Natural Product Sciences
20(1) : 7-12 (2014)

Cytotoxic Triterpenoids from the Fruiting Bodies of *Ganoderma lucidum*

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Abstract – Twelve triterpenoids (**1** - **12**) were isolated from $CHCl_3$-soluble fraction of fruiting bodies of *Ganoderma lucidum*. Extensive spectroscopic and chemical studies established the structures of these compounds as butyl lucidenate P (**1**), butyl lucidenate E_2 (**2**), butyl lucidenate D_2 (**3**), butyl lucidenate Q (**4**), ganoderiol F (**5**), methyl ganoderate H (**6**), methyl ganoderate J (**7**), lucidumol B (**8**), ganodermanondiol (**9**), methyl lucidenate N (**10**), methyl lucidenate A (**11**) and butyl lucidenate N (**12**). All of the compounds were examined for their cytotoxic activity against HL-60, HeLa, and MCF-7 cancer cell lines. Among them, compounds **4** and **8** showed cytotoxic activity with IC_{50} values of 6.6 and 1.6 μM against HL-60, respectively. In addition, compound **8** also showed cytotoxic activity with IC_{50} values of 2.0 μM against HeLa cancer cell line, other compounds were moderate or inactive.
Keywords – *Ganoderma lucidum*, Polyporaceae, Lanostadiene triterpene, Cytotoxic activity

Introduction

The fruiting bodies of *Ganoderma lucidum* Karst (Polyporaceae) are a well known Chinese crude drug component which has been used clinically in China, Japan and Korea for a long time as a home remedy. It is considered to promote longevity and maintain the vitality of humans.[1] It is also used for the treatment of neurasthenia, insomnia, anorexia, dizziness, chronic hepatitis, hypercholesterolemia, coronary heart disease, hypertension, and carcinomas.[2,3] Because of the potential medicinal value and wide acceptability, much attention has been paid to the search for bioactive compounds from this mushroom. It contains numerous bioactive natural components, polysaccharides, steroids, triterpenoids, proteins, unsaturated fatty acids, vitamins, and minerals, with properties conducive to normalizing and balancing the body. They can enhance health and help in relief of a multitude of diseases.[4]

Studies on *G. lucidum* have expanded its efficacy as an anti-tumor, anti-hypertensive, anti-cholesterolemic, anti-platelet aggregation,[5] anti-diabetic,[6] hepatoprotective[7] and anti-oxidant[8] agents. With respect to anti-tumor activities, *in vivo* and *in vitro* effects on various tumor cells had been ascertained[7,9-12] and numerous reports have shown that the high molecular weight *β*-(1,3), *β*-(1,6)-D-glucan exhibited anti-tumor activity.[13] Extracts from different parts (mycelia, spores and fruiting bodies) of *G. lucidum* have been used as a source of active compounds against tumor processes.[14-16] In order to elucidate the cytotoxic activity and evaluate the quality of *G. lucidum*, the guided isolation of triterpenoids and steroids from the fruit bodies of *G. lucidum* were carried out. In our previous study, we reported the isolation of five compounds from hexane-soluble fraction of *G. lucidum* and their cytotoxic activity.[17] Our present study reports the isolation of twelve triterpenoids from $CHCl_3$-soluble fraction of *G. lucidum* (Fig. 1). The structures of those isolated compounds were determined along with their cytotoxic activity.

Experimental

General experimental procedures – Mass spectroscopy was carried out with a JEOL JMS-700 Mstation mass spectrometer. The nuclear magnetic resonance (NMR) spectra were obtained on Varian Unity Inova 400 MHz spectrometer. Silica gel (Merck, 63 - 200 μm particle size) and RP-18 silica gel (Merck, 75 μm particle size) were used for column chromatography. TLC was carried out using Merck silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was performed using a Waters 600 Controller system with a UV 486 detector and an YMC Pak ODS-A

