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Cytotoxic and anti-angiogenic effects of lanostane triterpenoids from *Ganoderma lucidum*

Van Thu Nguyen [a,b], Nguyen The Tung [a], To Dao Cuong [a], Tran Manh Hung [a], Jeong Ah Kim [c], Mi Hee Woo [a], Jae Sue Choi [d], Jeong-Hyung Lee [e], Byung Sun Min [a,*]

[a] College of Pharmacy, Catholic University of Daegu, Gyeongbuk 712-702, Republic of Korea
[b] Vietnam Military Medical University, 160 Phung Hung, Ha Dong, Hanoi, Viet Nam
[c] College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea
[d] Faculty of Food Science and Biotechnology, Pukyung National University, Busan 608-737, Republic of Korea
[e] College of Natural Sciences, Kangwon National University, Gangwon-do 200-701, Republic of Korea

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ABSTRACT

Two new lanostane triterpenes, 3α,12β,15α-triacetoxy-5α-lanosta-7,9(11),24-trien-26-oic acid (**1**) and 5α-lanosta-8,24-diene-26,27-dihydroxy-3,7-dione (**2**), together with sixteen known compounds (**3**–**18**) were isolated from the fruiting bodies of the Vietnamese mushroom *Ganoderma lucidum*. Their chemical structures were determined by extensive spectroscopic (IR, HR-EI-MS, 1D and 2D NMR) analyses. Potential cytotoxic activities of these compounds were evaluated against human non-small cell lung adenocarcinoma (A549), breast adenocarcinoma (MCF-7), and prostatic small cell carcinoma (PC-3). Among the compounds, 3α,12β,15α-triacetoxy-5α-lanosta-7,9(11),24-trien-26-oic acid (**1**) showed significant cytotoxic activity against PC-3 cells with an IC_{50} of 11.5 μM. In studies of anti-angiogenesis activity, ganoderic acid F (**17**) was found to have the most potent inhibitory effect on the formation of capillary-like structures of human umbilical vein endothelial cells.

1. Introduction

Angiogenesis is the formation of new vessels from an existing vascular network. It is related to cancer, obesity, psoriasis, diabetic retinopathy, and arthritis. Angiogenesis plays an important part in the growth and metastasis of tumor by supplying oxygen and nutrients necessary for the growth of tumor cells (Folkman, 2006). In addition, angiogenesis is required for solid tumors to grow beyond a size of approximately 1–2 mm^3, which is sufficiently small to be treated with conventional chemotherapeutic agents (Folkman, 2006). Therefore, inhibitors of tumor angiogenesis are considered to be an effective strategy for the treatment of cancer.

Within the framework of our research project on Vietnamese traditional medicinal plants, the mushroom *Ganoderma lucidum* (Fr.) P. Karst (Polyporaceae), known locally as "Nam Lim Xanh", was selected. The fruiting bodies of *G. lucidum* are widely used in China, Japan, and Korea as a valuable crude drug, particularly in the treatment of chronic hepatitis, nephritis, hepatopathy, neurasthenia, arthritis, bronchitis, asthma, gastric ulcer, and insomnia (Namba, 1994). Triterpenoids are the main chemical constituents of *G. lucidum*, and these compounds have been shown to produce inhibitory effects on HIV-1 protease (Min et al., 1998), anti-tumor effects (Stanley et al., 2005; Sliva, 2006; Müller et al., 2006), inhibitory effects on histamine release (Kohda et al., 1985), as well as antimicrobial (Wang and Ng, 2006), anti-inflammatory (Tung et al., 2013; Dudhgaonkar et al., 2009), and antioxidant activities (Zhu et al., 1999). In addition, polysaccharides from *G. lucidum* have been shown to possess hypoglycemic (Hikino and Mizuno, 1989), immunostimulant (Kino et al., 1989; Soccol et al., 2010), anti-tumor, and anti-inflammatory activities (Joseph et al., 2011).

In continuing studies toward the discovery of anti-angiogenic agents from natural plants, further fractionation of the chloroform-soluble fraction prepared from the fruiting bodies of Vietnamese *G. lucidum* resulted in the isolation of two new triterpenes (**1** and **2**) along with sixteen known compounds (**3**–**18**). Here, we report on the isolation and structural elucidation of these compounds, as well as the evaluation of their anti-angiogenic effects and cytotoxic properties against some human cancer cell lines.

