Chemical Constituents from the Leaves of *Manglietia phuthoensis* and Their Effects on Osteoblastic MC3T3-E1 Cells

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Two new phenyl glycosides, mangliesides A and B (1, 2), a new ionol glycoside, manglieside C (3), two new lignan glycosides, mangliesides D and E (4, 5), were isolated from the leaves of *Manglietia phuthoensis*, along with two known lignans, 3-methoxymagnolol (6) and obovatol (7). Their structures were established by means of 1D and 2D NMR, electrospray ionization (ESI)-MS and HR-ESI-MS experiments. Among them, compounds 2 and 5 significantly ($p<0.05$) increased the growth and differentiation of osteoblastic MC3T3-E1 cells *in vitro*.

Key words *Manglietia phuthoensis*; manglieside A; manglieside B; manglieside C; manglieside D; manglieside E

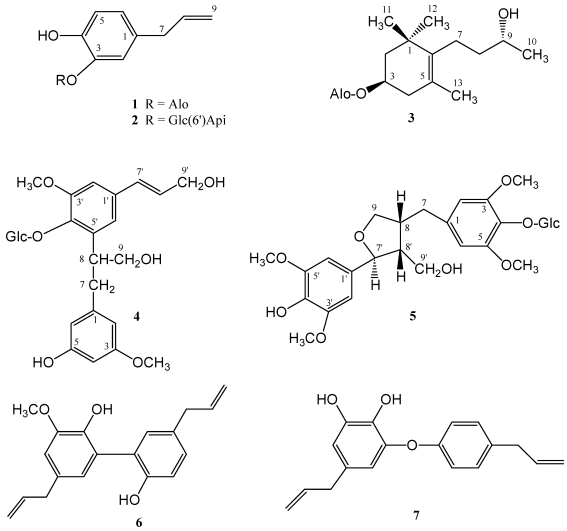
Osteoporosis is characterized by a reduced bone mass, which results in increased bone fragility and fracture risk. Many osteoporotic patients have already lost a substantial amount of bone; therefore, a method of increasing bone mass by stimulating new bone formation is required. Bone is composed of mineralized organic matrix and bone cells. Osteoblasts are the active mature bone cells that synthesize the organic matrix and regulate its mineralization. Accelerated osteoblast growth and protein synthesis are the key factors for efficient bone repair.[1] Hormones, which regulate the calcium balance in the body, are used for the treatment of osteoporosis. The main action mechanism of these agents is the inhibition of bone resorption. However, a substance that inhibits bone resorption while stimulating bone formation would be more suitable for the treatment of bone diseases. There are several reports demonstrating improvement in clinical association with the use of traditional medicines in the treatment of fractures.[2] Despite encouraging preliminary reports, basic sciences and clinical reports justifying the clinical application of specific traditional medicines are still not well established.

Many plant-derived substances have been used as drugs for the treatment of various diseases since ancient times, and traditional oriental therapies are rich in phytotherapeutic regimens. These medications have fewer side effects and are more suitable for long-term use as compared to chemically synthesized medications. Plants belonging to the family Magnoliaceae are well known as rich sources of phenyl derivatives type neolignan and biphenyl ethers, which showed antimicrobial, muscle-relaxant, antifungal and cytotoxic activities.[3—5] *Manglietia phuthoensis* DANDY (*Magnolia phuthoensis* DANDY), belonging to Magnoliaceae family, are woody trees distributed in northern Vietnam. In Vietnamese traditional medicine, the plant is used as an anti-inflammatory agent.[6] Up to date, no study on phytochemistry and bioactivities of this plant has been reported.

We report herein on the isolation and structural elucidation of two new phenyl glycosides, mangliesides A and B (**1**, **2**), a new ionol glycoside, manglieside C (**3**), two new lignan glycosides, mangliesides D and E (**4**, **5**), and two known compounds as 3-methoxymagnolol (**6**) and obovatol (**7**) from the leaves of *M. phuthoensis*. In an attempt to find pure lead compounds from plant origins to prevent bone loss without serious adverse side effects, we examined the effect of isolated compounds on the function of osteoblastic MC3T3-E1 cells, which has been well-characterized as an *in vitro* model for osteoblast differentiation.[7]

Results and Discussions

Manglieside A (**1**) was isolated as a white amorphous powder, its molecular formula was suggested as $C_{15}H_{20}O_7$ from the exhibition of the quasi-molecular ion peaks at *m*/*z* 311.1 $[M-H]^-$ (negative) and 335.1 $[M+Na]^+$ (positive) in the electrospray ionization (ESI) mass spectra, and further established by HR-ESI-MS (Found *m*/*z*: 335.1068 $[M+Na]^+$; Calcd 335.1107 for $C_{15}H_{20}O_7Na$). Its IR spectrum had typical absorptions of hydroxyl (3437 cm^{-1}) and ether (1056 cm^{-1}) functionalities. Compound **1** showed 15 signals, six of them attributable to a sugar moiety, and nine of them to an aglycon moiety in its ^{13}C-NMR spectrum. In particular, this spectrum showed an anomeric carbon at δ 102.30 and an aromatic ring



