New ursane-type triterpenoids from *Clerodendranthus spicatus*

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**ABSTRACT**

Five new ursane-type triterpenoids, spicatusoids A–E (1, 3–6), and three known ones (2, 7, and 8), and a known oleanane-type triterpenoid (9) were isolated from the aerial parts of *Clerodendranthus spicatus*. Their structures were elucidated by spectroscopic methods. In particular, the structure of 3 including its absolute configuration was confirmed by single-crystal X-ray diffraction analysis. Cell viability of all the compounds against rat kidney fibroblast cells (NRK-49F) with or without TGF-β1 induction and human cancer cells (HL-60, SMMC-7721, A-549, MCF-7, and SW-480) was examined by using MTT or MST assays. It was found that, with exception of 1, all the tested compounds could inhibit cell proliferation in TGF-β1 induced NRK-49F cells with compounds 2 being most active.

1. **Introduction**

*Clerodendranthus spicatus* is a plant species in the family of Labiatae found mainly in southern China including Hainan and Fujian provinces, Taiwan, and southern Guangxi and Yunnan provinces. It also spreads over India, Myanmar, Thailand, Indonesia, Philippines, and Australia [1]. The aerial parts of *C. spicatus* are medicinally used in China for the treatment of kidney associated disorders exemplified by nephritis and lithanguria [1]. Previous investigations on this species revealed the presence of > 200 substances including flavonoids [2], phenolic acids [2,3], diterpenes [4], triterpenoids [2,5,6], and essential oils [7]. We became interested in *C. spicatus* due to its pronounced effects on kidney diseases and its edible nature known as “Kidney Tea” in Dai nationality of Yunnan province of China, and characterized eighteen phenolic compounds thereof [8]. In our continuous study on this plant, eight ursane-type triterpenoids including five new ones (1, 3–6) and a known oleanane-type triterpenoid (9) (Fig. 1) were isolated and structurally characterized, subsequent biological evaluation against multiple human cancer cells and rat kidney fibroblast cells were carried out.

2. **Experimental**

2.1. **General**

Optical rotations were recorded on a JASCO P-1020 digital polarimeter. UV spectra were collected on a Shimadzu UV-2401PC spectrometer. NMR spectra were measured via a Bruker AV-400 MHz or a Bruker Avance III 600 MHz spectrometer, TMS was used as an internal standard. ESIMS and HRESIMS were obtained on an API QSTAR Pulsar 1 spectrometer. IR spectra were tested on a Bruker Tensor-27 spectrometer. RP-18 (20–45 μm; Daiso Co., Japan), MCI gel CHP 20P (75–150 μm, Mitsubishi Chemical Industries, Tokyo, Japan), and Sephadex LH-20 (Amersham Pharmacia, Uppsala, Sweden) were used for column chromatography. Silica gel (Qingdao Marine Chemical Inc., Qingdao, PR China) was used for preparative TLC. HPLC was carried out using a LC-3000 liquid chromatograph equipped with an Agilent Zorbax SB-C18 column (250 mm × 9.4 mm, i.d., 5 μm).

2.2. **Plant material**

The aerial parts of *C. spicatus* were collected from Xi-Shuang-Ban-Na of Yunnan province, PR China, in December 2013. The plant material was identified by Prof. Xun Gong at Kunming Institute of Botany, Chinese Academy of Sciences, and a voucher specimen (CHYX-0587) is...
kept at the State Key Laboratory of Photochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, PR China.

