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α-Amyrin Triterpenoids and Two Known Compounds with DNA Topoisomerase I Inhibitory Activity and Cytotoxicity from the Spikes of *Prunella vulgaris* var. *lilacina*

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Abstract – Three known α-amyrin triterpenoids, ursolic acid (**1**), 2α,3α-dihydro xyurs-12-ene-28-oic acid (**2**) and euscaphic acid (**3**), and β-amyrin triterpenoid, 3β-hydroxyolean-5,12-diene (**4**), and α-spinasterol (**5**) have been isolated from the fractionated *n*-butanol extracts of the spikes of *Prunella vulgaris* var. *lilacina*, guided by DNA topoisomerases I and II inhibitory activities and cytotoxic activity against human cancer cells. Their structures were elucidated on the basis of spectroscopic and chemical methods. Compound **4** exhibited significant cytotoxic activity against human colon adenoblastoma (HT-29), and **5** showed DNA topoisomerase I and II inhibitions.
Key words - *Prunella vulgaris* var. *lilacina*, DNA topoisomerases I and II inhibitory activities, cytotoxic activity, ursolic acid, 2α,3α-dihydroxyurs-12-ene-28-oic acid, euscaphic acid

Introduction

The DNA topoisomerases are the enzymes for relaxation of DNA during a number of critical cellular processes, including replication, recombination and transcription by transiently breaking one or two strands of DNA, passing a single- or double-stranded DNA through the break, and finally resealing the breaking (Wang, 1996; Pommier, 1993; D'Arpa, *et al*., 1989). Currently only a camptothecin (CPT) as DNA topoisomerase I-directed drugs has been introduced into the clinic to date (Slichenmeyer, *et al*., 1993; Potmesil, 1994), and many topoisomerase II-directed drugs have been in clinical use for many years (Chen, *et al*., 1994; Bodley, *et al*., 1988). The DNA topoisomerases have been established as important molecular targets for anticancer drugs (Liu, 1989). The spikes of *Prunella vulgaris* Linne var. *lilacina* Nakai (Labiatae) have been used as a traditional medicine for the treatment of fever, inflammation, dropsy, urinary disadvantage, gonorrhea and cancer (Namba, 1994). Several saponins, triterpenoids, flavonoids, sterols, fatty acids and polyphenolic compounds have been reported from the spikes of this plant (Okuda, *et al*., 1986; Wang, *et al*., 1999; Tian, *et al*., 2000).

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To find lead compounds for anticancer agent from Korean medicinal plants, cytotoxic activities of 200 species were screened, and inhibitory activities against DNA topoisomerase I were measured for MeOH extracts of 30 species posessing potent cytotoxic activity. In result, MeOH extracts of *Prunella vulgaris* var. *lilacina* showed potent cytotoxicities and DNA topoisomerase I inhibition. In this study, we report topoisomerases I and II inhibitory activities and cytotoxic activity against HT-29, HepG2 and MCF-7 cell lines for five compounds isolated from the spikes of *P. vulgaris* var. *lilacina*.

Experimental

Instruments and reagents – Melting points were determined on a Yanaco micro melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. IR spectra were measured on a Mattson Polaris FT/IR-300E spectrophotometer. NMR spectroscopy was taken on a Varian Unity INOVA-500S spectrophotometer in pyridine-d_5 using TMS as an internal standard. Mass spectra were recorded on a Quattro II spectrometer. Stationary phases for column chromatography were silica gel 60 (70 - 230 and 230 - 400 mesh, Merck). For TLC and HPTLC, silica gel 60 F_{254} (EM 5715, 5628) glass plates (0.25 mm) were

used and visualized by spraying with 10% H_2SO_4 and subsequent heating. All other chemicals and solvents were analytical grade and were used without further purification.

Plant material – The spikes of *Prunella vulgaris* var. *lilacina* were collected from the herb-market of Daegu Yangnyeongsi, Korea in July 2000. A voucher specimen has been deposited at the College of Pharmacy, Catholic University of Daegu, Korea.

