Chapter 9

Secoiridoid Glucosides from Fraxinus Excelsior with Effects on LPS-Induced Nitrite Production in RAW 264.7 Macrophages and Human Cancer Cell Lines

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Nine secoiridoid glucosides, excelside A (**1**) and excelside B (**2**), nuzhenide (**3**), GI3 (**4**), GI5 (**5**), ligstroside (**6**), oleoside-11-methyl ester (**7**), oleoside dimethyl ester (**8**), 1‴-*O*-*β*-D-glucosylformoside (**9**), and one phenylethanoid, salidroside (**10**), were isolated from the seeds of *Fraxinus excelsior*. The structures were established on the basis of NMR spectroscopic methods supported by MS or HRMS. HPLC method and a calibration curve was constructed for analysis the constituents of the extract. All isolated compounds were tested for cytotoxicity in human cancer cell lines (Hep G2, COLO 205, and HL-60) and anti-inflammatory activities in LPS-treated RAW264.7 macrophage cells. Among them, the water extract **11**, and the compounds **5**, **8**, **9**, **10**, and **4** were modestly active to inhibit nitrite production in macrophages, followed by compounds **3**, **2**, **1**, **6**, and **7**. Compound **4** was slightly effective as an anti-proliferative agent in HL-60 cells with IC_{50} of 82.0 μM. There was no cytotoxicity observed for any compounds except for **4** and the extract in these cells.

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Introduction

The plant of *Fraxinus excelsior* L. (Oleaceae) is known as 'common ash' or 'European ash' in temperate Asia and Europe (*1*, *2*). The genus Fraxinus (Oleaceae) is mainly distributed in the temperate and subtropics regions of the Northern hemisphere and the species have economical, commercial and medicinal importance (*3*, *4*). The characteristic chemical feature of Fraxinus species is the presence of coumarins, secoiridoids, and phenylethanoids. The herbs of Fraxinus species have been used in folk medicine in different area of the world for their diuretic and mild purgative effects, as well as for treatment of constipation, dropsy, arthritis, rheumatic pain, cystitis and itching scalp (*4*, *5*). The plant of *F. excelsior* is also widely distributed throughout the South-East of Morocco (Tafilalet), where it is locally known as "Lissan Ettir" and its seeds as "l'ssane l'ousfour". This region is a rich source of ethnobotanicals, and an area in which phytotherapy has been and remains to be well developed (*2*, *6*, *7*). The leaves and bark of *F. excelsior*, native in Europe and Asia, have been used as a diuretic and rheumatic remedy since olden times (*8*). The bark and the leaves of *F. excelsior* are applied in the folk medicine against various diseases, including wound healing, diarrhea and dysentery. Nowadays, the leaves of this species are mainly recommended against fever and rheumatism (*4*). The ethanolic extract of the bark of this plant is a component of the plant drug Phytodolor N with antiinflammatory and antirheumatic properties (*8*). Aqueous seed extract of *F. excelsior* (FE) has been shown to be highly potent in the reduction of blood glucose levels without significantly affecting insulin levels (*6*, *7*, *9*). The phlorizin-like effect of inhibiting renal glucose reabsorption is a potential mechanisms for the hypoglycemic effect of FE (*9*). Previous investigations on the chemical composition of FE led to the characterization of several compound classes including secoiridoid glucosides, coumarins, flavonoids, phenylethanoids, benzoquinones, indole derivatives, and simple phenolic compounds (*4*, *10–12*). Our previous study revealed that inhibition of adipocyte differentiation and PPARα-mediated mechanisms of the isolated secoiridoids might be relevant pathways for the anti-diabetic activity of *F. excelsior* extract (*13*).

It has been demonstrated that nitric oxide (NO) is involved in many inflammatory diseases when NO is produced in large amount. Overproduction of NO and its more reactive N-nitrosating agents such as peroxynitrite, may also represent an essential link between inflammation and carcinogenesis (*14*, *15*). The focus of the present study was to isolate and characterize the potential active principle(s) of *F. excelsior* and evaluate their biological activity in anti-inflammatory and cancer preventive assays. As a result, we reported herein the isolation of nine secoiridoids and one phenylethanoid, as well as the biological testing results on LPS-induced nitrite production in RAW 264.7 macrophage and human cancer cell lines of these compounds.

