Phytochemistry 96 (2013) 201–207

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Eudesmane-type sesquiterpene derivatives from *Laggera alata*

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ARTICLE INFO

Article history:
Received 8 December 2012
Received in revised form 20 February 2013
Available online 15 August 2013

Keywords:
Laggera alata
Compositae
Eudesmane
Sesquiterpene

ABSTRACT

Ten eudesmane-type sesquiterpene derivatives (**1**–**10**), including six cuauhtemone derivatives (**1**–**6**), one di-norsesquiterpene (3-oxo-di-*nor*-eudesma-4-en-11-oic acid, **7**), and three eudesmane glycosides (alatoside F–H, **8**–**10**) were isolated from the whole plants of *Laggera alata* together with 12 known compounds. Their structures were elucidated on the basis of extensive spectroscopic analysis, acid hydrolysis, and compounds **1** and **7** were studied by single-crystal X-ray diffraction analysis. The absolute configuration of **1** was determined by the application of the modified Mosher's method. All of the isolated eudesmane-type sesquiterpenes were evaluated for their cytotoxic activities on six human cancer cell lines, but all of the compounds were inactive on the tested cell lines in the concentration of 100 μg/mL.

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1. Introduction

The genus *Laggera* (Compositae), consisting of about 20 species, is mainly distributed in tropical Africa and Southeast Asia (Zheng et al., 2003b). *Laggera alata* (D. Don) Sch. Bip. ex Oliv. and *L. pterodonta* (DC.) Benth are the only two species of *Laggera* found in China, and both are employed as traditional herbal medicines due to their anti-inflammatory and anti-bacterial activities (Deng, 1963). Previous studies on the genus *Laggera* established that its plants contained a variety of compounds, whose major constituents were eudesmane-type sesquiterpenes (Ahmed and Mahmoud, 1998; Li et al., 2007; Onayade et al., 1990; Raharivelomanana et al., 1998; Zhao et al., 1997a, 1997b; Zheng et al., 2003a, 2003b). As part of our work on searching for bioactive constituents from traditional herbal medicines, 10 new (**1**–**10**) and 12 known (**11**–**22**) eudesmane-type sesquiterpene derivatives were isolated from the whole plants of *L. alata*. In 1addition, the cytotoxic effects of all the isolated compounds on a panel of human cancer cell lines were evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, but these were inactive. This paper describes the isolation, structure elucidation, and cytotoxic investigation results of the eudesmane-type sesquiterpene derivatives from *L. alata*.

2. Results and discussion

The 95% EtOH extract of the whole plants of *L. alata* was subjected to macrosporous resin, silica gel, and Sephadex LH-20 column chromatography (CC), as well as preparative purification procedures HPLC to give compounds **1**–**22**. The structures of the new compounds **1**–**10** (Fig. 1) were elucidated by the extensive spectroscopic analysis, acid hydrolysis, and single-crystal X-ray diffraction analysis. In addition to ten new compounds, the twelve known ones were identified as pterodonoic acid (**11**) (Cui et al., 1998), 15-hydroxyisocostic acid (**12**) (Dawidar and Metwally, 1985), β-isocostic acid (**13**) (Bohlmann et al., 1977), 1β-hydroxy-eudesma-4,11(13)-dien-12-oic acid (**14**) (Ohmoto et al., 1987), 5α-hydroxy-4α,15-dihydrocostic acid (**15**) (Zdero et al., 1990), 2α-hydroxylilicic acid (**16**) (Abu Zarga et al., 1998), 5β-hydroxylilicic acid (**17**) (Zheng et al., 2003a), ilicic acid (**18**) (Herz et al., 1966), (4*S*∗,5*E*,10*R*∗)-7-oxo-tri-*nor*-eudesm-5-en-4β-ol (**19**) (Cheng et al., 2009), costic acid (**20**) (Herz et al., 1966), 5α-hydroxycostic acid (**21**) (Zdero et al., 1990), and cuauhtemone (**22**) (Nakanishi et al., 1974), respectively, by comparing their NMR and MS data with literature data.

The HRESIMS of **1** indicated a quasi-molecular ion peak at *m*/*z* 475.2310 $[M+Na]^+$ corresponding to the molecular formula of $C_{24}H_{26}O_8$ (calcd for $C_{24}H_{26}O_8Na$, 475.2302). The ^{13}C NMR spectrum showed the presence of 24 carbon resonances, consistent with the putative molecular formula, including an α,β-unsaturated ketone at δ_C 201.9, two olefinic carbons at δ_C 146.4, 130.4, and three ester carbonyls at δ_C 170.2, 169.8, 168.9. Analysis of the ^1H and ^{13}C NMR spectroscopic data of **1** (Tables 1 and 2) suggested that **1** was a cuauhtemone derivative. Four singlet methyls at δ_H 1.85, 2.06, 0.96 and 1.26 were assigned to H-12, H-13, H-14 and H-15 of the

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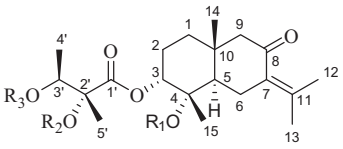
http://dx.doi.org/10.1016/j.phytochem.2013.07.014



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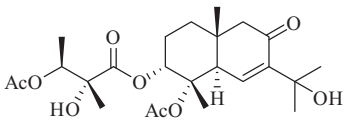