Alo: β-D-allopyranose, Api: β-D-apiofuranose, Glc: β-D-glucopyranose

Fig. 1. Structures of Compounds **1**—**7**

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at δ 119.16—146.77, suggesting that **1** was a phenyl glycoside. The ^{1}H-NMR spectrum of **1** exhibited signals of three aromatic protons at δ 7.01 (d, J=2.0 Hz), 6.77 (d, J=8.0 Hz), and 6.74 (dd, J=2.0, 8.0 Hz) suggesting the 1,3,4-trisubstituted aromatic ring; a broad doublet at δ 3.29 (2H, J=6.5 Hz), a multiplet at δ 5.94, as well as two doublet of doublet of doublet at δ 5.05 (J=17.0, 3.5, 1.5 Hz) and 5.02 (J=9.0, 3.5, 1.5 Hz) defined the presence of a propenyl group.[5] The ^{13}C-NMR data of the sugar moiety of **1** (δ_C 102.30, 75.88, 72.84, 72.19, 68.50) and spin-coupling pattern of its proton signals $J_{1'-2'}$=8.0 Hz, $J_{2'-3'}$=3.0 Hz, $J_{3'-4'}$=3.0 Hz (Table 1) suggesting a β-D-allopyranosyl unit[8,9] with *axial* configuration of 3′-OH. The partial structures of **1** were deduced from the ^{1}H–^{1}H COSY, HMQC spectra, and were further connected based on long-range correlations in the HMBC spectrum (Table 1). In the HMBC spectrum, H-1′ (δ 5.11) correlated to C-3 (δ 146.77), H-2 (δ 7.01) correlated to C-1 (δ 133.11)/C-3 (δ 146.77)/C-4 (δ 146.66)/C-6 (δ 124.55), and to C-7 (δ 40.51). This evidence and comparison of the NMR spectral data of **1** with those of 3,4-dihydroxyallylbenzene-4-*O*-β-D-glucoside[10] confirmed that the propenyl group attached to C-1 and the sugar linked to C-3 by an ether linkage. The configuration of the sugar unit was assigned as D-allopyranose after hydrolysis of **1** with 1 N HCl. The hydrolysate was trimethylsilylated, and GC retention time compared with that of authentic D-allopyranose (Merck) prepared in the same manner. On the basis of all the above evidence, compound **1** was determined to be the new natural product 3,4-dihydroxyallylbenzene-3-*O*-β-D-allopyranoside named manglieside A.

The NMR spectra of **2** showed close similarities to those of **1** except for the additional signals of a sugar unit (Table 1). Signals at δ_C 110.95, 78.14, 80.57, 75.08, 65.82/δ_H 5.00 (d, J=2.0 Hz), 3.93 (d, J=2.0 Hz), 3.78 and 3.98 (d, J=10.0 Hz), 3.60 (s) suggested the appearance of a β-apiofuranosyl sugar, and signals at δ_C 104.69, 74.90, 77.58, 71.46, 77.06, and 68.41 were very typical of a β-glucopyranosyl having an ether linkage at C-6′.[11,12] These evidence led to suggest the molecular formula of **2** as $C_{20}H_{28}O_{11}$, which was further confirmed by the ESI mass spectra {*m*/*z* 445.1 $[M+H]^+$, 467.1 $[M+Na]^+$ (positive), 443.2 $[M-H]^-$ (negative)}, and the HR-ESI mass spectrum (Found *m*/*z*: 467.1396, Calcd 467.1365 for $C_{20}H_{28}O_{11}Na$). Moreover, hydrolysis of **2** with 1 N HCl in methanol gave D-apiofuranose and D-glucopyranose, which were identified by GC experiment (see Experimental). In the HMBC spectrum, H-1″ proton at δ 5.00 has correlation to C-6′ at δ 68.41, H-1′ proton at δ 4.71 correlated to C-3 at δ 146.80 confirming that the apiofuranosyl moiety linked to C-6′ of the glucopyranose, and the glucopyranosyl moiety attached to C-3 of the aglycon. Thus, the structure of **2** was established to be 3,4-dihydroxyallylbenzene-3-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside, a new natural product named manglieside B.

