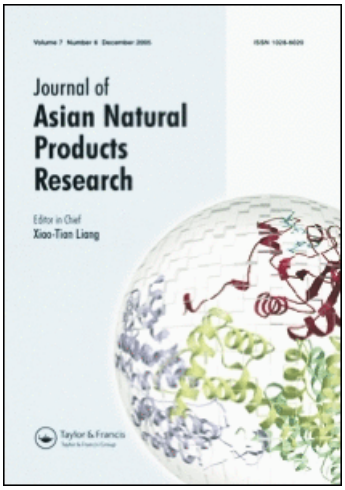

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[a] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, The Chinese Academy of Sciences, Kunming, China [b] Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China [c] Natural Products Resource Research Centre, College of Life Sciences, Northwest A and F University, Yangling, China

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Speciosaperoxide, a new triterpene acid, and other terpenoids from *Chaenomeles speciosa*

Ya-Ling Song[ab], Li Zhang[c], Jin-Ming Gao[b], Guan-Hua Du[c] and Yong-Xian Cheng[a]*

[a]*State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, The Chinese Academy of Sciences, Kunming 650204, China;* [b]*Natural Products Resource Research Centre, College of Life Sciences, Northwest A and F University, Yangling 712100, China;* [c]*Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing 100050, China*

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Investigation on the EtOH extract of the fruits of *Chaenomeles speciosa* led to the isolation of a new triterpene acid bearing an unusual hydroperoxyl substitute group at C-11, speciosaperoxide (**1**), along with six known triterpenoids, 3β-acetoxyurs-11-en-13β,28-olide (**2**), 3-*O*-acetyl ursolic acid (**3**), oleanolic acid (**4**), ursolic acid (**5**), masilinic acid (**6**), and tormentic acid (**7**), and three known norsesquiterpenoids, roseoside (**8**), vomifoliol (**9**) and (6*S*,7*E*,9*R*)-6,9-dihydroxy-4,7-megastigmadien-3-one 9-*O*-[β-D-xylopyranosyl (1 → 6)-glucopyranoside] (**10**). Their structures were elucidated on the basis of spectroscopic data and comparison with reference data. Besides compound **1**, compounds **2**, **8**–**10** were obtained from this genus for the first time. None of these compounds exhibited inhibitory activity against T-and B-lymphocyte proliferation.

Keywords: *Chaenomeles speciosa*; Rosaceae; peroxide triterpene acid; norsesquiterpenoid; lymphocyte proliferation

1. Introduction

Disease prevention is increasingly becoming a public concern of modern healthcare.[1] Nature manufactures a great deal of diverse structures that may serve as natraceuticals providing medical or health benefits including the treatment and prevention of disease. Edible plants containing nutrients, dietary supplements or secondary metabolites may play an essential role in preventing the incidence of cardio-and cerebrovascular diseases, cancer and various chronic diseases.[2] C*haenomeles speciosa* (Sweet) Nakai (Rosaceae) is a shrub originally growing in China, whose matured fruits are used as both famous herbal medicine for the treatment of rheumatism and arthritis as well as widespread foods.[3–5] Previous investigation revealed that amino acids, carbohydrates, proteins, organic acids, phenolics, triterpenoids and their glycosides and vitamin C are the main components.[6–12] To explore additional secondary metabolites and extend our understanding of their structural features and roles in rheumatoid arthritis disease, a phytochemical investigation followed by bioassay was performed, and ten terpenoids were obtained. Among them, compound **1** was a novel triterpene acid with an unusual hydroperoxyl group at C-11; compounds **2**, **8**–**10** were first isolated from this genus. All of these isolates showed no inhibitory effect on T-and B-lymphocyte proliferation.

2. Results and discussion

The dried fruits of *Chaenomeles speciosa* were extracted with 95% EtOH and partitioned with petroleum ether, EtOAc in H_2O. A combination of normal phase and gel chromatography of petroleum ether extract afforded a new peroxide triterpene acid, speciosaperoxide (**1**), along with six triterpenoids 3β-acetoxyurs-11-en-13β,28-olide (**2**),[13] 3-*O*-acetyl ursolic acid (**3**),[14,15] oleanolic acid (**4**),[16] ursolic acid (**5**),[14,15] masilinic acid (**6**),[17] tormentic acid (**7**),[18] and three norsesquiterpenoids, roseoside (**8**),[19] vomifoliol (**9**)[20] and (6*S*,7*E*,9*R*)-6,9-dihydroxy-4,7-megastigmadien-3-one 9-*O*-[β-D-xylopyranosyl (1 → 6)-glucopyranoside] (**10**)[21] (Figure 1).

