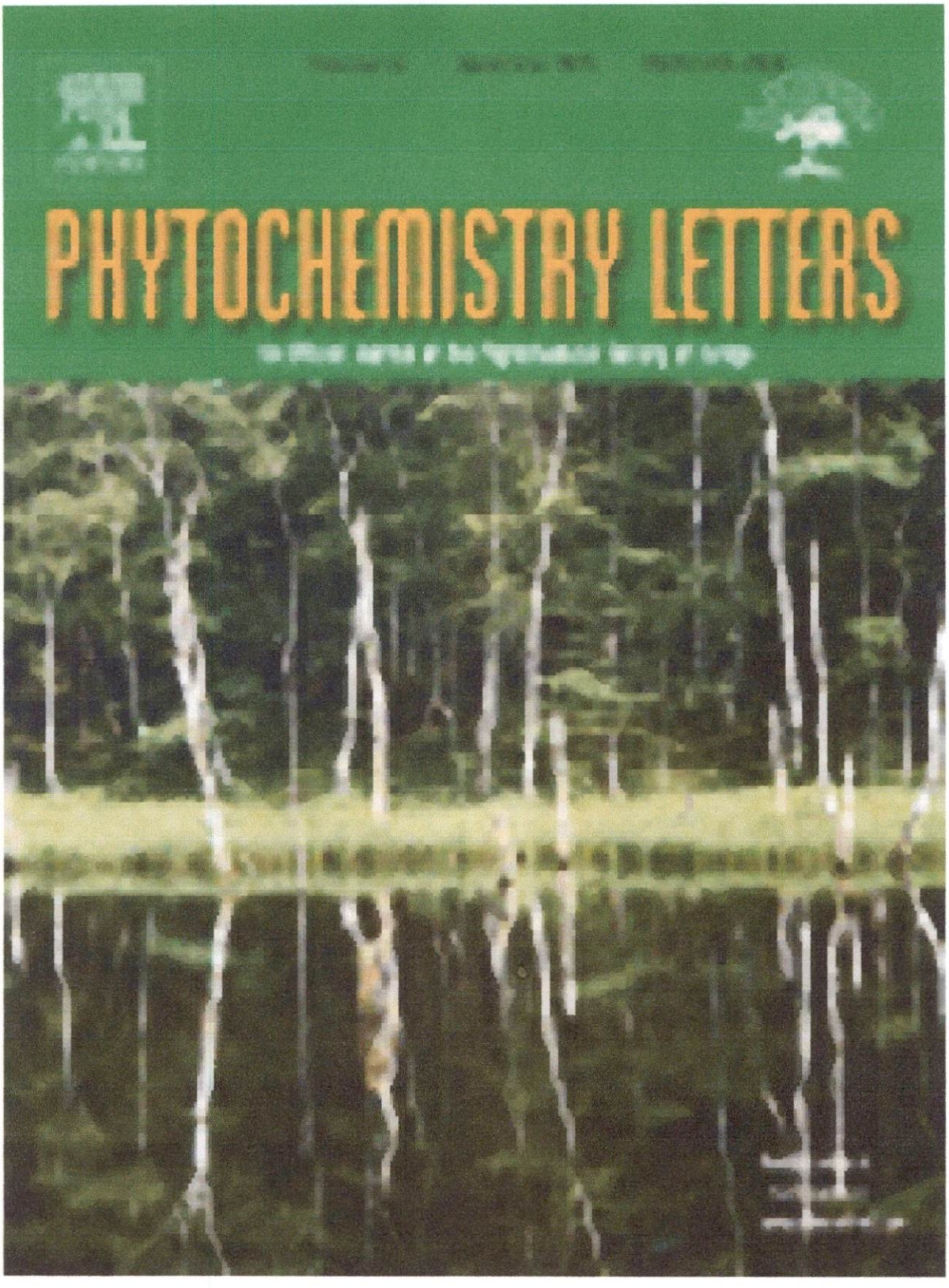
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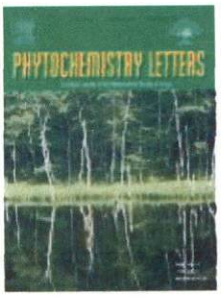

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1. Invited Mini Reviews

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Short communication

New *cis-ent*-clerodanes from *Linaria japonica*

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ABSTRACT

Five new *cis-ent*-clerodanes, linarenones A–E (**1–5**) and two known compounds (**6** and **7**) were isolated from whole plant of *Linaria japonica*. The structures of these compounds were determined by various spectroscopic analyses (UV, IR, HR–ESI–MS, 1D and 2D NMR). The absolute configuration of five new diterpenoids was confirmed by circular dichroism (CD) analysis and chemical conversion. Cytotoxicity of the isolated compounds against A549 cell lines and *Leishmania major* were evaluated. The new *cis-ent*-clerodane **3** was found to be moderately active against A549 cell lines, and new *cis-ent*-clerodanes **1**,**6** and desacetyl-linarienone (**7**) were active against *L. major*.

1. Introduction

Linaria japonica Miq. is a perennial plant that grows in seashore areas, and is used in folk medicine as a diuretic and a purgative. The isolation of flavonoid glycosides (Morita et al., 1974), iridoid glucosides (Kitagawa et al., 1972, 1973), and diterpenoids (Kitagawa et al., 1975, 1976, 1977, 1978, 1980) from *L. japonica* Mig. have reported earlier. On our investigation of the same plant, collected in sandy seashore areas of Tottori Prefecture, new flavonoids, phenylethanoids, iridoid and monoterpene glucosides have been isolated (Otsuka, 1992, 1993a,b, 1994a,b, 1995).

As a result of further investigation of the non-polar fraction, i.e., a mixture of hexane and ethyl acetate layers of the same plant, five new diterpenoids (**1–5**) along with two known diterpenoids (**6** and **7**) were isolated by various chromatographic techniques. The structures of these compounds were determined as follows by spectrometric analysis (Fig. 1). Herein, described are the isolation and structural elucidation of them as well as the cytotoxic activities against lung cancer cell lines (A549) and *Leishmania major*.

