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Bioactive *ent*-kaurane diterpenoids from *Isodon serra*

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ABSTRACT

Nine 7,20-epoxy-*ent*-kaurane diterpenoids (15-acetylmegathyrin B, serrin E, 14β-hydroxyrabdocoestin A, serrin F, serrin G, 11-*epi*-rabdocoestin A, serrin H, serrin I, and 15-acetylenanderianin N), along with seven known ones, were isolated from the aerial parts of *Isodon serra*. Their structures were elucidated by extensive spectroscopic analysis, and the absolute configuration of 15-acetylmegathyrin B was determined by signal-crystal X-ray diffraction. All of these compounds were evaluated for their cytotoxic activities against five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, SW480). Serrin F, rabdocoestin B and 1α,11β-dihydroxy-1α,11β-acetonide-7α,20-epoxy-*ent*-kaur-16-en-15-one showed cytotoxic activities against all cell lines, with IC_{50} values ranging from 0.7 to 4.6 μM; serrin F also strongly inhibited NO production in LPS-stimulated RAW264.7 cells. Otherwise, 14β-hydroxyrabdocoestin A, serrins H and I, as well as enanderianin N and megathyrin B, also exhibited inhibitory effects towards NO production, while no cytotoxicity against five cell lines was detected.

1. Introduction

The genus *Isodon*, including about 150 species, has been investigated for its abundant diterpenoid constituents and their biological activities for over half a century since the determination of its enmein structure (Sun et al., 2001, 2006; Ikeda and Kanatomo, 1958; Iitaka and Natsume, 1964; Fukita et al., 1965). *Isodon serra* (Maxim.) Hara, also called 'Xihuangcao' in Chinese folk medicine, is mainly distributed in Guangdong, Fujian and Jiangxi Provinces of China, and has long been used to treat jaundice hepatitis, acute cholecystitis, and enteritis (Zhong Yao Da Ci Dian, 1997; Flora of China, 1977; Wong et al., 2015). Previous phytochemical investigations of *I. serra* from different regions of China led to the isolation of a variety of *ent*-kaurane types, including enmein, spirolactone, C-20-non-oxygenated, and C-20-oxygenated-kauranoids types (Jin et al., 1985; Zhao et al., 2004; Yan et al., 2007; Du et al., 2011; Zheng et al., 2011; Yan et al., 2012). Among them, serrins A–C showed immunosuppressive effects towards T-lymphocytes (Zhao et al., 2004), and rabdoserrin B exhibited inhibitory activity to human retinoblastoma cells (Jin et al., 1987; Hai et al., 2010).

In an ongoing search for bio-active diterpenoids from the genus *Isodon*, the chemical constituents of *I. serra*, collected from the E'mei mountain, Sichuan Province, were investigated. This study led to isolation of nine new *ent*-kauranoids, 15-acetylmegathyrin B (**1**), serrin E (**2**), 14β-hydroxyrabdocoestin A (**3**), serrin F (**4**), serrin G (**5**), 11-*epi*-rabdocoestin A (**6**), serrin H (**7**), serrin I (**8**), and 15-acetylenanderianin N (**9**), as well as seven known ones (**10**–**16**), rabdocoestin A (**10**) (Xu et al., 1993), rabdocoestin B (**11**) (Chen et al., 1990), enanderianin N (**12**) (Xiang et al., 2003), 1α,11β-dihydroxy-1α,11β-acetonide-7α,20-epoxy-*ent*-kaur-16-en-15-one (**13**) (Chen et al., 1990), megathyrin A (**14**) (Sun et al., 1994), enanderianin L (**15**) (Xiang et al., 2003) and megathyrin B (**16**) (Qiu et al., 1998) (Fig. 1). Due to the potent antitumor activities of the *ent*-kauranoids isolated from this genus (Sun et al., 2006), all of these compounds were evaluated for both their cytotoxic activities against five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, SW480) (Table 3) and their NO production inhibitory activities in LPS-stimulated RAW264.7 cells (Table 4). In this paper, the isolation, structure elucidation, cytotoxic activities and NO production inhibitory activities of these diterpenoids are reported.

2. Results and discussion

A 70% aqueous acetone extract of the air-dried and powdered

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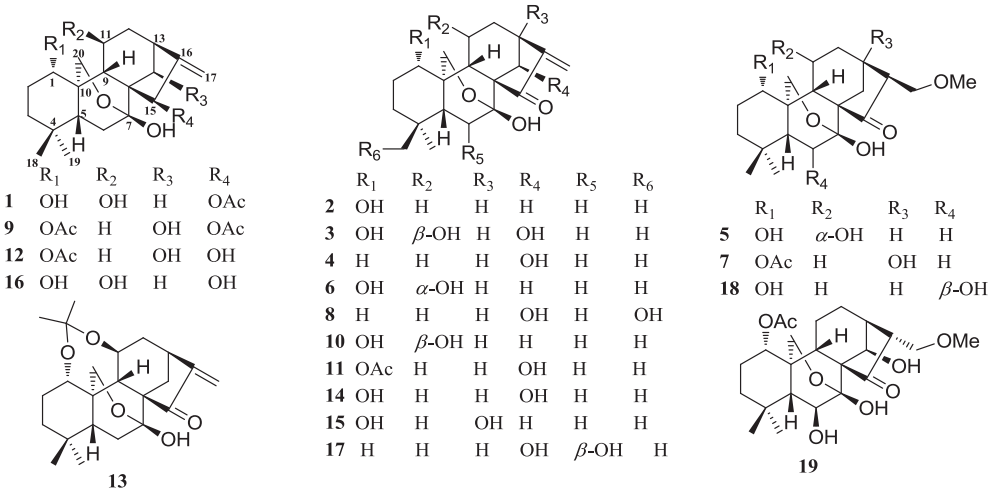

Fig. 1. Structures of compounds **1**–**19**.

aerial parts of *I. serra* was partitioned between EtOAc and H_2O. The EtOAc layer was subjected to repeated column chromatography and then HPLC to afford 9 new 7,20-epoxy-*ent*-kauranoids, named 15-acetylmegathyrin B (**1**), serrin E (**2**), 14β-hydroxyrabdocoestin A (**3**), serrin F (**4**), serrin G (**5**), 11-*epi*-rabdocoestin A (**6**), serrin H (**7**), serrin I (**8**), and 15-acetylenanderianin N (**9**), respectively.

