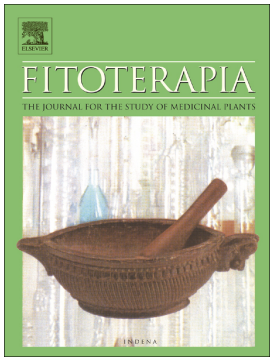

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New ursane-type triterpenoids from *Clerodendranthus spicatus*

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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**−**6** being most active.

Keywords:

Clerodendranthus spicatus; ursane-type triterpenoids; human cancer cells; kidney fibroblast cells

1. Introduction

*Clerodendranthus spicatu*s 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. spicatu*s are medicinally used in China for the treatment of kidney associated disorders exemplified by nephritis and lithangiuria [1]. Previous investigations on this species revealed the presence of more than 200 substances including flavonoids [2], phenolic acids [2,3], diterpenes [4], triterpenoids [2,5,6], and essential oils [7]. We became interested in *C. spicatu*s 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**) 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 $CHCl_3$ and EtOAc to afford respective $CHCl_3$ 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 three fractions (Frs. A4.1–A4.3). Among them, Fr. A4.2 (6.0 g) was submitted to a RP-18 column eluted with gradient aqueous MeOH (30%–100%) to further produce three fractions (Frs. A4.2.1–A4.2.3). Fr. A4.2.2 (1.6 g) was purified by preparative TLC ($CHCl_3$–MeOH, 8:1) followed by semi-preparative HPLC (CH_3CN–H_2O, 42%) to get compound **1**

(2.3 mg, t_R = 18min). Part B (0.14 kg) was separated by MCI gel CHP 20P eluted with gradient aqueous MeOH (30%–100%) to yield seven parts (Frs. B1–B7). Fr. B4 (14.5 g) was submitted to a RP-18 column eluted with aqueous MeOH (45%–80%) to yield two fractions (Frs. B4.1–B4.2). Fr. B4.2 (0.87 g) was passed through Sephadex LH-20 (MeOH) to afford six fractions (Fr. A4.2.1–A4.2.6). Fr. A4.2.2 (0.27 g) was purified by semi-preparative HPLC (CH_3CN–H_2O, 30%) to yield compounds **2** (3.0 mg, t_R = 26.8 min), **3** (9.8 mg, t_R = 40 min), **4** (1.0 mg, t_R = 31 min), **5** (15.1 mg, t_R = 45 min), and **6** (2.6 mg, t_R = 30 min). Similarly, Fr. B6 (14.5 g) was subjected to a RP-18 column (MeOH–H_2O, 60%–100%) 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, t_R = 24 min, MeOH–H_2O, 72%), **8** (6.4 mg, t_R = 34 min, MeOH–H_2O, 67%), and **9** (5.2 mg, t_R = 24 min, CH_3CN–H_2O, 40%).

Spicatusoid A (**1**): yellowish solid; $[\alpha]_D^{26}$ –13.8 (*c* 0.22, MeOH); IR (KBr) ν_{max} 3428, 2929, 2875, 1708, 1629, 1452, 1384, 1261, 1038, 563 cm^{-1}; UV (MeOH) λ_{max} (log ε): 206 (4.31); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 485.3253 $[M-H]^-$ (calcd for $C_{30}H_{45}O_5$, 485.3272).

Spicatusoid B (**3**): colorless crystal (MeOH); $[\alpha]_D^{25}$ –48.7 (*c* 0.18, MeOH); IR (KBr) ν_{max} 3425, 2939, 2885, 1726, 1630, 1457, 1382, 1118, 1055, 1034, 577 cm^{-1}; UV (MeOH) λ_{max} (log ε): 207 (3.87); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 517.3156 $[M-H]^-$ (calcd for $C_{30}H_{45}O_7$, 517.3171).

Spicatusoid C (**4**): white solid; $[\alpha]_D^{26}$ –16.0 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3436, 2935, 2879, 1728, 1630, 1457, 1383, 1120, 1036, 583 cm^{-1}; UV (MeOH) λ_{max} (log ε): 207 (4.13); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 547.3258 $[M+COOH]^-$

(calcd for $C_{31}H_{46}O_8$, 547.3276).

