Bioorganic & Medicinal Chemistry Letters 18 (2008) 5006–5009

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

ortho-Dihydroxyisoflavone derivatives from aged Doenjang (Korean fermented soypaste) and its radical scavenging activity

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ARTICLE INFO

Article history:
Received 28 April 2008
Revised 22 July 2008
Accepted 6 August 2008
Available online 9 August 2008

Keywords:
ortho-Dihydroxyisoflavone
Doenjang
Antioxidant

ABSTRACT

One new *ortho*-dihydroxyisoflavone, 7,3′,4′-trihydroxyisoflavone (**2**), and two known *ortho*-dihydroxyisoflavone derivatives were isolated from 5-year-old *Doenjang* (Korean fermented soypaste), and evaluated as potent antioxidant by comparing with other known isoflavones. 7,8,4′-Trihydroxyisoflavone (**1**), 7,3′,4′-trihydroxyisoflavone (**2**), and 6,7,4′-trihydroxyisoflavone (**3**) inhibited DPPH (Diphenyl-1-picryl hydrazyl) formation by 50% at a concentration of 21.5 ± 0.2, 28.7 ± 0.4 and 32.6 ± 0.6 (IC_{50}), respectively, whereas three isoflavones showed weak DPPH radical scavenging activity. In xanthine oxidase (XO) system, in which both inhibition of xanthine oxidase and superoxide scavenging effect were measured in one assay. Compound **1** (IC_{50} = 6.6 ± 0.4 μM) and **2** (IC_{50} = 16.8 ± 1.2 μM) show significant inhibitory activity and greater effect than allopurinol. But, compound **3** and other isoflavones showed lower inhibition activity. This study shows that the position of hydroxyl substituent at the aromatic ring of isoflavone plays an important role in radical scavenging effect.

In recent years, cosmetic, pharmaceutical, and chemical industries have become increasingly interested in antioxidants.[1–4] Recent research efforts on antioxidants have focused on flavonoids that show strong free radical scavenging effects and metal ion chelating properties. In addition to their antioxidant activity, flavonoids have been reported to inhibit various enzymes such as cyclooxygenase and lipoxygenase related to inflammation.[5–7] Evidence for the presence of flavonoids in ancient remedies for burns and inflammation has been reported and these substances, which have been isolated, are presently used in commercial products.[5–7] Thus, dietary flavonoids have attracted attention for potential beneficial effects on humans.

Fermented foods are important components of traditional diets in some areas of the world. *Doenjang* (Korean fermented soybean paste) 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.[8,9] Epidemiological studies suggest that the consumption of *Doenjang* provides protection against cancers in humans. Also, a highly aged Doenjang showed greater biological activity.[9,10] Though free isoflavones, such as daidzein, genistein and glycitein, that are produced during fermentation are generally known to be active compound in *Doenjang*, the effect of aging on biological activity of Doenjang is not clear.[10] Because the longer fermentation or aging time of *Doenjang* may be an important factor in its biological activity, we focused on the newly formed compounds in aged Doenjang to discover and determine new antioxidants.

The 5-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) that has several new peaks, as shown in Figure 1. The *Doenjang* extract (15.8 g) 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% in 60 min at a flow rate of 30 mL/min. The pressure of system was 3 Mpa. A 300 × 37 mm id, 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 and 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. 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. 2), on the basis of their LC/MS and NMR spectral data, which were in good agreement with those published previously.[11,12] Compound **2** was identified as 7,3′,4′-trihydroxyisoflavone, which is isolated for the first time as natural constituent.[13]

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doi:10.1016/j.bmcl.2008.08.016

