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Anti-inflammatory triterpenes from the apical bud of *Gardenia sootepensis*

Ui Joung Youn [a,b], Eun-Jung Park [a,c], Tamara P. Kondratyuk [a], Tawanun Sripisut [a], Surat Laphookhieo [d], John M. Pezzuto [a,c], Leng Chee Chang [a,*]

[a] Department of Pharmaceutical Sciences, Daniel K. Inouye College of Pharmacy, University of Hawai'i at Hilo, Hilo, HI 96720, United States
[b] Division of Life Sciences, Korea Polar Research Institute, KIOST, Incheon 21990, Republic of Korea
[c] Arnold & Marie Schwartz College of Pharmacy and Health Sciences, Long Island University, Brooklyn, NY 11201, United States
[d] Natural Products Research Laboratory, School of Science, Mae Fah Luang University, Tasud, Muang, Chiang Rai 57100, Thailand

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ABSTRACT

Bioassay-guided fractionation of the dichloromethane extract from the apical bud of *Gardenia sootepensis* Hutch. (Rubiaceae) led to the isolation of four new cycloartane triterpenes, sootepins F–I (**1**–**4**), along with four known derivatives (**5**–**8**). The structures of the new compounds were determined by 1D and 2D NMR experiments and by comparison of the physicochemical data with published values. The isolates were evaluated for cancer chemopreventive potential based on their ability to inhibit nitric oxide (NO) production and tumor necrosis factor-alpha (TNF-α)-induced nuclear factor kappa (NF-κB) activity. Compounds **6**–**8** inhibited TNF-α-induced NF-κB activity with half maximal inhibitory concentration (IC_{50}) values of 8.3, 5.6, and 6.0 μM, respectively; compounds **7** and **8** showed significant NO-inhibitory activity with IC_{50} values of 3.2 and 2.0 μM, respectively.

Keywords:
Gardenia sootepenesis
Rubiaceae
Cycloartane triterpenes
Anti-inflammatory activity

1. Introduction

Several species in the *Gardenia* genus (Rubiaceae) have been used pharmacologically for abortifacient [1] and contraceptive [1–3] purposes, primarily in subtropical regions. Some have been used as a febrifuge [4] and a larvicide [5], as well as for the treatment of headaches [3], asthma [6] and malaria [7]. Leaves and apical buds of most *Gardenia* plants are covered with exudate (resin), some of which was used as antiplasmodic, carminative, diaphoretic, expectorant, and antihelmintic agents [8]. *Gardenia sootepensis* Hutch. is mainly distributed in the northern part of Thailand. In previous investigations, cycloartane triterpenes [9,10], sesquiterpenes [11], flavonoids [11], and benzoic acid derivatives [11] have been reported in this plant, with biological activities, such as cytotoxicity and angiogenic effects [9,10]. However, no studies have been performed to assess anti-inflammatory potential.

As described herein, while continuing our search for novel plant-derived cancer chemopreventive agents, the dichloromethane (CH_2Cl_2)-soluble extract of the apical bud of *G. sootepenesis* was found to inhibit TNF-α-induced NF-κB activity with transfected human embryonic kidney cells 293, and lipopolysaccharide (LPS)-induced NO production using murine macrophage RAW 264.7 cells. This paper reports the activity, isolation, and structure elucidation of new cycloartane triterpenes **1**–**4** isolated from the apical bud of *G. sootepensis* (Fig. 1).

2. Experimental section

2.1. General experimental procedures

Optical rotations were measured on a Rudolph Research AUTOPOL IV multiwavelength polarimeter. UV spectra were recorded on a Shimadzu PharmaSpec-1700 UV–visible spectrophotometer. IR spectra were measured on a Bruker Tensor-27 spectrometer. 1D and 2D NMR spectra were recorded on a Bruker AVANCE (400 MHz) spectrometer.

* Corresponding author.
E-mail address: lengchee@hawaii.edu (L.C. Chang).

