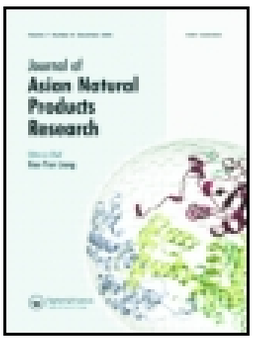

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A new triterpene ester and other chemical constituents from the aerial parts of *Anodendron paniculatum* and their cytotoxic activity

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ABSTRACT

The aim of the research was to study the active constituents of *Anodendron paniculatum* Roxb. (Apocynaceae). A new triterpene ester, named anopaniester (**1**), and cycloartenol (**2**), ursolic acid (**3**), esculenic acid (**4**), bis-(2-ethylhexyl) phthalate (**5**), desmosterol (**6**), stigmasterol (**7**), vaniline (**8**), and (*E*)-phytol (**9**), were isolated from the aerial parts of *A. paniculatum*. Compounds **3** and **6** showed the significant inhibitory effect (IC_{50} values ranging from 30.89 ± 3.60 to 44.37 ± 5.40 µg/ml) against tested human cancer cell lines LU-1 and MKN-7. The compounds **1**–**4**, and **6** were isolated from this genus *Anodendron* for the first time.

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KEYWORDS
Anodendron paniculatum; anopaniester; ursolic acid; desmosterol; cytotoxicity; anticancer activity

1. Introduction

Anodendron paniculatum Roxb. is an woody climber belonging to the family Apocynaceae and broadly distributed in Sri Lanka, India, Bangladesh, Burma, and South-East Asia [1]. The roots of this plant have been used in traditional medicine as remedy for vomiting and cough [2]. The knowledge about the chemical composition of *A. paniculatum* is rather poor and previous studies have led to the isolation and characterization of a large number of cardenolides [3,4].

As part of an on-going research for novel and anticancer compounds from Vietnam's medicinal plants, we have observed that the methanol (MeOH) extract of *A. paniculatum* exhibited potent *in vitro* cytotoxicity toward LU-1, human lung cancer, and MKN-7, stomach cancer cell lines. Therefore, this plant was selected for bioassay-guided fractionation to clarify its active constituents. In a previous investigation, we reported the isolation of kaempferol-3-*O*-rutinoside, rutin, and sargentol from the water-soluble portion of methanol extract [5]. In the current study, one new triterpene ester, anopaniester (**1**), along with eight known compounds (**2**–**9**), were isolated from the aerial parts of *A. paniculatum*. This article

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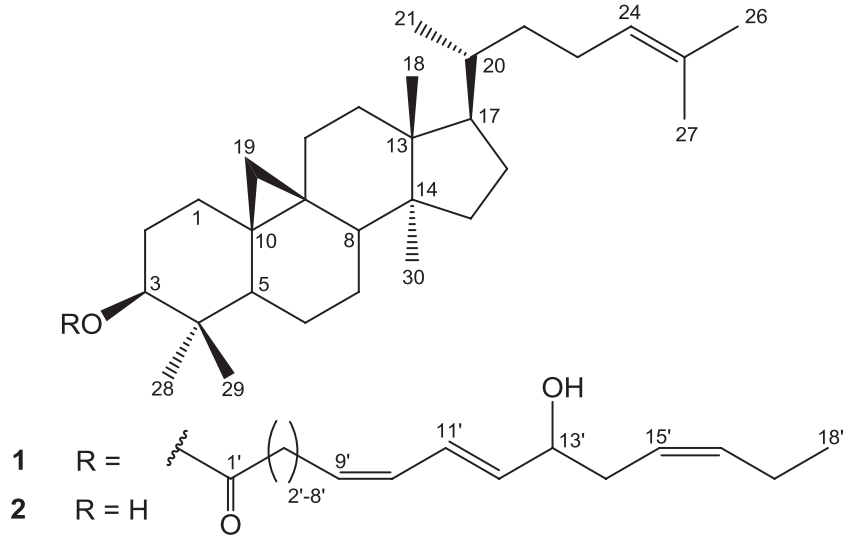

Figure 1. Chemical structures of isolated compounds **1** and **2** from the aerial parts of *Anodendron paniculatum*.

aims to report the isolation and the structural elucidation of the new compound (**1**) as well as the *in vitro* cytotoxic activity of isolated compounds toward LU-1 and MKN-7 cell lines.

