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Chisopanins A–K, 11 new protolimonoids from *Chisocheton paniculatus* and their anti-inflammatory activities

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ABSTRACT

Eleven new protolimonoids, chisopanins A–K (**1**–**11**), were isolated from the twigs of *Chisocheton paniculatus*, as well as thirteen known (**12**–**24**) protolimonoids. The structures were elucidated on the basis of spectroscopic analysis, X-ray crystallographic analysis, and chemical methods. Chisopanins A and B (**1** and **2**) possessing uncommon hemiketal tetrahydropyran ring at C-17 showed the most potent inhibitory activities on lipopolysaccharide-stimulated inflammation factor-release with IC_{50} values at 5.4 and 7.9 μM for NO, and at 26.9 and 30.7 μM for TNF-α, respectively. In addition, compounds **5**–**7**, **9**, **12**, **13**, and **20** were potent to inhibit NO production with IC_{50} value lower than 10 μM.

1. Introduction

Protolimonoids are precursors of limonoids, mainly distributed in the family Meliaceae and Rutaceae, and some of which exhibited antifeedant, antimalarial and cytotoxic activities.[1–4] Recently two protolimonoids have been reported with marked anti-inflammatory activities.[5,6] They significantly inhibited the production and gene expression of pro-inflammatory mediators in vitro and also showed activities in animal tests in vivo, which combined with the fact that a large number of other triterpenoids were principles responsible for anti-inflammatory effects in plants,[7] prompted us to investigate the anti-inflammatory activities of protolimonoids.

In our initial evaluation of the anti-inflammatory activities of *Chisocheton paniculatus* Hiern (Meliaceae), a plant abound with protolimonoids,[8,9] both the extract of its twigs (CPA) and subsequent $CHCl_3$ partitioned phase (CPC) significantly suppressed edema formation in carrageenan-induced mouse paw edema model of inflammation (Fig. 1). Further chemical investigation on the $CHCl_3$ partitioned phase resulted in the isolation of 11 new (chisopanins A–K, **1**–**11**) and 13 known (**12**–**24**) protolimonoids along with 7 new C-*seco*-type tetranortriterpenoids,[10] which were reported previously. In this study, we describe the isolation and structural elucidation of these protolimonoids from *C. paniculatus* as well as their effects on the inflammation factor-release (NO and TNF-α).

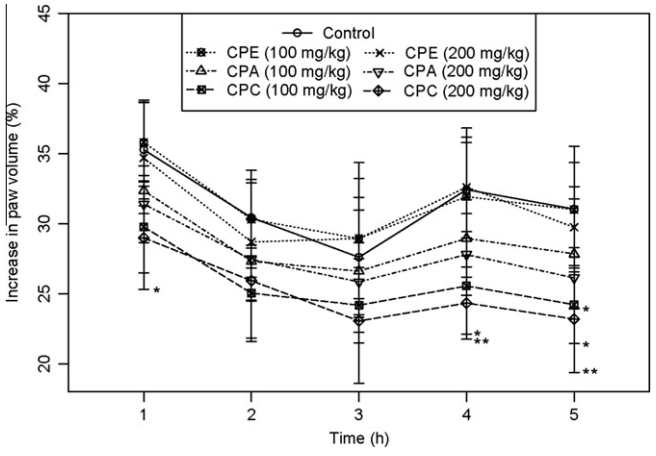

Figure 1. Effects of CPA, CPC, and CPE on carrageenan-induced paw edema in mice. CPA, crude ethanol extract; CPC, $CHCl_3$-soluble extract; CPE, EtOAc-soluble extract. Rats were orally administrated with different extracts (100 and 200 mg/kg) for 5 consecutive days, with the same volume of PEG (15 mL/kg, i.g.) as control. The volume of the paw was measured before and 1, 2, 3, 4, and 5 h after the injection of carrageenan. Each point represents the percent increase of paw volume, which was calculated based on the volume of the paw before carrageenan injection. The values are expressed as the mean ± SD ($n = 6$). $^{*}P$ <0.05 and $^{**}P$ <0.01 compared with the control group.

2. Results and discussion

2.1. Identification and structure determination

The $CHCl_3$ partitioned phase of the twigs of *C. paniculatus* was subjected to column chromatography on silica gel, ODS, and

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Sephadex LH-20, as well as preparative HPLC to yield 11 new (**1**–**11**) and 13 known (**12**–**24**) protolimonoids (Fig. 2). Their structures were determined by 1D and 2D NMR experiments, X-ray crystallographic analysis, and chemical methods.

Chisopanin A (**1**) was isolated as colorless crystals (MeOH/$CHCl_3$). Its molecular formula of $C_{34}H_{54}O_8$ was determined by the quasi-molecular ion peak at 613.3731 $[M+Na]^+$ (calcd for $C_{34}H_{54}O_8Na$, 613.3711) in HRESIMS. Its IR spectrum revealed absorption bands for hydroxyls (3451 m^{-1}), carbonyl groups (1730 cm^{-1}), and a double bond (1640 cm^{-1}). The ^1H NMR spectrum (Table 1) showed seven tertiary methyl groups (δ_H 0.77, 0.93, 0.97, 1.13, 1.14, 1.22, and 1.33, each 3H, s), two acetoxyl groups (δ_H 1.97, 2.07, each 3H, s), one olefinic proton (δ_H 5.23), and five oxygenated protons (δ_H 3.58, 3.84, 3.88, 4.64, and 5.14). In addition to two acetoxyl groups, the ^{13}C NMR spectra (Table 2) showed the presences of seven methyls, eight methylenes (one oxygenated), eight methines (three oxygenated, one olefinic), seven quaternary carbons (one hemiketal and one oxygenated). These data indicated that **1** was an *apo*-tirucallol protolimonoids with a modified eight-carbon side chain,[11] which was determined by the HMBC experiment. The HMBC correlations (Fig. 3) originated from oxymethylene protons [δ_H 3.84, 3.58 (H-21)] to a hemiketal carbon [δ_C 96.5 (C-24)] revealed the existence of an ether bridge between C-21 and C-24 of the side chain, forming a tetrahydropyrane ring. The cross peaks from two methyl protons Me-26 (δ_H 1.33, s) and Me-27 (δ_H 1.22, s) to the hemiketal carbon (C-24) also confirmed the connection of a 2-hydroxyisopropyl group at C-24. In combination with the chemical shift, the HMBC correlations from H-23 (δ_H 3.88, t, 2.5) to C-24 (δ_C 96.5) and C-22 (δ_C 34.3) placed a hydroxyl at C-23. Thus, the side chain was assigned as 21,24-epoxy-23,24,25-triol. Moreover, two acetyl groups were placed at C-3 and C-7 due to the observed HMBC cross peaks from H-3 (δ_H 4.64, t, 2.5) and H-7 (δ_H 5.14, t, 2.0) to the acetyl carbonyl at δ_C 172.5 and 172.0, respectively. Extensive analysis of its HMBC spectrum further confirmed the planar structure.

