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Cytotoxic androstane derivatives from *Sarcococca ruscifolia*

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ABSTRACT

Sarcorusones A–D (**1–4**), four new androstane (C19-steroid) derivatives were characterized from *Sarcococca ruscifolia* along with five known compounds. Their structures were elucidated on the basis of extensive MS and NMR spectroscopic analysis. All the new structures share common 14-hydroxyl and 17-ketone functional groups, and compounds **2–4** feature a seneciamide group connecting to C-3 position. The inhibitory activities of all the isolates against melanoma cell B16F10 and lung cancer cell H1299 were evaluated, and compounds **2**, **3**, **5**, and **6** exhibited moderate cytotoxic activities against B16F10 and H1299 cell lines with IC_{50} values 2.7–8.0 μM.

1. Introduction

The plants of *Sarcococca*, nicknamed of "The friend of stomach", have been used as a folk medicine to treat ulcer, gastritis, diarrhoea, tumor, and malaria et al. [1]. *Sarcococca* species accumulate various steroids with diverse bioactivities, such as antitumor [2], immunosuppressive [3], hepatoprotective [4], and antileishmanial activities [5]. Especially, *Sarcococca* plants are a rich source of pregnane alkaloids, a special kind of C23-steroid derivatives with promising antitumor and Cholinesterase inhibiting activities [6–8]. *Sarcococca ruscifolia*, mainly distributed in south of China [9], is a Chinese herbal medicine being used to treat stomach pain, rheumatism, and bruises [10]. Previous studies on this species have led to the characterization of diverse pregnane alkaloids [11–15]. In a continuous search for new and bioactive plant-derived alkaloids from Yunnan local medicinal plants [16–21], the phytochemical investigation of *S. ruscifolia* was carried out. As a result, a known pregnane alkaloid, sarsaligenine A (**5**) [22], a new androstane, sarcorusone A (**1**), and three new nitrogen-containing androstane (C19-steroid) derivatives, sarcorusones B–D (**2–4**), were isolated together with the known compounds: ponasterone A (**6**) [23], posterone (**7**) [24], calonysterone (**8**) [25], lupeol (**9**) [26]. All the new structures share common 14-hydroxyl and 17-ketone functional groups (Fig. 1), and compounds **2–4** feature a seneciamide group connecting to C-3 position. Herein are described the isolation and structural elucidation of the new metabolites **1–4** and the cytotoxic activities of all compounds obtained against B16F10 and H1299 cell lines.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured with an Autopol VI automatic polarimeter (Rudolph, Hackettstown, NJ, USA). UV spectra were obtained on a Shimadzu UV2700 spectrometer. IR spectra were obtained on a Nicolet iS10 infrared spectrometer (KBr pellets). NMR spectra were recorded on a Bruker AVANCE III 400 M spectrometer with TMS as the internal standard in DMSO. Unless otherwise specified, chemical shifts (δ) were expressed in ppm with reference to solvent signals. The 1D and 2D NMR (COSY, HMQC, HMBC, ROESY) spectra using standard pulse programs were recorded at room temperature (298 K) operating at 400 (^{1}H) or 100 (^{13}C) MHz (NS: 16 for ^{1}H and 1024 for ^{13}C spectra). HRESIMS analyses were measured on Agilent 1290 UPLC/6545 Q-TOF mass spectrometer (Ionization mode: ESI; Gas temperature 325 °C; Flow: 11 L/min; Nebulizer: 35 psig; Vcap: 4000 V; Fragmentor voltage: 175 V; Skimmer: 65 V). Column chromatography (CC) was performed on silica gel (100–200 and 200–300 mesh, Qingdao Marine Chemical Co. Ltd., P.R. China), Sephadex LH-20 (GE Healthcare Bio-Sciences AB), and RP-18 gel (20–45 μm, Fuji Silysia Chemical Ltd., Japan). Fractions