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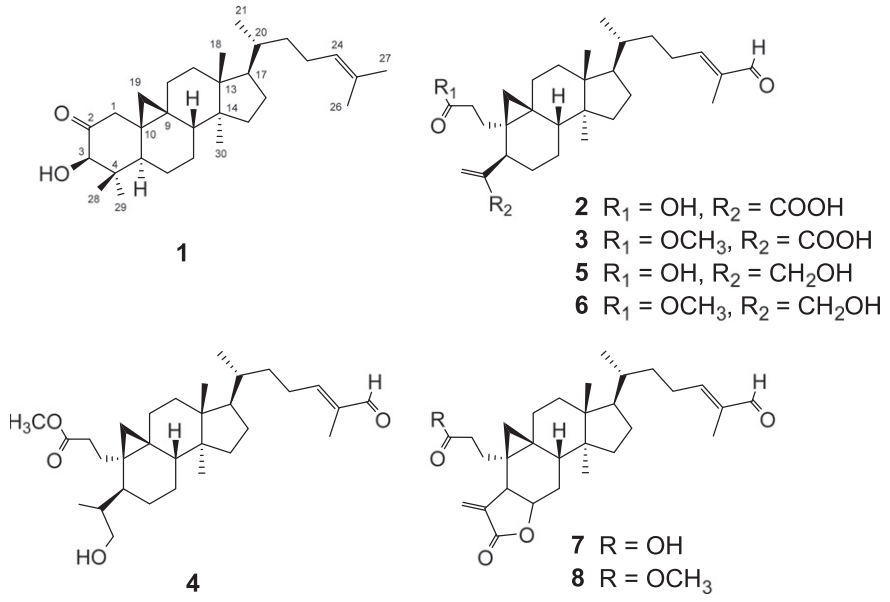

Fig. 1. Structures of compounds 1–8 isolated from *G. sootepensis*.

Mass spectra and high-resolution MS spectra were performed with a JEOL JMS-700 mass spectrometer. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (0.25 mm, Merck, Germany). Silica gel (230–400 mesh, Merck, Germany) and RP-18 (YMC·GEL ODS-A, 12 nm, S-150 μm) were used for column chromatography. The semipreparative HPLC was conducted on a Beckman Coulter Gold-168 system equipped with a photodiode array detector using an Alltech reversed-phase Econosil C-18 column (10 μm, 10 × 250 mm) with a flow rate of 2 mL/min.

2.2. Plant material

The fresh apical buds of *G. sootepensis* were collected from Mae Fah Luang University, Chiang Rai Province, Northern Thailand in June 2012. The plant was identified by Dr. Surat Laphookhieo, School of Science, Mae Fah Luang University, Tasud, Muang, Chiang Rai, Thailand. A voucher specimen (no. GS001) was deposited at the Natural Product Chemistry Laboratory, Daniel K. Inouye College of Pharmacy, University of Hawai'i at Hilo.

2.3. Extraction and isolation

The fresh apical buds of *G. sootepensis* (238 g) were extracted with CH_2Cl_2 (3 × 2 L) at room temperature. The solvent was concentrated *in vacuo* to yield a CH_2Cl_2 extract (150 g). The crude extracts (140 g) were subjected to silica gel column chromatography (CC; ϕ 15 cm; 230–400 mesh, 2.5 kg) using a gradient solvent system of hexane-ethyl acetate (50:1 to 0:100), to afford 10 fractions (Fc1–Fc10). Fraction Fc9 (1 g) was subjected to silica gel CC (ϕ 10 cm; 230–400 mesh, 200 g), with hexane-ethyl acetate (50:0 to 1:1) as the solvent system, yielding seven subfractions (Fc9s1 to Fc9s7). Subfraction Fc9s6 (300 mg) was separated by a C-18 gel column and eluted with H_2O–MeOH (30:70 to 0:100), to afford five subfractions (Fc9s6.1 to Fc9s6.5). Subfraction Fc9s6.5 (100 mg) was subjected to semiprep. HPLC (MeOH–H_2O/0.1% acetic acid = 90:10 to 100:0), to yield **2** (2 mg, t_R 70 min), **3** (1 mg, t_R 78 min), **5** (0.5 mg, t_R 82 min), and **6** (1 mg, t_R 115 min). Subfraction Fc9s6.4 (50 mg) was subjected to semiprep. HPLC (MeOH–H_2O/0.1% acetic acid = 90:10 to 100:0), to yield **7** (t_R 54 min, 4 mg), **3** (t_R 71 min, 0.5 mg), **5** (t_R 78 min, 1 mg), **8** (t_R 82 min, 1.5 mg), **6** (t_R 131 min, 0.8 mg), and **4** (t_R 160 min, 0.8 mg). Compound **1** (10 mg) was recrystallized from subfraction Fc9s7 using a solvent mixture, hexane-ethyl acetate (1:1).