The molecular formula of **3** was identified to be $C_{19}H_{34}O_7$

Table 1. NMR Data of **1**—**3**

C	**1**		**2**		**3**	
	δ_C[a),b)]	δ_H[a),c)] [mult. (*J* in Hz)]	δ_C[a),b)]	δ_H[a),b)] [mult. (*J* in Hz)]	δ_C[a),b)]	δ_H[a),b)] [mult. (*J* in Hz)]
1	133.11	—	133.18	—	38.77	—
2	119.16	7.01 d (2.0)	119.70	7.02 d (2.0)	47.56	1.82 dt (12.5, 2.0)
						1.49 m
3	146.77	—	146.80	—	73.23	4.05 m
4	146.66	—	146.59	—	39.87	2.02 dd (16.5, 10.0)
						2.33 dd (16.5, 4.5)
5	116.89	6.77 d (8.0)	117.08	6.78 d (8.0)	125.15	—
6	124.55	6.74 dd (8.0, 2.0)	124.98	6.80 dd (8.0, 2.0)	138.05	—
7	40.51	3.29 d (6.5)	40.49	3.30 br d (6.5)	25.54	1.94 m
						2.22 m
8	139.24	5.94 m	139.29	5.96 m	40.72	1.52 m
9	115.57	5.02 ddd (9.0, 3.5, 1.5)	115.67	5.03 ddd (9.0, 3.5, 1.5)	69.20	3.71 m
		5.05 ddd (17.0, 3.5, 1.5)		5.06 ddd (17.0, 3.5 1.5)		
10					23.24	1.16 d (6.0)
11					28.82	1.04 s
12					30.29	1.05 s
13					20.01	1.64 s
	Alo		Glc		Alo	
1′	102.30	5.11 d (8.0)	104.69	4.71 d (7.5)	99.76	4.80 d (8.0)
2′	72.19	3.65 dd (8.0, 3.0)	74.90	3.47 dd (8.0, 7.5)	72.41	3.27 dd (8.0, 3.0)
3′	72.84	4.18 t (3.0)	77.58	3.49 dd (8.0, 8.0)	72.97	4.03 t (3.0)
4′	68.50	3.63 dd (10.0, 3.0)	71.46	3.42 dd (8.0, 8.0)	68.98	3.48 dd (10.0, 3.0)
5′	75.88	3.82 m	77.06	3.56 ddd (8.0, 5.5, 2.0)	75.40	3.71 m
6′	62.72	3.88 dd (12.0, 2.0)	68.41	4.03 dd (12.0, 2.0)	63.14	3.82 dd (12.0, 3.0)
		3.73 dd (12.0, 5.0)		3.66 dd (12.0, 5.5)		3.65 dd (12.0, 5.0)
			Api			
1″			110.95	5.00 d (2.0)		
2″			78.14	3.93 d (2.0)		
3″			80.57	—		
4″			75.08	3.78 d (10.0)		
				3.98 d (10.0)		
5″			65.82	3.60 s		

a) Measured in CD_3OD, *b*) at 125 MHz, *c*) at 500 MHz.

by means of ESI-MS (at *m/z* 397.4 $[M+Na]^+$) and HR-ESI-MS (Found *m/z* 375.4785, Calcd 375.4827 for $C_{19}H_{35}O_7$), which was the same as that of linarionoside A.[13] The NMR spectral data of **3** were nearly identical to those of linarionoside A. The differences between the two compounds were only in the signals of the sugar moiety. The ^{13}C-NMR spectral data of the sugar moiety of **3** (δ 99.76, 75.40, 72.97, 72.41, 68.98, 63.14) were similar to those of **1**, suggesting for the presence of a β-D-allopyranosyl unit. The absolute configuration of the sugar unit was determined as reported for compound **1**. All the NMR spectral data of **3** were assigned by comparison of them with the corresponding data of linarionoside A[13] and further confirmed by the HSQC and HMBC experiments. The attached position of the sugar moiety at C-3 was confirmed by HMBC cross peak from the anomeric proton (δ 4.80, 1H, d, J=8.0 Hz) to carbon C-3 (δ 73.23). From all above, **3** was identified to be 5-megastigmene-3β,9α-diol 3-*O*-β-D-allopyranoside, which was named as manglieside C.

Compound **4** was isolated as a white amorphous powder. The molecular formula, $C_{26}H_{34}O_{11}$, was determined by ESI-MS (at *m/z* 545.2 $[M+Na]^+$) and HR-ESI-MS (Found *m/z* 523.2147, Calcd 523.2180 for $C_{26}H_{35}O_{11}$) data. The NMR spectral data of **4** resembled to those of icariside E_5,[14,15] except for the signals from C-1 to C-6. The ^{1}H-NMR spectrum of **4** exhibited signals at δ 6.76 (1H, br s, H-2), 6.67 (1H, br s, H-4), and 6.68 (1H, br s, H-6), indicating that the vanillyl moiety (4-hydroxy-3-methoxybenzyl) in icariside E_5 was changed to a 5-hydroxy-3-methoxybenzyl moiety in **4**. This was further confirmed by the HMBC experiment. Moreover, acid hydrolysis of **4** afforded D-glucopyranose (see Experimental). The above evidence led to the conclusion that **4** was 3,3′-dimethoxy-4′,5,9,9′-tetrahydroxy-5′,8-biligin-7′-ene 4′-*O*-β-D-glucopyranoside which was named manglieside D.

