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Cytotoxicity and DNA Topoisomerases Inhibitory Activity of Constituents from the Sclerotium of *Poria cocos*

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The bioactivity-guided fractionation of the methylene chloride extract of the sclerotium of *Poria cocos* led to the isolation of (*S*)-(+)-turmerone (**1**), ergosterol peroxide (**2**), polyporenic acid C (**3**), dehydropachymic acid (**4**), pachymic acid (**5**), and tumulosic acid (**6**). Compounds **4-6** exhibited moderate cytotoxicities, with IC_{50} values of 20.5, 29.1, and 10.4 μM, respectively, against a human colon carcinoma cell line. However, **3-6** not only showed inhibitory activities as potent as etoposide used as a positive control on DNA topoisomerase II (36.1, 36.2, 43.9 and 66.7% inhibition at a concentration of 20 μM, respectively), but also inhibition of DNA topoisomerase I (55.8, 60.7, 43.5, and 83.3% inhibition at a concentration of 100 μM, respectively).

Key words: *Poria cocos*, Polyporaceae, Cytotoxicity, DNA topoisomerases I and II inhibition

INTRODUCTION

The sclerotium of *Poria cocos* Wolf (Polyporaceae) has been used as a diuretic, sedative and tonic drug (Tai *et al.*, 1995a), and induces relaxation of the intestine, protects against ulcer formation, reduces the acidity of gastric juice and has an antinephritic effect (Hattori *et al.*, 1992). Various lanostane-type triterpene acids have been reported (Tai *et al.*, 1991, 1992, 1993, 1995a~c, 1996; Kaminaga *et al.*, 1996a~b; Nukaya *et al.*, 1996; Yasukawa *et al.*, 1998), some of which have been revealed to possess strong inhibitory effects against 12-*O*-tetradecanoylphorbol-13-acetate-induced and arachidonic acid-induced ear inflammation in mice (Tai *et al.*, 1995a; Kaminaga *et al.*, 1996a~b; Nukaya *et al.*, 1996; Yasukawa *et al.*, 1998; Giner *et al.*, 2000). The cytotoxicities of poricoic acid A and poricoic acid G against human cancer cell lines have been described (Ukiya *et al.*, 2002). As a preliminary search for new cytotoxic compounds from natural products, the solvent extracts of more than 100 Korean folk medicines were screened for their cytotoxic activity. Of these, the CH_2Cl_2 extract of the sclerotium of *P. cocos* showed strong cytotoxicity (96.4% inhibition against a human colon carcinoma cell line at 25 μg/mL). In this study, the results of the bioactivity-guided fractionation of the CH_2Cl_2 extract of the sclerotium of *P. cocos*, and evaluation of the isolated compounds for their cytotoxicities against human carcinoma cell lines and DNA topoisomerases I and II inhibitory activities are reported.

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MATERIALS AND METHODS

General experimental procedures

The melting points were measured using a Fisher-Johns melting point apparatus and are uncorrected. 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 the Bruker' standard pulse program. Samples were dissolved in pyridine-d_5 or $CDCl_3$, and the chemical shifts reported in ppm downfield from TMS. FAB-MS spectra were measured by a VG TRIO 2A mass spectrometer. The stationary phases for 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}, Merck) were purchased from Merck KGaA (Darmstadt, Germany). 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). Supercoiled pBR 322 plasmid DNA and calf thymus topoisomerase I were purchased from MBI Fermentas Inc. (Hanover, MD, USA). Human topoisomerase II was purchased from TopoGEN Inc. (Columbus, OH, USA). All other chemicals and solvents were of analytical grade and used without further purification.

Plant materials

The sclerotium of *P. cocos* was purchased in September 1999 from a folk medicine market, "Yak-ryong-si" in Daegu, Republic of Korea. This material was confirmed taxonomically by Professor Ki-Hwan Bae, of Chungnam National University, Daejeon, Republic of Korea. A voucher specimen (YNPC-02) has been preserved at the College of Pharmacy, Yeungnam University.