Extraction and isolation – The spikes of *Prunella vulgaris* var. *lilacina* (10 kg) were extracted three times with MeOH by reflux for 8 hours. The MeOH extract was concentrated under reduced pressure to yield of a black syrup (F001, 1037.7 g). F001 was partitioned between CH_2Cl_2-H_2O (1 : 1) to yield the CH_2Cl_2-soluble fraction (F002, 224.5 g), interface (F003), and the H_2O-soluble fraction I (F004). F003 was partitioned between *n*-BuOH saturated with H_2O to give the *n*-BuOH-soluble fraction I (F005, 44.0 g) and the H_2O-soluble fraction II (F006). The combined H_2O-soluble fraction (F004 and F006) was then partitioned between *n*-BuOH saturated with H_2O to yield the *n*-BuOH-soluble fraction II (F007, 181.7 g) and the H_2O-soluble fraction III (F008, 587.1 g). F001, F002, F005, F007 and F008 were assayed for cytotoxic activities against HT-29 and HepG2, and the inhibitory activity against DNA topoisomerase I. The most active fraction was F005 (see Table 1 and Fig. 1). F005 was subjected to flash column chromatography on silica gel, eluted with a gradient of CH_2Cl_2/MeOH/H_2O. Fractions were collected into 18 pools (Fr. I - Fr. XVIII) according to their similar TLC patterns. The combined fraction (Fr. II and III; 5.73 g) were further purified by silica gel open column eluted with CH_2Cl_2/MeOH/H_2O to afford compounds **1** (10.45 g), **2** (135 mg), and **3** (120 mg). F002 was subjected to flash column chromatography on silica gel, eluted with a gradient of Hexane/EtOAc. Fractions were collected into 17 pools (Fr. A - Fr. Q) according to their similar TLC patterns. Fr. F (5.62 g) was further resolved on a silica gel open column, eluted with a gradient of Hexane/EtOAc to afford compounds **4** (50 mg). Fr. J (2.59 g) was purified on a silica gel open column, eluted with a gradient of Hexane/EtOAc to yield **5** (140 mg).

Ursolic acid (1) – white amorphous powder; mp 288.2 -290.1 °C; $[\alpha]_D^{25}$: +67.5° (*c* 1.0, MeOH); IR (KBr) ν_{max} cm^{-1}: 3418 (OH), 1691 (COOH), 1637 (C = C); Mass (EI) *m/z*: 456 $[M]^+$, 438, 248 (100), 208, 203, 190; ^1H and ^{13}C NMR data are consistent with literature data (Lin, *et al*., 1987).

2α,3α-dihydroxyurs-12-ene-28-oic acid (2) – white needle; mp 236.7 - 238.3 °C; IR (KBr) ν_{max} cm^{-1}: 3418 (OH), 1689 (COOH), 1651 (C = C); Mass (EI) *m/z*: 472 $[M]^+$, 454, 426, 409, 408, 248 (100), 223, 203, 205; ^1H and ^{13}C NMR data are consistent with literature data (Wang, *et al*., 1999).

Euscaphic acid (3) – white amorphous powder, mp 268.5 - 270.4 °C; IR (KBr) ν_{max} cm^{-1}: 3418 (OH), 1689 (COOH), 1652 (C = C); Mass (EI) *m/z*: 488 $[M]^+$, 442, 264, 244, 219, 218, 206, 146 (100); ^1H and ^{13}C NMR data

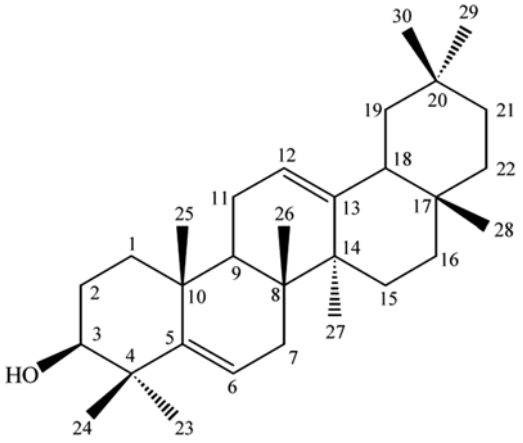

Fig. 1. Chemical structure of compound **4**.

