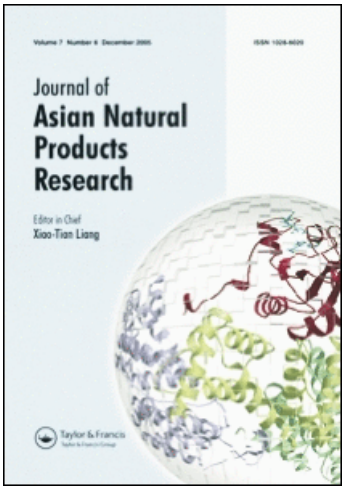

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Inhibitory effects of sesquiterpenes from *Saussurea lappa* on the overproduction of nitric oxide and TNF-α release in LPS-activated macrophages

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Nitric oxide (NO), derived from L-arginine, is produced by two types (constitutive and inducible) of nitric oxide synthase (NOS: cNOS and iNOS). The NO produced in large amounts by the iNOS is known to be responsible for inflammation, the vasodilation, and hypotension observed in septic shock and cancer metastasis. The inhibitors of the overproduction of NO, thus, may be useful candidates for the treatment of inflammatory diseases. We have found that the petroleum ether extract of *Saussurea lappa* Decne, which is a wild species wildly distributed in India, can strongly inhibit the overproduction of NO in mouse macrophage RAW 264.7 cells. Through bioassay-guided fractionation, 13 sesquiterpenes were isolated from the active petroleum ether extract. Furthermore, another five sesquiterpenes were synthesized by chemical methods. In the present study, their effects on LPS-induced NO production and TNF-α release are reported. Compounds **1**, **3**, **9**, **17**, and **18** showed significant inhibitory activities on the production of NO and release of TNF-α with IC_{50} values lower than 1 μmol/l. SAR studies suggest that the exocyclic double bond ($\Delta^{11(13)}$) is necessary for the inhibitory activities of sesquiterpenes on the NO production.

Keywords: *Saussurea lappa* Decne; sesquiterpene; macrophage; nitric oxide; TNF-α release

1. Introduction

Saussurea lappa introduced from India has been cultivated in southwestern China. It has been a traditional Chinese medicine and also an important spice since ancient times, which is now in common use in China and Japan. It possesses the activities of anti-inflammation, spasmolysis, anti-hypertension, and anti-bacteria. Its chemical constituents have been studied by some groups [1–5]. Sesquiterpenes and sesquiterpene lactones, which are identified as the main constituents of *S. lappa* Decne, have been discussed regarding their biological activities such as the inhibition on the VEGFR KDR/Flk-1 signaling pathway [6], cytotoxic activity [7], and inhibition on PTP1B [8]. *S. lappa* extract has also been reported to induce G2-growth arrest and apoptosis in AGS gastric cancer cells [9]. Recently, the methanolic extract of the roots of this plant has been reported to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages [10]. Two main sesquiterpene lactones (costunolide and dehydrocostus lactone) potently inhibited LPS-induced NO production. In order to clarify the medicinal value of *S. lappa* clearly, we have investigated the inhibitory activity of extracts from *S. lappa* on the NO overproduction induced by LPS. The petroleum ether extract exhibited the strongest activity on the NO production. We then focused on the isolation and semi-synthesis of

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sesquiterpene lactones and their inhibitory activities on the NO production and structure–activity relationships.