Materials and Methods

General Experimental Procedures

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. FT-IR was performed on a Perkin-Elmer spectrum BX system (PerkinElmer Instruments, Norwalk, CT). UV spectra were acquired on a Shimadzu, UV-1700 UV-Visible Spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded on an Inova-400 (^{1}H at 400 MHz) instrument (Varian Inc., Palo Alto, CA) with CD_3OD (*reference 3.30* ppm) and D_2O as the solvent (Aldrich Chemical Co., Allentown, PA). The 2D correlation spectra were obtained using standard gradient pulse sequences of Varian VNMR software and performed on 4-nuclei PFG AutoSwitchable or PFG Indirect Detection probes. HRFAB-MS was run on a JEOL HX-110 double focusing mass spectrometer. Both negative and positive electrospray ionization-mass spectrometric spectra (ESI-MS) were obtained on an LCQ ion trap (Thermo-Finnigan, San Jose, CA). GC-MS analysis was carried out on an Agilent HP 6890 Series Gas Chromatograph system and *Agilent HP 5973 Mass Spectrometer (*Santa Clara, CA) *with Rxi®-1ms capillary GC column (60 m × 0.25 mm ID × 1.0 μm).*

HPLC Analysis Conditions

HPLC analysis was performed on an Agilent 1100 LC Series using Prodigy ODS3 column (5 micron, 4.6 mm ID × 25 cm) with a flow rate of 1.0 mL/min. Solvent system consisted of 0.1% triflouroacetic acid/water (A) and acetonitrile (B) in the following manner: 0-5 min, 0-20% B; 5-15 min, 20-30% B; 15-25 min, 30-100% B. At the end of the run, 100% of acetonitrile was allowed to flush the column for 10 min, and an additional 10 min of post run time were set to allow for equilibration of the column with the starting eluant. The UV detector was operating at 238 nm, and the column temperature was ambient.

Calibration Curves

Methanol stock solutions containing the two active compounds [nuzhenide (**3**), and GI3 (**4**)] were prepared and diluted to five different final concentrations. A calibration curve was constructed for each of the compounds by plotting peak areas versus compound concentrations.

Plant Material

The seeds of *Fraxinus excelsior* (Oleaceae) were collected in Morocco. A voucher specimen (J02/02/A7) was deposited in the Herbarium of Naturex, Inc.

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Extraction and Isolation

A total of 2.5 kg of air-dried and powered seeds of *F. excelsior* was extracted twice with 15 L of water at 95° C for 2 hours. The combined extract was concentrated and dried into powder (**11** or FE994702, 500 g). The powder was re-extracted with MeOH two times (3.5 L each) and the MeOH was evaporated in vacuum. The obtained extract (54 g solid) was reconstituted in 0.5 L of water and was loaded on a C-18 (1 L) (Sigma Chemical Co., St. Louis, MO) column (8.0 cm i.d. × 70 cm) eluted with water (5 L) and 10% MeOH/water (3 L). Fractions with similar HPLC chromatograms were combined and concentrated *in vacuo*. The combined water fractions (21 g solid) were separated over silica gel (Sorbent Technologies, Inc.) by column chromatography (500 g, 3.5 cm × 60 cm), eluting with a step gradient consisting of CH_3Cl-MeOH (10:1, 8:1, 5:1, 3:1, 2:1); In each gradient step, 1.5 L of eluent were used, collecting of 0.5 L . A total of 15 fractions was collected and labeled as W-fractions. These fractions were subjected to column chromatography over MCI GEL CHP-20P (Mitsubishi Kasei Co.) (100 mL, 2.5 cm × 40 cm) and/or Sephadex LH-20 (100 mL, 2.5 cm × 40 cm), eluting with a water-MeOH (4:6) system to yield **3** (210 mg from fraction W-9, t_R = 12.6 min in HPLC), **6** (46 mg from W-2, t_R = 19.3 min), **7** (28 mg from W-11, t_R = 9.4 min), **8** (41 mg from W-10, t_R = 12.7 min), **9** (21 mg from W-8, t_R = 13.4 min), and **10** (22 mg from W-4, t_R = 8.1 min). In a similar manner, the 10% MeOH-water eluates (12 g solid) from the C-18 column were chromatographed over a silica gel column, collecting a total of 15 M-fractions. These fractions were chromatographed over MCI GEL CHP-20P and/or Sephadex LH-20 to yield **1** (16 mg from fraction M-8, t_R = 10.4 min), **2** (33 mg from M-6, t_R = 15.6 min), **4** (238 mg from M-3, t_R = 17.9 min), and **5** (36 mg from M-5, t_R = 20.4 min).

