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RESEARCH ARTICLE

Chemical components from the leaves of *Ardisia insularis* and their cytotoxic activity

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Abstract One new oleanane triterpene glycoside, ardinsuloside (**1**), and twelve known compounds, demethoxybergenin (**2**), norbergenin (**3**), bergenin (**4**), 4-*O*-galloylbergenin (**5**), quercitrin (**6**), myricitrin (**7**), myricetin 3-*O*-(3″-O-galloyl)-α-L-rhamnopyranoside (**8**), desmanthine-2 (**9**), epicatechin 3-*O*-galloyl ester (**10**), 3′-methoxyepicatechin 3-*O*-galloyl ester (**11**), gallic acid (**12**), and methyl galloate (**13**) were isolated from the leaves of *Ardisia insularis*. Their structures were established on the basis of spectral and chemical evidence, which were in agreement with those reported in literature. The cytotoxic activities of these compounds were evaluated on three cancer cell lines namely A-549 (human lung cancer), HT-29 (Human colon adenocarcinoma), and OVCAR (human ovarian carcinoma). The results revealed that compound **1** inhibited A-549, HT-29, and OVCAR cell lines with IC_{50} values of 8.5 ± 1.2, 16.4 ± 3.1, and 13.6 ± 2.4 μM, respectively. The remaining compound showed weak cytotoxic activity. This result indicated that compound **1** could be useful in the treatment of cancer disease.

Keywords *Ardisia insularis* · Myrsinaceae · Ardinsuloside · Cytotoxic activity

Introduction

Traditional Chinese medicines have attracted increasing interest as potential sources of novel drugs with a wide range of biological and pharmacological activities, including anticancer activities (Vickers 2002). In 2014, there will be an estimated 1,665,540 new cancer cases diagnosed and 585,720 cancer deaths in the United States. Cancer remains the second most common cause of death in the US, accounting for nearly 1 of every 4 death (Cancer Facts & Figs. 2014). More than half of currently available drugs are natural compounds or are related to them, and in the case of cancer this proportion surpasses 60 % (Newman and Cragg 2007). To find new medications, more attention has been focused on natural compounds in plants, marine organisms, and microorganisms.

The genus *Ardisia* is the largest in the family of Myrsinaceae, and approximately 500 species of evergreen shrubs and trees are found throughout the subtropical and tropical regions of the world. The leaves of *Ardisia insularis* Mez. have been used in the folk medicine to treat rheumatism, diarrhea, and liver diseases (Chi 2012). A number of phytochemical studies of genus *Ardisia* has reported the isolation of oleanane saponins (Mu et al. 2012b), bergenin derivatives (Mu et al. 2012a), and quinones (Kobayashi and de Mejía 2005). However, chemical components of *A. insularis* have not been studied yet. We report herein the isolation, structural elucidation, and

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evaluation of the in vitro cytotoxic activities of one new and twelve known compounds from the leaves of *A. insularis.*

Materials and methods

General procedures

Optical rotations were determined on a Jasco DIP-370 digital polarimeter. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on an Agilent 400-MR NMR spectrometer and TMS was used as an internal standard. Data processing was carried out with the MestReNova 6.0.2 program. HR-ESI–MS spectra were obtained using an Agilent 6550 iFunnel Q-TOF LC/MS system. GC was recorded on a Shimadzu GC-2010 plus. Preparative HPLC was carried out using an Agilent 1290 HPLC system. Column chromatography was performed on silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck) and YMC RP-18 resins.

Plant material

The leaves of *A. insularis* were collected in Quang Khe, Dakglong, Daknong, Taynguyen, Vietnam in March, 2012, and identified by Dr. Nguyen Quoc Binh, Vietnam National Museum of Nature, VAST, Vietnam. A voucher specimen (BMN-B0001532) was deposited at the Herbarium of Institute of Natural Products Chemistry.