Table 1. Cytotoxic activities of solvent fractions from the spikes of *P. vulgaris* var. *lilacina*

Solvent Fraction	Topoisomerase I [a] (μg/ml)		Cytotoxicity (%)			
			HT-29 [b]		HepG2 [c]	
	50	5	50 μg/ml	5 μg/ml	50 μg/ml	5 μg/ml
MeOH (F001)	++	–	0	0	0	0
CH_2Cl_2 (F002)	++	–	–	–	11.4	7.9
n-BuOH (F005)	++	–	48.6	1.2	1.5	–
n-BuOH (F007)	++	±	–	–	–	–
H_2O (F008)	++	++	–	–	–	–

[a] Degree of inhibition: ++; positive, +; medium, ±; slight, –; negative
[b] HT-29: human colon adenocarcinoma; [c] HepG2: human liver carcinoma

are consistent with literature data (Atsushi, *et al*., 1989).

3β-hydroxyolean-5,12-diene (**4**) – white amorphous powder, mp 164.2 - 166.1 °C; $[\alpha]_D^{25}$: +76° (*c* 0.05, pyridine); IR (KBr) ν_{max} cm^{-1}: 3418 (OH), 1638 (C = C); Mass (EI) *m/z*: 424 $[M]^+$ (9), 258 (2.5), 218 (100), 206 (11), 188 (13), 148 (8); ^{13}C NMR (125 MHz, pyridine-d_5) δ: 16.26 (C-26), 16.43 (C-27), 17.06 (C-11), 17.55 (C-23), 17.60 (C-16), 18.27 (C-25), 24.03 (C-15), 24.30 (C-2), 26.68 (C-28), 29.09 (C-24), 29.26 (C-30), 31.70 (C-29), 33.24 (C-20), 34.48 (C-17), 37.66 (C-10), 37.75 (C-21), 39.90 (C-22), 40.61 (C-7), 40.81 (C-1), 42.42 (C-8), 42.80 (C-4), 48.04 (C-14), 48.52 (C-9), 56.23 (C-19), 59.85 (C-18), 78.23 (C-3), 122.75 (C-6), 125.64 (C-12), 140.36 (C-13), 145.71 (C-5)

***α*-spinasterol** (**5**) – white amorphous powder, mp 153.5 - 155.1 °C; $[\alpha]_D^{25}$: –52° (*c* 0.05, pyridine); IR (KBr) ν_{max} cm^{-1}: 3418 (OH), 1637 (C = C); Mass (EI) *m/z*: 412 $[M]^+$, 397, 368, 300, 273, 255, 112, 97; ^1H and ^{13}C NMR data are consistent with literature data (Kim, *et al*., 1999).

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*. (Fukuda, *et al*., 1996) with minor modifications. Topoisomerase I activity was measured by measuring 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 DTT, 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 were terminated by adding 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 an 1% agarose gel in TBE (Tris-borate-EDTA) running buffer at 1.5 V/cm for 10 hrs. The gels were stained in ethidium bromide (0.5 μg/mL) for 30 min, then destained in water for 30 min. For visualization and quantitative analyses of 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. 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 were terminated by addition of 5 μL of a mixture [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 an 1% agarose gel in TBE running buffer at 1.5 V/cm for 10 hrs. The gels were stained in 0.5 μg/mL ethidium bromide for 30 min, and were destained in water for 30 min. For visualization and quantitative analyses of DNA topoisomerase II activity, the gels were directly scanned with an image analyzer, and the area representing supercoiled DNA was calculated.