* Corresponding author. Tel.: +82 53 850 3613; fax: +82 53 850 3602.
E-mail address: bsmin@cu.ac.kr (B.S. Min).

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2. Results and discussion

The MeOH extract of the fruiting bodies of *G. lucidum* was partitioned into *n*-hexane-, $CHCl_3$-, EtOAc-, and *n*-BuOH-soluble fractions, as well as an H_2O layer. Chromatographic purification of the $CHCl_3$-soluble fraction led to the isolation of two new (**1** and **2**) and sixteen known compounds (**3**–**18**). Known compounds were identified as ganoderic acid DM (**3**) (Wang et al., 1997), ergosta-7,22-dien-2β,3α,9α-triol (**4**) (Lin and Tome, 1991), ganodermanontriol (**5**) (Fujita et al., 1986), ganodermanondiol (**6**) (Fujita et al., 1986), ganoderitriol M (**7**) (Chen et al., 2009), lucidenic acid A (**8**) (Nishitoba et al., 1985), lucidenic acid C (**9**) (Kikuchi et al., 1986), ganoderic acid S_1 (**10**) (Morigiwa et al., 1986), methyl lucidenate Q (**11**) (Kenji et al., 2003), methyl lucidenate L (**12**) (Nishitoba et al., 1987), methyl lucidenate C (**13**) (Kikuchi et al., 1986), lucidadiol (**14**) (González et al., 2002), ganoderiol F (**15**) (Nishitoba et al., 1988), ganoderic acid A (**16**) (Kubota et al., 1982), ganoderic acid F (**17**) (Kikuchi et al., 1986), and methyl lucidenate A (**18**) (Nishitoba et al., 1985) (Fig. 1). The structures of these known compounds were identified by comparison of their spectroscopic data with that reported in the literature.

Compound **1** was obtained as colorless oil with an optical rotation of +22.8 (*c* 0.12, $CHCl_3$). Its HR-EI-MS spectrum gave a molecular ion peak at an *m/z* value of 612.3662, which corresponded to the molecular formula $C_{36}H_{52}O_8$. The IR spectrum showed the presence of OH (3424 cm^{-1}), and C=O (1718 cm^{-1}) absorptions. The ^1H NMR spectrum of compound **1** (Table 1) displayed signals for five tertiary methyls at δ_H 0.67 (3H, s, H-18), 1.04 (3H, s, H-19), 0.99 (3H, s, H-28), 0.98 (3H, s, H-29), and 0.89 (3H, s, H-30), a secondary methyl at δ_H 0.98 (d, *J* = 3.6 Hz), an allyl methyl at δ_H 1.87 (3H, s, H-27), three *O*-acetyl methyls at δ_H 2.06 (3H, s), 2.07 (3H, s), and 2.09 (3H, s), three oxymethine protons [δ_H 4.68 (1H, s, H-3), 5.04 (1H, t, *J* = 7.2 Hz, H-12), 5. 09 (1H, dd, *J* = 4.4, 10.0 Hz, H-15)], and three olefinic protons [δ_H 5.49 (1H, brs, H-7), 5.32 (1H, d, *J* = 6.4 Hz, H-11), and 6.78 (1H, t, *J* = 7.2 Hz, H-24)]. The ^{13}C NMR spectrum, combined with the DEPT data, showed that **1** had 36 carbon signals consisting of eleven methyls, six methylenes, eight methines and eleven quaternary carbons. Among them, **1** contained distinctively three oxygenated methines [δ_C 78.3 (C-3), 74.7 (C-12), and 77.4 (C-15)], three acetoxy groups [δ_C 170.8, 21.5 (3-OAc), 171.0, 21.6 (12-OAc), and 171.3, 21.3 (15-OAc)], three olefinic quaternary carbons [δ_C 140.2 (C-8), 146.2 (C-9), and 129.5 (C-25)], three olefinic methine carbons [δ_C 121.6 (C-7), 115.6 (C-11), and 139.2 (C-24)], and one carbonyl carbon [δ_C 171.6 (C-26)] (Table 1).