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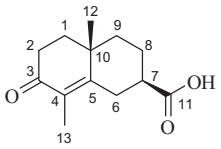

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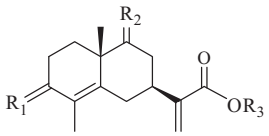

8 R_1 = O R_2 = H R_3 = β-D-Glc
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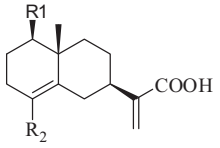

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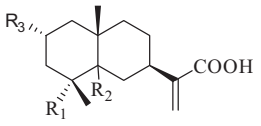
12 R_1 = H R_2 = CH_2OH
13 R_1 = H R_2 = CH_3
14 R_1 = OH R_2 = CH_3



15 R_1 = H R_2 = α-OH R_3 = H
16 R_1 = OH R_2 = α-H R_3 = OH
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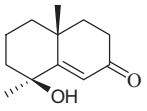
18 R_1 = OH R_2 = α-H R_3 = H



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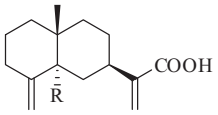

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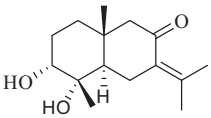

22

Fig. 1. Chemical structures of compounds **1**–**22**.

Table 1
^{1}H NMR spectroscopic data of compounds **1**–**6** (J in Hz).

No.	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[b]	**6**[b]
1	1.32 m	1.19 m	1.26 m	1.29 m	1.29 m	1.26 m
	1.48 m	1.31 m	1.39 m	1.45 m	1.54 m	1.50 m
2	1.79 m	1.81 m	1.71 m	1.81 m	1.81 m	1.81 m
	1.81 m	1.90 m	2.08 m	2.04 m	1.90 m	1.90 m
3	5.01br s	5.83 br s	5.81 br s	4.91 br s	5.91 br s	5.90 br s
5	1.91 dd (13.5, 4.0)	2.15 m	2.14 m	1.93 m	2.25 m	3.00 d (1.5)
6	2.21 m	2.20 m	2.22 m	2.18 m	2.20 m	6.88 d (1.5)
	3.00 dd (15.4, 3.3)	2.84 d (12.9)	2.89d (14.8)	2.94 dd (15.3, 3.8)	2.89 d (12.0)	
9	2.21 m	2.18 m	2.21 m	2.23 m	2.25 m	2.34 m
12	1.85 s	1.80 s	1.85 s	1.83 s	1.86 s	1.45 s
13	2.06s	2.00 s	2.07 s	2.04 s	2.07 s	1.45 s
14	0.96 s	0.93 s	0.97 s	0.95 s	1.00 s	0.99 s
15	1.26 s	1.53 s	1.58 s	1.28 s	1.60 s	1.55 s
3′	5.23 q (6.4)	5.04 q (6.2)	5.12 q (8.1)	5.13 q (6.4)	3.95 q (6.4)	5.11 q (6.4)
4′	1.25 d (6.4)	1.25 d (6.2)	1.17 d (8.1)	1.31 d (6.4)	1.25 d (6.4)	1.30 d (6.4)
5′	1.66 s	1.29 s	1.62 s	1.41 s	1.30 s	1.33 s
AcO-4	2.07 s	1.91 s	2.07 s		1.96 s	1.98 s
AcO-2′	2.07 s		2.05 s			
AcO-3′		1.93 s	2.05 s	1.99 s		1.99 s

[a] Measured at 500 MHz in $CDCl_3$.
[b] Measured at 400 MHz in $CDCl_3$.

presumed cuauhtemone moiety on the basis of HMBC correlations, respectively. The downfield shifted proton at δ_H 5.01 (H-3) indicated that the hydroxyl group at C-3 was esterified, which was further confirmed by the correlation between H-3 and C-1′ (δ_C 168.9) in the HMBC spectrum. In addition, the HMBC correlation from H-3′ (δ_H 5.23) to carbonyl group (δ_C 169.8), and the comparison of downfield shifted signals at δ_H 5.23 (H-3′) and δ_C 82.1 (C-2′) with the reported data (Ahmad et al., 1991) indicated the side-chain was a 2′,3′-diacetoxy-2′-methylbutyryloxy group. The methyl groups of the ester side-chain showing a singlet at δ_H 1.66 and a characteristic doublet at δ_H 1.25 (J = 6.4 Hz) were attributed to H-5′ and H-4′, respectively. The NOESY correlations of H_β-1/H-14, H_β-1/H-3, H-3/H-15 and H-14/H-15 suggested that H-3, H-14 and H-15 were on the same side of the cuauhtemone skeleton. Similarly, the NOESY correlation between H_α-1 and H-5 placed H-5 on the opposite side of the cuauhtemone skeleton. The structure and relative configuration of **1** were further confirmed by single crystal X-ray diffraction analysis (Fig. 2). The absolute configuration of C-3 in **1** was

Table 2
^{13}C NMR spectroscopic data of compounds **1**–**10**.

