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Flavonoids from *Dracocephalum tanguticum* and their cardioprotective effects against doxorubicin-induced toxicity in H9c2 cells

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ABSTRACT

Two new flavonoids, ladanetin-6-*O*-β-(6″-*O*-acetyl)glucoside (**1**) and pedalitin-3′-*O*-β-glucoside (**2**), together with 15 known compounds (**3–17**), were isolated from the whole plants of *Dracocephalum tanguticum*. Their structures were established on the basis of extensive spectroscopic (IR, MS, 2D NMR) data analysis and by the comparison with spectroscopic data reported in the literature. Antioxidant capacities of the isolated substances were determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferrous ions, and $ABTS^{\cdot+}$ radical in vitro assay, and their cytoprotective activities were also tested on doxorubicin (DOX)-induced toxicity in H9c2 cardiomyocytes. Among all the tested compounds, luteolin-7-*O*-β-D-glucopyranoside (**7**) exhibited both strong antioxidative effect and high protective activity against DOX-induced toxicity. Further investigation found **7** could decrease DOX-induced death of H9c2 cell, reduce LDH and CK level, and inhibit the elevated intracellular concentration of ROS and $[Ca^{2+}]_i$. The preliminary structure–activity relationships (SAR) of these compounds revealed the $\Delta^{2,3}$ double bond on C-ring and 3′,4′-di-OHs on B-ring with a flavone skeleton such as luteolin and its derivatives, were necessary for their cardioprotective effects.

Doxorubicin (DOX) is an anthracycline antibiotic with a broad spectrum of activity and high potency against human malignant neoplasms. However, long-term clinical usefulness is limited by a cumulative dose-dependent irreversible chronic cardiotoxicity, which manifests itself as congestive heart failure.[1–3] Oxidative stress is now considered a major contributor as a trigger for cardiomyocytes death by apoptosis or cell necrosis.[4] Treatment with antioxidants or natural phenolic compounds has been found to protect against DOX-induced cardiotoxicity.[5,6]

In a continuation of our search for the antioxidant agents from natural products, we have investigated *Dracocephalum tanguticum* Maxim, a folk medicine in Tibet with the anti-hypoxia activity.[7–9] Herein, we report the isolation, structure elucidation and evaluation of antioxidant and cytoprotective activity of 17 flavonoids (Fig. 1), along with their preliminary structure–activity relationships.

The air-dried whole plant of *D. tanguticum* Maxim was collected in Qinghai Province, People's Republic of China, in March 2007. The sample was identified by Gui-Fa Luo (Qinghai Institute for Drug Control) and a voucher specimen (No. DRA0703) was deposited in School of Pharmaceutical Sciences, Shandong University.