This evidence clearly indicated that **1** was a triacetoxy-ganoderic acid of the 7,9(11),24-triene type (Hirotani et al., 1986). The full NMR assignments and connectivity of **1** were determined by analysis of HMQC and HMBC spectroscopic data. The position of three acetoxyl groups at C-3, C-12, and C-15 were decided by the key HMBC correlations; from H-3 (δ_H 4.68) to C-2/C-4/C-29, and δ_C 170.8, from H-12 (δ_H 5.04) to C-9/C-11, and δ_C 171.0,

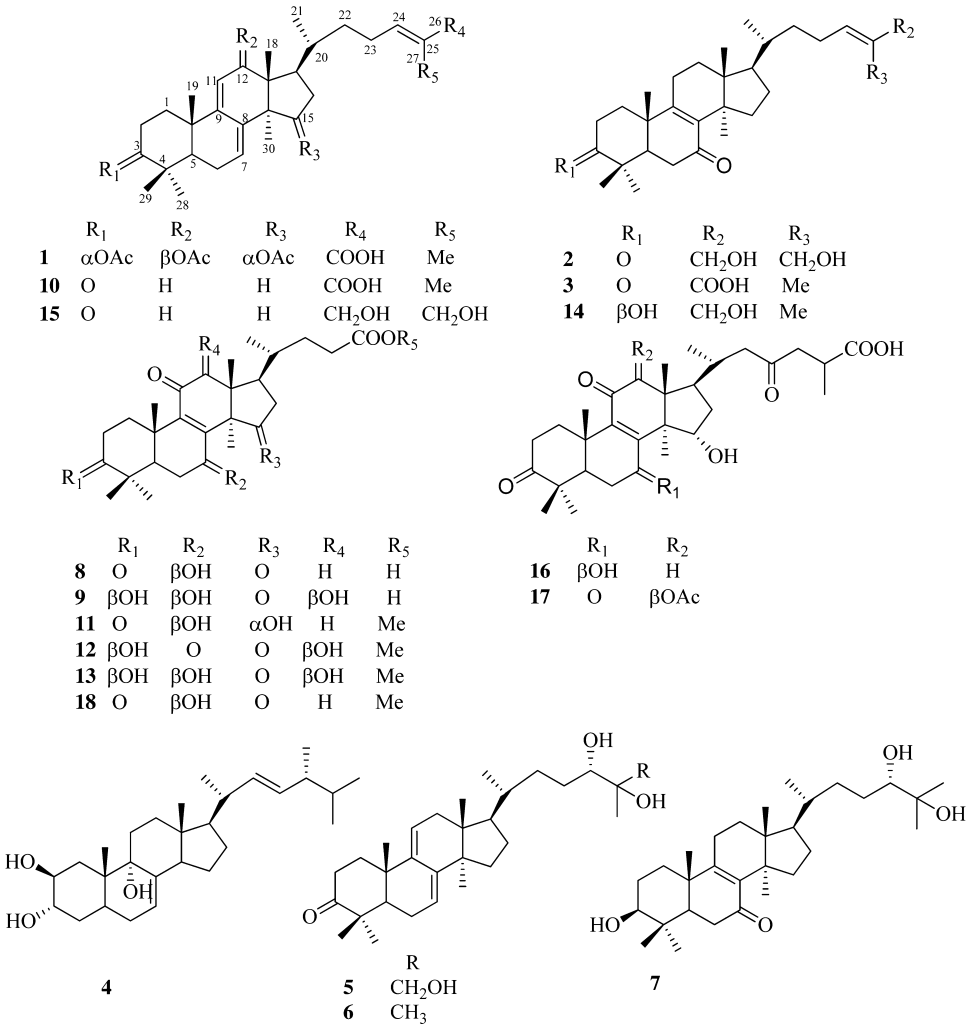

Fig. 1. Chemical structures of isolated compounds (**1**–**18**).

Table 1
^{1}H and ^{13}C NMR data of compounds **1** and **2**.