No.	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[b]	**6**[b]	**7**[a]	**8**[c]	**9**[c]	**10**[c]
1	33.8	32.3	33.0	33.3	33.0	31.7	37.2	38.4	36.2	79.0
2	24.0	22.9	22.4	23.9	22.8	23.0	33.7	34.5	28.2	27.9
3	78.4	74.5	74.2	79.0	71.7	73.9	198.9	201.5	33.0	33.0
4	72.1	82.9	82.7	72.3	83.1	81.5	129.9	129.9	145.8	134.6
5	47.6	45.0	45.2	46.9	45.3	48.6	159.1	164.5	126.7	125.5
6	25.5	25.7	25.8	25.5	25.8	145.4	29.4	34.6	32.7	28.7
7	130.4	130.0	130.0	130.5	130.2	140.7	43.1	41.5	41.5	41.4
8	201.9	201.7	201.3	201.9	201.5	200.0	24.3	28.2	37.0	32.7
9	60.1	59.7	60.4	59.9	60.2	57.7	40.8	43.0	218.3	40.1
10	35.9	35.8	35.7	36.0	35.9	39.0	35.6	37.0	48.4	40.6
11	146.4	145.5	146.3	145.5	145.7	71.7	179.6	145.4	134.9	146.3
12	23.1	22.7	23.0	22.8	22.8	29.1	22.3	166.8	167.0	167.2
13	23.7	23.5	23.6	23.6	23.6	28.7	11.0	125.9	125.3	125.0
14	18.7	18.8	19.1	18.6	19.0	18.1		22.7	22.5	17.9
15	17.2	17.8	17.9	21.5	18.0	18.9		11.0	19.1	19.2
1′	168.9	173.7	168.0	174.8	175.0	173.6		96.1	96.0	95.9
2′	82.1	75.7	82.2	76.2	77.2	75.8		74.0	74.0	74.0
3′	72.8	74.2	72.4	74.3	74.5	74.3		78.9	78.9	78.8
4′	14.6	13.1	14.4	13.3	16.7	13.2		71.1	71.1	71.0
5′	20.8	22.1	16.1	22.3	22.0	22.1		78.2	78.2	78.1
6′								62.3	62.3	62.3
AcO-4		169.4	169.4		169.5	169.2				
		22.1	23.6		22.1	22.1				
AcO-2′	170.2		170.1							
	21.1		21.1							
AcO-3′	169.8	170.0	169.8	169.8		170.0				
	21.1	20.9	21.1	21.0		21.0				

[a] Measured at 125 MHz in $CDCl_3$.
[b] Measured at 100 MHz in $CDCl_3$.
[c] Measured at 125 MHz in CD_3OD.

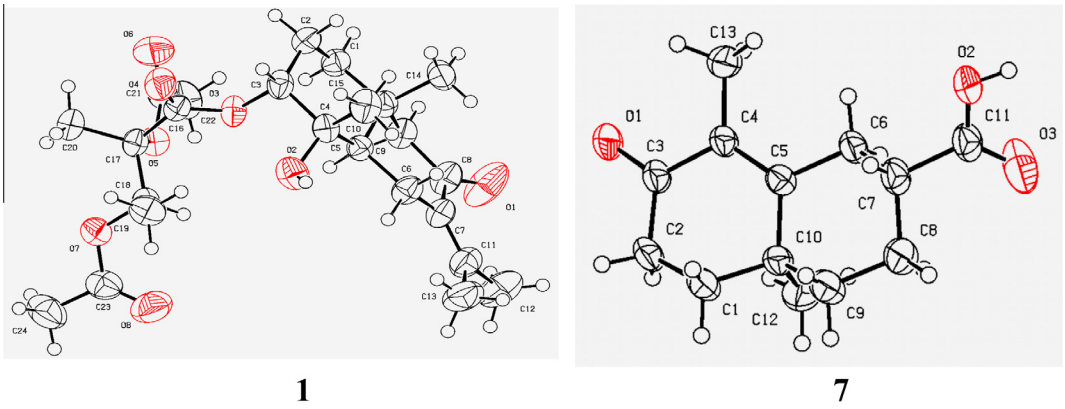

Fig. 2. X-ray crystal structures of **1** and **7**.

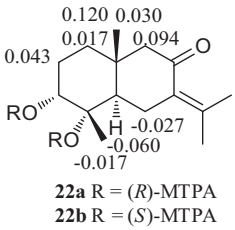

Fig. 3. $\Delta\delta$ Values (δ_S–δ_R) of the MTPA Esters **22a** and **22b**.

determined as *R* by acid hydrolysis and the modified Mosher's method (Fig. 3) (Su et al., 2002). Accordingly, compound **1** was defined as 3-*O*-(2′,3′-diacetoxy-2′-methylbutyryl)-cuauhtemone.

Compound **2** was isolated as a brown oil. The HRESIMS indicated that **2** was an isomer of **1**. The ^{13}C NMR spectroscopic data (Table 2) of the two compounds were similar, except that C-4 of **1** (δ_C 72.1) was shifted downfield to δ_C 82.9 in **2**, and likewise, C-2′ of **1** (δ_C 82.1) was shifted upfield to δ_C 75.7 in **2**. This suggested that the two acetoxy groups in **2** were at the C-4 and C-3′ positions. Therefore, compound **2** was determined as 4-*O*-acetyl-3-*O*-(3′-acetoxy-2′-hydroxy-2′-methylbutyryl)-cuauhtemone.