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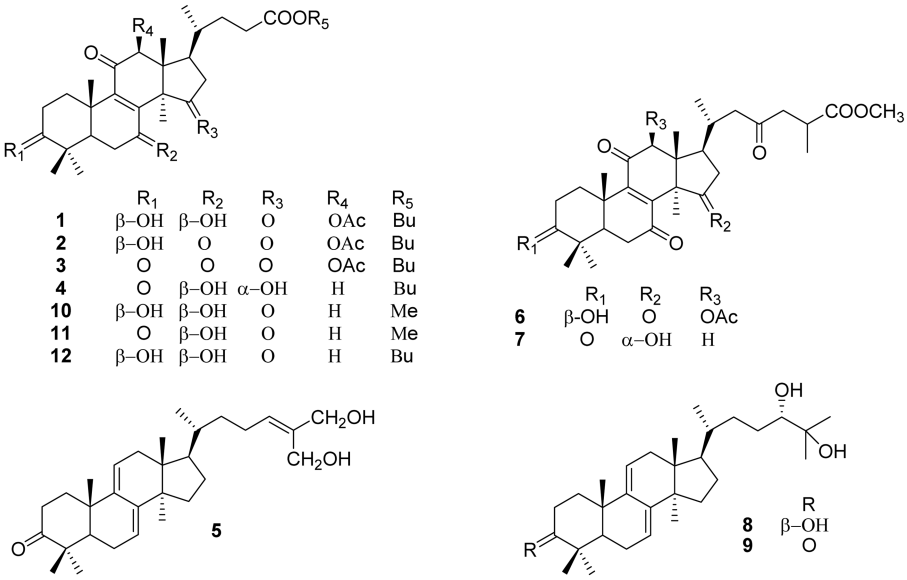

Fig. 1. Chemical structure of the isolated compounds.

column (20 × 250 mm, 5 μm particle size, YMC Co., Ltd., Japan) and HPLC solvents were from Burdick & Jackson, USA.

Plant material – The dried fruiting bodies of *G. lucidum* (cultured at Nagano, Japan) were supplied from Prof. Masao Hattori, Toyama University, Japan, and identified by Prof. Byung-Sun Min, Catholic University of Daegu, Korea. A voucher specimen (CUD-3170) was deposited at the herbarium of the college of Pharmacy, Catholic University of Daegu, Korea.

Extraction and isolation – The dried fruit bodies (10 kg) were extracted four times with refluxing MeOH. After the solvent was removed under reduced pressure, the residue (392.2 g) was suspended in H_2O and then partitioned with *n*-hexane, $CHCl_3$ and EtOAc, successively. The $CHCl_3$-soluble fraction (130.0 g) was separated into ten fractions (Fr.1-10) on silica gel column chromatography (80 × 12 cm, 63 - 200 μm particle size, Merck) using a stepwise gradient elution of $CHCl_3$-MeOH (100 : 1 to 1 : 1) according to their TLC profiles. Fraction 3 (5.7 g) was subsequently subjected to silica gel column chromatography (60 × 6.5 cm) eluting with hexane-EtOAc (5 : 1) to yield thirteen sub-fractions (Fr.3.1 to Fr.3.13). Fraction 3.5 (400 mg) was re-chromatographed on a silica gel column (60 × 3.5 cm) using a gradient solvent system of hexane-acetone (20 : 1 to 1 : 1) to give compound **9** (30 mg). Fr 3.6 (600 mg) was further chromatographed on a YMC RP-18 column with MeOH-H_2O (50 : 50 to 95 : 5) as an eluent to yield compounds **2** (15 mg), **10** (10 mg) and **11** (30 mg). Further purification of Fr 3.7 (250 mg) by semi-preparative HPLC systems [using an isocratic solvent system of 80% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min; UV detection at 254 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size] resulted in the isolation compounds **3** (16 mg; t_R = 30.2 min), **4** (12 mg; t_R = 41.5 min) and **12** (8 mg, t_R = 45.8 min). Fraction 3.8 (250 mg) was subjected to silica gel column chromatography (60 × 2.5 cm) eluting with a gradient solvent system of $CHCl_3$-EtOAc (15 : 1 to 2 : 1) to yield compounds **1** (28.5 mg), **6** (5 mg) and **7** (5 mg). Fr 3.9 (460 mg) was further purified over an YMC RP-18 column (50 × 3.5 cm) using a gradient solvent system of MeOH-H_2O (65 : 45 to 80 : 20) to afford compounds **5** (60 mg) and **8** (18 mg) (Fig. 1).