Chopped and air-dried whole plants of *D. tanguticum* (5.0 kg) were extracted at room temperature with 95% EtOH (24 h × 3) and concentrated under reduced pressure to give a crude extract (132.8 g). The crude extract was suspended in H_2O, filtered, and then fractionated with petroleum ether (PE), CH_2Cl_2, and *n*-BuOH (each three times) successively. The CH_2Cl_2 extract (52.1 g) was divided by a silica gel column, eluted with a PE–EtOAc, gradient (50:1 → 1:1) to give eight fractions (P1–P7). Fraction P2 (5.3 g) was subjected to silica gel chromatography with PE–Me_2CO (8:1 → 1:1), and Sephadex LH-20 eluted with CH_2Cl_2–MeOH (1:1) to give naringenin (**14**, 26 mg),[10] and 7-*O*-methyl-seutellarein (**17**, 21 mg).[11] Fraction P3 (3.3 g) was subjected to a silica gel column, eluted with a CH_2Cl_2–MeOH gradient (50:1 → 20:1) to afford eriodictyol (**13**, 38 mg),[12] and 3′,5,5′,7-tetrahydroxyflavanone (**15**, 17 mg).[13] Fraction P5 (4.0 g) was subjected to a silica gel column and eluted with a CH_2Cl_2–MeOH to afford luteolin (**16**, 22 mg),[14] and ladanetin-6-*O*-β-(6″-*O*-acetyl)glucoside (**1**, 60 mg).[15] Fraction P6 (5.4 g) was purified by silica gel eluted with a CH_2Cl_2–MeOH and Sephadex LH-20 to afford pedalitin-6-*O*-glucoside (**11**, 38 mg),[16] and ladanetin-6-*O*-β-D-glucopyranoside (**12**, 40 mg).[11] The *n*-BuOH extract (263.7 g) was purified using a polyamide column with a H_2O–EtOH gradient (1:0–1:4) as eluent to give six fractions (B1–B6). Fraction B2 (20.1 g) was successively subjected to silica gel column eluted with a CH_2Cl_2–MeOH gradient (10:1 → 4:1) and Sephadex LH-20 eluted with EtOH to afford 5,7,3′,5′-tetrahydroxyflavanone-7-*O*-β-D-glucopyranoside (**4**, 330 mg),[17] apigenin-7-*O*-β-D-glucopyranoside (**6**, 370 mg),[18] and luteolin-7-*O*-β-D-glucopyranoside (**7**, 180 mg).[19] Fraction B3 (20.3 g) was repeatedly purified on silica gel and Sephadex LH-20 to yield

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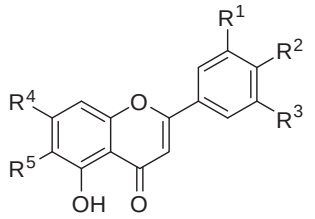
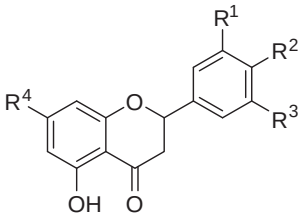


	R1	R2	R3	R4	R5
1	H	OH	H	OCH3	Y
2	H	OH	X	OCH3	OH
4	OH	H	OH	X	H
6	H	OH	H	X	H
7	H	OH	OH	X	H
8	H	OCH3	H	X	H
9	H	OH	OH	Z	H
10	H	OH	OH	OCH3	Y
11	H	OH	OH	OCH3	X
12	H	OH	H	OCH3	X
16	H	OH	OH	OH	H
17	H	OH	H	OCH3	OH



	R1	R2	R3	R4
3	H	OH	OH	X
5	H	OH	H	X
13	H	OH	OH	OH
14	H	OH	H	OH
15	OH	H	OH	OH

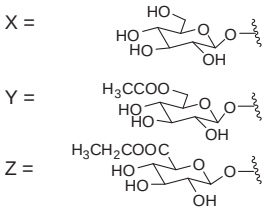


Figure 1. Structures of isolated flavonoids (**1**–**17**) from *D. tanguticum*.

eriodictyol-7-*O*-β-D-glucopyranoside (**3**, 165 mg).[20] Fraction B4 (17.2 g) was subjected to silica gel eluted with a CH_2Cl_2–MeOH to give subfractions $B4_1$ and $B4_2$. Subfraction $B4_1$ (1.1 g) was further purified using Sephadex LH-20 eluted with EtOH to yield tilianin (**8**, 105 mg).[21] The purification of Subfraction $B4_2$ (3.6 g) was carried out by repeated preparative HPLC on an ODS C_{18} column, using MeOH–H_2O (53:47) to afford pedalitin-3′-*O*-glucoside (**2**, 20 mg),[22] and pedaliin-6″-acetate (**10**, 16 mg).[23] Fraction B6 (30.4 g) was repeatedly purified on silica gel and Sephadex LH-20 to yield naringenin-7-*O*-β-D-glucopyranoside (**5**, 215 mg),[24] and luteolin-7-*O*-β-D-glucuronide ethyl ester (**9**, 135 mg).[25] These isolates were all flavonoid derivatives and their structures were identified on the basis of extensive spectroscopic (IR, MS, 2D NMR) data analysis and by the comparison with spectroscopic data reported in the literature. Compounds 1 and **2** were found to be new.