The molecular formula of compound **3** was established as $C_{26}H_{38}O_9$ by a quasi-molecular ion at *m/z* 517.2406 $[M+Na]^+$ (calcd for $C_{26}H_{38}O_9Na$, 517.2408) in HRESIMS. The NMR spectroscopic data (Tables 1 and 2) of **3** were very similar to those of **1**, except that **3** had the signals of an additional acetyl group (δ_C 170.1, 21.0), and the carbon resonance at C-4 shifted downfield from δ_C 72.1 in **1** to δ_C 82.7 in **3**, suggesting the hydroxyl group at C-4 was esterified. The configuration of **3** was determined as same as that of **1** by the coupling constants and NOESY correlations. So the structure of **3** was elucidated as 4-*O*-acetyl-3-*O*-(2′,3′-diacetoxy-2′-hydroxy-2′-methylbutyryl)-cuauhtemone.

Table 3
^{1}H NMR spectroscopic data of compounds **7**–**10** (*J* in Hz).

No.	**7**[a]	**8**[b]	**9**[b]	**10**[b]
1	1.78 m	1.81 m	1.47 td (13.1, 4.3)	3.40 m
			1.81 dt (13.1, 3.0)	
2	2.42 m	2.35 dt (16.7, 3.8)	1.73 m	1.68 m
	2.53 m	2.57 m	2.38 m	
3			1.68 td (13.1, 3.0)	2.15 m
				1.98 m
6	2.29 d (15.0)	2.13 m	1.95 m	1.57 m
	2.99 d (15.0)	2.93 m	2.72 d (14.3)	1.68 m
7	2.46 m	2.61 m	2.41 m	2.35 tt (12.3, 3.1)
8	1.87 m	1.81 m	2.43 m	1.80 t (13.2)
	1.98 m	1.74 m	2.65 m	2.67 dt (13.2, 2.5)
9	1.44 td (13.6, 3.9)	1.75 m		1.20 td (13.2, 4.5)
	1.72 dt (13.6, 3.2)	1.51 m		2.06 dt (13.2, 3.1)
12	1.24 s			
13	1.79 s	6.39 s	5.78 s	6.32 s
		5.84 s	6.34 s	5.74 s
14		1.28 s	1.26 s	1.02 s
15		1.75 s	1.73 s	1.59 s
1′		5.58 d (7.8)	5.57 d (7.9)	5.57 d (7.9)
2′		3.40 m	3.39 m	3.39 m
3′		3.42 m	3.38 m	3.41 m
4		3.38 m	3.37 m	3.38 m
5′		3.41 m	3.43 m	3.41 m
6′		3.84 dd (12.2, 1.6)	3.84 dd (12.2, 1.7)	3.84 dd (12.2, 1.8)
		3.68 dd (12.2, 4.6)	3.68 dd (12.2, 4.7)	3.69 dd (12.2, 4.8)

[a] Measured at 500 MHz in $CDCl_3$.
[b] Measured at 500 MHz in CD_3OD.

The HRESIMS of compounds **4** and **5** showed that they had a same molecular formula of $C_{22}H_{34}O_7$ by quasi-molecular ions at *m/z* 433.2191 and 433.2195 $[M+Na]^+$ (calcd for $C_{22}H_{34}O_7Na$, 433.2197). The NMR spectroscopic data of **4** and **5** (Tables 1 and 2) were also similar to those of **2**, except for the presence of only one acetyl group in **4** and **5**. Comparison of the chemical shifts at C-4 and C-3′ in **4** (δ_C 72.3, 74.3) and **5** (δ_C 83.1, 71.7) with those in **2** (δ_C 82.9, 74.2) suggested that the acetyl group was connected to C-3′ in **4** and C-4 in **5**, respectively. Thus, compounds **4** and **5** were determined as 3-*O*-(3′-acetoxy-2′-hydroxy-2′-methylbutyryl)-cuauhtemone and 4-*O*-acetyl-3-*O*-(2′,3′-dihydroxy-2′-methylbutyryl)-cuauhtemone, respectively.

The NMR spectroscopic data of compound **6** (Tables 1 and 2) were similar to those of **2**. The ^{1}H NMR spectrum showed the presence of one olefinic proton at δ_H 6.88 (H-6), and four methyl groups at δ_H 1.45 (H-12), 1.45 (H-13), 0.99 (H-14), and 1.55 (H-15) attributable to an eudesmane skeleton. The ^{13}C NMR and DEPT spectra displayed signals for two olefinic carbons at δ_C 145.4 and 140.7, and three oxymethines at δ_C 71.7, 73.9, and 81.5. Additionally, a methylene at δ_C 25.7 (C-6) in a cuauhtemone skeleton was missing. These data suggested that **6** had a skeleton of 11-hydroxy-6,7-dehydroeudesman-8-one (Vera et al., 2008). The NMR signals of the remaining ester side-chains resembled those in **2**, suggesting the locations and compositions of the side-chains. These were further confirmed by the HMBC correlations from H-3 (δ_H 5.90) to C-1′ (δ_C 173.6), and from H-3′ (δ_H 5.11) to the carbonyl group (δ_C 170.0). Therefore, **6** was elucidated as 4α-acetoxy-3α-(3′-acetoxy-2′-methylbutyryloxy)-11-hydroxy-6,7-dehydroeudesman-8-one.