2. Results and discussion

At first, the petroleum ether, EtOAc, *n*-butanol, and water-soluble fractions were evaluated for their inhibitory activity on the NO production induced by LPS. As shown in Table 1, the petroleum ether fraction exhibited the strongest activity and showed a good dose-dependent manner (IC_{50} value is 0.12 μg/ml). Further fractionation and isolation of this active fraction were then performed to obtain 13 sesquiterpenes and 10 other compounds. Five derivatives (**5**, **7**, **11**, **13**, and **14**) were synthesized by using compound **3** as the lead compound. The structures of the 18 sesquiterpenes are shown in Figure 1. In the present study, their inhibitory activities on the NO production induced by LPS were investigated to evaluate the potency of these natural compounds in the treatment of inflammation. Hydrocortisone, a clinical anti-inflammatory medicine, was used as the positive control. The IC_{50} values of compounds **1**–**18** and hydrocortisone are shown in Table 2. When LPS was added to RAW 264.7 cells, NO levels, measured as nitrite concentration, were dramatically increased to 34.78 μmol/l from the basal level of 1.65 μmol/l after 24 h incubation period ($n = 3$). As shown in Figure 2(A), compound **2** exhibited much weaker inhibitory activity than **1**, and **4** also exhibited much weaker inhibitory activity than **3**. The difference between IC_{50} values of compounds **1** and **2** is about 100 folds (1: 0.52 μmol/l; 2: 51 μmol/l). Compound **6** exhibited much weaker inhibitory activity than **5**. Compound **8** also exhibited much weaker inhibitory activity than **7** (Figure 2(B)). The same tendency between compounds **9** and **10** (Figure 2(C)), and **11** and **12** (Figure 2(D)) suggested that the exocyclic double bond ($\Delta^{11(13)}$) is necessary for the inhibitory activities of sesquiterpenes on the NO production. Compound **13** is the acetylated

Table 1. Inhibitory effects of *S. lappa* extracts on the NO production in RAW 264.7 cells.

	Inhibitory rate on the NO production[a]								
	Concentration (μg/ml)								IC_{50} (μg/ml)
	100	30	10	3	1	0.3	0.1	0.03	
Petroleum fraction	–	–	100.94 ± 0.63	101.25 ± 0.96	101.88 ± 1.25	80.00 ± 2.70	46.46 ± 3.29	23.75 ± 3.68	0.12
EtOAc fraction	–	–	103.13 ± 0.72	105.00 ± 1.77	60.00 ± 6.21	14.38 ± 7.25	9.69 ± 10.96	6.56 ± 4.72	0.86
n-Butanol fraction	100.63 ± 3.15	55.00 ± 4.33	19.06 ± 5.63	7.50 ± 2.70	–	–	–	–	27.2
Aqueous fraction	17.50 ± 9.24	6.88 ± 2.98	1.88 ± 6.57	1.56 ± 2.77	–	–	–	–	>100

[a]Data are expressed as mean ± SD from three separate experiments.
Cytotoxicity was evaluated by MTT assay. No cytotoxicity was observed in this experiment.