2. Results and discussion

2.1. Isolation and structural elucidation of new compounds

The mixture of hexane and ethyl acetate layers from methanol extract of aerial part of *L. japonica* was fractionated by silica gel and ODS column chromatography, then further purified by HPLC to afford seven compounds (Fig. 1). The known compounds were identified as linarienone (**6**) and desacetyl-linarienone (**7**) by comparing the physical and spectroscopic data in literature (Kitagawa et al., 1978). Desacetyl-linarienone (**7**) was isolated for the first time as naturally.

Linarenone A (**1**) was obtained as colorless powder with molecular formula of $C_{22}H_{34}O_4$ as determined by HR–ESI–MS at *m/z* 385.2348 $[M+Na]^+$ (calcd. 385.2349). The IR spectrum had absorption bands at 3340, 1732 and 1237 cm^{-1} indicating the presence of hydroxy and ester carbonyl group. The ^1H NMR spectrum (Table 1) displayed signals due to two tertiary methyls at δ_H 0.56 (s) and 1.23 (s), one secondary methyl at δ_H 0.84 (d, J = 6 Hz), two olefinic methyls at δ_H 1.64 (s) and 1.93 (s), eight methylene protons at δ_H 1.11 (br qd, J = 13, 3 Hz), 1.19 (ddd, J = 14,11, 3), 1.30 (dq, J = 13, 3 Hz), 1.61 (dd, J = 16, 2 Hz), 1.73 (dd, J = 16, 8 Hz), 2.06 (ddd, J = 14, 3, 3 Hz), 2.47 (br d, J = 18 Hz), and 2.71 (dd, J = 18, 6 Hz), two protons of oxygenated methylene at δ_H 4.15 (2H, d, J = 6 Hz), two methine protons at δ_H 1.42 (m) and 1.98 (br d, J = 6 Hz), and an oxygenated methine proton at δ_H 5.19 (dd, J = 8,

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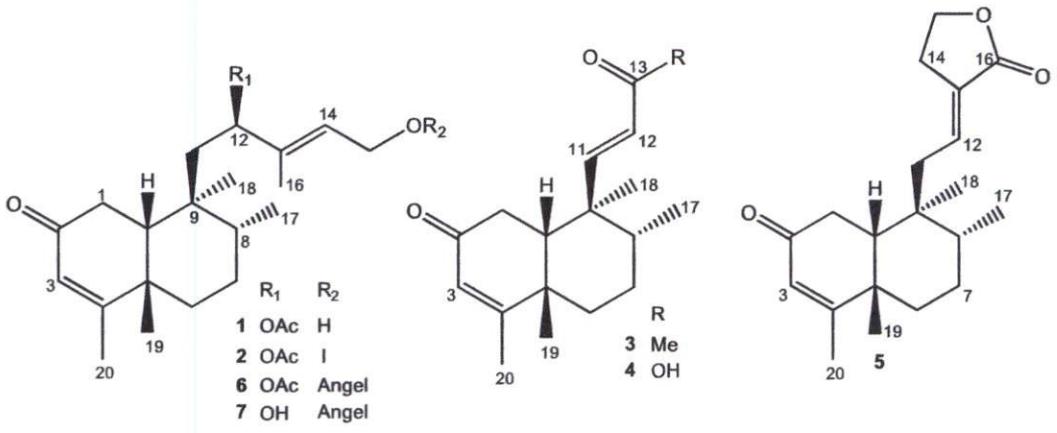

Fig. 1. Structures of compounds **1–7**.

Table 1
The ^{1}H NMR spectroscopic data for compounds 1–5.

Position	1	2	3	4	5
1-α	2.47 br d (18)	2.46 br d (18)	2.25 br d (18)	2.28 br d (18)	2.48 br d (18)
β	2.71 dd (18, 6)	2.71 dd (18, 6)	2.66 dd (18, 6)	2.68 dd (18, 6)	2.72 dd (18, 6)
2	–	–	–	–	–
3	5.83 s	5.83 s	5.85 s	5.86 s	5.84 s
4	–	–	–	–	–
5	–	–	–	–	–
6-α	2.06 ddd (14, 3, 3)	2.07 ddd (14, 3, 3)	2.16 ddd (14, 3, 3)	2.16 ddd (14, 3, 3)	2.07 ddd (14, 3, 3)
β	1.19 ddd (14, 11, 3)	1.18 ddd (14, 11, 3)	1.29 ddd (14, 11, 3)	1.30 ddd (14, 11, 3)	1.22 ddd (14, 11, 3)
7-α	1.11 br qd (13, 3)	1.09 br qd (13, 3)	1.14 br qd (13, 3)	1.14 br qd (13, 3)	1.12 br qd (13, 3)
β	1.30 dq (13, 3)	1.33 dq (13, 3)	1.46 dq (13, 3)	1.46 dq (13, 3)	1.38 dq (13, 3)
8	1.42 m	1.43 m	1.49 m	1.48 m	1.55 m
9	–	–	–	–	–
10	1.98 br d (6)	1.97 br d (6)	1.86 br d (6)	1.86 br d (6)	1.81 br d (6)
11	1.61 dd (16, 2)	1.62 dd (16, 2)	6.44 d (16)	6.72 d (16)	2.16 ddt (16, 6, 2)
	1.73 dd (16, 8)	1.73 dd (16, 8)	–	–	2.32 dd (16, 8)
12	5.19 dd (8, 2)	5.21 dd (8, 2)	6.03 d (16)	5.75 d (16)	6.75 ddt (8, 6, 3)
13	–	–	–	–	–
14	5.59 t (6)	5.53 t (6)	2.24 s	–	2.86 br t (7)
15	4.15 d (6)	4.56 d (6)	–	–	4.37 t, (7)
16	1.64 s	1.68 s	–	–	–
17	0.84 d (6)	0.84 d (6)	0.69 d (6)	0.70 d (6)	0.81 d (6)
18	0.56 s	0.56 s	0.75 s	0.76 s	0.66 s
19	1.23 s	1.23 s	1.24 s	1.24 s	1.20 s
20	1.93 s	1.93 s	1.95 s	1.95 s	1.93 s
12–OAc 2.01 s	2.01 s				
1′		–			
2′		2.52 septet (7)			
3′, 4′		1.14 d (7)			

Recorded at 600 MHz in $CDCl_3$. Chemical shifts (δ) are expressed in ppm and *J* values are presented in Hz in parenthesis.