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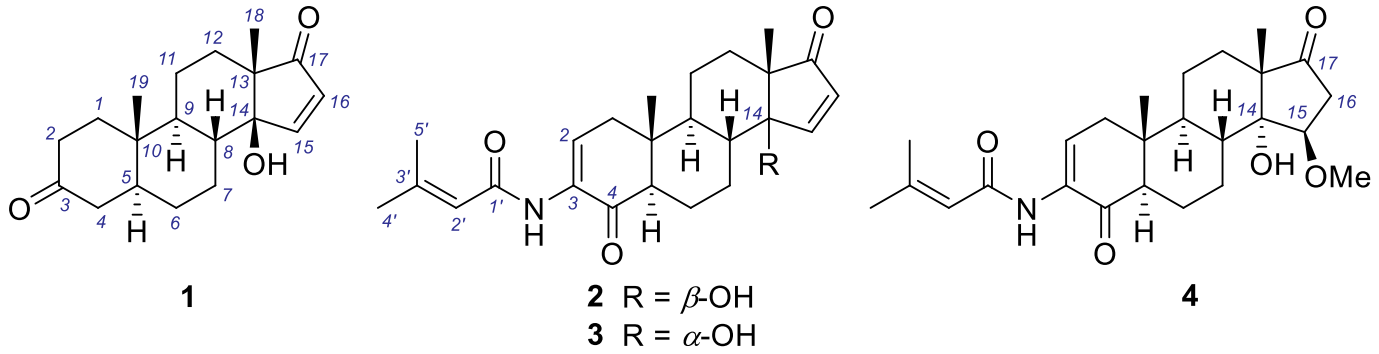

Fig. 1. Structures of sarcorusones A–D (**1**–**4**).

Table 1
^{1}H (400 MHz) NMR data of compounds **1**–**4** in DMSO-d_6.

No.	1	2	3	4
1	1.85, m	2.44, dd (18.5, 6.8)	2.44, m	2.45, m
	1.16, td (14.1, 4.9)	2.21, dd (18.5, 2.3)	2.36, m	2.35, dd (17.7, 6.1)
2	2.39, td (14.1, 6.4)	7.42, dd (6.8, 2.3)	7.46, m	7.45, brd (6.3)
	2.05, m			
4	2.28, t (14.3)			
	1.92, m			
5	1.25, overlap	2.33, dd (11.1, 3.3)	2.39, overlap	2.33, m
6	1.36, m	1.98, m	1.94, overlap	1.97, m
	1.25, overlap	1.25, overlap	1.34, m	1.30, overlap
7	2.14, m	2.24, overlap	1.81, overlap	1.64, overlap
	1.24, overlap	1.24, overlap	1.94, overlap	1.30, overlap
8	1.83, m	1.70, m	1.81, overlap	1.79, m
9	0.75, m	1.10, m	1.86, m	1.64, overlap
11	1.25, overlap	1.21, overlap	1.51, m	1.47, m
			1.38, overlap	1.23, m
12	1.42, overlap	1.42, brt (6.3)	1.98, m	1.75, m
			1.39, overlap	1.26, overlap
15	7.63, d (6.0)	7.63, d (5.9)	7.72, d (5.9)	3.76, d (6.1)
16	6.15, d (6.0)	6.17, d (5.9)	6.03, d (5.9)	2.46, overlap
				2.33, overlap
18	0.94, s	0.95, s	0.98, s	1.01, s
19	0.93, s	0.75, s	0.84, s	0.81, s
OMe				3.24, s
14-OH	4.93, s	4.97, s	4.97, s	4.50, s
NH		8.56, s	8.55, s	8.54, s
2′		6.02, s	6.03, s	6.02, s
4′		1.81, s	1.81, s	1.81, s
5′		2.08, s	2.09, s	2.09, s

were monitored by TLC (GF254, Qingdao Marine Chemical Co., Ltd., P. R. China) and spots were visualized by sulfuric acid-ethanol chromogenic agent and Dragendorff's reagent.

2.2. Plant material

The whole plants of *S. ruscifolia* were bought in Kunming, China, in March 2019. The materials were collected in Yunnan Province, China. The plant species was identified by Dr. W. Fang from Kunming Institute of Botany, and a voucher specimen (No. 20190320S) was deposited at Yunnan university.