2.3.1. Sootepin F (**1**)

White amorphous powder; $[\alpha]^{25}_D$ +48 (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 230 (4.2) nm; IR ν_{max} (KBr) 3361, 1710 cm^{-1}; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data, see Tables 1 and 2; HRFABMS *m/z* 439.3574 $[M - H]^-$, (calcd for $C_{30}H_{47}O_2$, 439.3576).

2.3.2. Sootepin G (**2**)

White amorphous powder; $[\alpha]^{25}_D$ +45 (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 232 (4.0) nm; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data, see Tables 1 and 2; HRFABMS *m/z* 483.3113 $[M - H]^-$, (calcd for $C_{30}H_{43}O_5$, 483.3116).

2.3.3. Sootepin H (**3**)

White amorphous powder; $[\alpha]^{25}_D$ +47 (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 232 (4.0) nm; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data, see Tables 1 and 2; HRFABMS *m/z* 497.3268 $[M - H]^-$, (calcd for $C_{31}H_{45}O_5$, 497.3267).

2.3.4. Sootepin I (**4**)

White amorphous powder; $[\alpha]^{25}_D$ +40 (*c* 0.20, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 230 (3.8) nm; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data, see Tables 1 and 2; HRFABMS *m/z* 485.3632 $[M - H]^-$, (calcd for $C_{31}H_{49}O_4$, 485.3631).

2.4. Tumor necrosis factor-α (TNF-α) activated nuclear factor-kappa B (NF-κB) assay

Human embryonic kidney cells 293 (Panomic, Fremont, CA) were used for monitoring changes occurring along the NF-κB pathway [12]. Stable constructed cells were seeded into 96-well plates at 20 × 10^3 cells/well. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Co.; Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. After 48 h of incubation, the medium was replaced and the cells were treated with various concentrations of test compounds. TNF-α (Human, Recombinant, *E. coli*, Calbiochem, Gibbstown, NJ, USA) was used as an activator at a concentration of 2 ng/mL (0.14 nM). The plate was incubated for 6 h. Spent medium was discarded and the cells were washed once with PBS. Cells were lysed using 50 μL (for 96-well plate) of Reporter Lysis Buffer from Promega, by incubating for 5 min on a shaker, and stored at

Table 1
^{1}H (400 MHz) NMR data (in $CDCl_3$, δ in ppm and J in Hz) for compounds **1–4**.