2.3. Extraction and isolation

The powders of *C. spicatus* (17.0 kg) were extracted by reflux with 80% EtOH (50 L × 1 h × 3) to give a crude extract (1.0 kg), which was subsequently partitioned with CHCl₃ and EtOAc to afford respective CHCl₃ soluble part (A) and EtOAc soluble part (B). Part A (0.67 kg) was divided into seven parts (Frs. A1–A7) by using a MCI gel CHP 20P column eluted with gradient aqueous MeOH (65%–100%). Fr. A4 (91.0 g) was further separated by Sephadex LH-20 (MeOH) to yield four fractions (Frs. B6.1–B6.4). Of which, Fr. B6.3 (1.8 g) was gel filtrated over Sephadex LH-20 (MeOH) to yield five fractions (Frs. A6.3.1–A6.3.5). Fr. A6.3.3 (0.45 g) was submitted to repeated semi-preparative HPLC to get compounds 7 (4.2 mg, tᵣ = 24 min, MeOH–H₂O, 72%), 8 (6.4 mg, tᵣ = 34 min, MeOH–H₂O, 67%), and 9 (5.2 mg, tᵣ = 24 min, CH₃CN–H₂O, 40%).

Spicatusoid A (1): yellowish solid; [α]²⁶⁺ = 13.8 (c 0.22, MeOH); IR (KBr) νmax 3428, 2929, 2875, 1708, 1629, 1452, 1384, 1261, 1038, 563 cm⁻¹; UV (MeOH) λmax (log ε): 206 (4.31); ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 485.3253 [M – H]⁻ (calcd for C₃₀H₄₅O₅, 485.3272).

Spicatusoid B (3): colorless crystal (MeOH); [α]²⁵⁺ = 48.7 (c 0.18, MeOH); IR (KBr) νmax 3436, 2935, 2879, 1728, 1630, 1457, 1382, 1118, 1055, 1034, 577 cm⁻¹; UV (MeOH) λmax (log ε): 207 (3.87); ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 517.3156 [M – H]⁻ (calcd for C₃₀H₄₅O₇, 517.3171).

Spicatusoid C (4): white solid; [α]²⁶⁺ = 16.0 (c 0.10, MeOH); IR (KBr) νmax 3436, 2935, 2879, 1728, 1630, 1457, 1383, 1120, 1036, 583 cm⁻¹; UV (MeOH) λmax (log ε): 207 (4.13); ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 547.3258 [M + COOH]⁺ (calcd for C₃₁H₄₉O₈, 547.3276).

Spicatusoid D (5): white solid; [α]²⁵⁺ = 26.9 (c 0.31, MeOH); IR (KBr) νmax 3430, 2940, 2881, 1716, 1630, 1451, 1384, 1268, 1235, 1208, 1131, 1096, 1037, 553 cm⁻¹; UV (MeOH) λmax (log ε): 207 (3.55); ¹H and ¹³C NMR data, see Table 2; HRESIMS m/z 549.3427 [M – H]⁻ (calcd for C₃₁H₄₉O₈, 549.3433).

Spicatusoid E (6): white solid; [α]²⁶⁺ = 23.1 (c 0.26, MeOH); IR (KBr) νmax 3427, 2941, 2882, 1715, 1629, 1605, 1452, 1384, 1268, 1235, 1206, 1134, 1095, 1048, 559 cm⁻¹; UV (MeOH) λmax (log ε): 283 (3.70), 207 (2.73); ¹H and ¹³C NMR data, see Table 2; HRESIMS m/z 549.3409 [M – H]⁻ (calcd for C₃₁H₄₉O₈, 549.3433).

2.4. Cell viability assay in NRK-49F without TGF-β1 induction

Rat normal renal interstitial fibroblast cells (NRK-49F) were obtained from ATCC (American Type Culture Collection). Cells were grown and maintained in DMEM with 10% FBS and 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco BRL, Grand Island, NY). Cells were incubated in an incubator with 5% CO₂ in air at 37 °C. Cell viability was carried out by the MTT assay as previous method[9].