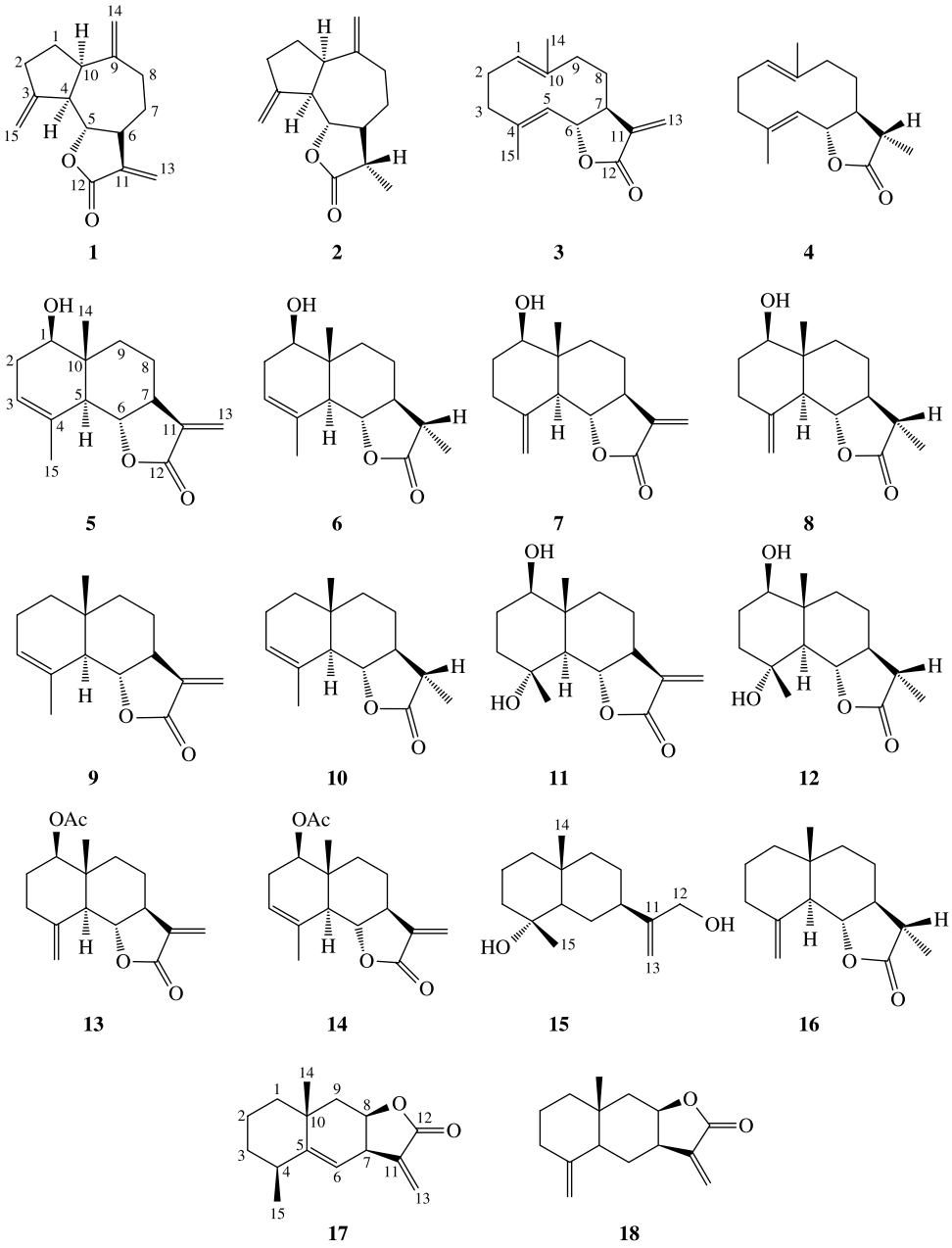

Figure 1. Chemical structures of sesquiterpenes **1–18**.

derivative of **7** at the position of 1-hydroxyl, as well as compound **14** is the acetylated derivative of **5** at the position of 1-hydroxy. Results in Figure 2(E) suggested that the acetylation of 1-hydroxyl might not cause improvement of NO production inhibitory activity. As shown in Figure 2(F), **9** (IC_{50}: 0.57 μmol/l) exhibited stronger inhibitory activity than **5** (IC_{50}: 2.3 μmol/l) because of the loss of 1-hydroxyl group. **10** (IC_{50}: 4.5 μmol/l) also exhibited stronger inhibitory activity than **6** (IC_{50}: 13 μmol/l). The same

Table 2. The IC_{50} values of compounds **1**–**18** on the overproduction of NO.

Compounds	IC_{50} (μmol/l)	Compounds	IC_{50} (μmol/l)
1	0.52	**10**	4.5
2	51	**11**	5.7
3	0.25	**12**	>100
4	5.1	**13**	12
5	2.3	**14**	1.6
6	13	**15**	93
7	3.2	**16**	16
8	20	**17**	0.25
9	0.57	**18**	0.49
Hydrocortisone	64		

tendency suggested that 1-hydroxyl group may decrease the inhibitory activity on the NO production. Namely, loss of 1-hydroxyl group may be helpful for the inhibitory activity.

