Anticancer Constituents from the Roots of *Rubia cordifolia* L.

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Activity-directed isolation of the methylene chloride fraction of the roots of *Rubia cordifolia* L. resulted in the identification of a new epoxymollugin (3) and eight known compounds (1, 2, 4—9). The structures of the compounds were elucidated from chemical and spectroscopic evidence. In addition, their topoisomerase I and II inhibitory activities and cytotoxicities were measured.

Key words *Rubia cordifolia* L.; epoxymollugin; topoisomerase; cytotoxicity

The roots of *Rubia cordifolia* L. have been used as a traditional Korean medicine to treat cough, bladder and kidney stones, joint inflammation, uterine hemorrhage and uteritis. Anthraquinone, anthraquinone glycoside, naphthoquinone, naphthoquinone glycoside, furomollugin, mollugin, alizarin, lucidin pimeveroside, ruberythric acid, purpurin, xanthopurpurin, cyclohexapeptide, alkaloid and lignan have been reported from *Rubia* species.[1—4)]

In the course of screening tests for topoisomerase I and II inhibitors and cytotoxicity of 40 species of Korean traditional anticancer plants, we found that the methylene chloride fraction from the roots of *Rubia cordifolia* L. (the methanol extract of this root was subsequently fractionated into four fractions; methylene chloride, ethyl acetate, *n*-butanol and water) showed strong cytotoxicity against HT-29 and MCF-7 cell lines, as well as DNA topoisomerase I and II inhibitory activities. In our previous studies of the methylene chloride fraction of this plant, we isolated seven compounds belonging to the lignan, anthraquinone, triterpenoid and sterol groups.[5)] As part of our continuing efforts to find anticancer constituents from the methylene chloride fraction of this plant, we have isolated compounds **1**—**9** on column chromatography over silica gel and RP C18, and investigated their DNA topoisomerase I and II inhibitory activities and cytotoxicities. Compound **3** was identified as epoxymollugin, and this marked its first isolation from a natural source. Compounds **1**, **2** and **4**—**9** were known compounds: furomollugin (**1**),[4)] mollugin (**2**),[1)] 3,3′-bis(3,4-dihydro-4-hydroxy-6-methoxy-2*H*-1-benzopyran) (**4**),[6)] 2-carbomethoxy-2,3-epoxy-3-prenyl-1,4-naphthoquinone (**5**),[4)] 1-acetoxy-3-methoxy-9,10-anthraquinone (**6**),[7)] 1,6-dihydroxy-2-methyl-9,10-anthraquinone (soranjidiol, **7**),[8—10)] 1-hydroxy-2-methoxy-9,10-anthraquinone (alizarin 2-methyl ether, **8**)[4)] and oleanolic acid (**9**).[11)] These compounds were identified by direct comparison with authentic samples or by comparing their physical and spectral data with those in the literature. Compound **6** had been reported on ^{13}C-NMR chemical shifts through the synthetic processes,[7)] but this marked its first isolation from