Cell Culture and Chemicals

The COLO 205 cell lines were isolated from human colon adenocarcinoma (ATCC CCL-222); human promyelocytic leukemia (HL-60) cells were obtained from American Type Culture Collection (Rockville, MD). The human HepG2 hepatocellular carcinoma cell lines (BCRC 60025) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). COLO-205 and HL-60 cell lines were grown at 37 °C in 5 % CO_2 atmosphere in RPMI. Hep G2 cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, Grand Island, NY), 100 units/mL of penicillin and 100 μg/mL of streptomycin, and kept at 37°C in a humidified atmosphere of 5% CO_2 in air. Selected compounds were dissolved in dimethyl sulfoxide (DMSO). Propidium iodide was obtained from Sigma Chemical Co. (St. Louis, MO).

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Determination of Cell Viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (*16*). Briefly, human cancer cells were plated at a density of 1×10^5 cells/mL into 24 well plates. After overnight growth, cells were pretreated with series of concentration of test compounds for 24 h. The final concentration of DMSO in the culture medium was < 0.05%. At the end of treatment, 30 μL of MTT was added, and the cells were incubated for a further 4 h. Cell viability was determined by scanning with an enzyme-linked immunosorbent assay reader with a 570 nm filter.

Nitrite Assay

The RAW264.7 cells were treated with selected compounds and LPS or LPS alone. The supernatants were harvested and the amount of nitrite, an indicator of NO synthesis, was measured by use of the Griess reaction. Briefly, supernatants (100 μL) are mixed with the same volume of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance at 570 nm is measured with an ELISA reader (Thermo Labsystems Multiskan Ascent, Finland).

Table I. HPLC Calibration Curve Data for Nuzhenide and GI 3 Compounds

Compounds	*Retention time (min)*	*Linear range (μg/mL)*	*Calibration eq*	*Correlation coefficient (R)*
Nuzhenide	12.6	1.9-1190	y = 9.6728x + 40.647	0.9999
GI 3	17.9	1.2-806	y = 12.29x + 6.1331	1.0

Results and Discussion

Chromatography of the hot water extract of *F. excelsior* as sequential combination of normal, reversed-phase, and gel permeation column chromatography now led to the isolation of nine secoiridoids including the new excelside A (**1**) and B (**2**) (*13*), the known nuzhenide (**3**) (*17*), GI3 (**4**) (*18*), GI5 (**5**) (*11*, *19*), ligstroside (**6**) (*20*), oleoside-11-methyl ester (**7**) (*21*), oleoside dimethyl ester (**8**) (*22*), 1‴-*O*-*β*-D-glucopyranosylformoside (**9**) (*23*), and one phenylethanoid, salidroside (**10**) (*24*, *25*) (Figure 1). The chemical structures were established by spectroscopic methods and compared with literature and the ^{13}C NMR data (Table II **&** III).