Butyl lucidenate P (1): colorless oil; HR-EIMS *m/z* 574.3503 $[M]^+$, $C_{33}H_{50}O_8$; ^1H and ^{13}C NMR date were in accordance with our previous paper.[18]

Butyl lucidenate E_2 (2): yellow oil; HR-FABMS *m/z* 573.343 $[M + H]^+$, $C_{33}H_{48}O_8$; ^1H and ^{13}C NMR date were in accordance with our previous paper.[18]

Butyl lucidenate D_2 (3): pale yellow oil; HR-FABMS *m/z* 571.3271 $[M + H]^+$, $C_{33}H_{46}O_8$; ^1H and ^{13}C NMR date were in accordance with our previous paper.[18]

Butyl lucidenate Q (4): colorless oil; HR-EIMS *m/z* 516.3453 $[M]^+$, $C_{31}H_{48}O_6$; ^1H and ^{13}C NMR date were in accordance with our previous paper.[18]

Ganoderiol F (5): white powder; EIMS *m/z* 454.3 $[M]^+$, $C_{30}H_{46}O_3$; ^1H-NMR (400 MHz, chloroform-*d*) δ: 5.54 (1H, t, J = 7.2 Hz, H-24), 5.50 (1H, d, J = 6.8 Hz, H-7), 5.38 (1H, d, J = 6.0 Hz, H-11), 4.32 (2H, s, H-27),

Table 1. ^{13}C NMR data (100 MHz) of compounds **5 - 12**

C	**5**[a]	**6**[b]	**7**[b]	**8**[c]	**9**[a]	**10**[a]	**11**[a]	**12**[a]
1	36.3	34.5	36.4	36.6	36.8	35.0	35.8	35.0
2	35.0	28.1	35.2	29.5	35.1	27.9	34.4	27.9
3	217.1	78.2	218.3	78.4	217.1	78.5	216.8	78.5
4	47.7	41.9	47.7	39.6	47.7	38.8	46.9	38.8
5	50.9	52.8	50.2	50.1	50.9	49.3	49.0	49.3
6	23.9	37.7	37.8	23.4	23.9	26.8	27.8	26.8
7	120.1	201.3	206.7	121.2	120.1	67.0	66.4	67.0
8	143.0	153.4	154.0	143.2	143.1	157.2	158.1	157.2
9	144.7	147.2	152.1	146.8	144.7	142.9	141.3	142.9
10	37.4	40.3	40.5	38.0	37.4	38.8	38.4	38.9
11	117.4	195.6	202.7	116.8	117.4	198.2	197.9	198.2
12	38.0	80.9	52.9	38.3	38.0	50.6	51.8	50.6
13	44.0	50.0	49.3	44.4	44.0	45.5	45.1	45.5
14	50.5	59.9	54.1	50.9	50.5	59.6	59.5	59.6
15	28.1	208.7	73.7	28.5	28.0	218.1	218.1	218.1
16	31.7	38.6	37.1	28.9	28.9	41.2	41.2	41.2
17	51.0	46.0	49.6	51.7	50.9	46.3	46.4	46.4
18	15.9	12.7	17.8	16.3	15.9	17.6	17.8	17.6
19	22.7	18.4	18.3	23.8	22.7	18.6	18.3	18.6
20	36.2	30.4	33.9	37.4	36.7	35.4	35.4	35.4
21	18.6	22.3	19.9	19.3	18.8	18.3	18.2	18.3
22	36.8	52.4	50.3	32.1	31.6	30.9	30.8	30.8
23	24.6	210.5	211.1	34.7	33.7	31.0	31.0	31.3
24	131.8	47.6	47.9	80.2	79.8	174.1	174.0	174.0
25	137.0	36.1	36.1	73.2	73.4			
26	67.9	178.1	178.2	26.4	25.7			
27	60.3	18.4	17.4	26.1	25.6			
28	25.6	28.4	27.7	23.4	23.4	28.3	27.2	28.3
29	22.3	16.3	20.6	29.1	26.8	15.6	20.9	15.6
30	25.5	21.6	20.8	16.9	22.3	24.6	24.8	24.6
CH_3OCO		171.8						
CH_3OCO		21.0						
$COOCH_3$		54.9	52.4			51.9	50.4	
Butyl 1'								64.7
2'								30.8
3'								19.3
4'								13.9

[a]in chloroform-d, [b]in methanol-d_4, [c]in pyridine-d_5

4.21 (2H, s, H-26), 1.20 (3H, s, H-19), 1.13 (3H, s, H-28), 1.09 (3H, s, H-29), 0.92 (3H, d, J = 6.3 Hz, H-21), 0.87 (3H, s, H-30), 0.59 (3H, s, H-18); ^{13}C NMR (chloroform-d, 100 MHz): see Table 1.

