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DNA Topoisomerases I and II Inhibitory Activity of Constituents Isolated from *Juglans mandshurica*

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Nine diarylheptanoids (**1-9**), one triterpene (**10**), one sesquiterpenoid (**11**), one naphthoquinone (**12**), four tetralones (**13-16**), one naphthalene carboxylic acid glucoside (**17**) and six naphthalenyl glycosides (**18-23**) were isolated from the roots of *Juglans mandshurica* Maximowicz (Juglandaceae), and their structures determined from the chemical and spectral data. Here, we report the inhibitory effects, on the DNA topoisomerases I and II activities, of all these compounds. Compounds **10** and **23** showed more potent inhibitory effects, on the DNA topoisomerases I and II (94.0 and 86.0% inhibitions at the concentration of 5 μg/mL, respectively), than the positive control compounds, camptothecin and etoposide.

Key words: *Juglans mandshurica,* DNA topoisomerases I and II inhibitors

INTRODUCTION

DNA topoisomerases are enzymes that relax 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 break (Wang, 1996; Pommier, 1993; D'Arpa and Liu, 1989). Currently, only the camptothecin (CPT) family of compounds, as DNA topoisomerase I-directed drugs, have been introduced into the clinic (Slichenmeyer *et al.*, 1993; Potmesil, 1994), and many topoisomerase II-directed drugs have been in clinical use for many years (Chen and Liu, 1994). Therefore, DNA topoisomerases have been established as important molecular targets for anticancer drugs (Liu, 1989).

The roots of *J. mandshurica* have been used as a folk medicine for the treatment of cancer in Korea. During our studies to find the leading compounds for an anticancer agent from Korean medicinal plants, we isolated 22 compounds from *J. mandshurica,* and evaluated their cytotoxicities (Son, 1995; Joe *et al.*, 1996; Kim *et al.,* 1998: Lee *et al.*, 2000; Lee *et al.*, 2002; Li *et al.*, 2003). In this paper, as a continuing biochemical study of these isolated compounds, their inhibitory activities against DNA topoisomerases I and II were evaluated.

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MATERIALS AND METHODS

General experimental procedure

The optical rotations were measured using a JASCO DIP-1000 (Tokyo, Japan) automatic digital polarimeter. The NMR spectra were recorded on a Bruker 250 MHz (DMX 250) spectrometer, using Bruker's standard pulse program. Samples were dissolved in acetone-d_6 and the chemical shifts reported in ppm downfield from TMS. The FAB-MS spectra were measured by a VG TRIO 2A mass spectrometer. The stationary phases for the column chromatography (Silica gel 60, 70-230 and 270-400 mesh and Lichroprep RP-18 gel, 40-63 μM, Merck) and TLC plates (Silica-gel 60 F_{254} and RP-18 F_{254}) were purchased from EM Scientific. The spots were detected under UV radiation and by spraying with 10% H_2SO_4, followed by heating. The camptothecin and etoposide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The supercoiled pBR 322 plasmid DNA and calf thymus topoisomerases I were purchased from MBI Fermentas INC. (Hanover, MD, USA). The human topoisomerases II was purchased from TopoGEN INC.

(Columbus, OH, USA). All other chemicals and solvents were of analytical grade, and used with no further purification.

Plant material

The roots of *Juglans mandshurica* were collected during September 1993 in a mountainous area of Pyongchang-goon, Gangwon-do, Korea, and dried at room temperature for two weeks. A voucher specimen (YNS 93001) has been preserved at the College of Pharmacy, Yeungnam University.

Extraction and isolation

The roots of *J. mandshurica* (3 kg) were extracted twice, with MeOH, by refluxing for 12 h. The MeOH solution was evaporated to dryness (300 g), and then partitioned between H_2O and hexane. The resulting H_2O layer was extracted with $CHCl_3$, and the $CHCl_3$ evaporated to dryness *in vacuo*. The $CHCl_3$ extract (50 g) was loaded onto a silica gel column (60×9 cm, Si-gel 70-230 mesh) and the 7th Fraction (4.3 g) underwent chromatography on a silica gel column (60×4 cm, Si-gel 70-230 mesh), using a hexane-EtOAc (gradient from 40:60 to 10:90) eluent, and the 3rd sub-fraction from the column was further purified on a reverse phase column (60×2.0 cm, LiChroprep RP-18) with a MeOH-H_2O (gradient from 2:8 to 9:1) eluent, affording compound **10**. The isolation and structural elucidation of the test compounds **1-9** and **11-23** have been reported previously (Son, 1995; Joe *et al.*, 1996; Kim *et al.*, 1998; Lee *et al.*, 2000; Lee *et al.*, 2002; Li *et al.*, 2003).