Compound **1** was isolated as a white oily solid. The molecular formula was determined as $C_{32}H_{50}O_6$ by HRFAB-MS and ^{13}C NMR and DEPT experiments. The ^1H NMR spectrum distinctly exhibited five singlet methyl groups (δ 0.81, 0.86, 0.88, 1.07, 1.17), two doublet methyl groups (δ 0.96, d, $J = 4.4$ Hz; 0.98, d, $J = 4.4$ Hz), one acetoxyl group (3H, δ 2.05, s), and one olefinic proton (δ 5.48, d, $J = 3.3$ Hz, H-12). The ^{13}C NMR and DEPT spectra showed signals for two carbonyl groups (δ 171.0 and 182.7), a double bond (δ 144.6 and 125.5), and two oxygenated carbons (δ 81.2 and 80.5). Comparison of these data with those of compound **3** indicated that the main difference between them was that a sp^3 methylene in **3** was replaced by an oxygenated sp^3

*Corresponding author. Email: yxcheng@mail.kib.ac.cn

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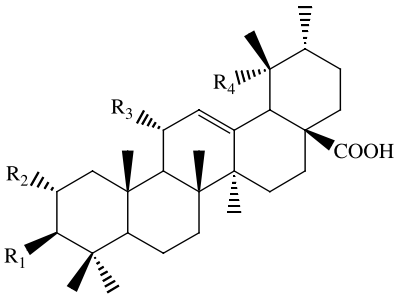
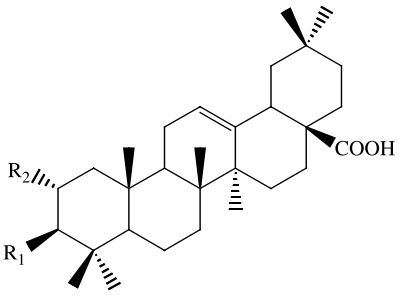
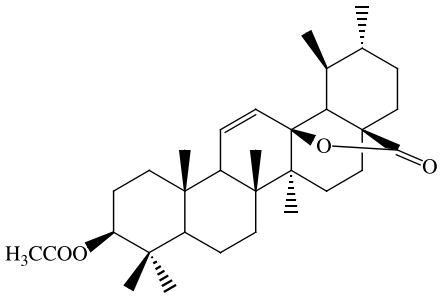


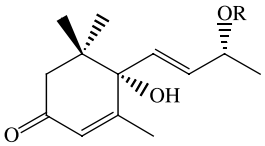
	R_1	R_2	R_3	R_4
1	OAc	H	OOH	H
3	OAc	H	H	H
5	OH	H	H	H
7	OH	OH	H	OH



	R_1	R_2
4	OH	H
6	OH	OH



2



	R
8	Glc
9	H
10	Glc (6-1) xyl

Figure 1. Structures of compounds **1**–**10**.

methine at δ 81.2, this oxygen bearing position was considered to be C-11 by the key HMBC correlations (Figure 2) of H-11 at δ 4.51 with C-9, 10, 12 and 13. A very downfield chemical shift of C-11 at δ 81.2 in **1** instead of δ 68.3 of C-11 in 3β-acetoxy-11α-hydroxy-12-ursene[22] supported the presence of a hydroperoxyl group at C-11 in **1** instead of a hydroxyl group. Further comparison of the NMR spectral data of **1** with those of 3β-acetoxy-11α-hydroxyperoxy-12-ursene[22] indicated that they bore the same substitute group at C-11, the main difference was the CH_3-17 in 3β-acetoxy-11α-hydroxyperoxy-12-ursene was substituted by COOH-17

