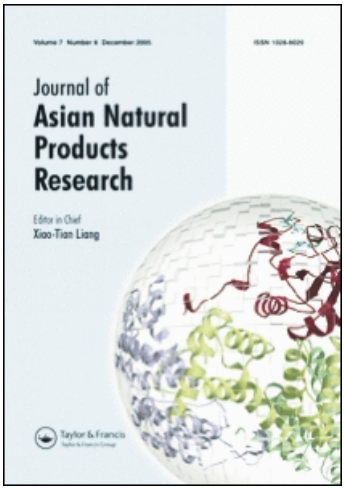

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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To cite this Article Liu, Li-Fang , Ma, Xiao-Li , Wang, Yu-Xin , Li, Feng-Wen , Li, Yun-Man , Wan, Zhu-Qing and Tang, Qiu-Ling(2009) 'Triterpenoid saponins from the roots of *Clematis chinensis* Osbeck', Journal of Asian Natural Products Research, 11: 5, 389 — 396
To link to this Article: DOI: 10.1080/10286020902867268
URL: http://dx.doi.org/10.1080/10286020902867268

Journal of Asian Natural Products Research
Vol. 11, No. 5, May 2009, 389–396

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Li-Fang Liu[a]*, Xiao-Li Ma[a], Yu-Xin Wang[b], Feng-Wen Li[b], Yun-Man Li[c], Zhu-Qing Wan[a] and Qiu-Ling Tang[a]

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(*Received 27 September 2008; final version received 25 February 2009*)

A new triterpenoid saponin named clematichinenoside AR_2, along with the six known compounds, was isolated and characterized from *Clematis chinensis* Osbeck (Ranunculaceae), a commonly used traditional Chinese medicine with anti-inflammatory and anti-rheumatoid activities. The structure of the new saponin was elucidated as 3-*O*-β-[(*O*-α-L-rhamnopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-ribopyranosyl-(1 → 3)-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)oxy]olean-12-*en*-21α-hydroxy-28-oic acid-*O*-α-L-rhamnopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**1**) by spectral analysis and chemical methods. The effects of two major saponins (clematichinenosides AR and AR_2) on the secretion of TNF-α in murine peritoneal macrophages induced by lipopolysaccharides were further investigated. The result indicated that a majority of triterpenoid saponins of this herb may be useful in the exploration of lead compounds for the treatment of some autoimmune diseases.

Keywords: Ranunculaceae; *Clematis chinensis* Osbeck; triterpenoid saponins; clematichinenoside AR; clematichinenoside AR_2; TNF-α

1. Introduction

Clematis chinensis Osbeck (Ranunculaceae), a plant widely distributed in China, is one of the three sources of a commonly used traditional Chinese medicine called "Wei-Lin-Xian" with anti-inflammatory and anti-rheumatoid activities [1]. During the last decade, more than 50 major triterpenoid saponins with molecular weight larger than 1300 kDa were characterized from the root extracts of Clematis species and some of them showed significant anti-inflammatory, antitumor, and analgesic activities [2–9]. Our preliminary pharmacological studies revealed that the total number of saponins prepared from the 50% alcoholic extract of this herb showed significant inhibitory activity on the Freund's complete adjuvant-induced paw edema in rats, suggesting the potential use of these components in the treatment of rheumatoid arthritis [10].

To investigate the major active constituents of the herbal extract, six triterpenoid saponins (**2**–**7**) with a new compound (**1**), designated clematichinenoside AR_2 (AR_2), were isolated from the total number of saponins by chromatographic methods. Their structures were elucidated on the basis of spectroscopic analysis, including extensive 1D and 2D NMR studies, and through comparison

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020902867268
http://www.informaworld.com

of the NMR and MS spectral data with those reported in the literatures. Meanwhile, the inhibitory activities on the TNF-α of these components were further evaluated by measuring their effects on the production of TNF-α in murine peritoneal macrophages induced by lipopolysaccharides (LPS).

