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Cycloartane-Type Triterpenes from the Leaves of *Homonoia riparia* with VEGF-Induced Angiogenesis Inhibitory Activity

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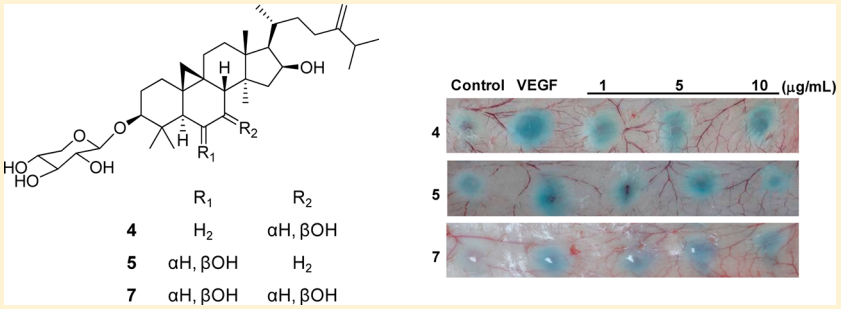
Supporting Information



ABSTRACT: Six new cycloartane-type triterpenes (**1**−**6**), 24-methylenecycloartane-3*β*,6*β*,7*β*-triol (**1**), 24-methylenecycloartane-3*β*,6*β*,7*β*,16*β*-tetraol (**2**), 24-methylenecycloartane-3*β*,6*β*,16*β*-triol (**3**), 24-methylenecycloartane-3*β*,7*β*,16*β*-triol 3-*O*-*β*-D-xylopyranoside (**4**), 24-methylenecycloartane-3*β*,6*β*,16*β*-triol 3-*O*-*β*-D-xylopyranoside (**5**), and 24-methylenecycloartane-3*β*,6*β*,7*β*-triol 3-*O*-*β*-D-xylopyranoside (**6**), were isolated from the leaves of *Homonoia riparia*, together with one known compound, 24-methylenecycloartane-3*β*,6*β*,7*β*,16*β*-tetraol 3-*O*-*β*-D-xylopyranoside (**7**). The structures of the new triterpenes were established by spectroscopic studies and from chemical evidence, and the inhibitory effects of compounds **1** and **3**−**7** on VEGF-induced vascular permeability were examined in vivo in rats using the Miles assay. In addition, the inhibitory effect of **7** on VEGF-induced tube formation by HUVECs in vitro was investigated.

Homonoia riparia Lour. is a small genus of shrubs or small trees of the family Euphorbiaceae that is widespread from India to mainland China, Taiwan, and throughout Malaysia to New Guinea, and is found commonly in groups at riverbanks.[1] Different parts of the plant have been used for the treatment of a wide range of ailments. The roots have laxative, diuretic, refrigerant, depurative, and emetic effects; a decoction of the roots is considered useful in the treatment of bladder stones, chest pain, gonorrhea, hemorrhoids, syphilis, and thirst.[2] The leaves and fruits are depurative and antiseptic and are used to treat wounds, ulcers, and skin diseases.[2] To date, few studies on the chemical constituents and biological activities of this plant have been carried out. A previous phytochemical investigation of the leaves resulted in the isolation of taraxerone, gallic acid, and quercetin glycoside.[3] Recently, it was reported that the crude extract of heartwood and stem bark of *H. riparia* yielded sterols, fatty acids, and triterpenoids.[4,5] Of these, a triterpenoid, aleuritolic acid, exhibited weak cytotoxicity against AGZY 83-a (human lung cancer) cells.[5]

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a critical process in physiological and pathological conditions, such as embryo development, wound repair, atherosclerosis, tumor growth, rheumatoid arthritis, and diabetic retinopathy.[6] Angiogenesis is tightly regulated by the balance between production of stimulators and inhibitors. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a potent angiogenic stimulator with endothelial cell-specific mitogenic activity and exerts a pivotal role in both normal and pathological angiogenesis.[7] The overexpression of VEGF is associated with chronic inflammation, tumor growth, and other angiogenesis-related diseases.[6,8] Thus, VEGF is a promising therapeutic target for the development of specific angiosuppressive drugs.

In a continuing effort to search for novel antiangiogenic agents from natural sources, six new cycloartane-type triterpenes (**1**−**6**), together with one known triterpene (**7**), were isolated from the leaves of *H. riparia*. Herein are described the isolation and

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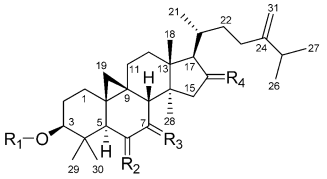
structure elucidation of these triterpenes and their inhibitory effects on VEGF-induced angiogenesis.