Extraction and isolation

The dried leaves of *A. insularis* (2.0 kg) were extracted with MeOH three times at room temperature to yield 150 g of a dark solid extract, which was then suspended in water and successively partitioned with *n*-hexane and ethyl acetate (EtOAc) to obtain *n*-hexane (AI1, 40.0 g), EtOAc (AI2, 45.0 g), and water (AI3, 65.0 g) extracts after removal solvent *in vacuo*. The AI2 extract (45.0 g) was chromatographed on a silica gel column and eluted with $CHCl_3$–MeOH gradient (50:1 → 1:1, v/v) to obtain four sub-fractions, AI2A (8.0 g), AI2B (7.5 g), AI2C (12.5 g), and AI2D (10.0 g). The AI2B fraction was chromatographed on an YMC RP-18 column eluting with acetone–water (1:1, v/v) to yield compounds **10** (80.0 mg) and **11** (19.0 mg). The AI2C fraction was chromatographed on an YMC RP-18 column eluting with acetone–water (0.8:1, v/v) to yield compounds **6** (42.0 mg) and **7** (55.0 mg). The water soluble fraction (AI3, 65.0 g) was chromatographed on a Diaion HP-20P column eluting with water containing increasing concentrations of MeOH (0, 25, 50, 75, and 100 %) to obtain five fractions,

Table 1 The NMR spectral data for compound **1**

Pos.	**1**	
	$\delta_C^{a,b}$	$\delta_H^{a,b}$ mult. (*J* in Hz)
Aglycone		
1	39.6	0.96 (m)/1.62 (m)
2	26.3	1.73 (m)/1.81 (m)
3	83.5	3.61 (br d, 10.4)
4	43.8	–
5	48.2	1.22 (m)
6	18.9	1.40 (m)/1.50 (m)
7	33.3	1.32 (m)/1.65 (m)
8	41.0	–
9	49.0	1.64 (m)
10	37.6	–
11	24.7	1.89 (m)
12	123.4	5.17 (br s)
13	145.7	–
14	42.9	–
15	26.6	1.32 (m)/1.84 (m)
16	22.9	1.19 (m)
17	38.1	–
18	43.9	1.97 (m)
19	47.8	1.04 (m)/1.77 (m)
20	31.8	–
21	35.3	1.12 (m)
22	32.3	1.35 (m)/1.52 (m)
23	65.2	3.33 (d, 10.4)/3.64 (d, 10.4)
24	13.4	0.73 (s)
25	16.6	1.00 (s)
26	17.4	0.99 (s)
27	26.6	1.20 (s)
28	69.8	3.10 (d, 11.2)/3.51 (d, 11.2)
29	33.8	0.87 (s)
30	24.0	0.88 (s)
3-*O*-Ara		
1′	106.1	4.35 (d, 7.6)
2′	72.1	3.68 (dd, 7.6, 8.0)
3′	84.2	3.64 (m)
4′	69.5	4.03 (br s)
5′	66.9	3.56 (d, 12.0)/3.86 (d, 12.0)
3′-*O*-Glc		
1″	105.5	4.54 (d, 7.6)
2″	75.3	3.30 (m)
3″	77.9	3.35 (m)
4″	71.1	3.30 (m)
5″	77.6	3.30 (m)
6″	62.3	3.65 (d, 4.8, 12.0)/3.83 (d, 12.0)

[a] Measured in CD_3OD, [b] 100 MHz, [c] 400 MHz, Assignments were done by HMQC, HMBC, ^{1}H-^{1}H COSY and ROESY experiments, Ara, L-arabinopyranosyl, Glc, D-glucopyranosyl

AI3A (30.0 g), AI3B (9.0 g), AI3C (13.0 g), AI3D (7.0 g), and AI3E (6.0 g). The AI3B fraction (9.0 g) was chromatographed on a silica gel column eluting with $CHCl_3$–MeOH–water (5:1:0.1, v/v/v) to give four fractions, AI3B1 (3.0 g), AI3B2 (2.4 g), AI3B3 (2.1 g), and AI3B4 (1.0 g). The AI3B2 fraction was chromatographed on an YMC RP-18 column eluting with MeOH–water (1:1, v/v) to yield compounds **12** (8.0 mg) and **13** (9.0 mg). The AI3B3 fraction was chromatographed on an YMC RP-18 column eluting with MeOH–water (2:1, v/v) to yield compounds **2** (5.0 mg), **3** (6.0 mg), and **4** (9.0 mg). The AI3C fraction was chromatographed on a silica gel column and eluted with $CHCl_3$–MeOH gradient (50:1 → 1:1, v/v) to obtain four fractions, AI3C1 (8.0 g), AI3C2 (7.5 g), AI3C3 (12.5 g), and AI3C4 (10.0 g). The AI3C2 fraction was chromatographed on an YMC RP-18 column eluting with MeOH–water (2:1, v/v) to obtain compounds **5** (10.0 mg), **8** (17.0 mg), and **9** (9.0 mg). The AI3C3 fraction was chromatographed on an YMC RP-18 column eluting with acetone–water (0.7:1, v/v) to yield compound **1** (6.0 mg).