Spicatusoid D (**5**): white solid; $[\alpha]_D^{25}$ +26.9 (*c* 0.31, MeOH); IR (KBr) ν_{max} 3430, 2940, 2881, 1716, 1630, 1451, 1384, 1268, 1235, 1208, 1131, 1096, 1037, 553 cm^{-1}; UV (MeOH) λ_{max} (log ε): 207 (3.55); ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 549.3427 $[M-H]^-$ (calcd for $C_{31}H_{49}O_8$, 549.3433).

Spicatusoid E (**6**): white solid; $[\alpha]_D^{26}$ +23.1 (*c* 0.26, MeOH); IR (KBr) ν_{max} 3427, 2941, 2882, 1715, 1629, 1605, 1452, 1384, 1268, 1235, 1206, 1134, 1095, 1048, 559 cm^{-1}; UV (MeOH) λ_{max} (log ε): 283 (3.70), 207 (2.73); ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 549.3409 $[M-H]^-$ (calcd for $C_{31}H_{49}O_8$, 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% CO_2 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 according to previously described methods [9].

2.6. Cell viability assay in human cancer cells

Human cancer cell lines including leukemia HL-60, hepatocarcinoma SMMC-7721, lung cancer A-549, breast carcinoma MCF-7, and colon cancer SW-480 cell lines were obtained from ATCC (Manassas, VA, USA) and used for cytotoxic assay. Cells were cultured in RMPI-1640 or DMEM medium (Biological Industries, Kibbutz

Beit-Haemek, Israel) supplemented with 10% fetal bovine serum (Biological Industries) at 37 °C in a humidified atmosphere with 5% CO_2. The cytotoxicity assay was performed by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) (Promega, Madison, WI, USA) assay as previously described method [10]. Briefly, cells were seeded into each well of a 96-well cell culture plate. After 12 h of incubation at 37 °C, the test compound (40 μM) was added. After incubated for 48 h, cells were subjected to the MTS assay. In this study, cisplatin and paclitaxel (Sigma) were used as two positive controls.

3. Results and discussion

Spicatusoid A (**1**) has a molecular formula $C_{30}H_{46}O_5$ on the basis of its negative HRESIMS at *m/z* 485.3253 $[M-H]^-$ (calcd for 485.3272, $C_{30}H_{45}O_5$), ^{13}C NMR, and DEPT spectra, indicating eight degrees of unsaturation. The ^1H NMR spectrum (Table 1) shows one olefinic proton (δ_H 5.64, t, J = 4.0 Hz, H-12), two oxygenated methine (δ_H 4.28, m, H-2; δ_H 3.74, brs, H-3), and six methyl singlets. The ^{13}C NMR and DEPT spectra (Table 1) displays 30 signals corresponding to six methyl, nine methylene (one oxygenated), six methine (one sp^2, and five sp^3 including two oxygenated), and nine quaternary carbons (one carbonyl, three sp^2, and five sp^3). These data prompted us to speculate that **1** is a triterpenoid similar to euscaphic acid (**7**) [11]. The main difference between **1** and **7** is that the presence of a $\Delta^{19(20)}$ double bond in **1** and a methyl group (C-30) in **7** is oxidized to hydroxymethyl in **1**.These alterations are supported by the HMBC correlations of H_3-29/C-18, C-19 (δ_C 131.5), C-20 (δ_C 129.5) and H-30/C-19, C-21 (Fig. 2). The ROESY correlations (Fig. 3) of H-2/H-25, H-3/H-24, H-5/H-23, H-5, H-9/H-27, and H-24, H-26/H-25 indicate that H-2, H-3, H-18, CH_3-24, CH_3-25, CH_3-26 are β-oriented and H-5, H-9, H-23, CH_3-27 are

α-oriented. With these, the structure of **1** was identified and named as spicatusoid A.

Spicatusoid B (**3**), obtained as a colorless crystal (MeOH), has a molecular formula $C_{30}H_{46}O_7$deduced from its HRESIMS (*m/z* 517.3156 $[M-H]^-$, calcd for $C_{30}H_{45}O_7$, 517.3171), ^{13}C NMR, and DEPT spectra. Careful analyses of 1D and 2D NMR data of compound **3** reveals that **3** extremely resembles vitexnegheterion H (**2**) [12] differing only in the configuration at C-3. This β-orientation of H-3 is secured by the observed ROESY correlations of H_3-24/H-2, H-3, H_3-25; and H-2/H_3-25 (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 2*R*,3*S*,4*R*,5*R*,8*R*,9*R*,10*R*,14*S*,17*R*,18*S*,19*S*,20*R*.