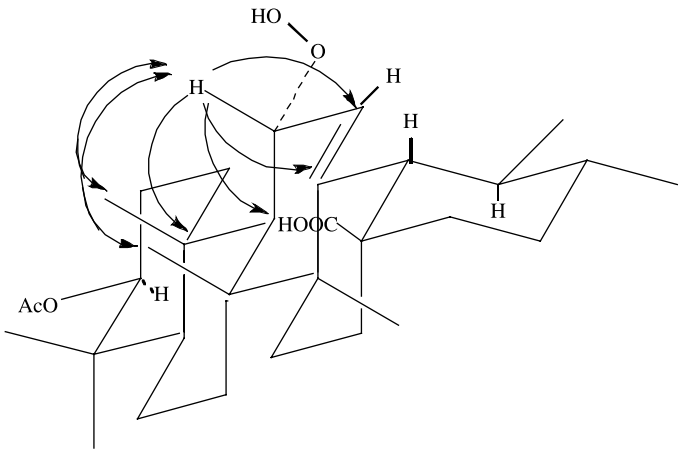


Figure 2. Important NOESY (↔) and HMBC (→) correlations for **1**.

in **1**, which hence led to the downfield shift of C-17 (from δ 33.6 to δ 47.4), and upfield shifts of C-16 (from δ 27.8 to δ 23.8), C-18 (from δ 58.4 to δ 51.9), and C-22 (from δ 41.3 to δ 36.5). A positive brown colour reaction of **1** with 1% KSCN-$FeSO_4$ reagent provided additional evidence for the existence of a hydroperoxyl moiety in **1**, although a downfield proton signal for active hydroperoxyl group was not detected in both DMSO-d_6 and $CDCl_3$. The proton of H-11 was positioned at a β-axial orientation because of the presence of NOESY correlations (Figure 1) of H-11 with CH_3-25 and CH_3-26. H-3 was assigned as α-orientation by comparison of ^{1}H NMR and ^{13}C NMR data with those of **3**. Taken together, the structure of compound **1** was elucidated as 3β-acetoxy-11α-hydroperoxy-12-ursen-28-oic acid.

The common functionality group at C-11 of ursane- and oleanane-type triterpenoids is ketone or hydroxyl. The hydroperoxyl group substitute at this position like compound **1** is unusual, and few similar examples have been isolated from *Ficus microcarpa*.[23,24] Amongst the five species of the genus *Chaenomeles*, only the fruit of *C. speciosa* is accepted by Chinese pharmacopoeia and considered as quality standard. Our present study indicated that as compounds **1**, **2**, **8**–**10** were first isolated from this genus, these components may be also related with the quality of *C. speciosa*.

Considering the medical uses of this herb relative with inflammation and immune response and *in vivo* experimental results on immunoregulatory activity of the total glucosides,[25] an *in vitr*o anti-proliferation assay against T and B lymphocytes was conducted in this study. Unfortunately, none of the isolates showed an inhibitory effect. Does the *in vivo* activity result from metabolic forms of these compounds or from other secondary metabolites present in total glucosides? This question could not be answered here.

3. Experimental

3.1 General experimental procedures

Optical rotation was measured on a P-1020 Polarimeter (JASCO, Tokyo, Japan). 1D and 2D NMR experiments were recorded in pyridine-d_5 or $CDCl_3$ on Bruker AM-400 and DRX-500 spectrometers with tetramethylsilane as the internal standard. Coupling constants are expressed in Hertz and chemical shifts are given on a δ (ppm) scale. FAB-MS and HRFAB-MS spectra were recorded on a VG Auto Spec-3000 (Micromass UK Ltd., Manchester) magnetic sector instrument. Column chromatography was performed on 200–300 mesh silica gel and silica gel H (Qingdao Marine Chemical Factory, China), Sephadex LH-20 (25–100 μm, Amersham Pharmacia Biotech AB) and RP-18 (40–63 μm, Daiso Co., Ltd., Japan). Fractions were monitored by thin-layer chromatography (TLC), with visualisation under UV (254 or 365 nm) or spots staining by spraying with 10% sulphuric acid in ethanol or anisaldehyde reagent, followed by heating.

3.2 Plant material

The dried fruits of *Chaenomeles speciosa* (Sweet) Nakai were commercially purchased from Yunnan Pharmaceutical Co. Ltd., Yunnan Province of China, in March 2005, and identified as *C. speciosa* by Mr Hong-Yan Sun. A voucher specimen (No. 00117) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, China.

