Tyrosinase Inhibitory Xanthones from *Cudrania tricuspidata*

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The methanolic roots bark extract of *Cudrania tricuspidata* (Carr.) Bureau was chromatographed, which yielded three xanthones 1-3 by tyrosinase inhibitory activity-guided fractionation. The structures were fully characterized by analysis of physical and spectral data. Among them, furano prenylxanthone 3, never reported as tyrosinase inhibitor, showed potent activity with IC₅₀ value of 16.5 μM, and appeared to inhibit the polyphenol oxidase activity of tyrosinase in an uncompetitive inhibitor (Kᵢ = 1.6 μM) when L-tyrosine was used as a substrate. Moreover, potent inhibitor furano prenylxanthone 3 had an extended lag time of 310 sec at 20 μM, while lag time of kojic acid as positive control was prolonged with 350 sec at the same concentration.

**Key words** — *Cudrania tricuspidata*, tyrosinase inhibitory activity, L-tyrosine, xanthone, Cudraxanthone M

**Introduction**

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme widely distributed in natural that catalyzes two distinct reactions of melanin biosynthesis, the hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones [13,21,22]. This enzyme is also known as a polyphenol oxidase [13,23], and the browning of some fruits, beverages, and vegetables due to tyrosinase cause a significant decrease in their nutritional and aesthetic value [1,14,15]. Especially, the enzymatic oxidation of L-tyrosine to melanin is of considerable importance since melanin has many functions, including light absorption and scattering. Therefore, the control of the tyrosinase is important in relation to browning control of fresh materials [20]. Additionally, this enzyme inhibitors have become increasingly important in medicinal [17] and constituents of cosmetic products [12] in relation to hyperpigmentation [17]. Although a large number of tyrosinase inhibitors have been described in the literature [2,16,18,20], the search for natural products and synthetic compounds with such activity still continues [7].

*Cudrania tricuspidata* (Carr.) Bureau, which belongs to the family *Morus*, has been used as traditional medicine in Korea and China as a remedy for anti-inflammatory, anti-cancer, gastritis, and live damage [4] as well as has also shown antioxidant activity [11]. Although the majority of phenolic compounds in *C. tricuspidata*, belong to the xanthone [3,5,8,19], which may help to offset chronic diseases related with ROS, there are no reports concerning tyrosinase inhibitory effects of xanthones in this species. Therefore, *C. tricuspidata* needs to be examined to identify its activities. Recently, we reported that xanthones and flavonoids from this species, and their respective cytotoxicity and antibacterial activity [8,10]. In the course of our investigation on this plant sources, we found that xanthones showed potent tyrosinase inhibitory activities.

We report here that tyrosinase inhibitory activity-guided fractionation of an extract of the root bark of this species resulted in the isolation of three xanthones 1-3. Among them, xanthone 3 showed potent tyrosinase inhibitory activity.

**Materials and Methods**

**Materials**

*C. tricuspidata* was collected in Hyoupchun (Korea) during the period of September 2-3, 2005, and identified by Prof. Jae-Hong Pak of Kyungpook National University. A voucher specimen (Park, K. H. 110) of this raw material is
deposited at Herbarium of Kyungpook National University (KNU).

Reagents
Tyrosinase (EC 1.14.18.1, Sigma Product T7755 with an activity of 6680 units/mg) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Also, potassium phosphate, L-tyrosine, kojic acid, EtOH, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Instruments
The purity of all compoundswere monitored by TLC (E. Merck Co., Darmstadt, Germany), using commercially available glass-backed plates and visualized under UV at 254 and 366 nm or sprayed with PMA solution. Column chromatography was carried out using 230-400 mesh silica gel (kieselgel 60, Merck, Germany). Melting points were measured on a Thomas Scientific capillary melting point apparatus (Electrothermal 9300, UK) and are uncorrected. IR spectra were recorded on a BrukerIFS66 (Bruker, Karlsruhe, Germany) infrared Fourier transform spectrophotometer (KBr) and UV spectra were measured on a Beckman DU650 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). 1H- and 13C-NMR along with 2D-NMR data were obtained on a Bruker AM 500 (1H-NMR at 500 MHz, 13C-NMR at 125 MHz) spectrometer (Bruker, karlsruhe, Germany) in CD3OD and CDCl3. EIMS was obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan). The tyrosinase inhibitory activity was measured as the optical density at 490 nm (BIO-RAD, Model 690).