Spicatusoid C (**4**) possesses a molecular formula $C_{30}H_{46}O_6$ on the basis of its HRESIMS (*m/z* 547.3258 $[M+COOH]^-$, calcd for $C_{31}H_{46}O_8$, 547.3276), ^{13}C NMR, and DEPT spectra, having 8 degrees of unsaturation. The ^1H NMR spectrum (Table 1) shows one olefinic proton (δ_H 5.52, m, H-12), two oxymethine signals (δ_H 3.90, m, H-2; δ_H 3.74, d, J = 1.9 Hz, H-3), and four methyl singlest and onemethyl doublet (δ_H 0.99, d, J = 6.8 Hz, H-29). The ^{13}C NMR and DEPT spectra of **4** reveals the presence of 30 carbons classified into five methyl, ten methylene (two oxygenated), seven methine (one sp^2, and six sp^3 including two oxygenated), and eight quaternary carbons (one carbonyl, a sp^2, and six sp^3). 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 H_3-29/C-18, C-19, C-20 and ^1H-^1H COSY correlations of H-18/H-19. Besides, ROESY correlations (Fig. 3) of H-3/H-5, H-24, H-2/H_3-23 (δ_H 1.22), H-25, indicated β-orientation for H-2 and H-23 and α-orientation for H-3, H-5, and H-24; ROESY correlations of H-19/H_3-27 and H_3-29/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 $C_{31}H_{50}O_8$ (8 degrees of unsaturation) derived by analysis of its HRESIMS, ^{13}C NMR, and DEPT spectra. The ^{1}H NMR spectrum (Table 2) gives one olefinic proton (δ_H 5.29, m, H-12), five methyl singlets, and one methoxyl (δ_H 3.59, s). The ^{13}C NMR and DEPT spectra (Table 2) reveal 31 carbons ascribed to six methyl (one oxygenated), ten methylene (two oxygenated), six methine (a sp^2 and five sp^3 including two oxygenated), and nine quaternary carbons (one carbonyl, one sp^2, and seven sp^3 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 correlationof H_3-31/C-28 indicate that ring F in **3** is absent in **5**, corresponding to variation of carbon chemical shifts around ring F. For relative configuration 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** (spicatusoid 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 spicatusoid E.

Four known compounds (**2** and **7**–**9**) were respectively identified as vitexnegheterion 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. However, 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/

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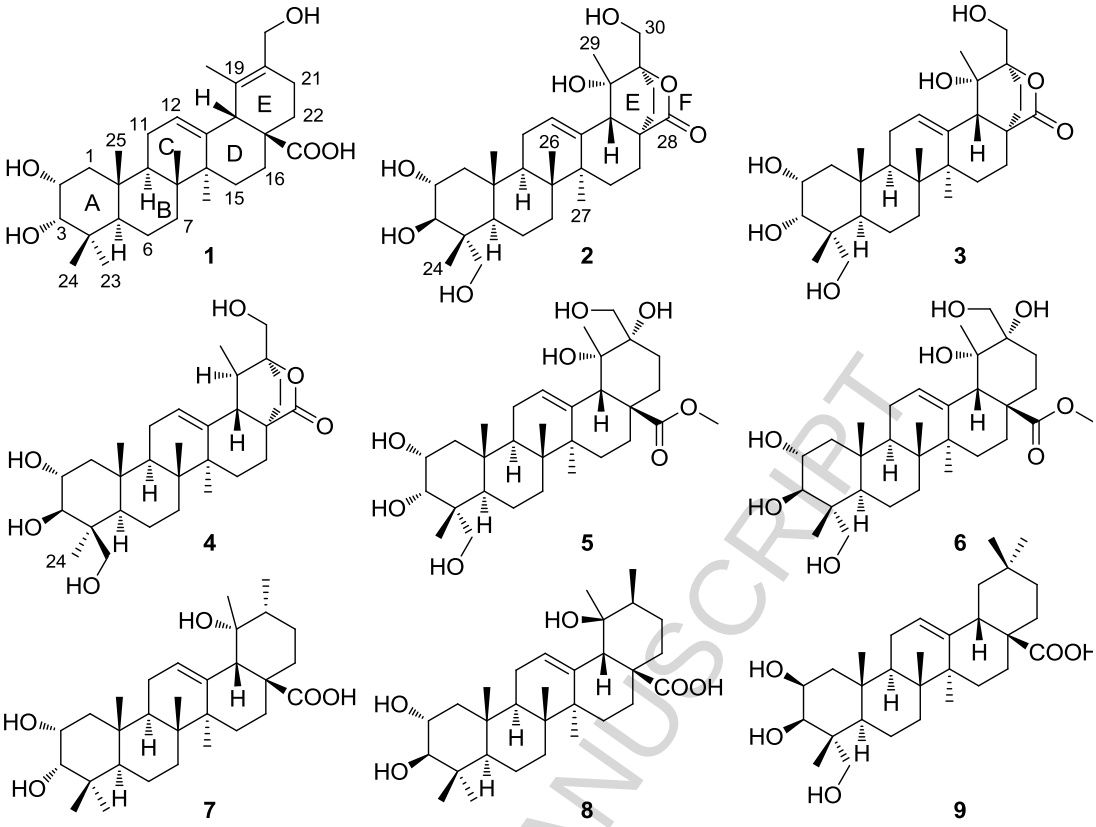