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Table II. ^{13}C NMR Data for Compounds 1-3 and 6-10 (CD_3OD)

	1	***2***	***3***	***6***	***7***	***8***	***9***	***10***
No.	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
1	94.8	94.7	95.0	95.0	95.3	95.0	95.2	71.3
3	155.2	155.2	155.1	155.1	154.5	155.1	155.3	36.2
4	109.3	109.3	109.2	109.2	110.7	109.2	109.2	129.3
5	31.9	32.0	31.6	31.7	32.9	31.8	31.7	130.7
6	41.1	41.3	41.2	41.1	45.0	40.9	41.0	116.7
7	173.7	173.4	172.9	173.2	179.6	173.5	171.6	158.2
8	124.7	124.8	124.9	124.8	123.8	124.8	125.1	116.7
9	130.4	130.1	130.2	129.9	131.1	130.3	130.5	
10	13.6	13.6	13.7	13.5	13.7	13.5	13.8	
11	168.7	168.7	168.5	168.6	169.0	168.6	168.6	
11-OCH_3	52.3	51.9	52.0	51.9	51.7	51.9	52.0	
7-OCH_3	51.9					52.1		
1′	100.6	100.4	100.6	100.7	100.7	100.8	100.9	104.1
2′	77.6	77.5	74.5	74.6	74.8	74.7	74.7	74.8
3′	77.8	77.8	78.1	78.2	78.4	78.3	77.9	77.7
4′	71.6	71.5	71.3	71.3	71.5	71.3	71.3	72.3
5′	75.2	75.1	77.7	77.7	77.8	77.8	78.3	77.6
6′	70.1	70.1	62.5	62.6	62.7	62.6	62.5	62.4
1″	105.2	105.2	104.2				104.3	
2″	74.7	74.7	74.8				75.0	
3″	77.7	77.6	77.7				78.0	
4″	71.5	71.4	71.2				71.5	
5″	77.8	77.6	74.9				77.8	
6″	62.7	62.7	64.9				62.7	
1‴		67.0	130.5	66.8			71.4	
2‴		35.2	130.8	35.0			36.5	
3‴		130.3	116.0	130.2			137.9	
4‴		131.1	156.6	130.9			131.0	
5″		116.4	116.0	116.2			122.5	
6‴		157.0	130.8	156.9			150.5	

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Table II. (Continued). ^{13}C NMR Data for Compounds 1-3 and 6-10 (CD_3OD)

	1	*2*	*3*	*6*	*7*	*8*	*9*	*10*
No.	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
7‴		116.4	36.2	116.2			122.5	
8‴		131.1	72.1	130.9			131.0	

Table III. ^{13}C NMR Data for Compounds 4 and 5 (CD_3OD)

	4	*5*		*4*	*5*
No.	δ_C	δ_C	*No.*	δ_C	δ_C
A			1‴	71.5	
1	95.2	95.2	2‴	36.4	
3	155.2	155.2	3‴	137.8	
4	109.2	109.2	4‴	130.9	
5	31.6	31.6	5‴	122.4	
6	41.1	41.1	6‴	150.4	
7	172.9	172.9	7‴	122.4	
8	125.1	125.1	8‴	130.9	
9	130.4	130.4	**B**		
10	13.8	13.8	1	95.0	95.0
11	168.5	168.5	3	155.1	155.1
OCH_3	52.0	52.0	4	109.1	109.1
1′	100.8	100.8	5	31.6	31.6
2′	74.6	74.6	6	40.9	40.9
3′	78.7	78.7	7	171.5	171.5
4′	71.3	71.3	8	124.9	124.9
5′	77.7	77.7	9	130.3	130.3
6′	62.6	62.6	10	13.7	13.7
1″	104.3		11	168.5	168.5
2″	74.8		OCH_3	52.0	52.0
3″	77.7		1′	100.7	100.7
4″	71.4		2′	74.6	74.6
5″	75.0		3′	78.7	78.7

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Table III. (Continued). ^{13}C NMR Data for Compounds 4 and 5 (CD_3OD)

	4	*5*		*4*	*5*
No.	δ_C	δ_C	*No.*	δ_C	δ_C
6″	64.9		4′	71.2	71.2
			5′	77.7	77.7
			6′	62.5	62.5

