ethyl acetate fraction as the active principles. Compared with arbutin (IC_{50}=0.223 mM), the IC_{50} values of these compounds were 0.023, 0.074, and 0.779 mM for *N*-feruloylserotonin, *N*-(*p*-coumaroyl)serotonin, and acacetin, respectively. It was also found that *N*-feruloylserotonin and *N*-(*p*-coumaroyl)serotonin strongly inhibited the melanin production of *Streptomyces bikiniensis* and B16 melanoma cells in comparison with a known melanogenesis inhibitor, arbutin.

Key words tyrosinase; melanin; safflower (*Carthamus tinctorius* L.) seed; skin whitening

The melanin synthesis inhibitors have been of interest as target molecules of natural product chemistry because they are related to localized hyper-pigmentation in humans such as lentigo, nevus, ephelis, post-inflammatory state and melanoma of pregnancy. Epidermal and dermal hyper-pigmentation can be dependent on either an increased number of melanocytes or activity of melanogenic enzymes.[1,2] Tyrosinase (EC 1.14.18.1) is the key enzyme in the undesirable browning of fruits and vegetables, and the coloring of skin, hair and eyes in animals.[3,4] This enzyme plays a role in oxidation from L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and from DOPA to DOPAquinone, which is the initial step in melanin synthesis.[5] This enzymatic oxidation of L-tyrosine to melanin is important because melanin has many functions, and alterations in melanin synthesis occur in many disease states. Therefore, tyrosinase inhibitors have become increasingly significant in the food industry as well as in medicinal and cosmetic products.[6,7] A number of tyrosinase inhibitors are reported from both natural and synthetic sources, but only a few of them are used as skin-whitening agents, primarily due to various safety concerns. For example, linoleic acid, hinokitiol, kojic acid, naturally occurring hydroquinones, and catechols were reported to inhibit enzyme activity but also exhibited side effects.[8]

In the course of research on the isolation of biologically active substances from safflower seeds, we discovered that the 80% aqueous methanol extract and ethyl acetate fraction possessed potent tyrosinase inhibitory activity. Safflower seeds rich in unsaturated fatty acid and α-linoleic acid have been commonly consumed as vegetable oils in the USA and Europe, and also used for clinical treatments of osteoporosis and rheumatism in Korea.[9] Although the biological activities of this plant have been reported extensively, there is no documentation related to inhibitory activity with respect to melanogenesis. Thus, the present study was undertaken to investigate the melanogenesis inhibitory activity of safflower seeds and to identify further the active compounds.

MATERIALS AND METHODS

Materials and Reagents Safflower seeds were purchased from a safflower suppler in Kimchun, Kyungbuk, Korea. Special grade organic solvents for HPLC work were purchased from Burdick & Jackson, USA. Mushroom tyrosinase, L-DOPA, and arbutin were purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

Extraction and Isolation Safflower seeds were washed several times with distilled water and stored in a 40 °C dry oven until the water was largely removed. Five volumes of 80% MeOH was added to the pulverized samples and the mixture was stirred for 24 h. The resulting mixtures were filtered and extracts were concentrated to dryness at 40 °C under vacuum to produce the MeOH extract. The MeOH extract was partitioned successively with $CHCl_3$, EtOAc, BuOH, and H_2O. The EtOAc extract was separated by silica gel column chromatography with a $CHCl_3$–MeOH system ($CHCl_3$–MeOH, 9 : 1 → 4 : 1 → $CHCl_3$–MeOH–H_2O, 5 : 4 : 1) to give seven fractions. Fr. 2 was further purified by recycling preparative HPLC (LC-908, JAI, Japan) on a JAIGEL W-252 column (ϕ20 mm×500 mm) using 100% MeOH at a flow rate of 3.8 ml/min, monitored at 280 nm to yield three compounds. The chemical structures of these compounds were identified as *N*-feruloylserotonin, *N*-(*p*-coumaroyl)serotonin, and acacetin (Fig.1), respectively, based on the spectral results of a previous study.[10]

Inhibition of Tyrosinase The tyrosinase assay was performed by the method of Masamoto *et al.* with slight modifications.[11] First, 65 μl of 2.5 mM L-DOPA solution, 10 μl of DMSO with or without a sample and 105 μl of 0.1 M phosphate buffer (pH 6.8) were mixed. The mixture was preincubated at 25 °C for 10 min. before 20 μl of 1380 units/ml tyrosinase in aqueous solution was added, and the reaction was monitored at 475 nm. A control reaction was conducted with DMSO. The O.D. values were measured by a UV spectrophotometer at 475 nm using an ELISA Microplate Reader (PowerWaveX, Bio-Tek, U.S.A.). Arbutin was used as a reference.

