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Antioxidant and cytotoxic activities of xanthones from *Cudrania tricuspidata*

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Abstract—The new catecholic xanthone, 1,3,7-trihydroxy-4-(1,1-dimethyl-2-propenyl)-5,6-(2,2-dimethylchromeno)-xanthone (**1**), was isolated from the root bark of *Cudrania tricuspidata* together with seven known xanthones. The structures were fully characterized by analysis of physical and spectral (UV, IR, mass, and NMR) data. Relationships between the structural characteristics of xanthones and their antioxidant activities (DPPH, superoxide, and hydroxyl radical) were studied. Among the range of catecholic xanthones, 6,7-dihydroxyl xanthones (**3**–**8**) exhibited a strong scavenging effect on the DPPH radical. When one of the catecholic hydroxyl groups was protected as in compounds **1** and **2**, DPPH radical scavenging activity was markedly decreased ($IC_{50} > 200$ μM). DPPH activities were consistent with electrochemical response by cyclic voltammetry. Interestingly, compounds (**1**, **2**) which had the weak activities on DPPH, exhibited both potent superoxide and hydroxyl radical scavenging activities. The strong activity on the hydroxyl radical of compounds (**1**, **2**) could be rationalized by their chelating effect with iron (Fe^{2+}) due to a redshift of its complex. The catecholic xanthones (**3**–**8**), being able to convert quinone methide intermediate, showed potent cytotoxicities against human cancer cell lines (HT-29, HL-60, SK-OV3, AGS, and A549). In particular, compounds **3**, **6**, and **7** had strong cytotoxic activities against AGS ($LD_{50} < 5$ μM). DNA fragmentation patterns induced by catecholic xanthones revealed that tumor cell death was due to apoptosis.

Catecholic polyphenols have been extensively exploited both because of their wide-ranging pharmacological properties and also because they serve as important units for donating electrons.[1] Their potent antioxidant activity plays a preventive role against disease by removing the reactive oxygen species (ROS) which cause destructive and irreversible damage to the components of a cell.[2] For example, among many phytochemicals, quercetin, catechin, and anthocyanidin have been focused on in the search for bioactive compounds in the phytochemical field because they possess a catecholic group.[3] The catecholic group could also be oxidized in an enzymatic or a non-enzymatic manner to yield a quinone or quinone-methide type prooxidant which is responsible for cancer prevention and apoptosis.[4]

Cudrania tricuspidata (Carr.) Burea has become one of the most important folk remedies for cancer in Korea during the past few decades and has also shown antioxidant and anti-inflammatory activities.[5] Although the majority of phenolic compounds in *C. tricuspidata* belong to the xanthone family, which may help to offset chronic diseases related with ROS, there are no reports concerning antioxidant activity of catecholic xanthones in this species. Therefore, *C. tricuspidata* needs to be examined to identify a correlation between its antioxidant and anticancer activities. Recently, we reported that isoprenylated xanthones were isolated from the root bark of *C. tricuspidata* and its cytotoxicities were evaluated against human cancer cell lines.[6] Antioxidant activity-guided fractionation of the extract of the roots of this species resulted in the isolation of eight catecholic xanthones (**1**–**8**) including a new xanthone (**1**) (Fig. 1).[7]

Keywords: Antioxidant activity; Catecholic xanthones; *Cudrania tricuspidata*; Cytotoxicity.
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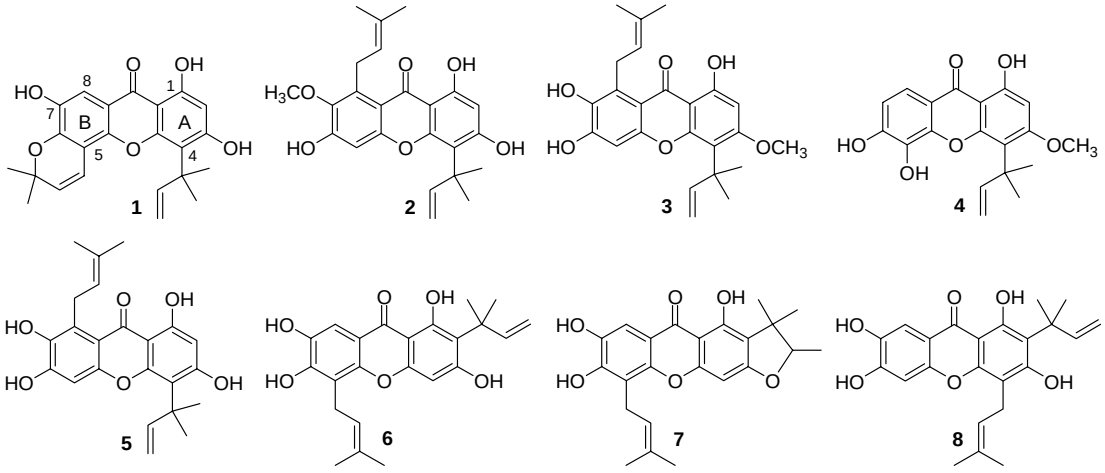