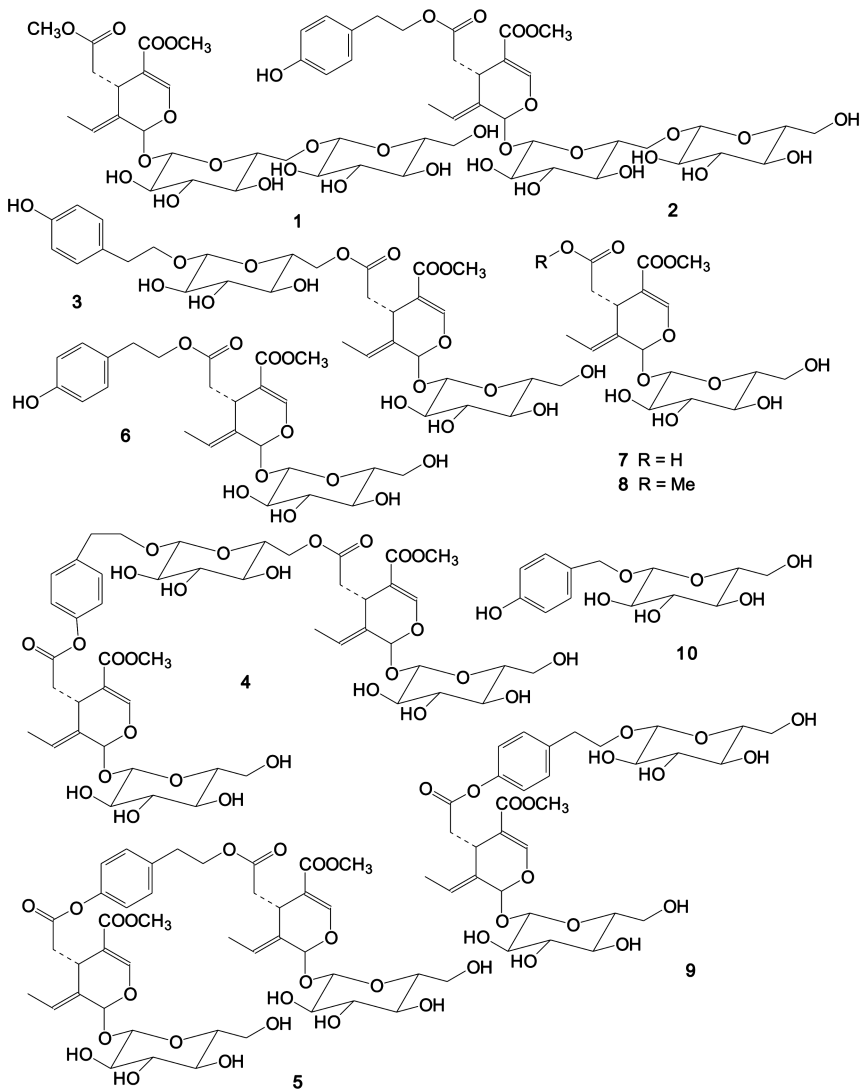

Figure 1. The compounds structure.

A calibration curve was constructed for nuzhenide (**3**), and GI3 (**4**) (Table I**)**. Based on HPLC analysis (Fig. 2), the compounds content in the extract 11 or FE994702 is listed as **2**, 0.41%; **3**, 11.42%; **4**, 6.15%; **5**, 0.63%; **7**, 0.19%; **9**, 1.35%; **10**, 0.20%; and total content of the identified compounds, 17.57%. The compounds were picked up and calculated by UV spectrum analogue comparing with that of the isolated compounds.

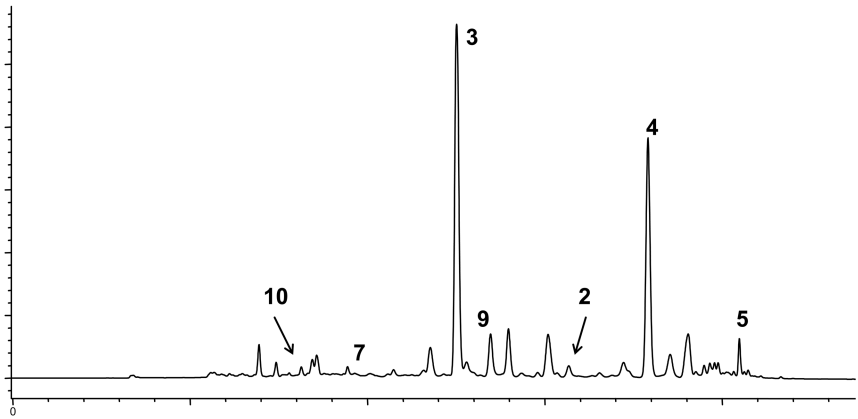

*Figure 2. HPLC chromatogram of the hot water extract **11** from the seeds of F. excelsior.*