	R_1	R_2	R_3	R_4
1	H	αH, βOH	αH, βOH	H_2
2	H	αH, βOH	αH, βOH	αH,βOH
3	H	αH, βOH	H_2	αH,βOH
4	Xyl	H_2	αH, βOH	αH,βOH
5	Xyl	αH, βOH	H_2	αH,βOH
6	Xyl	αH, βOH	αH, βOH	H_2
7	Xyl	αH, βOH	αH, βOH	αH,βOH

RESULTS AND DISCUSSION

An 80% EtOH extract of *H. riparia* leaves was suspended in H_2O and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc-soluble fraction was subjected to a series of chromatographic techniques, leading to the isolation of six new triterpenes (**1**–**6**), along with one known compound (**7**), which was identified as 24-methylenecycloartane-3*β*,6*β*,7*β*,16*β*-tetraol 3-*O*-*β*-D-xylopyranoside by comparing its observed and published physicochemical data.[9]

Compound **1** was obtained as a white powder with a positive specific rotation, $[\alpha]_D^{25}$ +90.4 (*c* 0.05, pyridine). The HRESIMS of **1** exhibited a molecular ion peak at *m*/*z* 495.3809 $[M + Na]^+$, corresponding to the molecular formula $C_{31}H_{52}O_3$. The IR spectrum exhibited absorption bands at 3430 cm^{-1} (hydroxy group) and 1640 and 890 cm^{-1} (1,1-disubstituted double bond). The ^1H NMR spectrum of **1** showed signals for four tertiary methyls [δ_H 1.78, 1.50, 1.31, and 1.21 (each s)], three secondary methyls [δ_H 1.08 (3H, d, *J* = 7.0 Hz), 1.07 (3H, d, *J* = 7.0 Hz), and 1.02 (3H, d, *J* = 6.5 Hz)], and three hydroxymethine protons [δ_H 4.51 (1H, br s), 3.85 (1H, dd, *J* = 11.5, 1.5 Hz), and 3.59 (1H, dd, *J* = 11.5, 4.0 Hz)]. The two high-field doublets observed at δ_H 1.60 (1H, d, *J* = 3.5 Hz) and 0.60 (1H, d, *J* = 3.5 Hz) are characteristic of the two germinal protons of a cyclopropane moiety.[10,11] In addition, two singlet olefinic protons at δ_H 4.97 (1H, s) and 4.87 (1H, s) indicated the presence of an olefinic methylene moiety. The ^{13}C NMR spectrum, combined with the DEPT data, showed that **1** has 31 carbons, consisting of seven methyls, 10 methylenes, eight methines, and six quaternary carbons. Of these, the signals at δ_C 79.1, 74.2, and 73.1 indicated the presence of three oxygenated methines, and two sp^2 carbons at δ_C 157.6 and 107.0 revealed that **1** contains an exocyclic (=CH_2) methylene group. These spectroscopic data suggested **1** to be a cycloartane-type triterpene with an exocyclic methylene group.[12−15] In addition, the ^1H and ^{13}C NMR data of **1** were very similar to those of the aglycone of **7**, except for the additional signals consistent with the presence of a methylene unit [δ_H 2.06 m, 1.34 m; δ_C 29.6 (t)] instead of a hydroxymethine group at C-16 [δ_H 4.74 (br q); δ_C 72.3 (d)] in **7**. This finding indicates that **1** differs from **7** in the substitution pattern of ring D, which was supported by analysis of the 2D NMR spectra. The $^1H-^1H$ COSY correlations between H-2/H-3, and H-5, H-6, H-7, and H-8, as well as the long-range $^1H-^{13}C$ coupling (HMBC) observed between H-3/C-4 and C-29, H-6/C-5 and C-7, and H-7/C-6 and C-8, confirmed the positions of the three hydroxy groups to be at C-3, C-6, and C-7, respectively (Figure 1). Furthermore, the HMBC correlations between H-19/C-1, C-9, C-10, and C-11 and between

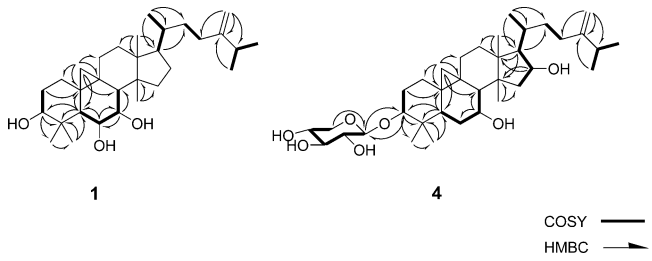


Figure 1. Key $^1H-^1H$ COSY and HMBC correlations of compounds **1** and **4**.

H-31/C-23, C-24, and C-25 indicated that the presence of an olefinic methylene group at C-24 and a cyclopropane moiety connected to C-9 and C-10, as found in **7**. The *β*-orientation of the hydroxy groups at C-3, C-6, and C-7 was deduced from the NOESY experiment, showing NOE correlations between H-3/H-5_{ax} and H_3-29, H-6/H-7 and H_3-29, and H-7/H-6 and H_3-28, respectively (Figure 2). Hence, the structure of **1** was established as 24-methylenecycloartane-3*β*,6*β*,7*β*-triol.