Figure 1. Structure of isoprenylated xanthones from *C. tricuspidata*.

Isolated xanthones were screened in view of antioxidant and cytotoxic activities.

The spectroscopic data for compound (**2**–**8**) agreed with those of cudraxanthone C (**2**), cudraxanthone D (**3**), isocudraniaxanthone B (**4**), 1,3,6,7-tetrahydroxy-4-(1,1-dimethyl-2-propenyl)-8-prenylxanthone (**5**), cudraxanthone L (**6**), cudraxanthone M (**7**), and macluraxanthone B (**8**).[6,8] Compound **1** was obtained as yellowish solid having the molecular formula $C_{23}H_{22}O_6$ established by HREIMS (*m*/*z* 394.1435 [M^+]). The UV spectrum of **1** (λ_{max} 244, 265, and 329 nm) resembled the spectra of 1,3,6,7-tetraoxygenated xanthone derivatives. The ^{1}H NMR showed signals for hydrogen-bonded hydroxyl group (δ_H 13.43), two aromatic protons (δ_H 6.20 and 6.77). The 1,1-dimethylallyl group was deduced from the connectivity between H-14 (δ_H 6.39) and vinyl protons (δ_H 5.30 and 5.39). The 1,1-dimethylallyl group was further deduced from the H-15$_{\alpha/\beta}$ (δ_H 5.30 and 5.39) in the ^{1}H–^{1}H COSY spectrum, and the correlation between C-12, 13, and H-14 in the HMBC experiment. The connectivity between H-16 (δ_H 7.93) and H-17 (δ_H 5.75) in a ^{1}H–^{1}H COSY spectrum, and HMBC correlation of H-19, 20 with C-17, 18 indicated the presence of a 2,2-dimethylpyran ring. The positions of the substituents on the ring system were determined by the HMBC correlations, as shown in Table 1.

Antioxidative activities of isolated compounds (**1**–**8**) were tested against various radical sources by UV–vis and ESR spectroscopy. Anti-radical property of catecholic xanthones (**1**–**8**) was examined with DPPH (1,1-diphenyl-2-dipicrylhydrazyl), of which DPPH is widely used for assessing the ability of polyphenols to transfer labile H-atoms to radicals.[9] Catecholic xanthones (**3**, **5**–**8**), which have dihydroxyl group in B-ring, exhibited strong free radical scavenging activities (Fig. 2). Xanthone **4** having a 5,6-dihydroxy group in the B-ring showed a lower activity (IC_{50} = 31.8 μM) compared to 6,7-dihydroxy xanthones. When one of the hydroxyl groups in vicinal diol was protected as in compounds **1** and **2**, anti-radical activities were decreased distinctively (IC_{50} > 200 μM). It can be rationalized that

Table 1. ^{1}H and ^{13}C NMR spectral data and HMBC correlations of **1** in $CDCl_3$[a]

