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Natural *ortho*-dihydroxyisoflavone derivatives from aged Korean fermented soybean paste as potent tyrosinase and melanin formation inhibitors

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ABSTRACT

Natural *o*-dihydroxyisoflavone (ODI) derivatives with variable hydroxyl substituent at the aromatic ring of isoflavone and three known isoflavones were isolated from five-year-old Korean fermented soybean paste (*Doenjang*) and evaluated as potent inhibitors on tyrosinase activity and melanin formation in melan-a cells comparing with other known isoflavones, 7,8,4′-trihydroxyisoflavone (**1**) and 7,3′,4′-trihydroxyisoflavone (**2**) inhibited tyrosinase by 50% at a concentration of 11.21 ± 0.8 μM and 5.23 ± 0.6 μM (IC_{50}), respectively, whereas, 6,7,4′-trihydroxyisoflavone (**3**), daidzein (**4**), glycitein (**5**) and genistein (**6**) showed very low inhibition activity. Furthermore, those compounds significantly suppressed the cellular melanin formation by 50% at a concentration of 12.23 ± 0.7 μM (**1**), 7.83 ± 0.7 μM (**2**), and 57.83 ± 0.5(**6**) and show more activity than arbutin. But, compounds **3**, **4**, and **5** showed lower inhibition activity. This study shows that the position of hydroxyl substituent at the aromatic ring of isoflavone plays an important role in the intracellular regulation of melanin formation in cell-based assay system.

Melanin synthesis is principally responsible for skin color and plays a key role in the prevention of UV-induced skin damages. However, increased levels of epidermal melanin synthesis can darken the skin and produce various dermatologic disorders, such as melasma, age spots or liver spots, and actinic damage, resulting in the accumulation of excessive levels of epidermal and dermal pigmentation.[1] Melanogenesis is the process of melanin synthesis and distribution by a cascade of enzymatic and chemical reactions in melanocytes.[2] Because melanin production is mainly controlled by the expression and activation of tyrosinase, tyrosinase inhibitors, such as kojic acid and arbutin, have been established as important constituents of cosmetic products and depigmenting agents for hyper pigmentation.[3]

Recently, the application of naturally occurring products as melanin synthesis inhibitors in cosmetics has attracted much interest.[3] For example, plant polyphenols have been the target of several studies,[3,4] resulting in repeated reviews of their classification, occurrence, structural aspects, reactivity, biochemistry, and biogenesis. Polyphenols is a broad term used in literature on natural products to refer to substances that possess one or more benzene ring(s) and a hydroxyl group, including functional derivatives. Extensive literature is available on the screening of tyrosinase inhibitors among phenolics of plant origin, and polyphenols are currently the target of numerous studies.

Korean fermented soybean paste (*Doenjang*) is a unique fermented food in Korea. It has been traditionally manufactured from soybeans, so called *JangYang* process, which are fermented by diverse microorganisms including fungi and bacilli.[5,6] Epidemiological studies suggest that the consumption of *Doenjang* provides protection against cancers in humans. Also, a highly aged *Doenjang* showed greater biological activity.[6,7] Though, free isoflavones such as daidzein, genistein, and gycitein are produced during fermentation, they are generally known to be active compounds in *Doenjang*. The effect of aging on biological activity of *Doenjang* is not clear.[7] To develop depigmentation agents, we focused on the newly being formed compounds in highly aged *Doenjang*.