The NMR data of **5** resembled to those of salvadoraside,[16] except for the absence of one sugar moiety signals. The HR-ESI mass spectrum showed a quasi-molecular ion at *m/z* 605.2183 $[M+Na]^+$ (Calcd 605.2210 for $C_{28}H_{38}O_{13}Na$), determining the molecular formula of $C_{28}H_{38}O_{13}$, confirming the loss of one sugar moiety of **5** comparing to salvadoraside. The structure of **5** was established by comparison its NMR spectral data with those of salvadoraside[16] and further confirmed by the HSQC, HMBC and ^{1}H–^{1}H COSY experiments (Fig. 2). The attached position of the remaining sugar moiety at C-4 was identified by HMBC cross peak from the anomeric proton (δ 4.82, d, J=7.5 Hz) to carbon C-4 (δ 134.79). The configuration of the sugar unit was determined as reported for compound **4**. Thus, the structure 5,5′-dimethoxylariciresinol 4-*O*-β-D-glucopyranoside was assigned to compound **5**, which was named manglieside E.

Compounds **6** and **7** were identified as 3-methoxymagnolol[3] and obovatol,[17] respectively by comparison of the ^{1}H-, ^{13}C-NMR and mass spectral data with those reported in the literature.

In the present study, we attempted to evaluate effects of compounds on the function of osteoblasts. Osteogenic MC3T3-E1 cells are able to differentiate into osteoblasts that express strong alkaline phosphatase (ALP) activity and to form a collagenous matrix organized in 3-dimensional nodules, which progressively become mineralized.[18] During the proliferative phase, these cells undergo DNA synthesis and

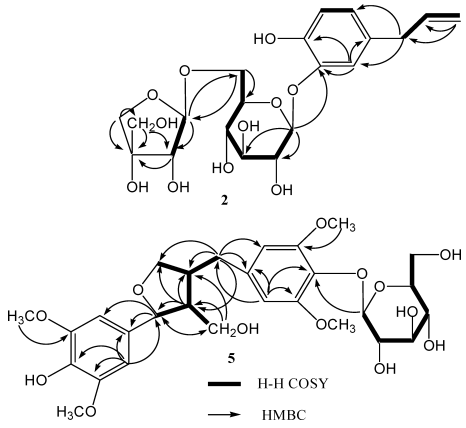

Fig. 2. Selected HMBC and COSY Correlations of **2** and **5**

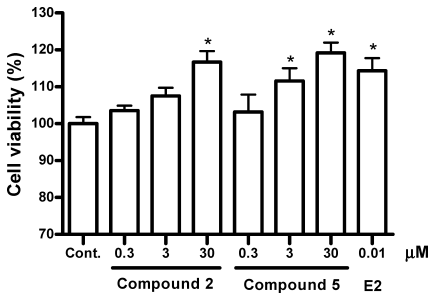

Fig. 3. Effects of Compounds **2** and **5** on the Viability of MC3T3-E1 Cells

E2: 17β-estradiol. Data are expressed as a percentage of the control. The control value was 0.4387±0.0077 OD. $*p<0.05$ *vs.* control.

cell division resulting in a rapid increase in cell number until the cultures become confluent. At this juncture, proliferation is down-regulated and increased expression of the osteogenic phenotype is observed indicating the presence of mature osteoblasts. It is known that osteoblasts produce ALP and type I collagen, which are associated with matrix maturation and mineralization.[19] Collagen expression is considered an early differentiation marker of the osteoblast phenotype.[20] Therefore, survival, ALP activity, collagen synthesis, and mineralization were examined to investigate the effects of compounds on the function of osteoblastic MC3T3-E1 cells. MC3T3-E1 cells were incubated with compounds (0.3—30 μM) and cell growth was measured (Fig. 3). MC3T3-E1 cell growth was elevated significantly by the presence of compounds **2** and **5** at concentrations of 30 μM. The effect of compounds on osteoblast differentiation was assessed by measuring the ALP activity, collagen content, and mineralization. Compounds **2** and **5** significantly increased the ALP activity at concentrations of 0.3—30 μM (Fig. 4). Moreover, compounds **2** and **5** significantly increased collagen synthesis at concentrations of 0.3 and 3 μM (Fig. 5). Next, we examined the effects of compounds on mineralization, another important process in differentiation, by measuring the calcium deposition by Alizarin Red staining. Compounds **2** and **5** showed significant stimulatory effect on mineralization at the concentration of 0.3 μM (Fig. 6). In bone formation, osteoblasts are key cell in bone matrix formation and calcification. Osteogenesis starts with osteoblasts producing and secreting type I collagen, which makes about 90% of the organic bone matrix, or the osteoid. Osteoblasts also become