Fig. 1. Chemical **s**tructures of compounds **1**–**9**.

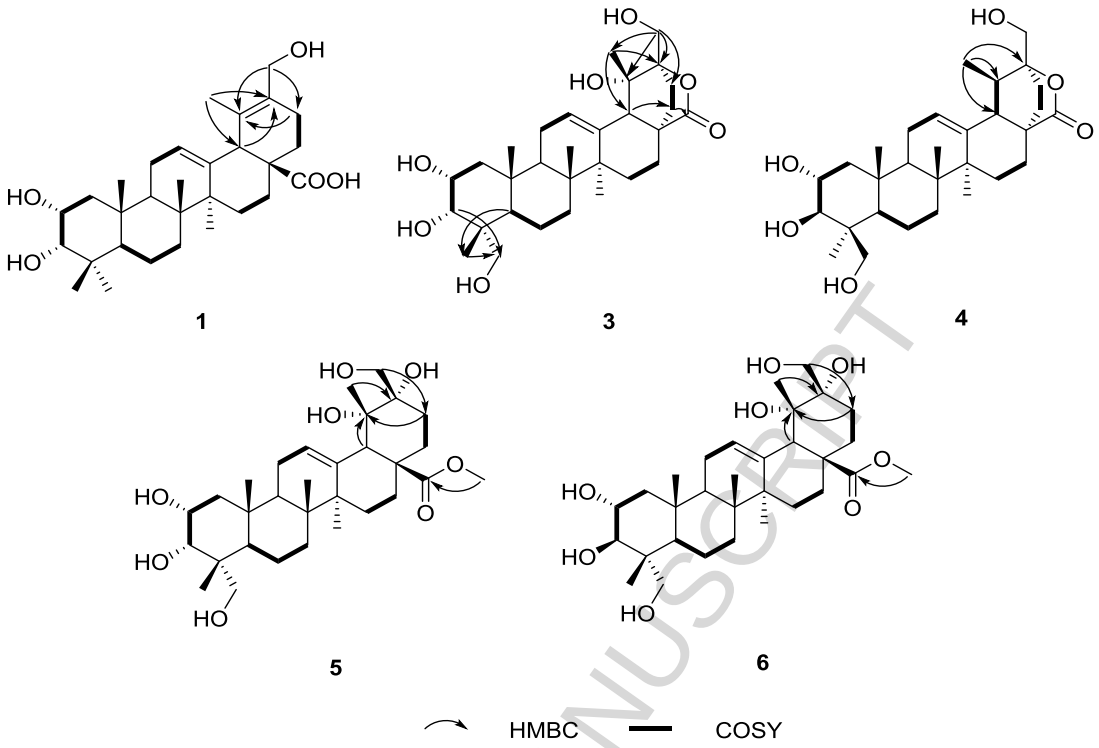


Fig. 2. Key HMBC and ^{1}H-^{1}H COSY correlations of compounds **1, 3–6.**

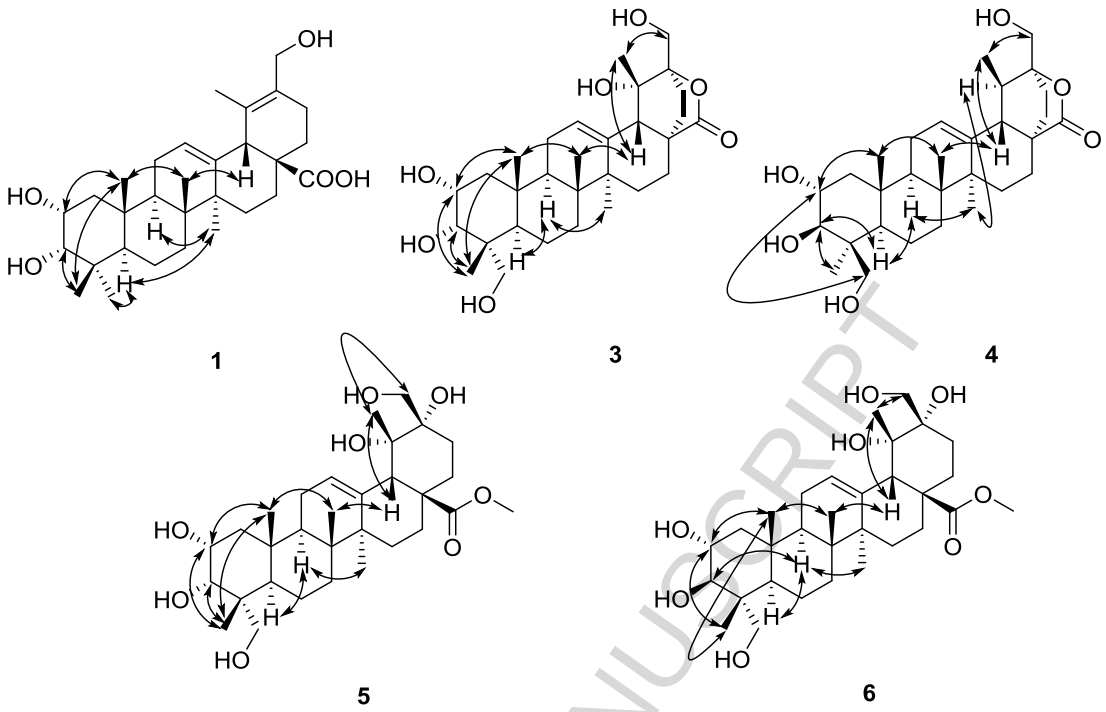


Fig. 3. Key ROESY correlations of compounds **1**, **3**–**6**.