Extraction and isolation

The sclerotium of *P. cocos* (9.6 kg) was extracted twice by refluxing with MeOH for 12 h. The MeOH was evaporated to dryness (450 g), partitioned between H_2O and CH_2Cl_2 and the CH_2Cl_2 solution evaporated to dryness *in vacuo*. The CH_2Cl_2 extract (33 g) was loaded onto a silica gel column (60 × 8 cm, Silica-gel 70-230 mesh) and the column was eluted with CH_2Cl_2-MeOH-H_2O (gradient from 350:2:0.1 to MeOH 100%). The eluent was combined on the basis of TLC, giving 16 fractions (F1-F16). The fraction F6 was obtained as amorphous powder of compound **2** (70 mg). Fraction F3 (200 mg) was subjected to further chromatography on a reverse phase RP-18 column (60 × 2.0 cm, LiChroprep RP-18) with MeOH-H_2O (gradient from 2:8 to 100% MeOH) as eluent, affording compound **1** (40 mg). Fraction F8 (5 g) was subjected to further chromatography on a silica gel column (60 × 3.0 cm) with $CHCl_3$-MeOH-H_2O (gradient from 150:2:0.1 to 100% MeOH) as eluent, giving 11 subfractions (F8-1-F8-11). The subfraction F8-6 (2.0 g) was subjected to chromatography on a reverse phase column (60 × 3.0 cm, LiChroprep RP-18) with MeOH-H_2O (gradient from 8:2 to 100% MeOH) as eluent, giving 8 subfractions (F8-6-1-F8-6-8). The subfractions F8-6-4, F8-6-5 and F8-6-7 were further purified, separately, on a reverse phase column (60 × 2.0 cm, LiChroprep RP-18) with MeOH-H_2O (gradient from 8:2 to 100% MeOH) as eluent, affording compounds **3** (25 mg), **4** (18 mg) and **5** (50 mg), respectively. Compound **6** (17 mg) was isolated from subfraction F8-8 (300 mg) by the same method (60 × 2.0 cm, LiChroprep RP-18, MeOH-H_2O gradient from 8:2 to 100% MeOH).

(*S*)-(+)-Turmerone (1)

Colorless oil, $[\alpha]_D^{25}$ +56.2º (*c* 0.2, $CHCl_3$), {lit. (Fuganti *et al.*, 1999), $[\alpha]_D^{20}$ +62.7(*c* 1.25, hexane)}; ^{1}H-NMR ($CDCl_3$, 250 MHz) δ 7.09 (4H, d, *J* = 7.2 Hz, H-2,3,5,6), 6.01 (1H, s, H-10), 3.27 (1H, m, H-7), 2.63 (2H, m, H-8), 2.29 (3H, s, H-15), 2.09 (3H, s, H-12), 1.84 (3H, s, H-13), 1.22 (3H, d, *J* = 6.9 Hz, H-14); ^{13}C-NMR ($CDCl_3$, 62.9 MHz) δ 199.8 (C-9), 155.1 (C-11), 143.6 (C-4), 135.5 (C-1), 129.1 (C-2,6), 126.6 (C-3,5), 124.0 (C-10), 52.6 (C-8), 35.2 (C-7), 27.6 (C-12 or 13), 21.9 (C-14), 20.9 (C-15), 20.6 (C-12 or 13). ^{1}H-NMR data are consistent with the literature values (Roth *et al.*, 1998; Fuganti *et al.*, 1999); positive FAB-MS *m/z* 217 $[M+H]^+$.

Ergosterol peroxide (2)

Amorphous power; mp. 178-180; $[\alpha]_D^{25}$ 22.8º (*c* 0.5, $CHCl_3$), {lit. (Nam *et al.*, 2001), $[\alpha]_D^{25}$ -26.0º (*c* 1, $CHCl_3$)}; ^{1}H- and ^{13}C-NMR data are consistent with the literature values (Kim *et al.*, 1997); positive FAB-MS *m/z* 429 $[M+H]^+$.