Cytotoxic Activity Bioassays – The tetrazolium-based colorimetric assay (MTT assay) was used for the *in vitro* assay of cytotoxicity to human colon adenoblastoma (HT-29), human breast adenocarcinoma (MCF-7), and human liver hepatoma (HepG2) cell lines (Rubinstein, *et al*., 1990; Mosman, 1983).

Results and Discussion

The MeOH extract of the spikes of *P. vulgaris* var. *lilacina* was partitioned between CH_2Cl_2 and H_2O, and the resulting interface layer was extracted with *n*-BuOH. Compounds **1 - 5** were isolated from the CH_2Cl_2 and *n*-BuOH fractions by column chromatography on silica gel. Their structures were elucidated by chemical and spectrascopic evidences, and they were identified as 3β-hydroxyurs-12-ene-28-oic acid (ursolic acid, **1**), 2α,3α-dihydroxyurs-12-ene-28-oic acid (**2**), 2α,3α,19α-trihydroxyurs-12-ene-28-oic acid (euscaphic acid, **3**), α-amyrin type triterpenoids, 3β-hydroxy-olean-5,12-diene (**4**), and α-spinasterol (**5**). Compounds **1**, **2** and **5** were previously isolated from this plant and reported. Compound **3** was known but first isolated from this plant. Compound **4** was registered only at chemical abstracts service (CAS), but was no evidence in the literature.

Compound **1** was isolated as a white amorphous powder. The molecular weight of **1** was indicated by a peak at *m/z* 456 $[M]^+$ in the EIMS spectrum. The presence of a hydroxyl group was indicated by the loss of one molecule of H_2O from $[M - H_2O]^+$ (*m/z*, 438) in the EIMS spectrum. Base peak was indicated at *m/z* 248 caused by characteristic retro-Diels-Alder reaction (Budzikiewicz, 1963) of Δ^{12}-unsaturated pentacyclic triterpenoid compounds. The ^1H NMR signals at δ 0.91, 1.04, 1.07,

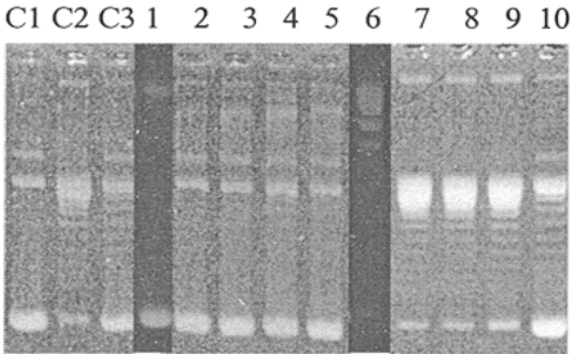

Fig. 2. Topoisomerase I inhibitory activity of solvent fractions from the spikes of *P. vulgaris* var. *lilacina*.

C1: supercoiled DNA alone; **C2**: supercoiled DNA + topoisomerase I (calf thymus) 0.3 U; **C3**: supercoiled DNA + topoisomerase I (calf thymus) 0.3 U + camptothecin (10 μM: 3.48 μg/mL); **1:** MeOH (F001, 50 μg/mL); **2**: CH_2Cl_2 (F002, 50 μg/mL); **3**: *n*-BuOH (F005, 50 μg/mL); **4**: *n*-BuOH (F007, 50 μg/mL); **5**: H_2O (F008, 50 μg/mL); **6**: MeOH (F001, 5 μg/mL); **7**: CH_2Cl_2 (F002, 5 μg/mL); **8**: *n*-BuOH (F005, 5 μg/mLl); **9**: *n*-BuOH (F007, 5 μg/mL); **10**: H_2O (F008, 5 μg/mL)