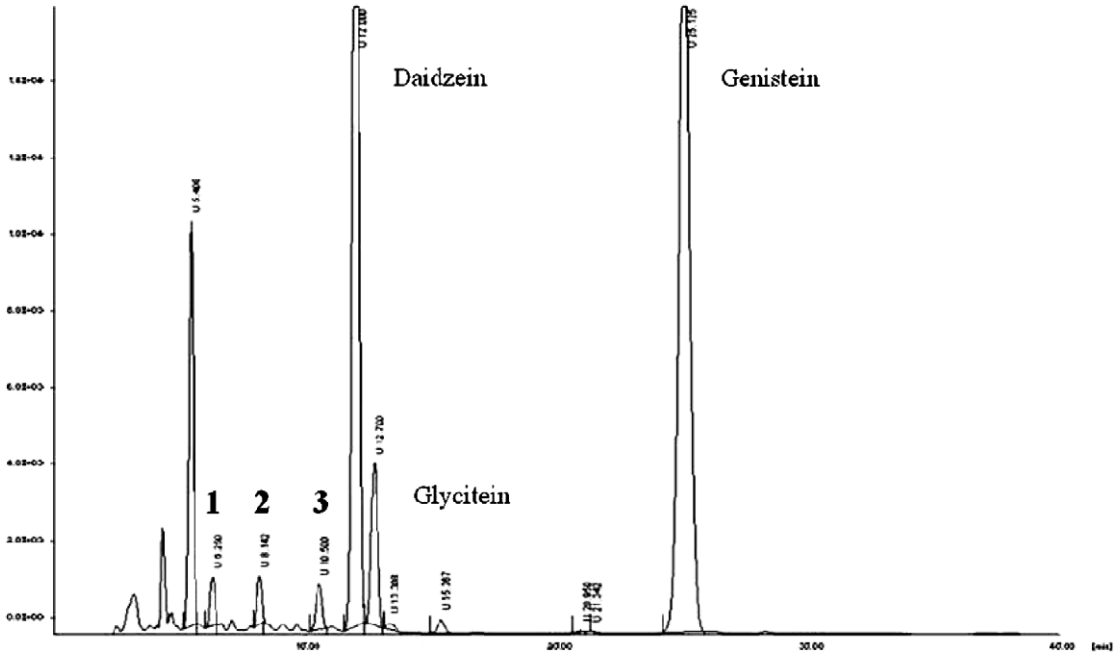
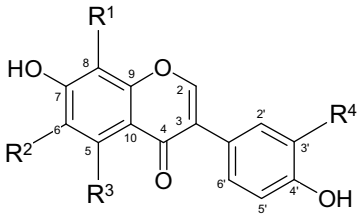


Figure 1. HPLC profile of 5-year-old *Doenjang* extract.



Compound	R^1	R^2	R^3	R^4
Compound **1**	OH	H	H	H
Compound **2**	H	H	H	OH
Compound **3**	H	OH	H	H
Daidzein	H	H	H	H
Glycitein	H	OMe	H	H
Genistein	H	H	OH	H

Figure 2. Structure of *ortho*-dihydroxyisoflavone isolated from 5-year-old *Doenjang* and other isoflavones.

Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities. The free radical scavenging activity of all isolated *ortho*-dihydroxyisoflavone derivatives from 5-year-old *Doenjang* was compared with that of known isoflavone such as daidzein, glycitein, and genistein by DPPH radical scavenging assay. The DPPH test is a non-enzymatic method currently used to provide basic information on the reactivity of compounds to scavenge free radicals.[14] Table 1 shows the DPPH radical scavenging activity of three *ortho*-dihydroxyisoflavones and isoflavones.[15] Compounds **1**, **2**, and **3** inhibited DPPH formation by 50% at a concentration of 21.5 ± 0.2, 28.7 ± 0.4, and 32.6 ± 0.6 (IC_{50}), respectively, whereas three isoflavones showed weak DPPH radical scavenging activity. The DPPH radical scavenging activities of *ortho*-dihydroxyisoflavone derivatives were more potent than that of Trolox and L-ascorbic acid which showed activity with IC_{50} values of 48.9 ± 0.6 and 52.3 ± 0.6. From the structure–activity point of view, we have found that *ortho*-dihydroxy group may contribute to the scavenging activity against DPPH radical.

Xanthine oxidase (XO) catalyzes the oxidation of xanthine to uric acid. During the reoxidation of XO, oxygen molecules act as electron acceptors, producing superoxide radicals and hydrogen peroxide. Consequently, XO is considered to be an important biological source of superoxide radicals. These are involved in many pathological processes such as inflammation, cancer, and aging.[16–18] To compare the anti-superoxide effect of *ortho*-dihydroxyisoflavone derivatives from 5-year-old *Doenjang*, both inhibition of XO and the scavenging effect on the superoxide anion were measured in one assay and compared with those of isoflavones.[19,20] For comparison of inhibition effect of these compounds, allopurinol, the most well-studied xan-

Table 1
DPPH radical scavenging activity of *ortho*-dihydroxyisoflavone derivatives and other isoflavones

Samples	IC_{50}[a] (μM)
Compound **1**	21.5 ± 0.2
Compound **2**	28.7 ± 0.4
Compound **3**	32.6 ± 0.6
Daidzein	>1000
Glycitein	>1000
Genistein	>1000
Trolox	48.9 ± 0.6
L-Ascorbic acid	52.3 ± 0.6

[a] IC_{50} denotes the antioxidant concentration causing 50% reduction of the free radical form of DPPH in methanol after 30 min with each sample, respectively. It was calculated from regression line using different concentrations in triplicate experiments.