Inhibition of Melanin Production in *S. bikiniensis* Melanin synthesis inhibitory activity was determined by a

* To whom correspondence should be addressed. e-mail: jkhwang@yonsei.ac.kr

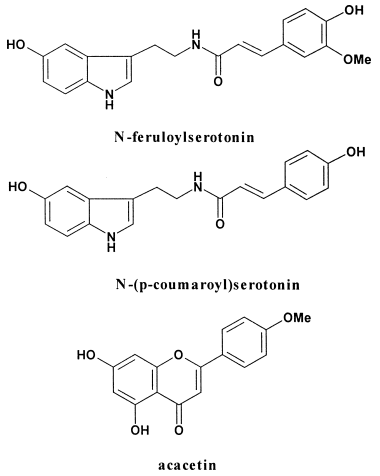

Fig. 1. Chemical Structures of Active Compounds Isolated from Safflower Seeds

paper-disc agar diffusion method using the inhibition of melanin production in *Streptomyces bikiniensis*.[12] A preserved culture of *S. bikiniensis* NRRL B-1049 (Korean Collection for Type Cultures, Daejon, Korea) was inoculated on a Papavizas' VDYA agar slant. After incubation at 28 °C for 2 weeks, the spore mass formed on the aerial mycelium was scraped with an inoculating loop. The spore suspension of *S. bikiniensis* was inoculated on an agar medium ISP No.7. After drying of the agar surface, a paper disc (8 mm) soaked with sample solution was placed on the agar plate. The plate was incubated at 28 °C for 48 h and the resulting zone of inhibition of melanin formation was measured from the reverse side of the plate.

Inhibition of Melanin Production in B16 Melanoma Cells B16 F1 melanoma cells, strain C57BL/6J, were purchased from the Korean Collection for Type Cultures (Daejon, Korea) and grown in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were placed in a 25 ml T-flask at a density of 1×10^5 cells/flask in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) containing 4.5 g/l of glucose, 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) antibiotic-antimycotic (Gibco, Auckland, N.Z.). After 24 h of cultivation, the medium with new DMEM medium containing test samples of various concentrations was replaced. After 5-d incubation, the adherent cells were washed with phosphate-buffered saline (PBS) and detached from the T-flask by trypsinization. The cells were collected in a test tube and washed twice with PBS. The number was determined by means of tryptan blue, and analyzed for melanin content. In order to extract melanin from the B16 F1 melanoma cells, 1.0 ml of the cell suspension was shaken with an equal volume of a mixture of 10% DMSO and 1N NaOH and stored at room temperature for 10 min. After centrifugation at 3000 rpm for 10 min, the melanin content was determined at 475 nm using the ELISA Microplate Reader (PowerWaveX, Bio-Tek, U.S.A.).

RESULTS AND DISCUSSION

We examined the inhibitory effect on mushroom tyrosinase activity using L-DOPA as a substrate. As shown in Fig. 2, the MeOH extract of safflower seeds clearly showed tyrosinase inhibitory activity in a concentration-dependent manner. Among the four fractions tested, the EtOAc fraction exhibited higher inhibitory activity for tyrosinase than the $CHCl_3$, BuOH, and H_2O fractions. Activity-guided isolation was carried out using the EtOAc fraction and recycling preparative HPLC finally led to the isolation of three active compounds, *N*-feruloylserotonin, *N*-(*p*-coumaroyl)serotonin, and acacetin as active principles. The IC_{50} values for *N*-feruloylserotonin, *N*-(*p*-coumaroyl)serotonin, and acacetin were 0.023, 0.074, and 0.779 mM, respectively (Table 1), while the IC_{50} value of the reference compound, arbutin, was 0.223 mM.