Compound **1** was obtained as colorless crystals (MeOH). Its molecular formula was deduced to be $C_{22}H_{32}O_6$ by its HRESIMS ($[M+Na]^+$ *m/z* 415.2093, calcd 415.2091), corresponding to seven degrees of unsaturation. Its IR spectrum disclosed the existence of hydroxy (3355 cm^{-1}), acetoxy carbonyl (1744 cm^{-1}), and olefinic groups (1632 cm^{-1}). The ^1H NMR (Table 1) spectrum showed resonances attributed to the characteristic AB methylene group at δ_H 4.57 and δ_H 4.81 (each 1H, d, J = 10.0 Hz), and three quaternary methyls at δ_H 0.77 (3H, s), δ_H 1.08 (3H, s), and δ_H 2.16 (3H, s). The ^{13}C NMR and DEPT (Table 2) spectra of **1** exhibited 22 carbon signals, including three methyls, seven methylenes (one oxygenated), six methines (three oxygenated) and six quaternary carbons (one oxygenated, one lactonic carbonyl carbon). These resonances suggested that **1** possessed a 7,20-epoxy-*ent*-kauranoid skeleton similar to that of megathyrin B (**16**), except that C-15 was substituted by an acetoxy group in **1**. Comparisons of the NMR spectroscopic data of **1** and **16** indicated that **1** differed from **16** by possessing one more methyl (δ_H 2.16, 3H, s) and acetyl carbon signals (δ_C 170.7, s), which was supported by the following 2D NMR correlations and its unsaturation degrees: (i) the $^1H-^1H$ COSY spectrum showed correlations of H-1/H_2-2/H_2-3 and H-11/H_2-12/H-13/H_2-14 (Fig. 2); (ii) HMBC correlations from H_2-2 (δ_H 1.90, 2H, m), H_2-3 (δ_H 1.44 and 1.25), H-5 (δ_H 1.69), H-9 (δ_H 2.47), and H_2-20 (δ_H 4.57, 4.81) to C-1 (δ_C 73.8, d), and from H-9 (δ_H 2.47), H_2-12 (δ_H 2.85 and 1.83), and H-13 (δ_H 2.68) to C-11 (δ_C 63.0, d) implied the positions of OH-1 and OH-11; (iii) HMBC correlations from H_2-14 (δ_H 2.09, 2H), H_2-17 (δ_H 5.12, 2H, br s), and CH_3-OAc (δ_H 2.16, 3H, s) to C-15 (δ_C 76.5, d) (Fig. 2), along with seven degrees of unsaturation, permitted assignment of an OAc group at C-15 in **1**. In addition, ROESY correlations (Fig. 3) of H-1/H-5β/H-9β, and H-11/H-13α/H-15 demonstrated that H-1 was β-oriented, while H-11 and H-15 were α-oriented.

To verify the structure of **1**, attempts to crystallize **1** from MeOH were made success and a X-ray diffraction experiment was performed. Single-crystal X-ray diffraction analysis using the anomalous scattering of Cu Kα radiation yielded a Flack parameter of 0.01 (14), and a Hoof parameter of 0.03 (3) for 1311 Bijvoet paris, confirming the assignment of absolute configuration of compound **1** to be *1S*, *5R*, *7R*, *8S*, *9S*, *10R*, *11S*, *13S*, *15R* (Fig. 3.). Therefore, the structure of **1** was established as 15β-acetoxy-7α,20-epoxy-1α,7β,11β-trihydroxy-*ent*-kaur-16-ene, and it was named 15-acetylmegathyrin B.

Compound **2** was obtained as a white amorphous powder and its molecular formula was determined to be $C_{20}H_{28}O_4$ by the HRESIMS ($[M+Na]^+$ *m/z* 355.1885, calcd 355.1880). Its IR spectrum gave absorption bands at 3482, 1722, and 1641 cm^{-1}, suggesting the presence of hydroxy, carbonyl, and olefinic groups, respectively. In the ^{13}C NMR and DEPT spectra (Table 2), 20 carbon signals were observed, which were assigned as two methyls, eight methylenes (one oxygenated and one olefinic), four methines (one oxygenated), and six quaternary carbons (one carbonyl), respectively. Detailed analysis of the NMR data (Tables 1 and 2) indicated that compound **2** was identical to rabdocoestin A (**10**), except for the absence of an OH substituent at C-11 in **2**. The structure of compound **2** was supported by its 2D NMR correlations. The $^1H-^1H$ COSY correlations of H-1/H_2-2/H_2-3, along with HMBC correlations from H_2-2 (δ_H 1.85, 2H, m), H_2-3 (δ_H 1.24 and 1.37), H-5 (δ_H 1.55), and H-9 (δ_H 1.71) to C-1 (δ_C 73.7, d), indicated an OH group at C-1. Meanwhile, the HMBC data showed correlations from H-1 (δ_H 3.64), H-5 (δ_H 1.55), and H-9 (δ_H 1.71) to C-20 (δ_C 64.3, t), and from H_2-20 (δ_H 4.52 and 4.80) to C-7 (δ_C 96.1, s), indicating an epoxy ring between C-7 and C-20. Therefore, compound **2** was determined to be a 7α,20-epoxy-*ent*-kauranoid. Additionally, ROESY correlations of H-5β/H-1/H-9β disclosed a β-orientation of H-1. Thus, the structure of compound **2** was defined as 7α,20-epoxy-1α,7β-dihydroxy-*ent*-kaur-16-en-15-one, and it was named serrin E.

