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Sesquiterpenoids from *Homalomena occulta* affect osteoblast proliferation, differentiation and mineralization *in vitro*

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ABSTRACT

Chemical investigation of rhizomes of *Homalomena occulta* (Lours) resulted in isolation and identification of two sesquiterpenoids (**6**,**7**), and one daucane ester **8**, together with five known sesquiterpenoids, oplodiol, oplopanone, homalomenol C, bullatantriol, and 1β,4β,7α-trihydroxyeudesmane. Their structures were elucidated using 1D and 2D NMR spectroscopic and X-ray analyses. The chloroform extract of this plant and compounds **1**–**7** were tested *in vitro* for their activities in stimulating osteoblast (OB) proliferation, differentiation and mineralization. Compounds **1**–**4** had a stimulative effect on significantly proliferation and differentiation of culture osteoblasts, while the chloroform extract and **1** significantly stimulated mineralization of cultured osteoblasts *in vitro*.

1. Introduction

In recent years, traditional Chinese medicinal plants have become important targets for exploration of potential therapeutic agents. As traditional Chinese medicine (TCM) represents an enormous reservoir of plants of tremendous diversity, various strategies have been applied to select TCMs for phytochemical and/or pharmacological analyses. Those based on therapeutic theories and botanical classifications have been the most widely adopted. Postmenopausal osteoporosis in women has for long been an issue of concern in health care sectors. Insufficient production of the hormone estrogen has been implicated as one of the most important causes of this health disorder (Brixen et al., 2004; Erdtsieck et al., 1995). Although advances in drug discoveries, especially of the large pharmaceutical companies have led to delivery of numerous therapeutic agents to markets, most are synthetic; there have also been safety concerns about the long term use of such medicines. In line with the primary focus to develop effective natural therapeutic agents for the treatment of osteoporosis, a TCM, *Homalomena occulta* (Lours) was chosen as a starting material in this study based on its well-known medical functions in China (Zhang and Xie, 2005). For example, this includes invigoration of the kidney and liver, strengthening of the muscles and bones, and treatment of rheumatoid arthritis. Further support for the choice of this medicinal herb arise from previous pharmacological reports which suggested that plants with the above-mentioned medical functions likely contain some estrogen-like bioactive phytochemicals (Guo et al., 1998; Li et al., 1998). This herb has only been examined for the anti-inflammatory and antibiotic activities of its essential oils (Chen et al., 1986; Zhou et al., 1991). To the best of our knowledge, there has been no prior report as to its effects on osteoblasts *in vitro*. In the present study, *H. occulta* was chromatographically fractionated using a bioactivity-guided approach that was based on the ability of the fractions to regulate cellular processes, such as proliferation, differentiation and mineralization of cultured osteoblasts. The final purified compounds, together with some crude extracts, were also assayed for their effects on these processes in order to verify their important roles in mediating the probable pharmacological functions.

2. Results and discussion

2.1. Isolation and identification of the constituents from *H. occulta*

Phytochemical analysis of the chloroform extract of *H. occulta* was conducted in this study. Following fractionation by silica gel

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chromatography, the effects of individual fractions on osteoblasts *in vitro* were measured. Two new sesquiterpenoids and one new daucane ester, together with other five known components, were isolated from the rhizomes of *H. occulta*. The structures of the purified compounds (see Fig. 1) were identified by direct comparisons of their melting points and spectroscopic data (^{1}H NMR and ^{13}C NMR) with literature data (Sung et al., 1992a,b). These include oplodiol (**1**), oplopanone (**2**), homalomenol C (**3**), bullatantriol (**4**), 1β,4β,7α-trihydroxyeudesmane (**5**), homalomenol **E** (**6**), (−)1β,4β,6α-trihydroxy-eudesmane (**7**) and homalomenol **F** (**8**). Compounds **6**–**8** have not been reported previously.

2.2. Structural identification of new compounds *6*–*8*

Compound **6**, m.p. 176–178 °C, was isolated as colorless needles. The features of the IR spectra at ν_{max} 3372 and 3319 cm^{-1} and the molecular and fragment ion peaks of the EIMS at *m*/*z* 256 $[M]^+$, 238 $[M-H_2O]^+$ and 202 $[M-3H_2O]^+$ suggested the presence of three hydroxyl groups. Analysis of **6** by HRESIMS gave a molecular ion peak at *m*/*z* 255.2234 $[M-1]^-$ (calculated 255.2158), indicating a molecular formula of $C_{15}H_{28}O_3$. The complete structural elucidation of this compound was based on 1D and 2D NMR spectroscopic (Table 1) analyses. From the methyl region of the ^{1}H NMR spectrum of compound **6**, the two singlet chemical shifts of three protons at δ 1.06 and 0.91 were assigned to the two tertiary methyl groups. The characteristic resonances of the two secondary methyl groups appeared at δ 0.84 (3H, *d*, *J* = 7.0 Hz) and 0.68 (3H, *d*, *J* = 6.9 Hz). The methine protons attached to oxygen-bearing carbons displayed a doublet peak at δ 3.90 (1H, *d*, *J* = 6 Hz). Assignments of 15 carbon signals including three quaternary carbons with two oxygenated carbons at δ 76.0, 75.3, three tertiary carbons with one oxygenated carbon at δ