2. Results and discussion

Compound **1** (Figure 1) was obtained as colorless oil. Its molecular formula was deduced to be $C_{48}H_{78}O_3$ by HRESIMS data in conjunction with NMR data analysis, which contains ten degrees of unsaturation. The IR spectrum of **1** revealed the presence of hydroxyl (3443 cm^{-1}), ester (1736 cm^{-1}), and olefinic (1630 cm^{-1}) functional groups.

The ^{1}H NMR spectrum of **1** measured in $CDCl_3$ showed typical singlet signals of six tertiary methyl groups [δ_H 0.84, 0.89, 0.89, 0.96, 1.60, 1.68 (3H each)], a secondary methyl group [δ_H 0.88 (3H, d, J = 7.0 Hz, H-21)], and a terminal methyl group [δ_H 0.97 (3H, t, J = 7.5 Hz, H-18′)]. The characteristic signals of two oxygenated methines [δ_H 4.20 (m, H-13′); 4.56 (dd, J = 11.0, 4.5 Hz, H-3)], seven olefinic protons [δ_H 5.10 (t, J = 6.0 Hz, H-24), 5.36 (td, J = 10.5, 8.0 Hz, H-15′), 5.43 (td, J = 10.5, 8.0 Hz, H-9′), 5.57 (td, J = 10.5, 7.5 Hz, H-16′), 5.69 (dd, J = 15.0, 6.5 Hz, H-12′), 5.97 (dd, J = 11.5, 10.5 Hz, H-10′), 6.51 (dd, J = 15.0, 11.5 Hz, H-11′)] were observed. In addition, the presence of a three-member ring was derived from the two methylene protons at δ_H 0.33 and 0.57 (each, 1H, br d, J = 4.0 Hz, H-19) in ^{1}H NMR spectrum.

Analysis of the ^{13}C NMR and HMQC spectra of **1** revealed forty-eight signals for eight methyl, twenty methylene, thirteen methine, and seven quaternary carbons. Moreover, the ^{13}C NMR spectrum contained signals corresponding to one carbonyl carbon [δ_C 173.8 (C-1′)], eight olefinic carbons (δ_C 123.9–135.3), and two oxygenated methine carbons [δ_C 72.3 (C-13′) and 80.5 (C-3)]. The ^{1}H and ^{13}C NMR spectroscopic data of **1** were indicative of an ester, which was composed of a cycloartane-*type* triterpenoid and an unsaturated fatty acid [6]. The complete assignment of all protons and carbons of **1** was conducted by analyses of the HMQC, HMBC, and ^{1}H–^{1}H COSY spectra (Table 1).

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data for compound **1** in $CDCl_3$ [δ (ppm), J (Hz)].