Compound **7** was isolated as a colorless block crystal. Its molecular formula was determined to be $C_{13}H_{18}O_3$ based on a quasi-molecular ion peak at *m/z* 245.1149 (calcd for $C_{13}H_{18}O_3Na$, 245.1148) in HRESIMS. Analysis of the ^{1}H and ^{13}C NMR spectra (Tables 2 and 3) indicated that **7** had a structure similar with pteodonoic acid (**11**) (Cui et al., 1998), except for missing two olefinic protons and two olefinic carbons. Comparison of the molecular formulae and degrees of unsaturation in these two compounds suggested that a carbonyl was connected to C-7 in **7** instead of an allylic acid moiety in **11**. This conclusion was confirmed by a single crystal X-ray diffraction analysis (Fig. 2). Thus, compound **7** was determined as 3-oxo-di-*nor*-eudesma-4-en-11-oic acid.

The molecular formula $C_{21}H_{30}O_8$ of compound **8** was deduced from HRESIMS which showed a quasi-molecular ion peak at *m/z* 433.1831 $[M+Na]^+$ (calcd for $C_{21}H_{30}O_8Na$, 433.1833). Comparison of the NMR spectroscopic data (Tables 2 and 3) of **8** with those of pteodonoic acid (**11**) (Cui et al., 1998) showed that they were very similar except for the presence of an additional glucose moiety (δ_C 96.1, 74.0, 78.9, 71.1, 78.2, 62.3) in **8**. The HMBC correlation from H-1′ (δ_H 5.58) to C-12 (δ_C 166.8) suggested that the sugar moiety was connected to C-12. Acid hydrolysis of **8** yielded D-glucose which was identified by reversed-phase HPLC after conversion of the sugar to thiocarbamoyl-thiazolidine derivative (Tanaka et al., 2007). The large *J* value (7.8 Hz) of the anomeric proton at δ_H 5.58 indicated the glucopyranosyl moiety was β configuration. Thus, compound **8** was determined to be β-D-glucopyranosyloxyl pteodonoate, and named alatoside **F**.

The HRESIMS of compound **9** exhibited a quasi-molecular ion peak at *m/z* 433.1835 $[M+Na]^+$ (calcd for $C_{21}H_{30}O_8Na$, 433.1833), indicating the molecular formula was $C_{21}H_{30}O_8$. The ^{1}H and ^{13}C NMR spectroscopic data (Tables 2 and 3) were similar to those of 9-oxo-4,11(13)-eudesmadien-12-oic acid (Guerreiro et al., 1990; Rustaiyan et al., 1987), except for the presence of an additional glucose moiety (δ_C 96.0, 74.0, 78.9, 71.1, 78.2, 62.3). The HMBC correlation from H-1′ (δ_H 5.57) to C-12 (δ_C 167.0) confirmed that the sugar moiety was connected to C-12. Acid hydrolysis of **9** yielded D-glucose which was identified by the method mentioned above (Tanaka et al., 2007). The *J* value (7.9 Hz) of the anomeric proton suggested the glucopyranosyl was also in a β configuration. Accordingly, **9** was determined as β-D-glucopyranosyloxyl 9-oxo-4,11(13)-eudesmadien-12-oate, and named alatoside G.

The ^{1}H NMR spectrum (Table 3) of compound **10** displayed signals of two olefinic protons at δ_H 5.74 and 6.32, an anomeric proton of sugar at δ_H 5.57 (*J* = 7.9 Hz), an oxygenated methine at δ_H 3.40, and two methyl groups at δ_H 1.02 and 1.59. The ^{13}C NMR spectrum (Table 2) showed resinances of four olefinic carbons (δ_C 134.6, 125.5, 146.3,

125.0), one carboxyl group (δ_C 167.2), two methyl groups (δ_C 17.9, 19.2) and one glucose moiety (δ_C 95.9, 74.0, 78.8, 71.0, 78.1, 62.3). These ^{1}H and ^{13}C NMR spectroscopic data were similar to those of an isolated known compound 1β-hydroxy-eudesma-4,11(13)-dien-12-oic acid (**14**) (Ohmoto et al., 1987), except for the presence of an extra sugar moiety, suggesting that **10** was another eudesmane glycoside. In addition, the correlation between H-1′ (δ_H 5.57) and C-1 (δ_C 79.0) in HMBC spectrum indicated that the sugar moiety was connected to C-1. Acid hydrolysis of **10** yielded D-glucose which was identified by the method mentioned above (Tanaka et al., 2007). The characteristic coupling constant of the anomeric proton (J = 7.9 Hz) indicated that it was a β-D-glucoside. Therefore, **10** was elucidated as 1β-*O*-(β-D-glucopyranosyloxyl)-eudesma-4,11(13)-dien-12-oic acid, and named alatoside H.

Recently, quite a few studies have focused on anticancer activities of sesquiterpenes, including eudesmane-type sesquiterpenes (Cui et al., 2011; Dai et al., 2009; Minakawa et al., 2012; Tanaka et al., 2012; Wang et al., 2007; Xu et al., 2010). The cytotoxic activities of some eudesmane-type sesquiterpene derivatives from *L. pterodonta* were also investigated (Xiao et al., 2003). Therefore, the present study tested the cytotoxic activities of all the isolated eudesmane-type sesquiterpenes with MTT assay on six human cancer cell lines including MFC-7 (human breast carcinoma cell), A375 (human melanoma cell), A549 (human lung carcinoma epithelial cell), Hep-2 (human larynx epidermoid carcinoma cell), CNE (human nasopharyngeal carcinoma epithelia cell), and Hela (human cervical carcinoma cell). However, all of the compounds were inactive on the tested cell lines in the concentration of 100 μg/mL.