2.3. Extraction and isolation

The milled dried whole plants of *S. ruscifolia* (10 kg) were extracted with 95% methanol (50 × 3, 24 h each) under reflux conditions, and the solvent was evaporated *in vacuum*. The residue was dissolved in 0.35% HCl and the solution was subsequently basified with 10% ammonia to pH 9. The basic solution was partitioned with ethyl acetate, affording a two-phase mixture including the aqueous phase and the ethyl acetate phase. Then, the ethyl acetate fraction (158 g) was dissolved in methanol, and the resulting solution was subjected to column chromatography over macroporous resin eluting with MeOH/H_2O (from 0%–100%) to afford six fractions (Fr. 1 to 6). Fr. 1 (18.6 g) was subjected to silica gel column chromatography (petroleum ether/acetone 5:1–1:1) to yield compounds **5** (54 mg) and **6** (41 mg). The subordinate Fr. 1–1 was chromatographed on RP-18 CC (MeOH/H_2O, 3:1) to afford compounds **7** (8 mg) and **8** (6 mg). Fr. 2 (42.3 g) was separated by RP-18 CC (H_2O/ MeOH, 4:1–1:9) and then purified by silica gel CC ($CHCl_3$/MeOH, 10:1–2:1) to obtain compounds **1** (14 mg), **2** (62 mg),**3** (25 mg), **4** (20 mg), and **9** (8 mg).

Sarcorusone A ***(1):*** white amorphous powder; $[\alpha]_D^{20}$ 195 (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 195 (3.86), 298 (1.59); IR (KBr) ν_{max} 3447, 2946, 2863, 1705, 1451, 1377, 1260, 1015, 806, 541 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; positive HRESIMS *m/z* 325.1780 [M + Na]$^+$ (calcd for $C_{19}H_{26}O_3Na$, 325.1780).

Sarcorusone B ***(2):*** white amorphous powder; $[\alpha]_D^{20}$ 296 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 195 (4.47), 225 (4.36), 280 (4.16); IR (KBr) ν_{max} 3430, 3380, 2940, 2869, 1665, 1644, 1515, 1372, 1159, 844, 632 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; positive HRESIMS *m/z* 398.2341 [M + H]$^+$ (calcd for $C_{24}H_{32}NO_4$, 398.2331).

Sarcorusone C ***(3):*** white amorphous powder; $[\alpha]_D^{20}$ 129 (c 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 195 (4.22), 206 (4.12), 227 (4.22); IR (KBr) ν_{max} 3443, 3358, 2933, 1725, 1660, 1636, 1518, 1370, 1338,1163, 818, 624 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; positive HRESIMS *m/z* 398.2342 [M + H]$^+$ (calcd for $C_{24}H_{32}NO_4$, 398.2331).

Sarcorusone D ***(4):*** white amorphous powder; $[\alpha]_D^{20}$ 72 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε) 195 (3.98), 207 (3.92), 234 (4.07); IR (KBr) ν_{max} 3464, 3365, 2941, 1745, 1638, 1519, 1367, 1336, 1166, 1052 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; positive HRESIMS *m/z* 430.2604 [M + H]$^+$ (calcd for $C_{25}H_{36}NO_5$, 430.2593).

2.4. Cytotoxcity bioassays

The melanoma cell B16F10 and lung cancer cell H1299 were cultured at 37 °C, 5% CO_2 incubator by the DMEM completed medium. The B16F10 and H1299 cells were seeded on 96-well-plate with 10,000 cells/well. Compounds were added with a serial dilution (10, 8, 6, 4, 2, 1, 0.5 μg/mL) and cultivated in cell incubator for 72 h. The MTS reagent was diluted 1:5 with fresh media, then added to 100 μL/well of fresh media, and the cells were incubated for another 1.5 h. Absorbance was measured by Hybrid Reader (BioTek Synergy H1) at 490 nm. The half-maximal inhibitory concentration (IC_{50}) value was measured and calculated by GraphPadPrism 5 software, Taxol was used as positive control.

Table 2
^{13}C (100 MHz) NMR data of compounds **1**–**4** in DMSO-d_6.

No.	1	2	3	4
1	37.2	37.8	38.3	38.6
2	37.4	127.0	127.3	127.3
3	210.3	131.9	131.7	131.7
4	44.1	195.4	195.6	195.6
5	45.4	53.7	54.2	54.0
6	28.1	20.5	20.2	20.1
7	26.1	24.9	24.1	23.3
8	39.9	39.3	33.9	33.0
9	45.2	45.2	45.8	46.0
10	36.3	40.0	39.8	39.9
11	19.8	19.0	18.3	18.6
12	32.9	32.9	23.3	25.6
13	51.4	51.3	53.5	51.3
14	81.5	81.3	80.0	80.1
15	163.1	162.9	158.9	80.6
16	131.4	131.5	131.9	40.1
17	213.2	213.1	211.8	216.9
18	17.7	17.5	22.7	17.7
19	10.3	12.3	12.4	12.8
OMe				56.7
1′		165.0	165.0	165.1
2′		119.1	119.1	119.1
3′		151.2	151.1	151.2
4′		26.9	26.9	27.0
5′		19.4	19.4	19.4