Compound **2** was obtained as a white powder. The molecular formula of **2** was found to be $C_{31}H_{52}O_4$ on the basis of a molecular ion peak at *m*/*z* 511.3750 $[M + Na]^+$ in the HRESIMS. The ^1H and ^{13}C NMR chemical shifts of the C-19 methylene group and C-18, C-28, C-29, and C-30 tertiary methyl groups were very similar to those of **1**, suggesting that **2** has the same cycloartane nucleus with an exocyclic methylene group. The difference in the ^1H NMR spectrum of **2** compared to that of **1** was the appearance of a signal at δ_H 4.75, which is assignable to the hydroxymethine proton instead of the 16-methylene protons in **1**. Inspection of the ^{13}C NMR spectrum when compared to **1** revealed a marked downfield shift of the C-16 signal (δ_C 72.3), suggesting the presence of a hydroxymethine group at C-16 in **2**, which was further supported by the $^1H-^1H$ COSY correlation between H-15, H-16, and H-17, coupled with the HMBC correlation of H-16 with C-14, C-17, and C-20. The *β*-configuration of the hydroxy groups at C-3, C-6, C-7, and C-16 was inferred from the NOE correlations between H-3/H-5_{ax} and H_3-29, H-6/H-7 and H_3-29, H-7/H-6 and H_3-28, and H-16/H-17_{ax} and H_3-28, respectively, in the NOESY spectrum. Thus, the structure of **2** was assigned as 24-methylenecycloartane-3*β*,6*β*,7*β*,16*β*-tetraol.

Compound **3** was obtained as a white powder and showed a molecular ion peak at *m*/*z* 495.3803 $[M + Na]^+$ in the HRESIMS, corresponding to the molecular formula $C_{31}H_{52}O_3$. The ^1H NMR spectrum of **3** was very similar to those of **1** and **2**, with the appearance of two typical high-field doublets at δ_H 1.61 and 0.60 (each 1H, d, *J* = 3.5 Hz) as well as two singlet olefinic protons at δ_H 4.92 and 4.85 (each 1H, s), suggesting that **3** is a cycloartane triterpene with the same skeleton as **1** and **2**. A comparison of the ^1H and ^{13}C NMR spectra of **3** with those of **2** revealed them to be very similar, with the only difference being the appearance of a set of proton signals at δ_H 1.70 and 1.46 (each 1H, m) corresponding to a methylene carbon signal at δ_C 35.8 in the HMQC experiment. These methylene proton signals (δ_H 1.70 and 1.46) revealed $^1H-^1H$ COSY correlations with H-6 and H-8, as well as HMBC correlations with C-6 and C-8, indicating the presence of a methylene group at C-7. The relative configuration of **3** was determined on the basis of the NOESY experiment and a comparison with that

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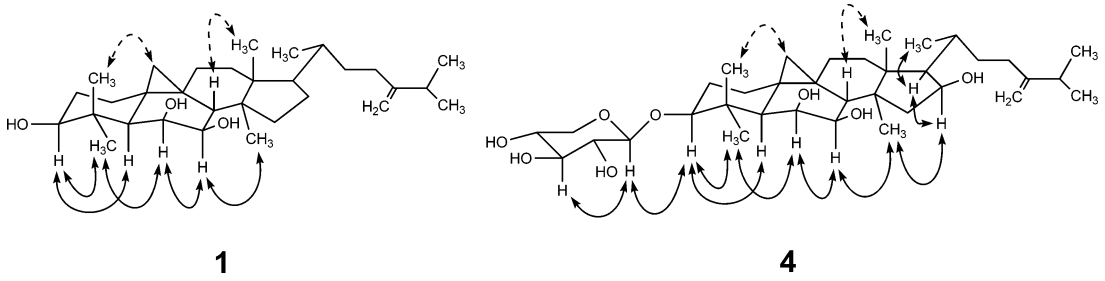

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Figure 2. Key NOESY correlations of compounds **1** and **4**.

of **2**. The NOE correlations observed between H-3/H-5_{ax} and H_3-29, H-6/H-7_{ax} and H_3-29, and H-16/H-17_{ax} and H_3-28 indicated a β-configuration of the hydroxy groups at C-3, C-6, and C-16, as found in **2**. Consequently, the structure of **3** was determined to be 24-methylenecycloartane-3β,6β,16β-triol.

Compound **4** was obtained as a white powder and gave a molecular ion peak at m/z 627.4243 $[M + Na]^+$ in the HRESIMS, establishing the molecular formula as $C_{36}H_{60}O_7$, which was 132 mass units greater than that of **1**. Acid hydrolysis of **4** yielded an aglycone and a monosaccharide unit. The ^{1}H and ^{13}C NMR spectroscopic data suggested the aglycone of **4** to be a cycloartane-type triterpene with an exocyclic methylene group. The carbon signals at δ_C 108.0, 79.0, 75.9, 71.6, and 67.5 and an anomeric proton signal at δ_H 4.88 (d, J = 7.5 Hz) of **4** were typical of a xylopyranosyl unit, which was identified as D-xylose following GC analysis. Moreover, the large coupling constant (J = 7.5 Hz) of the anomeric proton indicated that the xylopyranosyl unit was linked in the β-configuration. When the ^{13}C NMR spectrum of **4** was compared with those of **1**−**3**, a downfield shift by about 10 ppm was observed for C-3, suggesting that the xylopyranosyl linkage is at the C-3 hydroxy group in the aglycone, which was confirmed by observation of the HMBC cross-peak between the anomeric proton signal at δ_H 4.92 and the aglycone carbon signal at δ_C 88.7 (C-3) (Figure 1). Furthermore, the ^{1}H and ^{13}C NMR data of **4** were similar to those of **2**, except for the resonances of a methylene unit [δ_H 2.04 td (J = 8.5, 4.0 Hz), 1.26 m; δ_C 32.4 (t)] instead of a hydroxymethine group of C-6 [δ_H 4.51 (s); δ_C 73.0 (d)] in **2**, suggesting that the C-6 hydroxymethine group of **2** is replaced with a methylene group in **4**. This was supported by analysis of the 2D NMR spectra, including both the ^{1}H−^{1}H COSY and HMBC spectra. The β-orientation of the hydroxy groups at C-3, C-7, and C-16 was determined from the NOESY experiment (Figure 2). Therefore, the structure of **4** was determined to be 24-methylenecycloartane-3β,7β,16β-triol 3-*O*-β-D-xylopyranoside.