2 Hz), one acetyl methyl proton signal at δ_H 2.01 (s), two olefinic protons at δ_H 5.59 (t, *J* = 6 Hz) and 5.83 (s). The ^{13}C NMR spectrum (Table 2) of **1** showed 22 carbon resonances that were classified by analysis of its chemical shift values and its HSQC spectrum as five methyls (δ_C 12.3, 16.1, 18.2, 20.3, 31.8), four methylenes (δ_C 28.1, 35.5, 36.5, 39.7), an oxygenated methylene (δ_C 58.7), two methines (δ_C 37.6, 47.5), an oxygenated methine (δ_C 74.5), two olefinics methines (δ_C 125.3, 128.3), four quaternary carbons (δ_C 39.2, 40.5, 137.7, 168.8), an acetoxy carbon signals at δ_C 21.0 and δ_C 169.9, and a ketone (δ_C 198.6).

The NMR spectroscopic data of **1** was closely resembled that of linarienone (**6**), except for some differences in the chemical shift values at C-13, 14 and 15 (**6**: δ_C 140.0, 121.0 and 60.2, respectively). The proton (δ_H 4.15) and carbon resonance (δ_C 58.7) of **1** indicated that C-15 have hydroxy group. The acetate substituent was placed at C-12 on the basis of the HMBC correlation of H-12 (δ_H 5.19) to carbon signal at δ_C 169.9 (12-OAc) (Fig. 2). The A/B ring junction in **1** were deduced to be *cis* on the basis of the ^{13}C NMR chemical shifts of the angular methyl (δ_C 31.8 for C-19), which were found to be in the range δ_C 11-19 for *trans* and higher than δ_C 20 for *cis*-clerodanes (Manabe and Nishino, 1986).

Its relative configuration of **1** was established by NOESY analysis. The correlattion of H-15/Me-16 suggested as *E* configuration. Another correlations were observed between H-10/ Me-19, H-10/H-12, H18/H-20 and Me-17/Me-18, which suggested the orientation of H-10, C-11 and Me-19 to be β-oriented, and those of Me-17 and Me-18 to be α-oriented (Fig. 3). Next, the absolute stereochemistry of the ring moiety of **1** was determined by an analysis of the CD spectrum. The significant positive cotton effect at 331 nm (Δe = +1.02) showed the same absolute stereochemistry with linarienone (**6**). In addition, from the biogenetic point of view, the remaining stereochemistry at C-12 of **1** should be identical with co-occurring *cis*-clerodane derivatives (**6** and **7**). This hypothesis was confirmed by the alkaline hydrolysis of **1** and **6**.

Table 2
^{13}C NMR spectroscopic data for compounds 1–5.

Position	1	2	3	4	5
1	35.5	35.6	36.6	36.3	36.2
2	198.6	198.5	198.3	198.4	198.7
3	128.3	128.4	128.7	128.5	128.8
4	168.8	168.1	167.1	167.2	168.6
5	39.2	39.2	38.3	38.1	39.7
6	36.5	36.5	36.5	36.2	36.7
7	28.1	28.1	27.5	27.2	28.4
8	37.6	37.6	40.5	40.1	36.7
9	40.5	40.5	45.7	45.7	42.2
10	47.5	47.5	49.8	49.5	48.5
11	39.7	39.5	156.3	159.9	37.8
12	74.5	74.3	130.1	120.5	136.7
13	137.7	139.9	197.9	169.8	127.4
14	125.3	120.7	28.2	–	25.6
15	58.7	60.2	–	–	65.6
16	12.3	12.5	–	–	171.3
17	16.1	16.2	17	16.7	16.5
18	18.2	18.2	13.4	13.1	18.6
19	31.8	31.7	31.9	31.7	31.9
20	20.3	20.3	20.5	20.3	20.8
12-OAc	169.9	169.8			
	21.0	21.2			
1′		177.0			
2′		33.7			
3′, 4′		18.8			

Recorded at 125 MHz in $CDCl_3$. Chemical shifts (δ) are expressed in ppm.

The deacyl compounds (**1a** and **6b**) were revealed to be identical by spectroscopic and HPLC analysis. Hence, on the basis of above spectrum analysis, the structure of **1** was determined as (5*S*,8*R*,9*S*,10*R*,12*R*)-2-oxo-12,15-dihydroxy-*cis*-clerod-3*Z*,13(14)*E*-diene 12-acetate.

Linarenone B (**2**) was also a clerodane diterpenoid. It was obtained as colorless solid with molecular formula of $C_{26}H_{40}O_5$ as determined by HR–ESI–MS at *m/z* 455.2766 $[M+Na]^+$ (calcd. 455.2768). The ^{1}H and ^{13}C NMR spectrum (Tables 1 and 2) of **2** were similar to **1**, except for the appearance of isobutanoyl moiety, *i.e.*, an ester carbonyl at δ_C 177.0 (C-1′), a methine at δ_C 33.7 (C-2′), two methyls at δ_C 18.8 (C-3′ and C-4′), and an esterified methylene at δ_H 4.56 (d, *J* = 6 Hz, H-15), δ_C 60.2 (C-15). It suggested the presence of an isobutanoyl moeity at C-15, which was also supported by HMBC correlations of H-15 to C-1′ (Fig. 2). The relative and absolute stereochemistry of **2** was revealed to be the same as **1** by NOESY (Fig. 3), CD spectrum at 333 nm ($\Delta\varepsilon$ = +0.95) and a mild alkaline hydrolysis of **2** by which the deacyl derivative of **2** (**2a**) was identical to **1a** and **6b**, thus the structure of **2** was deduced as (5*S*,8*R*,9*S*,10*R*,12*R*)-2-oxo-15-isobutanoyl-*cis*-clerod-3*Z*,13(14)*E*-diene 12-acetate.