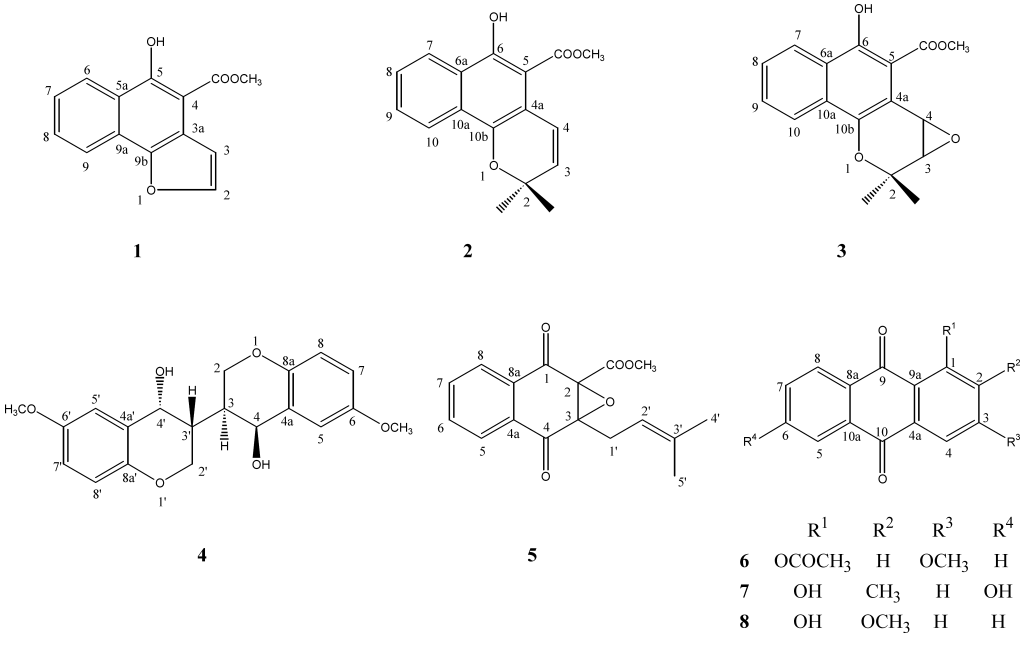

Fig. 1. Chemical Structures of Compounds **1**—**8**

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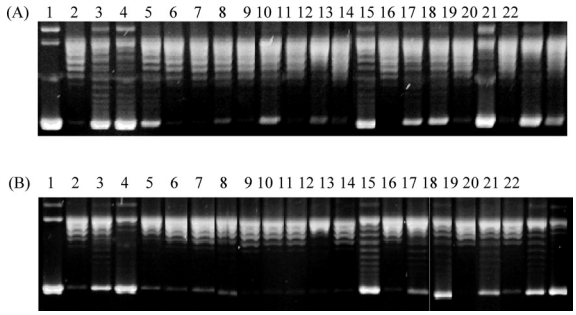

Fig. 2. DNA Topoisomerase I (A) and II (B) Inhibitory Activities of Compounds **1**—**9**

(A) 1: supercoiled DNA alone; 2: supercoiled DNA+topoisomerase I (calf thymus); 3: supercoiled DNA+topoisomerase I (calf thymus)+camptothecin (20 μM, positive control); 4: supercoiled DNA+topoisomerase I (calf thymus)+camptothecin (100 μM, positive control); 5—13: compounds **1**—**9** (20 μM); 14—22: compounds **1**—**9** (100 μM); (B) 1: supercoiled DNA alone; 2: supercoiled DNA+topoisomerase II (human); 3: supercoiled DNA+topoisomerase II (human)+etoposide (20 μM, positive control); 4: supercoiled DNA+topoisomerase II (human)+etoposide (100 μM, positive control); 5—13: compounds **1**—**9** (20 μM); 14—22: compounds **1**—**9** (100 μM).

natural sources. Compounds **4**, **7** and **9** were isolated for the first time from this plant.

HR-FAB-MS spectral analysis of compound **3** showed the $[M+H]^+$ at *m/z* 301.1072 (Calcd 301.1076) which corresponded to the molecular formula $C_{17}H_{17}O_5$. Its IR (ν_{max} 3433, 1653, 1238 cm^{-1}) data suggested the presence of a hydroxyl group and an ester band.

The ^{1}H- and ^{13}C-NMR data of compound **3** were identified by comparison to those of **2**. The ^{1}H-NMR spectrum (Table 1) of compound **3** showed doublets at δ 3.82 (1H, J=5.0 Hz) and δ 5.19 (1H, J=5.0 Hz), which were attributed to H-3 and H-4 of a pyran ring epoxidized between C-3 and C-4. There was also a multiplet at δ 7.57 (2H, H-8, 9) and doublets at δ 8.17 (1H, J=7.5 Hz, H-7) and δ 8.33 (1H, J=7.5 Hz, H-10). Accordingly, H-7, 8, 9 and H-10 of aromatic protons were closed, which was also confirmed from the HMBC spectrum. There were three singlets at δ 1.48 (6H), 4.03 (3H) and δ 11.15 (1H) for two methyl protons bonded at C-2, methyl proton of a methyl ester and a phenolic hydroxyl proton, respectively.

The ^{13}C-NMR and DEPT spectra showed three methyl, six methine and eight quaternary carbon signals including characteristic peaks of different methyl groups and ester group. In the ^{13}C-NMR spectrum of **3**, C-3 and C-4 signals were observed at δ 72.1 and δ 64.3 instead of δ 129.0 and δ 122.3 as in compound **2** due to the epoxidation of the double bond. That spectrum also indicated one aromatic carbon (δ 155.6) bearing hydroxyl group, and methyl carbon (δ 52.8) and carbonyl carbon (δ 171.2) of a methyl ester. The correlations in the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum displayed connectivities between H-4 (δ 5.19) and H-3 (δ 3.82), between H-7 (δ 8.17) and H-9 (δ 7.57), and between H-10 (δ 8.33) and H-8 (δ 7.57). In the HMBC spectrum of **3**, connectivities were observed between H-3 and C-2, and between H-4, and C-3, C-2, C-4a and C-10b, respectively. Accordingly, **3** was identified as epoxymollugin, the first isolation from a natural source.