Compound **10**: brown powder (40 mg); mp. 323-326; $[\alpha]_D^{25}$ +61.2° (*c* 0.2, MeOH) {lit. (Shaari and Waterman, 1996) $[\alpha]_D$ +57° (c 0.4, MeOH)}; ^{1}H- and ^{13}C-NMR data are consistent with the literature values (Shaari and Waterman, 1996); HR-FAB-MS *m/z* 641.3818 ([M+Na]$^+$, calcd. for $C_{39}H_{54}O_6Na$, 341.3818)

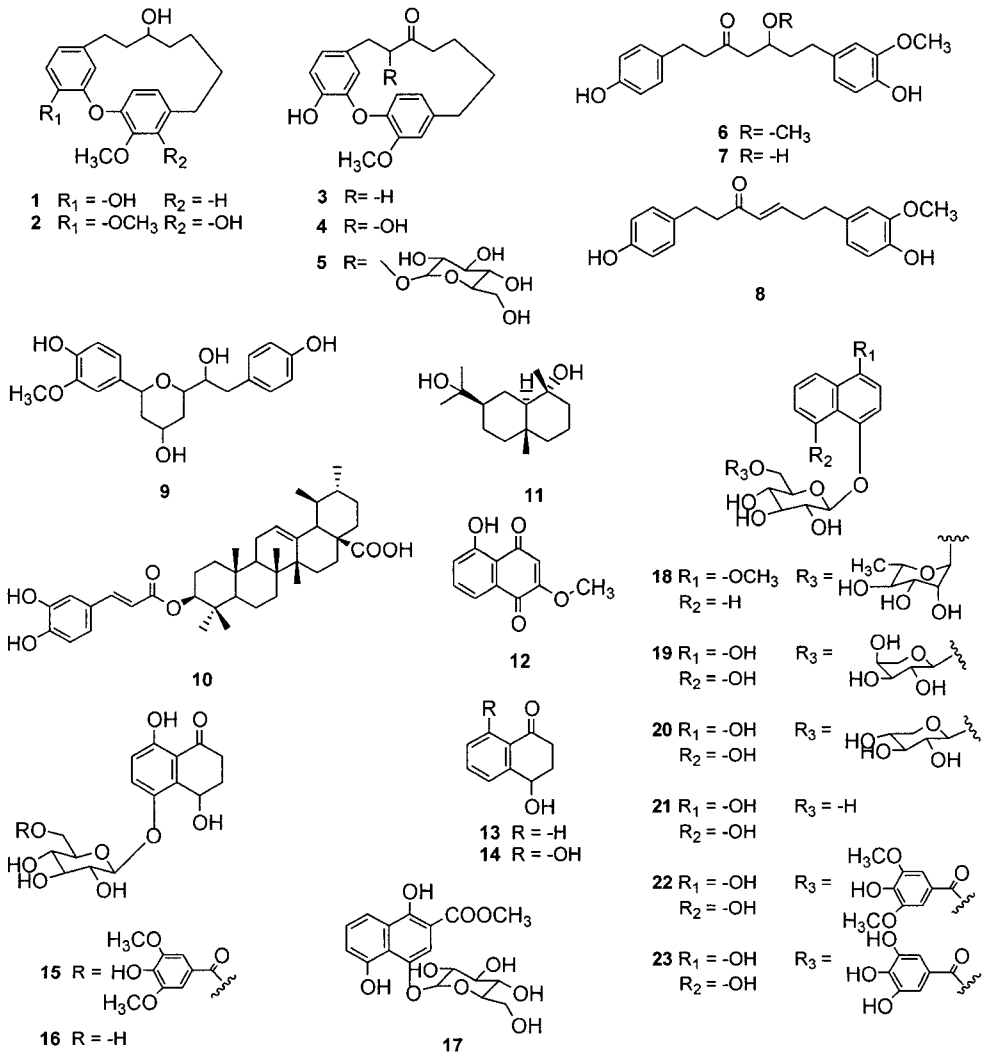

Fig. 1. Structures of compounds **1-23**

Assay for DNA topoisomerase I inhibition *in vitro*

A DNA topoisomerase I inhibition assay was carried out according to the method reported by Fukuda *et al.* (Fukuda *et al.*, 1996), with minor modifications. The 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 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 used for measuring the inhibition of the DNA relaxation by the topoisomerase I, in addition to a test compound solution (less than 0.25% DMSO), had a final volume of 10 μL. The reaction mixtures were incubated for 30 min at 37°C, and terminated by the addition of a dye solution comprised of 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 a 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 and destained in water for 30 min. For the visualization and quantitative analyses of the topoisomerase I activity, the gels were directly scanned with an image analyzer, and the area representing the supercoiled DNA calculated.

Assay for DNA topoisomerase II inhibition *in vitro*

The topoisomerase II activity was measured by assessing the relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixtures contained 50 mM Tris-HCl (pH 8), 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 the 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 a 1% agarose gel in TBE (Tris-borate-EDTA) 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 the visualization and quantitative analyses of the topoisomerase II activity, the gels were directly scanned with an image analyzer, and the area representing the supercoiled DNA calculated.

Cytotoxicity bioassays

The tetrazolum-based colorimetric assay (MTT assay) was used for the cytotoxicity towards human colon carcinoma (HT-29) and human breast carcinoma (MCF-7) cell lines (Rubinstein *et al.*, 1990).