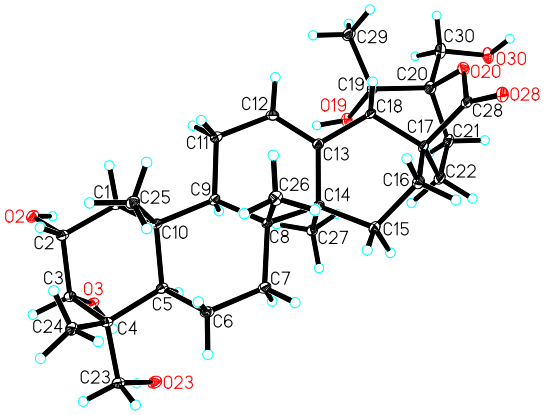


Fig. 4. Plot of X-ray crystallographic data of compound **3**.

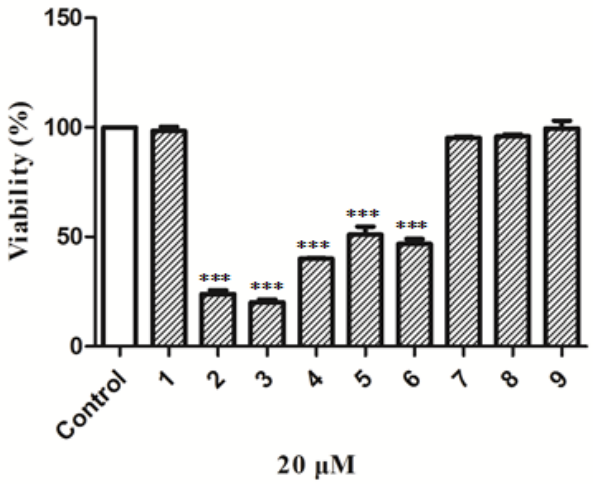


Fig. 5. Proliferation inhibition of compounds **1**–**9** towards normal NRK-49F cells. ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control.