Position	**1**	**2**	**3**	**4**
	δ_H	δ_H	δ_H	δ_H
1a	ax2.67 (d, 13.2)	2.15 (m)	2.08 (m)	1.40 (m)
1b	eq2.09 (d, 13.2)	1.23 (m)	1.22 (m)	
2a		2.46 (m)	2.61 (m)	2.52 (m)
2b		2.17 (m)	2.21 (m)	2.31 (m)
3	3.91 (s)			
4				1.94 (m)
5	1.93 (m)	3.01 (dd, 12.0, 4.8)	3.01 (dd, 12.0, 5.0)	2.14 (m)
6ax	1.74 (m)	1.59 (m)	–[a]	1.49 (m)
6eq	0.85 (m)			
7ax	1.45 (m)	1.29 (m)	1.27 (m)	1.28 (m)
7eq		1.22 (m)	1.12 (m)	1.10 (m)
8	1.62 (m)	1.60 (dd, 12.4, 4.0)	1.55 (dd, 12.8, 4.4)	1.49 (m)
11ax	1.96 (m)	2.14 (m)	1.95 (m)	1.91 (m)
11eq	1.20 (m)	1.22 (m)	1.27 (m)	1.34 (m)
12	1.65 (m)	1.71 (m)	1.72 (m)	1.64 (m)
15	1.33 (m)	1.34 (m)	1.34 (m)	1.34 (m)
16ax	1.93 (m)	1.29 (m)	1.30 (m)	1.28 (m)
16eq	1.33 (m)			
17	1.64 (m)	1.65 (m)	1.65 (m)	1.60 (m)
18	0.96 (s)	1.01 (s)	1.00 (s)	0.98 (s)
19a	0.49 (d, 4.8)	0.52 (d, 4.4)	0.55 (d, 4.2)	0.42 (d, 4.6)
19b	0.65 (d, 4.8)	0.79 (d, 4.4)	0.78 (d, 4.2)	0.65 (d, 4.6)
20	1.42 (m)	1.64 (m)	1.63 (m)	
21	0.90 (d, 6.4)	0.96 (d, 6.4)	0.96 (d, 6.4)	0.95 (d, 6.4)
22a	1.42 (m)	1.46 (m)	1.34 (m)	1.48 (m)
22b	1.09 (m)			
23a	2.07 (m)	2.45 (m)	2.41 (m)	2.42 (m)
23b	1.93 (m)	2.31 (m)	2.30 (m)	2.31 (m)
24	5.11 (t, 6.8)	6.52 (t, 7.4)	6.51 (t, 7.4)	6.51 (t, 7.2)
26	1.62 (s)	1.77 (s)	1.77 (s)	1.77 (s)
27	1.70 (s)	9.42 (s)	9.42 (s)	9.41 (s)
28a	1.15 (s)	6.42 (br s)	6.41 (br s)	0.85 (d, 6.8)
28b		5.91 (br s)	5.91 (br s)	
29	0.70 (s)			3.51 (d, 7.2)
30	0.95 (s)	0.96 (s)	0.97 (s)	0.95 (s)
OCH_3			3.65 (s)	3.68 (s)

[a] Not detected.

Table 2
^{13}C NMR (100 MHz) data in $CDCl_3$ (δ in ppm) for compounds **1–4.**

Position	**1**	**2**	**3**	**4**
	δ_C	δ_C	δ_C	δ_C
1	46.7 CH_2	27.3 CH_2	26.8 CH_2	26.9 CH_2
2	211.0 qC	31.9 CH_2	31.7 CH_2	31.7 CH_2
3	83.2 CH	181.0 qC	181.1 qC	174.7 qC
4	45.9 qC	144.0 qC	143.1 qC	35.7 CH
5	46.5 CH	37.1 CH	37.2 CH	37.0 CH
6	21.1 CH_2	21.7 CH_2	22.1 CH_2	21.3 CH_2
7	25.4 CH_2	25.2 CH_2	25.1 CH_2	25.1 CH_2
8	47.6 CH	48.1 CH	48.2 CH	48.3 CH
9	19.6 qC	–[a] qC	–[a] qC	21.0 qC
10	28.0 qC	28.0 qC	28.6 qC	27.1 qC
11	26.7 CH_2	27.3 CH_2	28.1 CH_2	28.1 CH_2
12	32.5 CH_2	33.0 CH_2	32.9 CH_2	33.0 CH_2
13	45.2 qC	45.3 qC	45.1 qC	45.1 qC
14	48.7 qC	49.3 qC	48.8 qC	48.9 qC
15	35.4 CH_2	35.7 CH_2	35.7 CH_2	35.7 CH_2
16	28.0 CH_2	25.2 CH_2	28.0 CH_2	28.0 CH_2
17	52.2 CH	52.4 CH	52.2 CH	52.2 CH
18	17.8 CH_3	18.1 CH_3	18.0 CH_3	18.0 CH_3
19	29.0 CH_2	29.8 CH_2	29.8 CH_2	30.2 CH_2
20	35.8 CH	34.6 CH	34.7 CH	34.7 CH
21	18.2 CH_3	18.2 CH_3	18.3 CH_3	18.2 CH_3
22	36.3 CH_2	35.9 CH_2	36.0 CH_2	35.9 CH_2
23	24.9 CH_2	26.0 CH_2	26.0 CH_2	26.0 CH_2
24	125.1 CH	138.8 CH	139.0 CH	139.1 CH
25	130.9 qC	155.7 qC	155.5 qC	155.5 qC
26	17.6 CH_3	9.1 CH_3	9.1 CH_3	9.1 CH_3
27	25.7 CH_3	195.6 CH	195.4 CH	195.4 CH
28	25.4 CH_3	126.3 CH_2	126.8 CH_2	11.8 CH_3
29	15.0 CH_3	173.5 qC	174.2 qC	67.1 CH_2
30	19.3 CH_3	19.4 CH_3	19.4 CH_3	19.4 CH_3
OCH_3			51.5 CH_3	51.6 CH_3

[a] Not detected.

