Note

Mushroom Tyrosinase Inhibitory Activity of Esculetin Isolated from Seeds of Euphorbia lathyris L.

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A tyrosinase inhibitor was isolated from the seeds of Euphorbia lathyris L. by bioassay-guided fractionation and purification, using silica gel column chromatography. It was identified as esculetin by comparing its physical properties and spectral data with those of an authentic sample. The IC50 value of esculetin in the mushroom tyrosinase activity test was 43 μM. The kinetic study indicates that esculetin exhibited competitive inhibition against the oxidation of 3-(3,4-dihydroxyphenyl)-alanine by mushroom tyrosinase. The structure-activity relationships among five esculetin analogs suggest that hydroxyl groups at the C6 and C7 positions of the coumarin skeleton played an important role in the expression of tyrosinase inhibitory activity.

Key words: esculetin; mushroom tyrosinase inhibitory activity; coumarin; Euphorbia lathyris L.

Melanin is a heteropolymer of indole compounds that is produced inside melanosomes by the action of the tyrosinase enzyme on the tyrosine precursor material in melanocytes. It has recently been shown that other factors such as metal ions and the TRP-1 and TRP-2 enzymes1-4 also contribute to the production of melanin. However, tyrosinase plays a critical regulatory role in melanin biosynthesis. Therefore, many tyrosinase inhibitors that suppress melanogenesis have been actively studied with the aim of developing preparations for the treatment of hyperpigmentation.5-13

We learned from the ancient Chinese medical books, “QianJin YiFang” and “WaiTai MiYao”, that crude drugs were applied as cosmetics for the treatment of hyperpigmentation such as melasma and ephelides. However, the reasons why such crude drugs were applied have not been clarified, and their pharmacological effects are also not well known. We therefore have attempted to isolate tyrosinase inhibitors from the crude drugs described in the books. In a previous screening with mushroom tyrosinase, we found that methanol extracts of the root of the umbelliferous plants, Angelica acutiloba var. sugiyamae and Gleichnia litoralis “Hamabofu”, and of the seeds of the euphorbiaceous plant, Euphorbia lathyris L. “Xusuizi”, showed high inhibitory activity.13

We report in this paper the purification and identification of the tyrosinase inhibitor contained in the seeds of Euphorbia lathyris L.

The seeds of Euphorbia lathyris L. produced in China’s Henan province were purchased from Shinwa Bussan Co. (Osaka, Japan). The active compound was separated by measuring the 50% tyrosinase inhibitory concentration (IC50) as a guide, and was identified as esculetin (Fig. 1) by a comparison of its spectral data with those of an authentic sample. Esculetin has already been reported to be

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\text{Esculetin}
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\begin{center}
\begin{tabular}{c}
\text{Coumarin} \\
\text{Umbelliferone} \\
\text{Escluln} \\
\text{(Glc=β-D-Glucopyranosyl)}
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Fig. 1. Chemical Structures of Esculetin, Coumarin, Umbelliferone, Escluln and Scopeletin.

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contained in *Euphorbia lathyrus* L.,*14-16 Fraxinus japonica* Blume and *Cichorium intybus* L. Many scientific papers have also reported the pharmacological properties of esculetin such as anti-inflammatory effects,*17,21* antioxidative,*22* antiviral,*23* antimutagenic,*24* and antiproliferative activities,*25* ultraviolet absorption effect,*26* and inhibition of lipoygenase activity.*27-29* However, there is no report on the effect of esculetin against tyrosinase activity. Esculetin inhibited mushroom tyrosinase with an IC_{50} value of 43 \mu M. This inhibitory activity of esculetin is weaker than that of kojic acid (Table 1). In addition, the inhibition kinetics of esculetin were analyzed by a Lineweaver-burk plot (Fig. 2). The three lines for 0, 12.5 and 50 \mu M esculetin intersected the ordinate at the same point. This result indicates that esculetin exhibited competitive inhibition of 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) oxidation by mushroom tyrosinase.

Esculetin is a coumarin analog with two hydroxyl groups at C6 and C7. The tyrosinase inhibitory activity of five compounds with a coumarin skeleton (Fig. 1) was measured, and the obtained IC_{50} values are listed in Table 1. Esculetin showed the strongest activity among them, the inhibitory activity of esculetin and umbelliferone being about 200 and 20 times stronger than that of coumarin, respectively. On the other hand, the inhibitory activity of scopoletin and esculetin were dramatically lower. These results suggest that the hydroxyl groups at the C6 and C7 positions of the coumarin skeleton may have played an important role in exhibiting the tyrosinase inhibition activity.