The ROESY experiment of **1** (Fig. 4) gave the relative configuration of the tetracyclic core as shown in Fig. 2. ROESY cross peaks of Me-29/H-3 and Me-30/H-7 also revealed a β-orientation for H-3 and H-7. The characteristic coupling constants between H-20 and H-21 ($J_{21a,20}$ = 11.5 Hz, $J_{21b,20}$ = 4.6 Hz) revealed axial orientations for H-20 and H-21a. The ROESY correlations between H-21α (δ_H 3.84, dd, 11.0, 4.6) with Me-18 (δ_H 1.13, s) and H-11α (δ_H 1.75, m), and between H-21β (δ_H 3.58, dd, 11.0, 11.5) with H-17 (δ_H 1.35, m) were observed, which was possible only when **1** had C-20*S* configuration (*apo*-tirucallol skeleton) according to a molecular model.[11] The assignment of C-23*R* was made by NOE correlations of H-23/H-21β. Since the configuration of C-24 was uncertain, a single-crystal X-ray diffraction analysis (Fig. 5) was performed

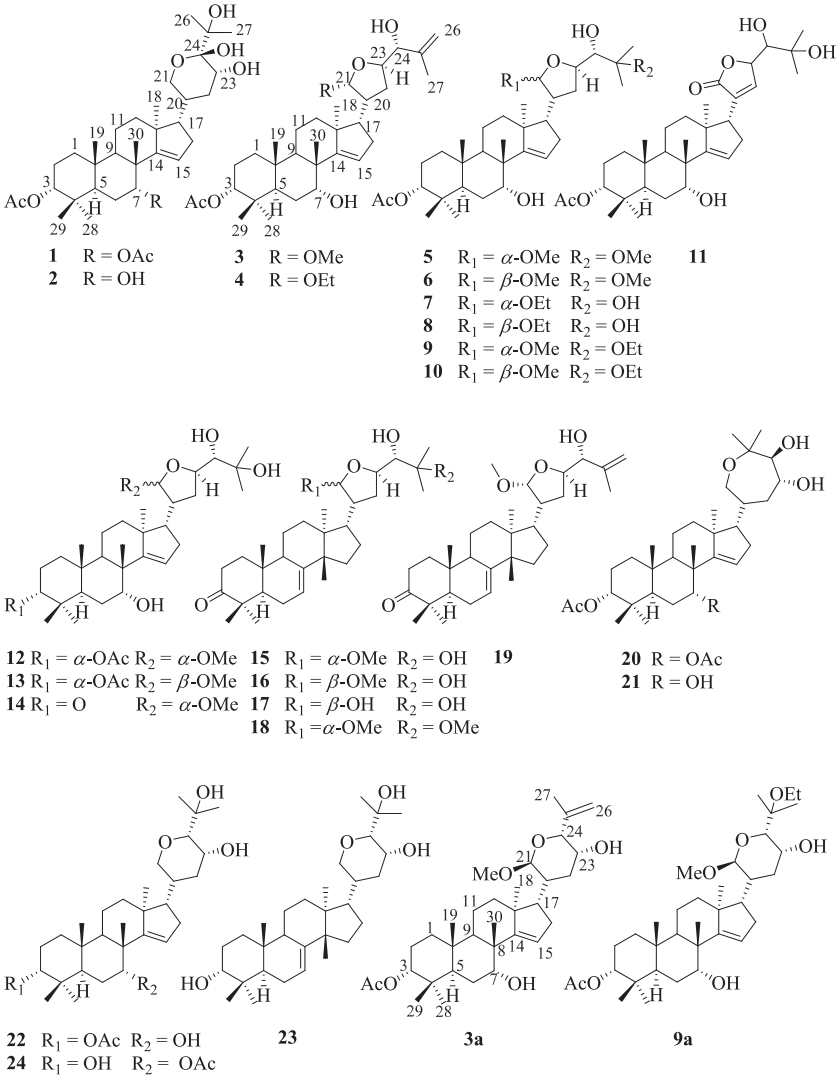

Figure 2. Chemical structures of compounds **1**–**24**, **3a**, and **9a**.

Table 1
^{1}H NMR spectroscopic data for compounds **1**–**12** (*J* in Hz within parentheses)

No.	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[b]	**6**[b]	**7**[b]	**8**[b]	**9**[b]	**10**[b]	**11**[b]	**12**[b,c]
1a	1.45 m	1.45 m	1.83 m	1.84 m	1.36 m	1.39 m	1.37 m	1.35 m	1.36 m	1.36 m	1.36 m	1.36 m
1b	1.81 m	1.83 m	1.44 m	1.46 m	1.25 m	1.25 m	1.28 m	1.23 m	1.26 m	1.26 m	1.24 m	1.23 m
2a	2.17 m	1.95 m	1.88 m	1.90 m	1.90 m	1.89 m	1.90 m	1.90 m	1.87 m	1.87 m	1.87 m	1.89 m
2b	1.60 m	1.58 m	1.59 m	1.60 m	1.58 m	1.60 m	1.60 m	1.59 m	1.59 m	1.58 m	1.57 m	1.59 m
3	4.64 (t, 2.5)	4.63 br s	4.64 s	4.66 (t, 2.6)	4.65 (d, 2.7)	4.65 (d, 2.6)	4.65 br s	4.65 br s	4.65 (t, 3.0)	4.66 br s	4.66 s	4.64 (dd, 2.6, 2.8)
5	2.08 m	2.04 m	2.05 m	2.03 m	2.02 m	2.01 m	1.95 m	1.95 m	1.95 m	1.98 m	1.95 m	1.98 m
6a	1.86 m	1.83 m	1.72 m	1.74 m	1.76 m	1.74 m	1.76 m	1.75 m	1.73 m	1.75 m	1.75 m	1.70 m
6b	1.70 m	1.70 m	1.69 m		1.70 m	1.70 m	1.72 m	1.71 m				1.73 m
7	5.14 (t, 2.0)	3.93 br s	3.90 br s	3.91 s	3.91 (d, 2.7)	3.91 (d, 2.7)	3.92 s	3.91 br s	3.91 br s	3.91 br s	3.95 s	3.91 (dd, 2.5, 2.6)
9	1.88 m	2.08 m	1.95 m	1.97 m	2.06 m	1.99 m	2.03 m	1.96 m	2.03 m	2.02 m	2.01 m	2.02 m
11a	1.75 m	1.74 m	1.72 m	1.72 m	1.73 m	1.70 m	1.70 m	1.70 m	1.70 m	1.70 m	1.70 m	1.72 m
11b	1.53 m	1.52 m	1.49 m	1.53 m	1.50 m	1.51 m	1.52 m	1.49 m	1.52 m	1.49 m	1.42 m	1.51 m
12a	1.47 m	1.39 m	1.91 m	1.93 m	1.83 m	1.60 m	1.80 m	1.62 m	1.83 m	1.58 m	1.90 m	1.79 m
12b	1.29 m	1.29 m	1.38 m	1.36 m	1.43 m	1.51 m	1.46 m	1.51 m	1.46 m	1.52 m	1.63 m	1.45 m
15	5.23 (t, 3.5)	5.44 (t, 2.0)	5.44 br s	5.45 (d, 2.2)	5.45 (d, 2.3)	5.51 (d, 2.1)	5.46 (d, 2.4)	5.48 br s	5.45 (t, 2.4)	5.47 s	5.54 (d, 2.3)	5.44 m
16a	1.96 m	2.11 m	2.16 m	2.18 m	2.18 (ddd, 4.7, 8.2, 12.1)	2.15 m	2.22 m	2.19 m	2.20 m	2.15 m	2.69 (dd, 11.7, 15.0)	2.22 (ddd, 3.5, 7.5,15.5)
16b	2.17 m	2.23 m	2.06 m	2.10 m	2.15 m	2.13 m	2.13 m	2.15 m	2.12 m		2.38 (ddd, 6.9, 10.5, 15.1)	2.13 m
17	1.35 m	1.36 m	1.70 m	1.69 m	1.68 m	1.98 m	1.72 m	2.04 m	1.70 m	1.98 m	2.85 (dd, 7.2, 10.7)	1.73 m
18	1.13 s	1.12 s	1.07 s	1.10 s	1.09 s	1.04 s	1.11 s	1.05 s	1.10 s	1.05 s	0.90 s	1.08 s
19	0.97 s	0.95 s	0.90 s	0.91 s	0.91 s	0.91 s	0.92 s	0.92 s	0.91 s	0.91 s	0.92 s	0.90 s
20	2.25 m	2.26 m	2.37 m	2.40 m	2.34 m	2.16 m	2.36 m	2.19 m	2.34 m	2.18 m		2.34 m
21a	3.58 (dd, 11.0, 11.5)	3.58 (dd, 11.0, 11.5)	4.82 m	4.93 (d, 3.6)	4.80 (d, 3.7)	4.76 (d, 4.3)	4.90 (d, 4.2)	4.87 (d, 4.3)	4.80 (d, 3.6)	4.75 (d, 4.3)		4.79 (d, 3.7)
21b	3.84 (dd, 11.0, 4.6)	3.84 (dd, 11.0 4.5)										
22a	1.67 m	1.70 m	1.34 m	1.36 m	1.96 m	1.93 m	1.90 m	1.98 m	1.97 m	1.92 m	7.17 s	1.93 m
22b			1.25 m	1.24 m	1.63 m	1.81 m	1.70 m	1.87 m	1.63 m	1.83 m		1.81 m
23	3.88 (t, 2.5)	3.88 (t, 2.5)	4.07 m	4.10 m	4.22 (ddd, 2.3, 4.8, 7.0)	4.46 (ddd, 2.3, 6.8, 8.4)	4.29 (dd, 4.2 10.2)	4.44 (ddd, 1.9, 7.2, 9.2)	4.23 m	4.48 m	5.22 s	4.25 (ddd, 1.7, 5.0, 10.7)
24			3.92 br s	3.90 (d, 2.5)	3.37 (d, 2.3)	3.26 (d, 2.3)	3.25 s	3.17 (d, 2.0)	3.38 (d, 3.6)	3.27 (d, 2.7)	3.54 (d, 2.5)	3.24 (dd, 1.7, 9.7)
26	1.33 s	1.33 s	4.99 br s; 4.89 br s	5.00 br s; 4.90 br s	1.24 s	1.25 s	1.30 s	1.26 s	1.25 s	1.25 s	1.33 s	1.26 s
27	1.22 s	1.22 s	1.76 s	1.77 s	1.15 s	1.18 s	1.27 s	1.26 s	1.16 s	1.19 s	1.43 s	1.29 s
28	0.77 s	0.84 s	0.85 s	0.86 s	0.86 s	0.86 s	0.87 s	0.86 s	0.86 s	0.86 s	0.86 s	0.85 s
29	0.93 s	0.93 s	0.89 s	0.90 s	0.89 s	0.89 s	0.90 s	0.90 s	0.90 s	0.90 s	0.91 s	0.89 s
30	1.14 s	1.08 s	1.05 s	1.07 s	1.06 s	1.06 s	1.07 s	1.08 s	1.06 s	1.06 s	1.12 s	1.05 s
OAc	1.97 s; 2.07 s	2.06 s	2.06 s	2.07 s	2.11 s	2.07 s	2.08 s	2.07 s	2.10 s	2.07 s	2.07 s	2.07 s
OMe			3.36 s		3.23 s; 3.36 s	3.25 s, 3.37 s			3.37 s	3.37 s		3.35 s
OEt				3.78 m; 3.45 m 1.22 (t, 7.1)			3.73 m; 3.44 m 1.23 (t, 7.2)	3.75 m; 3.42 m 1.23 (t, 7.2)	3.44 (2H m); 1.15 (t, 6.6)	3.45 (2H m); 1.14 (t, 6.9)		