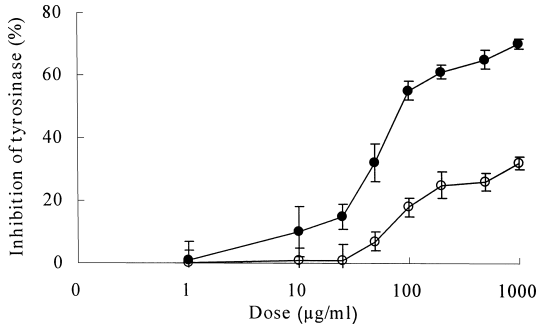

Fig. 2. Tyrosinase Inhibitory Activity of Safflower Seeds

(○) MeOH extract, (●) EtOAc fraction. Each value represents the mean ±standard error in triplicate.

Table 1. Inhibitory Activity on Tyrosinase and Melanin Formation of *S. bikiniensis*

Compound	*S. bikiniensis*	Tyrosinase
	Inhibition zone (mm)[a]	IC_{50} (mM)
N-Feruloylserotonin	35	0.023
N-(*p*-Coumaroyl)serotonin	28	0.074
Acacetin	11	0.779
Arbutin	0	0.223

a) compound 30 mg/paper disk.

We also examined the inhibitory effects of active compounds isolated from safflower seeds on melanogenesis by a paper-disc diffusion method using the inhibition of melanin production in *S. bikiniensis*. *N*-Feruloylserotonin and *N*-(*p*-coumaroyl)serotonin potently inhibited melanin production of *S. bikiniensis* in comparison with the known melanogenesis inhibitor arbutin (Table 1).

According to Nakajima *et al.*,[13] arbutin at concentrations in the range of 0.5—8 mM increased the pigmentation of cultured melanocytes. As cosmetics are usually used daily, the effects of the ingredients of cosmetics applied at concentrations in the high-dose range should be examined. Thus, the pigmentation-promoting effect of arbutin at concentrations higher than 1 mM should be given attention to.

A general strategy was described by Dooley for the discovery and development of novel topical skin-lightening products.[14] A desirable skin-lightening agent 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. Table 2 presents the assessment of active compounds in melanoma cells. The IC_{50} values of inhibition of cultured B16 melanoma cell pigmentation due to melanin synthesis were compared to the cytotoxicity effects of the compounds. *N*-Feruloylserotonin and

Table 2. Effects of Active Compounds on Melanin Synthesis in B16 Melanoma Cells

Compound	Melanin	Cytotoxicity
	IC_{50} (mM)	IC_{50} (mM)
N-Feruloylserotonin	0.191	>20
N-(*p*-Coumaroyl)serotonin	0.245	>20
Acacetin	>20	0.423
Arbutin	0.122	>20

N-(*p*-coumaroyl)serotonin were very effective agents, with IC_{50} of 0.191 and 0.245 mM, respectively. In addition, these compounds did not show any cytotoxicity, while acacetin exhibited some cytotoxicity with an IC_{50} of 0.423 mM. According to Curto *et al.*,[15] a potentially efficacious skin depigmentation agent is one that inhibits tyrosinase with an $IC_{50}<25\,\mu$g/ml, inhibits melanocyte pigmentation with an $IC_{50}<100\,\mu$g/ml, and is noncytotoxic to cells with an $IC_{50}>100\,\mu$g/ml. Of the compounds tested in the present study, *N*-feruloylserotonin and *N*-(*p*-coumaroyl)serotonin satisfied these requirements.

The inhibitory effects on the DOPA oxidase activity of tyrosinase and the melanin production of *S. bikiniensis* and B16 melanoma cells by *N*-feruloylserotonin and *N*-(*p*-coumaroyl)serotonin were evaluated in order to examine the relationship between structure and activity. Although *N*-feruloylserotonin and *N*-(*p*-coumaroyl)serotonin bear the same serotonin skeleton, the aromatic group is different. On the basis of the inhibitory activity on tyrosinase and melanin formation in Tables 1 and 2, *N*-feruloylserotonin bearing a methoxy group in the aromatic ring at the 3′ position stronger than that bearing a hydrogen group.

