DNA Topoisomerases I and II Inhibition and Cytotoxicity of Constituents from the Roots of *Rubia cordifolia*

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The roots of *Rubia cordifolia* L. have been used in the Korean traditional medicine for the treatment of cough, the stone in the bladder and kidney, inflammation of the joints, uterine hemorrhage and uteritis. Several anthraquinones, anthraquinone glycosides, naphthoquinones, naphthoquinone glycosides, furomollugin, mollugin, alizarin, lucidin pimeveroside, ruberythric acid, purpurin, xanthopurpurin, cyclohexapeptide, alkaloids and lignans from *Rubia* species have been reported.[1-4]

The methanol extract of the roots of *R. cordifolia* L. was partitioned between methylene chloride and water. The methylene chloride fraction yielded compounds **1-7** on column chromatography over silica gel and RP C18, and PTLC. Compounds **2-7** are known: 2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-5-ω-hydroxypropyl-7-methoxybenzofuran (**2**),[5] 1,2,4,6-tetrahydroxyanthraquinone (**3**), 3β,7β,19α,28-tetrahydroxyarbor-9(11)-ene (Rubiarbonol A, **4**),[6] 3β,7β,19α-trihydroxyarbor-9(11)-ene (Rubiarbonol B, **5**),[6] sitost-4-en-3-one (β-sitostenone, **6**)[7-10] and sterol mixture (β-sitosterol, stigmasterol and campesterol, **7**)[11] by direct comparison with authentic samples, or by comparing their physical and spectral data with those in the literature. Compound **3** was first isolated from natural sources. Compounds **2**, **6** and **7** were isolated the first time from *Rubia* species, and **5** was previously reported from *R. oncotricha* but was first isolated from *R. cordifolia* L.

The molecular weight of compound **1** was suggested by the mass peak at *m/z* 360 $[M]^+$ in the EIMS. Its HREIMS showed the $[M]^+$ at *m/z* 360.1570 (calcd. 360.1573) which corresponded the molecular formula $C_{20}H_{24}O_6$. Its UV [λ_{max} (MeOH) 282.0 nm (ε 7801)] and IR (ν_{max} 3427, 1529, 1269, 1030 cm^{-1}) data suggested the hydroxyl group and ether band.

Its assignments of ^{1}H and ^{13}C-NMR chemical shifts were made by comparison of the spectral data of 3-(α-4-dihydroxy-3-methoxybenzyl)-4-(4-hydroxy-3-methoxybenzyl) tetrahydrofuran.[12] The ^{1}H-NMR spectrum of compound **1** showed a double triplets at δ 2.37 (1H, J = 7.0, 14.0), double doublets at δ 2.48 (1H, J = 12.2, 12.5) and 2.93 (1H, J = 4.5, 12.5) and one multiplet at δ 2.74 (1H), which were attributed to H-8', Ha-7, Hb-7, and H-8. Four double doublets were observed at δ 3.72 (1H, J = 5.9, 7.5 Hz), 3.97 (1H, J = 6.4, 7.5 Hz), 3.62 (1H, J = 6.4, 10.8 Hz) and 3.82 (1H, J = 7.0, 10.5 Hz), which were assigned to Ha-9, Hb-9, Ha-9' and Hb-9', respectively. There were two singlets at δ 3.82 (3H) and δ 3.83 (3H), for two methoxy groups, and one doublet at δ 4.73 (1H, J = 7.0 Hz), assigned to H-7'. The aromatic region extended from δ 6.63-6.89 (6H) showed two singlets at δ 6.78 (H-2) and δ 6.89 (H-2'), an overlapped singlet at δ 6.75 (2H, H-4', 6'), one doublet at δ 6.71 (1H, J = 8.0, H-5) and one double doublet at δ 6.63 (1H, J = 2.0, 8.0, H-6).