[a] Measured at 500 MHz in CD_3OD.
[b] Measured at 500 MHz in $CDCl_3$.
[c] Literature data, see Ref. 7.

and demonstrated the 24*S* configuration and the proposed structure of **1**. So the structure of **1** was thus elucidated as 3α,7α-diacetoxyl-17α-20*S*-21,24-epoxyapotirucall-14-ene-23*R*,24*S*,25-trisol.

Chisopanin B (**2**) had the molecular formula $C_{32}H_{52}O_7$ as established from its positive HRESIMS (*m*/*z* 571.3591 $[M+Na]^+$, calcd for $C_{32}H_{52}O_7Na$, 571.3605), 42 mass units less than **1**. ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2) of **2** were very similar to those of **1**, except for the existence of only one acetoxyl group. In comparison with **1**, the H-7 signal was shifted upfield by 1.21, suggesting the absence of an acetoxyl at C-7 in **2**. The observed HMBC correlation between H-3 and the acetoxyl carbonyl at δ_C 172.7 placed this acetoxyl at C-3. ROESY experiments indicated that the relative stereochemistry and configuration of **2** was the same as that of **1**. Therefore, **2** was determined as 3α-acetoxyl-17α-20*S*-21,24-epoxyapotirucall-14-ene-7α,23*R*,24*S*,25-tetraol.

Chisopanin C (**3**) was isolated as a white, amorphous powder. The molecular formula of $C_{33}H_{52}O_6$ was established by a peak at *m*/*z* 567.3668 for the $[M+Na]^+$ ion (calcd for $C_{33}H_{52}O_6Na$, 567.3656) in HRESIMS. The IR spectrum showed absorbance bands at 3457 cm^{-1} (hydroxy groups) and 1727 cm^{-1} (carbonyl group). The ^{1}H NMR spectrum (Table 1) showed six tertiary methyl groups (δ_H 0.85, 0.89, 0.90, 1.05, 1.07, and 1.76, each 3H, s), one acetoxyl (δ_H 2.06, 3H, s), one methoxyl (δ_H 3.36, 3H, s), three olefinic protons (δ_H 4.89, 4.99, 5.44, each 1H, br s), and five protons (δ_H 3.90, 3.92, 4.07, 4.64, 4.82) bonded to oxygenated carbons. A total of 33 carbon resonances were observed in the ^{13}C NMR spectrum (Table 2). Carbon resonances for one trisubstituted double bond, one terminal double bond, six methyls, seven sp^3 methylenes, nine sp^3 methines, and four sp^3 quaternary carbons were assigned by ^{13}C NMR and HSQC experiments, in addition to those for the

Table 2
^{13}C NMR spectroscopic data for compounds **1–12**

No.	1[a]	2[a]	3[b]	4[b]	5[b]	6[b]	7[b]	8[b]	9[b]	10[b]	11[b]	12[b,c]
1	35.9	35.7	32.8	32.9	33.2	33.3	33.2	33.2	33.1	33.2	33.2	33.1
2	23.7	23.8	22.7	22.8	22.8	22.8	22.7	22.8	22.8	23.3	22.8	22.7
3	79.6	79.8	78.0	78.1	78.1	78.1	78.0	78.1	78.1	78.1	78.1	78.1
4	37.2	37.4	36.1	36.2	36.2	36.2	36.1	36.2	36.1	36.2	36.2	36.1
5	44.8	43.2	41.6	41.8	41.7	41.7	41.8	41.9	41.8	41.8	41.9	41.8
6	24.3	25.4	23.6	23.6	23.6	23.6	23.5	23.6	23.5	23.6	23.7	23.5
7	77.3	73.9	72.2	72.2	72.2	72.2	72.1	72.2	72.1	72.2	72.3	72.1
8	43.5	45.2	44.4	44.5	44.5	44.5	44.4	44.5	44.4	44.4	44.7	44.4
9	44.4	43.0	41.8	41.6	41.9	41.9	41.6	41.6	41.6	41.6	41.6	41.6
10	38.6	38.7	37.6	37.6	37.6	37.6	37.6	37.7	37.6	37.6	37.7	37.5
11	17.6	17.6	16.3	16.3	16.3	16.4	16.3	16.4	16.3	16.4	16.3	16.2
12	34.6	34.5	34.0	34.2	32.7	32.9	32.7	32.9	32.6	32.9	32.5	32.7
13	47.9	47.9	47.0	47.1	47.0	46.7	47.0	46.7	47.0	46.6	47.8	46.9
14	161.4	162.4	162.5	162.5	162.5	162.1	162.3	162.1	162.4	162.1	161.7	162.3
15	119.5	120.6	119.1	119.2	119.3	119.7	119.3	119.7	119.2	119.7	119.3	119.2
16	34.8	34.9	34.7	34.8	34.7	35.0	34.8	35.0	34.7	34.9	33.7	34.7
17	58.6	58.6	57.6	57.8	57.7	52.5	57.5	52.3	57.6	52.5	50.8	57.4
18	19.8	19.2	19.4	19.4	19.9	19.9	19.3	20.0	19.4	19.9	20.4	19.4
19	15.9	15.8	15.1	15.2	15.2	15.2	15.2	14.2	15.2	15.1	15.2	15.1
20	31.1	31.0	46.7	46.9	46.2	44.8	45.9	44.6	46.2	44.8	134.3	45.8
21	66.3	66.3	109.2	108.1	109.3	104.6	108.4	103.4	109.2	104.5	173.3	109.5
22	34.3	34.3	33.2	33.2	35.5	32.4	33.8	31.6	35.6	32.4	148.9	33.7
23	69.1	69.1	78.8	78.8	75.1	77.8	76.8	78.6	75.2	77.9	81.0	76.9
24	96.5	96.5	78.2	78.3	76.4	77.1	75.3	76.2	76.4	77.2	76.2	75.4
25	77.4	77.4	144.5	144.5	76.8	76.9	73.1	72.9	76.8	76.8	72.5	73.0
26	25.2	25.2	113.2	113.2	21.6	22.5	26.4[d]	26.3[d]	22.4	22.8	26.2	26.3[d]
27	23.5	23.5	18.2	18.2	20.1	20.1	26.5[d]	26.4[d]	20.7	20.6	27.1	26.4[d]
28	28.1	28.0	27.6	27.6	27.6	27.6	27.6	27.6	27.6	27.6	27.6	27.6
29	22.0	22.2	21.7	21.8	21.8	21.8	21.8	21.8	21.8	21.8	21.8	21.7
30	27.9	28.6	27.8	27.8	27.8	27.9	27.9	27.9	27.8	27.8	28.0	27.8
OAc	172.5, 21.2[d]; 172.0, 21.1[d]	172.7, 21.2	170.9, 21.4	170.1, 21.4	170.9, 21.5	170.9, 21.5	171.0, 21.5	171.0, 21.5	171.1, 21.5	170.9, 21.4	170.9, 21.5	171.0, 21.5
OMe			55.4		55.4; 49.2	54.9; 49.3			55.5	54.8		55.6
OEt				63.7, 15.4			64.1; 15.4	63.8; 14.2	56.5 16.1	56.6 16.2		