−80 °C. The luciferase assay was performed using the Luc assay system from Promega (Madison, WI). The gene product, luciferase enzyme, reacts with luciferase substrate, emitting light which was detected using a luminometer (LUMIstar Galaxy, BMG, Otrenberg, Germany). Data for NF-κB constructs were expressed as IC_{50} values (i.e. the concentration required to inhibit TNF-activated NF-κB activity by 50%). As a positive control, two known NF-κB inhibitors were used: TPCK, IC_{50} 3.8 μM and BAY-11, IC_{50} 2.0 μM.

2.5. Inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS)-activated murine macrophage RAW 264.7 cell assay

The level of nitrite, the stable end product of NO, was estimated as described previously [13]. Briefly, RAW 264.7 cells were seeded at a density of 1×10^5 cells/well and incubated in 96-well culture plates at 37 °C, 5% CO_2 in humidified air for 24 h. The cultured medium was replaced with phenol red-free medium containing various concentrations of compounds for 15 min prior to 1 μg/mL of LPS exposure for 20 h. The amount of nitrite in the culture media was measured by using Griess reagent. Under the same experimental conditions, sulforhodamine B assays were performed to evaluate the cytotoxic effect of compounds toward RAW 264.7 cells. L-N^G-monomethyl arginine citrate (L-NMMA), a positive control of this assay showed an IC_{50} value of 25.1 μM.

3. Results and discussion

Compound **1**, $[\alpha]^{25}_D$ +48 (*c* 0.25, $CHCl_3$), was obtained as a white amorphous powder and its molecular formula was established as $C_{30}H_{48}O_2$ by molecular ion at *m/z* 439.3574 $[M - H]^-$, (calcd for $C_{30}H_{47}O_2$, 439.3576) in the negative HRFABMS, implying seven degrees of unsaturation. The IR spectrum of compound **1** showed broad bands at 3361 cm^{-1} and 1710 cm^{-1}, indicating one or more hydroxyl and ketone carbonyl group(s), respectively. Inspection of the ^{13}C NMR spectrum (Table 2), together with the molecular formula indicated a triterpene. The ^{1}H NMR spectrum (Table 1) showed a characteristic pair of methylene doublets at δ_H 0.65 (1H, d, J = 4.8 Hz, H-19b) and 0.49 (1H, d, J = 4.8 Hz, H-19a), indicating the methylene protons of the cyclopropane of a cycloartane triterpene [9,10], an oxygenated methine at δ_H 3.91 (1H, s, H-3), a trisubstituted olefinic proton at δ_H 5.11 (t, J = 6.8 Hz, H-24), two singlet terminal methyls attached at an olefinic double bond at δ_H 1.62 (s, CH_3-26)/1.70 (s, CH_3-27), a doublet methyl at δ_H 0.90 (d, J = 6.4 Hz, CH_3-21), and four quaternary methyl protons at δ_H 0.96 (s, CH_3-18)/0.95 (s, CH_3-28)/0.70 (s, CH_3-29)/1.15 (s, CH_3–30). The ^{13}C NMR and HSQC spectra revealed the presence of ten methylenes, six methines (an olefinic), six tertiary methyls, one secondary methyl, six quaternary carbons, and one ketone carbonyl carbon at δ_C 211.0 (C-2), which were close to those of cycloartan-24-ene-1α,2α,3β-triol [14], except for a methylene at δ_H 2.09 (1H, d, J = 13.2 Hz) and 2.67 (1H, d, J = 13.2 Hz)/δ_C 46.7 (C-1), an oxygenated singlet methine group at δ_H 3.91/δ_C 83.2 (C-3), and a carbonyl carbon at δ_C 211.0 (C-2) instead of 1α,2α,3β-triols [14]. These observations were supported by the HMBC correlations from H-1 to C-2/C-3/C-5 and from H-3 to C-2/C-30 (Fig. 2). The orientation of hydroxyl group at C-3 was determined as β form based on NOESY correlations from H-5 to H-1a/CH_3-28 and from H-3 to H-1a/CH_3-29/H-5 (Fig. 3), as well as the comparison of its physicochemical data with published values [9,10]. Accordingly, **1** was confirmed as a new compound, namely sootepin F.