2. Results and discussion

2.1 Chemical structure analysis

Compound **1** was obtained as a white amorphous solid (MeOH), mp 235.5–238.5°C. The optical rotation ($[\alpha]_D^{20}$) was −31.6 ($c = 0.15$, H_2O). The IR spectrum showed the presence of hydroxyl groups at 3419 cm^{-1} and carbonyl groups at 1658 cm^{-1}. A high-resolution mass spectrum (ESI) gave a strong double charge ion with the *m/z* value of 934.4039, indicating a pseudo-molecular formula of $C_{82}H_{134}O_{44}Na_2$ (calcd 934.4016) and therefore the molecular formula was found to be $C_{82}H_{134}O_{44}$. This is consistent with the ^{13}C and ^{1}H NMR spectral data. The ^{1}H NMR spectrum of **1** exhibited nine signals of the sugar anomeric protons at δ 4.82 (1H, m, H-1 of Ara), 6.23 (1H, s, H-1 of Rha), 5.82 (1H, m, H-1 of Rib), 4.93 (1H, m, H-1 of Glc), 5.08 (1H, m, H-1 of Glc′), 5.40 (1H, m, H-1 of Rha′), 6.23 (1H, m, H-1 of Glc″), 4.96 (1H, m, H-1 of Glc‴), 5.82 (1H, m, H-1 of Rha″) and methyl signals of the three rhamnose at δ 1.57 (3H, d, $J = 6.0$ Hz, Me-6 of Rha″), 1.67 (3H, d, $J = 6.2$ Hz, Me-6 of Rha′), 1.51 (3H, d, $J = 6.0$ Hz, Me-6 of Rha). Also there are seven methyl signals of aglycone shown in the ^{1}H NMR spectrum at δ 1.26 (3H, s, Me-23), 1.32 (3H, s, Me-27), 1.12 (3H, s, Me-24), 1.06 (3H, s, Me-26), 2.21 (3H, s, Me-29), 0.99 (3H, s, Me-30), and 0.86 (3H, s, Me-25). The ^{13}C NMR spectral data were reported in Table 1, which showed the oleanolic acid as the aglycone. However, the downfield shift of C-3 and the upfield shift of C-28 suggested that the hydroxyl group of C-3 and the carbonyl group of C-28 were both glycosidated. The chemical shift of C-21 moved to the downfield at δ 73.4 because of the hydroxylation. For the aglycone, the ROESY spectrum showed the correlation of H-21 and H-30, so the hydroxy group was located in the α-position. The acid hydrolysis of compound **1** showed the presence of arabinose, glucose, rhamnose, and ribose. The TOCSY and COSY spectral data supported the above result. Compared with the data in the literatures [2,7], the exact sugar sequence and its linkage position to the aglycone were solved by a detailed analysis of the 2D NMR spectra. The HMBC spectrum showed the correlations from H-Rha′-1 to C-Glc′-6, H-Glc′-1 to C-Glc-6, H-Glc-1 to C-Rib-4, H-Rib-1 to C-Rha-3, H-Rha-1 to C-Ara-2, H-Ara-1 to C-3, H-Glc″-1 to C-Glc‴-4, and H-Glc‴-1 to C-28. So, the structure of this compound was elucidated as 3-*O*-β-[(*O*-α-L-rhamnopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-ribopyranosyl-(1 → 3)-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)oxy]olean-12-*en*-21α-hydroxy-28-oic acid-*O*-α-L-rhamnopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**1**), designated as clematichinenoside AR_2 (AR_2).

Six known compounds were identified as 3-*O*-β-[(*O*-α-L-rhamnopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-ribopyranosyl-(1 → 3)-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)oxy]olean-12-*en*-28-oic acid (**2**) [2]; clematichinenoside C, 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**3**) [5]; huzhangoside B, 3-*O*-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-

Table 1. ^{1}H and ^{13}C NMR spectral data for compound **1** (pyridine-d_5).