Linarenone C (**3**) was also colorless amorphous powder and displayed an $[M+Na]^+$ ion peak at *m/z* 297.1828 (calcd. 297.1825) corresponding to a molecular formula of $C_{18}H_{26}O_2$. The ^{1}H and ^{13}C NMR spectrum (Tables 1 and 2) displayed two *trans* coupled doublets with *J* = 16 Hz at δ_H 6.03 (H-12) and δ_H 6.44 (H-11), a singlet methyl at δ_H 2.24 (Me-14), a carbonyl group at δ_C 197.9 (C-13), which is characteristic as a conjugated methyl ketone. The planer structure of **3** was determined by COSY and HMBC spectra as 2-oxo-14,15-bisnor-3,11*E*-kolavadien-13-one (Kabir et al., 2010) (Fig. 4), however, the chemical shift of C-19 has quite different (δ_C 31.9 for **3**, δ_C 18.9 for 2-oxo-14,15-bisnor-3,11*E*-kolavadien-13-one). It indicated that **3** is an epimer of C-19, *i.e.*, a *cis*-clerodane diterpenoid. The relative configuration of **3** was deduced on the basis of NOESY correlations of H-10/H-11 and H-10/Me-19, which indicated β-orientation, and the correlation between Me-17/Me-18 for α-orientation (Fig. 5). The significant positive cotton effect of CD spectrum at 334 nm ($\Delta\varepsilon$ = +1.16) is similar to **1**. Thus the absolute stereochemistry of **3** should be (5*S*,8*R*,9*S*,10*R*,3*Z*,11*E*)-2-oxo-14,15-bisnor-3*Z*,11*E*-kolavadien-13-one.

Linarenone D (**4**), a colorless amorphous powder, was exhibited a HR–ESI–MS $[M+Na]^+$ ion peak at *m/z* 299.1618 (calcd. 299.1618) suggesting to a molecular formula of $C_{17}H_{24}O_3$. The NMR spectroscopic data (Tables 1 and 2) of **3** and **4** indicated differences regarding the C-13 substituent. The carbon signal at δ_C 169.8 (C-13), the up-field shift to δ_H 5.75 (d, *J* = 16 Hz, H-12) and δ_C 120.5 (C-12) and the downfield shift to δ_H 6.72 (d, *J* = 16 Hz, H-11) and δ_C 159.9 (C-11) indicated a carboxylic acid at the C-13 position. The relative and absolute configuration of **4** was assigned to be the same as that of **3**, based on 1D and 2D NMR analysis including NOESY (Figs. 4 and 5) and CD spectrum at 325 nm ($\Delta\varepsilon$ = +1.22) as described for **3**. Thus, the structure of **4** was elucidated as (5*S*,8*R*,9*S*,10*R*)-2-oxo-14,15-bisnor-3*Z*,11*E*-kolavadien-13-carboxylic acid.

Linarenone E (**5**), isolated as a colorless powder, was assigned the molecular formula of $C_{20}H_{28}O_3$ as determined by HR–ESI–MS at *m/z* 339.1929 $[M+Na]^+$ (calcd. 339.1931). The IR absorption band at 1754 cm^{-1} was indicative of the presence of γ-lactone moiety. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) also revealed a *cis*-clerodane framework, with two tertiary methyls [δ_H/δ_C 0.66 (s)/18.6 (Me-18) and 1.20 (s)/31.9 (Me-19)], a secondary methyl [δ_H 0.81 (d, *J* = 6 Hz); δ_C 16.5 (Me-17)], an olefinic methyl [δ_H 1.93 (s); δ_C 20.8 (Me-20)], an olefinic methine [δ_H 6.75 (ddt, *J* = 8, 6, 3 Hz); δ_C 136.7 (C-12)], a typical γ-lactone ring [δ_C 127.4 (C-13), δ_H 2.86 (br t,

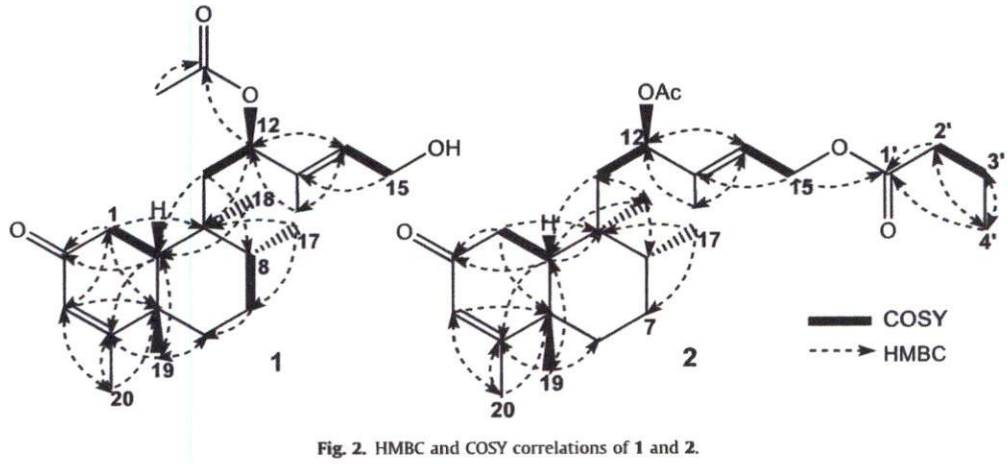

Fig. 2. HMBC and COSY correlations of **1** and **2**.