Triterpenoids possessing a ketone group in C-3 position are commonly found in nature [15–17], and 1,2-diol, 1,3-diol, 2,3-diol, and 1,2,3-triol types of triterpenoids have also been reported from natural sources [14,18–20]. The oxidized products, 1,3-dioxo and 3-oxo-2-hydroxy triterpenoids have been synthesized from 1,3-diol and 2,3-diol

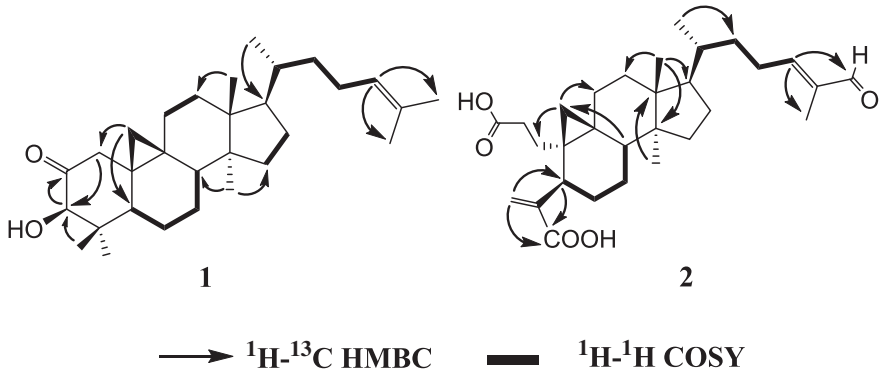

Fig. 2. Important ^{1}H-^{13}C HMBC and ^{1}H-^{1}H COSY correlations for **1** and **2**.

triterpenes, respectively, using a pyridinium chlorochromate (PCC) catalyst [18,21]. The unique structure, **1** possessing a 2-oxo-3-hydroxy group seems to be occurred from 2,3-diol [22] and is reported first time in this study.

Compound **2** was obtained as a white amorphous powder. The molecular formula was established as $C_{30}H_{44}O_5$ by the negative HRFABMS (*m/z* 483.3113 [M – H]$^-$, calcd for $C_{30}H_{43}O_5$, 483.3116), indicating nine degrees of unsaturation. The structure of **2** was also assigned as the cycloartane triterpene compare to those of **1**. When considering the number of degrees of unsaturation and functional groups of **2** compare to those of **1**, compound **2** must have of a less ring system than **1** (Tables 1 and 2). Accordingly, the skeleton of **2** was assigned as shown in Fig. 1. The ^{1}H and ^{13}C NMR of compound **2** showed additional signals for two ester carbonyl carbons at [δ_C 181.0 (C-3) and δ_C 173.5 (C-29)], an aldehyde group at δ_H 9.42 (1H, s)/δ_C 195.6 (C-26), and an *exo*-methylene group at [δ_H 6.42 (1H, s), 5.91 (1H, s)/δ_C 126.3 (C-28) and δ_C 144.0 (C-4)], which is comparable to that of coronalonic acid (**5**) [23], replacing the carbonyl group instead of methylene group at C-29. This observation was supported by three-bond HMBC correlations from H-5 and H-28 to C-29 (Fig. 2). The relative configuration of **2** was determined as the same with those of **1** and **5**, based on the NOESY correlations of H-5/CH_3-30, H-8/H-19b, and H-8/CH_3-18 (Fig. 3). Consequently, **2** was determined as a new compound, sootepin G.

Compound **3** was obtained as a white amorphous powder. The molecular formula was established as $C_{31}H_{46}O_5$ by the negative HRFABMS (*m/z* 497.3268 [M – H]$^-$, calcd for $C_{31}H_{45}O_5$, 497.3267). The NMR spectra of **3** were quite similar to those of **2**, except for an additional methoxy group at δ_H 3.65 (3H, s)/δ_C 51.5, indicating the attachment to carbonyl carbon (C-3), confirmed by HMBC correlation (Fig. S15). The NOESY correlations and physicochemical data of **3** were similar to those of **2** and **5**, thus the relative configuration of **3** was suggested as shown in Fig. 1. On the basis of above data, **3** was confirmed as a new compound, sootepin H.

