Chemical Constituents and Biological Activity of the Stems of

Adinandra hainanensis Hayata

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Abstract: Phytochemical study of *Adinandra hainanensis* stems led to the isolation of a new triterpene lupan-3β,20-dihydroxy-28-carbaldehyde (**1**) and eleven known compounds **2**-**12**. The stems of *A. hainanensis* was rich in lupane-type triterpenes, especially betulinic acid (**9**) was abundant (around 0.45% w/w). The isolated triterpenes, including new compound **1** showed strong α-glucosidase inhibitory activity with IC_{50} values ranging of 2.27 ± 0.05 μg/mL to 12.25 ± 0.36 μg/mL. In the cytotoxic evaluation, betulinic acid, the major component, showed good cytotoxic activity against all tested cancer cell lines while new compound **1** was weakly active.

1. Introduction

The genus *Adinandra* comprises over 100 species distributed in South East Asian countries, China, southern Japan, Bangladesh, India and African tropical forests. *Adinandra* genus has been previously classified in the Theaceae family, and the current botanical databases classified it as a member of the Pentaphylacaceae [1-2]. Plants of the genus have been used in Vietnamese traditional medicine for the treatment of snake bites and stomachaches [3]. In China, tea (Shiyacha) prepared from leaves of *Adinandra nitida* was reported to have many pharmacological activities such as reducing blood pressure, antioxidant, antibacterial and anti-inflammatory effects [4-5]. Several other *Adinandra* plants including *A. milletti*, *A. latifolia* and *A. jubata* were reported to have antioxidant and antiproliferation activities [6]. Phytochemical studies on *Adinandra nitida* leaves revealed the presence of triterpene saponins and flavonoids with camellianin A, which was found as the main compound [7-9]. However, so far, there is no report on the chemical composition of different *Adinandra* species.

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Plant *Adinandra hainanensis* Hayata (synonym: *A. rubropunctata* Merr. & Chun) was a species found in northern mountains of Vietnam. This plant was used in folk medicine for the treatment of throat cancer [3]. In the present study, we reported the isolation and identification of a new triterpene (**1**) together with eleven known compounds **2-12** from the *A. hainanensis* stems. Several isolated components were evaluated for cytotoxicity and α-glucosidase enzyme inhibitory activity.

2. Materials and Methods

2.1. Plant Materials

The plant stems were collected in Van Ban, Laocai province, Vietnam in 2008 and identified as *Adinandra hainanensis* Hayata (Pentaphylacaceae family) by Dr. Nguyen Quoc Binh, Vietnam Museum of Nature, VAST and Dr. Bui Thu Ha, Faculty of Biology, Hanoi National University of Education. A voucher specimen (VN-1968) was deposited at the Institute of Marine Biochemistry, Vietnam Academy of Science and Technology.

2.2. General Experimental Procedures

Optical rotation was recorded on JASCO P-2000 polarimeter. IR spectra were obtained on a Bruker TENSOR 37 FT-IR spectrometer. The NMR experiments were recorded on a Bruker AM500 FT-NMR spectrometer with tetramethylsilane (TMS) was used as an internal standard. High-resolution mass spectra (HR-ESI-MS) were obtained with a Thermo LTQ Orbitrap XL mass spectrometer. Open column chromatography (CC) was performed on silica gel 60 (70-230 mesh, Merck) or Sephadex LH-20® (Sigma-Aldrich) Thin-layer chromatography (TLC) was performed on DC-Alufolien silica gel 60 F_{254} plates (Merck, Germany). Spots were visualized under UV lamp at 254 nm or spraying with H_2SO_4 10% reagents, followed by heating.

2.3. Extraction and Isolation

The stems of *A. hainanensis* were dried and ground into powder. The plant material (3.3 kg) was macerated with MeOH (10L x 4 times x 24 h) at room temperature. The combined filtrate was evaporated under reduced pressure to obtain 157 g crude extract. It was suspended in distilled water, then successively partitioned with *n*-hexane and EtOAc (each solvent, 3 times x 1 L/time extraction). The organic solvent layers were separated and evaporated *in vacuo* to obtain *n*-hexane residue (6.9 g) and EtOAc residue (41 g), respectively. The *n*-hexane residue was subjected to open normal-phase silica gel CC (4 cm size) and eluted with gradient solvent mixture of *n*-hexane/EtOAc (100:1 – 0:1) to result in 23 fractions (H1 – H23). Fraction H3 (51 mg) was purified by silica gel CC (1 cm size) using *n*-hexane/CH₂Cl₂ (3/7, v/v) as eluent to yield **2** (8.3 mg). Compound **3** (27 mg) was obtained from H9 fraction (0.167 g) by crystallization in acetone. Fraction H12 (601 mg) was fractionated to 4 subfractions H12A-H12D by open silica gel CC (2.5 cm size), eluting with *n*-hexane/EtOAc (85/15, v/v). Subfraction H12B (110 mg) was purified by silica gel CC (1.5 cm size) and eluted with *n*-hexane/EtOAc (85/15, v/v) to afford **4** (6.5 mg). Fraction H16 (199 mg) was separated by silica gel CC (2.0 cm size), eluted with *n*-hexane/EtOAc (8/2, v/v) to give 3 subfractions H16A–H16C. Subfraction H16B (45 mg) was further purified by open Sephadex LH-20 CC (1.5 cm size), eluted with MeOH/CH₂Cl₂ (9: 1) to yield **5** (3.4 mg). Subfraction H16C (121 mg) was purified by Sephadex LH-20 CC (2 cm size), eluted with MeOH/CH₂Cl₂ (9: 1) to afford compound **6** (6 mg). Compound **7** (19 mg) was obtained from H19 fraction (205 mg) by crystallization in acetone. Fraction H22 (171 mg) was fractionated to 2 fractions H22A–H22B by Sephadex LH-20 CC (2 cm size), eluted with MeOH/CH₂Cl₂ (9/1, v/v). The fraction H22B (86 mg) was purified in open silica gel CC (1.5 cm size), eluted with *n*-hexane/EtOAc (7/3, v/v) to afford **8** (7.5 mg). The H23 (389 mg) was separated by Sephadex LH-20 CC (2.5 cm size), eluted with MeOH/CH₂Cl₂ (9:1, v/v) to give 4 subfractions H23A – H23D. Subfraction H23B (166.3 mg) was purified on silica gel CC (2 cm size) using CH₂Cl₂/acetone (9/1, v/v) to afford compound **1** (6.9 mg). Compound **9** (15 g) was obtained from the EtOAc residue (41 g) by crystallization in acetone. The remained EtOAc residue (25 g) was subjected to silica gel CC (5 cm size)