Position	^{1}H (*J* in Hz)	^{13}C[b]	HMBC
1		162.2 s	OH-1
2	6.20 s	100.6 d	OH-1
3		162.4 s	H-2
4		109.4 s	H-2, H-12
4a		155.6 s	
4b		137.4 s	H-8, H-16
5		120.0 s	H-16, H-17
6		151.4 s	H-8, H-16
7		153.0 s	H-8
8	6.77 s	102.3 d	
8a		108.5 s	H-8, H-16
9		183.2 s	H-2, H-8
9a		105.0 s	OH-1, H-2
11		41.3 s	H-12, H-14, H-15
12	1.43 s	28.4 q	H-14, H-15
13	1.43 s	28.4 q	H-14, H-15
14	6.39 dd (17.8, 10.5)	149.7 d	H-12
15$_\alpha$	5.30 d (10.5)	113.7 t	
15$_\beta$	5.39 d (17.8)		
16	7.93 d (10.2)	121.3 d	
17	5.75 d (10.2)	132.8 d	H-19, H-20
18		77.5 s	H-16, H-19
19	1.62 s	27.7 q	H-16, H-17
20	1.63 s	27.7 q	H-16, H-17
OH-1	13.43 s		

[a] Recorded at 500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively.
[b] Multiplicity was established from the DEPT experiment.

O-protected catechol could not transfer to quinone, while vicinal dihydroxyl group could transfer to quinone easily by releasing two electrons in Figure 3.

The oxidation potential of two representative compounds, dihydroxylated xanthone **6** and O-protected xanthone **2**, was measured by cyclic voltammetry. Both compounds were readily oxidized in the potential range from 200 to 1000 mV (Ag/AgCl) in 0.1 M tetraethylammonium perchlorate (TEAP) solution of methanol as shown in Figure 4. Dihydroxylated xanthone **6** had peak potentials of 680 mV, up to 100 mV lower than that of O-protected xanthone **2** (Fig. 4). Moreover compound

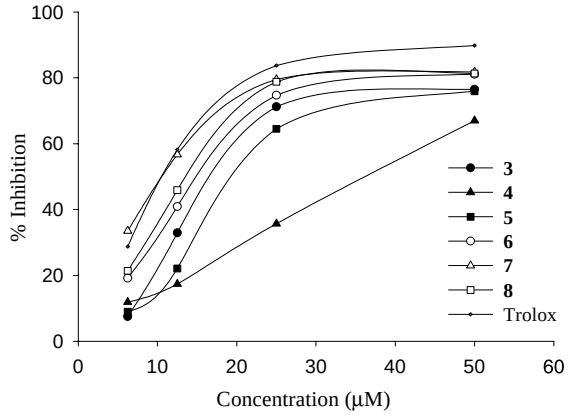

Figure 2. DPPH radical scavenging activities of compounds **3**–**8** and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

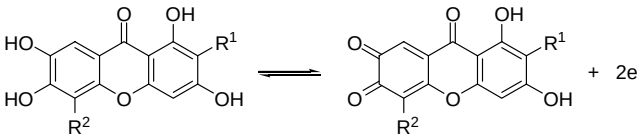

Figure 3. Scheme of oxidation of xanthones to convert into corresponding quinones.

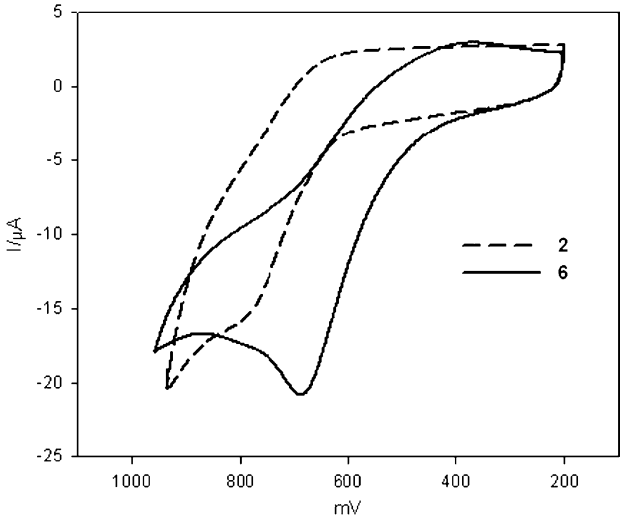

Figure 4. Cyclic voltammograms of 0.1 mM compounds **2** and **6** measured at a glass carbon electrode at 50 mV/s in 0.1 M TEAP.