We isolated in this study esculetin as a tyrosinase inhibitor from *Euphorbia lathyrus* L. seeds. In addition to its anti-inflammatory and ultraviolet absorption effects, the tyrosinase inhibitory activity of esculetin may be useful for treating dermal hyperpigmentation. Furthermore, the study on the structure-activity relationship of esculetin derivatives on the tyrosinase inhibitory activity may be useful for the design and discovery of new tyrosinase inhibitors.

### Experimental

**General methods.** \(^1\)H-NMR spectra were recorded by a Varian Unity Inova-500 spectrometer with tetramethylsilane (TMS) used as an internal standard. HR-EL-MS spectra were obtained with a Hitachi M-4100H (70 eV) mass spectrometer. UV and IR spectra were recorded by Shimadzu UV-2100 and Perkin Elmer FT-IR 1720 spectrophotometers, respectively. Thin-layer chromatography (TLC) used Merck silica gel F_{254} plates (0.25 mm), spots being detected by UV irradiation (254 or 365 nm).

**Materials.** Coumarin, umbelliferone, esculetin, esculetin and scopoletin were purchased from Aldrich Chemical Co. (Wisconsin, U.S.A). Kojic acid was obtained from Wako Pure Chemical Industries (Osaka, Japan). These purchased chemicals were used as received.

**Assay of the tyrosinase activity.** The assay was performed as previously described\(^13\) with slight modifications. One milliliter of a 1.5 mm L-DOPA solution, 0.1 ml of dimethyl sulfoxide (DMSO) with or without a sample and 1.8 ml of a 1/15 M phosphoric acid buffer solution (pH 6.8) were mixed. The mixture was preincubated at 25°C for 10 minutes, before 0.1 ml of an aqueous solution of mushroom tyrosinase (1000 U/ml, Sigma Chemical Co.) was added, and the reaction was monitored at 475 nm. A control reaction was conducted with DMSO. The percentage of inhibition of tyrosinase activity was calculated as inhibition (\%) = (A - B)/A \times 100, where A represents the difference in the absorbance of the control sample between an incubation time of 0.5 and 1.0 min, and B represents the difference in the absorbance of the test sample between the incubation time of 0.5 and 1.0 min. Each result is the mean of three concurrent readings. Kojic acid was used as a positive standard.

**Isolation and identification of the tyrosinase inhibitor.** One hundred g of *Euphorbia lathyrus* L. seeds

| Table 1. Inhibitory Effect of Esculetin Analogs and Kojic Acid on Mushroom Tyrosinase Activity (substrate: L-DOPA) |
| --- | --- | --- |
| Test sample | IC_{50} (\mu M) | Mode of inhibition |
| Coumarin | 8.1 | — |
| Umbelliferone | 0.42 | — |
| Esculetin | 0.043 | competitive |
| Esculin | >14 | — |
| Scopoletin | 2.6 | — |
| Kojic acid | 0.010 | mixed |

* Not tested.
* Obtained from data in ref. 12.
was finely powdered and extracted twice with 50% methanol (300 ml) under reflux. The combined extract was evaporated to yield 7.7 g (IC50 = 0.28 mg/ml). This extract was suspended in distilled water, and the oily components were extracted with n-hexane. No inhibitory activity was apparent with the n-hexane extract. The remaining aqueous layer was extracted with ethyl acetate and concentrated to dryness in vacuo to give 1.7 g (IC50 = 0.035 mg/ml). Next, the ethyl acetate extract was subjected to column chromatography on silica gel (Merck 70–230 mesh, 80 g) with chloroform/methanol (10:1), and the eluate was separated into 18 fractions. Each fraction was measured for its tyrosinase inhibitory activity and analyzed by TLC with chloroform/methanol (10:1). High inhibitory activity was observed in fractions 5–8 with identical Rf values (0.3), exhibiting blue-white fluorescence under UV light. The active fractions were combined and concentrated under reduced pressure to yield 0.47 g (IC50 = 0.016 mg/ml). The active compound was then isolated from hot methanol to yield 0.12 g of a pure product (IC50 = 0.0078 mg/ml) as light yellow-brown needle-shaped crystals which reacted with the FeCl3 reagent to exhibit a dark brown coloration. Mp 269–271°C; HR-El-MS m/z (M+): calcd. for C10H14O6, 178.0265; found, 178.0270. UV λmax (MeOH) nm (log ε): 330 (4.06), 300 (3.75). IR (KBr) cm⁻¹: 3330 (–OH), 1667 (lactone carbonyl), 1620, 1609, 1567 (aromatic ring). ¹H-NMR δ (acetone-d6): 6.14 (1H, d, J = 9.5 Hz, H-3), 7.77 (1H, d, J = 9.5 Hz, H-4), 6.78 (1H, s, H-5), 7.02 (1H, s, H-8), 8.20, 9.00 (each 1H, br s, OH).

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References

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