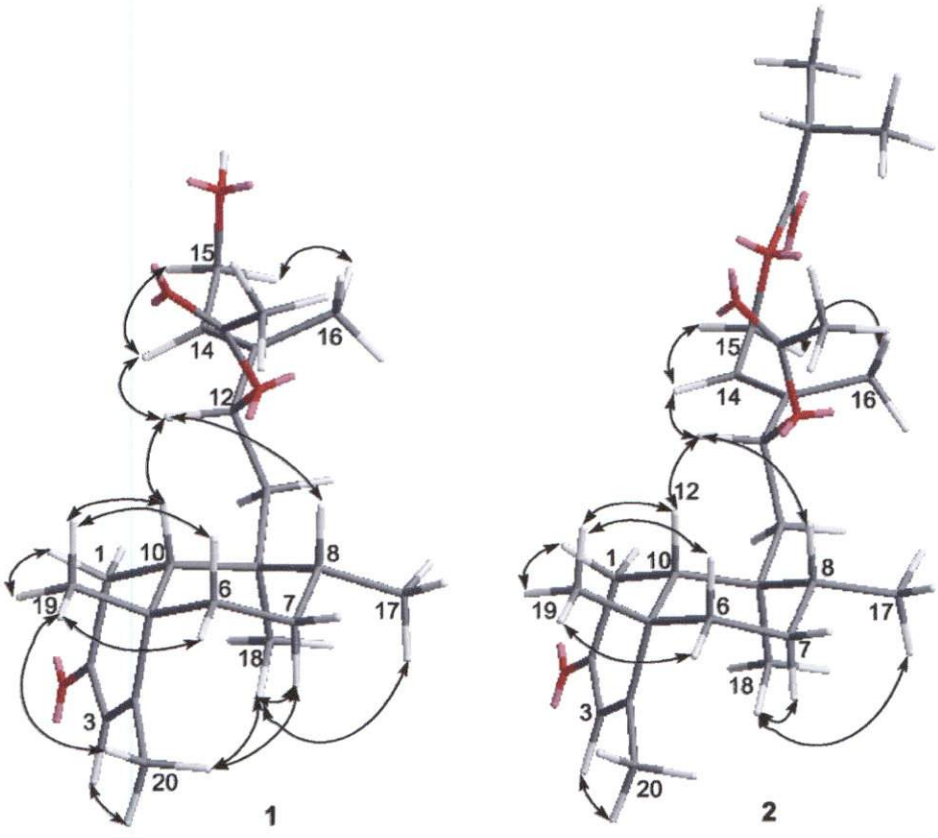

Fig. 3. Key NOESY correlations of **1** and **2**.

J = 7 Hz, H-14), δ_C 25.6 (C-14), δ_H 4.37 (t, J = 7 Hz, H-15), δ_C 65.6 (C-15) and δ_C 171.3 (C-16)] and a six membered ring ketone [δ_C 198.7 (C-2)]. The γ-lactone was connected to C-12 according to the HMBC correlations of H-12 to carbon at δ_C 171.3 (C-16) and 25.6 (C-14), together with the correlations of H-11, 14 and 15 to the carbon at δ_C 127.4 (C-13) (Fig. 6). The NOESY correlations of H-10/Me-19, H-10/H-12, H-3/H-18 and Me-17/Me-18 indicated that H-10, C-11 and Me-19 were β-oriented and Me-17 and Me-18 were α-oriented (Fig. 6), and the absolute stereochemistry of **5** was established by CD spectrum ($\Delta\varepsilon$ = +1.13 at 327 nm) that was similar to **6**. Accordingly, the structure of **5** was determined as (5*S*,8*R*,9*S*,10*R*)-3*Z*,12*E*-2-oxo-dihydrofuran-2(3H)-one.

2.2. *Cytotoxic and anti-*Leishmania major *activity of isolated compounds*

Several clerodane type diterpenoids have been proven to be potential drug leads most notably with anti-cancer effects (Wang et al., 2013, 2012, 2010, and Yang et al., 2010) and anti-*Leishmania* activity (Andres et al., 1997 and Nikolas et al., 2006). Therefore, the

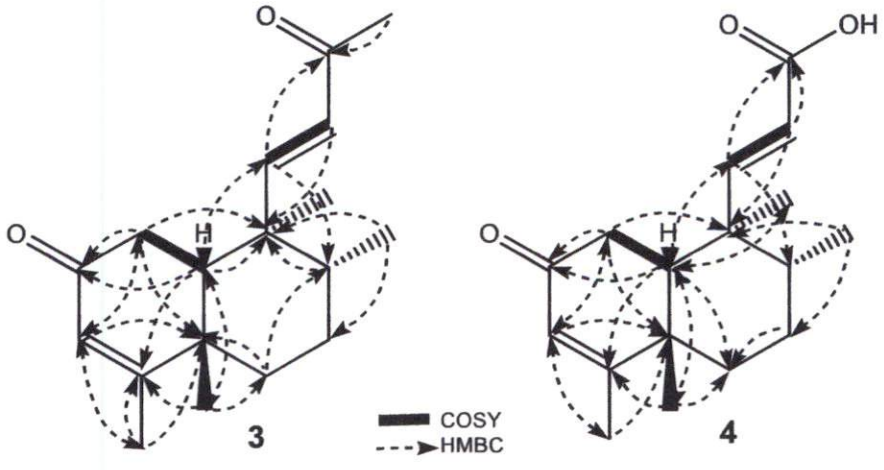

Fig. 4. HMBC and COSY correlations of **3** and **4**.

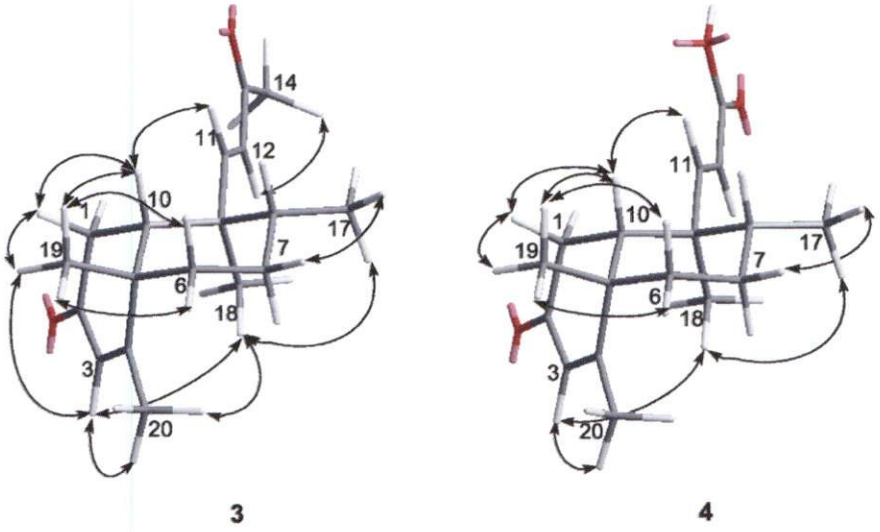

Fig. 5. Key NOESY correlations of **3** and **4**.

isolated compounds (**1-7**) were evaluated for their cytotoxicity against A549 (human lung cancer) cell lines and *L. major*. For A549, linarenone C (**3**) showed moderate inhibition (IC_{50} = 51.2 μM) and linarenone E (**5**) and linarienone (**6**) found to be active (IC_{50} 86.5 and 79.0 μM, respectively). The presence of an acetyl group at C-12 of **3** may improve the activity compared with **4** (inactive) that have carboxylic acid at this position. For *L. major*, linarenon A (**1**), linarienone (**6**) and desacetyl-linarienone (**7**) showed moderate inhibition (IC_{50} 56.7, 50.3 and 52.7 μM, respectively) and linarenon B (**2**) and linarenone E (**5**) found to be active (IC_{50} 89.4 and 97.3 μM, respectively). It is noteworthy that **1** and **7** were relatively selective against *L. major* than cytotoxicity.