Extraction and isolation
The air-dried root bark of C. tricuspidata (3.5 kg) were cut into pieces and were extracted at room temperature with MeOH (5L x 3) for 7 days, and then the methanolic extract was evaporated in vacuo to give a crude extract (250 g). The concentrated extract was suspended in water:MeOH (9:1) mixture and extracted successively with n-hexane (2.0 L), CHCl3 (2.0 L), and n-BuOH (2.5 L) to give three fractions, n-hexane (25.4 g), CHCl3 (31.2 g), and n-BuOH (46.4 g) fractions, respectively. The CHCl3 fraction exhibited activities against tyrosinase with 81% inhibition at 100 mg/mL. Therefore, this fraction was subjected to flash silica gel column chromatography with a gradient of CHCl3-MeOH (30:1→2:1) to give 10 fractions (Fr 1-10).

Fraction 7 (Fr. 7, 3.1 g) was applied to a silica gel column (4.0 × 65 cm, 230-400 mesh, 200 g) chromatography with CHCl3-MeOH (15:1→1:1) and then purified by a second flash silica gel column (3.0 × 50 cm, 230-400 mesh, 150 g) using a gradient of CHCl3-MeOH [12:1 (300 mL), 10:1 (300 mL), 8:1 (250 mL), 6:1 (250 mL), 4:1 (250 mL), and 2:1 (250 mL)] to yield compound 1 (49 mg). Fraction 3 (Fr. 3, 1.8 g) was submitted to silica gel column (2.0 × 50 cm, 230-400 mesh, 100 g) chromatography and eluted with a CHCl3-acetone (20:1→2:1) resulting in 42 subfractions; subfractions 24-32 were rechromatographed on silica gel with CHCl3-acetone (16:1→4:1) to yield compounds 2 (78 mg). Fraction 4 (Fr. 4, 1.3 g) was chromatographed using a stepwise gradient of CHCl3-acetone [12:1 (200 mL), 10:1 (200 mL), 8:1 (200 mL), 6:1 (200 mL), and 4:1 (200 mL)], then purified by second flash silica gel column (1.5 × 30 cm, 230-400 mesh, 75 g) using a gradient of CHCl3-acetone to yield compound 3 (38 mg).

Cudraxanthone L (1): yellowish needle mp 202°C; UV (CH3OH) \(\lambda_{max} (\log \epsilon) 232 (4.53), 259 (4.57), 319 (4.37)\) nm IR (KBr) \(\nu_{max} 3332, 1657, 1614, 1598, 1467 \text{ cm}^{-1}\) EIMS m/z 396 [M]+ (67), 381 (100), 355 (29), 341 (9), 325 (49), 297 (18), 285 (12), 272 (7), 257 (4), 241 (2), and 203 (2) \(\text{H}-\text{NMR} (500 \text{ MHz, CD3OD}) \delta 1.59 (3H, s, 12-CH3), 1.59 (3H, s, 13-CH3), 1.65 (3H, s, 19-CH3), 1.85 (3H, s, 20-CH3), 3.51 (2H, d, J = 7.1 Hz, H-16), 4.81 (1H, d, J = 10.6 Hz, H-15a), 4.90 (1H, d, J = 17.5 Hz, H-15b)], 5.23 (1H, m, H-17), 6.28 (1H, s, H-4), 6.33 (1H, dd, J = 17.5, 10.6 Hz, H-14), and 7.32 (1H, s, H-8); \(^{13}C\)-NMR (125 MHz, CD3OD): see Table 1.