Compound **4** was obtained as a white amorphous powder. The molecular formula was established as $C_{31}H_{50}O_4$ by the negative HRFABMS (*m/z* 485.3632 [M – H]$^-$, calcd for $C_{31}H_{49}O_4$, 485.3631), supported by the ^{13}C NMR spectrum. The structure of **4** was also assigned as a cycloartane triterpene by comparison of its ^{1}H and ^{13}C NMR spectra with those of compounds **2** and **3**, however **4** displayed additional signals for a methyl doublet at δ_H 0.85 (3H, d, J = 6.8 Hz, CH_3-28) and an oxygenated methylene group at δ_H 3.51 (2H, d, J = 7.2 Hz)/δ_C 67.1 (C-29), in replacement of an *exo*-methylene group and a carbonyl carbon at C-4 and C-29 in **3**, respectively, indicated the presence of 2-hydroxy-1-methylethyl group [24]. This was supported by the HMBC correlations from CH_3-28 to C-5/C-29 (Fig. S20). The relative configuration of **4** was determined as the same with that of compound **3**, as a result of NOESY correlations of H-5 with CH_3-30 and of H-8 with H19b/CH_3-18 (Fig. S21). Thus, **4** was elucidated as a new compound, sootepin I.

The other four isolates were identified as coronalonic acid (**5**) [23], sootepin D (**6**) [10], coronalolide (**7**) [23], and coronalolide methyl ester (**8**) [23], by comparison of their physical and spectroscopic data with those of published values.

NF-kB transcription factors and corresponding signalling pathways have been shown to mediate inflammation and suppress apoptosis. Aberrantly active NF-κB has been found in various inflammatory diseases, including rheumatoid arthritis, septic shock, and myocardial ischemia [25,26]. Recent work has demonstrated that specific inhibitors of NF-κB might serve as chemical leads for developing effective therapeutic agents for the treatment of inflammation and cancer [27].

The free radical nitric oxide (NO), synthesized by a family of enzymes termed NO-synthases (NOS), acts as a host defense by damaging pathogenic substances and as a regulatory molecule with homeostatic activity [28]. However, excessive production has detrimental effects on many organ systems of the body, leading to tissue damage, and possibly fatal consequences such as septic shock [29]. It is generally know that inhibition of aberrantly active NF-κB activity correlates with the inhibition of NO-production. Similarly, inducible nitric oxide synthases is consistently associated with chronic inflammation. Therefore, inhibition of NO production may be of therapeutic benefit in various diseases induced by pathological levels of NO.

In studying plant-derived cancer chemopreventive agents, a CH_2Cl_2 partition of this plant demonstrated appreciable inhibitory effects against TNF-α-induced NF-κB activity using stably-transfected human embryonic kidney cell NF-κB Luc-293, and NO production in LPS-

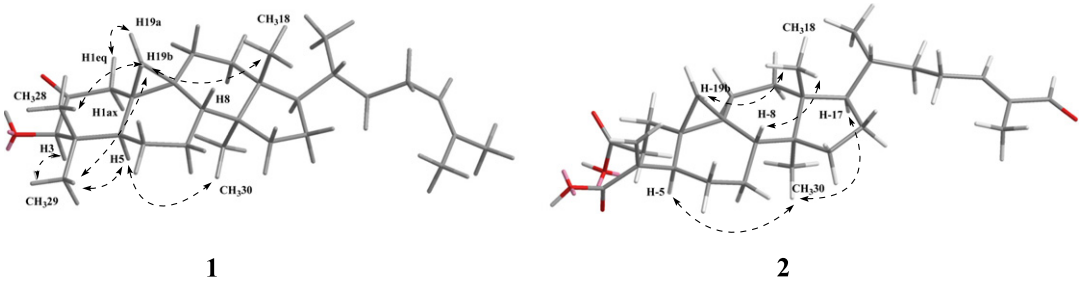

Fig. 3. Key NOESY correlations for **1** and **2**.