2.5. TGF-β1 induced proliferation inhibitory assay

Biological evaluation of proliferation inhibition of the compounds towards NRK-49F cells with TGF-β1 induction was carried out accord-
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5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-

1. Salt (MTS) (Promega, Madison, WI, USA) assay as previously described

2. 1640 or DMEM medium (Biological Industries, Kibbutz Beit-Haemek,

3. Colon cancer SW-480 cell lines were obtained from ATCC (Manassas,

4. 2.6. Cell viability assay in human cancer cells

5. Human cancer cell lines including leukemia HL-60, hepatocarcinoma

6. SMMC-7721, lung cancer A-549, breast carcinoma MCF-7, and

7. and DEPT spectra (Table 1) displays 30 signals corresponding to six

8. that the presence of a

9. In methanol-d₄.

10. its negative HRESIMS at m/z 485.3253 [M − H]⁻ (calcd for 485.3272,

11. C₅H₆O₅), ¹³C NMR, and DEPT spectra, indicating eight degrees of

12. its MPCI using ESI. This indicates that there are two oxygenated

13. 2, (4-sulfophenyl)-2H-tetrazolium, inner

14. and a methyl group (C-30) in

15. 3-30, CH₃

16. δᵣ(4H, 1H-CH₃)

17. of 3-(4,5-dimethylthiazol-2-yl)-2-(3-carboxymethoxyphenyl)-2-(4-

18. 2-(4-sulfophenyl)-2H-tetrazolium, inner

19. deoxytetracycline (β-H) and trimethoprim (β-S). The ¹³C NMR and

20. 1, 2′, 3′, 4′-tetrahydrospicatin (Spicatusoid B (Spic.)

21. 3, 4, 5′-O-dimethyl ether, and a methyl group (C-30) in

22. 1H-CH₃)

23. 485.3253 [M − H]⁻ (calcd for 485.3272, C₅H₆O₅), ¹³C NMR, and DEPT spectra, indicating eight degrees of

24. 1H-CH₃)

25. 3, 4, 5′-O-dimethyl ether, and a methyl group (C-30) in

26. 1H-CH₃)

27. 3-(4,5-dimethylthiazol-2-yl)-2-(3-carboxymethoxyphenyl)-2-(4-

28. 1H-CH₃)

29. 485.3253 [M − H]⁻ (calcd for 485.3272, C₅H₆O₅), ¹³C NMR, and DEPT spectra, indicating eight degrees of

30. 485.3253 [M − H]⁻ (calcd for 485.3272, C₅H₆O₅), ¹³C NMR, and DEPT spectra, indicating eight degrees of

31. in methanol-d₄.
secured by the observed ROESY correlations of H3–24/H-2, H-3, H3–25; and H-2/H3–25 (Fig. 3). This conclusion is further confirmed by X-ray diffraction analysis using CuKα irradiation (Fig. 4 and Supplementary data), which also permits the absolute configuration determination of 3 as 2R,35R,5R,8R,9R,10R,14S,17R,18S,19S,20R.

Spicatusoid C (4) possesses a molecular formula C30H46O6 on the basis of its HRESIMS (m/z 547.3258 [M + COOH]−, calcld for C31H46O8, 547.3276), 13C NMR, and DEPT spectra, having 8 degrees of unsaturation. The 1H NMR spectrum (Table 1) shows one olefinic proton (δH 5.52, m, H-12), two oxymethine signals (δH 3.90, m, H-2; δH 3.74, d, J = 1.9 Hz, H-3), and four methyl singlets and onemethyl doublet (δH 0.99, d, J = 6.8 Hz, H-29). The 13C NMR and DEPT spectra of 4 reveals the presence of 30 carbons classified into five methyl, ten methylene (two oxygenated), seven methine (one sp2, and six sp3 including two oxygenated), and eight quaternary carbons (one carbonyl, a sp3, and six sp3). These data are extremely similar to those of 3, suggesting their structural resemblance. The structure 4 is different from 3 in that 19-OH in 3 is absent in 4, which is evident from HMBC correlations of H3–29/C-18, C-19, C-20 and 1H–1H COSY correlations of H-18/H-19. Besides, ROESY correlations (Fig. 3) of H-19/H3–27 and H3–29/H-30 allow the relative configuration determination at C-19. As a result, the structure of 4 was determined and named as spicatusoid C.