Position	**1** (in $CDCl_3$)		**2** (in $CDCl_3$)	
	δ_H (J in Hz)[a]	δ_C[b]	δ_H (J in Hz)[a]	δ_C[b]
1	1.7 (m), 2.18 (m)	30.8	1.80 (m), 2.08 (m)	36.2
2	2.17 (m), 2.60 (m)	23.0	2.45 (dd, 1.4), 2.7 (m)	34.6
3	4.68 (s)	78.3		214.9
4		36.9		47.4
5	1.45 (m)	44.1	2.09 (m)	50.6
6	2.03 (m)	22.8	2.37 (m), 2.51 (m)	37.3
7	5.49 (s)	121.6		198.4
8		140.2		139.7
9		146.2		163.1
10		37.5		39.6
11	5.32 (d, 6.4)	115.6	2.30 (m)	24.0
12	5.04 (t, 7.2)	74.7	1.77 (m), 1.80 (m)	30.3
13		44.2		45.1
14		51.6		48.0
15	5.09 (dd, 4.4, 10.0)	77.4	1.64 (m), 2.04 (m)	32.1
16	1.72 (m), 1.81 (m)	36.7	1.39 (m), 1.99 (m)	28.9
17	1.51 (m)	45.7	1.47 (m)	49.2
18	0.67 (s)	16.0	0.68 (s)	16.1
19	1.04 (s)	23.0	1.34 (s)	18.1
20	1.34 (m)	39.8	1.40 (m)	36.3
21	0.98 (d, 3.6)	12.9	0.95 (d, 4.4)	18.8
22	1.10 (m), 1.51 (m)	36.9	1.09 (m), 1.52 (m)	35.6
23	2.26 (m), 2.60 (m)	23.3	1.90 (m), 2.06 (m)	24.5
24	6.78 (t, 7.2)	139.3	5.55 (t, 7.2)	131.8
25		129.5		137.0
26		171.6	4.32 (s)	67.9
27	1.87 (s)	12.5	4.22 (s)	60.3
28	0.99 (s)	28.0	1.10 (s)	25.6
29	0.98 (s)	22.9	1.12 (s)	21.6
30	0.89 (s)	18.7	0.93 (s)	25.1
3-O$\underline{C}$OCH$_3$		170.8		
3-OCO$\underline{CH}_3$	2.06[c]	21.5		
12-O$\underline{C}$OCH$_3$		171.0		
12-OCO$\underline{CH}_3$	2.07[c]	21.6		
15-O$\underline{C}$OCH$_3$		171.3		
15-OCO$\underline{CH}_3$	2.09[c]	21.3		

[a] Recorded at 400 MHz.
[b] Recorded at 100 MHz.
[c] Assignments may be interchanged.

and from H-15 (δ_H 5.09) to C-14/C-16, and δ_C 171.3 (Fig. 2). From ^{1}H NMR spectrum, a broad single signal of H-3 indicated a α-orientation of the acetyl groups at C-3 (Lin et al., 1997). Additionally, orientations of 12β-OAc and 15α-OAc were deduced from NOEs of H-12/H-21 and H-15/H-18, respectively. Thus, the structure of **1** was determined to be 3α,12β,15α-triacetoxy-5α-lanosta-7,9(11),24-trien-26-oic acid.

Compound **2** was isolated as a white amorphous powder with optical rotation of +2.6 (c 0.13; $CHCl_3$). The molecular formula of **2** was found to be $C_{30}H_{46}O_4$ on the basis of a molecular ion peak at an m/z value of 470.3395 $[M]^+$ in the HR-EI-MS. Its IR spectrum disclosed absorption bands at 3328 and 1728 cm^{-1} assignable to hydroxyl (OH) and carbonyl (C=O) groups, respectively. The ^{1}H NMR spectrum of **2** contained signals for five tertiary methyls at δ_H 0.68 (s, H-18), 1.34 (s, H-19), 1.10 (s, H-28), 1.12 (s, H-29), and 0.93 (s, H-30), a secondary methyl at δ_H 0.95 (d, J_Y= 4.4 Hz, H-21), two allyl hydroxymethylenes at δ_H 4.22 (2H, s, H-27) and 4.32 (2H, s, H-26)], and a vinyl proton at δ_H 5.55 (t, J_Y= 7.2 Hz, H-24). The ^{13}C NMR (Table 1), HMQC and DEPT spectra of compound **2** showed 30 carbon signals, which were recognized as six methyls, eleven methylenes [including two allyl hydroxymethylenes at δ_C 67.9 (C-26) and 60.3 (C-27)], four methines [including an olefinic methine at δ_C 131.8 (C-24)], and nine quaternary carbons [including three olefinic quaternary carbons at δ_C 139.7 (C-8), 163.1 (C-9), and 137.0 (C-25), and two carbonyl carbons at δ_C 214.9 (C-3), and 198.4 (C-7)]. Comparison of the NMR data (Table 1) of **2** with those of ganoderone A indicated that they were closely related to their structures, except of a methyl group at C-27 in ganoderone A being replaced by an allyl hydroxymethylene group (δ_C 60.3) in **2** (Timo et al., 2005). The difference was proved by the significant change of the chemical shift value for C-27 from δ_C 13.6 of ganoderone A to δ_C 60.3 of **2**, which was consistent with its molecular formula. The linkage position of the allyl hydroxymethylene on C-27 was supported by significant HMBC correlations from δ_H 4.32 (H-27) to δ_C 131.8 (C-24) and δ_C 137.0 (C-25) (Fig. 2). Additional HMBC correlations between H-2, H-28, H-29, and δ_C 214.9 (C-3), and between H-6, and δ_C 198.4 (C-7), 139.7 (C-8) indicated the presence of two ketone groups at C-3 and C-7 (Fig. 2). On the basis of the above evidence, the structure of **2** was identified as 5α-lanosta-8,24-diene-26,27-dihydroxy-3,7-dione.