Table 3
Inhibition effect of compounds **1–8** against the TNF-α-induced NF-κB activity and NO production in LPS-stimulated RAW 264.7 cells.

Compounds	Nitrite assay			NF-κB assay		
	% inhib.[a]	IC_{50} (μM)	% surv.[b]	% inhib.[c]	IC_{50} (μM)	% surv.[d]
1	66.2 ± 2.6	43.6 ± 1.2	73 ± 1.2	69.0 ± 3.8	20.3 ± 0.4	100 ± 1.1
2	94.6 ± 0.3	18.4 ± 1.5	62.8 ± 1.6	35.0 ± 2.8		89.9 ± 5.6
3	100 ± 0.1	15.1 ± 0.8	39.3 ± 1.3	64.2 ± 3.3	42.3 ± 1.6	68.3 ± 12
4	87.9 ± 2.0	19.7 ± 3.2	40.9 ± 5.6	60.1 ± 0.9		78.0 ± 14
5	100 ± 0.1	15.0 ± 1.1	38.0 ± 2.0	84.3 ± 2.4	15.4 ± 2.0	84.0 ± 3.3
6	93.9 ± 0.7	17.7 ± 1.0	40.4 ± 4.1	92.5 ± 0.9	8.3 ± 1.1	85.9 ± 5.6
7	100 ± 0.5	3.2 ± 0.2	38.1 ± 0.6	95.4 ± 3.0	5.6 ± 0.5	88.6 ± 4.2
8	98.8 ± 0.2	2.0 ± 0.1	31.1 ± 1.2	82.3 ± 1.8	6.0 ± 1.3	101 ± 3.6
L-NMMA[e]		25.1 ± 2.3				
TPCK[f]					3.8 ± 0.6	
BAY-11[f]					2.0 ± 0.3	

[a] % Inhibition at a concentration of 50 μM.
[b] % Survival at a concentration of 50 μM.
[c] % Inhibition of NF-κB at 50 μM.
[d] % Survival at concentration of 50 μM.
[e] Positive control for NO.
[f] Positive control for NF-κB.

stimulated RAW 264.7 cells. The isolates from the plant were also evaluated for anti-inflammatory activity with these models. Among the isolates, compounds **7** and **8** exhibited significant NO inhibitory activity with IC_{50} values of 3.2 and 2.0 μM, respectively. These isolates are more potent than a positive control, L-N^G-monomethyl arginine citrate (IC_{50} 25.1 μM). A cytotoxic response was also observed in this model system, but only at a significantly higher concentration (50 μM). Thus, inhibition of NO production was selective. Compounds **2**–**6** also showed inhibitory effects with IC_{50} values in the range of 15.0 to 19.7 μM, but again, cytotoxicity was observed. In addition, compounds **7** and **8** displayed a considerable inhibitory effect on TNF-α-induced NF-κB activity with IC_{50} values of 5.6 and 6.0 μM, respectively, with less or no cytotoxicity at 50 μM. Compound **6** exhibited moderate inhibition with an IC_{50} value of 8.3 μM (Table 3). Although the structure–activity relationships of cycloartane triterpenes have not been investigated thoroughly, our results suggest the *exo*-methylene γ-lactone ring system in the structure may contribute to the inhibitory effects on TNF-α-induced NF-κB activity and NO-production. It is generally accepted that Michael-acceptor reactions form covalent bonds with the active site of the cysteine of proteases to elicit a biological effect [30,31]. Examples of cysteine proteases include papains, calpains and caspases, which are important proteins for therapeutic targeting of inflammatory disease and tumors [32,33]. In addition, the α-methylene-γ-lactone moiety present in damsin, a natural pseudoguaianolide sesquiterpene, exhibits its activity by interacting with the thiol group of cysteine-38 in Rel A gene via a Michael-acceptor which results in inhibition of HIV-1 LTR production which is activated by TNF-α through a NF-κB-dependent mechanism [34].

In conclusion, the anti-inflammatory activity of cycloartane triterpenes isolated from the CH_2Cl_2-soluble extract of the apical bud of *G. sootepenesis* can be attributed, at least in part, to inhibition of TNF-α-induced NF-κB activity and NO-production.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fitote.2016.08.012.

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