[a] Measured at 125 MHz in CD_3OD.
[b] Measured at 125 MHz in $CDCl_3$.
[c] Literature data, see Ref. 7.
[d] Maybe exchanged in the same column.

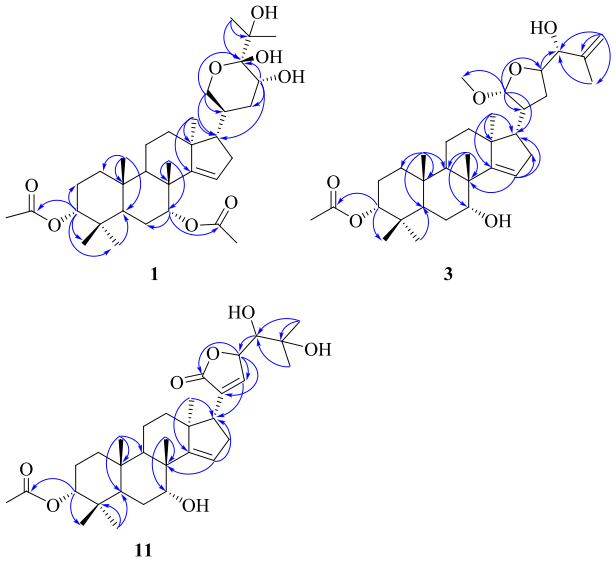

Figure 3. Key HMBC (H→C) correlations of compounds **1**, **3**, and **11**.

substituents. A comparison of the NMR data of **3** with those of chisiamols D[8] (**12**) (Tables 1 and 2) indicated that **3** was an analogue of **12**, an *apo*-tirucallol protolimonoid. Further analysis suggested that the two compounds shared the same tetracyclic core with one alkenyl at C-14 and one acetoxyl at C-3, but differed in the side chain. In the HMBC spectrum of **3** (Fig. 3), correlations of C-23/H-21 and C-21/OMe demonstrated the existence of the ether bridge between C-21 and C-23 to form a tetrahydrofuran ring and the location of a methoxyl at C-21 to form an acetal group. The HMBC correlations from two protons of the terminal double bond (δ_H 4.99, 4.89, each 1H, br s) to C-24 (δ_C 78.2) and C-27 (δ_C 18.2) revealed the existence of a double bond between C-25 and C-26 in **3**.

The relative configuration of the tetracyclic core in **3** was assigned by a ROESY experiment (Fig. 4), which was identical to that of **12**. The small coupling constants and NOE correlations of H-3/Me-29 and H-7/Me-30 also indicated a β-orientation for H-3 and H-7. When compound **3** was treated with boron trifluoride diethyl etherate in $CHCl_3$, compound **3a** (Fig. 2) was produced. In the ROESY spectrum of **3a** (Fig. 4), correlations were detected between H-21/H-17, H-21/H-12a, H_3-18/H-20, and H_3-18/H-21, which was possible only when **3a** had the 20*S* configuration.[2] The NOE correlations between H-23/H-17, H-23/H-24, MeO-21/H-24, H-22b/H-23 and H-22b/H-24 showed that the configurations at C-23 and C-24 were both in *R*. Moreover, in the ROESY spectrum of **3**, cross peaks between H-21/H-17, MeO-21/H-23 and MeO-21/H-20 revealed the *R* configuration of C-21 since the 20*S* configuration was determined. Therefore, compound **3** was concluded to be 3α-acetoxyl-21*R*-methoxyl-17α-20*S*-21,23*R*-epoxyapotirucall-14,25-dien-7α,24*R*-diol.

Chisopanin C (**4**) gave a molecular formula of $C_{34}H_{54}O_6$ as established by HRESIMS, exhibiting a quasi-molecular ion at *m/z* 581.3842 $[M+Na]^+$ (calcd for $C_{34}H_{54}O_6Na$, 581.3813). Comparison

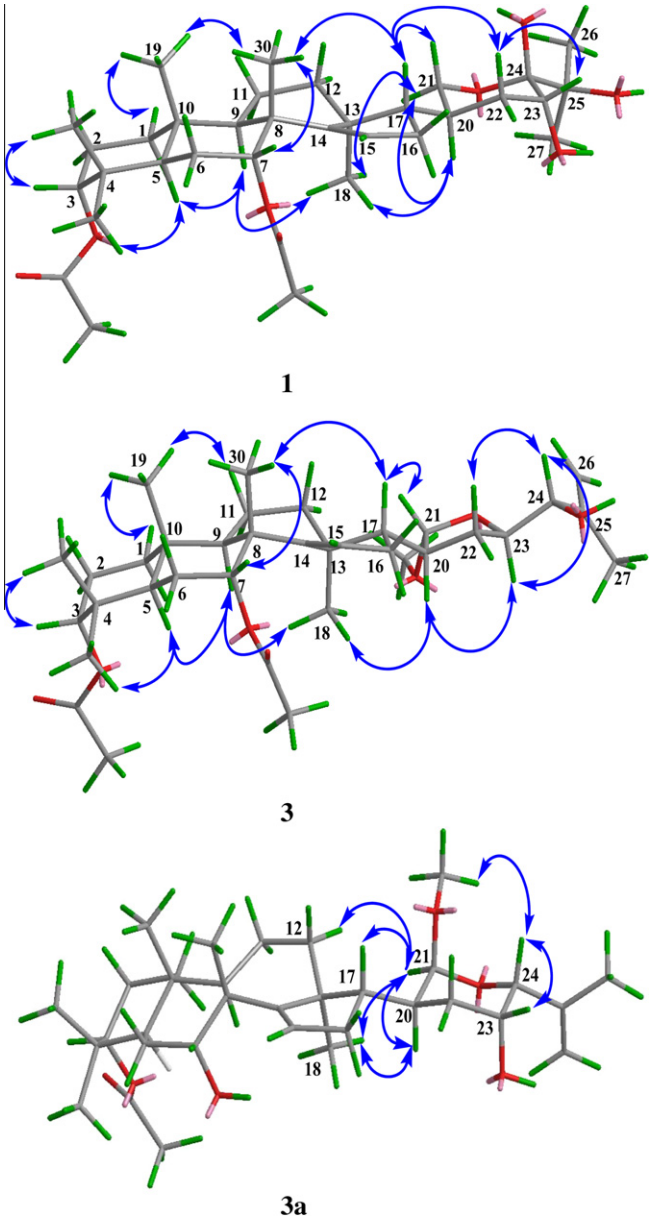

Figure 4. Selected NOESY correlations of compounds **1**, **3**, and **3a**.