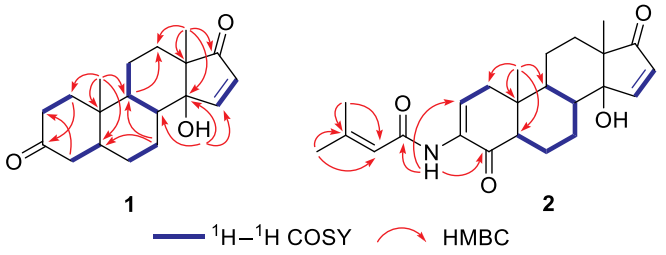

Fig. 2. Key ^{1}H-^{1}H COSY and HMBC correlations of compounds **1** and **2**.

3. Results and discussion

Sarcorusone A (**1**) was obtained as a white amorphous powder. Its molecular formula was assigned as $C_{19}H_{26}O_3$ by ^{13}C NMR and positive HRESIMS ion peak at *m/z* 325.1780 ([M + Na]$^+$, calcd for 325.1780). The UV spectrum exhibited the existence of conjugated groups by the observed maximum absorptions at 195 and 298 nm, and the IR spectrum showed absorption bands due to hydroxyl (3447 cm^{-1}) and carbonyl (1705 cm^{-1}) groups. The ^{1}H NMR spectrum (Table 1) presented a pair of mutual coupling olefinic protons at δ_H 7.63 and 6.15 (d, J = 6.0 Hz), a downfield singlet at δ_H 4.93, and two singlet methyls (δ_H 0.94 and 0.93). The ^{13}C and DEPT NMR spectra (Table 2) showed the presence of 19 carbon resonances corresponding to two ketones (δ_C 213.2 and 210.3), three quaternary carbons (δ_C 36.3, 51.4, and an oxygenated one at δ_C 81.5), five methines (δ_C 39.9, 45.2, 45.4, and two olefinic ones at δ_C 131.4 and 163.1), seven methylenes (δ_C 19.8–44.1), and two methyls (δ_C 10.3 and 17.7). The observations above indicated that compound **1** could be a C19-steroid derivative. Comparison of the ^{13}C NMR data of **1** (Table 2) and 5*α*-androstane-3,17-dione [27], a known C19-steroid, indicated that they are structurally similar. Instead of a methine (C-14) and two methylenes (C-15 and C-16) in 5*α*-androstane-3,17-dione, a downfield quaternary carbon at δ_C 81.5 and double-bond signals at δ_C 131.4 and 163.1 appeared in **1**, consistent with oxidation of C-14 and formation of C-15/16 double bond in **1**. In the HMBC spectrum of **1** (Fig. 2), the correlations of singlet methyl at δ_H 0.94 (Me-18) with δ_C 32.9 (C-12), 51.4 (C-13), 81.5 (C-14), and 213.2 (C-17), as well as correlations of a hydroxyl proton at δ_H 4.93 with δ_C 32.9 (C-8), 163.1 (C-15) and C-14, confirmed the difference. Other moieties of planar structure of **1** were determined to be the same as those of 5*α*-androstane-3,17-dione [27] by detailed analysis of the ^{1}H-^{1}H COSY and HMBC spectroscopic data.

The NOE contacts of Me-19 (δ_H 0.93)/H-4$_{ax}$ (δ_H 2.28) and OH-14/Me-18/H-8 (δ_H 1.83) in the ROESY spectrum, in combination with the large coupling constant (14.3 Hz) of H-4$_{ax}$ and H-5, defined the *α*-orientation of H-5 and *β*-orientation of OH-14 (Fig. 3). Thus, the structure of **1** was elucidated as shown in Fig. 1.