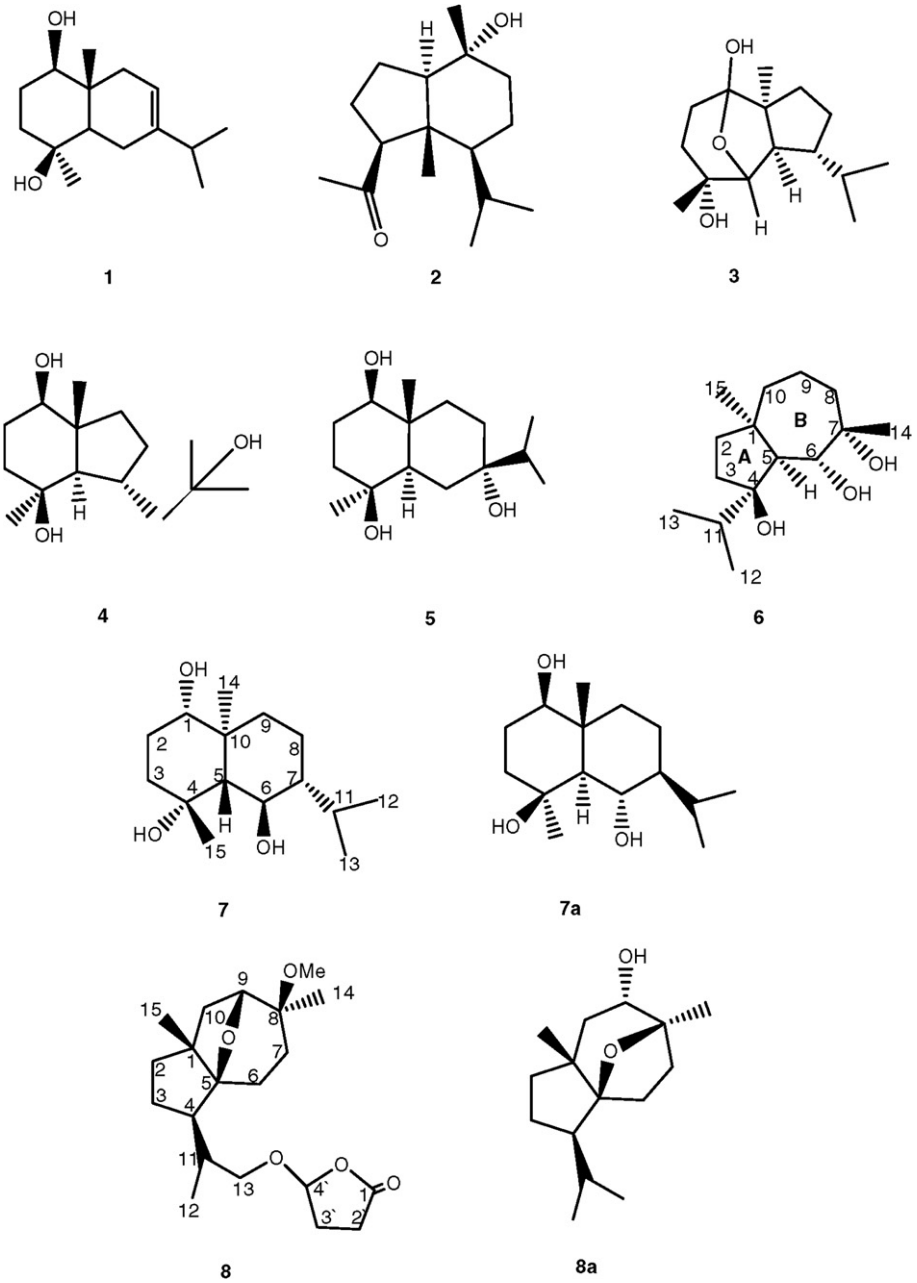

Fig. 1. Structures of compounds **1**–**8a**.

Table 1
NMR spectroscopic data of compound **6** (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR, DMSO-d_6, TMS as standard)

Atom number	^{13}C (δ)	^{1}H (δ, mult, J [Hz])	HMBC (atom number of ^{13}C)
1	48.2		
2	37.8	1.33, *m*	C-1, 4, 5, 10, 15
		1.43, *m*	
3	26.7	1.21, *m*	C-1, 5, 15
		1.45, *m*	
4	76.0		
5	47.2	1.32, *d* (6)	C-1, 4, 6, 7, 10, 11, 15
6	76.4	3.90, *d* (6)	C-1, 4, 7, 14
7	75.3		
8	33.5	1.34, *m*	C-6, 9
		1.57, *m*	
9	29.7	1.42, *m*	C-1, 7, 8
		1.54, *m*	
10	43.1	1.26, *m*	C-2, 5, 15
		1.42, *m*	
11	33.9	1.97, *m*	C-3, 4, 5, 12, 13
12	20.3	0.68, *d* (6.9)	C-4, 11, 13
13	26.4	0.84, *d* (7.0)	C-4, 11, 12
14	25.8	1.06, *s*	C-6, 8
15	25.4	0.91, *s*	C-1, 2, 5, 10

76.4, five secondary and four primary carbon atoms were generally established by interpretation of the ^{13}C NMR and HSQC spectra. Signals from several spin coupling units, namely –C–CH_2–CH_2–CH_2–C–, –CH–CH(OH)–C– and –C–CH_2–CH_2–C–, were used for assignment of proton signals in the ^{1}H–^{1}H COSY and HSQC spectra. Inter-residue linkages and positions of the attachments of the carbohydrate units were at last established by the long-range correlation experiment, HMBC (Table 1).

The relative stereochemistry of **6** was determined using NOESY spectra (Fig. 2) as well as the coupling constant. The stereo-correlation of the proton H-5 (δ 1.32) with H-15 (δ 0.91) was assigned to a *cis*-configuration for ring A/B. The resonance of H-6 (δ 3.90) was correlated to the proton signal H-14 (δ 1.06), implicating that the hydroxyl groups at C-6 and C-7 possessed the *cis*-form. With reference to the literature (Tchuendem et al., 1999), H-5 and H-15 were assigned as being in the α-form. Consequently, the hydroxyl group at C-4 was assumed to have a β-form and the hydroxyl groups at C-6 and C-7 were assigned to an α-form based on the observed coupling constant of $J_{5,6}$ = 6 Hz. Thus, **6** was 4,6,7-trihydroxy-isodaucane (1α,4β-OH,5α,6α,7α) (Fig. 1) and named homalomenol **E**.