Polyporenic acid C (3)

Amorphous power; mp. 271-272; $[\alpha]_D^{25}$ +8.4º (*c* 0.2, pyridine), {lit. (Kawagishi *et al.*, 1997), $[\alpha]_D^{25}$ +17º (*c* 0.5, $CHCl_3$)}; ^{1}H- and ^{13}C-NMR data are consistent with the literature values (Kawagishi *et al.*, 1997); positive FAB-MS *m/z* 483 $[M+H]^+$.

Dehydropachymic acid (4)

White needles; mp. 267-269; $[\alpha]_D^{25}$ +52.2º (*c* 0.3, pyridine) {lit. (Tai *et al.*, 1992), $[\alpha]_D^{26}$ +41º (*c* 1.0, pyridine)}; ^{1}H- and ^{13}C-NMR data are consistent with the literature values (Tai *et al.*, 1992); positive FAB-MS *m/z* 527 $[M+H]^+$.

Pachymic acid (5)

White needles; mp. 296-298; $[\alpha]_D^{25}$ +6.2º (*c* 0.2, pyridine), {lit. (Tai *et al.*, 1992), $[\alpha]_D^{26}$ +6º (*c* 1.0, pyridine)}; ^{1}H- and ^{13}C-NMR data are consistent with the literature values (Tai *et al.*, 1992); positive FAB-MS *m/z* 529 $[M+H]^+$.

Tumulosic acid (6)

White needles; mp. 304-306; $[\alpha]_D^{25}$ +5.4º (*c* 0.1, pyridine), {lit. (Buckingham, 1994), $[\alpha]_D$ +8.1º (*c* 3.3, pyridine)}; ^{1}H- and ^{13}C-NMR data are consistent with the literature values (Tai, 1996); positive FAB-MS *m/z* 487 $[M+H]^+$.

Cytotoxicity bioassays

A tetrazolum-based colorimetric assay (MTT assay) was used to determine the cytotoxicity on human colon carcinoma (HT-29) cell line (Rubinstein *et al.*, 1990).

Assay for DNA topoisomerase I inhibition *in vitro*

The 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 DNA topoisomerase I activity was determined by measuring

the relaxation of supercoiled pBR 322 plasmid DNA. The reaction mixture 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 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 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 1% agarose gel in TBE (Tris-borate-EDTA) running buffer at 1.5 V/cm for 10 h. Gels were stained in ethidium bromide (0.5 μg/mL) for 30 min, and 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 calculated.

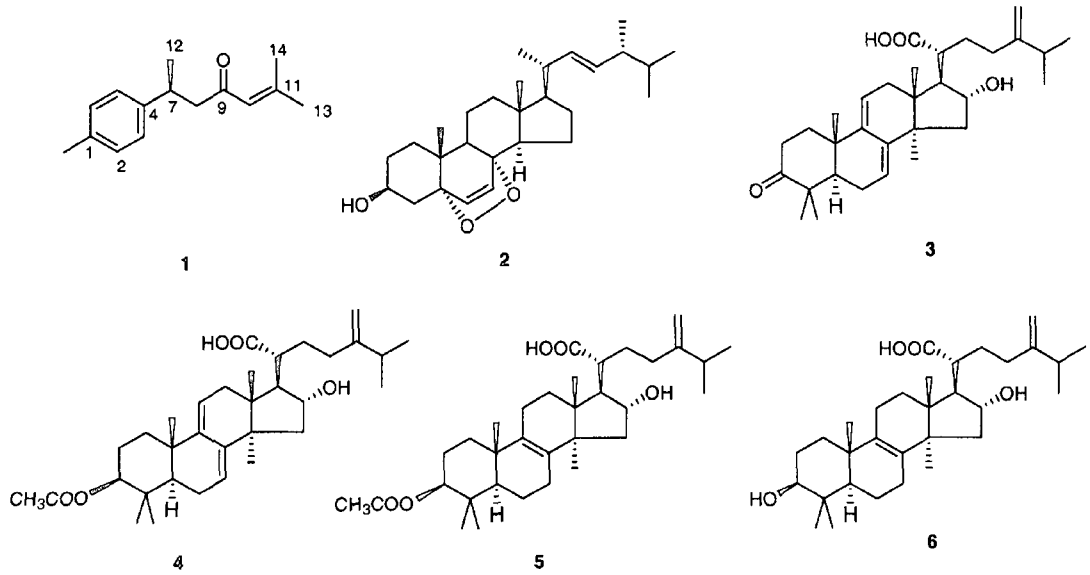
Assay for DNA topoisomerase II inhibition *in vitro*