RESULTS AND DISCUSSION

Compound **10** was isolated for the first time from *J. Mandshurica*, and identified by comparing the physical and spectroscopic data with the literature values (Shaari and Waterman, 1996).

The conversion of the supercoiled pBR 322 plasmid DNA to relaxed DNA, by the action of calf thymus topoisomerase I and human topoisomerase II, was examined in the presence of twenty three compounds (**1-23**) (Table I). In the DNA topoisomerase I assays, compounds **5, 9-11** and **13** showed 100% inhibition of the catalytic reaction, at the concentration of 50 μg/mL and when these five compounds were assayed at the concentration of 5 μg/mL, only **10** showed a significant inhibition, 94.0%, which was

Table I. Inhibitory effects of compounds **1-23** on topoisomerases I and II enzymes (% inhibition ratio of relaxation), and their IC_{50} values against HT-29 and MCF-7 cell lines

Compounds	Inhibition ratio for topoisomerase I (%)		Inhibition ratio for topoisomerase II (%)		Cytotoxicity IC_{50} (μg/mL)	
	50 μg/mL	5 μg/mL	50 μg/mL	5 μg/mL	HT-29[a]	MCF-7[b]
1	0	NA[c]	97.5	10.0	>50	>50
2	0	NA	0	NA	23.9	>50
3	0	NA	0	NA	5.3	>50
4	33.1	NA	95.7	8.5	>50	>50
5	100.0	10.3	0	NA	>50	>50
6	0	NA	0	NA	>50	>50
7	5.0	NA	24.2	NA	41.3	>50
8	37.7	NA	40.8	NA	8.2	22.1
9	100.0	4.1	50.6	1.7	>50	>50
10	100.0	94.0	89.9	33.3	22.1	38.6
11	100.0	5.9	31.6	NA	12.9	>50
12	5.0	NA	14.5	NA	2.6	1.2
13	100.0	4.6	33.3	NA	>50	>50
14	19.0	NA	8.1	NA	>50	>50
15	47.3	NA	0.4	NA	>50	>50
16	0	NA	0	NA	>50	>50
17	37.0	NA	95.0	6.0	8.1	>50
18	0	NA	0	NA	>50	>50
19	0	NA	100.0	0.	>50	>50
20	3.3	NA	100.0	0.	>50	>50
21	11.3	NA	18.0	NA	>50	>50
22	21.5	NA	59.6	0.	>50	>50
23	0	NA	100.0	86.0	>50	>50
CPT[d]	74.7[e]		NA		0.035	3.5
etoposide[f]	NA		84.7[g]		NA	NA

[a]HT-29: human colon carcinoma; [b]MCF-7: human breast carcinoma; [c]NA: not applicable; [d]camptothecin: positive control for topoisomerase I; [e]at 6.96 μg/mL (20 μM) concentration; [f]positive control for topoisomerase II; [g]at 29.43 μg/mL (50 μM) concentration.