Compound **1** was obtained as a yellow amorphous solid with the molecular formula of $C_{24}H_{24}O_{12}$ as deduced from HRESIMS ($[M+H]^+$ at *m/z* 505.1325). The ^{13}C NMR spectrum of **1** showed resonances for all 24 carbons present in the molecule. The IR spectrum of **1**, apart from hydroxyl (3233 cm^{-1}) and flavone carbonyl

Table 1
The ^1H and ^{13}C NMR data for **1** and **2** in DMSO-d_6, δ in ppm, *J* in Hertz

Position	1		2	
	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]
2		163.9		163.6
3	6.87 (s)	102.5	6.86 (s)	104.0
4		182.1		182.6
5	13.05 (s)	151.7	12.59 (s)	146.5
6		127.7	8.75 (s)	130.4
7		152.7		154.9
8	6.94 (s)	91.4	6.96 (s)	91.7
9		158.6		150.1
10		104.7		105.6
1′		121.0		125.3
2′	7.98 (d, *J* = 8.8)	128.4	7.55 (s)	116.4
3′	6.94 (d, *J* = 8.8)	115.9		148.9
4′	10.39	161.2	9.05 (s)	147.3
5′	6.94 (d, *J* = 8.8)	115.9	7.25 (d, *J* = 9.2)	114.3
6′	7.98 (d, *J* = 8.8)	128.4	7.55 (s)	118.9
1″	4.97 (*J* = 7.26)	101.9	4.89 (d, *J* = 7.3)	101.7
2″	3.27 (m)	73.6	3.41 (m)	73.7
3″	3.21 (m)	76.2	3.39 (m)	76.7
4″	3.16 (m)	69.9	3.18 (m)	70.2
5″	3.89 (m)	73.6	3.47 (m)	77.7
6″	4.11 (dd, *J* = 11.9, 2.3)	63.1	3.80 (m)	61.1
	4.07 (dd, *J* = 11.9,6.4)		3.72 (m)	
7-OMe	3.88 (m)	56.4	3.92 (s)	56.7
CO		169.9		
Me	1.85 (m)	20.4		

[a] Measured at 600 MHz.
[b] Measured at 150 MHz.

Table 2
DPPH., $ABTS^{\cdot+}$ radical, Ferrous ions scavenging activities, and cytoprotective effect of compounds **1**–**17**[a]

Compound	DPPH. EC_{50}[b] (μM)	Fe^{2+} EC_{50}[c] (mM)	$ABTS^{\cdot+}$ EC_{50}[d] (μM)	Cytoprotection EC_{50}[e] (μM)
1	113	4.51	12.3	None
2	31	0.06	15.72	13.1
3	15.6	212.90	7.26	38.23
4	32	415.70	9.65	None
5	40.6	380.20	18.06	None
6	20.37	515.10	13.09	12.3
7	3.94	1.10	56.46	5.04
8	95	420.00	400	None
9	13.15	3.31	87.84	6.34
10	95.24	1.13	76.19	19.1
11	81.17	27.60	86.12	None
12	159.1	621.10	581.2	None
13	98.77	8.37	1.15	25.5
14	72.33	21.40	13.48	None
15	32.15	161.20	16.32	None
16	6.12	12.00	105.3	5.53
17	20.02	500.00	13.37	None

None: ineffective.
[a] Each experiment was performed at least three times independently.
[b] Effective concentration needed to scavenge (DPPH.) free radical to 50%.
[c] Effective concentration needed to chelate ferrous ions to 50%.
[d] Effective concentration needed to scavenge $ABTS^{\cdot+}$ cation radical to 50%.
[e] Effective concentration needed to protect cell viability to 50%.

(1659 cm^{-1}) absorption bands, showed an additional carbonyl absorption band at 1737 cm^{-1}, indicating the presence of an ester group.