Cytotoxic activities of isolated compounds were tested using a modified MTT assay in several cancer cell lines (A549, MCF-7, and PC-3). Among them, five compounds, 3α,12β,15α-triacetoxy-5α-lanosta-7,9(11),24-trien-26-oic acid (**1**), 5α-lanosta-8,24-diene-26,27-dihydroxy-3,7-dione (**2**), ganodermanondiol (**6**), lucidenic acid A (**8**), and lucidadiol (**14**) showed cytotoxic activity against PC-3 cells with IC_{50} values ranging from 11.5 to 44.0 μM. Compound **1** also exhibited significantly cytotoxic activity against A549 cells with an IC_{50} value of 16.1 μM (Table 2). The other compounds showed weak inhibitory effects against the tested human cancer cell lines.

To assess the anti-angiogenesis effect of isolated compounds, we firstly investigated their cytotoxic effects on human umbilical vein endothelial cells (HUVECs), using MTT assay with various concentrations of **1**–**18** (3–30 μg/mL) and 0.1% DMSO as a negative control. After 48 h incubation, none of the isolates significantly affected the viabilities of the HUVECs (data not shown). To investigate the manner by which the isolated compounds produced anti-angiogenesis effects and suppress HUVEC proliferation, the formation of tube-like structures (which is a step in the angiogenic process) was further examined. Capillary tube structures were observed in the control group after HUVECs were placed in the wells, whereas the presence of compounds **1**–**18** at 3 μM significantly reduced formation of tube-like structures (Fig. 3A). Curcumin (3 μM), a non-toxic natural compound used to treat chronic diseases

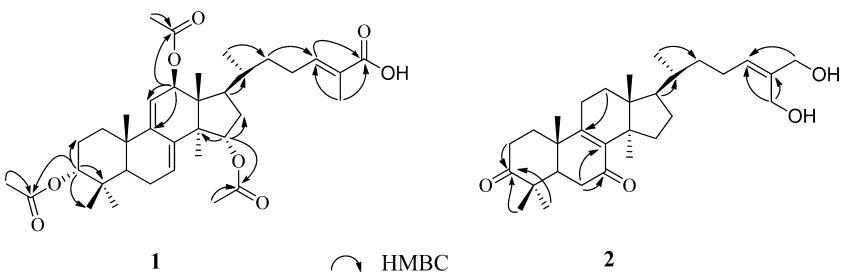

Fig. 2. Key HMBC correlations (H → C) for the new compounds **1** and **2**.

Table 2
Cytotoxic activity of compounds **1**–**18**.

Compound	IC_{50} (μM)[a]		
	A549	MCF-7	PC-3
1	16.1 ± 2.3	49.2 ± 4.4	11.5 ± 2.3
2	49.3 ± 4.2	50.0 ± 3.6	21.3 ± 3.1
3	>50	>50	>50
4	>50	>50	>50
5	>50	>50	>50
6	>50	>50	44.0 ± 5.2
7	>50	>50	>50
8	>50	>50	35.0 ± 4.1
9	>50	>50	>50
10	>50	>50	>50
11	>50	>50	>50
12	>50	>50	>50
13	>50	>50	>50
14	>50	>50	32.0 ± 3.2
15	>50	>50	>50
16	>50	>50	>50
17	>50	>50	>50
18	>50	>50	>50
Adriamycin[b]	2.4 ± 2.5	3.4 ± 1.3	3.4 ± 3.6