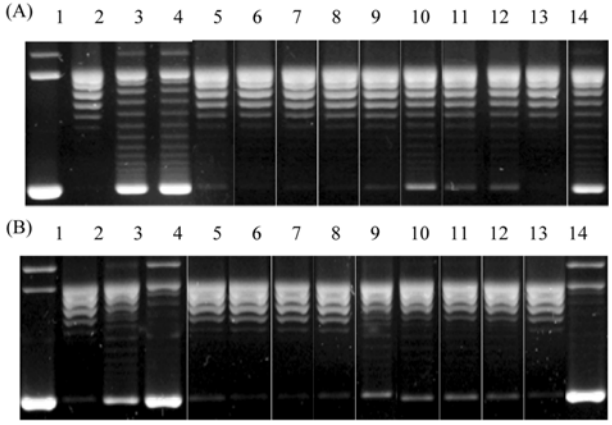

Fig. 3. DNA topoisomerases I (A) and II (B) inhibitory activity of compounds 1-5.

(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); 5-9: compounds **1 - 5** (20 μM); 10-14: compounds **1 - 5** (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 - 9: compounds **1 - 5** (20 μM); 10 - 14: compounds **1 - 5** (100 μM).

1.25 and 1.26 (23-, 24-, 25-, 26-, 27-CH_3) were indicated the presence of the five angular methyl groups. Doublet signals at δ 0.97 (29-CH_3), 1.02 (30-CH_3) and 2.65 (H-18), broad singlet signal at δ 5.51 (H-12), and two carbon resonances at δ 125.67 and 139.28 in the ^1H and ^{13}C NMR spectra were typical urs-12-ene type signals (Numata, *et al*., 1989). A carbon signal at δ 179.89 (C-28) was suggested the presence of a carboxyl group. The reason of upfield shift of C-13 chemical shift in comparison with β-amyrin type is that C-13 is influenced of γ-gauche effect by induction of methyl group at C-19. The arrangement of H-3 was assigned as α because of proton double triplet at δ 3.47 and carbon resonances at *d* 78.14 (C-3) (Kang, *et al*., 1986). From these results, compound **1** was identified as 3*b*-hydroxyurs-12-ene-28-oic acid, ursolic acid.

Compound **2** was a white needle. The molecular weight of **2** was indicated by a peak at *m/z* 472 $[M]^+$ in the EIMS spectrum. The presence of two hydroxyl groups was indicated by the loss of a molecule of H_2O from $[M - H_2O]^+$ (*m/z*, 454) in the EIMS spectrum. Base peak was indicated at *m/z* 248 caused by characteristic retro-Diels-Alder reaction (Budzikiewicz, 1963) of Δ^{12}-unsaturated pentacyclic triterpenoid compounds. The arrangements of H-2 and H-3 were assigned as H-2β and H-3β because of signal splitting patterns, coupling constants and $\Delta\delta_{2\text{-}3}$ of two signals, and proton multiplet at δ 4.30 and proton doublet at δ 3.76, and carbon resonances at *d* 66.09 (C-2) and δ 79.31 (C-3) (Grover, *et al*., 1973; Kojima, *et al*., 1989). Thus, this compound was identified as 2α,3α-dihydroxyurs-12-ene-28-oic acid.

Compound **3** was isolated as a white amorphous powder. The molecular weight of **3** was indicated by a peak at *m/z* 488 $[M]^+$ in the EIMS spectrum. Base peak was indicated at *m/z* 146 caused by characteristic retro-Diels-Alder reaction (Budzikiewicz, 1963) of Δ^{12}-unsaturated pentacyclic triterpenoid compounds. The presence of a hydroxyl group at D/E ring and two hydroxyl groups at A/B ring was suggested from mass fragmentation. The arrangements of H-2 and H-3 were assigned as H-2β and H-3β because of signal splitting patterns, coupling constants and $\Delta\delta_{2\text{-}3}$ of two signals, and proton multiplet at δ 4.30 and proton doublet at δ 3.76, and carbon resonances at δ 66.11 (C-2) and 79.36 (C-3) (Grover, *et al*., 1973; Kojima, *et al*., 1989). Another hydroxyl group exists at C-19 because of proton singlet signal at δ 3.04 (H-18) and carbon signal at δ 72.69 (C-19). Finally, this compound was identified as 2α,3α,19α-trihydroxyurs-12-ene-28-oic acid, euscaphic acid.