The ^{1}H NMR spectrum of **1** was very similar to that of ladanetin-6-*O*-β-D-glucopyranoside (**12**), except for an additional signal at δ_H 1.83 (s, 3H), which, in conjunction with two carbon resonances at δ_C 169.9 and 20.4 in its ^{13}C NMR spectrum, indicated the presence of an acetyl moiety in **1** (Table 1). The acetyl moiety in **1** was found to be linked to the C-6″-hydroxyl of glucose, as the carbon signal was shifted downfield by 1.73 ppm, while the C-5″ signal was shifted upfield by 3.41 ppm compared to **12**.[11] The acetyl moiety at C-6″ in **1** was further supported by the presence of a cross-peak between the C-6″ methylene protons (δ_H 4.11 and 4.07) of the glucose residue and carbonyl (δ_C 169.9) of the acetyl moiety in the HMBC spectrum. The structure of compound **1** was established as ladanetin-6-*O*-β-(6″-*O*-acetyl) glucoside.

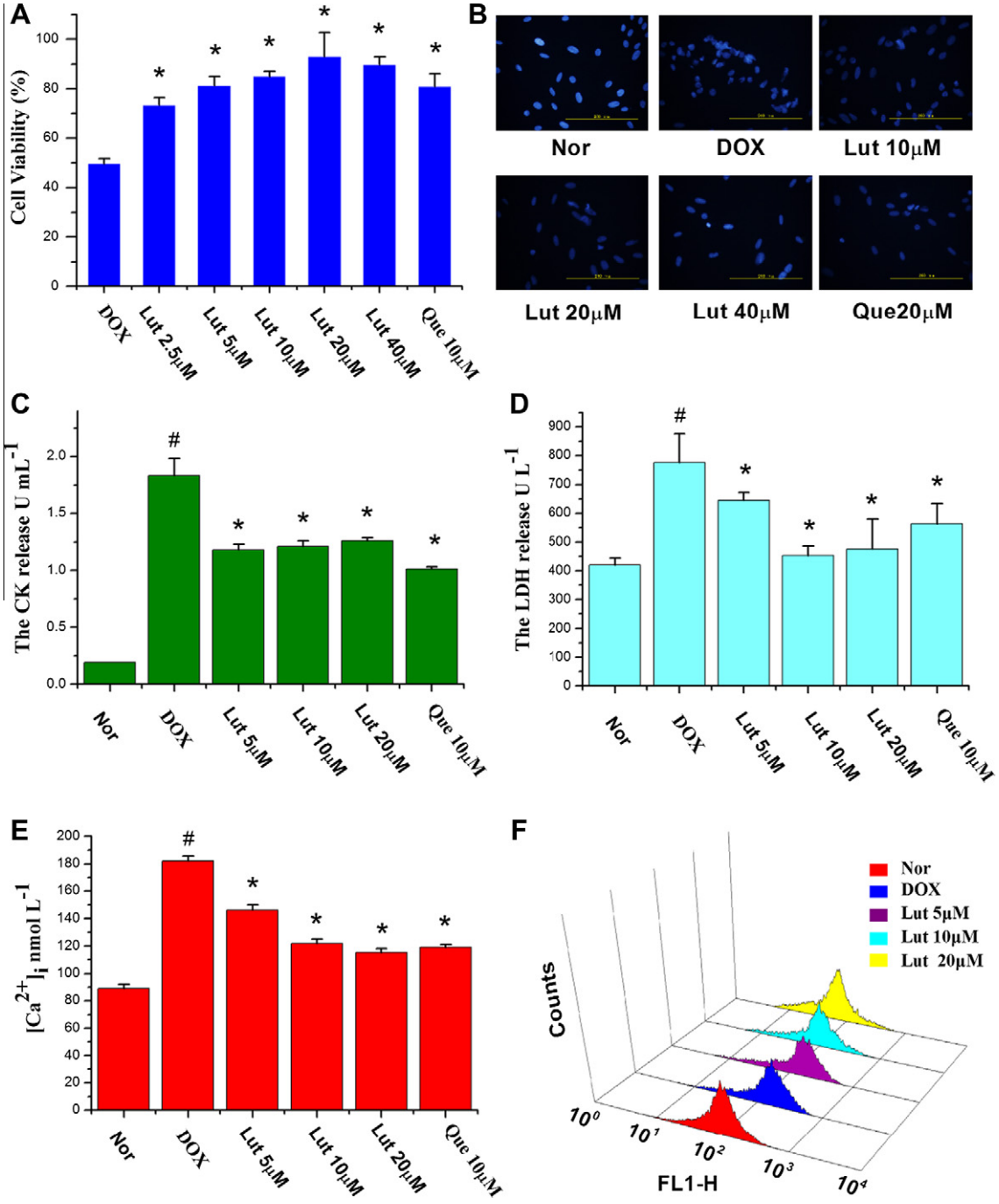


Figure 2. Protective effects of **7** on DOX-mediated cytotoxicity in H9C2 cells. H9c2 cells were preincubated with different concentrations of **7** for 24 h. Then H9c2 cells were incubated with 20 μM DOX for 24 h. (A) Protective effects of **7** on DOX-mediated cytotoxicity in H9C2 cells by MTT Assay. (B) Effects of **7** on DAPI nuclear staining in DOX-induced H9c2 cell injury. DAPI nuclear staining was observed under fluorescence microscopy. Bar = 200 μm. (C) Effects of **7** on release of CK in DOX-induced H9c2 cell. (D) Effects of **7** on release of LDH in DOX-induced H9c2 cell. (E) Effects of **7** on the intracellular Ca^{2+} concentration in DOX-induced H9c2 cell. $[Ca^{2+}]_i$ was measured using Ca^{2+} indicator fura-2 pentakis(acetoxymethyl) ester. (F) Effects of **7** on the intracellular ROS production in DOX-induced H9c2 cell, by measuring the cellular fluorescence using flow cytometry with FL1-H filter. Data are mean ± SD ($n = 6$); Nor: normal cells; DOX: doxorubicin; Lut: luteolin-7-*O*-β-D-glucopyranoside (**7**); Que: quercetin, as positive control drug. $^{\#}P$ <0.05 compared to the normal group; $^{*}P$ <0.05 compared to DOX group.