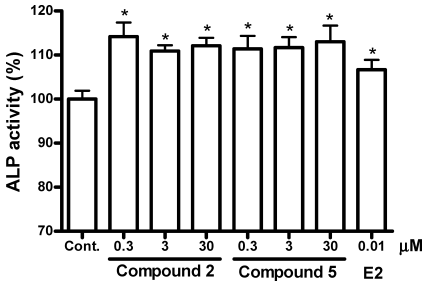

Fig. 4. Effects of Compounds **2** and **5** on the Alkaline Phosphatase Activity of MC3T3-E1 Cells

E2: 17β-estradiol. Data are expressed as a percentage of the control. The control value was 0.977±0.018 Unit/10^6 cells. $*p<0.05$ *vs.* control.

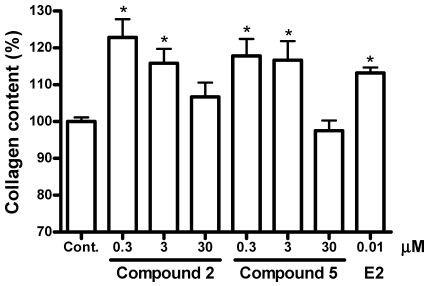

Fig. 5. Effects of Compounds **2** and **5** on the Collagen Content of MC3T3-E1 Cells

E2: 17β-estradiol. Data are expressed as a percentage of the control. The control value was 14.54±0.166 μg. $*p<0.05$ *vs.* control.

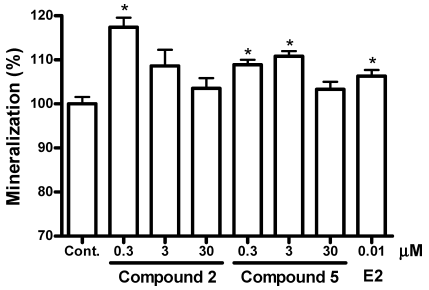

Fig. 6. Effects of Compounds **2** and **5** on the Mineralization of MC3T3-E1 Cells

E2: 17β-estradiol. Data are expressed as a percentage of the control. The control value was 0.391±0.006 OD. $*p<0.05$ *vs.* control.

high in alkaline phosphatase, a phosphate-splitting enzyme. Alkaline phosphatase is released into the osteoid to initiate the deposit of minerals. After mineralization, the complete bone becomes hard and rigid with necessary mechanical properties to withstand the external forces to support the body and protect the internal organs.[19] Our results demonstrate that compounds **2** and **5** significantly increase the proliferation and differentiation of osteoblastic MC3T3-E1 cells, indicating their anabolic effect.

Osteoporosis associated with estrogen deficiency is the most common cause of age-related bone loss. Hormone replacement therapy (HRT) can resolve most postmenopausal problems. However, compliance with HRT is poor because of its associated risks of breast and endometrial cancers with long-term use.[21] In the search for an alternative treatment, the potential health benefits of phytoestrogens have been suggested. There is considerable evidence indicating that phytoestrogens, like certain selective estrogen receptor modulators, have estrogenic action on bone and the cardiovascular system, and have antiestrogenic action on the breast and uterus.[22] In the present study, we show that a stimulation of proliferation and differentiation by compounds **2** and **5** occurs in osteoblasts, accompanied by increases in cell growth, ALP activity, collagen content, and calcium deposition. These results suggest that mangliesides B and E newly isolated from the leaves of *Manglietia phuthoensis* may modulate bone disorders in post-menopausal women.