Position	δ_C (ppm)	δ_H (ppm)
1	39.0	0.93, 1.51
2	26.6	1.83, 2.07
3	88.7	3.26
4	39.5	
5	56.0	0.75
6	18.4	1.19, 1.39
7	33.2	1.21, 1.36
8	39.8	
9	48.1	1.63
10	37.0	
11	23.8	1.90
12	122.7	5.46
13	144.4	
14	42.4	
15	28.3	1.14, 2.21
16	27.0	2.35, 3.06
17	47.4	
18	41.7	3.37
19	41.4	1.19, 2.53
20	35.7	
21	73.4	3.66
22	39.6	2.24, 2.27
23	28.1	1.26 s
24	17.1	1.12 s
25	15.7	0.86 s
26	17.5	1.06 s
27	25.6	1.32 s
28	176.5	
29	28.7	2.21 s
30	25.0	0.99 s
3-*o*-sugar		
Ara-1	105.1	4.82
2	75.4	4.52
3	74.5	4.24
4	69.2	4.22
5	65.5	3.81, 4.28
Rha-1	101.4	6.23
2	71.9	4.65
3	81.8	4.14
4	71.9	4.86
5	69.7	4.28
6	18.4	1.57 d, 6.0 Hz
Rib-1	104.6	5.82
2	72.5	4.38
3	69.8	4.54
4	76.4	3.85
5	61.9	4.40
Glc-1	103.2	4.93
2	74.0	4.28
3	76.6	4.28
4	82.0	4.64
5	76.6	4.15
6	61.7	4.25
Glc′-1	104.8	5.08
2	74.8	4.00
3	78.1	4.38
4	71.9	4.72
5	76.7	4.01
6	68.5	3.92
Rha′-1	102.7	5.40
2	71.7	3.90
3	72.7	4.51
4	73.9	4.18
5	69.8	4.28
6	18.5	1.67 d, 6.2 Hz
28-*o*-sugar		
Glc″-1	95.6	6.23
2	73.8	4.07
3	78.7	4.14
4	70.9	4.24
5	78.0	4.07
6	69.3	4.63
Glc‴-1	104.8	4.96
2	75.2	3.87
3	76.4	3.85
4	78.3	4.38
5	77.0	3.63
6	61.3	4.09
Rha″-1	102.7	5.82
2	72.5	4.07
3	72.6	4.63
4	74.1	3.85
5	70.2	4.92
6	18.5	1.51 d, 6.0 Hz

(1 → 6)-β-D-glucopyranosyl ester (**4**) [11]; hederasaponin B, 3-*O*-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**5**) [11]; clematichinenoside AR (AR), 3-*O*-β-[(*O*-α-L-rhamnopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-ribopyranosyl-(1 → 3)-α-*O*-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)oxy]oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**6**) [11]; clematomandshurica

saponin C, 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**7**) [7] by comparison of their spectral data with those reported in the literature (Figure 1).

2.2 Inhibitory effects of AR and AR_2 on LPS-induced TNF-α secretion of murine peritoneal macrophages

The murine peritoneal macrophages (10^5 cells/well) were cultured in 96-well plates, stimulated with LPS at a final concentration of 10 μg/ml for 2 h, and co-incubated with different doses of AR and AR_2 (0.5, 1.0, 5, 10, and 20 μg/ml, respectively) or cyclophosphamide (as the positive control; 0.1 μg/ml) at 37°C for 24 h. The secretion of TNF-α was measured according to the method of the ELISA kit from the manufacturer. The amount of TNF-α was obtained by a standard curve from plotting concentration versus the value of optical density, which was $Y = 0.8993 \ln(X) - 3.3644$ and the corresponding coefficient was 0.9973.

The effects of AR and AR_2 on LPS-induced TNF-α excretion of mouse peritoneal macrophages were shown in Figure 2. Compared with the model control (242.0 ± 8.2) and the positive control, the secretion of TNF-α in the AR- and AR_2-treated groups were significantly reduced at the dosage of 5, 10, and 20 μg/ml ($P < 0.01$) and the IC_{50} value of each compound was 2.96 μM (5.4 μg/ml) and 3.02 μM (5.5 μg/ml), respectively. As shown in this study, these two saponins can significantly inhibit the LPS-induced TNF-α secretion in murine peritoneal macrophages.