The DNA 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 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 (Tris-borate-EDTA) running buffer at 1.5 V/cm for 10 h. Gels were stained in 0.5 μg/mL ethidium bromide for 30 min, and then 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 calculated.

RESULTS AND DISCUSSION

The methanol extract of sclerotium of *Poria cocos* was partitioned by solubility to different solvents, giving methylene chloride, ethyl acetate and water extracts, with the methylene chloride extract repeatedly subjected to normal-phase silica gel and reverse-phase gel chromatography to afford one known sesquiterpene ketone (**1**), one known sterol (**2**) and four known lanostane-type triterpene acids (**3-6**). By comparison of their optical rotation values, ^1H- and ^{13}C-NMR spectra and MS data with public values, compounds 1-6 were characterized as (*S*)-(+)-turmerone (**1**) (Roth *et al.*, 1998; Fuganti *et al.*, 1999), ergosterol peroxide (**2**) (Nam *et al.*, 2001; Kim *et al.*, 1997), polyporenic acid C (**3**) (Kawagishi *et al.*, 1997), dehydropachymic acid (**4**) (Tai *et al.*, 1992), pachymic acid (**5**) (Tai *et al.*, 1992) and tumulosic acid (**6**) (Tai, 1996). Compounds **1** and **2** were isolated for the first time from this plant, and the ^{13}C-NMR data of **1**, as far as could be determined, are presented here for the first time.

The cytotoxic potentials of compounds **1-6** were tested on a cultured human colon carcinoma cell line (HT-29), using the MTT assay. As shown in Table I, compounds **2,**



1 2 3 4 5 6

Fig. 1. Chemical structures of compounds **1-6**

Table I. Inhibitory effects of compounds **1-6** from *Poria cocos* on topoisomerases I and II enzymes (% inhibition ratio of relaxation) and their IC_{50} values against HT-29 cell line

Compounds	(%) Inhibition ratio for Topoisomerase I		(%) Inhibition ratio for Topoisomerase II		Cytotoxicity IC_{50} (μM)
	100 μM	20 μM	100 μM	20 μM	HT-29[a]
1	0	0	0	0	>50
2	0	0	0	0	47.3
3	55.8	0	76.2	36.1	>50
4	60.7	20.4	76.9	36.2	20.5
5	43.5	0	74.7	43.9	29.1
6	83.3	23.4	75.5	66.7	10.4
CPT[b]	94.6	83.6	NA[c]		0.1
etoposide[d]	NA		76.8	47.2	NA