Table 2. NMR Data of **4** and **5**

C	**4**		**5**	
	δ_C [a),b)]	δ_H [a),c)] [mult. (*J* in Hz)]	δ_C [a),b)]	δ_H [a),b)] [mult. (*J* in Hz)]
1	133.48	—	139.10	—
2	114.24	6.76 br s	107.82	6.60 br s
3	148.55	—	154.24	—
4	115.84	6.67 br s	136.04	—
5	145.54	—	154.24	—
6	122.81	6.68 br s	107.82	6.60 br s
7	38.21	2.86 dd (10.0, 5.0)	34.40	3.01 dd (13.0, 5.0)
		2.97 dd (10.0, 3.0)		2.58 dd (13.0, 11.5)
8	43.54	3.82 m	43.66	2.71 m
9	65.87	3.65 dd (10.5, 5.5)	73.50	4.05 m
		3.76 dd (10.5, 3.0)		3.77 t (7.0)
1′	134.78	—	135.03	—
2′	109.67	6.97 d (2.0)	104.37	6.65 br s
3′	153.20	—	149.29	—
4′	144.75	—	134.79	—
5′	138.59	—	149.29	—
6′	120.23	6.91 d (2.0)	104.37	6.65 br s
7′	131.50	6.56 d (16.0)	84.17	4.78 d (6.0)
8′	129.41	6.29 m	54.13	2.40 m
9′	63.67	4.23 d (6.0)	60.54	3.70[e)]
		4.22 d (6.0)		3.87[e)]
3-OMe	56.48	3.78 s	57.07[d)]	3.86 s
5-OMe	—	—	57.07[d)]	3.86 s
3′-OMe	56.58	3.88 s	56.82[d)]	3.86 s
5′-OMe	—	—	56.82[d)]	3.86 s
	Glc		Glc	
1″	104.55	5.01 d (7.5)	105.62	4.82 d (7.5)
2″	76.09	3.52 dd (8.0, 7.5)	75.77	3.50 dd (8.0, 7.5)
3″	77.92	3.46 dd (8.0, 8.0)	77.83	3.45 dd (8.0, 8.0)
4″	71.85	3.36 dd (8.0, 8.0)	71.38	3.44 dd (8.0, 8.0)
5″	78.33	3.24 m	78.33	3.24 m
6″	62.81	3.82 dd (10.0, 3.0)	62.64	3.82[e)]
		3.66 dd (10.0, 5.0)		3.72[e)]

a) Measured in CD_3OD, *b*) at 125 MHz, *c*) at 500 MHz, *d*) Values are interchangeable, *e*) Overlapped signals.

Experimental

General Melting points were determined using an Electrothermal IA-9200 and were uncorrected. The IR spectra were obtained from a Hitachi 270-30 type spectrometer with KBr discs. Optical rotations were determined on a Jasco DIP-1000 KUY polarimeter. The electrospray ionization (ESI) mass spectra were obtained using an AGILENT 1100 LC-MSD Trap spectrometer. The HR-ESI-MS spectra were obtained using a Waters Q/TOF premier equipped with an electrospray ion source. The ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a Bruker AM500 FT-NMR spectrometer and TMS was used as an internal standard. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70—230 mesh and 230—400 mesh, Merck) and YMC RP-18 resins.

Plant Material The leaves from *M. phuthoensis* were collected at Tamdao National Botanical Park, Vinhphuc province, Vietnam during December 2006 and identified by Dr. Tran Huy Thai, Institute of Ecology and Biological Resources, VAST, Vietnam. A voucher specimen (VN20061712) was deposited at the herbarium of the Institute of Natural Products Chemistry,

VAST, Vietnam.

Extraction and Isolation Air-dried leaves of *M. phuthoensis* (5.0 kg) were powdered and extracted three times with hot MeOH to give the methanol extract (120.0 g), which was then suspended in water and extracted with chloroform. The chloroform fraction (45.0 g) was chromatographed on a silica gel column, using $CHCl_3$–MeOH gradient (from 100 : 0 to 0 : 100) as eluent, to yield four fractions (Fr. A—D). Fraction B (15.0 g) was chromatographed on a silica gel column using *n*-hexane–ethyl acetate (from 100 : 0 to 0 : 100) to give four subfractions [Fr. B1 (2.0 g), Fr. B2 (4.0 g), Fr. B3 (3.0 g), Fr. B4 (5.8 g)]. Subfraction B2 (4.0 g) was chromatographed on a silica gel column using *n*-hexane–acetone (80 : 1) as eluent to yield **6** (75.0 mg) and **7** (37.0 mg) as white crystals. The residue of the water fraction was adsorbed on highly porous polymer resin (Dianion HP-20, Mitsubishi Chem. Ind. Co. Ltd., Tokyo, Japan) and eluted with water containing increasing concentrations of MeOH [100% H_2O (2 l), 25% MeOH (2 l), 50% MeOH (2 l), 75% MeOH (2 l), and 100% MeOH (2 l)]. The 100% MeOH fraction (12.0 g) was chromatographed on a silica gel column using $CHCl_3$–MeOH–H_2O (80 : 20 : 2) as eluent to give five subfractions [SF. A (2.1 g), SF. B (3.5 g), SF. C (3.0 g), SF. D (1.5 g), SF. E (1.4 g)]. The subfraction SF. B (3.5 g) was repeatedly chromatographed on a YMC RP-18 column using a MeOH–H_2O (8 : 2) system to yield **1** (32.0 mg) and **3** (15.5 mg) as white amorphous powders. The 75% MeOH fraction (17.0 g) was chromatographed on a silica gel column using $CHCl_3$–MeOH–H_2O (70 : 30 : 4) as eluent to give five subfractions (SF. F—J: 1.7 g, 5.1 g, 4.3 g, 2.5 g, 2.5 g, respectively). The subfraction SF. G (5.1 g) was repeated chromatographed on a YMC RP-18 column using a MeOH–H_2O (7 : 3) as eluent to yield **2** (47.0 mg), **4** (7.0 mg), and **5** (20.0 mg) as white amorphous powders.