Position	δ_C	δ_H	Position	δ_C	δ_H
1	31.7	1.22*, 1.60*	25	131.0	–
2	27.0	1.61*, 1.74*	26	25.9	1.68 s
3	80.5	4.56 dd (11.0, 4.5)	27	17.8	1.60 s
4	39.6	–	28	25.6	0.84 s
5	47.3	1.42*	29	15.4	0.89 s
6	21.1	0.79*, 1.57*	30	19.4	0.89 s
7	26.0	1.08*, 1.31*	1′	173.8	–
8	48.0	1.52 dd (12.0, 4.5)	2′	35.0	2.30*
9	20.3	–	3′	25.2	1.61*
10	26.1	–	4′	29.1[a]	1.30*
11	26.6	1.11*, 1.98*	5′	29.2[a]	1.30*
12	33.0	1.61*	6′	29.6[a]	1.30*
13	45.4	–	7′	29.8	1.36*
14	48.9	–	8′	27.8	2.17 td (8.0, 7.0)
15	35.7	1.28*	9′	133.1	5.43 td (10.5, 8.0)
16	28.3	1.29*, 1.92*	10′	127.9	5.97 dd (11.5, 10.5)
17	52.4	1.58*	11′	126.0	6.51 dd (15.0, 11.5)
18	18.1	0.96 s	12′	135.2	5.69 dd (15.0, 6.5)
19	29.9	0.33 br d (4.0); 0.57 br d (4.0)	13′	72.3	4.19–4.23 m
20	36.0	1.38*	14′	35.4	2.33*
21	18.4	0.88 d (7.0)	15′	123.9	5.36 td (10.5, 8.0)
22	36.5	1.04*, 1.41*	16′	135.3	5.57 td (10.5, 7.5)
23	25.1	1.86*, 2.03*	17′	20.9	2.02–2.10 m
24	125.4	5.10 t (6.0)	18′	14.4	0.97 t (7.5)

Notes: Assignments were done by HMQC, HMBC, COSY, and NOESY experiments.
*Overlapped signals.
[a]The assignments may be exchanged.

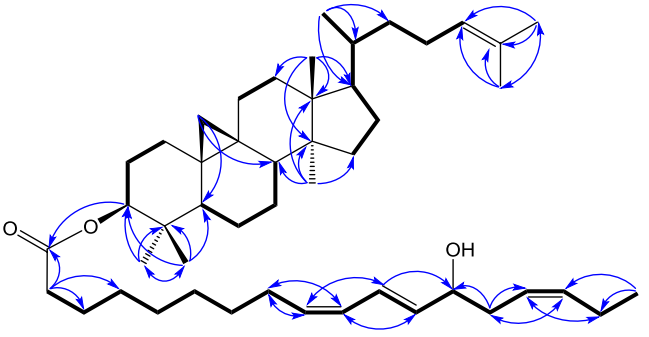

Figure 2. Key HMBC (^{1}H→^{13}C, arrows) and COSY (bold lines) correlations of compound **1**.

The signal of the oxygenated methine group at δ_C 80.5 was assigned to C-3 based on the HMBC correlations from H_3-28 (δ_H 0.84)/H_3-29 (δ_H 0.89) to C-3 (δ_C 80.5). Similarly, the HMBC correlations between H_3-26 (δ_H 1.68)/H_3-27 (δ_H 1.60) and C-24 (δ_C 125.4)/C-25 (δ_C 131.0) confirmed the double bond position at C-24/C-25 of the side chain. On the basis of the ^{1}H NMR and NOESY experiments, the relative stereochemistry of the triterpene moiety in **1** was determined as shown in Figure 2. The NOESY correlations between H-3 (δ_H 4.56) and H_3-28 (δ_H 0.84)/H-5 (δ_H 1.42), as well as the coupling constant values of 11.0 and 4.5 Hz between H-2 (δ_H 1.61 and 1.74) and H-3, suggested the α orientation of H-3 and H-5. Based on the previously mentioned evidence, the structure for the triterpene moiety of compound **1** was assigned to be cycloartenol (**2**) [7] which were coexistent in *A. paniculatum*.