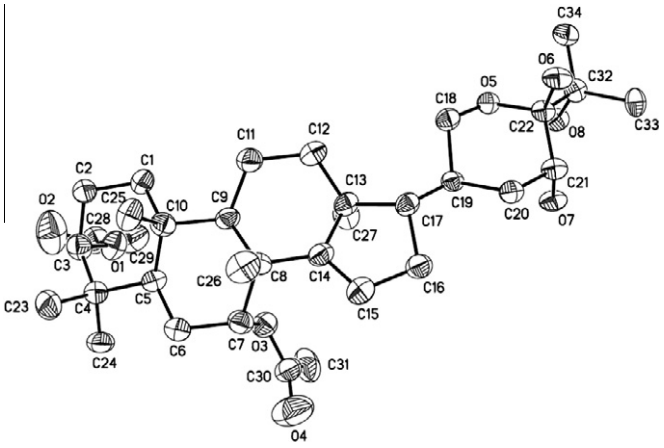

Figure 5. X-ray crystallographic structure of **1**.

of its NMR data with **3** revealed that they share the same carbon skeleton. The difference was the appearance of an ethoxyl rather than a methoxyl at C-21, which was demonstrated by the HMBC correlations from the ethoxylic methylene (δ_C 63.7) to the ethoxylic methyl (δ_H 1.22, 3H, t, 7.1), and to H-21 (δ_H 4.93, d, 3.6). The ROESY cross-peaks of H-24/H-22 and H-24/H-23 and the small coupling constant ($J_{23,24}$ = 2.5 Hz) revealed the 24*R* configuration.[12] 2D NMR experiments, including HSQC, HMBC, and ROESY spectra, further confirmed the structure of **4**. Consequently, compound **4** was assigned as 3α-acetoxyl-21*R*-ethoxyl-17α-20*S*-21,23*R*-epoxy-apotirucall-14,25-dien-7α,24*R*-diol.

Chisopanins E (**5**) and F (**6**) with the same molecular formula of $C_{34}H_{56}O_7$ (at *m/z* 599.3923 $[M+Na]^+$ and 599.3917 $[M+Na]^+$, respectively) as determined by HRESIMS showed the presence of one more CH_2 unit than that of **12**. Their NMR spectra consisted of signals similar to those of **12** except the presence of an additional methoxyl [δ_H 3.23 (3H, s) for **5**; 3.25 (3H, s) for **6**]. The downfield shift of C-25 from δ_C 72.9 to δ_C 76.8 in **5** and to δ_C 76.9 in **6** when compared with **12** suggested the attachment of a methoxy group to C-25, which was confirmed by the HMBC correlation from methoxy group to C-25. NOE correlations of H-21/H-17, H-24/H-22 and H-24/H-23 and the small coupling constant of H-23/H-24 were also found in **5** to affirm the 21*R* and 24*S* configurations. Thus, compound **5** was characterized as 3α-acetoxyl-21*R*,25-dimethoxyl-17α-20*S*-21,23*R*-epoxyapotirucall-14-ene-7α,24*S*-diol. The observed upfield shifts of C-21 ($\Delta\delta$ 4.7) and C-17 ($\Delta\delta$ 5.2) in **6** implied that it was the 21*S* epimer of **5**,[12] which was further confirmed by the NOE correlations of H-21/Me-18 and H-21/H-20. Therefore, compound **6** was determined to be 3α-acetoxyl-21*S*,25-dimethoxyl-17α-20*S*-21,23*R*-epoxyapotirucall-14-ene-7α,24*S*-diol.

Chisopanin G (**7**) and chisopanin H (**8**) displayed quasi-molecular ion at *m/z* 599.3936 $[M+Na]^+$ and 599.3889 $[M+Na]^+$, respectively, in positive-ion HRESIMS, corresponding to the same molecular formula of $C_{34}H_{56}O_7$ as **5** and **6**. Their NMR spectroscopic data (Tables 1 and 2) were generally similar to those of **12** except for the presence of an ethoxy group, which was placed at C-21 by correlations of ethoxylic methylene proton signals with the acetal carbon signals in their HMBC spectrum. The observed NOE correlation between H-21 (δ_H 4.90, d, 4.2) and H-17 (δ_H 1.72, m) in **7** implied the 21*R* configuration, while **8** had the C-21*S* configuration, based on the same elucidation process in **6**. By detailed analysis of 2D NMR experiments, including HSQC, HMBC, and ROESY spectra, compound **7** was then determined as 3α-acetoxyl-21*R*-ethoxyl-17α-20*S*-21,23*R*-epoxyapotirucall-14-ene-7α,24*S*,*25*-trisol, and compound **8** was elucidated as 3α-acetoxyl-21*S*-ethoxyl-17α-20*S*-21,23*R*-epoxyapotirucall-14-ene-7α,24*S*,*25*-trisol.

Chisopanin I (**9**) and chisopanin J (**10**) were also stereo isomers, as deduced by the same molecular formula of $C_{35}H_{58}O_7$ and their similar NMR spectra, which were closely related to those of **5** and **6** except the presence of an ethoxy group instead of a methoxyl. Since HMBC correlations from ethoxylic methylene protons to C-25 were both observed in **9** and **10**, the ethoxy group was assigned at C-25. The configurations at C-21, C-23 and C-24 of **9** were determined to be *R*, *R* and *S*, respectively, by acid-catalyzed formation of **9a** (Fig. 2). Therefore, compound **9** was assigned as 3α-acetoxyl-21*R*-methoxyl-25-ethoxyl-17α-20*S*-21,23*R*-epoxyapotirucall-14-ene-7α,24*S*-diol. Similarly to **6**, the NOE correlations of H-21/Me-18, along with the upfield shifts of C-17 and C-21, revealed the C-21*S* configuration in **10**. Thus, the structure of **10** was determined as 3α-acetoxyl-21*S*-methoxyl-25-ethoxyl-17α-20*S*-21,23*R*-epoxy-apotirucall-14-ene-7α,24*S*-diol.

Chisopanin K (**11**) had the molecular formula $C_{32}H_{48}O_7$ as established by HRESIMS at *m/z* 567.3277 $[M+Na]^+$ (calcd for $C_{32}H_{48}O_7Na$, 567.3292), indicating nine degrees of unsaturation. The NMR data of **11** (Tables 1 and 2) indicated that it was similar to those compounds above, however, with difference in ring E: the absence of signals due

to the tetrahydrofuran ring and hemiacetal group and, instead, the appearance of those for an α,β-unsaturated-γ-lactone ring[13] [δ_H 5.22 (1H, s), and 7.17 (1H, s); δ_C 81.0, 134.3, 148.9, and 173.3]. Furthermore, in its HMBC experiment (Fig. 3), the correlations were observed from the proton at H-22 (δ_H 7.17, s) to C-21 (δ_C 173.3), C-23 (δ_C 81.0), and C-17 (δ_C 50.8), and from the proton at H-23 (δ_H 5.22, s) to C-21 (δ_C 173.3), C-22 (δ_C 148.9), and C-24 (δ_C 76.2), which established unequivocally the existence of the unsaturated lactone ring in **11**. The structure of **11** was further confirmed by 2D NMR experiments, and determined to be 3α-acetoxyl-7α,24,25-trihydroxy-17α-apotirucall-14,20(22)-dien-21,23-olide.

The hemiketal tetrahydropyrane ring at C-17 described in compounds **1** and **2** was uncommon and rarely reported in protolimonoids.[11,14,15] When measured in different solvents, protolimonoids possessing this unusual structure had an interesting NMR phenomenon due to the existence of an equilibrium system:[11] when recorded in $CDCl_3$, their ^1H and ^{13}C NMR spectra seemed like mixtures with many unassignable signals, which disappeared when measured in CD_3OD.