Sarcorusone B (**2**) demonstrated a positive alkaloidal test with Dragendorff's reagent and showed a quasi-molecular ion peak [M + H]$^+$ at *m/z* 398.2341 (calcd for 398.2331) in the HRESIMS, which corresponds to the molecular formula $C_{24}H_{31}NO_4$. Comparing the ^{1}H and ^{13}C NMR data of **2** (Tables 1 and 2) to those of **1** suggested that compound **2** possessed an additional seneciamide group (δ_C 165.0, C-1′; 119.1, C-2′; 151.2, C-2′; 26.9, C-4′; and 19.4, C-5′). This group was further assigned by the correlations from both δ_H 1.81 (Me-4′) and 2.08 (Me-5′) to C-2′ and C-3′ and from a downfield active proton at δ_H 8.56 (*NH*) to C-1′ in the HMBC spectrum of **2**. The connection of the seneciamide group to C-3 was deduced by the HMBC correlations of δ_H 8.56 (*NH*) with an olefinic methine at δ_C 127.0 (C-2) and a conjugated carbonyl at 195.4 (C-4). Other parts of the steroid moiety of **2** were determined to be the same as those of **1** by detailed analysis of the ^{1}H-^{1}H COSY and HMBC spectroscopic data (Fig. 2). In the ROESY spectrum of **2**, the proton spin coupling system of Me-19/H-8/Me-18/OH-14 and H-5/H-9 defined the relative configuration of **2** (Fig. 3).

Sarcorusone C (**3**) shared the same carbon skeleton as **2** on the basis of analysis of its HRESIMS and 1D and 2D NMR spectroscopic data. The ^{13}C NMR data of **3** (Table 1) were nearly the same to those of **2** except for the carbon resonances of C-8 (Δ − 5.4 ppm), C-12 (Δ − 9.6 ppm), and C-18 (Δ + 5.2 ppm), which indicated that compound **3** should be

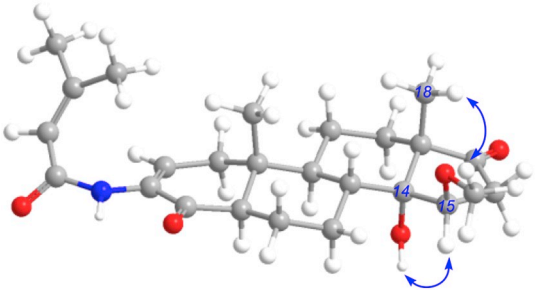

Fig. 4. Key ROESY correlations of compound **4**.

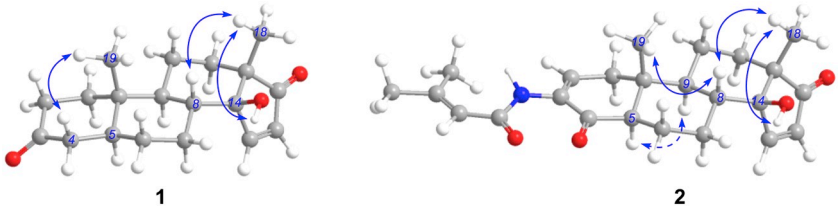

Fig. 3. Key ROESY correlations of compounds **1** and **2**.