Compound **7**, m.p. 186–188 °C, was isolated as colorless needles. The absorption peaks of IR spectrum at ν_{max} 3410 and 3321 cm^{-1} and the three fragment ion peaks in the EIMS at *m*/*z* 238 [M−H_2O]$^+$, 220 [M−3H_2O]$^+$ and 202 [M−3H_2O]$^+$ were again indicative of three hydroxyl groups. Application of high-resolution ESI-MS (found *m*/*z* 255.1933 [M−H]$^-$, calculated 255.1822) confirmed a structural composition of $C_{15}H_{28}O_3$. The chemical shifts at δ 0.97 (3H, *s*) and 1.42 (3H, *s*) in the ^{1}H NMR spectrum featured the presence of two primary methyl groups. The proton signals at δ 0.93 (3H, *d*, *J* = 6.9 Hz) and 0.87 (3H, *d*, *J* = 6.9 Hz) indicated the presence of an isopropyl group. Two methine protons at δ 3.13 (1H, *dd*, *J* = 4.0 and 11.8 Hz) and 3.85 (1H, *t*, *J* = 10 Hz) were correlated to the carbon resonances at δ 80.1 (C-1) and 70.0 (C-6), respectively, in the HSQC spectra. Analysis of the ^{13}C NMR and DEPT spectra indicated the presence of two quaternary, five tertiary, four secondary and four primary carbon atoms. A detailed study of the ^{1}H–^{1}H COSY spectrum in conjunction with the HSQC spectrum established the presence of several spin coupling units, namely –CH(OH)–CH_2–CH_2–, –C–CH–CH(OH)–CH(CH_3)$_2$ and –C–CH_2–CH_2–. The HMBC spectrum interpreted some main coupling peaks of proton H-1 (δ 3.14) with C-2, C-5, C-10 and C-14, and H-5 (δ 1.03) with C-4, C-6, C-10 and C-14, and H-15 with C-3, C-4 and C-5 (Table 2). Based on above spectroscopic analysis, the skeleton of **7** was designed to be 1,4,6-trihydroxy-eudesmane.

The relative configuration of **7** was proposed based on the NOESY spectrum (Fig. 3) as well as the coupling constant. Stereo-correlations of H-1 to H-5, H-7 and H-15, and H-6 to H-11, H-12 and H-14 were elucidated from the NOESY experiment. The equatorial position of the hydroxyl group at C-1 was supported by the coupling constant of $J_{1,(2a,2b)}$ = 4 and 11.8 Hz in the ^{1}H NMR spectrum (Nyasse et al., 1988). Another coupling constant (*J* = 10 Hz) that was attributed to H-6 implicated a *trans*-diaxial spin–spin interaction between H-6 and H-7 protons. Assuming a chair–chair conformation as frequently found for eudesmanolides with a β-oriented axial angular methyl group, and considering the equatorial arrangement of the three hydroxyl groups at C-1, C-4 and C-6, it is appropriate to propose the compound's structure as **7a** (Fig. 2) (Izbosarov et al., 1998). Comparison of the spectroscopic data between compounds **7** and **7a** showed almost identical NMR

Table 2
NMR spectroscopic data of compound **7** (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR, CD_3OD, TMS as standard)

Atom number	^{13}C (δ)	^{1}H (δ, mult, *J* [Hz])	HMBC (atom number of ^{13}C)
1	80.1 *d*	3.13, *dd* (4, 11.8)	C-2, 5, 10, 14
2	27.6 *t*	1.50, 1.93, *m*	C-1, 3, 4, 10
3	42.4 *t*	1.61, 1.66, *m*	C-2, 4, 5, 15
4	72.8 *s*		
5	57.8 *d*	1.03, *d* (11)	C-1, 4, 6, 10, 14, 15
6	70.0 *t*	3.85, *t* (10)	C-5, 7, 10
7	53.0 *s*	1.27, *m*	
8	19.4 *d*	1.30, 1.47, *m*	
9	39.2 *t*	0.96[a], 1.86, *m*	C-8, 10, 14
10	42.1 *s*		
11	27.0 *d*	2.31, *m*	C-6, 7, 12, 13
12	21.7 *q*	0.93, *d* (6.9)	C-7, 11, 13
13	16.1 *q*	0.87, *d* (6.9)	C-7, 11, 12
14	13.9 *q*	0.97, *s*	C-1, 5, 10
15	34.5 *q*	1.42, *s*	C-3, 4, 5

[a] Obscured by methyl peaks.

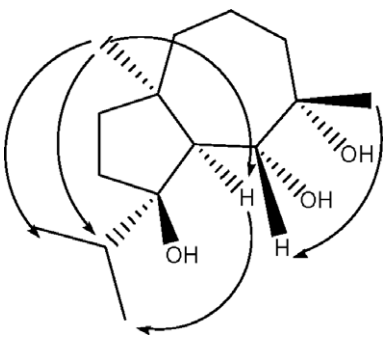

Fig. 2. Pertinent NOESY correlations observed with **6**.

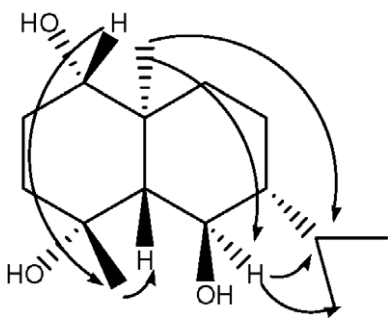

Fig. 3. Pertinent NOESY correlations observed with **7**.

spectroscopic data (Menezes et al., 2004), indicating the same molecular skeleton of the two compounds.

Further confirmation of the configuration of **7** was completed by an X-ray crystallographic analysis. The X-ray structural elucidation showed that **7** possessed 1α,4α,6β-trihydroxyl and 7α-isopropyl group (Fig. 4). The opposite optical rotation of **7** ($[\alpha]_D^25$ −39° (MeOH; *c* 0.05)) to that of **7a** ($[\alpha]_D^25$ +42° (MeOH; *c* 0.05)) indicated that **7** is an enantiomer of **7a**, Thus, compound **7** was elucidated as (−)1β,4β,6α-trihydroxy-eudesmane.