[a] The values are mean ± SD (n = 3); a compound is considered inactive with IC_{50} > 50 μM.
[b] Positive control.

associated with extensive neovascularization (Gururaj et al., 2002), reduced the formation of tube-like structures by 45% compared with control (Fig. 3A). These results indicated that ganoderic acid F (**17**) effectively inhibited formation of tube-like structures (≤37.9% of the control). Treatment with **17** (3–30 μM) also significantly reduced the formation of tube-like structures. At a concentration 30 μM, **17** effectively inhibited tube-like structure formation (4.0% vs control) (Fig. 3B).

Here, we showed, the first time, that two new triterpenoids, 3α,12β,15α-triacetoxy-5α-lanosta-7,9(11),24-trien-26-oic acid (**1**) and 5α-lanosta-8,24-diene-26,27-dihydroxy-3,7-dione (**2**), were isolated from the fruiting bodies of Vietnamese *G. lucidum*. Compound **1** showed potential cytotoxic activity against A549 and PC-3 cells. In addition, ganoderic acid F (**17**) displayed potent anti-angiogenesis activity, and its angiogenic effect was found to occur via suppression of proliferation of endothelial cells and inhibition of formation of tube-like capillary structures. These findings could suggest that lanostane triterpenes from *G. lucidum* are involved in attenuating the proliferation and migration of vessel endothelial cells in HUVECs. Thus, this is the first report showing ganoderic acid F may be a potential therapeutic strategy for the treatment of cancer by the inhibition of angiogenesis.