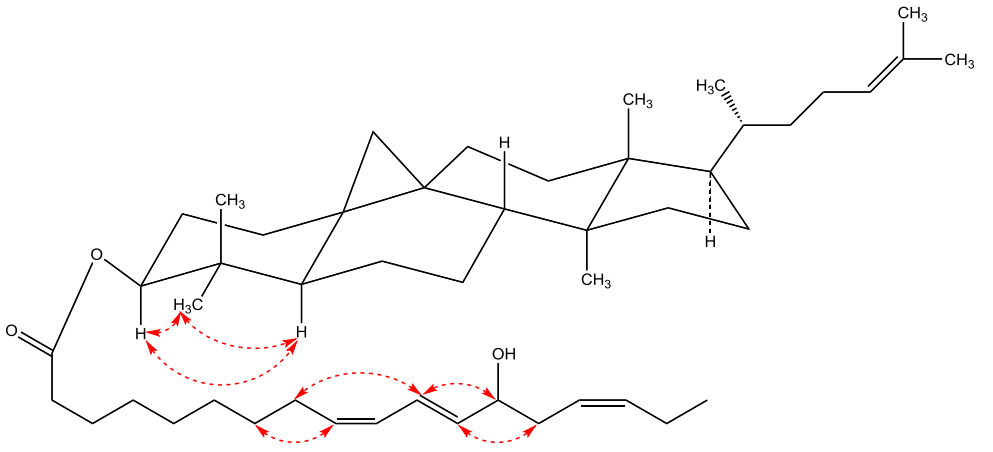

Figure 3. Key NOESY correlations (dash arrows) of compound **1**.

Detailed analyses of the ^{1}H-^{1}H COSY and HMBC correlations (Figure 2) led to the assignment of three double bonds at C-9′/C-10′, C-11′/C-12′, and C-15′/C-16′ as well as the hydroxyl group at C-13′ of fatty acid moiety. The vicinal coupling constant ($^{3}J_{H/H}$) of 10.5 Hz between H-9′ and H-10′ suggested the *Z* configuration of C-9′/C-10′ double bond. This was supported by the key correlation between H-8′ (δ_H 2.17) and H-11′ as well as the absence of cross-peaks of H-8′/H-10′ and H-9′/H-11′ in NOESY spectrum. Similarly, the large coupling constant of 15.0 Hz between H-11′ and H-12′, together with the NOESY correlation cross-peaks of H-10′/H-12′, H-11′/H-13′, and the lack of cross-peak of H-10′/H-13′, confirmed the *E* configuration of C-11′/C-12′ double bond. The stereochemical configuration of C-15′/C-16′ double bond was assigned to be *Z* based on the $^{3}J_{H\text{-}15'/H\text{-}16'}$ (10.5 Hz) as well as the lack of NOESY correlations between H-14′ (δ_H 2.33) and H-16′, between H-15′ and H-17′ (δ_H 2.06) [6,8] (Figure 3). The HMBC correlation from H-3 to C-1′ indicated the esterification had occurred at C-3. It accounted for a significant downfield shift of C-3 (δ_C 80.5) comparing to that of cycloartenol (δ_C 78.9) [7]. Due to the deficient amount of compound **1**, the absolute configuration of C-13′ was not established. Consequently, the chemical structure of **1** was elucidated to be 3β-*O*-(13-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoyl) cycloart-24-ene, named anopaniester.

The other compounds were identified as ursolic acid (**3**) [9], esculenic acid (**4**) [10], bis-(2-ethylhexyl) phthalate (**5**) [11], desmosterol (**6**) [12], stigmasterol (**7**) [13], vaniline (**8**) [14], and (*E*)-phytol (**9**) [15] (Figure 1). Their structures were established based on spectra which were agreed with previous studies. Among them, compounds **1**–**4** and **6** were isolated from this genus for the first time.

The cytotoxicity of the isolated compounds (**2**–**4**, **6**, and **9**) against the growth of LU-1 and MKN-7 cell lines was tested by a sulforhodamine B assay [16], and the results are described in Table 2. These data revealed that compounds **3** and **6** exhibited the moderate inhibitory effect against tested human cancer cell lines including LU-1 and MKN-7 with IC_{50} values ranging from 30.89 ± 3.60 to 44.37 ± 5.40 μg/ml respectively. Compounds **1, 5, 7,** and **8** were not tested on the cytotoxicity because the amount (4.5 mg) of compound **1** is no longer sufficient for testing activity, and other compounds are rather well known.