6 generated a high current response at around 680 mV. The potential values, reducing strength, correlated with anti-radical properties of DPPH.[10]

Catecholic xanthones (**1**–**8**) were screened against the superoxide radical which is known to be very harmful to cellular components as a precursor of the more reactive oxygen species.[11] Superoxide radicals generated in vitro by irradiated riboflavin/EDTA system[12] were trapped as the 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin adduct and gave rise to the ESR signal shown in Figure 5. Experimental data revealed that most catecholic xanthones exhibited strong scavenging

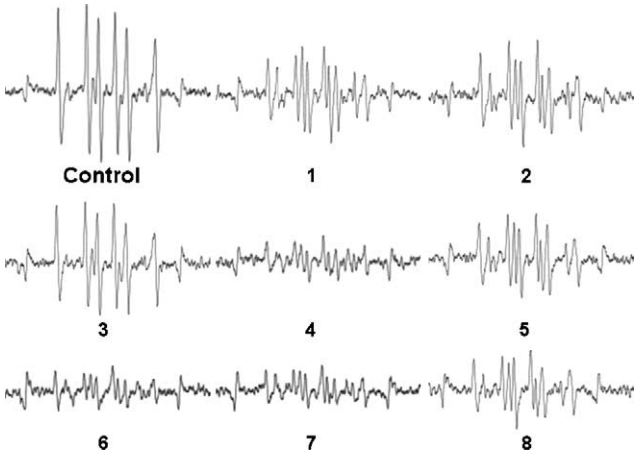

Figure 5. ESR spectra of DMPO radical adducts obtained in the irradiated riboflavin/EDTA system in the absence and presence of 50 μM compounds. The spectra were obtained after irradiation for 60 s on the sample containing 0.3 mM riboflavin, 5 mM EDTA, 0.1 M DMPO in 0.05 M (pH 7.4) phosphate buffer.

activities. Interestingly, compound (**1** and **2**) showed much stronger activities in superoxide radical system compared with DPPH, which requires only an electron donating effect (Fig. 6). It could be explained that activity of superoxide radical is affected not only by concerted H-atom abstraction (electron donation) but also by catalyzing the dismutation of $O_2^{\cdot -}$ (protonation effect).[13]

Hydroxyl radicals were generated in a Fenton-type system ($Fe^{2+} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$).[14] Therefore, hydroxyl radical scavenging activity could be influenced by electron-donating and metal-chelating abilities of catecholic xanthones. The general tendency of hydroxyl radical activities of catecholic xanthones was correlated with the DPPH one (Table 2). Unexpectedly, a strong activity of O-protected xanthones **1** and **2** may be due to their chelating effect with iron as well as their electron-donating properties, because 8 nm of redshift was observed at UV–vis spectra of the complex formed between compound **2** and iron (80 μM) (Fig. 7).

Xanthone-antioxidants were assayed for cytotoxic activity against human cancer cell lines (HT-29, HL-60,

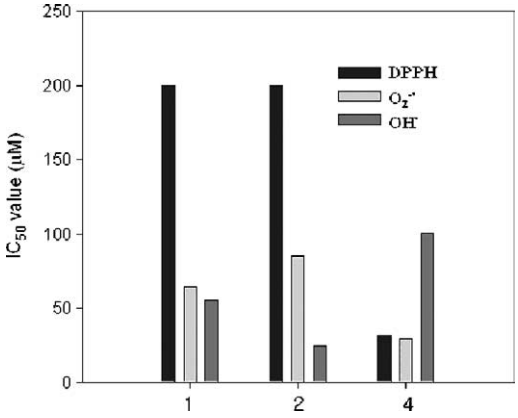

Figure 6. IC_{50} values of compounds (**1**, **2**, and **4**) on DPPH, superoxide, and hydroxyl radical.

Table 2. Radical scavenging activities of compounds **1**–**8** from *Cudrania tricuspidata*[a]

Compound	DPPH	$O_2^{\cdot -}$	$^{\cdot}OH$
1	200>	64.5	55.5
2	200>	85.1	24.3
3	17.4	92.8	25.2
4	31.8	29.5	100.7
5	21.3	51.0	21.2
6	13.7	23.6	50.3
7	10.4	26.0	42.6
8	15.4	47.8	74.1
Trolox	10.6	33.6	48.2

[a] Results are expressed as IC_{50} values (μM) by ESR signal intensity.