Compound **3** was obtained as a white amorphous powder that gave a molecular formula of $C_{20}H_{28}O_6$ by the HRESIMS ($[M-H]^-$ *m/z* 363.1813, calcd 363.1813), implying seven degrees of unsaturation. The NMR data of **3** suggested its structure resembled rabdocoestin A (**10**), except for an OH locating at C-14 in **3**. This was supported by the chemical shift of C-13 ($\Delta\delta$ 9.5 ppm) and C-8 ($\Delta\delta$ 2.5 ppm) in **3** relative to those of **10**. The 2D NMR spectra of **3** further verified that inference: the $^1H-^1H$ COSY data exhibited H-9/H-11/H-12/H-13/H-14 correlations (Fig. 2), and the HMBC data showed key correlations from H-9 (δ_H 2.07), H_2-12 (δ_H 2.99, 1.92), and H-13 (δ_H 3.27) to C-14 (δ_C 73.1, d), confirming an OH group at C-14 (Fig. 2). Furthermore, ROESY correlations of H-5β/H-1/H-9β and H-11/H-20α/H-14 supported configurations of H-1, H-11 and H-14 as being β-, α-, and α-oriented, respectively. Thus, the structure of compound **3** was determined to be 7α,20-epoxy-1α,7β,11β,15β-tretrahydroxy-*ent*-kaur-16-en-15-one, and it was named 14β-hydroxyrabdocoestin A.

Table 1
^{1}H NMR spectroscopic data for compounds **1**–**9** in pyridine-d_5 (δ in ppm, J in Hz).

No.	1[a]	2[a]	3[c]	4[c]	5[c]	6[c]	7[c]	8[b]	9[c]
1a	4.21 (dd, 11.2, 5.4)	3.64 (overlap)	4.13 (m)	0.74 (overlap)	4.14 (m)	3.86 (m)	4.87 (overlap)	0.84 (m)	4.86 (dd, 11.5, 5.3)
1b				1.06 (m)				1.12 (m)	
2a	1.90 (2H, m)	1.85 (2H, m)	1.95 (overlap)	1.27 (2H, m)	1.95 (m)	1.9 (2H, m)	1.80 (m)	1.40 (2H, m)	1.82 (2H, m)
2b			1.87 (overlap)		1.86 (m)		1.56 (overlap)		1.50 (overlap)
3a	1.44 (br d, 13.3)	1.37 (dt, 12.5, 4.5)	1.42 (dt, 13.3, 3.2)	1.13 (br d, 13.8)	1.44 (dt, 13.2, 3.1)	1.43 (dt, 13.2, 3.2)	1.32 (br d, 13.5)	1.68 (overlap)	1.34 (br d, 13.3)
3b	1.25 (br t, 12)	1.24 (overlap)	1.24 (dt, 13.5, 3.2)	0.99 (m)	1.26 (dt, 13.6, 3.1)	1.29 (m)	1.19 (br t, 13.7)	1.55 (overlap)	1.24 (br d, 13.3)
5	1.69 (dd, 11.0, 7.6)	1.55 (dd, 11.5, 7.3)	1.59 (dd, 11.5, 6.6)	1.39 (dd, 11.2, 6.9)	1.63 (dd, 11.9, 5.6)	1.60 (m)	1.60 (overlap)	2.07 (overlap)	1.66 (m)
6a	2.13 (overlap)	3.67 (overlap)	3.74 (br t, 12.7)	3.56 (m)	3.52 (br t, 12.5)	2.16 (dd, 12.2,7.6)	3.49 (t, 12.4)	3.65 (t,12.2)	2.12 (t, 12.5)
6b	2.03 (overlap)	2.04 (dd, 12.9, 7.3)	2.05 (overlap)	1.99 (dd, 13.4, 6.9)	2.01 (dd, 12.5, 5.6)	3.78 (t, 12.2)	2.01 (overlap)	2.13 (overlap)	2.00 (overlap)
9	2.47 (d, 10.1)	1.71 (dd, 12.9, 5.5)	2.07 (overlap)	1.61 (overlap)	1.81 (overlap)	1.57 (d, 3.4)	1.75 (overlap)	1.61 (overlap)	2.45 (dd, 13.1, 5.9)
11a	4.67 (m)	2.43 (m)	4.91 (overlap)	1.58 (overlap)	4.79 (m)	4.58 (m)	2.38 (m)	1.64 (overlap)	1.90 (m)
11b		1.96 (m)		1.13 (m)			1.43 (m)		1.18 (m)
12a	2.85 (m)	2.23 (m)	2.99 (m)	2.32 (m)	2.48 (overlap)	1.79 (dd, 15.0, 5.3)	2.19 (2H, m)	2.34 (m)	2.33 (m)
12b	1.83 (overlap)	1.41 (overlap)	1.92 (overlap)	1.51 (m)	1.82 (overlap)	2.66 (dd, 15.0. 9.7)		1.50 (overlap)	1.56 (m)
13 a	2.68 (m)	2.88 (dd, 9.6, 3.8)	3.27 (br d, 10.0)	3.10 (br d, 9.6)	2.81 (m)	3.13 (dd, 4.2, 9.7)		3.11 (br d, 9.6)	2.80 (br d, 9.2)
14a	2.09 (2H, overlap)	2.46 (br d, 12.2)	5.46 (H, s)	5.17 (s)	2.43 (dd, 12.5, 3.3)	3.69 (dd, 11.5)	2.86 (2H, m)	5.19 (s)	5.04 (s)
14b		2.38 (dd, 12.2, 3.8)			2.51 (overlap)	2.52 (dd, 11.5, 4.3)			
15	6.35 (s)								6.85 (s)
16					2.94 (m)		3.10 (t, 4.8)		
17a	5.12 (2H, br s)	6.00 (s)	6.29 (s)	6.27 (s)	3.88 (dd, 10.3, 4.6)	6.02 (s)	3.98 (2H, m)	6.26 (s)	5.33 (s)
17b		5.23 (s)	5.46 (s)	5.41 (s)	3.71 (dd, 10.3. 4.6)	5.25 (s)		5.42 (s)	5.25 (s)
18	0.77 (3H, s)	0.79 (3H, s)	0.78 (3H, s)	0.76 (3H, s, overlap)	0.75 (3H, s)	0.83 (3H, s)	0.72 (3H, s)	3.63 (d, 10.6)	0.77 (3H, s)
18b								3.54 (2H, d, 10.6)	
19	1.08 (3H, s)	1.11 (3H, s)	1.13 (3H, s)	1.03 (3H, s)	1.12 (3H, s)	1.12 (3H, s)	1.06 (3H, s)	1.15 (3H, s)	1.05 (3H, s)
20a	4.81 (d, 10.0)	4.80 (d, 10.0)	4.87 (overlap)	4.29 (d, 9.9)	4.75 (d, 10.3)	5.29 (2H, dd, 9.4,	4.54 (d, 10.1)	4.39 (d, 9.8)	4.57 (d, 9.9)
20b	4.57 (d, 10.0)	4.52 (d, 10.0)	4.67 (dd, 10.3, 1.8)	3.98 (d, 9.9)	4.61 (d, 10.3)	1.7)	4.43 (d, 10.1)	4.06 (d, 9.8)	4.45 (d, 9.9)
OAc-1							2.03 (3H, s)		2.03 (3H, s)
OAc-15	2.16 (3H, s)								2.28 (3H, s)
OMe					3.22 (3H, s)		3.3 (3H, s)		

[a] Recorded at 400 MHz.
[b] Recorded at 500 MHz.
[c] Recorded at 600 MHz.