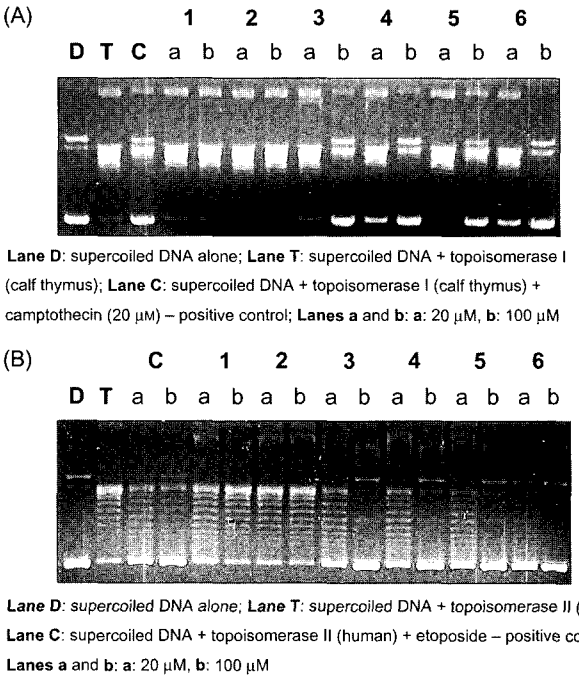
[a]HT-29: human colon carcinoma; [b]camptothecin: positive control for topoisomerase I; [c]NA: not applicable; [d] positive control for topoisomerase II

4, 5, and **6** exhibited moderate cytotoxicities against the HT-29 cell line: IC_{50}s of 47.3, 20.5, 29.1, and 10.4 μM, respectively.

DNA topoisomerases I and II have been established as important molecular targets for anticancer drugs, and inhibition of these enzymes is one of major mechanisms through which chemotherapeutic agents exert direct cytotoxicity to human cells (Liu, 1989).

To investigate whether these compounds (**1-6**) mediated cytotoxicities are related to the DNA topoisomerases inhibitory activites, the conversions of supercoiled pBR 322 plasmid DNA to relaxed DNA by calf thymus topoisomerase I were examined in the presence of the compounds. As illustrated in Fig. 2(A), compounds **3**, **4**, **5**, and **6** potently inhibited the supercoiled DNA relaxation induced by the DNA topoisomerase I by 55.8, 60.7, 43.5, and 83.3%, respectively, at a concentration of 100 μM compared to the control supercoiled DNA (Table I), even though the inhibition potencies were less than the camptothecin used as a positive control. To further elucidate the mechanism of action of these compounds for cytotoxicity, the topoisomerase II mediated DNA decatenation activity was also evaluated. When examined at a concentration of 100 μM, compounds **3-6** showed equivalent inhibitions to that of etoposide. When these compounds were assayed at a concentration of 20 μM, the inhibition of compound **6** was much more potent than that of etoposide [66.7% *versus* 47.2%, Fig. 2(B), Table I].

Compounds **3-6** were more effective against DNA topoisomerase II than DNA topoisomerase I at a concentration of 20 μM. These results suggest that these compounds have a selective inhibitory effect against the DNA topoisomerase II activity compared to topoisomerase I. No obvious correlation was observed between the



Lane D: supercoiled DNA alone; **Lane T**: supercoiled DNA + topoisomerase I (calf thymus); **Lane C**: supercoiled DNA + topoisomerase I (calf thymus) + camptothecin (20 μM) – positive control; **Lanes a** and **b**: **a**: 20 μM, **b**: 100 μM

Lane D: supercoiled DNA alone; **Lane T**: supercoiled DNA + topoisomerase II (human); **Lane C**: supercoiled DNA + topoisomerase II (human) + etoposide – positive control; **Lanes a** and **b**: **a**: 20 μM, **b**: 100 μM

Fig. 2. DNA topoisomerases I (A) and II (B) inhibitory activities of compounds **1-6**

cytotoxicity of these compounds and the inhibitory activity toward the DNA relaxation and decatenation by DNA topoisomerases I and II. The reason the cytotoxic potencies of these compounds are low, despite their potent inhibitory effects on the DNA topoisomerases I and II, might be that even though these compounds could directly inhibit the reactions of DNA topoisomerases I or II in the enzyme assays, their transportation through cell and nuclear membranes, in order to reach the target topoisomerases I and II, could be possible barriers in the MTT assay.

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