3. Concluding remarks

Eudesmane sesquiterpenes are the primary chemical constituents of *Laggera* species, which is consistent with the results of this study on *L. alata*. To the best of our knowledge, three cuauhtemone derivatives had been previously reported from *L. alata* (Zdero and Bohlmann, 1989), and this paper added 22 cuauhtemone derivatives into the current list of the compounds isolated from the plant. Moreover, there were no such compounds reported to be isolated from *L. pterodonta*, another *Laggera* plant found in China. This was helpful in chemotaxonomical classifications. It is noteworthy that the stereochemistry of the cuauhtemone derivatives determined in this paper by single crystal X-ray diffraction analysis and modified Mosher's method is consistent with that proposed by Nakanishi et al. (1974), rather than that described by Torres-Valencia et al. (2003). Quite a few reports concerned the anticancer activities of eudesmane-type sesquiterpenes (Cui et al., 2011; Dai et al., 2009; Minakawa et al., 2012; Tanaka et al., 2012; Xu et al., 2010); however, in the present study, the eudesmane sesquiterpenes isolated from *L. alata* possessed no cytotoxic effects on human cancer cell lines.

4. Experimental

4.1. General experimental procedures

Melting points were obtained on an X-5 micro-melting point apparatus. Optical rotations were recorded on a JASCO P-1020 polarimeter, whereas IR spectra were measured on a JASCO FT/IR-480 plus infrared spectrometer with KBr pellets. UV spectra were acquired on a JASCO V-550 UV/VIS spectrometer. HRESIMS data were determined on an Agilent 6210 ESI/TOF mass spectrometer. 1D NMR (^{1}H, ^{13}C, and DEPT) and 2D (^{1}H–1H COSY, HSQC, HMBC, and ROESY) NMR spectra were recorded on a Bruker Avance 400 or 500 NMR spectrometer with TMS as internal standard, and chemical shifts were expressed in δ values (ppm). Column chromatography (CC) employed silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, P.R. China), ODS (YMC, Kyoto, Japan) and Sephadex LH-20 (Pharmacia, NJ, USA), respectively. All solvents used in CC were of analytical grade (Tianjin Damao Chemical Plant, Tianjin, P.R. China). Preparative high-performance liquid chromatography (HPLC) was performed on a Varian chromatograph equipped with a Prostar 215 pump, a Prostar 325 UV–VIS detector, using a C_{18} reversed-phase column (Cosmosil, 20 × 250 mm, 5 μm).

4.2. Plant material

Whole plants of *L. alata* (D. Don) Sch. Bip. ex Oliv were collected from Guangxi Medicinal Plant Garden in September, 2008, and identified by Research Assistant You-Bao Huang of Guangxi Medicinal Plant Garden. A voucher specimen (No. 08091309) is deposited in the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University (Guangzhou, China).

4.3. Extraction and isolation

The air-dried whole plants of *L. alata* (4.5 kg) were powdered and percolated with EtOH–H_2O (95:5, v/v) at room temperature for three times. The extract was then concentrated *in vacuo* to yield a residue (540 g), which was dissolved in EtOH–H_2O (1:9, v/v) and subjected to Diaion HP-20 macrosporous resin CC successively eluted with EtOH–H_2O mixtures (1:9, 3:7, 6:4, 9:1, v/v). The fraction (70 g) eluted by EtOH–H_2O (6:4, v/v) was concentrated and then subjected to a silica gel CC ($CHCl_3$–MeOH, 100:0 → 50:50) to afford 10 fractions. Fraction 2 (8.7 g) was separated by ODS silica gel CC (MeOH–H_2O, 15:85 → 80:20) to give five subfractions (**2a**–**e**). Subfraction **2b** was purified by Sephadex LH-20 CC ($CHCl_3$–MeOH, 1:1) and preparative HPLC (MeOH–H_2O, 70:30, v/v) to yield compounds **1** (39 mg), **2** (28 mg), **3** (21 mg) and **19** (40 mg), respectively. Compound **7** (25 mg) was recrystallized from subfraction **3c**. Further purification of subfraction **3c** by Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) and preparative HPLC (MeOH–H_2O, 60:40, v/v) afforded compounds **12** (28 mg), **13** (12 mg) and **14** (15 mg). By similar procedures, compounds **11** (35 mg), **19** (29 mg) and **20** (18 mg) were obtained from subfraction **3d**. Fraction **5** (5.9 g) was subjected to silica gel CC using a petroleum ether (PE)–EtOAc gradient as eluent, and then purified by preparative HPLC (MeOH–H_2O, 60:40, v/v) to give compounds **4** (21 mg), **5** (12 mg) and **15** (18 mg). Fraction **6** (6.1 g) was separated by a silica gel CC eluting with gradient $CHCl_3$–MeOH, and further purified by Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) and preparative HPLC (MeOH–H_2O, 50:50, v/v) to afford compounds **6** (18 mg), **16** (16 mg) and **18** (24 mg), respectively. Compounds **9** (14 mg), **21** (20 mg) and **22** (25 mg) were isolated from fraction **7** (4.1 g) by ODS CC with a MeOH–H_2O gradient and preparative HPLC (MeOH–H_2O (40:60, v/v)). Fraction **9** (2.8 g) was purified by Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) and preparative HPLC (MeOH–H_2O, 35:65, v/v) to give compounds **8** (23 mg), **10** (16 mg) and **17** (34 mg).