Compound **6** had the molecular formula $C_{17}H_{12}O_5$ as determined from EI-MS and NMR data. Its UV [λ_{max} ($CHCl_3$) 461.0 nm (ε 1639)] and IR (ν_{max} 1737, 1677, 1594, 1026 cm^{-1}) data suggested the presence of carbonyl and aromatic double bonds and an ester band. The ^{1}H-NMR spectrum of compound **6** showed singlets at δ 2.36 and δ 4.01, which were attributed to methyl protons of acetyl and methoxyl groups. Multiplets were seen at δ 7.79 (2H, H-6 and 7), and δ 8.09 (2H, H-5 and 8), respectively. Accordingly, H-5, 6, 7 and H-8 of aromatic protons were closed, which was confirmed in the HMBC spectrum. The ^{13}C-NMR and DEPT spectra showed two methyl, six methine and nine quaternary carbon signals including characteristic peaks of ketonic carbonyl carbons and methoxy carbon. In the ^{13}C-NMR spectrum of **6**, ketonic carbonyl carbon signals and carbonyl carbon of acetyl group signals were shown at δ 183.2 and δ 182.0, and δ 157.6, respectively. The ^{1}H–^{1}H COSY spectrum displayed connectivities between H-2 (δ 6.26) and methyl proton of acetyl group (δ 2.36), between H-4 (δ 7.66), and H-2 (δ 6.26) and methoxyl proton (δ 4.01), respectively. This compound was reported on ^{13}C-NMR chemical shifts through the synthetic processes,[7] but was first isolated from natural source, and spectroscopic data such as UV, IR, ^{1}H-NMR and MS have never been reported.

Table 1. ^{1}H- (250 MHz) and ^{13}C-NMR (62.5 MHz) Data of Compounds **2** and **3** in $CDCl_3$[a]

Atom	**2**		**3**	
	δ_H	δ_C	δ_H	δ_C
2		74.6		77.2
3	5.67 d (10.0)	129.0	3.82 d (5.0)	72.1
4	7.10 d (10.0)	122.3	5.19 d (5.0)	64.3
4a		112.6		112.8
5		102.2		104.6
6		156.5		155.6
6a		125.0		125.5
7	8.17 d (8.5)	121.9	8.17 d (7.5)	122.5
8	7.50 dd (7.5, 7.5)	126.3	7.57 m	127.0
9	7.61 dd (7.0, 7.0)	129.3	7.57 m	129.4
10	8.36 d (8.0)	124.0	8.33 d (7.5)	123.8
10a		128.8		128.8
10b		141.6		140.8
$\underline{C}O_2Me$		172.5		171.2
CO_2Me	4.01 s	52.2	4.03 s	52.8
2-$(Me)_2$	1.49 s	26.8	1.48 s	22.1, 24.6
OH	12.16 s		11.15 s	

a) δ in ppm, J (in parentheses) in Hz.

Topoisomerase I and II inhibitory activities were measured by assessing the relaxation of supercoiled pBR 322 plasmid DNA. As shown in Table 2, **1**, **6** and **8** strongly inhibited DNA topoisomerase I at 100 μM, and when these three compounds were assayed at 20 μM, **1** and **6** showed 42 and 33% inhibition, respectively. In DNA topoisomerase II assays, **1**, **4** and **9** showed 65, 54 and 70% inhibition at 100 μM, respectively. The new compound, **3**, showed 42 and 23% inhibition for topoisomerases I and II at 100 μM, respectively. Camptothecin and etoposide for the positive control assays of topoisomerase I and II inhibited 81 and 86% at 100 μM and 70 and 58% at 20 μM, respectively.

The tetrazolium-based colorimetric assay (MTT assay) was used to assess 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 66.9, 51.6 and 61.4 μM were obtained for **1**, **8** and **9** in the HT-29 cell line, 41.3 μM for **3** in the MCF-7 cell

Table 2. Inhibitory Effects of Compounds **1**—**9** on DNA Topoisomerases I and II (%, Inhibition Ratio of Relaxation) and Their IC_{50} Values against HT-29, MCF-7 and HepG2 Cell Lines

Compd.	Topo I (%) (μM)		Topo II (%) (μM)		IC_{50} (μM)		
	100	20	100	20	HT-29[a]	MCF-7[b]	HepG2[c]
1	58	42	65	5	66.9	>100	>100
2	0	2	0	0	>100	>100	60.2
3	42	1	23	7	>100	41.3	76.3
4	51	11	54	7	>100	>100	>100
5	6	1	0	0	14.3	25.0	40.5
6	96	33	30	0	81.8	81.8	69.3
7	1	1	3	0	>100	>100	>100
8	75	17	49	0	51.6	>100	>100
9	37	4	70	0	61.4	>100	>100
CPT[d]	81	70	NA[f]				
VP-16[e]	NA		86	58			

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.

line, and 60.2 and 69.3 μM for **2** and **6** in the HepG2 cell line. Notably, the IC_{50} values for **5** were 14.3, 25.0 and 40.5 μM in the HT-29, MCF-7 and HepG2 cell lines, respectively.