Compound **5** was obtained as a white powder. The HRESIMS showed a molecular ion peak at m/z 627.4236 $[M + Na]^+$, in accordance with the molecular formula, $C_{36}H_{60}O_7$. The ^{1}H and ^{13}C NMR spectra of **5** were superimposable with those of **3** except for the additional carbon signals at δ_C 107.9, 79.0, 76.0, 71.7, and 67.5 and an anomeric proton signal at δ_H 4.91, suggesting that **5** is a cycloartane triterpene monoglycoside. The sugar was identified as D-xylose by acid hydrolysis followed by GC analysis, and its configuration was elucidated as β from the large coupling constant (J = 7.5 Hz) of the anomeric proton. The linkage position of the β-D-xylopyranosyl unit on C-3 was confirmed on the basis of the HMBC cross-peak of the anomeric proton signal with C-3 (δ_C 89.4). The relative configuration of the aglycone moiety of **5** was assigned as being identical to that of **3** on the basis of NOE correlations. Thus, the structure of **5** was elucidated as 24-methylenecycloartane-3β,6β,16β-triol 3-*O*-β-D-xylopyranoside.

Compound **6**, a white powder, exhibited a $[M + Na]^+$ peak at m/z 627.4230 in the HRESIMS, consistent with a molecular formula of $C_{36}H_{60}NO_7$. The ^{13}C NMR spectrum of **6** revealed 36 carbon signals, of which five were assigned to a pentose residue and the remaining 31 to an aglycone. In addition, the ^{1}H and ^{13}C NMR spectra of **6** were almost identical to those of **1**, except for the sugar moiety, indicating that **1** and **6** have the same aglycone. The sugar moiety was elucidated as D-xylose by comparison of the NMR signals for the sugar unit of **6** with those of **4** and **5** and confirmed by GC analysis after acid hydrolysis. The large coupling constant (J = 7.5 Hz) of the anomeric proton at δ_H 4.91 indicated that D-xylose is in the β-configuration. The β-D-xylopyranosyl unit was shown to be attached at C-3 by observation of an HMBC correlation between the anomeric proton signal and C-3 (δ_C 89.2). The relative configuration of the aglycone moiety of **6** was deduced as being identical to that of **3** from the NOESY spectrum. Hence, the structure of **6** was established as 24-methylenecycloartane-3β,6β,7β-triol 3-*O*-β-D-xylopyranoside.

The inhibitory effects of the isolated compounds (**1** and **3**−**7**) on VEGF-induced vascular permeability in vivo were examined in rats using the Miles assay. The extravasation of Evans blue induced by VEGF was reduced by treatment with **4**, **5**, and **7**, in a dose-dependent manner (Figure 3A). In contrast, **1**, **3**, and **6** did not alter the vascular permeability induced by VEGF, demonstrating that the presence of both the C-3 xylopyranosyl and C-16 hydroxy groups may modulate the inhibitory potential of these triterpenoids. Quantitative analysis revealed that the known compound **7** reduced the VEGF-induced dermal extravasation most effectively. This compound, at the highest concentration used (10 μg/mL), exhibited considerable inhibition of VEGF-induced dermal extravasation, with 64% inhibition versus the control group (Figure 3B).

An in vitro angiogenesis model was used to determine the effect of **7** on VEGF-induced endothelial cell tube formation, a critical step in angiogenesis. Capillary-like tube formation was assessed with human umbilical vein endothelial cells (HUVECs) on Matrigel. Treatment of HUVECs with **7** resulted in a significant inhibition of VEGF-induced tube formation (Figure 4A), and cytotoxicity was not observed at the concentrations used in this study. Quantitative analysis showed that **7** inhibited tube formation by about 28.5% and 59.5% at 12.5 and 25 μM, respectively (Figure 4B). This result suggested that **7** inhibited in vitro angiogenesis by suppressing the activation of VEGF-induced tube formation.