3. Experimental

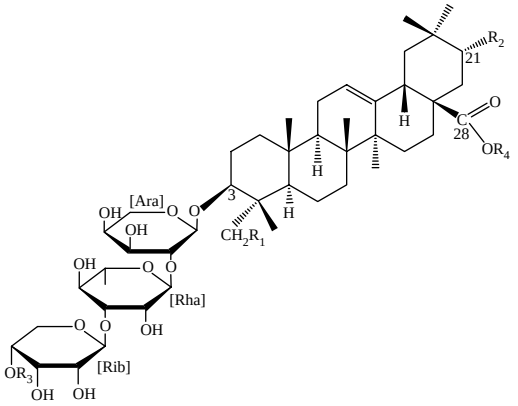
3.1 General experimental procedures

IR spectra were performed with Nicolet Impact 410 infrared spectrometer detector (Waltham, MA, USA). Optical rotations were measured using a PE-241MC (Norwalk, OH, USA) automatic digital polarimeter. NMR spectra were measured on a Bruker ACF-500 NMR spectrometer (Fallanden, Switzerland) with TMS as an internal standard and pyridine-d_5 was used as a solvent. MS spectra were recorded on an LC-MSD-Trap-SL and LC/MSD TOF system (Agilent, Santa Clara, CA, USA). HR-MS measurements were performed in the positive ion mode with a scan range 100–3000 *m/z* and the flow rate of drying gas was 8 l/min; temperature of the dry gas was 310°C; the capillary voltage was set to 4000 V. ODS silica gel (FUJI Silysia Chemical, Kasugai, Japan) and HPD100 macroporous absorption resin (CangZhou Bonchem, Hebei, China) were used for column chromatography. TLC was carried out on precoated Kieselgel 60 F254 (0.25 mm; Merck, Darmstadt, Germany) and on RP-18 F254S (0.25 mm; Merck) plates. TLC spots were visualized by spraying the plates with 10% H_2SO_4 solution followed by heating. Cyclophosphamide was obtained from Jiangsu Hengrui Pharmaceutical Co. (Lot: 07110621; Lianyungang, China). LPS were from Sigma Co. (St Louis, MO, USA) and murine TNF-α ELISA kit (96T) was purchased from ADL Co. (Lot: RT110371; Fremont, CA, USA). All other chemicals used were of analytical reagent grade.

3.2 Plant material and animals

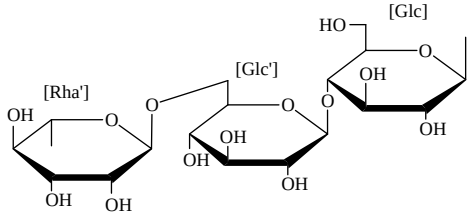
The roots of *C. chinensis* (050318) were obtained from Bozhou City, Anhui Province of China and identified by one of the authors (Liu). A voucher specimen (050318) has been deposited in the China Pharmaceutical University.

SPF male mice (ICR), weighing 14–16 g, were purchased from the Comparative Medicinal Center of Yangzhou University (Jiangsu, China). All surgical and experimental procedures were performed in



	R_1	R_2	R_3	R_4
1	H	OH	S-1	S-3
2	H	H	S-1	H
3	H	H	S-2	S-3
4	H	H	H	S-3
5	OH	H	H	S-3
6	H	H	S-1	S-3
7	H	H	S-4	S-3

S-1=



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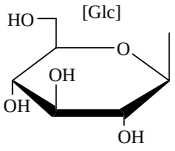
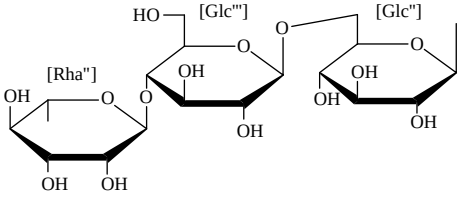


S-3=



S-4=

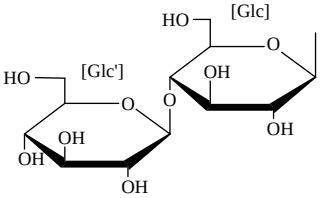


Figure 1. Structures of compounds **1**–**7**.