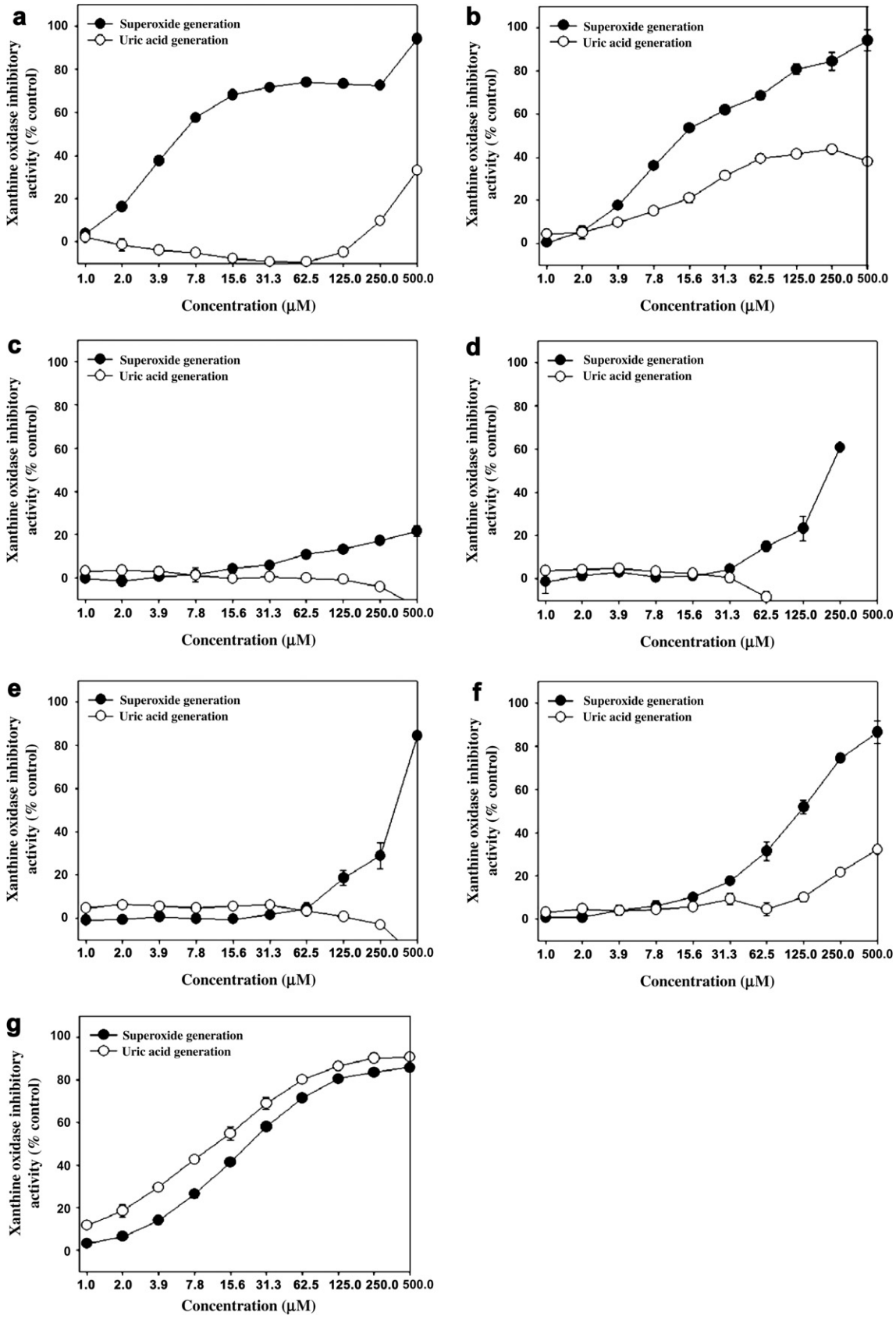


Figure 3. Antioxidation effect of *ortho*-dihydroxyisoflavones and isoflavones on xanthine/xanthine oxidase system. (a) Compound **1** (7,8,4′-trihydroxyisoflavone), (b) compound **2** (7,3′,4′-trihydroxyisoflavone), (c) compound **3** (6,7,3′-trihydroxyisoflavone), (d) daidzein, (e) glycitein, (f) genistein, (g) allopurinol. Open circles indicate the uric acid generation activity and closed circles indicate the superoxide generation activity.