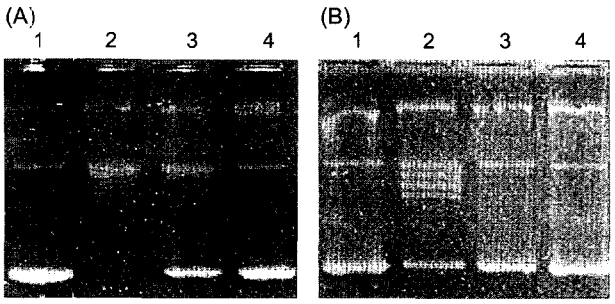

Fig. 2. Topoisomerase I inhibitory activity of compound **10** (**A**, 5 μg/mL), and topoisomerase II inhibitory activity of compound **23** (**B**, 5 μg/mL). **(A)** 1: supercoiled DNA alone; **2**: supercoiled DNA+topoisomerase I (calf thymus); **3**: supercoiled DNA + topoisomerase I (calf thymus) + camptothecin (20 μM) positive control; **4**: compound **10**. **(B) 1**: supercoiled DNA alone; **2**: supercoiled DNA+topoisomerase II (human); **3**: supercoiled DNA+topoisomerase II (human)+etoposide (50 μM) positive control; **4**: compound **23**.

more potent than the positive control compound, camptothecin (74.7% inhibition at a concentration of 6.96 μg/mL) (Fig. 2). In the DNA topoisomerase II assays, compounds **1**, **4**, **9**, **10**, **17**, **19**, **20**, **22** and **23** showed more than 50% inhibition at the concentration of 50 μg/mL, but only **23** showed 86.0% inhibition at the concentration of 5 μg/mL, which was more potent than etoposide (84.7% inhibition at a concentration of 29.43 μg/mL) (Fig. 2). In the cytotoxicity assays, the IC_{50} values of compounds **2**, **3**, **8**, **10**, **11**, **12** and **17**, against the HT-29 cell line, were 23.9, 5.3, 3.2, 22.1, 12.9, 2.6 and 8.1 μg/mL, respectively, and those of compounds **8**, **10** and **12**, against the MCF-7 cell line, were 22.1, 38.6 and 1.2 μg/mL, respectively (Table I).

Considering the selectivity between the inhibitory activities of topoisomerases I and II, compounds **5**, **11** and **13** had selective inhibitory effects against the topoisomerase I activity at a concentration of 50 μg/mL, but only compound **11** showed moderate cytotoxicity against the HT-29 cell line (Lee *et al.*, 2002). Compounds **1**, **4**, **17**, **19**, **20** and **23** had selective inhibitory effects against the topoisomerase II activity at a concentration of 50 μg/mL, but only compound **17** showed cytotoxicity against the two cell lines (Kim *et al.*, 1998). Compounds **9** and **10** caused inhibitory effects towards both topoisomerases I and II at a concentration of 50 μg/mL, and **10** showed not only a greater inhibitory effect against topoisomerase I (94.0% inhibition at 5 μg/mL) than camptothecin, but a significant inhibition against topoisomerase II (33.3% inhibition at 5 μg/mL), with only weak cytotoxicity. The reason that inhibition potencies of the above compounds, on the topoisomerases I and II, were not in parallel with the cytotoxicities on these cell lines might be speculated as; even though these compounds could directly inhibit the reactions of topoisomerases I and II in the enzyme assays, the transportation of these compounds through cell and nuclear membranes, in order to reach the target topoisomerases I and II, could be possible barriers in the MTT assay. Additional studies on the inhibition mechanisms of topoisomerases I and II, due to compounds **10** and **23**, are in progress.

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