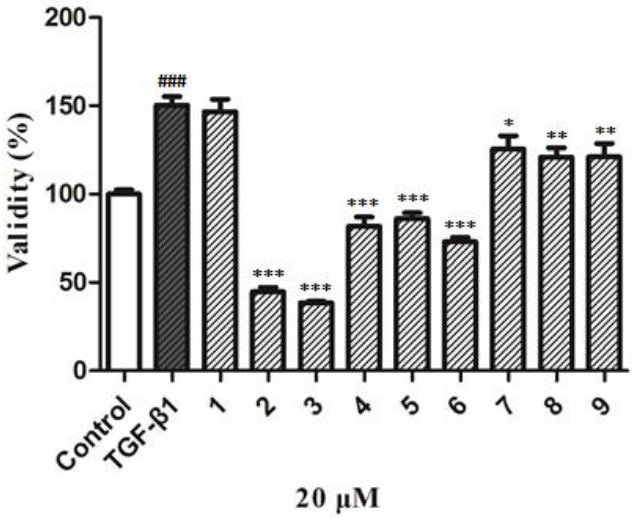


Fig. 6. Proliferation inhibition of compounds **1**–**9** towards TGF-β1 stimulated NRK-49F cells. ANOVA, $^{\#\#\#}p < 0.001$ versus control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus only TGF-β1 treatment group.

Table 1. ^{1}H (400 MHz) and ^{13}C NMR (150 MHz) data of **1**, **3**, and **4** (δ in ppm, J in Hz).

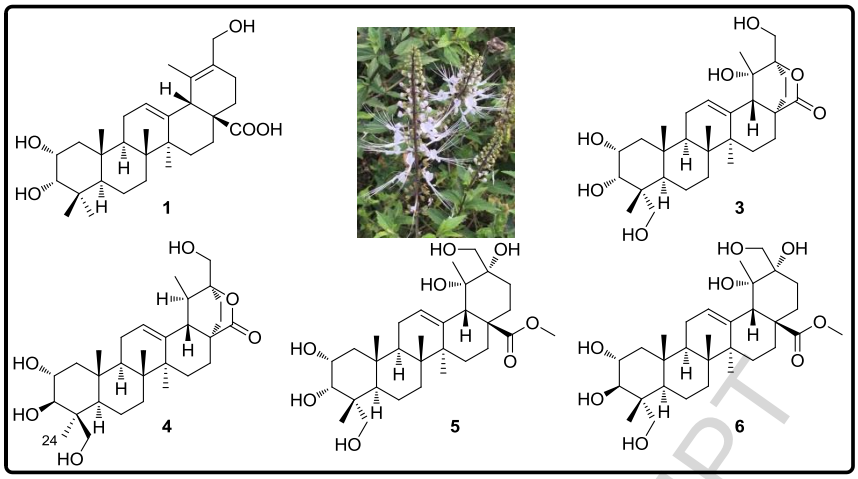
no	**1**[a]		**3**[b]		**4**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.93, m 1.73, m	43.1	1.58, m 1.33, m	41.9	1.55, overlap 1.34, m	42.1
2	4.28, m	66.0	3.89, m	67.2	3.90, m	66.9
3	3.74, br s	79.1	3.62, br s	78.7	3.74, d, 1.9	74.7
4		38.6		41.1		45.4
5	1.58, m	48.6	1.59, m	44.5	1.40, overlap	50.2
6	1.42, m 1.27, m	18.1	1.45, m 1.43, overlap	19.2	1.57, m 1.41, overlap	19.7
7	1.56, m 1.35, m	34.1	1.46, m 1.43, overlap	33.8	1.35, m	34.1
8		39.7		41.7		40.9
9	1.69, m	47.9	1.97, overlap	48.6	2.02, m	49.3
10		38.4		39.5		39.7
11	1.94, m 1.91, m	23.5	2.21, m 1.98, overlap	25.1	2.23, m 1.96, m	25.4
12	5.64, t, 4.0	127.4	6.01, t, 4.0	126.9	5.52, m	122.8
13		138.0		136.1		141.3
14		43.8		43.1		42.9
15	2.21, m 1.19, overlap	28.5	1.68, m 1.17, m	25.6	1.63, m 1.11, m	24.2
16	2.06, overlap	23.8	2.40, overlap 1.80, m	25.86	2.83, dd, 15.1, 4.9 1.38, m	24.3
17		46.8		42.5		41.0
18	3.64, s	50.8	2.24, s	51.5	1.77, overlap	50.1
19		131.5		73.8	2.11, overlap	36.1
20		129.5		88.1		85.3
21	2.54, m	24.5	1.45, overlap 1.43, overlap	26.7	1.86, m 1.74, overlap	30.4
22	2.09, overlap 2.00, overlap	33.5	2.38, overlap	26.5	2.13, overlap 1.52, overlap	28.9
23	1.22, s	29.4	3.53, d, 11.1 3.40, d, 11.1	71.3	1.08, s	23.2
24	0.85, s	22.2	0.79, s	17.4	3.64, d, 11.4 3.42, d, 11.4	65.7
25	0.93, s	17.0	1.01, s	17.4	0.94, s	17.3
26	0.99, s	17.8	0.83, s	16.7	0.74, s	16.6
27	0.99, s	21.9	1.18, s	23.4	1.11, s	21.9
28		179.8		181.0		181.1
29	1.76, s	16.8	1.38, s	25.8	0.99, d, 6.8	17.1
30	4.52, d, 12.2 4.31, d, 12.2	63.0	3.84, d, 12.0 3.76, d, 12.0	63.2	3.66, d, 12.0 3.62, d, 12.0	64.6