Compound **2** was also obtained as a yellow solid. The HRESIMS of **2** showed a peak at *m/z* 479.1169 $[M+H]^+$ corresponding to a molecular formula $C_{22}H_{22}O_{12}$. The ^1H NMR spectrum of compound **2** showed the typical pattern of a flavonol with a pedalitin aglycon together with signals ascribable to a sugar moiety.[23] The HMBC experiment indicated correlation between δ_H 4.89 (H-1″) and δ_C 148.9 (C-3′). The site of glycosylation in **2** was also revealed by downfield shifts of $\Delta\delta$ 3.6, 3.0, and 3.2 observed for C-1′, C-2′, and C-3′, and upfield shifts of $\Delta\delta$ 2.6 and 1.6 observed for C-4′ and C-5′, respectively, compared with pedalitin.[23] Accordingly, compound **2** was established as pedalitin-3′-*O*-β-glucoside.[26]

The mechanisms of DOX cardiotoxicity have been the subject of considerable controversy, and dozens of various potential pathways have been proposed and studied. Nevertheless, the iron-mediated formation of ROS and promotion of myocardial oxidative stress remain by far the most frequently proposed mechanism.[4] Thus removal of excess ROS or suppression of their generation by antioxidants may be effective in preventing DOX cardiotoxicity. In the present study, 17 flavonoids were subjected to in vitro antioxidative activities testing using the DPPH, ferrous ions, and $ABTS^{\cdot+}$ radical-scavenging assay as previously reported.[27,28] As shown in Table 2, all the compounds displayed moderate to good antioxidative activity. In addition, those compounds were subsequently evaluated for their cytoprotective effects on against DOX-induced myocardial toxicity in H9c2 cardiac muscle cells using the MTT colorimetric method.[29] The protective effect of 17 flavonoids at 20 μM against DOX-induced myocardial toxicity are summarized in Table 2. It should be noted that compounds **7**, **9**, and **16** with the 2,3-double ($\Delta^{2,3}$) bond on C-ring and 3′,4′-dihydroxy groups on B-ring showed strong cytoprotective activity.