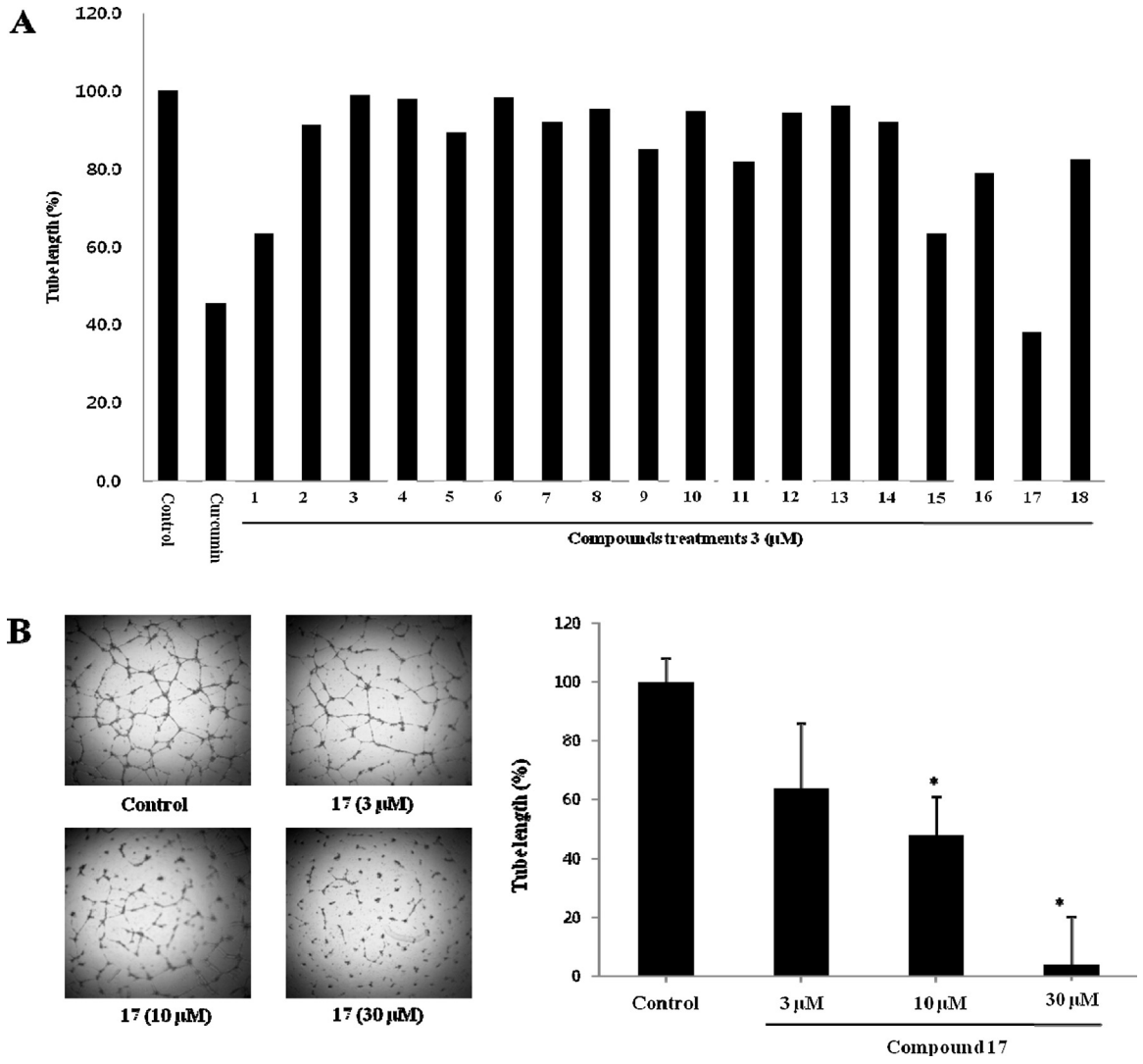

Fig. 3. Effect of isolated compounds (**1**–**18**) on HUVEC tube-like structure formation.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured using a JASCO DIP 370 digital polarimeter. UV spectra were recorded in MeOH using a Thermo spectrometer. 1D and 2D NMR spectra were obtained using a Varian Unity Inova 400 MHz spectrometer with tetramethylsilane (TMS) as the internal standard, and the chemical shifts were recorded in δ values (ppm). Mass spectra were recorded using a JEOL JMS-AX 300L spectrometer. Silica gel (Merck, 63–200 μm particle size) and RP-18 (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 carried out using a Gilson Trilution system with a UV detector (UV/VIS – 156) and a YMC-Pack ODS-A column (250 × 20 mm, 5 μm particle size, YMC Co. Ltd., Japan). HPLC solvents were purchased from Burdick & Jackson (USA).

3.2. Plant material

Dried fruiting bodies of *G. lucidum* were collected in Quang Nam province, central land of Vietnam, in May 2012. Professor Tran Cong Luan of Hochiminh City University of Medicine and Pharmacy performed the botanical identification and a voucher specimen (CUD-3177-2) was deposited at the Herbarium of the College of Pharmacy, Catholic University of Daegu, Korea.

3.3. Extraction and isolation

The dried fruiting bodies of *G. lucidum* (4 kg) were extracted 3 times by reflux with MeOH. After the solvent was removed under reduced pressure, the residue (164 g) was suspended in H_2O and then partitioned with *n*-hexane, $CHCl_3$, and EtOAc, successively. The $CHCl_3$-soluble fraction (98.2 g) was separated into 8 fractions (Fr. 1–8) with silica gel column chromatography (80 cm × 12 cm) using a stepwise gradient elution of $CHCl_3$–MeOH (100:1–1:1) according to their TLC profiles. Fraction 4 was subjected to a silica gel column eluted with *n*-hexane–EtOAc (10:1–1:1 gradient system) to afford four sub-fractions (Fr. 4–1 to 4–4). Fraction 4-2 (280 mg) was rechromatographed on a silica gel column (60 cm × 3.5 cm) using a gradient solvent system of *n*-hexane–acetone (20:1–1:1) to give compounds **6** (13 mg), **8** (32 mg), and **14** (23 mg). Fraction 4-3 (600 mg) was further chromatographed on an YMC RP-18 column with MeOH–H_2O (65:35–95:5) as an eluent to yield **3** (12 mg), **13** (9.0 mg), **15** (11 mg) and **17** (15 mg). Chromatography of fraction 6 (5.2 g) on the silica gel column (60 cm × 6.5 cm) using a gradient solvent system of $CHCl_3$–MeOH (50:1–10:1) yielded five subfractions (Fr. 6–1 to 6–5). Subfraction 6-2 (800 mg) was further purified by extensive preparative RP-HPLC [Gilson Trilution system; YMC Pak ODS-A column (20 mm × 250 mm, 5 μM particle size) column; ultraviolet (UV) detection at 210 nm] using MeOH–H_2O (65:35, v/v) at a flow rate of 5 mL/min as the mobile phase. This procedure yielded compounds **7** (8 mg), **9** (12 mg), **4** (7 mg), and **5** (12 mg). Fraction 6-3 (255 mg) was subjected to silica gel column chromatography (60 cm × 3.5 cm) eluting with a gradient solvent system of $CHCl_3$–EtOAc (15:1–2:1) to yield compounds **2** (21.5 mg), **16** (8 mg), **10** (7.8 mg), and **12** (13 mg). Fraction 6-4 (340 mg) was further purified over a YMC RP-18 column (50 cm × 3.5 cm) using a gradient solvent system of acetonitrile–H_2O (50:50–75:25) to afford **1** (7 mg), **11** (43 mg) and **18** (8.2 mg).