Cudraxanthone D (2): yellowish needle; mp 131°C; UV (CH3OH) \(\lambda_{max} (\log \epsilon) 240 (4.23), 258 (4.90), 316 (3.98), 370 (3.88)\) nm; IR (KBr) \(\nu_{max} 3380, 2990, 1638, 1620,\) and 1565 cm\(^{-1}\) EIMS m/z 410 [M]+ (7), 395 (100), 367 (47), and 355 (28); \(\text{H}-\text{NMR} (500 \text{ MHz, CDCl3}) \delta 1.62 (3H, s, 12-CH3), 1.62 (3H, s, 13-CH3), 1.77 (3H, s, 19-CH3), 1.88 (3H, s, 20-CH3), 3.82 (3H, s, 3-OCH3), 4.30 (2H, d, J = 6.8 Hz, H-16), 4.84 (1H, d, J = 10.6 Hz, H-15a), 4.90 (1H, d, J = 17.4 Hz, H-15b)], 5.30 (1H, m, H-17), 6.25 (1H, dd, J = 17.4, 10.6 Hz, H-14), 6.32 (1H, s, H-2), 7.26 (1H, s, H-5), and 13.79 (1H, s, 1-OH); \(^{13}C\)-NMR (125 MHz, CDCl3): see Table 1.

Cudraxanthone M (3): yellowish power; mp 119°C; UV (CH3OH) \(\lambda_{max} (\log \epsilon) 245(4.48), 258(4.86), 323 (4.28),\) and 3.64 (3.98); IR (KBr) \(\nu_{max} 3483, 2983, 1632, 1600,\) and 1457 cm\(^{-1}\) EIMS m/z 396 [M]+ (16), 381 (100), and 325 (47).
Table 1. $^{13}$C-NMR of compound 1, 2, and 3 (I, cudraxanthone L; 2, cudraxanthone D; 3, cudraxanthone M) at 125 MHz (ppm, m)

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*The chemical shifts of compound 1 was determined in CD$_3$OD and compounds 2 and 3 were measured in CDCl$_3$.

1H-NMR (500 MHz, CDCl$_3$) δ 1.28 (3H, s, 12-CH$_3$), 1.42 (3H, d, J = 6.6 Hz, 15-CH$_3$), 1.51 (3H, s, 13-CH$_3$), 1.65 (3H, s, 19-CH$_3$), 1.81 (3H, s, 20-CH$_3$), 3.25 (2H, m, H-16), 4.53 (1H, q, J = 6.6 Hz, H-14), 5.14 (1H, m, H-17), 6.33 (1H, s, H-4), 7.50 (1H, s, H-8), and 12.77 (1H, s, 1-OH); $^{13}$C-NMR (125 MHz, CDCl$_3$): see Table 1.

Inhibition of tyrosinase activity

Potassium phosphate buffer (0.07 mL, 50 mM) at pH 6.5, 0.03 mL tyrosinase (333 units/mL) and 2 μL of the tested compounds (5-200 μM), were dissolved in absolute ethanol, and inserted into 96 well plates. After 5 min incubation at room temperature, 0.1 mL L-tyrosine (2 mM) was added. The optical density at 490 nm was measured (BIO-RAD, Model 690) [6]. The inhibitory activity of the compound was expressed as the concentration at which 50% of the enzyme activity was inhibited (IC$_{50}$). Inhibition constant (K) of the tyrosinase was determined by Cornish-Bowden's plot using various concentrations of L-tyrosine.

Results and Discussion

The MeOH extract obtained from root bark of C. tricuspidata was fractionated into n-hexane, CHCl$_3$, and n-BuOH layer through solvent fractionation; three xanthones were isolated by the repeated chromatographic separation of CHCl$_3$ fraction. Structural identifications of three xanthones were carried out by interpretation of several spectral data and comparison with the data described in the literatures [5,10] (Fig. 1).

Compound 1 was obtained as yellowish needle, with a molecular ion peak at m/z 396, as revealed by EIMS. UV spectrum showed absorption maximum at 319 nm and IR spectrum showed strong hydroxyl and carbonyl group absorption bands at 3532 and 1657 cm$^{-1}$, respectively. The $^1$H and $^{13}$C-NMR data with DEPT experiment showed the presence of twenty three carbon atoms as one carbonyl group, one sp$^2$ methylene, one sp$^3$ methylene, four methins, four methyl, and twelve quaternary carbons. The $^1$H NMR data showed evidence for two aromatic protons ([6 6.28, s, 1H]) and ([6 7.32, s, 1H]), 3,3-dimethylallyl group ([methyl proton at δ 1.65 and 1.85] and [vinyl proton δ 5.23]), and 1,1-dimethylallyl group [two methyl protons (δ 1.59, s, 6H)], three protons [ABX system, (δ 4.81, 1H, d, J = 10.6 Hz), (δ 4.99, 1H, d, J = 17.5 Hz), and (δ 6.33, dd, J = 17.5 and 10.6 Hz)]. The positioning of the substituents on the ring system was based on the results of HMBC experiments (Fig. 2). The HMBC correlations of H-8 with C-9.