The ^{13}C-NMR and DEPT spectra showed two methyl, three methylene, nine methine and six quaternary carbon signals including methoxy group. In the ^{13}C-NMR spectrum of **1**, the aromatic region extended from δ 110.6-149.2 (12C), and C-7' signal showed at δ 84.1 shifted downfield due to hydroxyl group. The correlations in the ^{1}H-^{1}H COSY spectrum displayed connectivities between H-7' (δ 4.73) and H-8' (δ 2.37), between H-9a (δ 3.72) and H-9b (δ 3.97), and H-8 (δ 2.74), between H-9'a (δ 3.62) and H-9'b (δ 3.82), and H-8' (δ 2.37). In the HMBC spectrum of **1**, the connectivities of the two aromatic rings with tetrahydrofuran ring were indicated by the cross peaks between H-2' (δ 6.89), and C-6' (δ 119.8) and C-7' (δ 84.1), between H-2 (δ 6.78), and C-6 (δ 122.2) and C-7 (δ 33.6), between H-5 (δ 6.71), and C-1 (δ 133.3), between H-6 (δ 6.63), and C-7 (δ 33.6) and C-2 (δ 113.3), respectively.

The relative stereochemistry of THF moiety in **1** was identified as *cis* from the positive NOE effect between H-8 and H-8'. The absolute stereochemistry of the carbinol center in **1** was determined using Mosher ester methodology based on the differences between the ^{1}H-NMR chemical shifts of (*S*)- and (*R*)-MTPA ester derivatives (**1**$_R$, **1**$_S$). ^{1}H-NMR data were assigned based on the ^{1}H-^{1}H COSY spectra of **1**$_R$ and **1**$_S$ (Table 1). Ikuko *et al.* reported that the modified Mosher's method has to be applied carefully to α-aromatic secondary alcohols.[13] In this case, irregularities of $\Delta\delta_H$ (δ_S-δ_R) values were only observed on β-protons (H-8' in **1**$_R$ and **1**$_S$). The negative value of $\Delta\delta_H$ at H-7a, 8, 9a, 9b and H-9'b suggested a *R* configuration at C-7' in compound **1**.

Compound **3** had the molecular formula of $C_{14}H_8O_6$

Table 1. Characteristic ^{1}H-NMR Data of Mosher Esters of **1** for Determination of Stereochemistry

Position	**1**$_S$	**1**$_R$	$\Delta\delta$
	δ_S	δ_R	$\delta_S - \delta_R$
7a	2.53	2.59	−0.06
7b	2.86	2.86	0
7'	6.70	6.74	R
8	2.74	2.76	−0.02
8'	2.62	2.62	0
9a	3.708	3.710	−0.002
9b	4.02	4.03	−0.01
9'a	4.55	4.47	10.08
9'b	4.66	4.72	−0.06

determined from its EIMS and NMR data. Its UV [λ_{max} (MeOH) 277.2, 282.0, 344.0 nm (ε 2781, 5660, 4705)] and IR (ν_{max} 3404, 2937, 1722, 1583 cm^{-1}) data suggested the presence of hydroxyl group, aromatic double bond and carbonyl band. In the ^{1}H-NMR spectrum of compound **3**, aromatic singlet proton at δ 7.70 (H-3) was indicative of a 1,2,4-trisubstituted A-ring, while the signal at δ 7.99 (1H, d, J = 2.4 Hz), δ 7.44 (1H, dd, J = 2.5, 8.5 Hz) and δ 8.40 (1H, d, J = 8.5 Hz) were attributed to the H-5, H-7 and H-8 of B-ring, respectively. The ^{13}C-NMR signals were unambiguously assigned using HMQC and HMBC correlations. In the HMBC spectrum of **3**, the connectivities of anthraquinone ring were indicated by the cross peaks between H-3 (δ 7.70), and C-9a (δ 109.8), C-4a (δ 118.9) and C-10 (δ 186.7), between H-5 (δ 7.99), and C-10 (δ 186.7) and C-7 (δ 121.8), between H-7 (δ 7.44), and C-5 (δ 113.5) and C-8a (δ 125.8), between H-8 (δ 8.40), and C-10a (δ 136.3), C-6 (δ 164.6) and C-9 (δ 183.0), respectively. This compound was used as stabilization agent for agrochemical emulsion with metal compounds,[14] but was first isolated from natural sources. Furthermore its spectral data of UV, IR and NMR have never been reported.

Topoisomerases I and II inhibitory activities was measured by assessing the relaxation of supercoiled pBR 322 plasmid DNA. As shown in Table 2, **3** and **6** showed 38 and 28% inhibiton at the concentration of 100 uM, while **4** and **7** exhibited week inhibition at the concentration of 100 uM in DNA topoisomerase I assay. In DNA topoisomerase II assay, **3**, **4** and **6** showed 56, 28 and 41% inhibition at the concentration of 100 uM, respectively, while **1**, **2**, **5** and **7** exhibited week inhibition at the concentration of 100 uM.