Compound **4** was isolated as a white amorphous powder. The molecular weight of **4** was indicated by a peak at *m/z* 424 $[M]^+$ in the EIMS spectrum. By retro-Diels-Alder reaction from C ring, the fragment ions of A/B and D/E rings were indicated at *m/z* 206 and 218 (base peak), respectively. In addition, by retro-Diels-Alder reaction from B ring the fragment ions of C/D/E ring, and

Table 2. Inhibitory effects of compounds **1-5** on DNA topoisomerases I and II (%, inhibition ratio of relaxation) and cytotoxic activity against HT-29, MCF-7 and HepG2 cell lines

Comp.	Topo I (%)		Topo II (%)		Cytotoxicity IC_{50} (μM)		
	20 (μM)	100 (μM)	20 (μM)	100 (μM)	HT-29 [a]	MCF-7 [b]	HepG2 [c]
1	5	28	1	7	64.0	82.0	79.0
2	1	13	0	10	>100	>100	>100
3	1	13	0	4	46.0	>100	79.0
4	1	0	0	0	26.3	63.0	93.0
5	4	70	7	76	>100	>100	>100
CPT [d]	76	82	NA [f]				
VP-16 [e]	NA		37	78			

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.

dehydrate of A ring were shown at *m/z* 258 and 148, separately. Accordingly, the presence of a hydroxyl group at A ring and double bonds at B and C rings, were suggested from mass fragmentation. The ^{1}H-NMR spectrum of **4** showed eight singlet angular methyl protons at δ 0.99-1.27. That showed double triplet at δ 3.47 (1H, dt, J = 3.0, 7.5 Hz, H-3α), and olefinic multiplet protons at δ 5.23 (1H, H-6) and 5.27 (1H, H-12), respectively. These results then indicated that compound **3** was olean type of triterpenoid, having two double bonds and hydroxyl group (Nakanish, *et al*., 1982). In the ^{13}C-NMR and DEPT spectra of **4**, signals including characteristic peaks of olefinic carbon showed quaternary carbon at δ 145.71 (C-5) and 140.36 (C-13), and methine carbon at δ 122.75 (C-6) and 125.64 (C-12), thus **4** suggested β-amyrin type from the ^{13}C-NMR chemical shift values of C-12 and C-13 (Kang, *et al*., 1987). The assignment was made on the basis of ^{13}C-NMR data by comparison of the literature data of β-amyrin (Kang, *et al*., 1987). Accordingly, this compound was identified as 3$\hat{a}$-hydroxyolean-5,12-diene.

The conversion of supercoiled pBR 322 plasmid DNA to relaxed DNA by calf thymus topoisomerase I and human topoisomerase II was examined in the presence of five compounds (Table 2). As shown in Table 2, **5** showed 70 and 76% inhibitions for DNA topoisomerases I and II at the concentration of 100 μM, respectively. The tetrazolium-based colorimetric assay (MTT assay) was used for the cytotoxic activity against 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 **1, 3**, and **4** were 64.0, 46.0, and 26.3 μM for HT-29 cell line, and those of **1** and **4** were 82.0 and 63.0 μM for MCF-7 cell line. Also IC_{50} of **1**, **3**, and **4** for HepG2 cell line were 79.0, 79.0, and 93.0 μM, respectively.

Considering the selectivity between the inhibitory activities of topoisomerases I and II, **5** showed the strongest inhibitory activity against DNA topoisomerases I and II at the concentration of 100 μM, and **3** and **4** showed moderate cytotoxic activity against HT-29 cell line. However, another compounds showed weak cytotoxic activity against HT-29, MCF-7 and HepG2 cell lines. These results indicate that no obvious correlation can be observed between the cytotoxic activity and the inhibitory activity of DNA relaxation and decatenation by DNA topoisomerases I and II.

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