At 100 μM, **6** showed the strongest inhibitory activity toward DNA topoisomerase I, and **8**, the second most effective inhibitor, has selective inhibitory activity against DNA topoisomerase I compared with DNA topoisomerase II. Against DNA topoisomerase II, the first and second most effective inhibitors, **9** and **1**, had selective inhibitory activity against DNA topoisomerase II compared with DNA topoisomerase I. However, these four compounds showed weak cytotoxicity against HT-29, MCF-7 and HepG2 cell lines. Compound **5** showed strong cytotoxicity against HT-29 and MCF-7 cell lines, but weak inhibitory activity against DNA topoisomerases I and II. These results indicate no obvious correlation between the cytotoxicity of these compounds and their inhibitory activity against DNA relaxation and decatenation by DNA topoisomerases I and II. Compound **1**, with its naphthofuran moiety, exhibited stronger inhibitory activity against DNA topoisomerases I and II than **2**, with its naphthopyran ring. The DNA topoisomerase I and II inhibitiory activities and cytotoxicity against MCF-7 cell line of **3** (epoxymollugin) were stronger than those of **2** (mollugin) due to epoxidation. Based on the inhibitory activities against DNA topoisomerases I and II of anthraquinones (**6**—**8**), the acetyl group on C-1 and the methoxyl group on C-2 or C-3 are considered to enhance the inhibition of DNA topoisomerases I and II.

Experimental

Plant Material The roots of *Rubia cordifolia* L. were purchased in November 2002 from a folk medicine market, "Yak-ryong-si", in Daegu, Republic of Korea. These materials were confirmed taxonomically by Professor Chong Won Kim, College of Pharmacy, Catholic University of Daegu, Korea. A voucher specimen has been deposited at the College of Pharmacy, Catholic University of Daegu, Korea.

General Procedures Melting points were measured using a Yanaco micro melting point apparatus. Optical rotation was measured using a JASCO DIP-370 digital polarimeter. UV spectra were measured on a Shimadzu UV-160A spectrometer. IR (KBr disk) spectra were measured on Mattson Genesis II and JASCO-300E FT-IR spectrophotometers, EI-MS and HR-FAB-MS were performed with a Quattro II spectrometer. The NMR spectra were recorded in CD_3OD, $CDCl_3$, and C_5D_5N on Varian Unity INOVA-500 and Bruker DMX 250.

Extraction and Isolation Roots of *Rubia cordifolia* L. (10 kg) were cut into small pieces and extracted four times with MeOH at 60 °C. MeOH extract (1048.4 g) was dissolved in H_2O (2.2 l) and fractionated with CH_2Cl_2 (2 l×5, 360.0 g). The H_2O layer was extracted sequentially with EtOAc (2 l×5, 214.2 g) and *n*-butanol (2 l×5, 117.6 g). One hundred and eighty grams of CH_2Cl_2 extract were subjected to flash 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 to afford 34 fractions, RA-MC-1 to RA-MC-34.