The five-year-old *Doenjang* (100 g) was defatted three times with *n*-hexane (3 L) for 3 h. After removal of the solvent by filtration, it was extracted with 2 L of MeOH in a Soxhlet apparatus for approximately 6 h and was then filtered. Evaporation of the solvent under reduced pressure provided the *Doenjang* extract (15.8 g). The *Doenjang* extract was subjected to medium-pressure liquid chromatography (MPLC) system (Yamazen Co., Japan) using a gradient elution system of distilled water (DW) and acetonitrile gradient with acetonitrile from 20% to 80% acetonitrile in 60 min at a flow rate of 30 mL/min. The pressure of system was 3 Mpa. A

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300 × 37 mm i.d., 50 μm, Ultra pack-ODS-S-50C column (Yamazen Co., Japan) was used. The detector wavelength was set at 263 nm. The fraction volume was 30 mL. Twelve fractions were collected and monitored by HPLC. The fractions were combined [fr. 1–4 (11.5 g), fr. 5 (0.06 g), fr. 6 (0.03 g), fr. 7 (0.02 g), fr. 8–9 (0.54 g), fr. 10–12 (1.30 g)] and compound **1** (fr. 5), compound **2** (fr. 6) and compound **3** (fr. 7) were isolated in yields of 0.06 g, 0.03 g and 0.02 g, respectively. Fr. 8–9 (0.54 g) were subjected to preparative TLC developed with CH_2Cl_2/methanol/water (5:4:1). From subfraction 4, 5 and 9, compound **4**, compound **5**, and compound **6** were isolated in yields of 0.07 g, 0.02 g and 0.03 g, respectively. To determine the structure of the isolated compounds, LC/MS and NMR analysis was conducted. Compounds **1** and **3** were identified as 7,8,4′-trihydroxyisoflavone and 6,7,4′-trihydroxyisoflavone, respectively (Fig. 1), on the basis of their LC/MS and NMR spectral data, which were in good agreement with those published previously.[8,9] Compound **2** was identified as 7,3′,4′-trihydroxyisoflavone[10] compounds **4**–**6** were identified as known isoflavones daidzein, glycitein, and genistein with comparing the authentic standard purchased from Sigma Co., respectively.[17]

Six isolated *o*-dihydroxyisoflavone (ODI) and isoflavone derivatives were subjected to tyrosinase inhibition assay using melan-a cell line as cell-based assay system.[22] After incubation for 3 days with various concentrations of compounds in cultured cell, a cell lysate containing tyrosinase was prepared and the inhibitory activity with L-tyrosine and L-Dopa measured. Arbutin isolated from the bearberry is well-known tyrosinase inhibitor and it is therefore used as a skin-lightening agent and a positive standard.[11] 7,8,4′-trihydroxyisoflavone (**1**) and 7,3′,4′-trihydroxyisoflavone (**2**) inhibited tyrosinase by 50% at a concentration of 11.21 ± 0.8 μM and 5.23 ± 0.6 μM(IC_{50}), respectively, whereas 6,7,4′-trihydroxyisoflavone (**3**), daidzein (**4**), glycitein (**5**) and genistein (**6**) showed low inhibition activity. The inhibitory activity of compounds 1 and **2** was more potent than that of which arbutin showed tyrosinase activity with IC_{50} values of 30.26 ± 0.4 μM. (Table 1) From the structural–activity point of view, we have found that *o*-dihydroxy group may contribute to the inhibitory activity against tyrosinase. It is suggested that the presence of the OH group at C-3′or C-8 increases the activity. However, C-6 position replacement with OH or OCH_3 decreases the activity very much.

The tyrosinase catalyzes the oxidation of L-Dopa to *ortho*-dopaquinone, and antioxidants may prevent the oxidation step by tyrosinase reaction in melanogenesis.[12] We measured antioxidation effect of ODI derivatives by superoxide radical scavenging effect. The superoxide scavenging test is a nonenzymatic method currently used to provide basic information on the reactivity of compounds to scavenge free radicals.[23] Table 2 shows the superoxide radical scavenging activity of three *o*-dihydroxyisoflavones and isoflavones. Compounds 1 and **2** showed significant antioxidant activity with IC_{50} values of 18.10 ± 0.2 and 10.54 ± 0.4 μM, respectively. The superoxide radical scavenging activities of ODI derivatives were more potent than that of which L-ascorbic acid (65.10 ± 3.5 μM), known tyrosinase inhibitor.[18] This result shows that the inhibitory effect of ODI derivatives against tyrosinase reaction is evidence for direct correlation with antioxidant activity.