4.3.1. (3R,4S,5R,6R,2′R,3′S)-3-O-(2′,3′-Diacetoxy-2′-methylbutyryl)-cuauhtemone (**1**)

Colorless blocks; mp 118–120 °C, $[\alpha]_D^{25}$ +21.6 (*c* 0.2, MeOH); UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3473, 2942, 2867, 1739, 1449, 1372, 1197, 1143, 952, 607, 526 cm^{-1}; for ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) spectroscopic data, see Tables 1 and 2; HRESIMS *m/z*: 475.2310 [M+Na]$^+$ (calcd for $C_{24}H_{26}O_8Na$, 475.2302).

4.3.2. 4-O-Acetyl-3-O-(3′-acetoxy-2′-hydroxy-2′-methylbutyryl)-cuauhtemone (**2**)

Brown oil, $[\alpha]_D^{25}$ +21.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3475, 2939, 2867, 1736, 1448, 1375, 1195, 1145, 942, 615 cm^{-1}; for ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$,

125 MHz) spectroscopic data, see Tables 1 and 2; HRESIMS *m/z* 475.2302 $[M+Na]^+$ (calcd for $C_{24}H_{26}O_8Na$, 475.2302).

*4.3.3. 4-O-Acetyl-3-O-(2′,3′-diacetoxy-2′-hydroxy-2′-methylbutyryl)-cuauhtemone (**3**)*

Brown oil, $[\alpha]_D^{25}$ +21.7 (*c* 0.2, MeOH); UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3473, 2942, 2837, 1740, 1448, 1372, 1242, 1139, 1016, 949, 674 cm^{-1}; for ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectroscopic data, see Tables 1 and 2; HRESIMS *m/z* 517.2406 $[M+Na]^+$ (calcd for $C_{26}H_{38}O_9Na$, 517.2408).

*4.3.4. 3-O-(3′-Acetoxy-2′-hydroxy-2′-methylbutyryl)-cuauhtemone (**4**)*

Brown oil, $[\alpha]_D^{25}$ +23.2 (*c* 0.2, MeOH); UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3464, 2941, 2828, 1735, 1453, 1374, 1264, 1200, 1143, 990, 952, 851, 672 cm^{-1}; for ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectroscopic data, see Tables 1 and 2; HRESIMS *m/z* 433.2191 $[M+Na]^+$ (calcd for $C_{22}H_{34}O_7Na$, 433.2197).

*4.3.5. 4-O-Acetyl-3-O-(2′,3′-dihydroxy-2′-methylbutyryl)-cuauhtemone (**5**)*

Brown oil, $[\alpha]_D^{25}$ +24.7 (*c* 0.2, MeOH); UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3503, 2929, 2860, 1729, 1461, 1379, 1270, 1171, 1115, 989, 731, 675 cm^{-1}; for ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectroscopic data, see Tables 1 and 2; HRESIMS *m/z* 433.2195 $[M+Na]^+$ (calcd for $C_{22}H_{34}O_7Na$, 433.2197).

*4.3.6. 4α-Acetoxy-3α-(3′-acetoxy-2′-methylbutyryloxy)-11-hydroxy-6,7-dehydroeudesman-8-one (**6**)*

Brown oil, $[\alpha]_D^{25}$ +23.4 (*c* 0.2, MeOH), UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3449, 2954, 1744, 1638, 1459, 1376, 1239, 1134, 1105, 951, 873, 607 cm^{-1}; for ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectroscopic data, see Tables 1 and 2; HRESIMS *m/z* 491.2242 $[M+Na]^+$ (calcd for $C_{24}H_{36}O_9Na$, 491.2251).

*4.3.7. 3-Oxo-di-nor-eudesma-4-en-11-oic acid (**7**)*

Colorless blocks, mp 120–122 °C, $[\alpha]_D^{25}$ +23.8 (*c* 0.2, MeOH); UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3449, 2954, 1744, 1638, 1459, 1376, 1239, 1134, 1105, 950, 872, 607 cm^{-1}; for ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) spectroscopic data, see Tables 2 and 3; HRESIMS *m/z* 245.1149 $[M+Na]^+$ (calcd for $C_{13}H_{18}O_3Na$, 245.1148).

*4.3.8. Alatoside F (**8**)*

Colorless oil, $[\alpha]_D^{25}$ +26.2 (*c* 0.2, MeOH), UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3419, 2929, 1715, 1650, 1453, 1402, 1384, 1332, 1274.1, 1244, 1155, 1079, 674 cm^{-1}; for ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic data, see Tables 2 and 3; HRESIMS *m/z* 433.1831 $[M+Na]^+$ (calcd for $C_{21}H_{30}O_8Na$, 433.1833).

*4.3.9. Alatoside G (**9**)*

Colorless oil, $[\alpha]_D^{25}$ +20.8 (*c* 0.2, MeOH), UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3421, 2933, 1711, 1631, 1453, 1401, 1384, 1252, 1076, 708, 601 cm^{-1}; for ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic data, see Tables 2 and 3; HRESIMS *m/z* 433.1835 $[M+Na]^+$ (calcd for $C_{21}H_{30}O_8Na$, 433.1833).

*4.3.10. Alatoside H (**10**)*

Colorless oil, $[\alpha]_D^{25}$ +20.8 (*c* 0.2, MeOH), UV (MeOH) λ_{max}: 254 nm; IR (KBr) ν_{max}: 3418, 2933, 1722, 1676, 1406, 1245, 1076.4, 956, 873, 607 cm^{-1}; for ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic data, see Tables 2 and 3; HRESIMS *m/z* 435.1981 $[M+Na]^+$ (calcd for $C_{21}H_{30}O_8Na$, 435.1989).