The first steps in the biosynthetic pathway of melanin are the hydroxylation of monophenol tyrosine to *o*-diphenol DOPA and the oxidation of DOPA to DOPAquinone, both using molecular oxygen. Tyrosinase catalyzes these steps, and antioxidants may inhibit these oxidation steps. We have previously reported that *N*-feruloylserotonin and *N*-(*p*-coumaroyl)serotonin showed the highest free radical scavenging activity and the inhibition of lipid peroxidation induced by ascorbic acid/Fe^{2-} and ADP/Fe^{3-}/NADPH in rat liver microsomes.[10] These results indicate that *N*-feruloylserotonin and *N*-(*p*-coumaroyl)serotonin exhibited a potent antioxidant effect as well as melanin synthesis inhibition. Therefore, the strong effects of *N*-feruloylserotonin and *N*-(*p*-coumaroyl)serotonin on melanin synthesis inhibition may be attributed to the combined antioxidant and tyrosinase inhibition activities.

It is meaningful to note the present investigation revealed for the first time that *N*-feruloylserotonin and *N*-(*p*-coumaroyl)serotonin are potent melanin synthesis inhibitors and thus may be considered as promising candidates for novel whitening agents.

REFERENCES

1) Griffiths C. E., Finkel L. J., Ditre C. M., Hamilton T. A., Ellis C. N., Voorhees J. J., *Br. J. Dermatol.*, **129**, 415—421 (1993).
2) Kanwar A. J., Dhar S., Kaur S., *Dermatology*, **188**, 170 (1994).
3) Kubo I., Kinst-Hori I., Chaudhuri S. K., Kubo Y., Scanchez Y., Ogura T., *Bioorg. Med. Chem.*, **8**, 1749—1755 (2000).
4) Perez-Gilabert M., Garcia-Carmona F., *Biochem. Biophys. Res. Commun.*, **285**, 257—261 (2001).
5) Shin N. H., Ryu S. Y., Choi E. J., Kang S. H., Chang I. M., Min K., Kim Y., *Biochem. Biophys. Res. Commun.*, **243**, 801—803 (1998).
6) Mosher D. B., Pathak M. A., Fitzpatrick T. B., "Update: Dermatology in General Medicine," ed. by Fitzpatrick T. B., Eisen A. Z., Wolff K., Freedberg I. M., Austen K. F., McGraw-Hill, New York, 1983, pp. 205—225.
7) Maeda K., Fukuda M., *J. Soc. Cosmet. Chem.*, **42**, 361—368 (1991).
8) Seo S. Y., Sharma V. K., Sharma N., *J. Agric. Food Chem.*, **51**, 2837—2853 (2003).
9) Kim H. J., Bae Y. C., Park R. W., Choi S. W., Cho S. H., Choi Y. S., Lee W. J., *Calcif Tissue Int.*, **71**, 88—94 (2002).
10) Roh J. S., Sun W. S., Oh S. U., Lee J. I., Oh W. T., Kim J. H., *Food Sci. Biotecnol.*, **8**, 88—92 (1999).
11) Masamoto Y., Ando H., Murata Y., Shimoishi Y., Tada M., Takahata K., *Biosci. Biotechnol. Biochem.*, **67**, 631—634 (2003).
12) Tomita K., Oda N., Ohbayashi H., Kamei T., Miyaki T., Oki T., *J. Antibiot.*, **43**, 1601—1605 (1990).
13) Nakajima M., Shinoda I., Fukuwatari Y., Hayasawa H., *Pigment Cell Res.*, **11**, 12—17 (1998).
14) Dooley T. P., *J. Dematol. Treat.*, **7**, 188—200 (1997).
15) Curto E. V., Kwong C., Hermersdorfer H., Glatt H., Santis C., Virador V., Hearing V. J., Jr., Dooley T. P., *Biochem. Pharmacol.*, **57**, 663—672 (1999).