Compounds **5** and **7** have the same backbone structure at B and C rings, but difference in A ring. Compound **5** owns an endocyclic double bond ($\Delta^{3(4)}$) but **7** owns an exocyclic double bond ($\Delta^{4(15)}$). In the same way, compounds **6** and **8** differ in A ring with the same B and C rings. Compound **6** owns an endocyclic double bond ($\Delta^{3(4)}$) but **8** owns an exocyclic double bond ($\Delta^{4(15)}$). There were no significant changes of activity observed between these two pairs of compounds (Figure 2(B)), which may provide us with a conclusion that the position of the double bond in A ring (endocyclic or exocyclic) may not be important. MTT assay was simultaneously performed to evaluate the cytotoxicity of test compounds. All the compounds did not show cytotoxic effect on RAW 264.7 cells at the concentration of 0.3–10 μmol/l.

Macrophages play major roles in inflammation and host defense mechanisms against bacterial and viral infections [11]. During acute and chronic inflammation, excessive production of NO may cause severe injury to host cells and tissues [12]. LPS-mediated excessive and prolonged NO generation has attracted wide attention because of its relevance to inflammatory disease. Our data indicated that above sesquiterpenes could inhibit LPS-induced NO production, especially compounds **1**, **3**, **9**, **17**, and **18**. Furthermore, their inhibitory effects on the tumor necrosis factor alpha (TNF-α) release induced by LPS were also investigated. As shown in Table 3, when RAW 264.7 cells were treated with 1 μg/ml LPS for 6 h, TNF-α level in the supernatant increased to 3389.97 pg/ml from the basal level of 63.57 pg/ml. Treatment with 0.25–2 μmol/l of sesquiterpene lactones **1**, **3**, **9**, **17**, and **18** significantly blocked the TNF-α release induced by LPS.

In summary, sesquiterpene lactones from *S. lappa* strongly inhibited the NO overproduction and proinflammatory cytokine TNF-α release induced by LPS in macrophages. Despite compounds **1** and **3** have been reported to possess the inhibitory activity on the NO production, there are no reports about the activities of other sesquiterpene lactones. Our results suggest the potency of these sesquiterpene lactones in the treatment of inflammatory disease and may provide an appropriate explanation of the medicinal value of this plant or its extract.

3. Experimental

3.1 Extraction and isolation

About 25 kg of dry roots of *S. lappa* was extracted with 95% ethanol to obtain 5.7 kg of crude extract. The extract was suspended in water and partitioned with petroleum ether, EtOAc, and *n*-butanol. Evaporation of the solvent yielded the petroleum ether