Ardinsuloside **(1)** A white amorphous powder, $[\alpha]_D^{25}$: +58 ($c = 0.2$, MeOH), $C_{41}H_{68}O_{12}$, HR-ESI–MS found m/z 787.4394 $[M + Cl]^-$ (Calcd $C_{41}H_{68}O_{12}Cl^-$ for 787.4405), ^{1}H- and ^{13}C-NMR: see Table 1.

Acid hydrolysis of 1

Compound **1** (2.0 mg) was dissolved in 1 N HCl (dioxane-H_2O, 1:1, 1 Ml) and then heated to 80 °C in a water bath for 3 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under N_2 gas overnight. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2 gas. The residue was dissolved in 0.1 Ml of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06, 0.1 Ml) was added to the solution. The reaction mixture was heated at 60 °C for 2 h, and 0.1 Ml of trimethylsilylimidazole solution was added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with *n*-hexane and H_2O (0.1 Ml each), and the organic layer was analyzed by gas liquid chromatography (GC): column SPB-1 (0.25 mm × 30 m); detector FID, column temp 210 °C, injector temp 270 °C, detector temp 300 °C, carrier gas He (2.0 Ml/min). Under these conditions, standard sugars gave peaks at t_R (min) 8.55 and 9.25 for D- and L-glucose, 4.72 and 9.16 for D- and L-arabinose, respectively. Peaks at t_R (min) 8.55 and 9.16 of D-glucose and L-arabinose for compound **1**, respectively, were observed.

Cytotoxicity assay

The effect of compounds **1**–**13** on the growth of human cancer cells was determined by measuring the cytotoxic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Nhiem et al. 2012). The A-549 (human lung cancer), HT-29 (human colon cancer) and OVCAR (human ovarian cancer) cell lines were obtained from the korea cell line bank (KCLB) and were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum and penicillin/streptomycin (100 U/Ml and 100 g/Ml, respectively) at 37 °C in a humidified 5 % CO_2 atmosphere. The MTT assays were performed as follows: 1.5 ~ 2.5 × 10^5 cells/Ml of human cancer cells were treated for 3 days with isolated compounds at concentration of 100 μM. After incubation, 0.1 mg (50 Ml of a 2 mg/Ml solution) MTT (Sigma, Saint Louis, MO, USA) was added to each well and the cells were then incubated at 37 °C for 4 h. The plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. Dimethylsulfoxide (DMSO) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech., USA). The compounds which result in more than 50 % human cancer cells dead will be further tested using dose concentration: 1, 10, 50 and 100 μM. Mitoxantrone was used to final concentrations of 1, 3, 10 and 20 μM as a reference and DMSO 0.1 % was used as negative control. All the experiments were performed three times and the mean absorbance values were calculated. The results are expressed as the relative cell viability percentage presented by a reduction in the absorbance after the treatment of the tested compounds compared to the untreated controls. A dose–response curve was generated and the IC_{50} was determined for each compound as well as each cell line.