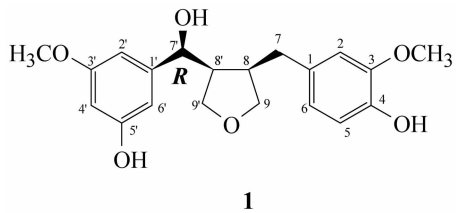
The tetrazolium-based colorimetric assay (MTT assay) was used for the cytotoxicity towards human colon carcinoma (HT-29), human breast carcinoma (MCF-7) and human liver carcinoma (HepG2) cell lines. As shown in Table 2, IC_{50} values of **4** and **5** were obtained at 5.0 and 53.0 uM for HT-29 cell line, and **7** was obtained below 12.5 uM for HT-29 cell line. Also IC_{50} values of **4** was obtained at 69.0 and 40.0 uM for MCF-7 and HepG2 cell lines, respectively.

At the concentration of 100 μM, **3** and **6** showed the strong inhibitory activity on DNA topoisomerase II compared with DNA topoisomerase I. However, these two compounds showed weak cytotoxicity against HT-29, MCF-7 and HepG2 cell lines. Compounds **4** and **7** showed cytotoxi-

Table 2. Inhibitory Effects of Compounds **1-7** for DNA Topoisomerases I and II (%, Inhibition Ratio of Relaxation) and Their IC_{50} Values against HT-29, MCF-7 and HepG2 Cell Lines

Comp.	Topo I (%)		Topo II (%)		Cytotoxicity IC_{50} (μM)		
	100 (μM)	20 (μM)	100 (μM)	20 (μM)	HT-29[a]	MCF-7[b]	HepG2[c]
1	0	0	13	0	>100	>100	>100
2	0	0	10	0	>100	>100	>100
3	38	6	56	13	>100	>100	>100
4	2	0	28	9	5.0	69.0	40.0
5	0	0	12	2	53.0	100	>100
6	28	4	41	4	>100	>100	>100
7	3	0	1	0	<12.5	>100	>100
CPT[d]	74	58	NA[f]				
VP-16[e]	NA		63	40			

[a]HT-29: Human colon carcinoma. [b]MCF-7: Human breast carcinoma. [c]HepG2: Human liver carcinoma. [d]Camptothecin: positive control for topoisomerase I. [e]Etoposide: positive control for topoisomerase II. [f]NA: not applicable.



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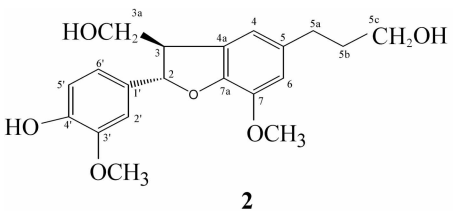

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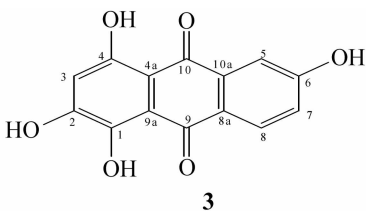

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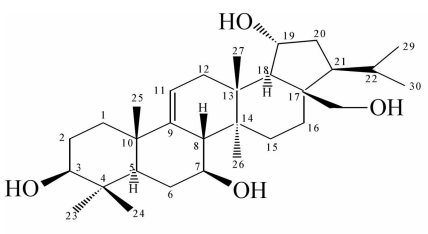

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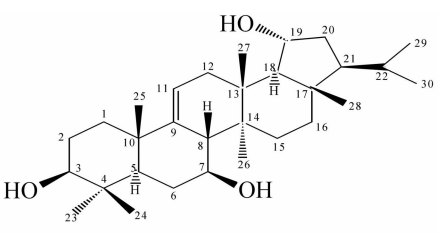

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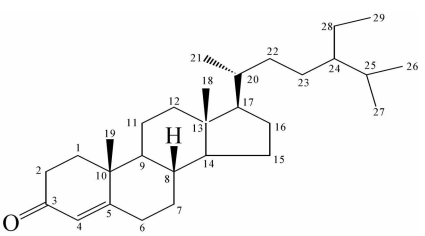

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city against HT-29 cell line but weak inhibitory activity against DNA topoisomerases I and II. These results indicate that no obvious correlation can be observed between the cytotoxicity and the inhibitory activity of DNA relaxation and decatenation by DNA topoisomerases I and II.