Cycloartane-type triterpenes have been reported to exhibit a number of biological properties, including cytotoxic,[15] anti-HIV,[16] antiviral,[17] immunostimulant,[18] and antiprotozoal activities.[19] Recently, it has been reported that a series of 3,4-*seco*-cycloartane triterpenes possess antiangiogenic activity in both in vitro and ex vivo models.[20] However, the inhibitory effects of cycloartane

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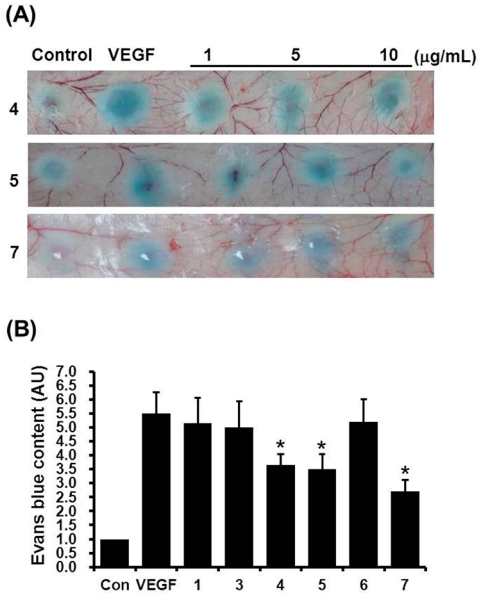

Figure 3. Antiangiogenic activity of **1** and **3**–**7** in rats in vivo. (A) Effects of **4**, **5**, and **7** on VEGF-induced vascular permeability in male Sprague–Dawley rat skin dermis; 15 μL of PBS as a control with or without VEGF (100 ng/mL) or **4**, **5**, and **7** (each 1, 5, and 10 μg/mL) was intradermally injected. (B) Quantitative analysis of extravasation of Evans blue dye in the skin after injection of 15 μL of PBS with or without VEGF (100 ng/mL) or **1** and **3**–**7** (each 10 μg/mL) (means ± SD, $n = 3$). *$p < 0.05$ vs VEGF alone.

triterpenes on vascular permeability in vivo are reported here for the first time. The present results suggest that the cycloartane-type triterpenes isolated in this study contributed to the inhibitory effect of *H. riparia* leaves on VEGF-induced angiogenesis. Further studies are needed to better understand the specific mechanism(s) involved in the mediation of their antiangiogenic effects.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-2000 digital polarimeter. IR spectra were recorded on a JASCO 100 IR spectrometer. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained using a Bruker DRX-300 spectrometer with TMS as an internal standard. 2D NMR experiments (COSY, HMQC, HMBC, and NOESY) were run on a Bruker Avance 500 NMR spectrometer. HRESIMS were recorded on a Shimadzu LCMS-IT-TOF spectrometer. Column chromatography was performed using silica gel (70–230 mesh and 230–400 mesh, Merck), YMC-gel ODS-A (12 nm, S-75 μm, YMC), and Sephadex LH-20 (Amersham Pharmacia Biotech). Thin-layer chromatography was performed on precoated silica gel 60 F_{254} (0.25 mm, Merck) and RP-18 F_{254s} plates (0.25 mm, Merck). Spots were detected by UV light (254 nm) and spraying with 10% H_2SO_4 followed by heating.

Plant Material. The leaves of *H. riparia* were collected in Hanoi, Vietnam, in June 2009, and identified by Prof. J.-H. Kim, Gachon University, Republic of Korea. A voucher specimen (No. TBRC-VN-118) has been deposited in the Herbarium of the Diabetic Complications Research Team, Korea Institute of Oriental Medicine, Republic of Korea.

Extraction and Isolation. Dried *H. riparia* leaves (6.0 kg) were extracted with 80% EtOH (3 × 30 L) at room temperature for seven days by maceration, filtered, and concentrated to give an 80% EtOH extract (700 g). This extract (200 g) was suspended in H_2O (4 L) and then partitioned successively with *n*-hexane (3 × 4.0 L), EtOAc (3 × 4.0 L), and *n*-BuOH (3 × 4.0 L), to afford *n*-hexane- (3.98 g), EtOAc- (56.01 g), and *n*-BuOH-soluble fractions (41.42 g), respectively. The EtOAc-soluble fraction (50.0 g), which significantly inhibited the VEGF-induced vascular permeability, was subjected to silica gel column chromatography (70–230 μm, 40 × 9.5 cm) and eluted with a gradient solvent system of $CHCl_3$–MeOH (50:1 → 0:1) to generate 11 fractions (A–K).

Fraction E (2.89 g) was chromatographed on a Sephadex LH-20 column (60 × 6.5 cm) using a $CHCl_3$–MeOH (1:1) solvent system to yield three subfractions (E1–E3). Of these, E2 (1.2 g) was further chromatographed on a YMC RP-18 column (50 × 4.5 cm) and eluted using a MeOH–H_2O gradient solvent system (70:30 → 90:10) to yield