Results and discussion

Using various chromatographic techniques, one new and twelve known compounds were isolated from the methanol extract of the leaves of *A. insularis.*

Compound **1** was obtained as a white amorphous powder. The molecular formula, $C_{41}H_{68}O_{12}$, was determined on the HR-ESI–MS at m/z 787.4394 $[M + Cl]^-$ (Calcd. 787.4405 for $C_{41}H_{68}O_{12}Cl$). The ^{1}H-NMR spectrum of compound **1** showed the following signals: one olefinic proton at δ_H 5.17, one oximethine proton at δ_H 3.61, and six tertiary methyl groups at δ_H 0.73, 0.87, 0.88, 0.99, 1.00, and 1.20 (each 3H, s), assigned to oleane-*type* triterpene aglycone; two anomeric protons at δ_H 4.35 (d, $J = 7.6$ Hz) and 4.54 (d, $J = 7.6$ Hz), assigned to two sugar moieties. The ^{13}C-NMR and DEPT spectra of compound **1** revealed 41 carbon signals including seven quaternary, fourteen methine, fourteen methylene, and six methyl carbons. The ^{1}H- and ^{13}C-NMR data of compound **1** were similar to those of assamicoside A except for the disappearance of

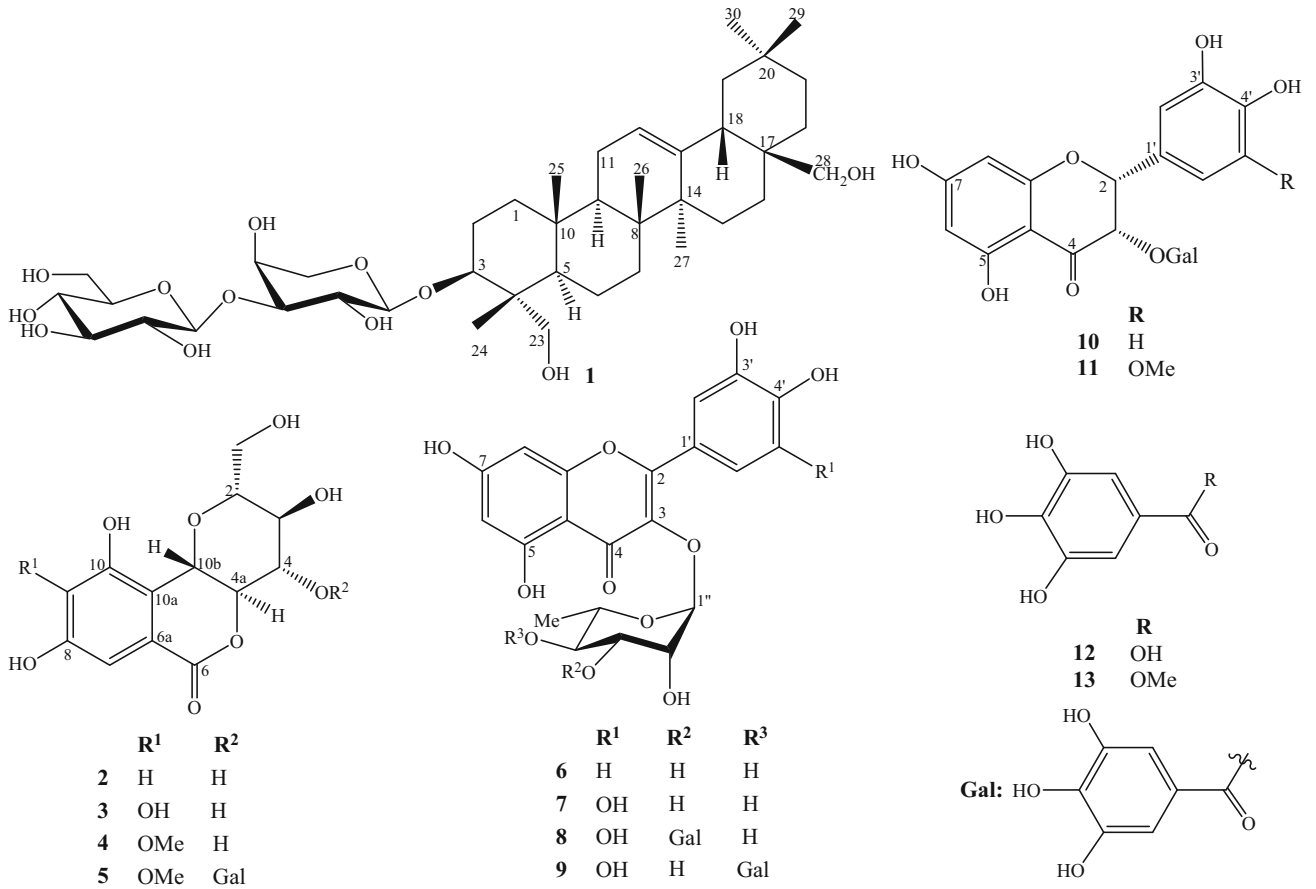

Fig. 1 Structures of isolated compounds **1**–**13** from *Ardisia insularis*

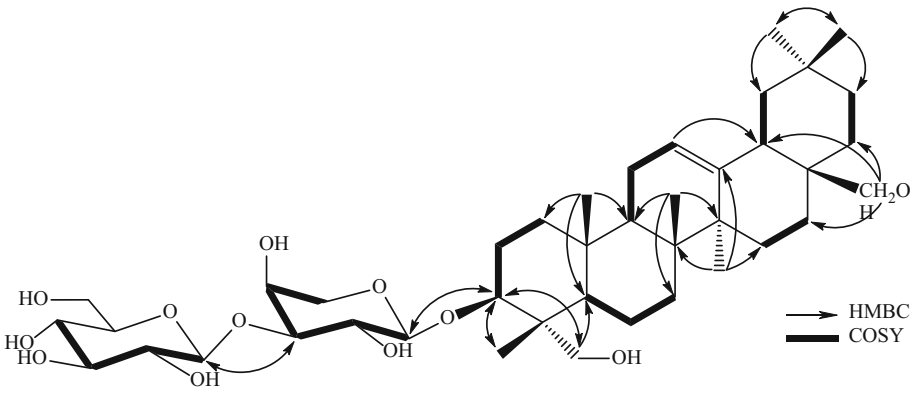