In conclusion, **3** of anthraquinone with four hydroxyls and **6** (*β*-sitostenone) showed the strong inhibitory activity on DNA topoisomerase II. Compound **4**, triterpenoid of arborane type, and **7** (sterol mixture) exhibited strong cytotoxicity against HT-29 cell line. In comparison of **4** and **5**, induction of hydroxyl group on C-28 seems to enhance the cytotoxicity, especially against HT-29 cell line.

Experimental Section

General Experimental Procedures. Mps were determined on a Yanaco micro melting point apparatus and are uncorrected. Optical rotations were taken on a JASCO DIP-370 digital polarimeter. IR spectra were measured on a JASCO FT/IR 300E spectrophotometer. UV spectra were obtained on a Shimadzu UV-1601PC spectrophotometer. ^{1}H, ^{13}C, COSY, HMQC, and HMBC NMR spectra were recorded on Varian Unity INOVA-500 and Bruker DMX 250 spectrometer using TMS as an internal standard. Low- and high-resolution EIMS data were collected on a Quattro II spectrometer. For TLC, silica gel 60 F-254 (EM 5717) glass plates (0.25 mm) were used and visualized by spraying with 10% sulfuric acid and heating.

Plant Material. *R. cordifolia* L. roots were collected from the herbmart of Daegu, Korea in November 2002. This plant was verified by Dr. Chong Won Kim, Professor, College of Pharmacy, Catholic University of Daegu, Korea. A voucher specimen (CUDP 02001) deposited at the College of Pharmacy, Catholic University of Daegu, Korea.

Extraction and Isolation. Roots of *R. cordifolia* L. (10 kg) were cut into small pieces and refluxed four times with MeOH. MeOH extract (1048.4 g) was suspended in H_2O (2.2 L) and fractionated with CH_2Cl_2 (2 L × 5, 360.0 g). H_2O layer was extracted with EtOAc (2 L × 5, 214.2 g) and *n*-butanol (2 L × 5, 117.6 g), respectively. 180 g of CH_2Cl_2 (360 g) extract were subjected to column chromatography over 4 Kg silica gel using 100% hexane to 100% CH_2Cl_2 in 2-15% stepwise elutions and then 99% CH_2Cl_2/1% MeOH to 100% MeOH in 1-25% stepwise elutions which afforded 44 fractions, RA-MC-1 to RA-MC-44. Activity-directed Isolation of RA-MC-1, -17, -29 and -34 resulted in the identification of compounds **1-7**.

RA-MC-17 fraction (1.5 g) was chromatographed on a reverse-phase column (LiChroprep Rp-18, using 45% MeOH/55% H_2O to 100% MeOH in 5% stepwise elutions), and then RA-MC-17-31 and -35 fractions (0.1 and 0.13 g) were chromatographed on a silica gel column (Si-gel under 70 mesh, elution with hexane : EtOAc = 5 : 0.1, 1 : 0.1, respectively) to yield sitost-4-en-3-one (*β*-sitostenone, **6**, 16.7 mg) and sterol mixture (*β*-sitosterol, stigmasterol and campesterol, **7**, 46.4 mg). RA-MC-29 fraction (1.5 g) was chromatographed on a reverse-phase column (using 10% MeOH/90% H_2O to 100% MeOH in 5% stepwise elutions) and then RA-MC-29-16 fraction (0.36 g) was chromatographed on a reverse-phase column (using 15% MeOH/85% H_2O to 100% MeOH in 5% stepwise elutions) and then RA-MC-29-16-4 fraction (60 mg) was chromatographed on a PTLC (20 × 20 cm, using CH_2Cl_2 : MeOH = 10 : 0.1) to yield 3-(4-hydroxy-3-methoxybenzyl)-4-[(7*R*),5'-dihydroxy-3'-methoxybenzyl]-tetrahydrofuran (**1**, 13.6 mg). RA-MC-29-36 fraction (58.2 mg) was chromatographed on a silica gel column (elution with CH_2Cl_2 : MeOH = 12 : 0.1) to yield 1,2,4,6-tetrahydroxy-anthraquinone (**3**, 14.6 mg), and RA-MC-29-39 fraction (98.4 mg) was chromatographed on a silica gel column (elution with CH_2Cl_2 : MeOH = 8 : 0.1) and then RA-MC-29-39-4 fraction (98.4 mg) was chromatographed on a silica gel column (elution with CH_2Cl_2 : MeOH = 12 : 0.1) to yield 3*β*,7*β*,19*α*-trihydroxyarbor-9(11)-ene (Rubiarbonol B, **5**, 8.5 mg).