3.3.1. 3α,12β,15α-Triacetoxy-5α-lanosta-7,9(11),24-trien-26-oic acid (**1**)

Colorless oil; $[\alpha]_D^{25}$ 22.8 (*c* 0.12; $CHCl_3$); IR (KBr) ν_{max} 3424, 1718 cm^{-1}. For ^1H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR spectroscopic data, see Table 1. HR-EI-MS *m*/*z* 612.3662 $[M]^+$ (calcd. for $C_{36}H_{52}O_8$, 612.3664).

3.3.2. 5α-Lanosta-8,24-diene-26,27-dihydroxy-3,7-dione (**2**)

White amophous powder; $[\alpha]_D^{25}$ 2.6 (*c* 0.13; $CHCl_3$); IR (KBr) ν_{max} 3328, 1728 cm^{-1}; For ^1H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) spectroscopic data, see Table 1. HR-EI-MS *m*/*z* 470.3395 $[M]^+$ (470.3398, calcd. for $C_{30}H_{46}O_4$).

3.4. In vitro cytotoxicity assay

Three human cancer cell lines were used in the cytotoxicity assay: A549 (non-small cell lung adenocarcinoma), MCF-7 (breast adenocarcinoma), PC-3 (prostatic small cell carcinoma). Cells were maintained in RPMI 1640 supplemented with 10% FBS and 2% (v/v) penicillin-streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. In vitro cytotoxicity against human cancer cell lines (A549, MCF-7 and PC-3) was evaluated using a modified MTT assay with adriamycin as the positive control. The tests were performed according to the protocols described in the literature (Van et al., 2009). The IC_{50} value was defined as the concentration of sample that reduced absorbance by 50% relative to the vehicle-treated control.

3.5. Anti-angiogenesis activity

3.5.1. Cell culture

HUVECs were cultured in M199 medium supplemented with 20% fetal bovine serum (Hyclone, Logan, UT, USA), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), 0.01% heparin (Sigma–Aldrich, St. Louis, MO, USA), and 30 μg/mL endothelial cell growth supplement (ECGS) (Sigma–Aldrich), and maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded on plates coated with 0.2% gelatin (Sigma–Aldrich) and allowed to grow. Cell media was changed every other day.

3.5.2. Cell viability assay

The colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was modified and performed to quantify the effects of isolated compounds on cell viability. Briefly, HUVECs were seeded in a 96-wellmicrotiter plate (Falcon, Franklin Lakes, NJ, USA), and allowed to reach confluency approximately 80% after which various doses of isolates were treated for 48 h. After completion of treatment, MTT stock solution (0.25%) (Sigma, St. Louis, MO, USA) was added to the cells to a final concentration of 0.05% and the cells were incubated for 3 h at 37 °C. Next, the MTT solution was removed and replaced with 50 μL DMSO, and the plates were shaken for 3 min. The optical density of each condition was determined using a microplate reader at a wavelength of 570 nm with a reference wavelength of 630 nm. The percentage of cell viability was calculated against untreated cells.

3.5.3. In vitro capillary tube formation assay

A modified Matrigel assay was used to evaluate in vitro angiogenesis activity by quantifying the formation of HUVEC capillary tubes as described in the protocol of the Chemicon In Vitro Angiogenesis Assay Kit (ECM625). HUVECs (1×10^4 cells) were suspended in 50 μl of media containing various concentrations of isolated compounds and then added to the polymerized Matrigel. After incubation at 37 °C for 2–10 h, each culture was photographed at a magnification of 100× with a microscope video system (Carl Zeiss, Chester, VA).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2015.02.012.

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