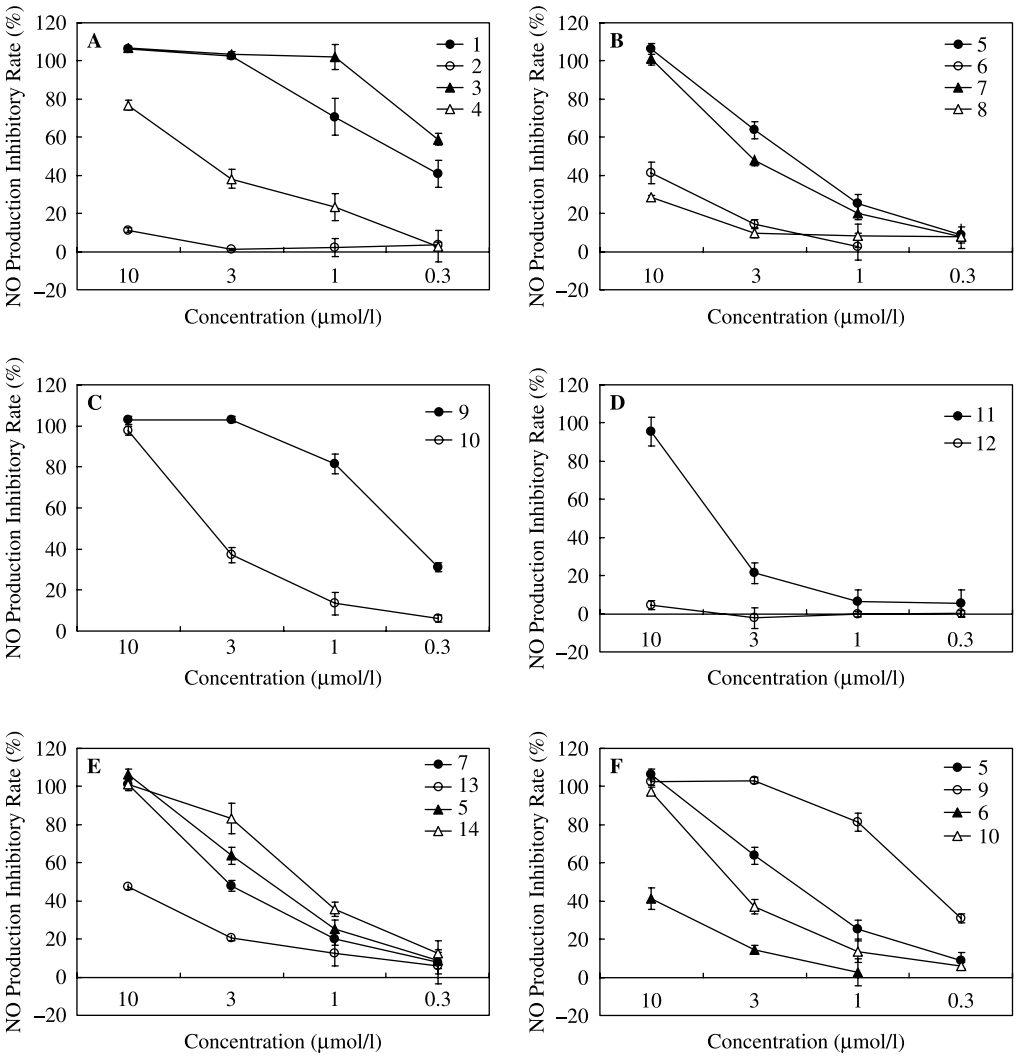

Figure 2. Structure–activity relationship. RAW 264.7 cells were treated with 1 μg/ml of LPS with or without various concentrations (10, 3, 1, 0.3 μmol/l) of test compounds for 24 h. As a parameter of NO synthesis, the nitrite concentration was measured in the supernatant of RAW 264.7 cells by the Griess reaction as described in the experimental. The concentration of NO_2^- was calculated by a working line from sodium nitrite solutions. The inhibitory rate on NO production induced by LPS was calculated by the NO_2^- levels and shown in the figure.

fraction, EtOAc fraction, *n*-butanol fraction, and water-soluble fraction. Sesquiterpenes **1–4**, **6**, **8–10**, **12**, and **15–18** were obtained from the petroleum ether fraction by a combination of silica gel, Sephadex LH-20, ODS column chromatography, and PTLC. Compounds above were identified as dehydrocostus lactone (**1**) [13], 11β,13-dihydrodehydrocostus lactone (**2**) [14], costunolide (**3**) [13], 11β,13-dihydrocostunolide (**4**) [15], 11β,13-dihydrosantamarin (**6**) [16–17], 11β,13-dihydro-reynosin (**8**) [16–17], α-cyclocostunolide (**9**) [13], 11β,13-dihydro-α-cyclocostunolide (**10**) [18–19], 1β-hydroxycolartin (**12**) [20–21], ilicol (**15**) [21–22], 11β,13- dihydro-β-cyclocostunolide (**16**) [18–19], alantolactone (**17**) [22], and isoalantolactone (**18**) [22] by comparison of their

Table 3. Inhibitory effects of compounds **1**, **3**, **9**, **17**, and **18** on the TNF-α release induced by LPS.