As melanin formation is the most important factor to determine the mammalian skin color, the inhibition of melanin formation may result in a reduction of skin darkness. To evaluate the inhibitory potency against the melanin formation, three ODI derivatives and daidzein were assessed for their inhibitory effect in the cultured melan-a cells.[24] After treatment of compound for 5 days, a melanin fraction from a cell lysate was prepared and the melanin contents determined. A standard curve for melanin determination was prepared using synthetic melanin purchased from Sigma Co. Among those compounds, compounds **1** (IC_{50} = 12.23 ± 0.7 μM), **2** (IC_{50} = 7.83 ± 0.7 μM), and **6** (IC_{50} = 57.83 ± 0.5 μM) significantly suppressed the cellular melanin formation as compared to the inhibitory activity of arbutin (IC_{50} = 98.05 ± 0.4 μM) as shown in Table 1, whereas compounds **3**, 4 and **5** showed low inhibition activity. From the above data, it is suggested that the presence of the dihydroxyl group is very important in order to exhibit anti-

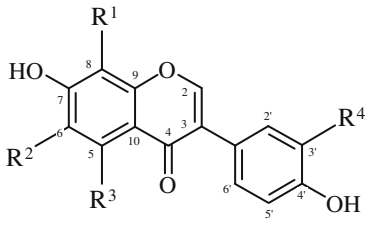
Table 1
Inhibitory effects of *o*-dihyroxyisoflavones and isoflavones on tyrosinase and melanin formation in melan-a cells

Compound	Tyrosinase IC_{50} (μM)	Melanin formation IC_{50} (μM)
1	11.21 ± 0.8	12.23 ± 0.7
2	5.23 ± 0.6	7.83 ± 0.7
3	>500	>500
4 (Daidzein)	>500	>500
5 (Glycitein)	>500	>500
6 (Genistein)	>500	57.83 ± 0.5
Arbutin	30.26 ± 0.4	98.05 ± 0.4

Table 2
Superoxide radical scavenging activity of *o*-dihyroxyisoflavones and isoflavones

Compound	IC_{50}[a] (μM)
1	18.10 ± 0.2
2	10.54 ± 0.4
3	>500
4 (Daidzein)	>500
5 (Glycitein)	>500
6 (Genistein)	489.00 ± 36.8
L-Ascorbic acid	65.10 ± 3.5

[a] IC_{50} denotes the antioxidant concentration causing 50% reduction of the free radical form of superoxide radical. It was calculated from regression line using different concentrations in triplicate experiments.



Compound	R^1	R^2	R^3	R^4
1	OH	H	H	H
2	H	H	H	OH
3	H	OH	H	H
4 (Daidzein)	H	H	H	H
5 (Glycitein)	H	OMe	H	H
6 (Genistein)	H	H	OH	H

Figure 1. Structure of *o*-dihydroxyisoflavone and isoflavones isolated from five-year-old *Doenjang*.

melanin formation activity. Compounds **1**, and **2** have an OH group in C-8 or C-3′ positions resulted in dihydroxyl group. This makes them potential candidates for melanin formation inhibition reaction, whereas the substitution of C-6 with OH or OCH_3 reduced inhibition activity. This type of inhibition is due to the direct tyrosinase inhibition binding of dihydroxyl group to the copper ion like arbutin.[19] The activity of compound **6** (genistein), which has very low tyrosinase inhibition and antioxidation activity, has no dihydroxyl group. It has another mechanism of melanin formation inhibition such as tyrosinase glycosylation inhibition and cell growth suppression.[20,21] These results suggest that compounds 1 and **2** can be utilized for the development of new candidate for tyrosinase and melanin formation inhibitors.