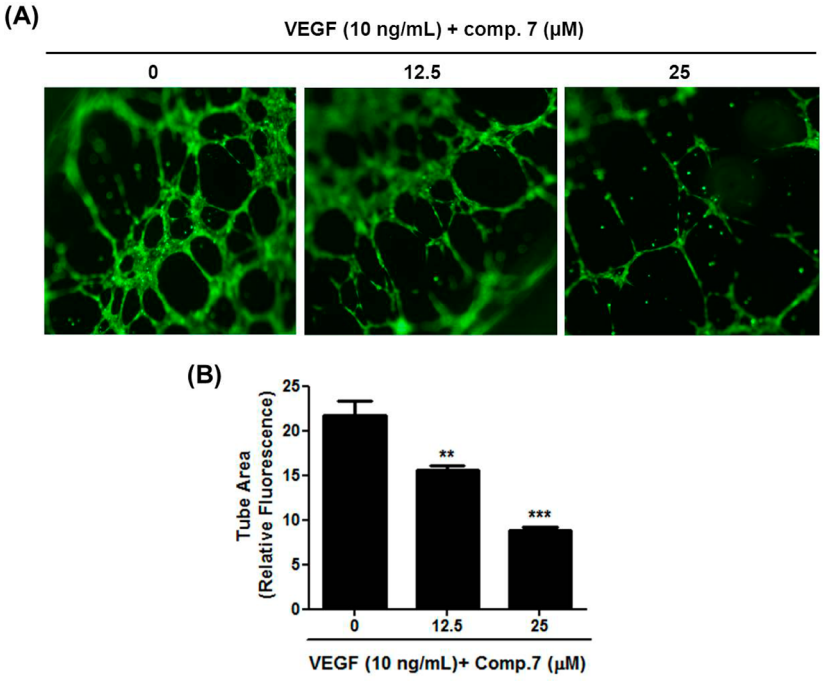

Figure 4. Antiangiogenic activity of **7** on HUVECs in vitro. (A) Effect of **7** on VEGF-induced tube formation by HUVECs (×20); HUVECs were plated on a BME-coated 24-well plate with medium containing VEGF (10 ng/mL) with or without **7** (12.5 or 25 μM) (B) Quantitative analysis of tube formation using Image J and GraphPad Prism 5.0 (means ± SD, $n = 3$). **$p < 0.01$ and ***$p < 0.001$ vs VEGF alone, respectively.

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Table 1. ^{1}H NMR (300 MHz) Data of Compounds 1−6 (J in Hz) in Pyridine-d_5

H	1	2	3	H	4	5	6
1	1.63 m, 1.26 m	1.64 m, 1.28 m	1.65 m, 1.30 m	1	1.64 m, 1.26 m	1.64 m, 1.26 m	1.61 m, 1.23 m
2	2.28 m, 2.04 m	2.28 m, 2.05 m	2.35 m, 2.13 m	2	2.23 m, 1.94 m	2.37 m, 2.13 m	2.36 m, 2.10 m
3	3.59 dd (11.5, 4.0)	3.58 dd (11.5, 4.0)	3.60 dd (11.5, 4.5)	3	3.54 dd (11.5, 4.0)	3.56 dd (11.5, 4.5)	3.53 dd (11.5, 4.0)
5	1.56 d (3.5)	1.56 d (3.5)	1.78 m	5	1.63 d (3.0)	1.72 m	1.55 d (3.5)
6	4.51 br s	4.51 br s	4.63 m	6	2.04 td (8.5, 4.0), 1.26 m	4.59 m	4.48 br s
7	3.85 dd (11.5, 1.5)	3.86 dd (11.5, 1.5)	1.70 m, 1.46 m	7	3.85 m	1.65 m, 1.45 m	3.92 dd (11.5, 1.5)
8	2.49 d (11.5)	2.64 d (11.5)	2.62 dd (13.5, 4.0)	8	2.09 d (9.0)	2.58 dd (13.5, 4.0)	2.45 d (11.5)
11	1.15 m, 2.12 m	1.18 m, 2.20 m	1.18 m, 2.22 m	11	1.14 m, 2.14 m	1.14 m, 2.15 m	1.15 m, 2.14 m
12	1.73 m	1.88 m, 1.78 m	1.84 m, 1.78 m	12	1.76 m	1.75 m, 1.80 m	1.72 m
15	2.10 m, 1.84 td (12.6, 6.0)	2.90 dd (13.5, 8.0), 2.28 m	2.18 m	15	2.80 dd (13.5, 8.0), 2.32 m	2.14 m	2.08 m, 1.82 td (12.6, 6.0)
16	2.06 m, 1.34 m	4.75 m	4.70 m	16	4.75 m	4.68 m	2.07 m, 1.34 m
17	1.68 m	1.90 dd (10.5, 8.0)	1.90 dd (10.5, 8.0)	17	1.87 dd (10.5, 8.0)	1.87 dd (10.5, 8.0)	1.67 m
18	1.21 s	1.64 s	1.60 s	18	1.54 s	1.55 s	1.18 s
19	1.60 d (3.5), 0.60 d (3.5)	1.65 d (3.5), 0.61 d (3.5)	1.61 d (3.5), 0.60 d (3.5)	19	0.79 d (4.2), 0.30 d (4.2)	1.55 d (3.5), 0.55 d (3.5)	1.57 d (3.5), 0.55 d (3.5)
20	1.44 m	2.34 m	2.32 m	20	2.23 m	2.26 m	1.42 m
21	1.02 d (6.5)	1.16 d (6.5)	1.13 d (6.5)	21	1.14 d (6.5)	1.10 d (6.5)	1.12 d (6.5)
22	1.70 m, 1.25 m	2.26 m, 1.42 m	1.70 m, 1.46 m	22	2.24 m, 1.41 m	1.70 m, 1.46 m	1.68 m, 1.23 m
23	2.25 m, 2.04 m	2.38 m, 2.21 m	2.38 m, 2.20 m	23	2.38 m, 2.20 m	2.36 m, 2.18 m	2.22 m, 1.98 m
25	2.28 m	2.31 m	2.28 m	25	2.31 m	2.30 m	2.25 m
26	1.08 d (7.0)	1.06 d (7.0)	1.06 d (7.0)	26	1.06 d (7.0)	1.05 d (7.0)	1.07 d (7.0)
27	1.07 d (7.0)	1.05 d (7.0)	1.04 d (7.0)	27	1.05 d (7.0)	1.04 d (7.0)	1.06 d (7.0)
28	1.31 s	1.33 s	1.08s	28	1.20 s	1.06 s	1.28 s
29	1.50 s	1.49 s	1.60 s	29	1.08 s	1.68 s	1.60 s
30	1.78 s	1.76 s	1.77 s	30	1.35 s	1.73 s	1.73 s
31	4.97 s, 4.87 s	4.93 s, 4.86 s	4.92 s, 4.85 s	31	4.93 s, 4.86 s	4.89 s, 4.82 s	4.91 s, 4.87 s
Xyl-1				Xyl-1	4.88 d (7.5)	4.91 d (7.5)	4.91 d (7.5)
Xyl-2				Xyl-2	4.04 t (8.5)	4.07 t (8.5)	4.07 t (8.5)
Xyl-3				Xyl-3	4.17 t (9.0)	4.18 t (9.0)	4.17 t (9.0)
Xyl-4				Xyl-4	4.24 td (9.0, 5.0)	4.25 td (9.0, 5.0)	4.25 td (9.0, 5.0)
Xyl-5				Xyl-5	4.39 dd (11.0, 5.0), 3.76 t (10.5)	4.40 dd (11.0, 5.0), 3.78 t (10.5)	4.40 dd (11.0, 5.0), 3.77 t (10.5)