	Concentration of TNF-α (pg/ml)			
Untreated	63.57 ± 6.41			
1 μg/ml LPS	3389.97 ± 39.11			
	2 μmol/l	1 μmol/l	0.5 μmol/l	0.25 μmol/l
LPS + **1**	218.31 ± 22.47***	729.93 ± 14.64***	1856.05 ± 347.58*	2327.03 ± 296.97*
LPS + **3**	287.05 ± 14.81***	771.96 ± 18.99***	1629.49 ± 122.50**	2377.41 ± 45.73**
LPS + **9**	963.30 ± 137.74**	1204.94 ± 78.10***	2980.34 ± 188.67	3448.47 ± 78.91
LPS + **17**	845.52 ± 116.52***	1262.65 ± 32.00***	2520.41 ± 309.31*	3059.42 ± 159.65
LPS + **18**	815.65 ± 97.60***	1352.65 ± 192.50**	2248.04 ± 98.41**	3032.55 ± 96.11*

Note: *Significant difference compared with LPS group. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

spectral data with those reported in the literature, respectively.

3.2 Semi-synthesis of sesquiterpene derivatives

Lactone **3** was converted to a series of oxidation products by previously described methods [23]. In detail, a solution of **3** (costunolide, 530 mg, 2.284 mmol) in CH_2Cl_2 (10 ml) was stirred with 85% *m*-CPBA (556.2 mg, 2.741 mmol) at 0°C until TLC indicated that all of **3** had reacted (about 1.5 h). Then the solution was washed successively with 10% aqueous solution of $NaHCO_3$ (10 ml) and a saturated aqueous solution of NaCl (10 ml). The organic phase was dried (anhydrate $MgSO_4$) and concentrated to give a crude powder product (690 mg), which was subjected to silica gel column chromatography using hexane–EtOAc (3:1) as the mobile phase to yield pure **5**, **7**, and **11**. Compounds **7** and **5** were further reacted in the usual manner with pyridine–acetic anhydride to afford acetates **13** and **14**, respectively. All the chemical structures of the semi-synthesized derivatives were determined on the basis of physiochemical properties and spectroscopic analysis.

3.2.1 Santamarin (**5**)

$C_{15}H_{20}O_3$ (Mr: 248) colorless needles, m.p. 137–138°C, $[\alpha]_D^{20} + 95.8$ (*c* 0.50, $CHCl_3$). IR (KBr) ν_{max} cm^{-1}: 3380 (OH); EI-MS *m/z* (%): 248 (M^+, 95), 230 ($M^+ - H_2O$, 39), 215 ($M^+ - H_2O - Me$, 16); ^{1}H NMR (400 MHz, in $CDCl_3$) δ: 6.03 (1H, d, $J = 3.2$ Hz, H-13a), 5.37 (1H, d, $J = 3.2$ Hz, H-13b), 5.31 (1H, br.s, H-3), 3.91 (1H, t, $J = 11.0$ Hz, H-6β), 3.63 (1H, dd, $J = 9.9$, 6.6 Hz, H-1α), 1.79 (3H, br.s, H-15), 0.84 (3H, s, H-14). ^{13}C NMR spectral data see Table 4.

3.2.2 Reynosin (**7**)

$C_{15}H_{20}O_3$ (Mr: 248) colorless needles, m.p. 145–146°C, $[\alpha]_D^{20} + 178$ (*c* 0.20, $CHCl_3$). IR

Table 4. ^{13}C NMR spectral data (100 MHz, $CDCl_3$, δ) of compounds **5**, **7**, **11**, **13,** and **14**.