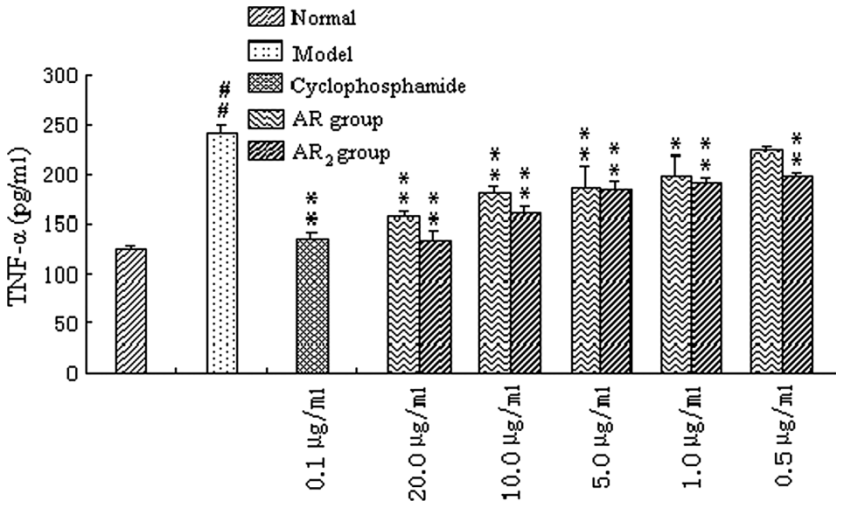


Figure 2. Effects of AR and AR_2 on LPS-induced TNF-α secretion of murine peritoneal macrophages. Each bar is mean ± SD ($n = 3$). The murine peritoneal macrophages (10^5 cells/well) were stimulated with LPS in a final concentration of 10 μg/ml for 2 h and co-incubated with different doses of AR and AR_2 (0.5, 1.0, 5, 10, and 20 μg/ml, respectively) or cyclophosphamide (0.1 μg/ml). The concentration of TNF-α was obtained by a standard curve. Compared with the model control (242.0 ± 8.2), the secretion of TNF-α in the AR and AR_2 treated groups were significantly reduced at the dose of 5, 10, and 20 μg/ml. The IC_{50} value of each compound was 2.96 μM (5.4 μg/ml) and 3.02 μM (5.5 μg/ml), respectively. $*P < 0.05$, $**P < 0.01$, when compared with the model group.

accordance with the Institutional Animal Care Guidelines of the University.

3.3 Extraction and isolation

The roots of *C. chinensis* (5.0 kg) were extracted with 50% EtOH twice (10 l and 3 h each) at 90°C. The EtOH extract was concentrated under reduced pressure at 70°C, and the viscous concentrate (707 g) was passed through a HPD100 macroporous adsorption resin column, successively eluting with water, 20, 50, and 95% EtOH. After collecting the part of 50% EtOH eluant and partitioning it with *n*-BuOH, the concentrate (230 g) was subjected to silica gel column eluting with a mixture of chloroform–MeOH–water (13:7:1). The eluant was concentrated and passed through an ODS silica gel column chromatography eluting with a stepwise gradient mixture of water–acetonitrile (8:2; 7:3; 6:4; and 5:5). Compounds **1** (80 mg), **2** (0.5 g), **4** (80 mg), **5** (40 mg), **6** (12 g) and a mixture of compounds **3**, **6**, and **7** (1 g) were obtained. Compound **3** (0.2 g) was obtained through further ODS silica gel column eluted with acetonitrile–water (30:70) and compound **7** (40 mg) was isolated by preparative HPLC with acetonitrile–water (30:70).

*3.3.1 Compound **1***

It was obtained as a white amorphous solid (MeOH); mp 235.5–238.5°C; $[\alpha]_D^{20}$ −31.6 ($c = 0.15$, H_2O); IR (KBr) cm^{-1}: 3419 (OH), 2933 (C—H), 1658 (C═O), 1061, 739; ^1H NMR (pyridine-d_5): δ 4.82 (1H, m, H-1 of Ara), 6.23 (1H, s, H-1 of Rha), 5.82 (1H, m, H-1 of Rib), 4.93 (1H, m, H-1 of Glc), 5.08 (1H, m, H-1 of Glc′), 5.40 (1H, m, H-1 of Rha′), 6.23 (1H, m, H-1 of Glc″), 4.96 (1H, m, H-1 of Glc‴), 5.82 (1H, m, H-1 of Rha″), 1.57 (3H, d, $J = 6.0$ Hz, Me-6 of Rha″), 1.67 (3H, d, $J = 6.2$ Hz, Me-6 of Rha′), 1.51 (3H, d, $J = 6.0$ Hz, Me-6 of Rha), 1.26 (3H, s, Me-23), 1.32 (3H, s, Me-27), 1.12 (3H, s, Me-24), 1.06 (3H, s, Me-26), 2.21 (3H, s, Me-29), 0.99 (3H, s,