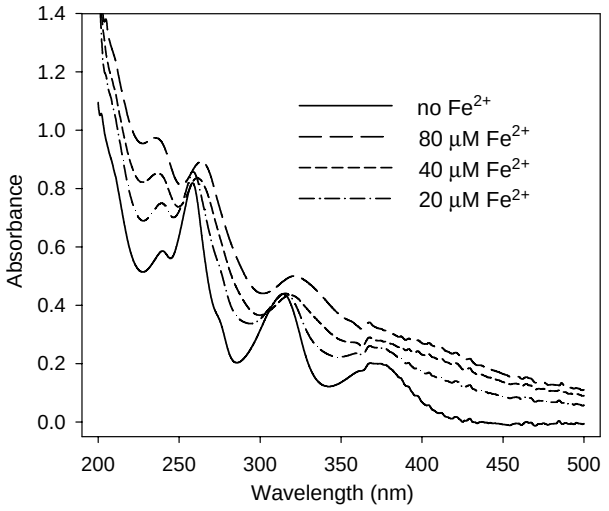

Figure 7. Spectra of compound **2** (40 μM) with several concentration of Fe^{2+} in MeOH.

Table 3. In vitro cytotoxicity of prenylated xanthones and adriamycin against human cancer cell lines[a]

Compound	HT-29	HL-60	SK-OV3	AGS	A549
1	46.3	35.9	70.4	44.7	61.9
2	50.7	40.8	73.5	49.5	61.7
3	20.7	6.2	23.8	4.7	16.3
4	65.0	45.2	71.3	43.9	57.8
5	41.4	32.8	43.2	32.8	45.8
6	11.4	8.6	38.0	3.9	33.5
7	12.1	8.2	14.6	4.1	11.8
8	28.0	29.5	23.1	15.2	25.8
Adriamycin	1.8	1.1	14.2	1.2	1.3

[a] Results are expressed as LD_{50} values (μM).

SK-OV3, AGS, and A549) using a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[15] Catecholic xanthones (**1**–**8**) were able to convert quinone methide intermediates after auto-oxidation showed a quite cytotoxic activity (Table 3). It correlated very well with DPPH activities.

Especially, compounds **3**, **6**, and **7** had strong cytotoxic activity against AGS ($LD_{50} < 5$ μM). In addition, a DNA fragmentation assay was used to verify that the mode of cell death induced by xanthones (**1**–**8**) on HL-60 was apoptosis (Fig. 8). [16] Results indicated that the

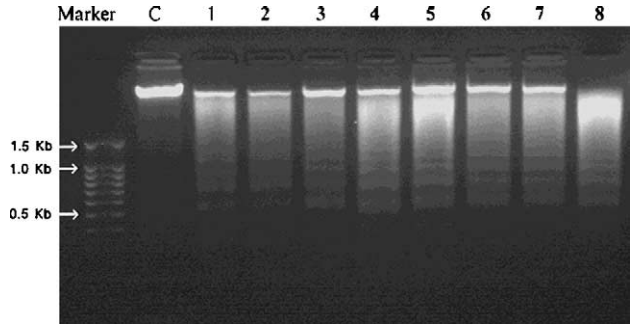

Figure 8. DNA fragmentation of HL-60 cells induced by LD_{50} of xanthones after treating for 24 h.

tumor cell death induced by xanthones was due to apoptosis.

In conclusion, a new isoprenylated xanthone **1** and known xanthones (**2**–**8**) were isolated by bioassay-guided fractionation. Antioxidant analysis of isolated xanthones provides information relevant to their structural feature and antioxidant activity. The observed radical scavenging properties correlate with cytotoxic activities due to the formation of prooxidant-derived antioxidant.