Compound **4** was obtained as a white amorphous powder, and it had a molecular formula $C_{20}H_{28}O_4$ as deduced from the HRESIMS ($[M+H]^+$ m/z 333.2062, calcd 333.2060), including seven degrees of unsaturation. The NMR spectroscopic data of **4** were similar to those of longikaurin A (**17**) (Fig. 1) (Fujita et al., 1980), except for the absence of an oxygenated carbon (δ_C 64.5, d) and the presence of one more methylene (δ_C 32.2, t) in **4**. The position of the OH substituent in **4** was deduced by the ^{1}H—^{1}H COSY and HMBC correlations. The ^{1}H—^{1}H COSY correlations of H_2-11/H_2-12/H_2-13/H-14 and HMBC correlations from H-9 (δ_H 1.61), H_2-12 (δ_H 1.51, 2.32), and H-13 (δ_H 3.10) to C-14 (δ_C 73.2, d) indicated an OH group at C-14. The configuration of H-14 was established as α-oriented due to the correlation between H-20 with H-14 in its ROESY spectrum. Compound **4** was then established as 7α,20-epoxy-7β,14β-dihydroxy-*ent*-kaur-16-en-15-one, and it was named serrin F.

Compound **5** possessed a molecular formula of $C_{21}H_{32}O_6$, corresponding to its molecular ion peak m/z 379.2127 ($[M-H]^-$, calcd 379.2126), implying six degrees of unsaturation. The NMR data of **5** resembled those of nervosanin A (**18**) (Fig. 1) (Wang et al., 1994) except for one OH group at C-11 in **5** relative to C-6 in nervosanin A. The ^{1}H—^{1}H COSY correlations of H-9/H-11/H-12/H-13/H-14, as well as HMBC correlations from H-9 (δ_H 1.81), H_2-12 (δ_H 1.82, 2.48), and H-13 (δ_H 2.81) to C-11 (δ_C, 63.9, d), confirmed a hydroxy substituent at C-17 in **5**. The β-orientation of H-1, H-11, and MeO-17 were determined by the ROESY correlations of H-1/H-5β/H-9β, H-11/H-1 and H-17/H-9β, respectively. Consequently, compound **5** was assigned as 7α,20-epoxy-1α,7β,11α-trihydroxy-17β-methoxy-*ent*-kaur-15-one, and it was named serrin G.

Compound **6** was obtained as a white amorphous powder and it had a same overall structure as rabdocoestin A (**10**) according to the HRESIMS ($[M-H]^-$, m/z 347.1865; calcd 347.1864) and NMR spectra (Tables 1 and 2). The ^{13}C NMR spectroscopic data showed chemical shifts of C-9 ($\Delta\delta$ 2.2 ppm) and C-11 ($\Delta\delta$ 4.2 ppm) in **6** compared to those of **10**, which might be caused by the inverse orientation of OH-11 in **6**. The ROESY correlations of H-11/H-1β/H-9β verified the β-orientation of H-11 in **6**. Thus, **6** was assigned to be 7α,20-epoxy-1α,7β,11α-trihydroxy-*ent*-kaur-16-en-15-one, and it was named 11-*epi*-rabdocoestin A.

Compound **7** was obtained as a white amorphous powder and its molecular formula was established to be $C_{23}H_{34}O_7$ by the HREIMS ($[M]^+$ m/z 422.2284, calcd 422.2305). Comparisons of the ^{1}H and ^{13}C NMR spectroscopic data of **7** with those of lasiokaurinin

Table 2
^{13}C NMR spectroscopic data for compounds **1**–**9** in pyridine-d_5 (δ in ppm).

Position	1[a]	2[a]	3[c]	4[c]	5[c]	6[c]	7[c]	8[b]	9[c]
1	73.8 d	73.7 d	73.8 d	30.5 t	74.1 d	73.9 d	76.6 d	30.9 t	76.9 d
2	28.4 t	30.5 t	28.9 t	18.8 t	28.6 t	29.8 t	25.7 t	18.6 t	25.9 t
3	39.5 t	39.1 t	39.7 t	40.8 t	39.9 t	39.2 t	38.6 t	35.2 t	38.6 t
4	34.2 s	34.0 s	34.7 s	33.8 s	34.6 s	34.4 s	34.1 s	35.9 s	34.1 s
5	48.3 d	49.2 d	48.6 d	48.7 d	49.5 d	50.1 d	49.9 d	42.8 d	49.0 d
6	34.5 t	33.1 t	32.9 t	32.2 t	32.9 t	33.7 t	33.6 t	32.3 t	34.6 t
7	95.4 s	96.1 s	98.8 s	98.1 s	96.5 s	96.9 s	96.2 s	98.2 s	98.5 s
8	53.6 s	57.9 s	59.9 s	59.5 s	59.1 s	57.6 s	61.3 s	59.7 s	53.8 s
9	52.5 d	52.0 d	60.0 d	52.3 d	58.7 d	55.3 d	50.3 d	52.4 d	48.0 d
10	41.3 s	41.2 s	42.5 s	35.9 s	42.3 s	43.1 s	39.8 s	39.0 s	39.2 s
11	63.0 d	20.4 t	62.4 d	16.4 t	63.9 d	67.6 d	20.7 t	16.6 t	17.3 t
12	42.7 t	30.7 t	40.5 t	30.6 t	31.1 t	40.2 t	32.3 t	30.7 t	33.3 t
13	37.1 d	34.5 d	43.8 d	43.0 d	29.8 d	34.6 d	74.5 s	43.2 d	46.2 d
14	27.1 t	25.8 t	73.1 d	73.2 d	28.9 t	27.0 t	37.1 t	73.4 d	75.3 d
15	76.5 d	205.7 s	204.5 s	203.6 s	218.4 s	206.5 s	216.5 s	203.7 s	75.5 d
16	158.6 s	155.3 s	153.9 s	153.6 s	58.6 d	155.6 s	63.7 d	153.7 s	158.0 s
17	108.5 t	113.5 t	117.3 t	116.9 t	70.0 t	113.4 t	69.3 t	117.0 t	111.3 t
18	32.0 q	31.8 q	32.2 q	32.2 q	32.3 q	32.5 q	31.8 q	70.8 t	32.1 q
19	21.2 q	20.7 q	21.4 q	20.7 q	21.3 q	21.3 q	20.7 q	17.1 q	21.0 q
20	64.6 t	64.3 t	65.4 t	66.3 t	65.6 t	66.6 t	64.3 t	67.2 t	63.9 t
OAc-1							170.6 s		107.6 s
							21.8 q		21.9 q
OAc-15	170.7 s								171.3 s
	21.3 q								21.8 q
OMe					59.1 q		59.2 q		