Me-30), 0.86 (3H, s, Me-25); ^{13}C NMR spectral data, see Table 1. HR-ESI-MS: *m/z* 934.4039 $[M+2Na]^{2+}$ (calcd for $C_{82}H_{134}O_{44}Na_2$, 934.4016).

3.4 Acid hydrolysis of saponins

Each compound (30.0 mg) was subjected to acid hydrolysis in 1.0 mol/l HCl and heated at 90°C for 1 h under reflux. After cooling, the mixture was neutralized and then extracted with chloroform. The aqueous layer was analyzed by HP-TLC to confirm the sugar components with the standard preparation. TLC was carried out on precoated Kieselgel 60 F254 (0.25 mm; Merck) and on RP-18 F254S (0.25 mm; Merck) plates. The following two solvent systems were used to detect the sugars by HP-TLC: (1) $CHCl_3$–MeOH–H_2O (70:3:3) and (2) pyridine-*n*-butanol–H_2O (6:4:1). TLC spots were visualized by spraying the plates with 10% H_2SO_4 solution followed by heating [3].

3.5 The isolation and culture of murine peritoneal macrophages

Macrophages play a significant role in host defense mechanisms. Reactive oxygen and nitrogen species, such as superoxide anion, peroxide, hydroxy radical, and nitrite radical, are generated when these cells are activated. They can act as mediators of inflammation when over produced [12,13]. In addition, overproduction of proinflammatory cytokines, such as interleukine-6 and TNF-α, plays a key role in the onset and progress of inflammatory diseases [14–16]. Compounds effective in suppressing macrophages response to activation can significantly reduce inflammation processes. The aim of this study was to determine whether these two main saponins (AR and AR_2) play a protective effect against TNF-α secretion in stimulated murine macrophages.

The murine peritoneal macrophages were isolated and cultured as previously described [17]. The cells were cultured in the conditions of 37°C, with 5% of carbon dioxide (CO_2) after the inoculation on a 96-well plate with a concentration of 2×10^6 cell/ml. The adherent cells were used to perform the experiments.

4. Concluding remarks

The TNF-α is a major proinflammatory cytokine, which regulates inflammation and related disorders [18,19]. Several protein-based TNF-α inhibitors have been demonstrated for their efficacy. However, some potentially serious adverse effects that include greater predisposition towards infection, congestive heart failure, neurological changes, and problems related to autoimmunity were also reported. Thus, it has become important and essential to develop safer and perhaps more cost-effective TNF-α inhibitors. The past decades have witnessed that natural products have been, and continue to be a major source of pharmacologically active substances from which drugs can be developed. Actually, many natural compounds belonging to various classes have been found to reduce TNF-α levels. These natural compounds have been found to interfere with various proinflammatory mediators and upstream targets, such as nuclear transcription factor-κB and other signaling molecules, involved in TNF-α expression. Therefore, it could provide an alternative means of treating inflammatory diseases by modulating production, rather than the activity of TNF-α [20].

Our study reports the isolation and purification of seven main triterpenoid saponins from the roots of *C. chinensis*, a commonly used Chinese herb. The two major saponins have high efficacy on inhibiting the production of TNF-α in murine peritoneal macrophages induced by LPS. These findings warrant further studies on the potential therapeutic value of these components as lead compounds for

the more cost-effective TNF-α inhibitors for the treatment of some autoimmune diseases.

Acknowledgements

Financial supports from the National Natural Science Foundation of China (Project No. 30772770) and High Technology Foundation Program of Jiangsu Province (No. BG2007613) are gratefully acknowledged.

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