In summary, chemical investigation of non-polar fraction of *L. japonica* led to the isolation of seven compounds (**1–7**), including five new diterpenoids (**1–5**). These isolated compounds were evaluated for their cytotoxicity against A549 cell lines and *L. major*. Linarenone C (**3**) showed the moderate inhibition toward A549 cell lines, and linarenone A (**1**), linarienone (**6**) and desacetyl-linarienone (**7**) had activity toward *L. major*.

3. Experimental

3.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were taken on a Bruker Ultrashield 600 spectrometer at 600 MHz and 150 MHz, respectively, with TMS as an internal standard. IR and UV spectra were measured on a HORIBA FT-720 and JASCO V-520 UV/vis spectrophotometer, respectively. Optical rotations and CD spectra were measured on a JASCO P-1030 digital polarimeter and a JASCO J-720 spectropolarimeter, respectively. Positive ion HR–ESI–MS was performed with an Applied Biosystems QSTAR XL NanoSprayTM System. Silica

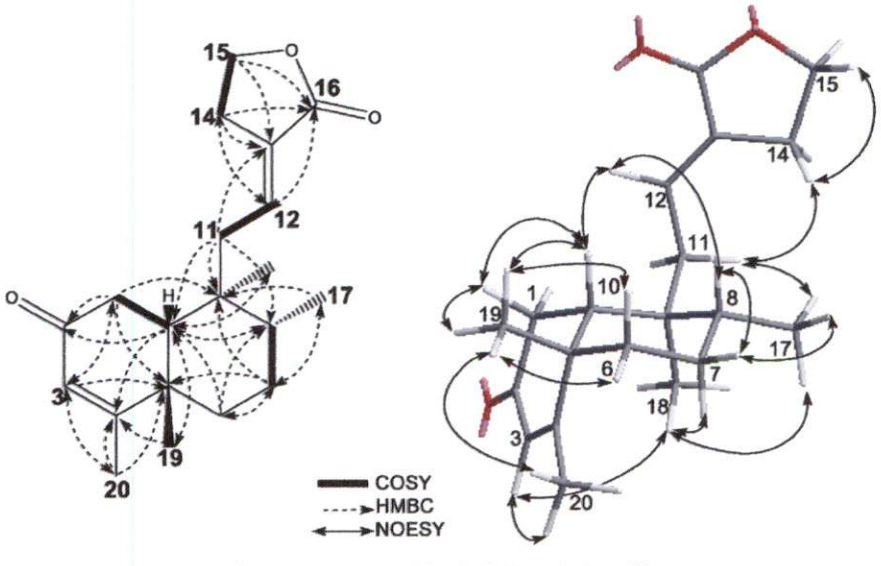

Fig. 6. HMBC, COSY and key NOESY correlations of **5**.

gel open column chromatography (CC) and reversed phase [octadecyl silylated silica gel (ODS)] CC were performed on silica gel 60 (E. Merck, Darmstadt, Germany), and Cosmosil 75C18-OPN (Nacalai Tesque, Kyoto, Japan; $\Phi = 35$ mm, $L = 350$ mm), respectively. HPLC was performed on an ODS column (Inertsil ODS-3, GL Science, Tokyo, Japan; $\Phi = 6$ mm, $L = 250$ mm) and the eluate was monitored with a JASCO RI-930 intelligent refractive index detector and a JASCO PU-1580 intelligent pump.

3.2. Plant material

Whole plants of *L. japonica* were collected in late July 1990 in seashore areas of Tottori Prefecture, and a voucher specimen (90-LJ-Tottori) was deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Hiroshima University.

3.3. Extraction and isolation

The air-dried plants (2.30 kg) were extracted with MeOH (15 L × 2). The MeOH solution was concentrated and adjusted to 95% aqueous MeOH by the addition of H_2O and then partitioned with *n*-hexane (1.5 L × 2, 35.0 g). The remaining aqueous MeOH layer was evaporated and re-suspended in 1.5 L of H_2O and then partitioned with EtOAc (1.5 L × 2, 49.7 g) and 1-BuOH (1.5 L × 3, 151 g), successively.

The non-polar fraction (60.5 g, as a mixture of EtOAc and *n*-hexane layer) was proceeded on silica gel (300 g) CC with increasing polarity [Hexane–$CHCl_3$ (1:1), 4l, $CHCl_3$–MeOH (50:1, 40:1, 30:1, 20:1, 15:1, 10:1, 7:1, 5:1, 3:1, 2:1, MeOH, each 2 L)] yielding 12 fractions (Fr. Lj1–Lj12). The fraction Lj4 (10.1 g) was subjected to reversed phase (ODS) CC with 10% aq. MeOH to 100% MeOH, step gradient, led 19 fractions (Fr. Lj4-1–Lj4-19). The residue of fraction Lj4-10 (54.6 mg) was purified by HPLC (65% aq. MeOH) to give linarenone A (**1**, 7.58 mg). The fraction Lj4-12 (178 mg) was also purified by HPLC (67.5% aq. MeOH). Two peaks appeared at 18 and 35 min were collected to give desacetyl-linarienone (**7**, 5.01 mg) and linarenone B (**2**, 3.07 mg). The other residue of fraction Lj4-9 (91.9 mg) was purified by prep. HPLC (45% aq. acetone) to give **3** (linarenone C, 2.83 mg) and **5** (linarenone E, 12.7 mg). The fraction Lj4-14 (238 mg) was purified by prep. HPLC (65% aq. acetone) to give **4** (linarenone D, 6.64 mg). Then the fraction Lj4-13 (491 mg) was purified by prep. HPLC (80% aq. MeOH) to give **6** (linarienone, 20.6 mg).