Methyl ganoderate H (6): white powder; EIMS m/z 586.3 $[M]^+$, $C_{33}H_{46}O_9$; ^{1}H-NMR (400 MHz, methanol-d_4) δ: 5.72 (1H, s, H-12), 3.68 (3H, s, COOMe), 3.25 (1H, dd, J = 11.0, 5.0 Hz, H-3), 2.24 (3H, s, 12-OAc), 1.76 (3H, s, H-30), 1.37 (3H, s, H-19), 1.18 (3H, d, J = 7.2 Hz, H-27), 1.04 (3H, s, H-28), 0.99 (3H, d, J = 6.6 Hz, H-21), 0.91 (3H, s, H-18), 0.84 (3H, s, H-29); ^{13}C NMR (methanol-d_5, 100 MHz): see Table 1.

Methyl ganoderate J (7): white powder; EIMS m/z 528.3 $[M]^+$, $C_{31}H_{44}O_7$; ^{1}H-NMR (400 MHz, methanol-d_4) δ: 4.37 (1H, dd, J = 6.0, 2.8 Hz, H-15), 3.67 (3H, s, COOMe), 1.31 (3H, s, H-19), 1.22 (3H, s, H-30), 1.18

(3H, d, J = 7.2 Hz, H-27), 1.15 (3H, s, H-29), 1.14 (3H, s, H-28), 0.98 (3H, s, H-18), 0.89 (3H, d, J = 6.6 Hz, H-21); ^{13}C NMR (methanol-d_4, 100 MHz): see Table 1.

Lucidumol B (8): white powder; EIMS *m/z* 458.4 $[M]^+$, $C_{30}H_{50}O_3$; ^{1}H-NMR (400 MHz, pyridine-d_5) δ: 5.55 (1H, m, H-11), 5.39 (1H, m, H-7), 3.75 (1H, m, H-24), 3.47 (1H, m, H-3), 1.53 (3H, s, H-27), 1.20 (3H, s, H-26), 1.12 (3H, s, H-29), 1.08 (3H, s, H-19), 1.01 (3H, d, J = 6.6 Hz, H-21), 0.96 (6H, s, H-28, 30), 0.67 (3H, s, H-18); ^{13}C NMR (pyridine-d_5, 100 MHz): see Table 1.

Ganodermanondiol (9): white powder; EIMS *m/z* 456.4 $[M]^+$, $C_{30}H_{48}O_3$; ^{1}H-NMR (400 MHz, chloroform-*d*) δ: 5.52 (1H, d, J = 6.0 Hz, H-11), 5.40 (1H, d, J = 6.0, Hz, H-7), 3.30 (1H, m, H-24), 1.22 (3H, s, H-30), 1.20 (3H, s, H-27), 1.17 (3H, s, H-26), 1.13 (3H, s, H-29), 1.09 (3H, s, H-19), 0.92 (3H, d, J = 6.6 Hz, H-21), 0.88 (3H, s, H-28), 0.60 (3H, s, H-18); ^{13}C NMR (chloroform-*d*, 100 MHz): see Table 1.

Methyl lucidenate N (10): white powder; EIMS *m/z* 474.3 $[M]^+$, $C_{28}H_{42}O_6$; ^{1}H-NMR (400 MHz, chloroform-*d*) δ: 4.80 (1H, dd, J = 9.2, 8.4 Hz, H-7), 3.67 (3H, s, COOMe), 3.20 (1H, d, J = 10.5, 6.0 Hz, H-3), 1.34 (3H, s, H-30), 1.22 (3H, s, H-19), 1.03 (3H, s, H-28), 0.97 (3H, d, J = 6.6 Hz, H-21), 0.97 (3H, s, H-18), 0.86 (3H, s, H-29); ^{13}C NMR (chloroform-*d*, 100 MHz): see Table 1.