By comparison of the NMR and MS data with the published data, 13 known compounds were identified as chisiamol D (**12**),[8] chisiamol E (**13**),[8] 3-*O*-acetyl-21-*O*-methyltoosendanpentol (**14**),[16] 21α-methylmelianodiol (**15**),[5] 21β-methylmelianodiol (**16**),[5] 21β-melianodiol (**17**),[17] 21α,25-dimethylmelianodiol (**18**),[18] [21-α-methylmelianol (21*R*,23*R*)-epoxy-23-hydroxy-21α-methoxyl]triucalla-7,25-dien-3-one (**19**),[19] chisiamol C (**20**),[8] chisiamol B (**21**),[8] 3α-acetoxyl-21,24*R*-epoxyapotirucall-14-ene-7α,23*R*,25-triol (**22**),[20] bourjotinoline A (**23**),[21] paniculatin C (**24**).[22]

2.2. Anti-inflammatory activities

2.2.1. Determination of anti-inflammation—Carrageenan-edema test

Carrageenan-induced paw edema is highly sensitive to non-steroid anti-inflammatory drugs. Edema, one of the cardinal signs of inflammation, is an important parameter to be considered when evaluating drugs for their potential anti-inflammatory activity.[23] As shown in Fig. 1, the results indicated that the crude and the $CHCl_3$-soluble extracts of *C. paniculatus* significantly inhibited the mouse paw edema induced by carrageenan in a dose-dependent manner, while the EtOAc-soluble extract didn't. 5 h after edema induction, groups treated with $CHCl_3$-soluble extract at 100 and 200 mg/kg significantly suppressed edema formation with inhibitory rates at 21.9% and 25.3%, respectively.

2.2.2. Compounds reduced NO and TNF-α production from macrophages

Macrophages play a central role in the inflammatory response, and activated macrophages release a variety of inflammatory mediators. NO and TNF-α are key mediators in the early and late phases of carrageenan-induced paw inflammation.[24,25] Therefore, 11 new (**1**–**11**) and seven primary known (**12**–**15** and **20**–**22**) protolimonoids with amount over 10 mg were evaluated for their inhibitory activities on lipopolysaccharide-stimulated inflammation factor-release (NO and TNF-α) of mouse macrophages RAW 264.7 in vitro.

As shown in Table 3, all protolimonoids, except **4**, **8**, and **22**, inhibited LPS-stimulated NO expression to various degrees. Nine compounds (**1**, **2**, **5**–**7**, **9**, **12**, **13**, and **20**) exhibited the striking inhibitory abilities with IC_{50} value lower than 10 μM, and compounds **10**, **14** and **21** showed moderate inhibitory effects with IC_{50} values of 12.3, 18.2 and 16.5 μM, respectively. Interestingly, concluding from the three pairs of epimers (**5**/**6**, **7**/**8**, and **9**/**10**), protolimonoids bearing 21*R* tetrahydrofuran ring worked better than the 21*S* epimers. Terminal double bonds at C-25 in compounds **3** and **4** reduced inhibitory activities comparing with other analogues. Meanwhile, compounds **1** and **2** with characteristic six-membered hemiacetal also significantly inhibited LPS-induced TNF-α release in RAW 264.7 macrophages (IC_{50} at 26.9 and 30.7 μM), superior to **22**, which revealed that the hemiketal formation at C-24 and the presence of hydroxyl group could obviously improve their anti-inflammatory activities. In addition, compounds **11**, **14**, **15** and **22** displayed temperate activities in inhibition of LPS-induced TNF-α expression with IC_{50} values range from 45 to 75 μM.

Table 3
Effects of different compounds on NO and TNF-α production in LPS-stimulated RAW 264.7 cells ($n = 4$)[a]

Compounds	IC_{50} (μM)	
	NO	TNF-α
Chisopanin A (**1**)	5.4 ± 0.2	26.9 ± 1.5
Chisopanin B (**2**)	7.9 ± 0.4	30.7 ± 1.7
Chisopanin C (**3**)	40.0 ± 2.7	>100
Chisopanin D (**4**)	>50	>100
Chisopanin E (**5**)	6.2 ± 0.3	>100
Chisopanin F (**6**)	6.9 ± 0.5	>100
Chisopanin G (**7**)	5.4 ± 0.2	>100
Chisopanin H (**8**)	>50	>100
Chisopanin I (**9**)	5.3 ± 0.2	>100
Chisopanin J (**10**)	12.3 ± 0.5	>100
Chisopanin K (**11**)	33.4 ± 2.1	47.4 ± 3.3
Chisiamols D (**12**)	6.5 ± 0.3	>100
Chisiamols E (**13**)	8.9 ± 0.5	>100
3-*O*-acetyl-21-*O*-methyltoosendanpentol (**14**)	18.2 ± 1.2	57.5 ± 10.8
21α-Methylmelianodiol (**15**)	29.4 ± 3.2	54.6 ± 7.2
Chisiamols C (**20**)	8.7 ± 0.5	>100
Chisiamols B (**21**)	16.5 ± 1.1	>100
Chisiamol B (**22**)	>50	73.5 ± 4.3
Dexamethasone[b]	0.86 ± 0.06	
Genistein[b]		19.1 ± 0.4

[a] A compound was regarded as inactive when IC_{50} >50 μM for NO, and IC_{50} >100 μM for TNF-α
[b] Positive controls.

3. Conclusion

Based on initial Carrageenan-induced paw edema test of crude extracts, further fractionation on active $CHCl_3$-soluble extract has led to the discovery of 11 new (**1**–**11**) and 13 known (**12**–**24**) protolimonoids from *C. paniculatus*. Most of the tested protolimonoids showed inhibitory activities on LPS-stimulated inflammation factor-release (NO and TNF-α) of RAW 264.7 in vitro in various degrees with chisopanins A and B (**1** and **2**) being the most potent. Thus, we believe that protolimonoids are important anti-inflammatory constituents of *C. paniculatus*, and could be considered as a new class of triterpenoids that can be potential therapeutic drug against inflammation.

4. Experimental

4.1. General procedures

The melting point was recorded on an XT-4 micromelting point apparatus, uncorrected. Optical rotations were measured using a JASCO P-1020 polarimeter. IR (KBr disks) spectra were measured on a Bruker Tensor-27 spectrophotometer. NMR spectra were recorded on Bruker ACF-500 instrument (^1H: 500 MHz, ^{13}C: 125 MHz) using standard Bruker pulse programs. Chemical shifts are given as δ-value with reference to tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on an Agilent Micro Q-TOF mass spectrometer. Silica gel (Qingdao Marine Chemical Co. Ltd), Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), C_{18} reverse-phased silica gel (150–200 μm, Merck),

and MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd) were used for column chromatography. Silica gel GF_{254} plates (Qingdao Marine Chemical Co. Ltd) were used for thin-layer chromatography, and spots were visualized by spraying with 10% H_2SO_4 followed by heating. Preparative HPLC was performed using Agilent 1100 series instrument with a Shim-Park RP-C_{18} column (200 × 20 mm i.d.).

4.2. Plant material

The twigs of *C. paniculatus* were collected in Xishuangbanna, Yunnan Province, China, in May 2007. The botanical identification was made by Prof. Min-jian Qin, Department of Medicinal Plants, China Pharmaceutical University. A voucher specimen (No. 070705) is deposited in the Department of Natural Medicinal Chemistry, China Pharmaceutical University.