Manglieside A (**1**): White amorphous powder, $[\alpha]_D^{25}$ −63° (c=1.0, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3437 (OH), 2985 (CH), and 1056 (C–O–C); positive ESI-MS *m/z*: 335.1 $[M+Na]^+$; negative ESI-MS *m/z*: 311.1 $[M-H]^-$; HR-ESI-MS *m/z*: 335.1068 $[M+Na]^+$ (Calcd 335.1107 for $C_{15}H_{20}O_7Na$); ^{1}H- and ^{13}C-NMR are given in Table 1.

Manglieside B (**2**): White amorphous powder, $[\alpha]_D^{25}$ −85° (c=1.0, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3442 (OH), 2984 (CH), and 1052 (C–O–C); positive ESI-MS *m/z*: 445.1 $[M+H]^+$, 467.1 $[M+Na]^+$; negative ESI-MS *m/z*: 443.2 $[M-H]^-$; HR-ESI-MS *m/z*: 467.1396 (Calcd 467.1365 for $C_{20}H_{28}O_{11}Na$); ^{1}H- and ^{13}C-NMR are given in Table 1.

Manglieside C (**3**): White amorphous powder, $[\alpha]_D^{25}$ −55° (c=1.0, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3450 (OH), 2985 (CH), and 1046 (C–O–C); positive ESI-MS *m/z*: 397.4 $[M+Na]^+$; HR-ESI-MS *m/z*: 375.4785 (Calcd 375.4827 for $C_{19}H_{35}O_7$); ^{1}H- and ^{13}C-NMR are given in Table 1.

Manglieside D (**4**): White amorphous powder, $[\alpha]_D^{25}$ −125° (c=1.0, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3424 (OH), 2986 (CH), and 1025 (C–O–C); positive ESI-MS *m/z*: 545.2 $[M+Na]^+$; HR-ESI-MS *m/z*: 523.2147 (Calcd 523.2180 for $C_{26}H_{35}O_{11}$); ^{1}H- and ^{13}C-NMR are given in Table 2.

Manglieside E (**5**): White amorphous powder, $[\alpha]_D^{25}$ −92° (c=1.0, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3445 (OH), 2988 (CH), and 1017 (C–O–C); positive ESI-MS *m/z*: 605.2 $[M+Na]^+$; negative ESI-MS *m/z*: 581.2 $[M-H]^-$; HR-ESI-MS *m/z*: 605.2183 $[M+Na]^+$ (Calcd 605.2210 for $C_{28}H_{38}O_{13}Na$); ^{1}H- and ^{13}C-NMR are given in Table 2.

Acid Hydrolysis of 1—5 A solution of each compound (**1**—**5**, 2.0 mg each) in 1 N HCl in methanol (1 ml) was stirred at 80 °C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. Each residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 ml), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between water and chloroform. The chloroform layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm×25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected by comparison with the retention times of authentic samples of D-glucopyranose, D-allopyranose and D-apiofuranose (Merck) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

Cell Culture Osteoblastic MC3T3-E1 cells were cultured in plastic dishes containing alpha-minimum essential medium (α-MEM, GibcoBRL, Grand Island, NY, U.S.A.) plus 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, U.S.A.) in a CO_2 incubator (5% CO_2 in air) at 37 °C and subcultured every 3 d at a dilution of 1 : 5 using 0.05% trypsin–0.02% EDTA in Ca^{2+}, Mg^{2+} free phosphate buffered saline (DPBS). Compounds were dissolved in dimethylsulfoxide (DMSO) and diluted with serum-free media containing 0.3% bovine serum albumin (BSA, Sigma, St. Louis, MO, U.S.A.) (final DMSO concentration=0.01%).