[a] Recorded at 100 MHz.
[b] Recorded at 125 MHz.
[c] Recorded at 150 MHz.

(**19**) (Fig. 1) (Fujita et al., 1974) indicated that both compounds had an identical skeleton and groups, only differing in an OH group at C-13 in **7** instead of C-6 and C-14. This conclusion was confirmed by the key HMBC correlations from H_2-12 (δ_H 2.19, 2H, m), H_2-14 (δ_H 2.86, 2H, m), and H-16 (δ_H 3.10) to C-13 (δ_C 74.5, s) (Fig. 2). The stereogenic carbons were accordingly assigned as H-1β, HO-13α and H-16α, respectively, based on ROESY correlations of H-1/H-5β/H-9β and H_2-17/H-9β. The structure of 7 was determined to be 1α-acetoxy-7α,20-epoxy-7β,13α-dihydroxy-17β-methoxy-*ent*-kaur-15-one, and it was named serrin H. It was the first example of *ent*-kauranoid bearing an oxy-13 and oxy-17 moiety in D-ring isolated from *Isodon* species.

Compound **8** obtained as a white amorphous powder displayed a molecular ion peak at *m/z* 348.2013, in accordance with its molecular formula of $C_{20}H_{28}O_5$. The ^{13}C NMR and DEPT spectra suggested that **8** was a 7,20-epoxy-*ent*-kauranoid with one more hydroxy group at C-18 compared to serrin H (**4**). HMBC correlations from H_2-3 (δ_H 1.68, 1.55), H-5 (δ_H 2.07), and CH_3-19 (δ_H 1.15, 3H, s) to C-18 (δ_C 70.8, t) (Fig. 2), along with the up-field shift of C-5 ($\Delta\delta$ 5.9 ppm) due to the γ-gauche steric compression effect, confirmed an OH-18 fragment in **8**. The ROESY spectra of **8** indicated that the relative configurations of its stereogenic carbons were identical to those of **4**. Therefore, **8** was identified as 7α,20-epoxy-7β,14β,18-trihydroxy-*ent*-kaur-16-en-15-one, and it was named serrin I.

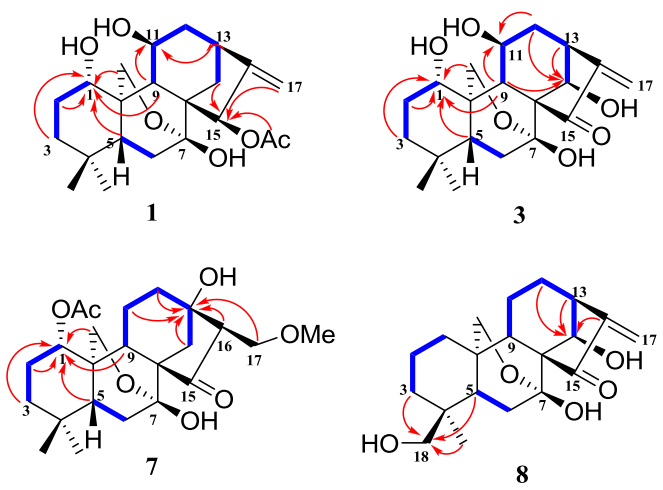

Fig. 2. Selected HMBC (arrows) and ^{1}H–^{1}H COSY (bold) correlations of **1**, **3**, **7** and **8**.

Compound **9** was obtained as a white amorphous powder that gave a molecular formula of $C_{24}H_{34}O_7$ by the HRESIMS ($[M+Na]^+$ *m/z* 457.2198, calcd 457.2197). It possessed a somewhat analogous structure compared to rabdocoestin B (**11**), except for an acetoxy group at C-15 in **9** instead of a carbonyl group in **11**. This was verified by HMBC correlations from H-14 (δ_H 5.04), H_2-17 (δ_H 5.25, 5.33), and CH_3-OAc (δ_H 2.28, 3H, s) to C-15 (δ_C 75.5, d). The orientations of the substituents were assigned to be AcO-1α, HO-14β, and AcO-15β, respectively, based on ROESY correlations of H-1/H-5β/H-9β, H-14/H-20b, and H-15/H-13α. Accordingly, the structure of **9** was determined to be 1α,15β-diacetoxy-7α,20-epoxy-7β,14β-dihydroxy-*ent*-kaur-16-en-15-one, and it was named to be 15-acetylenanderianin N.

The known compounds **10**–**16** were identified by the comparisons of their spectroscopic data with those reported in the literatures.

According to the cytotoxic activities of those diterpenoids isolated from *I. serra* previously, all isolates in this study were submitted to testing for their *in vitro* cytotoxicity against five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, SW-480) using the MTS method (Monks et al., 1991), with *cis*-platin and paclitaxel as positive controls. Compounds **4**, **11** and **13** exhibited weak cytotoxicity to all human tumor cell lines, with IC_{50} values ranging from 0.7 to 4.6 μM, compared with paclitaxel (IC_{50} values ranging from 0.09 $\times$ 10^{-3} to 0.89 $\times$ 10^{-3} μM), whereas compounds **2**, **6**, **10**, **11**, **14** and **15** showed even lower activities towards all five (Table 3).