3.3 Extraction and isolation

The dried fruits of *C. speciosa* (5 kg) were extracted with 95% EtOH under reflux three times (3 × 10 L). The combined EtOH extracts were evaporated to dryness *in vacuo* and suspended in water followed by partition with petroleum ether and EtOAc. The petroleum ether soluble extracts (18 g) were chromatographed on a silica gel column (900 g, 200–300 mesh) eluting with petroleum ether/EtOAc of increasing polarity, and four fractions were combined based on their TLC patterns. Repeated chromatography of fraction 1 over silica gel (10–40 μm) column with petroleum ether/^{i}PrOH (50:1) as eluent yielded compound **3** (38 mg). Compounds **1** (4 mg) and **2** (18 mg) were purified from fraction 2 by repeated column chromatography over silica gel (10–40 μm) and Sephadex LH-20 (25–100 μm) eluting with MeOH. The EtOAc soluble substance was dried to give 120 g of extract, an aliquot of which (50 g) was subjected to a silica gel column (2500 g, 200–300 mesh) eluting with $CHCl_3$/MeOH mixtures of increasing polarity to afford six fractions. Repeated column chromatography over silica gel, Sephadex LH-20 and RP-18 (40–63 μm) afforded compounds **4** (10 mg) and **8** (11 mg) from fraction 1, compounds **5** (9 mg) and **6** (7 mg) from fraction 2, compounds **7** (23 mg) and **9** (15 mg) from fraction 3, **10** (31 mg) from fraction 4, respectively.

3.3.1 Speciosaperoxide (1)

White oily solid; $[\alpha]_D^{23}$ + 8.3 (*c* 0.0055, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε): 241 (3.16) nm; IR (KBr) ν_{max} (cm^{-1}): 3426, 2923, 2853, 1734, 1691, 1640, 1461, 1369, 1252, 1033; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.10 (2H, m, H-1), 4.52 (1H, m, H-3), 0.90 (1H, m, H-5), 1.84 (1H, d, J = 8.8 Hz, H-9), 4.51 (1H, m, H-11), 5.48 (1H,

Table 1. ^{13}C NMR spectral data of **1** in $CDCl_3$ (100 MHz, δ in ppm).

Position	δ_C	Position	δ_C
1	39.0	17	47.4
2	23.6	18	51.9
3	80.5	19	38.6
4	37.8	20	38.6
5	55.2	21	30.4
6	18.1	22	36.5
7	33.2	23	28.1
8	42.7	24	16.6
9	49.1	25	16.7
10	37.9	26	18.6
11	81.2	27	22.4
12	125.5	28	182.7
13	144.6	29	17.1
14	41.9	30	21.3
15	29.7	CH_3CO	171.0
16	23.8	CH_3CO	21.1

d, $J = 3.3$ Hz, H-12), 2.28 (1H, d, $J = 11.2$ Hz, H-18), 1.78 (2H, m, H-22), 0.86 (3H, s, H-23), 0.88 (3H, s, H-24), 1.07 (3H, s, H-25), 0.81 (3H, s, H-26), 1.17 (3H, s, H-27), 0.96 (3H, d, $J = 4.4$ Hz, H-29), 0.98 (3H, d, $J = 4.4$ Hz, H-30), 2.05 (3H, s, CH_3CO); ^{13}C NMR (100 MHz, $CDCl_3$): see Table 1; FAB-MS (negative mode): *m/z* 529 $[M - H]^-$; HRFAB-MS *m/z*: 529.3524 $[M - H]^-$(calcd for $C_{32}H_{49}O_6$, 529.3529).

3.4 Assay for lymphocyte proliferation

Lymphocytes were isolated from the spleen of Balb/c mice. Proliferation of lymphocytes was analysed *in vitro* using an MTT colorimetric assay.[26] Lymphocytes were seeded in 96-well microculture plates at a cell density of 1×10^5 per well. T- and B-lymphocyte activation was induced by concanavalin A and lipopolysaccharide (final concentration 5 μg/ml, 15 μg/ml), respectively. A dose/response curve was drawn up using different concentrations of compounds **1**–**10**. Final concentrations of test compounds in the assay were 0.625, 1.25, 2.5, 5, and 10 μg/ml. Wells containing cyclosporine A (50 nM) and Medium 1640 were as positive and negative controls, respectively. All assays were carried out in duplicate. After 72 h of incubation, MTT reagent (final concentration of 4 mg/ml) was added and the cells were cultured for 4 h, then the cells were dissolved in dimethyl sulphoxide. Spectrophotometric measurement was carried out at 540 nm using a microplate reader.

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