The isolated compounds and the extract **1**-**11** were tested with regard to their effect on nitrite production in LPS-activated macrophages for anti-inflammatory screening. When RAW264.7 cells were treated with test compounds at 40 μg/mL and LPS (100 ng/mL), respectively, the potency of the inhibitory effects on nitrite production showed sequence as **5** > **8** > **9** > **10** > **4** > **3** > **2** > **1** > **6** > **7** (Table IV). Among them, compound **5**, **8**, **9**, and **10** were the strong inhibitors to nitrite production in macrophages and the extract **11** was active on this bioassay. The cytotxicity of compounds and the extract **1**-**11** was evaluated *in vitro* against HL-60, Hep G2, and COLO 205 cell lines. These cell lines were treated with different concentrations (5-100 μM) of selected compounds for 24 h, and the viability of the cells was determined by MTT assay. As shown in Table V, Compound **4** was slightly effective as an anti-proliferative agent in HL-60 cells with IC_{50} of 82.0 μM. However, there was no cytotoxicity observed for any compounds except for **4** and the extract in these cells. Therefore, the current study confirms that the secoiridoids are the major components responsible for the activities of anti-inflammatory and there are no cytotoxicities for the isolated secoiridoids. Thus, the seeds and the extract of *F. excelsior* are largely safe for normal use.

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Table IV. Effect of 1-11 on LPS-Induced Nitrite Production in RAW 264.7 Macrophages

	1	*2*	*3*	*4*	*5*	*6*
Control	0.0 ± 0.3	0.0 ± 0.3	0.0 ± 0.3	0.0 ± 0.3	0.0 ± 0.3	0.0 ± 0.3
LPS	22.8 ± 1.5	22.8 ± 1.5	22.8 ± 1.5	22.8 ± 1.5	22.8 ± 1.5	22.8 ± 1.5
20 μg/mL	23.2 ± 1.5	22.3 ± 0.8	19.4 ± 1.2	20.2 ± 2.3	13.1 ± 0.5	19.6 ± 0.8
40 μg/mL	19.7 ± 0.1	19.1 ± 1.1	17.0 ± 2.0	16.6 ± 1.3	10.3 ± 1.1	20.0 ± 1.3
	7	***8***	***9***	***10***	***11***	
Control	0.0 ± 0.3	0.0 ± 0.3	0.0 ± 0.3	0.0 ± 0.3	0.0 ± 0.3	
LPS	22.8 ± 1.5	22.8 ± 1.5	22.8 ± 1.5	22.8 ± 1.5	22.8 ± 1.5	
20 μg/mL	20.2 ±0.3	18.0 ± 0.6	16.4 ± 0.8	18.0 ± 0.1	16.4 ± 0.2	
40 μg/mL	19.9 ± 0.1	15.9 ±0.3	16.0 ± 0.7	15.6 ± 0.8	16.8 ± 0.8	

Table V. Effect of 1-11 on the Growth of Various Human Cancer Cells

Compound	*Cell line*		
IC_{50} (μM)	*HL-60*	*Hep G2*	*COLO 205*
1	> 100	> 100	> 100
2	> 100	> 100	> 100
3	> 100	> 100	> 100
4	82.0 ± 3.6 [b]	> 100	> 100
5	> 100	> 100	> 100
6	> 100	> 100	> 100
7	> 100	> 100	> 100
8	> 100	> 100	> 100
9	> 100	> 100	> 100
10	> 100	> 100	> 100
11	> 100	> 100	> 100
Doxorubicin[a]	5.0 ± 0.6	10.0 ± 4.3	11.9 ± 4.9

[a] Positive control. [b] Each experiment was independently performed three times and expressed as mean ± SE.

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