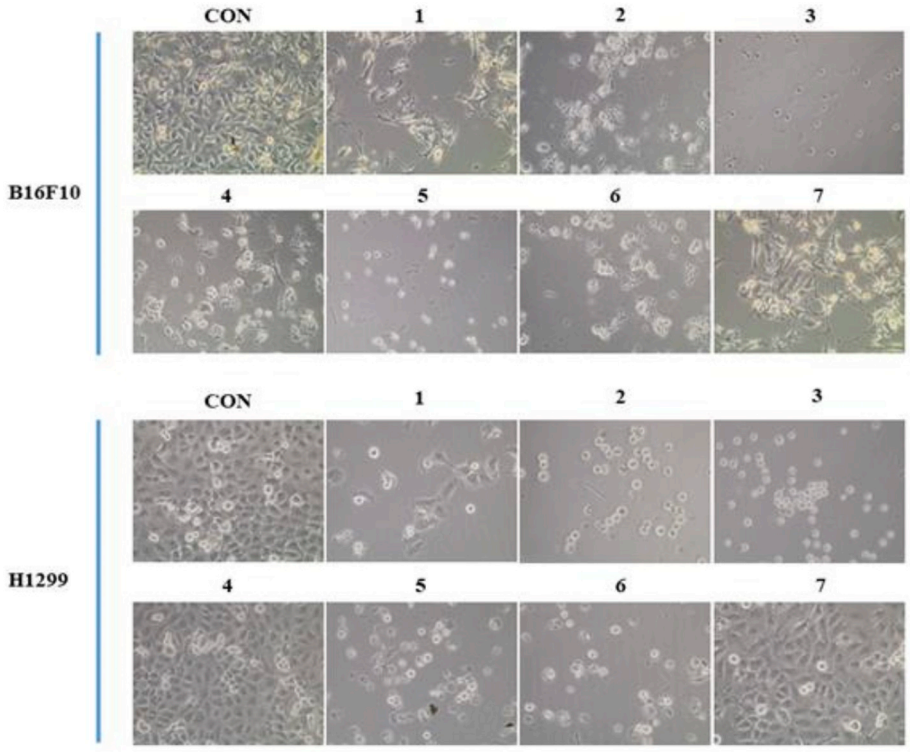

Fig. 5. Cytotoxic tests of compounds **1–7** (at the concentration of 10 μg/mL) against B16F10 and H1299 cells by phenotypic screening.

Table 3
IC_{50} values (μM) of compounds **1–7** worked against B16F10 and H1299 cell lines.

Compound	Taxol	1	2	3	4	5	6	7
B16F10	0.012	20.9	8.0	5.3	10.5	4.1	5.2	20.4
H1299	0.012	17.5	5.8	4.0	23.3	2.7	3.2	27.6

14-epimer of **2**. NOE contact of δ_H 4.97 (14-OH)/δ_H 1.98 ($H-12$) in the ROESY spectrum of **3** further confirmed such difference.

Sarcorusone D (**4**) was assigned the molecular formula $C_{25}H_{35}NO_5$ by its HRESIMS (*m/z* 430.2604, $[M + H]^+$) data, 32 mass units more than that of **3**. The ^1H and ^{13}C NMR data of **4** (Tables 1 and 2) were similar to those of **3** except that the signals of C-15/16 double bond were absent and an up-field methylene (δ_C 40.1), an oxygenated methine (δ_C 80.6), and a methoxyl group (δ_C 56.7) were present in **4**, implying methanol addition of C-15/16 double bond. This assumption was confirmed by the correlations from δ_H 4.50 (14-OH) to δ_C 51.3 (C-13), 80.1 (C-14), and 80.6 (C-15) and from δ_H 3.24 (OMe) to C-15 in the HMBC spectrum, together with correlation of H-15 (δ_H 3.76)/H-16 (δ_H 2.46 and 2.33) in the ^1H-^1H COSY spectrum. The NOE correlations of Me-18 (δ_H 1.01)/OMe and 14-OH/H-15 determined the α-configuration of OH-14 and β-configuration of the methoxyl group (Fig. 4). Other structural parts of **4** were confirmed to be the same as those of **3** by detailed analysis of the 2D NMR data.

The inhibitory activities of compounds **1–9** at the concentration of 10 μg/mL against melanoma cell B16F10 and lung cancer cell H1299 through phenotypic screening were preliminary evaluated [28,29]. As shown in Fig. 5, compounds **1–7** could effectively inhibit the growth of B16F10, while compounds **1**, **2**, **4**, **5**, and **6** could work against H1299 cell. Compounds **8** and **9** did not show inhibitory activity against both B16F10 and H1299 cell lines. IC_{50} values were further tested by cell viability assay, and compounds **2**, **3**, **5**, and **6** ultimately exhibited moderate cytotoxic activities against B16F10 and H1299 cell lines (Table 3).

4. Conclusions

A new C19-steroid, sarcorusone A (**1**), and three nitrogen-containing androstane (C19-steroid) derivatives, sarcorusones B–D (**2–4**) were isolated and characterized from *Sarcococca ruscifolia* together with five known analogues. In the bioassay, compounds **2**, **3**, **5**, and **6** exhibited moderate cytotoxic activities against B16F10 and H1299 cell lines with IC_{50} values 2.7–8.0 μM. Our findings enriched the structural and bioactive diversities of natural steroids.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix B. Supplementary data

The original MS and NMR spectra of the new isolates (Figs. S1–S28). Supplementary data to this article can be found online at DOI:XXX. Supplementary data to this article can be found online at [https://doi.org/10.1016/j.fitote.2020.104604].

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