Spicatusoid D (5) has a molecular formula C31H50O8 (8 degrees of
unsaturation) derived by analysis of its HRESIMS, 13C NMR, and DEPT spectra. The 1H NMR spectrum (Table 2) gives one olefinic proton (δH 5.29, m, H-12), five methyl singlets, and one methoxyl (δH 3.59, s). The 13C NMR and DEPT spectra (Table 2) reveal 31 carbons ascribed to six methyl (one oxygenated), ten methylene (two oxygenated), six methine (a sp2 and a sp3 including two oxygenated), and nine quaternary carbons (one carbyln, one sp2, and seven sp3 including two oxygenated). These data closely resemble those of compound 3. By carefully comparison of their NMR data, it was found that their main difference occur at C-17-C-19, C-20 and C-28-C-31. One degree of unsaturation less of 5 than 3 together with an HMBC correlation of H-23/C-28 indicate that ring F in 3 is absent in 5, corresponding to variation of carbon chemical shifts around ring F. For relative conformation of 5, ROESY correlations (Fig. 3) of H-2/H-24, H-25, H-3/H-24, H-9/H-5, H-27, H-25/H-24, H-26, H-18/H-26, H-29, and H-29/H-30 clearly indicate that H-2, H-3, H-18, H-24, H-25, H-26, H-29, and H-30 are spacially vicinal and H-5, H-9, H-23, and H-27 are at the same orientation. With these data in hand, the structure of 5 was assigned and named spicatusoid D.

Careful interpretation of HRESIMS, 1D and 2D NMR data of 6 (spicatudoid E) reveals that 6 resembles 5 differing only in the relative configuration at C-3. ROESY correlations (Fig. 3) of H-3/H-23, H-9, H-5/H-9, H-23, H-24/H-25 suggest that H-3 is α-oriented in 6. Thus, the structure of 6 was assigned and named as spicatudoid E.

Four known compounds (2 and 7–9) were respectively identified as vitexnagheteron H (2) [12], euscaphic acid (7) [11], 2α,3β,19β-trihydroxyurs-12-en-28-oic acid (8) [13], and arjunolic acid (9) [14], by comparison of their spectroscopic data with those literatures.

Proliferation of interstitial fibroblasts has been seen in renal pathogenesis such as fibrosis. Therefore, inhibition of interstitial fibroblast proliferation will be beneficial for kidney diseases [15]. Considering that the title species is used to treat kidney diseases, all the isolated triterpenoids were therefore evaluated for their biological potential towards TGF-β1 stimulated NRK-49F cells. The results (Figs. 5 and 6) show that, with exception of compound 1, all the other compounds could significantly inhibit kidney fibroblast proliferation at 20 μM with compounds 2 and 3 to be the most active. A subsequent experiment aimed to exclude the cellular toxicity of the active compounds disappointedly found that compounds 2–6 are toxic towards normal kidney fibroblasts at 20 μM, indicating that their effects might be caused by cellular toxicity or poor selectivity. Since these triterpenoids are toxic to kidney cells, we therefore examined their cytotoxicity towards human cancer cells (HL-60, SMMC-7721, A-549, MCF-7 and SW-480) with a maximum concentration of 20 μM. Whereas, none of them was found to be activity towards these cancer cell lines (data not shown).

Conflict of interest

We declare no conflict of interest for this study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2017.04.001.

References


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Fig. 4. Plot of X-ray crystallographic data of compound 3.

Fig. 5. Proliferation inhibition of compounds 1−9 towards normal NRK-49F cells. ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

Fig. 6. Proliferation inhibition of compounds 1−9 towards TGF-β1 stimulated NRK-49F cells. ANOVA, ***p < 0.001 versus control. *p < 0.05, **p < 0.01, ***p < 0.001 versus only TGF-β1 treatment group.