Methyl lucidenate A (11): white powder; EIMS *m/z* 472.3 $[M]^+$, $C_{28}H_{40}O_6$; ^{1}H-NMR (400 MHz, chloroform-*d*) δ: 4.84 (1H, dd, J = 9.0, 8.4 Hz, H-7), 3.66 (3H, s, COOMe), 1.33 (3H, s, H-30), 1.24 (3H, s, H-19), 1.11 (3H, s, H-28), 1.09 (3H, s, H-29), 0.99 (3H, s, H-18), 0.96 (3H, d, J = 6.6 Hz, H-21); ^{13}C NMR (chloroform-*d*, 100 MHz): see Table 1.

Butyl lucidenate N (12): white powder; EIMS *m/z* 516.3 $[M]^+$, $C_{31}H_{48}O_6$; ^{1}H-NMR (400 MHz, chloroform-*d*) δ: 4.80 (1H, dd, J = 9.2, 8.4 Hz, H-7), 4.06 (2H, t, J = 6.6 Hz, H-1'), 3.21 (1H, d, J = 11.0, 6.0 Hz, H-3), 1.34 (3H, s, H-30), 1.22 (3H, s, H-19), 1.04 (3H, s, H-28), 0.98 (3H, s, H-18), 0.97 (3H, d, J = 6.6 Hz, H-21), 0.93 (3H, t, J = 7.2 Hz, H-4'), 0.86 (3H, s, H-29); ^{13}C NMR (chloroform-*d*, 100 MHz): see Table 1.

Cytotoxic activity assay – The cancer cell lines (HL-60, HeLa and MCF-7) were maintained in RPMI 1640 that included L-glutamine (GIBCO) with 10% FBS (GIBCO) and 2% penicillin-streptomycin (GIBCO). Cells were cultured at 37 °C in a 5% CO_2 incubator. Cytotoxic activity was measured using a modified MTT assay (Mosmann, 1983). Viable cells were seeded in the growth medium (100 μL) into 96-well micro titer plates (1×10^4 cells per well) and incubated at 37 °C in a 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 5.0 to 150 μM by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to < 0.1%. After standing for 24 h, 10 μL of the test sample was added to each well. The same volume of DMSO was added to the control wells. On removing medium after 48 h of the test sample treatment, MTT (10 μL) was also added to the each well (final concentration, 5 mg/mL). Three hours later, the plate was centrifuged for 5 min at 1500 rpm, the medium was removed, and the resulting formazan crystals were dissolved with DMSO 150 μL. The optical density (O.D.) was measured at 570 nm using a Titertek microplate reader (Multiskan MCC/340, Flow). The IC_{50} value was defined as the concentration of sample which reduced absorbance by 50% relative to the vehicle-treated control.[19]

Results and Discussion

Phytochemical study of the $CHCl_3$ extract of *G. lucidum* led to the isolation of twelve triterpenoids (**1** - **12**). These compounds were identified as butyl lucidenate P (**1**), butyl lucidenate E_2 (**2**), butyl lucidenate D_2 (**3**), butyl lucidenate Q (**4**),[18] ganoderiol F (**5**),[20] methyl ganoderate H (**6**),[21] methyl ganoderate J (**7**),[22] lucidumol B (**8**),[23] ganodermanondiol (**9**),[23] methyl lucidenate N (**10**),[24] methyl lucidenate A (**11**)[25] and butyl lucidenate N (**12**).[26] All of the compounds were tested for their cytotoxic activity against HL-60 (human promyelocytic leukemia), HeLa (human cervival carcinoma), and MCF-7 (human breast adenocarcinoma) cancer cell lines, and the results are presented in Table 2. Compound **8** showed strong cytotoxic activity with IC_{50} values of 1.6 μM against HL-60 and 2.0 μM against HeLa cancer cell lines. Compound **4** also showed cytotoxic activity with IC_{50} values of 6.6 μM against HL-60. Compound **9** exhibited moderate cytotoxic activity against HL-60 and HeLa cancer cell lines with IC_{50} values as 9.6 and 10.2 μM, respectively. However, these two compounds, **8** and **9** presented very weak cytotoxic activity against MCF-7 cancer cell lines. The other compounds showed weak or no cytotoxic activity in all three kinds of cancer cell lines. In this experiment, adriamycin, an anticancer agent, was used as positive control with IC_{50} values as 1.0, 5.8 and 6.4 μM against HL-60, Hela and MCF-7 cancer cell lines, respectively.