Fig. 2 The important HMBC and COSY correlations of compound **1**

two hydroxyl groups at C-16 and C-21 of the aglycone (Tian et al. 2013). The carbons were assigned to the relevant protons by means of an HMQC experiment (Table 1). The HMBC correlations between H-23 (δ_H 3.33 and 3.64) and C-3 (δ_C 83.5)/C-4 (δ_C 43.8)/C-5 (δ_C 48.2)/C-24 (δ_C 13.4); H-24 (δ_H 0.73) and C-3 (δ_C 83.5)/C-4 (δ_C 43.8)/C-5 (δ_C 48.2)/C-23 (δ_C 65.2) suggested two hydroxyl groups at C-3 and C-23 of aglycone (Figs. 1, 2). The configurations of both the hydroxyl at C-3 and the methyl groups at C-4 were proved to be β-orientation by the observation of ROESY correlations of H-3 (δ_H 3.61)/H-23 (δ_H 3.33 and 3.64)/H-5 (δ_H 1.22) (Fig. 3) as well as of large coupling constant of H_a-2 and H_a-3, $J = 10.4$ Hz). The HMBC correlations between H-28 (δ_H 3.10 and 3.51) and C-16 (δ_C 22.9), C-17 (δ_C 38.1), C-18 (δ_C 43.9), and C-22 (δ_C 32.3) confirmed the position of hydroxyl group at C-28. Acid

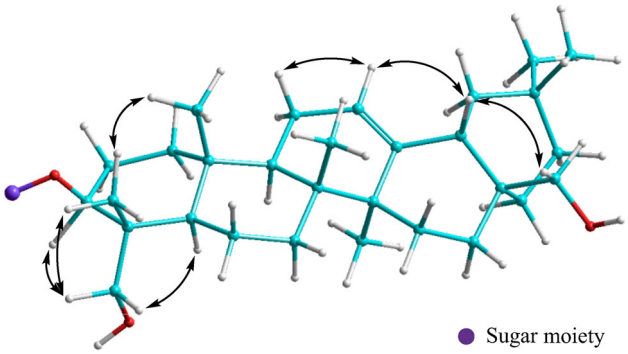

Fig. 3 The important ROESY correlations of compound **1**

Table 2 The effects of compounds **1**–**13** on the growth of human cancer cell lines at the concentration of 100 μM