3.3.1. Linarinone A (**1**)

Colorless amophous solid; $[\alpha]_D^{26}$ + 10.0 (c 0.50, $CHCl_3$); UV (EtOH) λmax (log ε) nm: 230 (3.72); CD (c 2.76 × 10^{-5} M, MeOH) Δε (nm): +1.02 (331); IR (film) ν_{max} cm^{-1}: 3340, 2929, 1732, 1716, 1653, 1456, 1375, 1237, 1029; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; positive HR-ESI-MS m/z 385.2348 $[M+Na]^+$ (calcd. for $C_{22}H_{34}O_4Na$: 385.2349).

3.3.2. Linarenone B (**2**)

Colorless amorphous solid; $[\alpha]_D^{31}$ + 5.7 (c 0.20, $CHCl_3$); UV (EtOH) λmax (log ε) nm: 224 (3.45); CD (c 1.94 × 10^{-5} M, MeOH) Δε (nm): +0.95 (333); IR (film) ν_{max} cm^{-1}: 2924, 1733, 1716, 1653, 1456, 1375, 1239, 1027; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; positive HR-ESI-MS m/z 455.2766 $[M+Na]^+$ (calcd. for $C_{26}H_{40}O_5Na$: 455.2768).

3.3.3. Linarenone C (**3**)

Colorless amorphous solid; $[\alpha]_D^{31}$ +15.5 (c 0.21, $CHCl_3$); UV (EtOH) λmax (log ε) nm: 232 (3.45); CD (c 2.33 × 10^{-5} M, MeOH) Δε (nm): +1.16 (334), −2.10 (246); IR (film) ν_{max} cm^{-1}: 2924, 1731, 1698, 1655, 1619, 1456, 1376, 1258, 1033; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; positive HR-ESI-MS m/z 297.1825 $[M+Na]^+$ (calcd. for $C_{18}H_{26}O_2Na$: 297.1825).

3.3.4. Linarenone D (**4**)

Colorless amorphous solid; $[\alpha]_D^{27}$ + 12.6 (c 0.44, $CHCl_3$); UV (EtOH) λmax (log ε) nm: 218 (3.62); CD (c 3.21 × 10^{-5} M, MeOH) Δε (nm): +1.22 (325), −3.32 (250); IR (film) ν_{max} cm^{-1}: 2925, 1715, 1698, 1649, 1456, 1376, 1260, 1034; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; positive HR-ESI-MS m/z 299.1618 $[M+Na]^+$ (calcd. for $C_{17}H_{24}O_3Na$: 299.1618).

3.3.5. Linarenone E (**5**)

Colorless amorphous solid; $[\alpha]_D^{27}$ + 48.8 (c 0.82, MeOH); UV (EtOH) λmax (log ε) nm: 239 (2.97); CD (c 5.21 × 10^{-5} M, MeOH) Δε (nm): +1.31 (327); IR (film) ν_{max} cm^{-1}: 2923, 1754, 1660, 1435, 1377, 1256, 1206, 1031; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; positive HR-ESI-MS m/z 339.1929 $[M+Na]^+$ (calcd. for $C_{20}H_{28}O_3Na$: 339.1931).

3.3.6. Linarienone (**6**)

Colorless amophous solid; $[\alpha]_D^{26}$ + 23.5 (c 0.17, MeOH); CD (c 2.77 × 10^{-5} M, MeOH) Δε (nm): +0.76 (334), −0.05 (271); ^{1}H NMR ($CDCl_3$, δ): 0.55 (3H, s, H_3-18), 0.82 (3H, d, J = 6 Hz, H_3-17), 1.08 (1H, br qd, J = 13, 3 Hz, H-7α), 1.18 (1H, ddd, J = 14, 11, 3 Hz, H-6β), 1.21 (3H, s, H_3-19), 1.32 (1H, dq, J = 13, 3 Hz, H-7β), 1.43 (1H, m, H-8), 1.62 (2H, m, H_2-11), 1.69 (3H, s, H_3-16), 1.71 (1H, dd, 16, 8 Hz, H-11), 1.85 (3H, s, H_3-5′), 1.92 (6H, br s, H_3-20 and H_3-4′), 1.95 (1H, br d J = 6 Hz, H-10), 2.00 (3H, s, 12-OAc), 2.05 (1H, ddd, J = 14, 3, 3 Hz, H-6α), 2.45 (1H, br d, J = 18 Hz, H-1α) and 2.70 (1H, dd, J = 18, 6 Hz, H-1β), 4.63 (2H, d, J = 6 Hz, H_2-15), 5.21 (1H, dd, J = 7, 2 Hz, H-12), 5.58 (1H, t, J = 6 Hz, H-14), 5.82 (1H, s, H-3), 6.02 (1H, dq, J = 7, 1 Hz, H-3′) and ^{13}C NMR ($CDCl_3$, δ): 12.7 (CH_3-16), 16.4 (CH_3-17), 18.4 (CH_3-18), 20.6 (CH_3-20), 20.6 (C-5′), 21.2 (C-4′ and CH_3CO), 28.3 (C-7), 31.9 (CH_3-19), 35.7 (C-1), 36.7 (C-6), 37.7 (C-8), 39.4 (C-11), 40.7 (C-5), 42.6 (C-9), 47.7 (C-10), 60.2 (C-15), 74.5 (C-12), 120.8 (C-14), 121.0 (C-3), 127.8 (C-3′), 128.5 (C-2′), 137.8 (C-4), 140.0 (C-13), 167.9 (CH_3CO), 170.0 (C-1′), 198.9 (C-2); positive HR-ESI-MS m/z 467.2773 $[M+Na]^+$ (calcd. for $C_{27}H_{40}O_5Na$: 467.2768).