Activity-directed isolation of RA-MC-1, -2, -15, -17 and -24 resulted in the identification of nine compounds. Furomollugin (**1**, 38 mg) in the first fraction was obtained directly. Fraction RA-MC-2 (5.0 g) was chromatographed on a silica gel column (3.2×60 cm, silica-gel under 70 mesh, elution with 100% hexane) to yield mollugin (**2**, 2.250 mg). Fraction RA-MC-15 (1.30 g) was chromatographed on a reverse-phase column (3.5×57 cm, LiChroprep Rp-18, using 30% CH_3CN/70% H_2O to 100% CH_3CN in 5% stepwise elutions) and then Fraction RA-MC-15-7 (0.25 g) was chromatographed on a silica gel column (2.3×53 cm, silica-gel under 70 mesh, elution with hexane : EtOAc=18 : 0.3) to afford 2-carbomethoxy-2,3-epoxy-3-prenyl-1,4-naphthoquinone (**5**, 157.2 mg). Fraction RA-MC-17 (1.45 g) was chromatographed on a reverse-phase column (3.5×57 cm, LiChroprep Rp-18, using 45% MeOH/55% H_2O to 100% MeOH in 5% stepwise elutions) and then RA-MC-17-8 (40.0 mg) was chromatographed on a silica gel column (2.3×53 cm, silica-gel under 70 mesh, elution with hexane : EtOAc=12 : 0.5) to give 1-acetoxy-3-methoxy-9,10-anthraquinone (**6**, 8.9 mg) and oleanolic acid (**9**, 8.0 mg). Fraction RA-MC-24 (1.30 g) was chromatographed on a reverse-phase column (3.5×57 cm, LiChroprep Rp-18, with 30% MeOH/70% H_2O to 100% MeOH in 5% stepwise elutions) and then fractions RA-MC-24-2, 8, 14 and 23 (75.0, 66.4, 65.2 and 62.7 mg, respectively) were chromatographed on a silica gel column (2.3×53 cm, silica-gel under 70 mesh, and elution with hexane : EtOAc=9 : 1, 9 : 1, 15 : 0.7 and 12 : 0.7, respectively) to afford 3,3′-bis(3,4-dihydro-4-hydroxy-6-methoxy-2*H*-1-benzopyran) (**4**, 22.7 mg), methyl 2,2-dimethyl-3,4-epoxy-6-hydroxy-2*H*-naphtho[1,2-b]pyran-5-carboxylate (epoxymollugin, **3**, 16.3 mg), 1,6-dihydroxy-2-methyl-9,10-anthraquinone (soranjidiol, **7**, 13.3 mg) and 1-hydroxy-2-methoxy-9,10-anthraquinone (alizarin 2-methyl ether, **8**, 18.9 mg).

The structures of these compounds were elucidated on the basis of the data that follows in the Results and Discussion and by comparison with authentic samples.

Compound **3** (Epoxymollugin): Brown powder, mp 135.2—137.1 °C. $[\alpha]_D^{22}$ +3.3° (*c*=0.02, $CHCl_3$). UV λ_{max} ($CHCl_3$) nm (log ε): 369.5 (3.57). IR ν_{max} (KBr) cm^{-1}: 3433 (OH), 1653 (C=O), 1447 (C=C), 1238 (ester C–O). EI-MS *m/z*: 318 $[M+H_2O]^+$, 300 $[M]^+$, 286, 215, 214, 158. HR-FAB-MS (pos.) *m/z*: 301.1072 for $C_{17}H_{17}O_5$ (Calcd 301.1076). ^{1}H- and ^{13}C-NMR data are shown in Table 1.

Compound **6** (1-Acetoxy-3-methoxy-9,10-anthraquinone): Yellowish powder, mp 101.2—102.9 °C. UV λ_{max} ($CHCl_3$) nm (log ε): 461.0 (3.21). IR ν_{max} (KBr) cm^{-1}: 1737, 1677 (C=O), 1594 (C=C), 1026 (ester C–O). EI-MS *m/z*: 296 $[M]^+$, 281 $[M-CH_3]^+$, 265 $[M-OCH_3]^+$, 253 $[M-COCH_3]^+$, 238. ^{1}H-NMR (δ, $CDCl_3$) 2.36 (3H, s, –OCOMe), 4.01 (3H, s, –OMe), 6.26 (1H, d, *J*=3.4 Hz, H-2), 7.66 (1H, d, *J*=3.5 Hz, H-4), 7.69—7.89 (2H, m, H-

6, 7), 8.06—8.12 (2H, m, H-5, 8). ^{13}C-NMR (δ, $CDCl_3$) δ: 14.0 (–OCOMe), 52.7 (–OMe), 111.0 (C-2), 124.1 (C-4), 126.2 (C-5), 126.8 (C-8), 129.7 (C-4a), 130.2 (C-9a), 131.3 (C-10a), 132.0 (C-8a), 133.9 (C-6), 134.3 (C-7), 144.0 (C-1), 157.6 (–OCOMe), 166.0 (C-3), 182.0 (C-10), 183.2 (C-9).

Assay for DNA Topoisomerase I Inhibition *in Vitro* DNA topoisomerase I inhibition assays were carried out according to the method reported by Fukuda *et al.*[12] with minor modifications. DNA topoisomerase I activity was measured by assessing the relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixture contained 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. This reaction mixture was used to measure the inhibition of DNA relaxation by the DNA topoisomerase I, by 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 containing 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 gels 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, and then destained in water for 30 min. For visualization and quantitative analyses of DNA topoisomerase I activities, 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 concentrations of test compounds (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 gels 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 The tetrazolium-based colorimetric assay (MTT assay) was used as an *in vitro* assay of cytotoxicity against human colon carcinoma (HT-29), human breast carcinoma (MCF-7) and human liver carcinoma (HepG2) cells .[13]

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