Compound **8**, m.p. 156–158 °C, was purified as colorless needles. The IR spectrum displayed large absorption bands at 1767 cm^{-1} (saturated lactone), 1117 and 1041 cm^{-1} (C–O). The ESI-MS established fragment ion peaks at *m*/*z* 353 $[M+H]^+$, 321 $[M-OMe]^+$, 336 $[M-O]^+$ and 102 $[C_4H_5O_3+H]^+$. The molecular formula, $C_{20}H_{32}O_7$ of compound **8** was determined based on positive HRESIMS at *m*/*z* 353.2256 for the $[M+H]^+$ ion (calculated for 353.2301) and NMR analyses. The ^1H and ^{13}C NMR spectra (Table 3) of the basic skeleton in **8** were similar to those of daucol (**8a**) (Mossa et al., 1992), except for additional proton signals at δ 4.92 (1H, *dd*, *J* = 3.5 and 6.0 Hz, H-4′), 2.03 (2H, *m*, H-2′), 1.88, 1.99 (2H, *m*, H-3′) and 3.23 (3H, *s*), and carbon resonances at δ 180, 104, 74, 54.6, 28.2 and 22.8, which suggest a five-carbon unit and a methoxy unit were attached to the *cis*-daucane skeleton of **8a**. Moreover, the signals of two geminal protons (δ 3.71 (1H, *dd*, *J* = 2.5 and 8.0 Hz, H-13a) and 3.57 (1H, *dd*, *J* = 2.5 and 7.9 Hz, H-13b)) instead of the methyl protons in the isopropyl group of **8a** implicated one methyl carbon being oxygenated in **8**. The daucane skeleton of **8** was unambiguously determined using the combinations of ^1H–^1H COSY, HMQC and HMBC spectroscopic data (Table 3). The ^1H–^1H COSY spectrum established spin coupling units of **8**, which involved –CH_2–CH_2–CH–CH(CH_2–O)CH_3, –CH_2–CH_2–, –CH_2–CH–O– and –CH_2–CH_2–CH–O–. The proton signal H-9 (δ 4.75) showed correlations with C-1, C-5, C-7, C-8 and C-14 (Fig. 5, Supplementary supporting information), implicating formation of an one five-membered tetrahydro-furan ring by the epoxy bridge between C-9 and C-5. Moreover, signals from the anomeric proton (δ 4.92) and the carbon (δ 104.0) were used as a starting point for the interpretation of the five-membered γ-lactone unit of **8** in the HMQC and HMBC spectra (Table 3). Correlations of H-4′ to C-1′ and C-2′ in the HMBC spectrum indicated formation of a γ-lactone via ring closure which involved an oxygen atom bridged to C-1′ and C-4′. The linkage sites of the five-membered γ-lactone unit and methoxy unit were deduced from an HMBC experiment. Correlations were observed between H-4′ (δ 4.92) and C-13, H-13 and C-4′; these indicated a junction between the γ-lactone unit and a daucane skeleton via the C-13-oxygen atom. Correlation of the methoxy proton signal δ 3.23 (3H, *s*) to C-8 (δ 88.1) in HMBC spectrum confirmed the methoxyl group at C-8.

Table 3
NMR spectroscopic data of compound **8** (400 MHz for ^1H NMR, 100 MHz for ^{13}C NMR, CD_3OD)

Atom number	^{13}C (δ)	^1H (δ, mult, *J* [Hz])	HMBC (atom number of ^{13}C)
1	45.2		
2	28.8	1.30/1.50, *m*	C-1, 4, 5, 10, 15
3	29.0	1.23/1.44, *m*	C-1, 4, 5, 11
4	47.6	1.78, *m*	C-1, 3, 5, 11, 12, 13
5	89.6		C-1, 4, 7, 11, 15
6	43.5	1.47, *ddd* (4, 10, 12), 1.98, *ddd* (3, 11, 12)	C-1, 5, 8
7	38.7	1.35, *ddd* (4, 10, 12), 1.90, *m*	C-5, 6, 9, 14
8	88.1		
9	75.6	4.75, *dd* (5.5, 10.8)	C-1, 5, 8, 10, 14
10	32.0	1.22, *dd* (5.5, 10.8, 12), 1.54, *m*	C-1, 2, 5, 9, 15
11	31.1	2.23, *ddd* (2.5, 6, 12)	C-4, 5, 12, 13
12	17.6	0.76, *d* (6)	C-4, 11, 13
13	74.7	3.71, *dd* (2.5, 8.0), 3.57, *dd* (2.5, 7.9)	C-4, 11, 12, 4′
14	22.9	1.20, *s*	C-7, 8, 9
15	16.9	0.91, *s*	C-1, 2, 5, 10
1′	180		
2′	28.2	2.03, *m*	C-3′, 4′
3′	22.8	1.99,1.88, *m*	C-2′, 4′
4′	104.0	4.92, *dd* (3.5, 6)	C-2′, 4′
Me	54.6	3.23, *s*	C-8

The stereochemistry of **8** was verified by analysis of its NOESY spectrum (Fig. 6). The correlations of H-15 to H-2β, H-11, H-12, H-13 and the methoxy proton as well as H-9 to H-7α and H-14 were found, supporting a *cis*-daucane form. Using the above information, **8** was elucidated as 8-methoxyl-13-(4′-oxy-butanolide)-5(9)-epoxy-daucane and named as homalomenol **F**.