RA-MC-34 fraction (3.0 g) was chromatographed on a silica gel column (elution with CH_2Cl_2 : MeOH = 15 : 0.1) and then RA-MC-34-9 and -10 fractions (0.19 and 0.94 g) chromatographed on a reverse-phase column (using 15% MeOH/85% H_2O and 10% MeOH/90% H_2O to 100% MeOH in 5% stepwise elutions, respectively). RA-MC-34-9-6 fraction (60 mg) was chromatographed on a PTLC (20 × 20 cm, using CH_2Cl_2 : MeOH = 10 : 0.1) to yield 2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-5-*α*-hydroxypropyl-7-methoxybenzofuran (**2**, 6.4 mg), and RA-MC-34-10-18 fraction (88.1 mg) was chromatographed on a silica gel column (elution with CH_2Cl_2 : MeOH = 12 : 0.1) to yield 3*β*,7*β*,19*α*,28-tetrahydroxyarbor-9(11)-ene (Rubiarbonol A, **4**, 13.1 mg).

3-(4-Hydroxy-3-methoxybenzyl)-4-[(7'*R*),5'-dihydroxy-3'-methoxybenzyl]tetrahydrofuran (1): yellowish crystal (13.6 mg); $[\alpha]_D$ +12° (*c* = 0.10, MeOH); UV (MeOH) λ_{max} 282.0; IR (KBr) ν_{max} 3427, 1529, 1269, 1030 cm^{-1}; ^{1}H-NMR (CD_3OD, 250 MHz) *δ*: 2.37 (1H, dt, *J* = 7.0, 14.0 Hz, H-8'), 2.48 (1H, dd, *J* = 12.2, 12.5 Hz, H-7a), 2.74 (1H, m, H-8), 2.93 (1H, dd, *J* = 4.5, 12.5 Hz, H-7b), 3.62 (1H, dd, *J* = 6.4, 10.8 Hz, H-9'a), 3.72 (1H, dd, *J* = 5.9, 7.5 Hz, H-9a), 3.82 (1H, dd, *J* = 7.0, 10.5 Hz, H-9'b), 3.82 (3H, s, 3-OMe), 3.83 (3H, s, 3'-OMe), 3.97 (1H, dd, *J* = 6.4, 7.5 Hz, H-9b), 4.73 (1H, d, *J* = 7.0 Hz, H-7'), 6.63 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), 6.71 (1H, d, *J* = 8.0 Hz, H-5), 6.75 (2H, overlapped s, H-4', 6'), 6.78 (1H, s, H-2), 6.89 (1H, s, H-2'); ^{13}C-NMR (CD_3OD, 62.5 MHz) *δ*: 33.6 (C-7a, 7b), 43.9 (C-8), 54.1 (C-8'), 56.3 (3, 3'-OMe), 60.4 (C-9'a, 9'b), 73.5 (C-9a, 9b), 84.1 (C-7'), 110.6 (C-2'), 113.3 (C-2), 116.1 (C-5), 116.2 (C-4'), 119.8 (C-6'), 122.2 (C-6), 133.3 (C-1), 135.3 (C-1'), 146.0 (C-4), 147.6 (C-5'), 149.1 (C-3'), 149.2 (C-3); HR-EI-MS *m/z* 360.1570 (Calcd. for $C_{20}H_{24}O_6$, 360.1573).