Inflammation is a systemic response aimed to decrease the

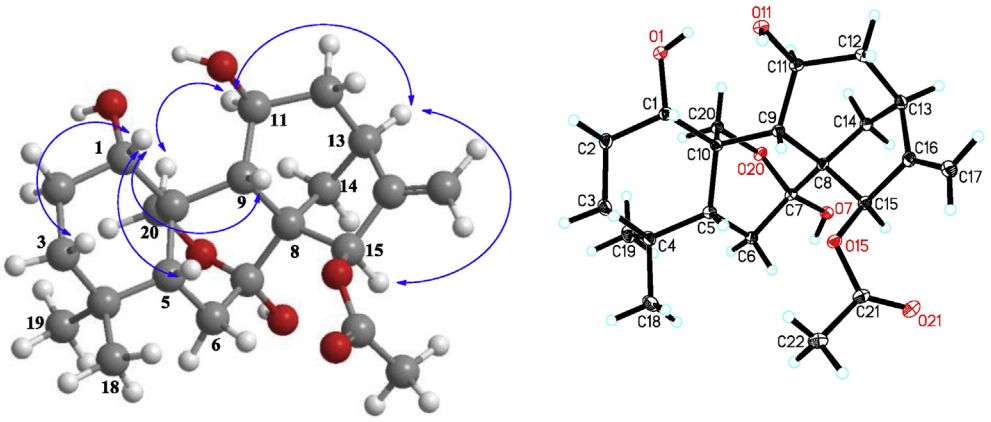

Fig. 3. Key ROESY correlations and X-ray crystallographic structure of compound **1**.

Table 3
Cytotoxic activities of several diterpenoids from *Isodon serra* against tumor cell lines, IC_{50} values (μM).

Compounds[a]	HL-60	SMMC-7721	A-549	MCF-7	SW480
2	3.6	>10	>10	4.1	4.1
4	0.7	2.3	3.7	2.2	3.4
6	5.3	9.5	>10	5.3	6.6
10	3.3	4.3	>10	4.1	3.3
11	2.7	1.4	4.6	2.4	2.2
13	0.8	1.7	4.4	2.4	2.6
14	5.0	8.2	>10	6.2	7.3
15	2.7	3.0	>10.	3.1	2.1
DDP[b]	1.9	11.8	12.4	18.3	18.1
Paclitaxel[b]	0.09×10^{-3}	0.64×10^{-3}	0.89×10^{-3}	0.37×10^{-3}	0.72×10^{-3}

[a] Compounds **1**, **3**, **5**, **7**, **8**, **9**, **12** and **16** had $IC_{50} > 10$ μM for all cell lines.
[b] DDP (*cis*-platin) and paclitaxel were used as positive controls.

toxicity of harmful agents and repair damaged tissue. A key feature of the inflammatory response is the activation of phagocytic cells involved in host defense. NO, a free radical produced by the inducible NO synthase (iNOS) isoform, is an essential component of the host innate immune and inflammatory responses to a variety of pathogens (McCartney-Francis et al., 2001). Due to the widespread folk use of *I. serra* for curing hepatitis and cholecystitis, all isolates except compounds **1** and **9** were tested for their inhibitory activities against NO production in LPS-stimulated RAW264.7 cells using the MTS assay. The experimental results showed that most compounds exhibited significant NO inhibitory effects: compound **4** exhibited a remarkable NO inhibitory effect with an IC_{50} value of 0.6 μM, while compounds **2**, **11**, **13** and **14** exhibited potent active effects with IC_{50} values of 1.4, 1.4, 1.3 and 1.5 μM, respectively (Table 4). Compounds **3**, **7**, **8**, **12** and **16** also exhibited inhibitory effects towards NO production, while no cytotoxicity against five cell lines was detected ($IC_{50} > 10$ μM).

Table 4
Inhibitory effects of compounds **2**–**4**, **6**–**8** and **10**–**16** on LPS-stimulated NO production in RAW264.7 cells.[a]

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
2	1.4	**11**	1.4
3	7.6	**12**	3.9
4	0.6	**13**	1.3
6	2.1	**14**	1.5
7	5.9	**15**	2.1
8	4.6	**16**	2.8
10	2.0	MG-132[b]	0.2

[a] Each value represents the mean ± SEM (n = 3).
[b] Positive control.

3. Concluding remarks

Ent-kaurane diterpenoids are the primary chemical constitutes of *Isodon* species responsible for the bioactivities of the plants (Wang et al., 2012; Wu et al., 2014; Yang et al., 2016), and the studies herein on *I. serra* added nine new ones. Among the isolates, two compounds exhibited weak cytotoxicity compared with paclitaxel, as well as a new one that showed remarkable effect against NO production. To some extent, the above bioactivity results gave a preliminary foundation for the interpretation of the folk effect of this species.

4. Experiments

4.1. General experimental procedures

X-ray data were collected using a Bruker APEX instrument. Optical rotations were measured in MeOH on a JASCO P-1020 digital Polarimeter, whereas UV spectra data were obtained on a Shimadzu UV2401PC spectrophotometer. A tensor 27 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. 1D- and 2D-NMR (δ_H 8.71, 7.55 and 7.19 for pyridine-d_5) spectra were recorded on Bruker AM-400, DRX-500 and DRX-600 spectrometers. Unless otherwise specified, chemical shifts (δ) were expressed in ppm with reference to the solvent signals. HRESIMS was performed on a VG Autospec-3000 spectrometer at 70 eV. HREIMS was performed on a VG Autospec-Premier P776 at 70 eV. Column chromatography (CC) was performed with silica gel

(100–200 mesh; Qingdao Marine Chemical, Inc., Qingdao, People's Republic of China). Semipreparative HPLC was performed on an Agilent 1100 and Agilent 1200 liquid chromatograph with a Zorbax SB-C_{18} (9.4 mm × 25 cm) column. Preparative HPLC was performed on an Agilent 1260 liquid chromatograph with a Zorbax SB-C18, 21 mm × 25 cm column. MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan), and Sephadex LH-20 gel (40–70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden), respectively. Lichroprep RP-18 gel (40–63 μM), Merck, Darmstadt, Germany. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 5% H_2SO_4 in EtOH. All solvents including petroleum ether were distilled prior to use.