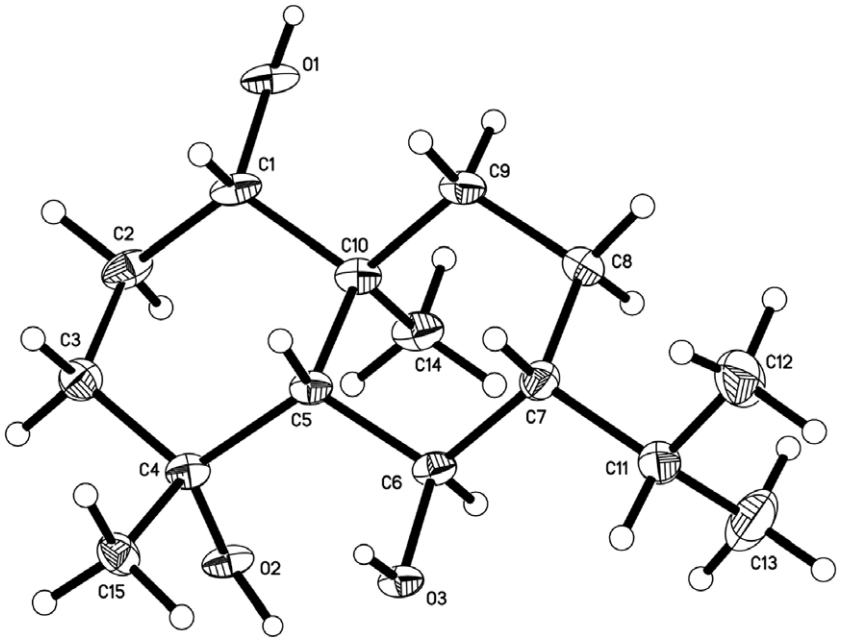

Fig. 4. Stereo view of **7**.

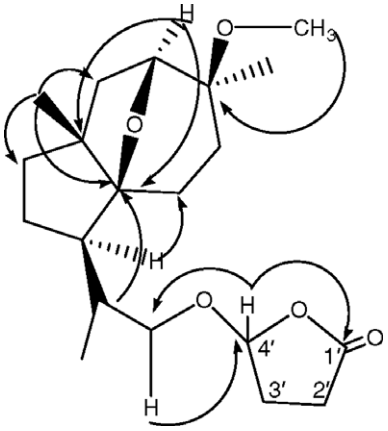

Fig. 5. Pertinent HMBC correlations observed with **8**.

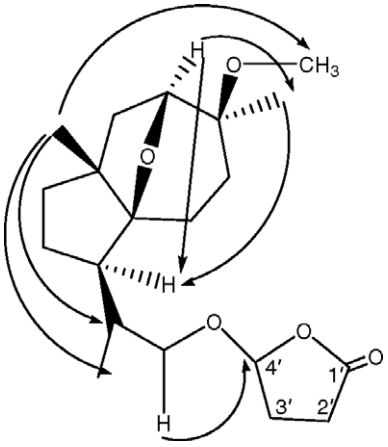

Fig. 6. Pertinent NOESY correlations observed with **8**.

2.3. Bioactivities of crude extracts and isolated constituents in cultured osteoblasts in vitro

Both the chloroform extract and the purified components were examined at micromolar levels for their effects on differentiation, proliferation and mineralization of osteoblasts *in vitro*. Activity of the aqueous extract was also tested to avoid loss of any active components in this fraction.

The effective concentrations of the crude chloroform extract to stimulate proliferation and differentiation of osteoblasts were in the range of 5×10^{-2} to 5×10^{-6} mg/ml (Table 4). In addition, a dose-dependent activity was observed within this concentration range. However, further increase in concentration of the extract to beyond 0.5 mg/ml was found to exhibit an inhibitory effect on cell proliferation and differentiation. This concentration-dependent dual behavior was similar to that observed *in vitro* with sodium fluoride. As shown in Table 5, the chloroform extract (5.0×10^{-4} g/l) was able to significantly increase ($P < 0.01$) the number of mineral nodes in cultured osteoblasts; the aqueous extract only exerted moderate effects on osteoblast mineralization.

An assessment of the effects of the isolated compounds from chloroform extract of *H. occulta* on osteoblast activity may generate useful information about their roles in mediating the pharmacological functions of this herb in traditional medicinal applications. As shown in Table 4, compounds **1**–**3** appeared to stimulate proliferation of osteoblasts in a concentration-dependent manner. Other compounds, except for compound **5** isolated from *H. occulta* exhibited moderate effects on osteoblast proliferation compared with the control group in the concentration range of $\sim 10^{-7}$ to $\sim 10^{-5}$ mol/l. Compounds **1**–**4** showed significant stimulative effects (Table 4) on differentiation (ALP (U/10L)) of osteoblasts, while other compounds exhibited moderate effects at the tested concentrations of $\sim 10^{-7}$ to $\sim 10^{-5}$ mol/l. Effects of the purified compounds on osteoblast mineralization are shown in Table 5. Among them, only compound **1** (1.3×10^{-4} mol/l) was able to significantly stimulate ($P < 0.01$) mineralization of cultured

Table 4
Effect of $CHCl_3$ extract and compounds **1**–**7** on osteoblastic proliferation and differentiation *in vitro* ($n = 6$, $X \pm SD$, *t*-test)