Table 2
Xanthine oxidase inhibitory activity of *ortho*-dihydroxyisoflavones and isoflavones

Samples	Xanthine oxidase inhibitory activity (IC_{50}[a], μM)	
	Superoxide generation inhibition	Uric acid generation inhibition
Compound **1**	6.6 ± 0.4	>1000
Compound **2**	16.8 ± 1.2	>1000
Compound **3**	>1000	>1000
Daidzein	429.8 ± 12.7	>1000
Glycitein	682.6 ± 41.2	>1000
Genistein	232.5 ± 8.7	>1000
Allopurinol	21.8 ± 0.8	11.4 ± 1.1

[a] For each test, two IC_{50} were calculated by linear regression analysis: 50% inhibition of xanthine oxidase activity (50% decrease in uric acid production and 50% decrease in superoxide level).

thine oxidase inhibitor, was used as positive control. Inhibition of XO results in a decreased production of uric acid, which can be measured spectrophotometrically, and a decreased production of superoxide was measured by the nitrite method (Fig. 3). For each compound tested, two IC_{50} values can be calculated by linear regression analysis: 50% inhibition of XO, which is calculated by 50% decrease of uric acid production and 50% reduction of the superoxide level. Each IC_{50} value of the compounds is listed in Table 2. Compounds **1** and **2** inhibited XO by 50% at a concentration of 6.6 ± 0.4 and 16.8 ± 1.2 (IC_{50}), respectively, two compounds show more activity than allopurinol. Daidzein, glycitein, and genistein showed a much lower inhibition of XO inhibition. Compound **3** showed no inhibition activity on XO inhibition. All tested isoflavone derivatives, except compound **2** and genistein, did not show inhibition of uric acid production as shown in Figure 3. It was concluded that the tested isoflavone derivatives were not capable of inhibiting xanthine oxidase. Therefore, the effects measured on superoxide in the presence of compound **1**, daidzein, and glycitein were considered as superoxide scavenging activities. Compound **2** and genistein show inhibition of superoxide production and inhibition of uric acid production at high concentration. This means that these compounds show superoxide scavenging activity and possess additional xanthine oxidase inhibition activity. Compounds **1**, **2**, and isoflavones have an OH group in both 7 and 4′ positions. This makes them potential candidates for superoxide reaction. Presence of an OH group in 8 or 3′ position makes them good candidates for superoxide reaction, whereas presence of an OH group in 6 position reduced the activity of superoxide reaction. These results suggest that *ortho*-dihydroxyisoflavone derivatives can be utilized for the development of new candidate of antioxidant.

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13. (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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19. Assay of superoxide anion generated by xanthine oxidase. Superoxide anions were generated by the xanthine/xanthine oxidase (XO) system, following the described procedure. The reaction mixture consisted of xanthine (0.5 mM), NBT (0.5 mM), and test samples at the concentrations of 31, 63, 125, 250, and 500 μM, in a final volume of 100 μL. Xanthine and NBT were dissolved in phosphate buffer 200 mM with 0.25 mM EDTA, pH 7.5. The reaction mixtures were preincubated at room temperature for 2 min, and reaction was initiated by the addition of 100 μL of XO (50 mU/mL). The mixtures (200 μL) were kept at 37 °C for 30 min. To detect superoxide, the coloring reagent (final concentration of 300 μg/mL sulfanilic acid, 5 μg/mL of *N*-(1-naphthyl)-ethylenediamine dihydrochloride, and 16.7% (v/v) acetic acid) was added after the incubation. The mixtures were allowed to stand at room temperature for 30 min, and the absorbance at 550 nm was measured (Ceres UV 900 Hdi).
20. Assay of uric acid generated by xanthine oxidase. The effect of compounds on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine. The reaction mixtures contained the same proportion of components as in assay for superoxide anion, except NBT. The reaction mixture consisted of 0.5 mM xanthine dissolved in 200 mM phosphate buffer with 0.25 mM EDTA, pH 7.5, and test samples at the concentrations of 31, 63, 125, 250, and 500 μM, in a final volume of 100 μL. The reaction mixture were preincubated at room temperature for 2 min, and reaction was initiated by the addition of 100 μL of XO (50 mU/mL). The mixture (200 μL) was kept for 30 min at 37 °C. The reaction was stopped with HCl (20 μL, 5 M). The production of uric acid was determined spectrophotometrically at 295 nm (Ceres UV 900 Hdi).