[a]In pyridine-d_5, [b]In methanol-d_4.

Table 2. ^{1}H (400 MHz) and ^{13}C NMR (150 MHz) data of **5** and **6** (δ in ppm, J in Hz).

Position	**5**[b]		**6**[b]	
	δ_H	δ_C	δ_H	δ_C
1	1.59, m 1.32, m	42.2	1.93, m 0.89, m	47.9
2	3.88, m	67.2	3.69, m	69.7
3	3.61, br s	78.9	3.36, br s	78.3
4		42.5		44.1
5	1.55, m	44.2	1.77, m	48.5
6	1.40, overlap 1.35, m	19.1	1.44, overlap 1.39, overlap	19.1
7	1.63, m 1.27, m	33.6	1.64, m 1.26, m	33.5
8		41.1		41.0
9	1.87, m	48.3	1.28, m	48.2
10		39.1		38.9
11	2.04, m	24.7	1.99, overlap	24.7
12	5.29, m	129.5	5.29, m	129.5
13		139.5		139.5
14		42.6		42.6
15	1.61, m 0.96, t, 4.4	29.5	1.61, m 0.99, m	29.5
16	2.74, dt, 13.2, 4.4 1.54, m	26.7	2.74, d, 13.2, 4.7 1.54, m	26.8
17		48.6		48.7
18	2.98, s	49.4	2.98, s	49.4
19		74.8		74.8
20		76.4		76.4
21	2.24, dt, 13.7, 4.0 1.41, overlap	27.0	2.24, dt, 13.6, 3.8 1.39, overlap	26.9
22	1.97, m 1.50, m	32.5	1.96, overlap 1.49, m	32.5
23	3,53, d, 11.0 3.39, d, 11.0	71.4	3.50, d, 11.0 3.26, d, 11.0	66.3
24	0.77, s	17.6	0.69, s	13.9
25	1.02, s	17.2	1.03, s	17.5
26	0.72, s	17.5	0.71, s	17.5
27	1.31, s	24.5	1.29, s	24.5
28		181.0		180.4
29	1.23, s	22.8	1.22, s	22.8
30	3.73, d, 12.0 3.55, d, 12.0	68.6	3.72, d, 10.9 3.54, d, 10.9	68.6
31	3.59, s	52.1	3.58, s	52.1

[a]In pyridine-d_5; [b]In methanol-d_4.



Graphical abstract