Table 2. Cytotoxicity of isolated compounds against human cancer cell lines.

	IC_{50} (μg/ml)	
Compounds	LU-1	MKN-7
Cycloartenol (**2**)	>100	>100
Ursolic acid (**3**)	44.37 ± 5.40	30.89 ± 3.60
Esculenic acid (**4**)	>100	>100
Desmosterol (**6**)	41.41 ± 2.31	38.06 ± 1.18
(*E*)-Phytol (**9**)	>100	>100
Ellipticine[#]	0.31 ± 0.07	0.39 ± 0.02

[#]Positive control.

Several previous studies indicated that ursolic acid (**3**) showed growth inhibitory activity against many human cancer cell lines such as HL-60 (leukemia cancer cell), BGC (gastric cancer cell), Bel-7402 (hepatocellular carcinoma cell), and Hela (cervical cell) [17], MGC-803 (gastric cancer cell) and Bcap-37 (breast cancer cell) [18]. In addition, desmosterol (**6**) showed significant cytotoxicity against NBT-T2 rat bladder epithelial cells [19] but was not cytotoxic to P-388 (mouse lymphocytic leukemia cell), KB (human nasopharyngeal carcinoma cell), A-549 (human lung carcinoma cell), and HT-29 (human colon carcinoma cell) [20].

Based on the obtained results, ursolic acid (**3**) and desmosterol (**6**) from the aerial part of *A. paniculatum* might be selected for further studies regarding their anticancer features. So, the data of ethnomedicine may be sometimes conceptional, by our experience the needles of *Pinus sylvestris* utilized against cancer in traditional medicine of Estonia [21] showed also cytotoxic effect to breast cancer cells [22]. Recently, it was shown by us [23] that *Sarcosperma affinis*, a plant from Vietnamese traditional medicine, really has biological activity.

3. Experimental

3.1. General experimental procedures

UV spectra were recorded on a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Infrared spectra were recorded on an IR Prestige-21 spectrometer. 1D and 2D NMR were carried out, using Bruker Avance 500 spectrometer (Bruker, Massachusetts, U.S.A.) with TMS as an internal reference. HRESIMS data were measured on a micrOTOF-Q 10187 mass spectrometer (Bruker, Massachusetts, U.S.A.). Column chromatography was performed using silica gel (60 N, spherical, neutral, 40–50 μm, Kanto Chemical Co., Inc., Tokyo, Japan), Cosmosil 75C18-OPN (Nacalai Tesque Inc., Kyoto, Japan), YMC RP-18 (Fuji Silysia Chemical Ltd, Kasugai, Aichi, Japan). Analytical TLC was performed on pre-coated silica gel $60F_{254}$ and RP-18 F_{254} plates (0.25 or 0.50 mm thickness, Merck KGaA, Darmstadt, Germany).

3.2. Plant material

The aerial parts of *Anodendron paniculatum* A.DC. were collected from Quang Tri province, Vietnam (17 °03′21.4″ N 107 °04′24.4″ E), in June 2014 and were identified by Dr.Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST, Vietnam. A voucher specimen (AV03) was deposited at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam.

3.3. Extraction and isolation

The dried aerial parts of *A. paniculatum* (2.5 kg) were extracted with MeOH (3 times, 10.0 L each) at room temperature to yield 105 g of a dark solid extract. This was then suspended in water and successively partitioned with chloroform ($CHCl_3$) and ethyl acetate (EtOAc) (3 times, 2.0 L each) to obtain the $CHCl_3$ (AC, 50.7 g), the EtOAc (AE, 10.2 g), respectively, and the water (AW, 27.5 g) layers after removal of the solvents *in vacuo*.