Fig. 1. Chemical structures of isolated xanthones 1-3 (1, cudraxanthone L; 2, cudraxanthone D; 3, cudraxanthone M).
C-8a, C-7, C-6 and C-4b, H-16 with C-4b and C-5 allowed
3,3-dimethylallyl group to site at C-5 on the B-ring. The
1,1-dimethylallyl group was placed at C-2 due to the corre-
lations of C-2 with H-4 and H-12, 13 in the HMBC ex-
periment. These data indicate that the structure of com-
ound 1 is Cudraxanthone L (1). Compound 2 was obtained
as yellowish needle, with its mass spectrum showing an ion
peak at m/z 410. The IR spectrum of 2 showed absorption
at 3380 and 1638 cm⁻¹, suggesting the presence of hydroxyl
and carbonyl moiety. The ¹H- and ¹³C-NMR data with DEPT
experiments showed the presence of twenty four carbon
atoms as one carbonyl group, one sp² methylene, one sp³
methylene, four methines, five methyls, and twelve quater-
nary carbons. In the ¹H-NMR spectrum, characteristic
signals were observed for a prenyl group [δ 1.77 (3H, s,
19-CH₃), δ 1.88 (3H, s, 20-CH₃), δ 4.30 (2H, d, J = 6.8 Hz),
and 5.30 (1H, m, H-17)], and 1,1-dimethylallyl group [δ 1.62
(6H, s), 4.48 (1H, d, J = 10.6 Hz), 4.90 (1H, d, J = 17.4 Hz)
and 6.25 (1H, dd, J = 17.4 and 10.6 Hz)]. The methoxyl
group was also observed at δ 3.82 (3H, s). The positioning
of the substituents on the ring system was based on the re-
sults of HMBC experiments (Fig. 2). In the HMBC ex-
periments, the following long-range correlations appeared:
hydrogen-bonded hydroxyl group at δ 13.79 ppm with C-1,
C-2, and C-9α; aromatic proton at δ 6.32 ppm (H-2) with C-3
and C-4; methoxyl proton at δ 3.82 (OMe) with C-3; methyl
proton at δ 1.62 ppm (H-12, 13) with C-4. These facts clari-
fied the structure of A-ring, on which the methoxyl and
1,1-dimethylallyl group were attached at C-3 and C-4,
respectively. Remaining prenyl group was located at C-8 on
B-ring because of the correlation of methylene proton at δ
4.30 ppm (H-16) with C-7 and C-8a, and aromatic proton
at 87.26 ppm (H-5) with C-4b, C-6, C-7, and C-8a. Thus,
compound 2 was identified as Cudraxanthone D.
Compound 3 was obtained as yellowish powder and
showed the presence of twenty three carbon atoms as one
carbonyl group, one sp² methylene, four methines, five
methyls, and twelve quaternary carbons. The ¹³C-NMR
spectral data enabled one carbonyl and seven double
bonds to be characterized, and these account for eight of
the total twelve degree of unsaturations. Hence, extra de-
grees of unsaturation were presumed to be due to a tetra-
cyclic ring included xanthone ring system. The prenyl
group was determined on the basis of successive con-
nectivities from C-16 to C-20 in ¹H-¹H COSY spectrum.
The HMBC correlation of H-16 with C-4b, C-5 and C-6, H-8
with C-4b, C-6, C-7 and C-8a allowed prenyl group to site
at C-5 on the B-ring obviously (Fig. 2). 2,3,3-Trimethyl-2,3-
dihydrofuran ring was deduced from the connectivity be-
tween H-14 (δ 4.53 ppm) and methyl proton H-15 (δ 1.42
ppm) in ¹H-¹H COSY spectrum, and the correlation be-
tween C-11 and H-12, 15 in the HMBC experiment. This
2,3,3-trimethyl-2,3-dihydrofuran ring was fused at C-2 and
C-3 on the A-ring due to the correlation of C-2 with H-14
and hydrogen-bonded hydroxyl group (δ 12.77 ppm), and
C-3 with H-14. Consequently, compound 3 was identified
as Cudraxanthone M.