Atom	5	7	11	13	14
1	75.3 d	78.3 d	78.4 d	79.2 d	76.7 d
2	32.8 t	31.3 t	28.4 t	27.7 t	29.4 t
3	121.3 d	33.5 t	39.0 t	33.2 t	120.9 d
4	133.5 s	142.4 s	71.3 s	141.9 s	133.3 s
5	51.2 d	53.0 d	56.6 d	53.0 d	50.9 d
6	81.5 d	79.6 d	80.9 d	79.3 d	81.1 d
7	51.1 d	49.6 d	50.4 d	49.4 d	51.0 d
8	21.3 t	21.5 t	38.1 t	21.3 t	21.0 t
9	34.3 t	35.7 t	21.8 t	35.4 t	34.1 t
10	40.9 s	43.0 s	41.9 s	41.9 s	39.7 s
11	139.0 s	139.3 s	138.1 s	139.1 s	138.8 s
12	170.7 s	170.6 s	169.6 s	170.4 s	170.6 s
13	116.8 t	117.0 t	118.1 t	117.1 t	116.9 t
14	11.1 q	11.6 q	13.1 q	11.6 q	12.3 q
15	23.3 q	110.7 t	24.3 q	111.1 t	23.3 q
CH_3CO				170.5 s	170.6 s
CH_3CO				21.1 q	21.1 q

(KBr) ν_{max} cm^{-1}: 3400 (OH); EI-MS *m/z* (%): 248 (M^+, 6), 230 ($M^+ - H_2O$, 100), 215 ($M^+ - H_2O - Me$, 9); ^1H NMR (400 MHz, in $CDCl_3$) δ: 5.99 (1H, d, $J = 3.2$ Hz, H-13a), 5.34 (1H, d, $J = 3.2$ Hz, H-13b), 4.89 (1H, br.s, H-15a), 4.76 (1H, br.s, H-15b), 3.95 (1H, t, $J = 10.8$ Hz, H-6β), 3.44 (1H, dd, $J = 7.6$, 4.3Hz, H-1α), 0.73 (3H, s, H-14). ^{13}C NMR spectral data see Table 4.

3.2.3 1β-Hydroxyarbusculin A (*11*)

$C_{15}H_{22}O_4$ (Mr: 248) colorless needles, m.p. 134–135°C. IR (KBr) ν_{max} cm^{-1}: 3390 (OH), 1770 (C = O); EI-MS *m/z* (%): 266 (M^+, 4), 248 ($M^+ - H_2O$, 21), 230 ($M^+ - 2H_2O$, 32), 215 ($M^+ - 2H_2O - Me$, 22); ^1H NMR (400 MHz, in $CDCl_3$) δ: 6.12 (1H, d, $J = 3.2$ Hz, H-13a), 5.46 (1H, d, $J = 3.2$ Hz, H-13b), 4.12 (1H, m, H-6), 3.45 (1H, m, H-1), 1.36 (3H, s, H-15), 0.98 (3H, s, H-14). ^{13}C NMR spectral data see Table 4.

3.2.4 Acetoxyreynosin (*13*)

$C_{17}H_{22}O_4$ (Mr: 290) colorless needles, m.p. 247–249°C. IR (KBr) ν_{max} cm^{-1}: 1780, 1735, 1645, 1245; EI-MS *m/z* (%): 290 (M^+, 3), 230 ($M^+ -$ AcOH, 100), 215 ($M^+ -$ AcOH − Me, 24); ^1H NMR (400 MHz, in $CDCl_3$) δ: 6.09 (1H, d, $J = 3.2$ Hz, H-13a), 5.41 (1H, d, $J = 3.2$ Hz, H-13b), 5.01 (1H, br.s, H-15a), 4.89 (1H, br.s, H-15b), 4.78 (1H, dd, $J = 11.7$, 4.7 Hz, H-1), 4.00 (1H, t, $J = 10.9$ Hz, H-6), 0.90 (3H, s, H-14), 2.06 (3H, s, H–OAc). ^{13}C NMR spectral data see Table 4.