4.3. Extraction and isolation

The air-dried twigs of *C. paniculatus* (30 kg) were crushed to pieces and extracted with 95% EtOH under reflux for 3 × 3 h. After concentration of the solution, the obtained dark crude extract (CPA, 1.5 kg), which was suspended in water, was partitioned with $CHCl_3$ (6 × 2 L) and EtOAc (6 × 2 L) to afford $CHCl_3$ layer (CPC, 300 g) and EtOAc layer (CPE, 205 g). The $CHCl_3$ extract was separated into 10 fractions (CS1–CS10) by chromatography on a silica gel column eluted with gradient PE/EtOAc (100:0 to 1:1). Fraction CS2 (11 g) was separated on a silica gel column chromatography using PE/EtOAc (100:1 to 2:1) to mainly afford three fractions, 2A–2C. Separation of fraction 2A on a reversed-phase C_{18} open column eluted with MeOH/H_2O (70:30 to 100:0) yielded **4** (75 mg) and **10** (82 mg). Purification of the fraction 2B by a C_{18} column chromatography with MeOH/H_2O (70:30 to 100:0) and followed by preparative HPLC (MeCN/H_2O, 85:15) afforded **3** (80 mg), **6** (13 mg), and **9** (70 mg). Purification of the fraction 2C by repeated preparative HPLC (MeCN/H_2O, 80:20) led to the isolation of **16** (4 mg). Fraction CS3 (9 g) was subjected to silica gel column chromatography using PE/EtOAc (50:1 to 2:1) as step gradient mixtures as eluents to afford four fractions (3A–3D). Purification of the fraction 3B by MCI gel with MeOH/H_2O (70:30 to 100:0) and further preparation by HPLC (MeCN/H_2O, 85:15) afforded **5** (8 mg) and **7** (21 mg). By the same ways, **8** (40 mg) and **21** (20 mg) were obtained from fraction 3D. Fraction CS4 (3 g) was chromatographed on a RP-18 column and eluted with MeOH and H_2O (70:30 to 100:0) to give white crude powder, which was further purified by silica gel column to afford **23** (200 mg). The remaining fraction CS4 was purified by chromatography over Sephadex LH-20 and then separated on a silica gel column to afford **17** (3 mg) and **18** (5 mg). Separation of fraction CS5 (12 g) was column chromatographed on MCI gel and further isolated by RP-18 column to obtain four fractions (5A–5D). Recrystallization of fraction 5B with MeOH and $CHCl_3$ afforded **1** (60 mg). Separation of fraction 5C on ODS column chromatography with MeOH/H_2O (60:00 to 100:0), followed by preparative HPLC (MeCN/H_2O, 80:20) led to the isolation of **11** (20 mg). By the same ways, **15** (16 mg) was obtained from fraction 5D. Fraction CS6 (26 g) was subjected to chromatography over silica gel ($CHCl_3$/MeOH, 200:1 to 50:1) and ODS (MeOH/H_2O 60:40 to 100:0) to afford fractions 6A–6E. By repeated preparative HPLC (MeCN/H_2O, 75:15 and/or MeOH/H_2O, 80:20), **19** (6 mg), **20** (3 mg), and **22** (110 mg) were obtained from fraction 6B. Similarly, **24** (6 mg) was isolated from fraction 6C, while **2** (18 mg), **13** (50 mg), and **14** (12 mg) were from fraction 6E.

Chisopanin A (**1**): colorless crystals (MeOH/$CHCl_3$); mp 152–155 °C; $[\alpha]_D^{27}$ −81.5 (*c* 0.30, $CHCl_3$); IR (KBr) ν_{max} 3451, 2947, 1730, 1640, 1379, 1249, 1038 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 613.3731 $[M+Na]^+$ (calcd for $C_{34}H_{54}O_8Na$, 613.3711).

Chisopanin B (**2**): white, amorphous powder; $[\alpha]_D^{27}$ -65.6 (*c* 0.14, $CHCl_3$); IR (KBr) ν_{max} 3450, 2942, 1711, 1640, 1377, 1249, 1030 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 571.3591 $[M+Na]^+$ (calcd for $C_{32}H_{52}O_7Na$, 571.3605).

Chisopanin C (**3**): white, amorphous powder; $[\alpha]_D^{27}$ −109.4 (*c* 0.32, $CHCl_3$); IR (KBr) ν_{max} 3457, 2942, 1727, 1641, 1376, 1248, 1034 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 567.3668 $[M+Na]^+$ (calcd for $C_{33}H_{52}O_6Na$, 567.3656).

Chisopanin D (**4**): white, amorphous powder; $[\alpha]_D^{27}$ −70.4 (*c* 0.18, $CHCl_3$); IR (KBr) ν_{max} 3455, 2973, 1724, 1641, 1378, 1247 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 581.3842 $[M+Na]^+$ (calcd for $C_{34}H_{54}O_6Na$, 581.3813).

Chisopanin E (**5**): white, amorphous powder; $[\alpha]_D^{27}$ −105.6 (*c* 0.14, $CHCl_3$); IR (KBr) ν_{max} 3536, 3460, 2956, 2936, 1725, 1640, 1382, 1270, 1034 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 599.3923 $[M+Na]^+$ (calcd for $C_{34}H_{56}O_7Na$, 599.3918).

Chisopanin F (**6**): white, amorphous powder; $[\alpha]_D^{27}$ −68.5 (*c* 0.12, $CHCl_3$); IR (KBr) ν_{max} 3457, 2943, 1731, 1641, 1464, 1250, 1034 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 599.3917 $[M+Na]^+$ (calcd for $C_{34}H_{56}O_7Na$, 599.3918).

Chisopanin G (**7**): white, amorphous powder; $[\alpha]_D^{27}$ −159.4 (*c* 0.16, $CHCl_3$); IR (KBr) ν_{max} 3536, 3455, 2973, 2936, 1725, 1641, 1461, 1372, 1270 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 599.3936 $[M+Na]^+$ (calcd for $C_{34}H_{56}O_7Na$, 599.3918).

Chisopanin H (**8**): white, amorphous powder; $[\alpha]_D^{27}$ −66.5 (*c* 0.13, $CHCl_3$); IR (KBr) ν_{max} 3453, 2972, 2940, 1710, 1641, 1462, 1386, 1249, 1033 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 599.3889 $[M+Na]^+$ (calcd for $C_{34}H_{56}O_7Na$, 599.3918).

Chisopanin I (**9**): white, amorphous powder; $[\alpha]_D^{27}$ −118.3 (*c* 0.45, $CHCl_3$); IR (KBr) ν_{max} 3456, 2975, 2940, 1727, 1639, 1388, 1247, 1035 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 613.4036 $[M+Na]^+$ (calcd for $C_{35}H_{58}O_7Na$, 613.4075.

Chisopanin J (**10**): white, amorphous powder; $[\alpha]_D^{27}$ −50.3 (*c* 0.21, $CHCl_3$); IR (KBr) ν_{max} 3462, 2975, 2941, 1729, 1641, 1462, 1388, 1248 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 613.4066 $[M+Na]^+$ (calcd for $C_{35}H_{58}O_7Na$, 613.4075).

Chisopanin K (**11**): white, amorphous powder; $[\alpha]_D^{27}$ −48.0 (*c* 0.05, $CHCl_3$); IR (KBr) ν_{max} 3460, 1741, 1643, 1386, 1252, 1101 cm^{-1}; ^1H NMR see Table 1 and ^{13}C NMR see Table 2; positive HRESIMS *m/z* found 567.3277 $[M+Na]^+$ (calcd for $C_{32}H_{48}O_7Na$, 567.3292).

4.4. Treatment of 3 and 9 with boron trifluoride diethyl etherate

Compound **3** (20 mg) in $CHCl_3$ (4 mL) mixed with boron trifluoride diethyl etherate (3 μL) were stirred at room temperature for 30 min. The mixture was diluted with 5 mL $CHCl_3$, and then washed with H_2O and brine respectively. After concentration, the residue was subjected to preparative HPLC (MeCN/H_2O, 85:15) to afford **3a** (2.4 mg).