We further investigated the effects of luteolin-7-*O*-β-D-glucopyranoside (**7**) on cell damage induced by doxorubicin. Pretreatment with different concentrations of **7** could not reverse DOX-induced cell death dramatically, but it could decrease DOX cytotoxicity (Fig. 2A). The dose-dependent manner of protective effect was observed at low doses. Cell damage was determined by release of CK and LDH enzyme, which increases with necrosis in cultured cells. There were significant increase in both CK and LDH levels (P <0.05) in cells exposed to DOX (20 μM) for 24 h. However, the increase of CK and LDH release were significantly reduced in cells pretreated with **7** (5, 10, and 20 μM) (Fig. 2C and D).[30,31]

To determine the effects of **7** on apoptosis induced by doxorubicin in H9c2 cells, we also evaluated nuclear condensation using DAPI staining. As shown in Figure 2B, normal cells showed no signs of morphological nuclear damage or chromatin condensation, and were seen as round-shaped nuclei with a homogeneous fluorescence intensity. The doxorubicin-induced rapid changes in the nuclear morphology of H9c2 cells, with heterogeneous intensity and chromatin condensation being apparent under fluorescence microscope. Pretreatment with 10, 20, and 40 μM of **7** significantly protected the cells from the morphological changes induced by doxorubicin.[32]

One potential mechanism for DOX-induced cell damage is the secondary generation of ROS. We analyzed the anti-reactive oxygen species (ROS) activity of **7**. As shown in Figure 2F, DOX significantly increased the intracellular level of ROS. Pretreatment with **7** (5, 10, and 20 μM) significantly inhibited the elevated intracellular concentration of ROS by DOX. DOX-mediated alteration of Ca^{2+} homeostasis is another possible mechanism of cardiotoxicity. Results of $[Ca^{2+}]_i$ were shown in Figure 2E. $[Ca^{2+}]_i$ level was quite low in normal cells. DOX treatment increased $[Ca^{2+}]_i$ and pretreatment of **7** inhibited DOX-induced $[Ca^{2+}]_i$ rise in a dose-dependent manner.[33,34]

Furthermore, K562, KB, MCF-7 and PC-3 cells were used to determine the effect of **7** on DOX's antineoplastic activity in MTT assay. As shown in Table 3, no significant difference could be found between DOX treatment group and combination group. Moreover, the apoptosis induced by DOX in K562 cell line was measured by Annexin V and propidium iodide (PI) staining (Fig. 3).[35] When DOX was combined with **7** (20 μM), total apoptotic cells was increased by 4.09%, compared with 0.3 μM of DOX treatment alone. These data are in agreement with the literature, which emphasizes flavonoids could improve therapeutic index of DOX.[36–38] Taken together, pretreatment of **7** did not decrease the antineoplastic activity of DOX.

Table 3
The effect of **7** (20 μM) on DOX's antineoplastic activity against human tumor cell lines[a] (means ± SE)[b]

	IC_{50} (μM)	
	DOX	DOX incubation with **7**
K562	0.32 ± 0.11	0.31 ± 0.09
KB	40.3 ± 2.4	38.5 ± 3.6
MCF-7	5.04 ± 0.39	4.84 ± 0.45
PC-3	0.31 ± 0.07	0.33 ± 0.12

[a] Cell lines: K562, leukemia; KB, epidermoid carcinoma; MCF-7, breast carcinoma; PC-3, human prostate.
[b] Each experiment was performed at three times independently.