Group	Sample	Concentration	OD_{490nm}	ALP (U/10L)
Blank	EtOH	–	0.77 ± 0.11	19.44 ± 2.23
	DMSO	–	0.76 ± 0.05	20.33 ± 5.60
	Substrate	–	0.70 ± 0.04	19.60 ± 1.36
Compound	**1**	6.09×10^{-5} mol/l	0.82 ± 0.04***	25.97 ± 0.45**
		6.09×10^{-6} mol/l	0.85 ± 0.05***	30.08 ± 5.38***
		6.09×10^{-7} mol/l	0.98 ± 0.09***	33.27 ± 8.86***
	2	6.09×10^{-5} mol/l	0.89 ± 0.12***	31.61 ± 7.37***
		6.09×10^{-6} mol/l	0.86 ± 0.05***	30.12 ± 2.71***
		6.09×10^{-7} mol/l	0.71 ± 0.03	29.93 ± 2.69***
	3	1.17×10^{-5} mol/l	0.75 ± 0.04**	26.40 ± 3.56***
		1.17×10^{-6} mol/l	0.75 ± 0.03**	24.31 ± 7.03*
		1.17×10^{-7} mol/l	0.84 ± 0.06***	20.10 ± 4.98
	4	5.16×10^{-5} mol/l	0.88 ± 0.07***	27.23 ± 4.11***
		5.16×10^{-6} mol/l	0.91 ± 0.13***	24.58 ± 9.45*
		5.16×10^{-7} mol/l	0.88 ± 0.16***	28.72 ± 2.64***
	5	5.16×10^{-5} mol/l	0.90 ± 0.11***	19.73 ± 5.25
		5.16×10^{-6} mol/l	0.90 ± 0.06***	19.87 ± 3.85
		5.16×10^{-7} mol/l	0.89 ± 0.12***	20.17 ± 9.05
	6	5.16×10^{-5} mol/l	0.86 ± 0.05***	22.19 ± 2.99*
		5.16×10^{-6} mol/l	0.85 ± 0.10**	21.72 ± 4.22
		5.16×10^{-7} mol/l	0.81 ± 0.06**	18.41 ± 6.27
	7	5.16×10^{-5} mol/l	0.65 ± 0.21	26.27 ± 8.61**
		5.16×10^{-6} mol/l	0.78 ± 0.16	18.84 ± 8.45
		5.16×10^{-7} mol/l	1.25 ± 0.21***	23.15 ± 16.70
Extracts	E	5.0 mg/ml	0.65 ± 0.21	26.27 ± 8.61**
		0.5 mg/ml	0.78 ± 0.16	18.84 ± 8.45
		0.05 mg/ml	1.25 ± 0.21***	23.15 ± 16.70
		5×10^{-5} mg/ml	0.98 ± 0.16***	24.61 ± 6.15*
		5×10^{-6} mg/ml	0.94 ± 0.22***	22.72 ± 8.54
		5×10^{-7} mg/ml	0.80 ± 0.17*	23.12 ± 7.63
Control	Estrogen	3.63×10^{-6} mol/l	0.76 ± 0.04*	38.81 ± 6.96***
		3.63×10^{-7} mol/l	0.84 ± 0.06***	24.74 ± 2.96*
		3.63×10^{-8} mol/l	0.81 ± 0.07**	31.01 ± 5.31***
	ED	3.06×10^{-5} mol/l	1.09 ± 0.11***	38.81 ± 6.96***
		3.06×10^{-6} mol/l	1.06 ± 0.08***	24.74 ± 2.96*
		3.06×10^{-7} mol/l	1.17 ± 0.10***	31.01 ± 15.31***
		3.06×10^{-8} mol/l	0.87 ± 0.08***	

vs, blank; *$P < 0.05$; **$P < 0.01$ (distinct diffentia); ***$P < 0.001$ (very distinct diffentia); E, the $CHCl_3$ extract; ED, etidronate disodium.

Table 5
Effect of $CHCl_3$ extract and purified compounds on the mineralization of cultured osteoblasts *in vitro* ($X \pm SD$, $n = 3$, *t*-test)

Group	Sample	Concentration	Mineral nodes/bore number
Blank		–	40 ± 18
Compound	**1**	1.3×10^{-4} mol/l	75 ± 20*
	2	2.5×10^{-4} mol/l	50 ± 23
	3	2.5×10^{-4} mol/l	49 ± 10
	4	2.5×10^{-4} mol/l	45 ± 17
	6	2.5×10^{-4} mol/l	39 ± 4
Extracts	E-1	5.0×10^{-4} g/l	59 ± 9
	E-2	5.0×10^{-4} g/l	78 ± 17*
Control	Estrogen	2.5×10^{-5} g/l	21 ± 11
	Etidronate disodium	3.1×10^{-5} mol/l	68 ± 21*
		3.1×10^{-6} mol/l	66 ± 13*

vs, blank; *$P < 0.01$ (distinct diffentia); E-1, the water extract; E-2, the $CHCl_3$ extract.

osteoblasts. Other compounds tested exerted moderate effects compared with the control group.

3. Conclusions

Compounds **1**–**4** isolated from the chloroform extract of *H. occulta* showed a stimulative effect on proliferation and differentiation of culture osteoblasts *in vitro*. The two new sesquiterpenoids, **6**, **7** were identified as 4,6,7-trihydroxy-isodaucane and (−)1β,4β,6α-trihydroxy-eudesmane, respectively. They both stimulated the proliferation of osteoblasts in a dose-dependent manner. On the other hand, their effects on osteoblast differentiation were moderate in the concentration range of $\sim 10^{-7}$ to $\sim 10^{-5}$ mol/l. Although the other new compound **8** did not exhibit obvious effects on osteoblasts, it has an interesting structural feature as it contains butanolide units and the linkage between the daucane skeleton (with a 5(9)-epoxy group) and the butanolide unit has not been reported for sesquiterpenoids to date.

4. Experimental

4.1. General procedures

Melting points were determined on an X-4 melting point apparatus and uncorrected. IR spectra were measured on a Nicolet Impact 410 spectrometer in KBr pellets. Specific optical rotations were measured on a Nicolet Impact-1000 polarimeter with MeOH as solvent. NMR spectra (^{1}H, ^{13}C, DEPT, ^{1}H–^{1}H COSY, NOE, HSQC and HMBC) were obtained on a Bruker Avance 400 spectrometer with chemical shifts reported in δ (ppm) using TMS as an internal standard and coupling constants (J) given in Hertz. EI-MS spectra were registered on a 50-400 HP5989A mass spectrometer. HRESI-MS spectra were obtained on an Applied Biosystems Mariner 5140 spectrometer. Column chromatography (CC) was run on silica gel (Qingdao Haiyang Chemical group Co. Ltd., China). TLC analyses were carried out on silica gel GF_{254} precoated plates with detection accomplished by spraying with 10% H_2SO_4 followed by heating at 105 °C.

4.2. Plant material

H. occulta (Lours) Schott was collected in Longsheng of Guangxi Province, PR China in July 2004, and authenticated by Dr. Min-Jian Qin (Department of Botany, China Pharmaceutical University, Nanjing, PR China). A voucher specimen (No. 202) was deposited in the Department of Traditional Chinese Medicine, Nanjing, PR China.