compounds **1** (10 mg), **5** (45 mg), and **6** (23 mg). Fraction C (1.9 g) was further purified over a Sephadex LH-20 column (60 × 6.5 cm) and eluted with $CHCl_3$−MeOH (1:1) to generate three subfractions (C1−C3). Compound **2** (5 mg) was isolated from fraction C3 (430 mg) using a YMC RP-18 column (50 × 3.5 cm) and eluted with a MeOH−H_2O gradient solvent system (80:20 → 100:0). Chromatography of fraction B (0.9 g) on a Sephadex LH-20 column (60 × 6.5 cm) using a $CHCl_3$−MeOH (1:1) solvent system yielded three subfractions (B1−B3). Fraction B2 (360 mg) was further purified on a YMC RP-18 column (50 × 3.5 cm) and eluted with a stepwise MeOH−H_2O (65:35 → 90:10) gradient to generate **3** (7 mg), **4** (18 mg), and **7** (50 mg).

*24-Methylenecycloartane-3β,6β,7β-triol (**1**):* white powder; $[\alpha]_D^{25}$ +90.4 (*c* 0.05, pyridine); IR (KBr) ν_{max} 3430, 2970, 1640, 1220, 890, 765 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 495.3809 [M + Na]$^+$ (calcd for $C_{31}H_{52}O_3Na^+$, 495.3809).

*24-Methylenecycloartane-3β,6β,7β,16β-tetraol (**2**):* white powder; $[\alpha]_D^{25}$ +92.1 (*c* 0.05, pyridine); IR (KBr) ν_{max} 3425, 2960, 1640, 1210, 895, 770 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 511.3750 [M + Na]$^+$ (calcd for $C_{31}H_{52}O_4Na^+$, 511.3758).

*24-Methylenecycloartane-3β,6β,16β-triol (**3**):* white powder; $[\alpha]_D^{25}$ +87.5 (*c* 0.05, pyridine); IR (KBr) ν_{max} 3430, 2975, 1645, 1220, 890, 770 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 495.3803 [M + Na]$^+$ (calcd for $C_{31}H_{52}O_3Na^+$, 495.3809).

*24-Methylenecycloartane-3β,7β,16β-triol 3-O-β-D-xylopyranoside (**4**):* white powder; $[\alpha]_D^{25}$ +64.2 (*c* 0.05, pyridine); IR (KBr) ν_{max} 3450, 2980, 1640, 1240, 1041, 890, 780 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 627.4243 [M + Na]$^+$ (calcd for $C_{36}H_{60}O_7Na^+$, 627.4231).

*24-Methylenecycloartane-3β,6β,16β-triol 3-O-β-D-xylopyranoside (**5**):* white powder; $[\alpha]_D^{25}$ +76.5 (*c* 0.05, pyridine); IR (KBr) ν_{max} 3460, 2980, 1640, 1240, 1040, 892, 775 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 627.4236 [M + Na]$^+$ (calcd for $C_{36}H_{60}O_7Na^+$, 627.4231).

*24-Methylenecycloartane-3β,6β,7β-triol 3-O-β-D-xylopyranoside (**6**):* white powder; $[\alpha]_D^{25}$ +69.8 (*c* 0.05, pyridine); IR (KBr) ν_{max} 3460, 2980, 1645, 1242, 1044, 895, 770 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 627.4230 [M + Na]$^+$ (calcd for $C_{36}H_{60}O_7Na^+$, 627.4231).