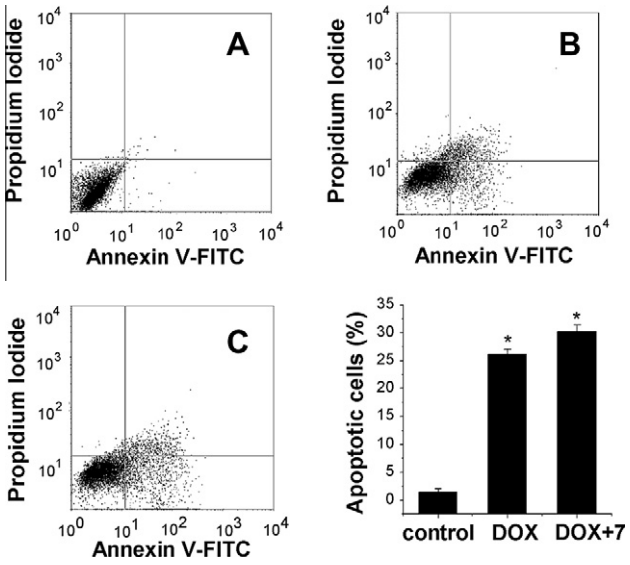


Figure 3. Effect of **7** on DOX-induced apoptosis of K562 cells. K562 cells were pretreated with 20 μM **7** for 24 h, before exposure to 0.3 μM Dox for additional 24 h. Dot-plot of flow cytometry analysis shows apoptotic cells detected with Annexin V/PI staining by flow cytometry. In each panel, the apoptotic cells were defined as the sum of cells in the lower right quadrant (early apoptotic cells) and the upper right quadrant (late apoptotic cells). Bar graph shows the percentage of apoptotic cells. (A) control; (B) the treatment of 0.3 μM DOX; (C) the treatments of 0.3 μM DOX in the presence of 20 μM **7**. Data represents mean ± SD, n = 3. $^*P$ <0.05 compared to control.

The present study concludes that flavonoids with the OH groups at 3′,4′-position in the B-ring, and a double bond between C-2 and C-3 displayed important roles for their protective effects against DOX-induced cardiotoxicity. Overall, the effective agent luteolin-7-*O*-β-D-glucopyranoside (**7**) showed cardioprotective effect by inhibiting the DOX-induced intracellular level of ROS and $[Ca^{2+}]_i$ rise and may act as a promising therapeutic agent for preventing the cardiotoxicity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.086.

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15. *Spectral data for* **1**: yellow powder; $[\alpha]_D^{25}$ −46.3 (*c* 0.1, MeOH). IR (KBr) ν_{max} 3233, 2913, 1737, 1659, 1603, 1508, 1497 cm^{-1}; HRESIMS *m*/*z* 505.1325 $[M+H]^+$ (calcd for $C_{24}H_{25}O_{12}$, 505.1341); ^1H and ^{13}C NMR see Table 1.
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22. *Spectral data for* **2**: yellow powder; $[\alpha]_D^{25}$ −54.1 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3327, 2923, 1671, 1608, 1508, 1076 cm^{-1}; HRESIMS *m*/*z* 479.1169 $[M+H]^+$ (calcd for $C_{22}H_{23}O_{12}$, 479.1184); ^1H and ^{13}C NMR see Table 1.
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    Surface exposure of phosphatidylserine in apoptotic cells was quantitatively detected using Annexin V/FITC and PI apoptosis detection kit (Becton Dickinson, USA). Briefly, cells (5×10^5 per well) were seeded into 6-well plates and then treated with **7** and DOX as mentioned above. After 48 h, the K562 cells were harvested and washed twice with ice-cold PBS (0.01 M, pH 7.2). After 5 min of centrifuging at 200*g*, Annexin V/FITC and PI double-staining were performed according to manufacturer's instruction. Cell apoptosis was analyzed on a FACScan flow cytometry (Becton Dickinson, USA).
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