The cancer cell cytotoxicity can be explained as the direct killing of the cells or the inhibition of cell proliferation. Using the tetrazolium (MTT) method, inhibition of proliferation has been shown in various

Table 2. Cytotoxic activity of compounds **1 - 12**

Compound	IC_{50} (μM)		
	HL-60	Hela	MCF-7
1	> 30	> 30	> 30
2	> 30	> 30	> 30
3	24.7 ± 2.2	> 30	> 30
4	6.6 ± 0.5	> 30	> 30
5	16.9 ± 1.5	> 30	> 30
6	> 30	> 30	> 30
7	20.6 ± 1.8	> 30	> 30
8	1.6 ± 0.1	2.0 ± 0.1	> 30
9	9.6 ± 0.7	10.2 ± 0.6	> 30
10	> 30	> 30	> 30
11	> 30	> 30	> 30
12	10.2 ± 0.8	22.0 ± 2.1	> 30
Adriamycin[a]	1.0 ± 0.1	5.8 ± 0.5	6.4 ± 0.6

[a] Positive control

cancer cell lines, including murine lymphocytic leukemia L1210 and Lewis lung carcinoma (LLC), human hepatoma PLC/PRF/5 and KB, human breast cancer MDA-MB-123, human prostate cancer PC-3, human breast cancer MCF-7, human cervix uteri tumor HeLa, and low-garde bladder cancer MTC-11.[27,28] *G. lucidum* extracts inhibit growth of human prostate and bladder cancer cell lines[15,29] and cell proliferation inducing apoptosis in human colon carcinoma and breast cancer cell lines.[30] Polysaccharides present in *G. lucidum* might responsible of growth delay of sarcoma cells.[31] In part, their anti-tumor activity may be a consequence of their immuno-modulatory properties.[13,32] The main group of *G. lucidum* component is triterpenoids. Their pharmacological effects are known as antioxidative, immune-modulating and antitumor. It has been demonstrated that ganoderic acids, lucidumols A - B, ganodermanondiol, ganoderiol F and ganodermanontriol exert cytotoxic-based carcinostatic effects on cancer cells, and many of them also possess anti-angiogenesis activity.[9,33] The cytotoxic activity of *Ganoderma* triterpenoids were reported to include inhibiting growth, inducing apoptosis and causing cell cycle arrest of cancer cells.[12,34,35] Also, triterpenoids present in *G. lucidum* inhibit growth of hepatoma cells by altering intracellular phosphorylation pathways.[7]

Recently, the effect of ganoderic acids such as ganoderic acid F on protein expression profile in HeLa cells were investigated. The twelve possible target-related proteins of ganoderic acids were identified using MALDI-TOF MS/MS. The results demonstrated that ganoderic acids might exert their cytotoxic activity by altering proteins involved in cell proliferation and/or cell death, carcinogenesis, oxidative stress, calcium signaling and ER stress.[36] In our previous study, ergosta-7,22-diene-2β,3α,9α-triol (EGDT) was reported to induce apoptosis in HL-60 human premyelocytic leukemia cells. EGDT activated the apoptotic process, including DNA fragmentation and caspase-3 activity. In immunoblotting analysis, treatment with EGDT resulted in the cleavage of procaspase-3 and poly(ADP-ribose) polymerase (PARP) into active forms.[37] Ganoderic acid T and three new derivatives, triterpenic acids produced by *G. lucidum*, have demonstrated therapeutic potential for tumor disease. Flow cytometry analysis revealed that those compounds caused cell cycle arrest at the G1 phase and induced apoptosis. Furthermore, they decreased the mitochondrial membrane potential and enhanced the activities of pro-apoptotic factors caspase-3 and caspase-9 in a dose-dependent manner. Accordingly, the apoptosis induction was presumed to occur through the endogenous pathway.[38]

By numerous studies about *G. lucidum* and its triterpenoids, ganoderic alcohols have potent cytotoxic activity than ganoderic acids.[39,40] Of the experiment results, compounds **8 - 9** and ganoderic alcohols also showed the most potent inhibitory activity. In the accordance with previous results, two compounds and other active components might play important role in the anti-cancer of *G. lucidum*.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF 2012R1A2A2A06046921 and 2012R1A1A1A 2003547) and BK21 Plus (22A20130000073).

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Received July 30, 2013
Revised August 19, 2013
Accepted Augsut 21, 2013