4.2. Plant material

The aerial parts of *I. serra* were collected in E'mei Mountain, Sichuan Province, People's Republic of China, in August 2008. The voucher specimen (KIB 2008091703) is deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, and identified by Prof. Xi-Wen Li.

4.3. Extraction and isolation

Air-dried aerial parts (6 kg) of *I. serra* were powdered and extracted with acetone–H_2O (3 × 20 L, 70:30, v/v, each for 3 days) at room temperature to give a crude extract. The extracts were combined and concentrated to about 2 L and then extracted with EtOAc (5 × 2 L), resulting in EtOAc soluble (ca. 300 g). This portion was then subjected to silica gel CC (1 kg, 100–200 mesh), eluting with $CHCl_3$–Me_2CO (1:0–0:1 gradient system) to produce seven fractions (Fr. 1–7). Each fraction was then decolorized on MCI gel, eluted with MeOH–H_2O (90:10, v/v).

Fr. 3 (37 g) was subjected to RP-18 CC (eluted with a MeOH–H_2O gradient 30:70–100:0) to give subfractions 3A–3H. Fr. 3F (1.0 g) was applied to preparative HPLC (CH_3CN–H_2O, 30:70 v/v) and then semi-preparative HPLC (CH_3CN–H_2O, 25:75 v/v) to give **3** (6.7 mg), **4** (5.0 mg) and **13** (6.6 mg). Due to the low solubility of the main component of **10**, Fr. 3E (30 g) was washed with MeOH repeatedly to give a pure white powder (10 g), and the corresponding solution (named Fr. 3Ea). Fr. 3Ea (8.436 g) was separated by silica gel, eluting with $CHCl_3$–Me_2CO (1:0–0:1 gradient system) to produce six portions (3Ea1–3Ea6). Fr. 3Ea3 (1.85 g) was applied to a RP-18 column, eluted with MeOH–H_2O (45:55–80:20 v/v) and by semi-preparative HPLC (CH_3CN–H_2O, 30:70 v/v) to give **5** (3 mg), **14** (8.3 mg), **15** (2 mg), respectively. Fr. 3Ea4 (2.58 g) was subjected to silica gel CC, eluting with $CHCl_3$–Me_2CO (1:0–0:1 gradient) and then one subfraction (83.5 mg) was applied to preparative TLC (petroleum ether–Me_2CO, 20:1 v/v) and to semipreparative HPLC (CH_3CN–H_2O, 30:70 v/v) to yield **2** (48.4 mg) and **12** (8.7 mg). Fr. 3Ea5 (2.73 g) was subjected to RP-18 CC with MeOH–H_2O (45:55–80:20 gradient), preparative HPLC (CH_3CN–H_2O, 35:65 v/v) and semipreparative HPLC (CH_3CN–H_2O, 28:72 v/v) to yield **1** (11 mg), **6** (3.7 mg) and **7** (3.5 mg). Fr. 2 (14 g) was washed repeatedly to yield **11** (8.0 g), with the wash solution subjected to RP–18 CC (MeOH–H_2O, 50:50–90:10 v/v) and then semi-preparative HPLC (CH_3CN–H_2O, 38:62 v/v) to afford **9** (12.6 mg). **8** (40 mg) and **16** (100 mg) were isolated from Fr. 4 (16 g) by silica gel (petroleum ether–Me_2CO, 30:1–0:1 gradient system), Sephadex LH-20 gel CC ($CHCl_3$–MeOH, 1:1 v/v) and preparative HPLC (MeOH–H_2O, 32:68 v/v).

4.3.1. 15-Acetylmegathyrin B (**1**)

Colorless needles; [α]18 D −62 (*c* 0.12, MeOH); UV (MeOH) λ_{max} nm (log ε): 201 (3.07), 254 (2.61); IR (KBr) ν_{max} 3355, 2941, 1744, 1632, 1369, 1233, 1050, 921 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS (positive-ion mode) *m/z* 415.2093 $[M+Na]^+$ (calcd for $C_{22}H_{32}O_6Na$, 415.2091).

4.3.2. Serrin E (**2**)

White amorphous powder; [α]18 D −108 (*c* 0.18, MeOH); UV (MeOH) λ_{max} nm (log ε) 234 (3.17), 343 (1.22); IR (KBr) ν_{max} 3482, 2951, 2863, 1722, 1641, 1364, 1167, 1057, 925 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS (positive-ion mode) *m/z* 355.1885 $[M+Na]^+$ (calcd for $C_{20}H_{28}O_4Na$, 355.1880).

4.3.3. 14β-Hydroxyrabdocoestin A (**3**)

White amorphous powder; [α]25 D −66 (*c* 0.10, MeOH); UV (MeOH) λ_{max} nm (log ε) 196 (2.81), 230 (3.15); IR (KBr) ν_{max} 3423, 2937, 2872, 1723, 1642, 1384, 1159, 1062, 968 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS (negative-ion mode) *m/z* 363.1813 $[M-H]^-$ (calcd for $C_{20}H_{27}O_6$, 363.1813).

4.3.4. Serrin F (**4**)

White amorphous powder; [α]25 D −100 (*c* 0.06, MeOH); UV (MeOH) λ_{max} nm (log ε) 229 (3.18), 334 (1.22); IR (KBr) ν_{max} 3420, 2927, 2869, 1727, 1645, 1477, 1273, 1169, 1059, 894 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS (positive-ion mode) *m/z* 333.2062 $[M+H]^+$ (calcd for $C_{20}H_{29}O_4$, 333.2060).

4.3.5. Serrin G (**5**)

White amorphous powder; [α]25 D −84 (*c* 0.07, MeOH); UV (MeOH) λ_{max} nm (log ε) 202 (2.44), 373 (0.93); IR (KBr) ν_{max} 3431, 2933, 2872, 1732, 1385, 1631, 1162, 1062, 612 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS (negative-ion mode) *m/z* 379.2127 $[M-H]^-$ (calcd for $C_{21}H_{31}O_6$, 379.2126).