We examined the inhibitory effects of isolated xanthones
1-3 on tyrosinase activity using L-tyrosine as the substrate
and this assay was also performed with kojic acid as posi-
tive control. The tyrosinase activity was inhibited by all
tested agents in a concentration-dependent manner. The fur-
nano prenylxanthone 3 showed potent tyrosinase inhibitory
activity with IC₅₀ value of 16.5 μM in comparison with kojic
acid (IC₅₀ = 14.6 μM) as positive control, while compounds
1 and 2 were not capable of activities against tyrosinase at
a concentration of 100 μM, showing less than 10% inhibitory
activities (Table 2) and as shown in Fig. 3, potent ty-
rosinase inhibitor 3 showed dose-dependent activity.

On the basis of tyrosinase inhibitory activities, figure 4
depicts the change in OD at 490 nm for 600 sec as a func-
tion of time due to the formation of o-quinone in the ab-
sence (control) and the presence of compound 3. As ex-
pected, in the presence of tyrosinase inhibitor, the lag time
was prolonged from 210 sec in the control to 410 sec with
the addition of xanthone 3 at 50 μM, whereas the lag time
of positive control showed 350 sec at 20 mM. Also, xanthone
Table 2. The inhibitory effects of compounds 1-3 (1, cudraxanthone L; 2, cudranaxanthone D; 3, cudraxanthone M) on tyrosinase activities

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<td>3</td>
<td>16.5, (1.6)</td>
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<td>kojic acid</td>
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*NT: not tested, Values are mean ± SD of three experiments.

Fig. 3. Tyrosinase inhibitory activity of compound 3 (cudraxanthone M). Each point represents the mean ± SD of three measurements.

Fig. 4. Lag period of L-dopaquinone formation. L-Tyrosine (2 mM) was incubated with tyrosinase in the absence (●, Control) or with the addition of compound 3 (cudraxanthone M) (○, 12 μM), (▼, 20 μM), (▲, 25 μM), (■, 50 μM), and (▲, Kojic acid), and the change in the OD at 490 nm were recorded as a function of time. Ethanol was used as a control. Values are mean ± SD of three experiments.

3 extended the lag time to 100 sec in comparison with control (210 sec) at 20 μM. These data revealed that furano prenylanthone 3 extended the lag time to similar that of kojic acid at 20 μM.

![Graph](image_url)

Fig. 5. Cornish-Bowden plots of tyrosinase and compound 3 (cudraxanthone M) with (●) 1.0 mM and (○) 0.5 mM L-tyrosine. [S]/[v] where [S] was concentration of substrate and [v] was oxidation rate of L-tyrosine by tyrosinase.

The inhibition kinetic of potent tyrosinase inhibitor 3 was analyzed by the Cornish-Bowden plot (Fig. 5). The two lines obtained from different concentrations of compound 3 (0.5 mM and 1.0 mM) and the inhibition kinetic analyzed by the Cornish-Bowden plot indicated xanthone 3 to be an uncompetitive inhibitor of tyrosinase when L-tyrosine was the substrate (Fig. 3 and Table 2).

In conclusion, three xanthones 1-3 were isolated by tyrosinase inhibitory activity-guided fractionation from roots bark of C. tricuspidata. Among them, furano prenylanthone 3 showed potent activity with IC_{50} value of 16.5 μM, and appeared to inhibit the polyphenol oxidase activity of tyrosinase in an uncompetitive inhibitor (K_{i} = 1.6 μM) when L-tyrosine was used as a substrate. Thus, the tyrosinase inhibitory activity of xanthone would contribute to enhance the value of C. tricuspidata and showed increasingly important in medicinal and constituents of cosmetic products in relation to hyperpigmentation.

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**References**