The AC extract was chromatographed on a silica gel column and eluted with the gradient of $CHCl_3$-MeOH solvent systems (100:0→0:100, v/v) to obtain eight fractions, AC1-AC8. The fraction AC2 (8.3 g) was applied on a silica gel column, using *n*-hexane–acetone gradient system (100:0→4:1) to obtain six sub-fractions, AC2.1-AC2.6. The fraction AC2.2 (1.2 g) was subjected to a silica gel column using *n*-hexane–EtOAc (10:1, v/v) as an eluent to yield compounds **1** (4.5 mg), **5** (60.6 mg), and **9** (100.5 mg). The fraction AC2.3 (0.75 g) was chromatographed on an YMC RP-18 column eluted with MeOH-acetone–water (10:5:1, v/v) to afford compounds **2** (7.5 mg), **6** (45.7 mg), and **7** (30.0 mg). The fraction AC2.4 (1.32 g) was chromatographed on a silica gel column eluted with *n*-hexane–acetone (5:1, v/v) to give compounds **3** (156.5 mg) and **8** (15.8 mg). Compound **4** (27.0 mg) was obtained from fraction AC2.5 (0.64 g) by a silica gel column using $CHCl_3$–MeOH (20:1, v/v) as a mobile phase.

3.3.1. Anopaniester (1)

Colorless oil; IR (KBr) ν_{max} (cm^{-1}): 3443, 2922, 2853, 1736, 1630, 1466, 1377, 1260, 1180, 1094; UV (MeOH) λ_{max} (nm): 201, 231; ^1H and ^{13}C NMR spectral data see Table 1; HRESIMS: *m/z* 725.5805 $[M + Na]^+$ (calcd for $C_{48}H_{78}O_3Na$, 725.5849).

3.4 SRB assay for evaluating cytotoxic activity

The cytotoxic activity of isolated compounds from the aerial parts of *A. paniculatum* was tested by sulforhodamine B assay against the growth of two monolayer human cancer cell lines, including LU-1 and MKN-7. Stock cultures were grown in T-75 flasks containing 50 ml of Dulbecco's modified eagle medium (DMEM) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum (FBS). Media were changed at 48-h intervals. The cells were dissociated with 0.05% Trypsin-EDTA, sub-cultured every 3–5 days with the ratio of (1:3), and incubated at 37 °C under humidified 5% carbon dioxide atmosphere. Tumor cells were cultivated in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h. Cell viability was examined by sulforhodamine B (SRB) method for cell density determination, based on the measurement of cellular protein content [16]. Viable cells were seeded overnight into 96-well microplates at a density of 4×10^4 cells/well in growth medium (180 µl/well). Tested samples were added carefully into each well of 96-well plates and the cultivation was continued under the same conditions for another 72 h. Thereafter, the medium was removed and the remaining cell monolayers are fixed with the cold 20% (w/v) trichloroacetic acid for 1 h at 4 °C and stained by 1X SRB staining solution at room temperature for 30 min, after which the unbound dye was removed by washing repeatedly with 1% (v/v) acetic acid. The protein-bound dye is dissolved in 10 mM Tris base solution for optical density determination at 515 nm on an ELISA Plate Reader (Bio-Rad). DMSO 10% was used as blank sample while ellipticine was used as positive control. The cytotoxicity was measured at doses of 100, 20,

4, and 0.8 μg/ml and estimated as a half maximal inhibitory concentration (IC_{50}), which was calculated by the program TableCurve Version 4.0. All experiments were prepared in triplicates. The inhibition rate (IR) of cells was calculated by the following formula IR% = {100% − [(absorbance$_t$ − absorbance$_0$)/(absorbance$_c$ − absorbance$_0$)] × 100}, in which: IR: Inhibition rate of cell growth, absorbance$_t$: average optical density value at day 3; absorbance$_0$: average optical density value at time-zero; absorbance$_c$: average optical density value of the blank DMSO control sample.

Disclosure statement

The authors declare no conflict of interest.

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