Compound	Relative cell viability (%)		
	A-549	HT-29	OVCAR
1	14.6 ± 3.1	21.7 ± 5.3	16.2 ± 2.8
2	94.4 ± 8.2	92.7 ± 5.9	97.0 ± 6.7
3	94.4 ± 7.4	87.5 ± 6.2	99.9 ± 6.6
4	99.3 ± 12.4	97.9 ± 9.9	80.8 ± 5.5
5	65.6 ± 10.4	88.9 ± 7.3	76.7 ± 4.5
6	71.5 ± 5.7	73.4 ± 5.6	93.6 ± 3.6
7	76.2 ± 4.5	86.4 ± 7.2	89.0 ± 2.1
8	53.1 ± 5.6	69.3 ± 3.9	59.4 ± 9.7
9	66.4 ± 8.8	76.0 ± 5.4	69.7 ± 12.1
10	99.2 ± 5.3	98.8 ± 8.9	91.4 ± 6.1
11	43.3 ± 2.6	56.2 ± 6.4	48.8 ± 4.6
12	79.7 ± 6.6	65.9 ± 7.1	81.8 ± 8.7
13	91.5 ± 7.5	83.7 ± 5.8	82.3 ± 4.3

hydrolysis of compound **1** gave D-glucose and L-arabinose (see Experimental). The coupling constants of the anomeric protons in two sugar moieties, $J_{\text{H-1}'\text{-H-2}'} = 7.6$ Hz and $J_{\text{H-1}''\text{-H-2}''} = 7.6$ Hz confirmed the presence of *β*-D-glucopyranoside and α-L-arabinopyranoside moieties. Moreover, the HMBC correlations between glc H-1′ (δ_H 4.54) and ara C-3′ (δ_C 84.2) as well as between ara H-3′ (δ_H 3.64) and glc C-1″ ((δ_C 105.5) confirmed the sugar linkage to be *β*-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranoside. This sugar linkage to C-3 of aglycone was confirmed by HMBC correlations from H-3 (δ_H 3.61) to ara C-1′ (δ_C 106.1); from ara H-1′ (δ_H 4.35) to C-3 (δ_C 83.5). The important HMBC and COSY correlations were shown in Fig. 2. Consequently, the structure of compound **1** was determined to be 3*β*,23,28-trihydroxyolean-12-ene 3-*O*-[*β*-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranoside], a new compound named ardinsuloside.

The remaining compounds were identified as demethoxybergenin (**2**) (Sumino et al. 2002), norbergenin (**3**) (Taneyama et al. 1983), bergenin (**4**) (Taneyama et al. 1983), 4-*O*-galloylbergenin (**5**) (Yoshida et al. 1982), quercitrin (**6**) (Kiem et al. 2010), myricitrin (**7**) (Kiem et al. 2010), myricetin 3-*O*-(3″-*O*-galloyl)-α-L-rhamnopyranoside (**8**) (Sun et al. 1991), desmanthine-2 (**9**) (Nicollier and Thompson 1983), epicatechin 3-*O*-galloyl ester (**10**) (Braca et al. 2003), 3′-methoxyepicatechin 3-*O*-galloyl ester (**11**) (Aihara et al. 2009), gallic acid (**12**), and methyl galloate (**13**) (see Fig. 1). Their structures were established on the basis of spectral and chemical evidences, which were in agreement with those of reported in literature. This is the first time all these compounds have been isolated from *A. insularis.*

The isolates **1**–**13** were evaluated for cytotoxic activity against three human cancer cell lines (A-549, HT-29, and OVCAR). Compound **1** exhibited the strongest cytotoxic activity with the relative cell viability of 14.6–21.7 % at the concentration of 100 μM. The remaining compounds showed weak cytotoxic activity (Table 2). Mitroxantrone was used as a positive control and inhibited A-549, HT-29, and OVCAR cell lines with the IC_{50} values of 7.2 ± 0.5, 3.1 ± 0.3, and 8.4 ± 0.9 μM, respectively. Whereas, compound **1** inhibited A-549, HT-29, and OVCAR cell lines with IC_{50} values of 8.5 ± 1.2, 16,4 ± 3.1, and 13.6 ± 2.4 μM, respectively. This result indicates compound **1** could be useful in the treatment of cancer disease and further study of the cytotoxic effects of these compounds by in vivo assay may be required.

Supporting information

The NMR and HR-ESI–MS spectra of compound **1** are available as Supporting information.

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Conflicts of interest The authors report no conflicts of interest.

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