3.2.5 Acetoxysantamarin (*14*)

$C_{17}H_{22}O_4$ (Mr: 290) colorless needles, m.p. 125–126°C. IR (KBr) ν_{max} cm^{-1}: 1770, 1735; EI-MS *m/z* (%): 290 (M^+, 4), 230 ($M^+ -$ AcOH, 100), 215 ($M^+ -$ AcOH − Me, 9); ^1H NMR (400 MHz, in $CDCl_3$) δ: 6.07 (1H, d, $J = 3.2$ Hz, H-13a), 5.39 (1H, d, $J = 3.2$ Hz, H-13b), 5.33 (1H, br.s, H-3), 4.88 (1H, dd, $J = 9.9$, 6.8 Hz, H-1), 3.92 (1H, t, $J = 11.0$ Hz, H-6), 1.84 (3H, s, H-15), 0.96 (3H, s, H-14), 2.06 (3H, s, H—OAc). ^{13}C NMR spectral data see Table 4.

3.3 Cell lines, chemicals, and biochemicals

Mouse macrophage RAW 264.7 was purchased from Chinese Academy of

Sciences, Beijing, PR China (ATCC: TIB-71). Lipopolysaccharide (LPS, from *E. coli*), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and hydrocortisone were purchased from Sigma Aldrich (MO, USA). RPMI 1640 medium and fetal bovine serum were purchased from Gibco Corporation (NY, USA). ELISA kit for Mouse TNF-α was purchased from R&D Systems (MN, USA).

3.4 Cell culture and treatments

Mouse macrophage RAW 264.7 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Sesquiterpenes **1–18** were dissolved in cell culture level dimethyl sulfoxide (DMSO), and were added to the culture medium to give a final DMSO concentration of 0.2% (v/v). This concentration of DMSO had no significant effect on the cell growth.

3.5 Determination of NO production [24]

Cell concentration was adjusted to 5×10^5 cells/ml and 200 μl was seeded in every well of a 96-well plate. After 1 h incubation, the cells were treated with 1 μg/ml of LPS with or without various concentrations of test compounds for 24 h. As a parameter of NO synthesis, the nitrite concentration was measured in the supernatant of RAW 264.7 cells by the Griess reaction as previously described. Briefly, 100 μl of culture medium in each well was taken out to another plate, and the level of NO was assessed by measuring the accumulation of nitrite (NO_2^-) using 100 μl of Griess agent (mixture of 0.1% *N*-[1-naphthyl]-ethylenediamine in 5% phosphoric acid and 1% sulfanilamide). The concentration of NO_2^- was calculated by a working line from 0, 1, 2, 5, 10, 20, 50, and 100 μmol/l sodium nitrite solutions. The inhibitory rate on NO production induced by LPS was calculated by the NO_2^- levels as follows:

$$\text{Inhibitory rates } (\%) = 100\text{cr}$$

3.6 MTT assay for cell cytotoxicity

After taking out 100 μl of supernatant in each well to determine the value of NO, 4 μl of MTT solution (5 mg/ml) was added to each well and cultured for another 4 h. The reduced MTT-formazan was solubilized with 100 μl of DMSO and the absorbance of MTT-formazan solution at 570 nm was measured by an immuno-reader using 630 nm as reference wave. The cell viability was calculated by comparing the delta OD values of sample-treated cells with that of non-treated cells.

3.7 Measurement of TNF-α concentration

Cell concentration was adjusted to 5×10^5 cells/ml and 200 μl was seeded in every well of a 96-well plate. After a 1-h incubation, cells were treated with LPS (1 μg/ml) and various concentrations of test samples for 6 h. About 100 μl of culture medium in each well was taken out and assayed by using a commercial enzyme-linked immunosorbent assay (ELISA) kit. The assays were performed according to the manufacturer's instructions.

3.8 Data analysis

Every experiment was performed in triplicate, and data are expressed as mean ± SD of three independent experiments. Statistical analysis of the data was performed using Student *t*-test. Differences with $*p < 0.05$ were considered as statistically significant.

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