1,2,4,6-Tetrahydroxyanthraquinone (3): yellow crystal (14.6 mg); UV (MeOH) λ_{max} 288.4, 344.0, 436.0, IR (KBr) ν_{max} 3404, 2937, 1722, 1583 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 250 MHz) *δ*: 7.44 (1H, dd, *J* = 2.5, 8.5 Hz, H-7), 7.70 (1H, s, H-3), 7.99 (1H, d, *J* = 2.4 Hz, H-5), 8.40 (1H, d, *J* = 8.5 Hz, H-8), 13.46, 13.98 (2H, s, -OH); ^{13}C-NMR (pyridine-d_5, 62.5 MHz) *δ*: 107.7 (C-3), 109.8 (C-9a), 113.5 (C-5), 118.9 (C-4a), 121.8 (C-7), 125.8 (C-8a), 130.0 (C-8), 133.0 (C-1),

134.7 (C-2), 136.3 (C-10a), 163.7 (C-4), 164.6 (C-6), 183.0 (C-9), 186.7 (C-10); MS *m/z* (rel. int., %) = 272 ([M]$^+$, 4), 270 (100), 242 (12).

Preparation of Mosher Esters.[15-17] To each 0.5 mg of **1** in 0.5 mL of CH_2Cl_2 was added sequentially 0.2 mL of pyridine, 0.5 mg of 4-(dimethylamino)pyridine, and 12.5 mg of (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl [(*R*)-MTPA] chloride. The mixture was left at room temperature overnight and purified over a microcolumn (0.6 × 6 cm) of silica gel (230-400 mesh) eluted with 3-4 ml of hexane-CH_2Cl_2 (1 : 3). The eluate was dried, CH_2Cl_2 (5 mL) was added, and the CH_2Cl_2 was washed using 1% $NaHCO_3$ (5 mL × 2) and H_2O (5 mL × 2). The washed eluate was dried *in vacuo* to give the *S*-Mosher ester (**1**$_S$) of **1**. Using (*S*)-MTPA chloride afforded the *R*-Mosher ester (**1**$_R$) of **1**. Their pertinent ^{1}H-NMR chemical shifts are given in Table 1.

Assay for DNA Topoisomerase I Inhibition *in vitro*. DNA topoisomerase I inhibition assay was carried out according to the method reported by Fukuda *et al.*[18] with minor modifications. DNA topoisomerase I activity was measured by measuring the relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixture was comprised of 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine, 0.01% bovine serum albumin (BSA), 250 ng pBR 322 plasmid DNA, and 0.3 U calf thymus DNA topoisomerase I. The reaction mixture was used for measuring the inhibition of DNA relaxation by the DNA topoisomerase I, in addition to a test compound solution (less than 0.25% DMSO) in a final volume of 10 μL. The reaction mixtures were incubated for 30 min at 37 °C, and terminated by adding a dye solution comprising 2.5% SDS, 15% ficoll-400, 0.05% bromophenol blue, 0.05% xylene cyanole and 25 mM EDTA (pH 8.0). The reaction products were determined by electrophoresis on 1% agarose gel in TBE (Tris-borate-EDTA) running buffer at 1.5 V/cm for 10 h. The gels were stained with ethidium bromide (0.5 μg/mL) for 30 min, then destained in water for 30 min. For visualization and quantitative analyses of the DNA topoisomerase I activity, the gels were directly scanned with an image analyzer, and the area representing supercoiled DNA was calculated.

Assay for DNA Topoisomerase II Inhibition *in vitro*. DNA Topoisomerase II activity was measured by assessing relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 300 ng pBR 322 plasmid DNA, 0.3 U human DNA topoisomerase II, and the indicated compound concentrations (less than 0.25% DMSO), in a final volume of 20 μL. The reaction mixtures were incubated for 30 min at 37 °C, and terminated by addition of 5 μL of a mixture containing 0.77% SDS, 77 mM EDTA (pH 8.0), 30% sucrose, 0.5% bromophenol blue and 0.5% xylene cyanole. The reaction products were determined by electrophoresis on 1% agarose gel in TBE running buffer at 1.5 V/cm for 10 h. The gels were stained with 0.5 μg/mL ethidium bromide for 30 min and destained in water for 30 min. For visualization and quantitative analyses of the DNA topoisomerase II activity, the gels were directly scanned with an image analyzer, and the area representing supercoiled DNA was calculated.

Cytotoxicity Bioassays. MTT assay was used for the *in vitro* assay of cytotoxicity against HT-29, MCF-7 and HepG2 cells.[19]

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