Acid Hydrolysis of 4−6. Compounds **4**−**6** (2 mg each) in 10% HCl−dioxane (1:1, 1 mL) were heated separately at 80 °C for 3 h in a water bath. The mixture was neutralized with Ag_2CO_3, filtered, and then extracted with EtOAc (20 mL). The aqueous layer was evaporated, and then the residue was treated with L-cysteine methyl ester hydrochloride (2 mg) in pyridine (0.5 mL) at 60 °C for 1 h. After the reaction was completed, the solution was treated with acetic anhydride (3 mL) at 60 °C for 1 h. Authentic samples were prepared using the same procedure. The acetate derivatives were treated with gas chromatography (GC) analysis. GC conditions: GC-2010 (Shimadzu) instrument; detector, FID; column, TC-1 capillary column (0.25 mm × 30 m; GL Science, Inc.); column temperature, 230 °C; programmed increase, 38 °C/min; carrier gas, N_2 (1 mL/min); injection and detector temperature, 270 °C; t_R 16.02 min (D-xylose), 16.80 min (L-xylose). D-Xylose was detected from **4**−**6**.

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Table 2. ^{13}C NMR (75 MHz) Data of Compounds 1−6 in Pyridine-d_5

C	1	2	3	4	5	6
1	34.0	33.9	33.7	32.3	33.5	33.8
2	31.7	31.7	31.8	30.4	30.6	30.6
3	79.1	79.0	79.2	88.7	89.4	89.2
4	42.0	41.9	42.2	41.4	42.5	42.2
5	50.5	50.4	50.4	46.8	50.4	50.4
6	73.1	73.0	66.7	32.4	66.6	72.8
7	74.2	74.1	35.8	70.7	35.7	74.1
8	47.6	47.8	40.9	55.7	40.9	47.5
9	19.9	19.9	20.2	20.7	20.2	19.9
10	25.6	25.7	25.2	27.3	24.9	25.3
11	26.9	26.8	26.8	27.2	26.8	26.8
12	33.8	33.9	34.0	33.6	34.0	33.7
13	46.9	47.0	46.6	46.7	46.5	46.9
14	48.9	47.1	47.2	47.5	47.2	48.8
15	39.4	53.3	50.4	51.9	50.4	39.3
16	29.6	72.3	71.8	72.1	71.8	29.6
17	52.7	57.1	57.8	56.9	57.7	52.7
18	19.3	20.5	20.5	20.4	20.5	19.3
19	32.7	32.9	33.5	29.0	33.4	32.5
20	36.9	31.3	31.4	31.3	31.3	36.9
21	19.1	18.9	18.7	18.9	18.7	19.1
22	35.9	36.1	36.0	36.1	36.0	35.8
23	31.7	32.8	32.7	32.8	32.7	32.1
24	157.3	157.6	157.6	157.6	157.6	157.2
25	34.5	34.5	35.0	34.5	34.5	34.5
26	22.4	22.4	22.4	22.4	22.4	22.4
27	22.6	22.5	22.5	22.5	22.5	22.5
28	19.8	20.6	21.3	20.4	21.3	19.7
29	25.7	25.7	25.8	26.2	25.3	25.2
30	16.9	16.9	16.7	15.7	17.4	17.6
31	107.0	106.7	106.7	106.7	106.7	107.0
Xyl-1				108.0	107.9	107.9
Xyl-2				75.9	76.0	75.9
Xyl-3				79.0	79.0	79.0
Xyl-4				71.6	71.7	71.6
Xyl-5				67.5	67.5	67.5

Vascular Permeability Assay. All experiments were performed in accordance with the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the Korea Institute of Oriental Medicine Institutional Animal Care and Use Committee (KIOM IACUC protocol number 11-019). To determine vascular permeabilities, the Miles assay[21] was performed. Male Sprague−Dawley rats (250−300 g) were anesthetized and shaved. After two to three days, the rats were again anesthetized and injected intravenously with 150 μL of 1% Evans blue dye (Sigma, St. Louis, MO, USA). After 15 min, intradermal injection of one of the following was performed: 15 μL of VEGF (100 ng/mL), the indicated amounts of test compounds, or PBS as a negative control. After 1 h, the skins were dissected and photographed. The dye was then eluted from the dissected samples with formamide at 56 °C, and the optical density at 620 nm was measured by spectrophotometry (Synergy HT, BioTek, Winooski, VT, USA).

Tube Formation Assay. HUVECs were obtained from Clonetics (Lonza #C2519A) and cultured according to Lonza's standard protocol. An in vitro tube formation assay was performed using an angiogenesis assay tube formation kit (Cultrex, #3470-096-K). Briefly, 5×10^4 HUVECs were plated on a basement membrane extract (BME)-coated 24-well plate with medium containing VEGF (10 ng/mL) with or without 7 (12.5 or 25 μM). Following 6 h of incubation, 2 μM Calcein AM (Cultrex, #4892-010-01) in PBS was added to the plate for 30 min, and cultures were examined for tube formation under a fluorescence microscope. The quantitative difference in tube formation in treated and untreated cultures was evaluated using NIH Image J (NIH Image, Bethesda, MD, USA) and GraphPad Prism, version 5.0 (GraphPad, San Diego, CA, USA).

Statistical Analysis. Data are expressed as means ± SD of three individual experiments. The significance of differences was analyzed using one-way ANOVA using SPSS software.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR spectra for the new compounds **1**−**6**. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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