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10. (a) Compound **1** (7,8,4′-trihydroxyisoflavone): ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.86 (d, J = 8.7 Hz, H-3′/5′), 7.09 (d, 8.5 Hz, H-6), 7.45 (d, J = 8.7 Hz, H-2′/6′), 7.46 (d, 8.7 Hz, H-5), 8.32 (s, H-2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 114.8 (C-6), 115.7 (C-3′, 5′), 116.4 (C-5), 117.8 (C-10), 123.2 (C-3), 123.4 (C-1′), 130.5 (C-2′, 6′), 133.4 (C-8), 147.2 (C-9), 150.5 (C-7), 153.1 (C-2), 157.1 (C-4′), 175.7 (C-4); LC/MS m/z 271.3 [M+H].
(b) Compound **2** (7,3′,4′-trihydroxyisoflavone): ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.85 (d, J = 2.5 Hz, H-8), 6.96 (d, J = 2.5 Hz, H-6), 7.47 (d, J = 8.0 Hz, H-5′), 7.61 (d, J = 2.5 Hz, H-2′), 7.64 (d, J = 8.0 Hz, H-6′), 8.08 (d, J = 8.8 Hz, H-5), 8.17 (s, H-2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 102.6 (C-8), 115.6 (C-6), 115.8 (C-2′), 117.1 (C-5′), 117.1 (C-10), 120.3 (C-3), 124.1 (C-1′), 127.8 (C-5), 145.3(C-3′), 145.8(C-4′), 153.2 (C-2), 157.9 (C-9), 162.9 (C-7), 175.2 (C-4); LC/MS m/z 271.2 [M+H].
(c) Compound **3** (6,7,4′-trihydroxyisoflavone): ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.86 (d, J = 8.7 Hz, H-3′/5′), 6.91 (s, H-8), 7.45 (d, J = 8.7 Hz, H-2′/6′), 7.53 (d, J = 7.51 Hz, H-5), 8.08 (s, H-2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 103.4 (C-8), 108.7 (C-5), 115.5(C-3′, 5′), 117.0 (C-10), 123.3 (C-3), 123.2 (C-1′), 130.5 (C-2′, 6′), 145.4 (C-6), 151.4 (C-9), 153.0 (C-2), 153.0 (C-7), 157.3 (C-4′), 174.9 (C-4); LC/MS m/z 271.2 [M+H].
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22. *Tyrosinase inhibition assay*: The tyrosinase activity was measured with a modification of the method reported by Martinez-Esparza et al.[13] Melan-a cells were cultured for 3 days with various concentrations of the synthetic compounds. The cells were collected by centrifugation, washed with ice-cold PBS (pH 7.4), and resuspended in 1 ml of homogenization buffer (80 mM PO^4 buffer + 1% Triton X-100 + 100 μg/ml PMSF). The cell lysate was centrifuged at 12,500 rpm for 15 min to remove insoluble material. The protein concentration was determined by the Bradford method using BSA as a standard. The cell homogenate was mixed and incubated with 0.8 mM L-Dopa for 1 h at 37 °C and then the activity was assayed at 475 nm using ELISA micro plate reader.
23. *Superoxide radical scavenging assay*: Superoxide scavenging activities of the compounds were determined by monitoring the competition of those with NBT for the superoxide anion generated by the PMS–NADH system by Liu et al.[14] Superoxide radicals were generated in 1 ml 20 mM Tris–HCl buffer pH 8.0 containing 0.05 mM nitroblue tetrazolium (NBT), 0.01 mM phenazine methosulfate (PMS) and test compounds were preincubated for 2 min. The reaction was initiated by the addition of 0.078 mM NADH. Blue chromogen, formed due to NBT reduction, was read at 560 nm.
24. *Melanin formation assay*: The melanin content for inhibition assay on melanin formation was measured using a modified method.[15] After incubation for 5 days with the compounds, the cells were washed with ice-cold PBS (pH 7.4) and the pellets were collected by trypsinization and centrifugation. The pellets were dissolved in 1 N NaOH solution and the melanin was measured for absorbance at 400 nm using an ELISA reader. The melanin content was determined in μg from a synthetic melanin standard curve and correlated to mg protein using method of Kalb and Bernlohr.[16]