4.3. Extraction and isolation of compounds

Air-dried and powdered rhizomes (5 kg) of *H. occulta* were extracted with EtOH–H_2O (95:5, v/v) three times to obtain the EtOH extract, which was partitioned successively against petroleum ether, $CHCl_3$ and *n*-BuOH. The crude $CHCl_3$ extract was subject to silica gel CC, using a $CHCl_3$–Me_2CO gradient system (100:0.5–2:1) and bioassay-guided based on the activities of the fractions in cultured osteoblasts. Fraction 6 eluted with $CHCl_3$–Me_2CO (6:1–4:1) was further purified by Sephadex LH-20 CC eluted with a MeOH–$CHCl_3$ system to give the new compounds **6** (23 mg) and **7** (8 mg). Fraction 2 eluted with $CHCl_3$–Me_2CO (50:1–20:1), was subjected repeatedly to silica gel CC, with a petroleum–EtOAc gradient to afford daucane ester **8** (12 mg).

4.4. Homalomenol **E** (4,6,7-trihydroxy-isodaucane (1α,4β-OH,5α,6α,7α) (**6**)

Colorless crystals from MeOH, m.p. 176–178 °C; $[\alpha]_D^2$5 +34.6 (MeOH; *c* 0.6); IR ν_{max}^{KBr}: 3372, 3319 (OH), 2977, 2946, 2872, 2336, 1461, 1386, 1370, 1022 (C–O), 904, 685, 607 cm^{-1}; EIMS 70 eV, *m*/*z* (rel. int.) 256 $[M]^+$, 238 $[M-H_2O]^+$ (4.5), 223 $[M-H_2O-Me]^+$ (2.0), 220 $[M-2H_2O]^+$ (13.8), 205 $[M-2H_2O-Me]^+$ (12.9), 202 $[M-3H_2O]^+$ (3.5), 195 $[M-H_2O-Me_2CH]^+$ (9.8), 187 $[M-3H_2O-Me]^+$ (2.2), 177 $[M-2H_2O-Me_2CH]^+$ (24.6), 159 $[M-3H_2O-Me_2CH]^+$ (38.9), 151 (21), 122 (12.5), 121(12.2), 107(22.9), 93 (29.8), 81 (29.5), 71 (35), 59 $[Me_2C(OH)]^+$ (10.1), 55 $[C_4H_7]^+$ (20.8), 43 $[C_3H_7]^+$ (100), 41 $[C_3H_5]^+$ (29.1), HRESIMS (*m*/*z* 255.2234 $[M-H]^-$, calculated 255.2158). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1.

4.5. (−)1β,4β,6α-trihydroxy-eudesmane (**7**)

Colorless crystals from MeOH, m.p. 186–188 °C; $[\alpha]_D^2$5 −39 (MeOH; *c* 0.05); IR ν_{max}^{KBr}: 3410, 3321 (OH), 2951, 2932, 2891, 2872, 1461, 1385, 1374, 1337, 1207, 1193, 1166, 1093, 1073, 1025 (C–O), 1004, 974, 938, 665 cm^{-1}; EIMS (probe) 70 eV, *m*/*z* (rel. int.) 256 $[M]^+$ (0.90), 241 $[M-Me]^+$ (4.87), 238 $[M-H_2O]^+$ (1.3), 223 $[M-H_2O-Me]^+$ (13), 220 $[M-2H_2O]^+$ (15.4), 205 $[M-2H_2O-Me]^+$ (10), 202 $[M-3H_2O]^+$ (14.5), 187 $[M-3H_2O-Me]^+$ (12.9), 180 (37), 159 $[M-3H_2O-Me_2CH]^+$ (39.2), 149 (17.3), 138 (20.9), 123 (37.5), 121 (30.7), 107 (49.4), 101 (100), 95 (44), 93 (39), 83 (34), 81 (54), 71 (2.5), 59 $[Me_2C(OH)]^+$ (31), 55 $[C_4H_7]^+$ (47), 43 $[C_3H_7]^+$ (85), 41 $[C_3H_5]^+$ (54), HRESIMS (*m*/*z* 255.1934 $[M-H]^-$, calculated 255.1822). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 2.

4.6. Homalomenol **F** (8-methoxyl-13-(4′-oxy-butanolide)-5(9)-epoxy-daucane) (**8**)

Colorless needles from MeOH, m.p. 156–158 °C; $[\alpha]_D^2$5 −41.5 (MeOH; *c* 0.6); IR ν_{max}^{KBr}: 2944, 2914, 2876, 2814, 1767 (C=O), 1463, 1389, 1368, 1327, 1262, 1242, 1117, 1041, 1032 (C–O), 984, 926, 899, 755 cm^{-1}; ESIMS 70 eV, *m*/*z* 353 $[M+H]^+$, 321 $[M-OMe]^+$, 336 $[M-O]^+$ and 102 $[C_4H_5O_3+H]^+$, HRESIMS (*m*/*z* $[M+H]^+$ 353.2256, calculated 353.2301). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 3.

4.7. X-ray crystallography of (−)1β,4β,6α-trihydroxy-eudesmane (**7**)

Colorless block-shaped crystals of **7** were grown from slow evaporation of MeOH. A selected crystal was mounted on a glass fiber and diffraction intensity data was collected with a Bruker CCD diffractometer with graphite monochromatized Mo K radiation (0.71073 Å) at 100 K under a stream of cold N_2. Lattice determination and data collection were carried out using SMART v.5.625 software. Data reduction and absorption correction were performed using SAINT v.6.26 and SADABS v.2.03. Structure solution and refinement were performed using SHELXTL v.6.10 software package. All non-hydrogen atoms were refined anisotopically. The hydrogen atoms were introduced at their geometric positions and refined as riding atoms.

4.8. In vitro activity in osteoblasts (OB)

4.8.1. Cell culture

Osteoblasts from calvaria of new born (<24 h) Sprague–Dawley (SD) rats were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Nanjing Biologics Inc., PR China) either alone or in the presence of different *H. occulta* constituents.