4.3.6. 11-epi-Rabdocoestin A (**6**)

White amorphous powder; [α]25 D −118 (*c* 0.14, MeOH); UV (MeOH) λ_{max} nm (log ε) 232 (3.17); IR (KBr) ν_{max} 3423, 2929, 1718, 1642, 1384, 1269, 1163, 1053, 921 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; ESIMS (negative-ion mode) *m/z* 347.1865 $[M-H]^-$ (calcd for $C_{20}H_{27}O_5$, 347.1864).

4.3.7. Serrin H (**7**)

White amorphous powder; [α]26 D −82 (*c* 0.10, MeOH); UV (MeOH) λ_{max} nm (log ε) 228 (3.05); IR (KBr) ν_{max} 3438, 2926, 2876, 1688, 1630, 1443, 1384, 1263, 1091, 1015 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HREIMS (positive-ion mode) *m/z* 422.2284 $[M]^+$ (calcd for $C_{23}H_{34}O_7$, 422.2305).

4.3.8. Serrin I (**8**)

White amorphous powder; [α]23 D −70 (*c* 0.18, MeOH); UV (MeOH) λ_{max} nm (log ε) 199 (2.73), 229 (3.00); IR (KBr) ν_{max} 3425, 2931, 2867, 1727, 1643, 1446, 1384, 1273, 1174, 1057, 893 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS (positive-ion mode) *m/z* 349.2013 $[M+H]^+$ (calcd for $C_{20}H_{29}O_5$, 349.2010).

4.3.9. 15-Acetylenanderianin N (**9**)

White amorphous powder; [α]23 D −45 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) nm 201 (2.96), 254 (2.31), 321 (1.43); IR (KBr) ν_{max} 3435, 2952, 2869, 1739, 1631, 1405, 1373, 1241, 1173, 1045, 603 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS (positive-ion mode) *m/z* 457.2198 $[M+Na]^+$ (calcd for $C_{24}H_{34}O_7Na$, 457.2197).

4.4. X-ray crystal structure analysis

Colorless crystals of **1** were obtained in MeOH. Intensity data

were collected at 100 k on a Bruker APEX DUO diffractometer equipped with an APEX II CCD, using Cu Kα radiation. Cell refinement and data reduction were performed with Bruker SAINT. The structures were solved by direct methods using SHELXS-97 (Sheldrick and Schneider, 1997). Refinements were performed with SHELXL-97 using full-matrix least-squares, with anisotropic displacement parameters used for all the non-hydrogen atoms. The H atoms were placed in the calculated positions and refined using a riding model. Molecular graphics were computed with PLATON. Crystallographic data (excluding structure factor tables) for the structures reported have been deposited with the Cambridge Crystallographic Data Center as supplementary publication no. CCDC 1443993 for **1**. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB 1EZ, UK [fax: Int. + 44 (0) (1223) 336 033; e-mail: deposi@ccdc.cam.ac.uk].

4.5. Crystallographic data for 15-acetylmegathyrin B (*1*)

$C_{22}H_{32}O_6$, $M_w = 392.48$, tetragonal, space group, P43212, $Z = 8$, $a = 11.1115(2)$ Å, $b = 11.1115(2)$ Å, $c = 31.8353(5)$ Å; $\alpha = \beta = \gamma = 90°$, $V = 3930.56(12)$ Å^3, $T = 100(2)$ K, μ (Cu Kα) = 0.778 mm^{-1}, 21,872 reflections measured, 3457 independent reflections ($R_{int} = 0.0355$). The final R_1 values were 0.0288 [$I > 2\sigma(I)$]. The final wR_2 (F^2) values were 0.0746 [$I > 2\sigma(I)$]. The final R_1 values were 0.0289 (all data). The final wR_2 (F^2) values were 0.0746 (all data). The goodness of fot on F^2 was 1.095. Flack parameter = 0.01(14). The Hooft parameter is 0.03(3) for 1311 Bijvoet pairs.

4.6. Cytotoxicity assays

The human tumor cell lines HL-60, SMMC-7721, A-549, MCF-7, and SW-480 were used, which were obtained from ATCC (Manassas, VA, USA). All cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere with 5% CO_2. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of MTS (Sigma, St. Louis, MO, USA) (Monks et al., 1991). Briefly, adherent cells (100 μL) were seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before test compound addition, while suspended cells were seeded just before test compound addition, both with an initial density of 1×10^5 cells/mL in 100 μL of 20% SDS-50% DMF after removal of 100 μL of medium. The optical density of the lysate was measured at 595 nm in a 96-well microtiter plate reader (Bio-Rad 680). The IC_{50} value of each compound was calculated by Reed and Muench's method (Reed and Muench, 1938).

4.7. Nitric oxide production in RAW 264.7 macrophages

Murine monocytic RAW264.7 macrophages were dispensed into 96-well plates (2×10^5 cells/well) containing RPMI 1640 medium (Hyclone) with 10% FBS under a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h pre-incubation, cells were treated with serial dilutions of the presence of 1 μg/mL LPS for 18 h. Each compound was dissolved in DMSO and further diluted in medium to produce different concentrations. NO production in each well was assessed by adding 100 μL of Griess reagent (reagent A and reagent B, respectively, Sigma) to 100 μL of each supernatant from LPS (Sigma)-treated or LPS- and compound-treated cells in triplicate. After 5 min incubation, the absorbance was measured at 570 nm with a 2104 Envision multitable plate reader (Perkin-Elmer Life Sciences, Inc., Boston, MA, USA). MG-132 was used as a positive control (Fan et al., 2010).

4.8. Determination of cytotoxic effects

The cytotoxicity of tested compounds was evaluated using an MTS assay (Monks et al., 1991). Briefly, RAW264.7 cells, 2×10^5 cells/well, were seeded in 96-well plates. After 24 h incubation, cells were treated with or without test compounds at given concentrations for 18 h. Then, MTS was added to each well and the plates were kept for 4 h. Testing compounds were dissolved in DMSO, and the absorbance was read at 490 nm. Cytotoxicity was calculated by cell viability of cells without compounds as 100%.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.05.014.

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