Compound **3a**: white, amorphous powders. The complete assignments of ^1H and ^{13}C NMR of **3a** were achieved by a comprehensive analysis of 2D NMR spectra. ^1H NMR ($CDCl_3$, 500 MHz) δ: 5.47 (1H, br s, H-15), 5.04 (1H, br s, H-26a), 4.92 (1H, br s, H-26b), 4.77 (1H, d, *J* = 4.3 Hz, H-21), 4.66 (1H, t, *J* = 2.8 Hz, H-3),

4.22 (1H, m, H-23), 3.92 (1H, t, J = 2.8 Hz, H-7), 3.82 (1H, d, J = 5.1 Hz, H-24), 3.36 (3H, s, OMe), 2.27 (1H, m, H-20), 2.18 (2H, m, H-16), 2.10 (3H, s, -CH_3 OAc), 2.05 (1H, m, H-5), 2.02 (1H, m, H-17), 2.00 (1H, m, H-9), 1.94 (1H, m, H-2a), 1.92 (1H, m, H-22a), 1.80 (3H, s, Me-27), 1.78 (2H, m, H-6), 1.75 (1H, m, H-22b), 1.72 (1H, m, H-11a), 1.64 (1H, m, H-12a), 1.55 (1H, m, H-11b), 1.54 (1H, m, H-12b), 1.52 (1H, m, H-2b), 1.38 (1H, m, H-1a), 1.27 (1H, m, H-1b), 1.09 (3H, s, Me-30), 1.07 (3H, s, Me-18), 0.96 (3H, s, Me-19), 0.94 (3H, s, Me-29), 0.88 (3H, s, Me-28). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 170.9 (–C=O, OAc), 162.2 (C-14), 144.8 (C-25), 119.6 (C-15), 112.6 (C-26), 104.5 (C-21), 80.5 (C-23), 78.1 (C-3, C-24), 72.2 (C-7), 54.7 (OMe), 52.6 (C-17), 46.7 (C-13), 45.4 (C-20), 44.5 (C-8), 41.9 (C-9), 41.6 (C-5), 37.7 (C-10), 36.2 (C-4), 34.9 (C-16), 33.3 (C-1), 32.9 (C-12), 30.9 (C-22), 27.9 (C-30), 27.6 (C-28), 23.7 (C-6), 22.8 (C-2), 21.8 (C-29), 21.5 (-CH_3, OAc), 19.9 (C-18), 18.4 (C-27), 16.4 (C-11), 15.1 (C-19).

Compound **9** (30 mg) was treated with boron trifluoride diethyl etherate (3 μL) in the same manner as described for **3** to give **9a** (3.1 mg).

Compound **9a**: white, amorphous powder. The complete assignments of ^{1}H and ^{13}C NMR of **9a** were achieved by a comprehensive analysis of 2D NMR spectra. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 5.46 (1H, br s, H-15), 4.85 (1H, d, J = 3.0 Hz, H-21), 4.67 (1H, s, H-3), 4.19 (1H, s, H-23), 3.92 (1H, t, J = 2.8 Hz, H-7), 3.59 (2H, m, -CH_2-, OEt), 3.35 (3H, s, OMe), 3.33 (1H, br s, H-24), 2.43 (1H, m, H-20), 2.18 (1H, m, H-16a), 2.10 (1H, m, H-16b), 2.09 (3H, s, -CH_3 OAc), 2.01 (1H, m, H-5), 2.00 (1H, m, H-9), 1.89 (1H, m, H-2a), 1.78 (1H, m, H-17), 1.73 (2H, m, H-6, H-22a), 1.71 (1H, m, H-11a), 1.69 (1H, m, H-12a), 1.62 (1H, m, H-2b), 1.55 (1H, m, H-22b), 1.54 (1H, m, H-12b), 1.53 (1H, m, H-11b), 1.38 (1H, m, H-1a), 1.37 (3H, s, Me-27), 1.34 (3H, s, Me-26), 1.26 (1H, m, H-1b), 1.23 (3H, t, J = 7.0 Hz, –CH_3, OEt), 1.10 (3H, s, Me-18), 1.07 (3H, s, Me-30), 0.92 (3H, s, Me-19), 0.91 (3H, s, Me-29), 0.87 (3H, s, Me-28). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 171.0 (–C=O, OAc), 162.2 (C-14), 119.6 (C-15), 100.6 (C-21), 78.6 (C-25), 78.1 (C-3), 74.0 (C-24), 72.2 (C-7), 65.1 (C-23), 57.7 (–CH_2–, OEt), 54.3 (C-17, OMe), 46.7 (C-13), 44.3 (C-8), 41.8 (C-9), 41.5 (C-5), 37.5 (C-10), 36.2 (C-4), 33.8 (C-16), 33.3 (C-1), 33.2 (C-20), 33.1 (C-12), 31.6 (C-22), 27.8 (C-30), 27.6 (C-28), 23.6 (C-6), 23.1 (C-27), 22.8 (C-2), 22.3 (C-26), 21.7 (C-29), 21.4 (–CH_3, OAc), 19.5 (C-18), 16.5 (C-11), 16.0 (–CH_3, OEt), 15.1 (C-19).

4.5. X-ray crystallographic study of 1

Crystal data for **1**: formula $C_{34}H_{54}O_8$; M_r = 590.77; orthorhombic crystalline system; space group $P2_12_12_1$; a = 7.3932(14) Å, b = 9.0434(15) Å, c = 26.310(3) Å; V = 1757.5(5) Å^3; Z = 2; d = 1.116 mg/m^3; crystal dimensions 0.46 × 0.31 × 0.12 mm^3; the final indices were R_1 = 0.0940 wR_2 = 0.1870.

Crystal data were obtained on a Bruker Smart-1000 CCD with a graphite monochromator with Mo Kα radiation at (λ = 0.71073 Å) 298(2) K. The structure was determined by direct methods using SHELX-97[26] and expanded using difference Fourier techniques, refined by SHELX-97.[27] CCDC-802253 contains the supplementary crystallographic data for this paper. Copies of the data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK [fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk].

4.6. Anti-inflammatory activities

4.6.1. Carrageenan-edema test

Male Kunming mice, weighing about 25–30 g, were randomly divided into several groups. Two concentrations (100 and 200 mg/kg) of *C. paniculatus* crude extracts (CPA, CPC, and CPE), dissolved in polyethylene glycol (PEG), were orally given for 5 days continuously. The same volume of PEG (15 mL/kg, i.g.) was administered to the control group of mice. One hour after carrageenan (Sigma) (1% 50 μL) was injected into the planar surface of left hind paw, the swelling of the foot was measured by a plethysmometer (YLS-7B, Beijing, China). The changes in the volume of the foot were detected every hour for 5 h. The percent increase of paw volume was calculated based on the volume of the paw before carrageenan injection. These animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

4.6.2. Cell culture

Mouse macrophage cell line (RAW 264.7) was obtained from the Chinese Academy of Science Cell Bank (Shanghai, China). The cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heated-deactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (15 μM) at 37 °C atmosphere and 5% CO_2.

4.6.3. Nitrite assay

RAW 264.7 cells were placed in 24-well cell culture plates at a density of 1 × 10^5 with 500 μL culture medium, and incubated for 24 h. The cells were pre-treated with different compounds (40, 20, 10, 5, 2.5 μM) for 2 h, which were solubilized with DMSO diluted with RPMI 1640 medium, and then stimulated with lipopolysaccharide (LPS) (Sigma) (2 μg/mL) for 18 h. The final concentration of DMSO should not exceed 0.1% in the culture medium. After incubation, the supernatants (100 μL) were added to a solution of 100 μL Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diamine dihydrochloride in 5% H_3PO_4). Using $NaNO_2$ to generate a standard curve, nitrite production was measured by a miciroplate reader (IQuantTM, BIO-TEK Instrument Inc., Winooski, VT) at 540 nm.

4.6.4. Pro-inflammatory cytokines (TNF-α) production

Before stimulation with LPS and test materials, RAW 264.7 cells were incubated for 24 h in 24-well plates under the same conditions. The test compounds (100, 50, 25, 12.5, 6.25 μM) and LPS were then added to the cultured cells as described in Section 4.6.3. The medium was used for TNF-α assay using mouse ELISA kit (R&D Systems Inc., MN, USA), according to the manufacturer's recommendations.

4.6.5. Statistical analysis

Results were expressed as mean ± SD and all statistical comparisons were made by means of a one-way ANOVA test followed by Dunett's t-test. A value of P <0.05 was considered significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.01.007. These data include MOL files and InChiKeys of the most important compounds described in this article.

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