Acknowledgments

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7. Procedure for extraction and isolation: the dried root bark of *C. tricuspidata* (2 kg) was air-dried, chopped, and extracted two times with $CHCl_3$ for 5 days at room temperature. The combined extract was concentrated in vacuo to afford a brown gum (62 g). The $CHCl_3$ extract was dissolved in 1.5 L of a mixture of water and methanol (9:1), and successively partitioned with hexane (1 L) and

chloroform (2 L), which yielded a hexane extract (24.7 g) and chloroform extract (46.4 g). The $CHCl_3$ extract was subjected to column chromatography over silica gel (500 g; 70–230 mesh) eluting initially with $CHCl_3$, then with $CHCl_3$–acetone mixtures of increasing polarity (40:1 → 1:1), yielding 11 fractions (F1–F11), based on the comparison of TLC profile. The Fraction F3 (1.6 g) was submitted to a flash silica gel CC (30 g, 230–400 mesh), eluted with a hexane–EtOAc gradient (40:1 → 8:1). Altogether, 90 fractions were collected. Subfraction 48–61 (0.184 g) was concentrated and submitted to preparative TLC [$CHCl_3$–acetone (8:1)] yielding compounds **1** (18 mg) and **2** (28 mg). The fraction F4 (1.2 g) was submitted to a flash silica gel CC (25 g, 230–400 mesh), eluted with a hexane–EtOAc gradient (40:1 → 2:1), resulting in 80 subfractions. Subfraction 22–32 was evaporated and recrystallized from hexane–chloroform to give compound **3** (84 mg). Subfraction 42–52 was concentrated and submitted to preparative TLC [$CHCl_3$–acetone (6:1)] affording compound **4** (16 mg). Fraction F5 (2.1 g) was subjected to a silica gel CC (6 g, 230–400 mesh), eluted with a hexane–EtOAc gradient (30:1 → 2:1) to give 110 subfraction. Subfraction 32–49 was pooled and rechromatographed on silica gel (5 g, 230–400 mesh) with a $CHCl_3$–acetone gradient (40:1 → 4:1). Subfraction 32–41 from this column was evaporated and recrystallized from chloroform to give compound **7** (26 mg). Isolations of compound **5**, **6**, and **8** were described as previously reported.[6]

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9. Braca, A.; Tommasi, N. D.; Bari, L. D.; Pizza, C.; Politi, M.; Morelli, I. *J. Nat. Prod.* **2001**, *64*, 892, Assay of DPPH radical scavenging activity: the various concentrations of catecholic xanthones (**1**–**8**) were added to a 0.15 mM DPPH in EtOH and the reaction mixture was shaken vigorously. The amount of remaining DPPH radical was determined at 517 nm after 30 min, and the radical scavenging effect was calculated as follows: $E = [(A_c - A_t)/A_c] \times 100$, where A_t and A_c are absorbances of samples with and without catecholic xanthones, respectively.
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12. Zhao, B. L.; Li, X. J.; He, R.; Cheng, S. J.; Xin, W. *J. Cell Biophys.* **1989**, *14*, 175, Assay of superoxide radical scavenging activity: the reaction mixture containing 0.3 mM riboflavin, 50 mM EDTA, 0.1 M DMPO and various concentrations of samples in 50 mM, pH 7.4 phosphate buffer was irradiated by UV lamp at 365 nm for 60 s, then transferred to a quartz capillary and fitted into the cavity of an ESR spectrometer (Jeol JES PX2300 spectrometer). ESR spectra were recorded immediately. The conditions of ESR measurement were as follows: microwave frequency 9.5 GHz, modulation frequency 100 KHz, microwave power 10 mW, modulation amplitude 2 G, time constant 0.03 s, and temperature 298 K. The scavenging effect was calculated as follows: $E = [(h_c - h_t)/h_c] \times 100$, where h_t and h_c are the ESR signal intensities of samples with and without catecholic xanthones, respectively.
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15. Cytotoxicity assay: cells (5×10^3 cells/well) were plated in 96-well plates and incubated in medium for 6 h. The various concentrations of catecholic xanthones were added. The cells were incubated for 3 days at 37 °C and then 10 μL of MTT (5 mg/mL) was added and incubated for an additional 4 h at 37 °C. Reduced MTT was measured spectrophotometrically at 550 nm after lysis of cells with 100 μL of 10% SDS in 0.01 M HCl. Inhibition of tumor growth was calculated as follows: $GI = (A_c - A_t)/A_c \times 100$, where A_t and A_c are absorbance of samples with and without catecholic xanthones, respectively.
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