4.4. X-ray data of **1** and **7**

*4.4.1. X-ray data of cuauhtemone A (**1**)*

Colorless blocks, $C_{24}H_{36}O_8$, M_r = 452.53, orthorhombic, space group $P2_12_12_1$, a = 8.24486(12) Å, b = 10.30986(18) Å, c = 28.5017 (5) Å, V = 2422.74(7) $Å^3$, Z = 4, d_x = 1.241 Mg/m^3, $F(000)$ = 976, μ(CuKα) = 0.762 mm^{-1}. Data collection was performed on a Gemini S Ultra using graphite-monochromated radiation (λ = 1.54184 Å); 3843 unique reflections were collected to θ_{max} = 62.77°, where 3652 reflections were observed [$F^2 > 2\sigma(F^2)$]. The structures were solved by direct methods (SHELXS 97) and refined by full-matrix least-squares on F^2. The final R = 0.0340, R_w = 0.0895, and S = 1.027.

*4.4.2. X-ray data of 3-oxo-di-nor-eudesma-4-en-11-oic acid (**7**)*

Colorless blocks, $C_{13}H_{18}O_3$, M_r = 222.27, monoclinic, space group $P2_1$, a = 7.6924(4) Å, b = 17.6450(8) Å, c = 9.0224(4) Å, V = 1179.05 (10) $Å^3$, Z = 4, d_x = 1.252 Mg/m^3, $F(000)$ = 480, μ(CuKα) = 0.710 mm^{-1}. Data collection was performed on a Gemini S Ultra using graphite-monochromated radiation (λ = 1.54184 Å); 2894 unique reflections were collected to θ_{max} = 60.88°, where 2590 reflections were observed [$F^2 > 2\sigma(F^2)$]. The structures were solved by direct methods (SHELXS 97) and refined by full-matrix least-squares on F^2. The final R = 0.0460, R_w = 0.1185, and S = 1.050.

The above crystallographic data have been deposited at the Cambridge Crystallographic Data Center under CCDC 909109 for cuauhtemone A (**1**) and CCDC 909110 for 3-oxo-di-nor-eudesma-4-en-11-oic acid (**7**). These data can be obtained free of charge via www.ccdc.cam.ac.uk/deposit (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

4.5. Acid hydrolysis and HPLC analysis of **8–10**

Compounds **8–10** (each 2.0 mg) were dissolved in 2 N HCl (10 ml) and heated at 80 °C for 5 h, respectively. The aglycone was extracted with CH_2Cl_2, and the aqueous layer was dried by N_2. Dry pyridine (0.5 ml) and L-cysteine methyl ester hydrochloride (2.0 mg) were added to the residue. The mixture was heated at 60 °C for 1 h and then dried by N_2. *O*-Toyl isothiocyomate (3–5 μl) was added, and the mixture was heated at 60 °C for 1 h. Then, the reaction mixture was directly analyzed by standard C_{18} HPLC and detected by a UV detector [column: Capcell pak C_{18} (4.6 × 250 mm, 5 μm); mobile phase: water (0.1% HCO_2H)-CH_3CN (75:25); follow rate: 0.8 mL/min, column temperature: 25 °C; detector: VWD; detector wavelength: 250 nm]. The standard sugars were treated by the same reaction and chromatographic conditions. As a result, D-glucose was detected from the hydrolyzate of compounds **8–10** with the same retention time of standard sugar derivatives, respectively.

4.6. Preparation of (*R*)- and (*S*)-MTPA esters of **22**

Compound **1** (10 mg) was dissolved in 2 N HCl (10 ml) and heated at 80 °C for 5 h. The solution was extracted with CH_2Cl_2 and then concentrated *in vacuo* to afford the aglycone, which was identical to the isolated compound **22** by comparison of their NMR and optical rotations. Compound **22** was dissolved in deuterated pyridine (0.5 ml) in a clean NMR tube under a gentle N_2 stream and a ^1H NMR spectrum was recorded as a reference. Then (*S*)-α-methoxy-α-(trifluoromethyl) phenylacetic chloride (5 μL) was added into the NMR tube under the N_2 gas stream and immediately shaken until uniformly mixed. After sealing with parafilm, the reaction NMR tube was kept for 8 h at room temperature.

The ^{1}H NMR spectrum, recorded directly from the reaction NMR tube, showed the production of the corresponding (*R*)-MTPA ester (**22a**). In an identical fashion, another portion of compound **22** (each 1.5 mg) were reacted in a second NMR tube with (*R*)-α-methoxy-α-(trifluoromethyl) phenylacetic chloride (5 μL) at room temperature for 8 h using deuterated pyridine (0.5 mL) as solvent, to afford the (*S*)-MTPA ester (**22b**).

5. MTT assay

Evaluation of the cytotoxic activities of all the isolated compounds on MFC-7, A375, A549, Hep-2, CNE, and Hela cells was carried out as described previously (Wu et al., 2012a,b). All the cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Acknowledgments

This work was supported by Grants from the Fundamental Research Funds for the Central Universities (21612417), the National Natural Science Foundation (Nos. 81072535, 81273390), and Research Team Program of Natural Science Foundation of Guangdong Province (No. 8351063201000003).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.07.014.

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