3.3.7. Desacetyllinarienone (**6a = 7**)

Colorless amorphous solid; $[\alpha]_D^{30.0}$ + 67.5 (c = 0.04, MeOH); UV (EtOH) λmax(log ε) nm: 247 (3.83); CD (c = 1.24 × 10^{-5} M, MeOH) Δε (nm): +8.61 (327), +0.10 (271); IR (film) ν_{max} cm^{-1}: 3430, 2926, 1714, 1700, 1650, 1456, 1225, 1151, 1080; ^{1}H NMR ($CDCl_3$, δ): 0.56 (3H, s, H_3-18), 0.86 (3H, d, J = 6 Hz, H_3-17), 1.12 (1H, br qd, J = 13, 3 Hz, H-7α), 1.22 (3H, s, H_3-19), 1.24 (1H, ddd, J = 14, 11, 3 Hz, H-6β), 1.35 (1H, dq, J = 13, 3 Hz, H-7β), 1.60 (2H, m, H-11), 1.72 (3H, s, H_3-16), 1.87 (3H, s, H_3-5′), 1.93 (3H, s, H_3-20), 1.96 (3H, dq, J = 7, 1 Hz, H_3-4′), 2.02 (1H, br d, J = 6 Hz, H-10), 2.06 (1H, ddd, J = 14, 3, 3 Hz, H-6α), 2.45 (1H, br d, J = 18 Hz, H-1α), 2.69 (1H, dd, J = 18, 6 Hz, H-1β), 4.16 (1H, br t, J = 6 Hz, H-12), 4.67 (2H, t, J = 7 Hz, H_2-15), 5.59 (1H, t, J = 7 Hz, H-14), 5.83 (1H, s, H-3), 6.04 (1H, dq, J = 7, 1 Hz, H-3′); positive HR-ESI-MS m/z 425.2661 $[M+Na]^+$ (calcd. for $C_{25}H_{38}O_4Na$: 425.2662).

3.3.8. Desdiacyl-linarienone (**6b = 1a = 2a**)

Colorless amorphous solid; $[\alpha]_D^{30}$ + 62.4 (c 0.11, MeOH); UV (EtOH) λmax (log ε) nm: 248 (4.50); CD (c 1.24 × 10^{-5} M, MeOH) Δε (nm): +1.62 (330), +0.00 (292); IR (film) ν_{max} cm^{-1}: 3382, 2927, 1730, 1717, 1649, 1456, 1379, 1258, 1004; ^{1}H NMR ($CDCl_3$, δ): 0.57 (3H, s, H_3-18), 0.86 (3H, d, J = 7 Hz, H_3-17), 1.10 (1H, br qd, J = 13, 3, H-7α), 1.23 (3H, s, H_3-19), 1.26 (1H, ddd, J = 13, 11, 3, H-6β), 1.35 (1H, dq, J = 13, 3, H-7β), 1.54 (2H, m, H_2-11), 1.67 (3H, s, H_3-16), 1.94 (3H, s, H_3-20), 2.07 (1H, ddd, J = 14, 3, 3, H-6α), 2.09 (1H, m, H-10), 2.44 (1H, br d, J = 18 Hz, H-1α), 2.68 (1H, dd, J = 18, 6 Hz, H-1β), 4.13 (1H, m, H-12), 4.17 (2H, m, H_2-15), 5.59 (1H, t, J = 7 Hz, H-14), 5.84 (1H, s, H-3) and ^{13}C NMR ($CDCl_3$, δ): 12.6 (C-16), 17.1 (C-17), 19.4 (C-18), 21.0 (C-20), 28.9 (C-7), 32.6 (C-19), 36.4 (C-1), 37.3 (C-6), 38.2 (C-8), 39.9 (C-5), 41.2 (C-9), 42.0 (C-11), 47.9 (C-10), 59.5 (C-15), 74.3 (C-

12), 124.4 (C-14), 129.1 (C-3), 142.9 (C-13), 168.9 (C-4), 199.3 (C-2); positive HR-ESI-MS m/z 343.2246 $[M+Na]^+$ (calcd. for $C_{20}H_{32}O_3Na$: 343.2244).

3.4. Mild alkaline hydrolysis

To a solution of linarienone (**6**) (11.5 mg) in MeOH–$CHCl_3$ (1:1, 1.8 ml) was added 1 mol/l NaOMe solution (0.2 ml), stirred at rt for 3 h. The mixture was neutralized with ion exchange resin IR-120B (ORGANO, H^+-form) and filtered off. The filtrate was evaporated to dryness under reduced pressure. Purification of the reaction product by preparative TLC (n-hexane:EtOAc = 3:2) furnished desacetyl-linarienone (**6a** = **7**) (0.3 mg, R_f = 0.45) and desdiacyl-linarienone (**6b**) (1.2 mg, R_f = 0.18). This procedure also performed for **1** and **2** to produce **1a** and **2a**, respectively, which were identical to **6b** by HPLC (65% aq. MeOH, ODS Φ = 6 mm × 250, UV–vis detector 248 nm, 1.5 ml/min, t_R = 16 min) and HR-ESI-MS analysis.

3.5. A549 growth inhibition assay

Human lung cancer cells (A549) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, kanamycin (100 μg/ml) and amphotericin B (0.5 μg/ml). Into a 96-well plate, aliquots of the DMSO solution of the test compounds (1% final concentration) were incubated with A549 cells (5×10^3 cells/well) in a CO_2 incubator at 37 °C for 72 h. MTT was added into each well and the plate was further incubated for 1.5 h. Absorbance was measured at 540 nm using a 2300 EnSpire Multimode plate reader (Perkin Elmer). DMSO was used as a negative control and doxorubicin as a positive control (IC_{50} = 0.70 μM). The viability was compared to that of control cells incubated in the same medium without the test compounds (Macahig et al., 2010).

$$\text{Inhibition (\%)} = [1 \times (A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

3.6. *Anti*-Leishmania major *assay*

L. major promastigotes were cultured in M199 medium supplemented with 10% heat-inactivated fetal bovine serum and kanamycin (100 μg/mL). Into a 96-well plate, aliquots of the DMSO solution of the test compounds (1% final concentration) were incubated with *L. major* cells (1×10^5 cells/well) at 27 °C for 72 h. MTT was added into each well and further incubated overnight. Absorbance was measured at 540 nm using a 2300 EnSpire Multimode plate reader (Perkin Elmer). DMSO was used as a negative and amphotericin B as positive control (IC_{50} = 0.58 μM). The viability was compared to that of control cells incubated in the same medium without the test compounds (Macahig et al., 2010).

$$\text{Inhibition (\%)} = [1 \times (A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

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