Cell Viability Cells were suspended in medium supplemented with 10% FBS, and cell suspension containing 5×10^3 cells was added to the individual wells of 48-well microplates. The plates were incubated at 37 °C in a CO_2 incubator for 48 h. After discarding the culture medium and washing the cells with DPBS, cells were transferred to serum-free medium containing 0.3% BSA and compounds, and the incubation was continued for a further 48 h. Control cells were cultured with serum-free medium containing 0.3% BSA and 0.01% DMSO. Surviving cells were counted by the MTT method. MTT 20 μl in 7.2 mM phosphate buffer solution, pH 6.5 (5 mg/ml), was added to each well, and the plates were incubated for an additional 2 h. After the removal of solutions in the well, DMSO was added to dissolve formazan products, and the plates were shaken for 5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

Alkaline Phosphatase Activity The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid, to initiate differentiation. The medium was changed every 2—3 d. After 8 d, the cells were cultured with medium containing 0.3% BSA and samples individually for 2 d. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. The cells were lysed with 0.2% Triton X-100, with the lysate centrifuged at 14000×g for 5 min. The clear supernatant was used to measure the ALP activity, which was determined using an ALP activity assay kit (Asan Co., Korea).

Collagen Content The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. The medium was changed every 2—3 d. After 8 d, the cells were cultured with medium containing 0.3% BSA and samples individually for 2 d. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. Collagen content was quantified by Sirius Red-based colorimetric assay. Cultured osteoblasts were washed with PBS, followed by fixation with Bouin's fluid for 1 h. After fixation, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained by Sirius Red dye reagent for 1 h under mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove non-bound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm.

Calcium Deposition Assay The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. After 12 d, the cells were cultured with medium containing 0.3% BSA and samples individually for 2 d. On harvesting, the cells were fixed with 70% ethanol for 1 h, and then stained with 40 mM Alizarin Red S for 10 min with gentle shaking. To quantify the bound dye, the stain was solubilized with 10% cetylpyridinum chloride by shaking for 15 min. The absorbance of the solubilized stain was measured at 561 nm.

Statistics The results are expressed as the mean±S.E.M. (n=5). Statistical analysis was performed using a one-way ANOVA ($p<0.05$) with the SAS statistical software.

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References

1) Yang Q., Populo S. M., Zhang J., Yang G., Kodama H., *Clin. Chim. Acta*, **324**, 89—97 (2002).
2) Huang H. F., You J. S., *Am. J. Clin. Med.*, **25**, 351—356 (1997).
3) Wantanabe K., Wantanabe H., Goto Y., Yamaguchi M., Yamamoto N., Hagino K., *Planta Med.*, **49**, 103—108 (1983).
4) Kim Y. K., Ryu S. Y., *Planta Med.*, **65**, 291—292 (1999).
5) Kijjoa A., Pinto M. M. M., Tantisewie B., Herz W., *Phytochemistry*, **28**, 1284—1286 (1989).
6) Ho P. H., "An Illustrated Flora of Vietnam," Tre Publishing House, Ho Chi Minh City, Vietnam, 1999.
7) Sudo H., Kodama H., Amagi Y., Yamamoto S., Kasai S., *J. Cell Biol.*, **96**, 191—198 (1983).
8) Toyota M., Saito T., Asakawa Y., *Phytochemistry*, **43**, 1087—1088 (1996).
9) Agrawal P. K., *Phytochemistry*, **31**, 3307—3330 (1992).
10) Yamamura S., Ozawa K., Ohtani K., Kasai R., Yamasaki K., *Phytochemistry*, **48**, 131—136 (1998).
11) Abe F., Chen R. F., Yamauchi T., *Phytochemistry*, **43**, 161—163 (1996).

12) Jiang J. S., Feng Z. M., Wang Y. H., Zhang P. C., *Chem. Pharm. Bull.*, **53**, 110—113 (2005).
13) Otsuka H., *Phytochemistry*, **37**, 461—465 (1994).
14) Miyase T., Ueno A., Takizawa N., Kobayashi H., Oguchi H., *Phytochemistry*, **28**, 3483—3485 (1989).
15) Iorizzi M., Lanzotti V., Marino S. D., Zollo F., Magdalena B. M., Antonio M., Eduardo M., *J. Agric. Food Chem.*, **49**, 2022—2029 (2001).
16) Kamel M. S., Ohtani K., Assaf M. H., Kasai R., El-Shanawani M. A., Yamasaki K., Ali A. A., Tanaka O., *Phytochemistry*, **31**, 2469—2471 (1992).
17) Kwon B. M., Kim M. K., Lee S. H., Kim J. A., Lee I. R., Kim Y. K., Bok S. H., *Planta Med.*, **63**, 550—551 (1997).
18) Wang D., Christensen K., Chawla K., *J. Bone Miner. Res.*, **14**, 893—903 (1999).
19) Stein G. S., Lian J. B., Owen T. A., *FASEB J.*, **4**, 111—123 (1990).
20) Stein G. S., Lian J. B., *Endocr. Rev.*, **14**, 424—442 (1993).
21) Persson I., Weiderpass E., Bergkvist L., Bergstrom R., Schairer C., *Cancer Causes Control*, **10**, 253—260 (1999).
22) Brzezinski A., Debi A., *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **85**, 47—51 (1999).