4.8.2. MTT and ALP-specific activity assay for osteoblast proliferation and differentiation in vitro

A cell Titer 96 assay Kit (Nanjing Biologics Inc., PR China) was used for the bioactivity assay. Third generation osteoblasts were cultured for 24 h, and then transferred to a fresh medium containing the test samples for further culturing of another 72 h. MTT [3-(4,5-dimethylthiazozyl)-2,5-diphenyl tetrazolium bromide] method was used to detect osteoblast proliferation in the cultured osteoblasts. Osteoblast differentiation was evaluated by measuring activities of ALP (alkaline phosphatase) using PNPP (*p*-nitrophenyl phosphate) method. Tested samples were diluted with DMSO, and 17β-estradiol (E_2) and etidronate disodium were used as positive controls. Results were summarized in Table 4.

4.8.3. Assay for mineralization of osteoblasts in vitro

Effects of extracts and isolated compounds on formation of mineral nodes in the cultured OB were tested. 0.1% ARS (Alizarin red S) was used to observe the number of mineral nodes in the cultured OB. The test samples were diluted with DMSO, and 17β-estradiol (E_2) and etidronate disodium were used for the controls. Results were summarized in Table 5.

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Appendix A. Supplementary material

Crystal data of **7** $C_{15}H_{28}O_3$ (256.37 g/mol), crystal size 0.40 × 0.35 × 0.30 mm^3, orthorhombic, space group $P2_12_12_1$, T = 100(2) K, a = 7.3285(8) Å, b = 10.2377(11) Å, c = 20.000(2) Å, V = 1500.6(3) $Å^3$, D_c = 1.135 Mg/m^3, Z = 4, $F_{(000)}$ = 568, $\mu_{(Mo\ K)}$ = 0.077 mm^{-1}. A total of 7487 reflections were collected in the range $2.04° < \theta < 25.00°$, with 1517 independent [R(int) = 0.0347], completeness to *max*. 98.2%; max. and min. transmission 1.00 and 0.81; data/restraints/parameters 1517/0/163; goodness-of-fit on F^2 = 1.025; final R indices [$I > 2\sigma(I)$], R_1 = 0.0439, wR_2 = 0.1024; R indices (all data), R_1 = 0.0501, wR_2 = 0.1055, largest difference peak and hole, 0.242 and −0.177 e/$Å^3$. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.05.023.

References

Brixen, K.T., Christensen, P.M., Ejersted, C., Langdahl, B.L., 2004. Teriparatide (biosynthetic human parathyroid hormone 1–34): a new paradigm in the treatment of osteoporosis. Basic Clin. Pharmacol. Toxicol. 94, 260–270.

Chen, Y.Z., Xue, D.Y., Li, Z.L., Han, H., 1986. Chemical constituents of the essential oil of *Homalomena occulta* (Lours) Schott. Sepu 4, 324–327.

Erdtsieck, R.J., Pols, H.A.P., Valk, N.K., van Ouwerkerk, B.M., Lamberts, S.W.J., Mulder, P., Birkenhager, J.C., 1995. Treatment of post-menopausal osteoporosis with a combination of growth hormone and pamidronate: a placebo controlled trial. Clin. Endocrinol. (Oxford) 43, 557–565.

Guo, S.Q., Dang, G.D., Wang, Z.G., 1998. Effect of sodium fluoride and *Dispacus asperoides* on osteoblastic proliferation. Zhongguo Guzhi Shusong Zazhi 18, 84–87.

Izbosarov, M.B., Yusupova, I.M., Abduazimov, B.K., Tashkhodzhaev, B., 1998. Structure of mucrolidin. Analysis of the influence of some oxygen-containing substituents on the chemical shifts of the protons of the angular methyl of sesquiterpenoids with the eudesmane type of skeleton. Chem. Nat. Compd. 34, 289–294.

Li, F.F., Li, E., Song, S.J., 1998. Effect of kidney-tonifying recipe and its different components on osteoblastic proliferation and differentiation in vitro. Zhongguo Guzhi Shusong Zazhi 4, 71–75.

Menezes, J.E.S.A., Machado, F.E.A., Lemos, T.L.G., Silveira, E.R., Braz, F.R., Pessoa, O.D.L., 2004. Sesquiterpenes and a phenylpropanoid from *Cordiatrichotoma*. Z. Naturforsch. C. J. Biosci. 59c, 19–22.

Mossa, J.S., Muhammad, I., El-Feraly, F.S., Hufford, C.D., McPhail, D.R., McPhail, A.T., 1992. Bisabolene and guaiane sesquiterpenes from *Pulicaria glutinosa*. Phytochemistry 31, 575–578.

Nyasse, B., Ghogomu, T.R., Sondengam, B.L., Martin, M.T., Bodo, B., 1988. Isolation of α-corymbolol, an eudesmane sesquiterpene diol from *Cyperus articulatus*. Phytochemistry 27 (1), 179–181.

Sung, T.V., Kutschabsky, L., Porzel, A., Steglich, W., Adam, G., 1992a. Sesquiterpenes from the roots of *Homalomena aromatica*. Phytochemistry 31 (5), 1659–1661.

Sung, T.V., Steffan, B., Steglich, W., Klebe, G., Adam, G., 1992b. Sesquiterpenoids from the roots of *Homalomena aromatica*. Phytochemistry 31 (10), 3515–3520.

Tchuendem, M.H., Mbah, J.A., Tsopmo, A., Ayafor, J.F., Sterner, O., Okunjic, C.C., Iwu, M.M., Schuster, B.M., 1999. Anti-plasmodial sesquiterpenoids from the African *Reneilmia cincinnata*. Phytochemistry 52, 1095–1099.

Zhang, J.G., Xie, S.G., 2005. Chinese medicinal composition for treating osteoarthritis and rheumatoid arthritis, and preparation method thereof. Faming Zhuanli Shenqing Gongkai Shuomingshu.

Zhou, C.M., Yao, C., Sun, H.L., Qiu, S.X., Cui, G.Y., 1991. Volatile constituents of the rhizome of *Homalomena occulta*. Planta Med. 57, 391–392.