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and eluted with gradient solvent mixture of CH_2Cl_2/MeOH (95/5, v/v) to result in 8 fractions (E1 – E8). Fraction E3 (250 mg) was purified by silica gel CC (2 cm size), eluted with *n*-hexane/EtOAc (7/3, v/v) to yield **10** (7.3 mg). Fraction E5 (2.1 g) was fractionated to 4 subfractions E5A – E5D by silica gel CC (3 cm size), eluted with *n*-hexane/EtOAc (1/1, v/v). Fraction E5C (450 mg) was purified by silica gel CC (2.5 cm size), eluted with CH_2Cl_2/acetone (8/2, v/v) to yield **11** (4.1 mg) and **12** (8.5 mg).

Lupan-3β,20-dihydroxy-28-carbaldehyde (1): White amorphous powder, $[\alpha]_D^{25}$ +23.5 (c 2.2, $CHCl_3$); IR v_{max} (KBr) 3354, 2942, 1726, 1451, 1383 cm^{-1}; HR-ESI-MS: *m/z* 493.3425 [M+Cl]$^-$ (calcd. for $C_{30}H_{50}O_3Cl$ 493.3448). ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) see the Table 1.
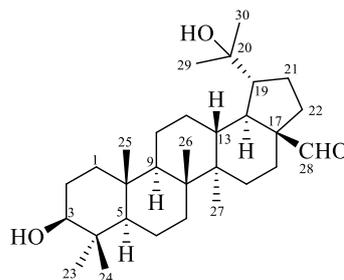
Figure 1. The structure of the new compound **1**

2.4. Cytotoxicity Assay

The cytotoxicity assays were carried out in triplicate against KB, MCF-7, and HepG-2 and LU cancer cell lines (ATCC) using a MTT method, that was described in previous study [10]. Ellipticine was used as a reference compound.

2.5. α-Glucosidase Enzyme Inhibition Evaluation

The assay for *in vitro* α-glucosidase enzyme (from *Saccharomyces cerevisiae*, G0660, Sigma-Aldrich) inhibition activity was performed in triplicate following the previously published method [11]. Briefly, 2 µL of sample solution prepared in DMSO and 40 µL of enzyme solution (0.5 unit/mL) in 120 mL of 0.1 M phosphate buffer (pH 7.0) were preincubated at 37 °C for 5 min. Then, 40 µL of 5 mM *p*-nitrophenyl-*β*-D-glucopyranoside (pNPG) solution prepared in phosphate buffer was added and incubated at 37 °C for another 30 min. The reaction was stopped by adding 80 µL of 0.2 M Na_2CO_3. The absorbance of the resulting mixture was recorded at 410 nm using a micro-plate reader. The α-glucosidase activity was determined by measuring the *p*-nitrophenol released from pNPG. Acarbose was used as the positive control.

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was isolated as a white amorphous powder with the molecular formula $C_{30}H_{50}O_3$, which was established from the negative HR-ESI-MS spectrum with the ion peak *m/z* 493.3425 [M+Cl]$^-$. Its IR spectrum showed absorption bands at 3354 and 1726 cm^{-1} due to hydroxy and carbonyl functions, respectively. The ^{1}H NMR spectrum revealed signals of a triterpene with an aldehyde group at δ_H 9.60 (1H, s, H-28), an oxymethine proton at δ_H 3.19 (1H, dd, *J* = 4.5; 11.5 Hz, H-3) and seven tertiary methyl groups at δ_H 1.25 (3H, H-29), 1.14 (3H, H-30), 0.99 (3H, H-27), 0.96 (3H, H-23), 0.90 (3H, H-25), 0.82 (3H, H-26) and 0.75 (3H, H-24). The ^{13}C NMR and DEPT spectra indicated the presence of 30 carbon signals including seven methines [among that a carbaldehyde was observed at δ_C 206.8 ppm and an oxymethine carbon was found at δ_C 79.0 (C-3)], ten methylenes, seven methyl groups, five quaternary carbons and a tertiary alcohol carbon at δ_C 73.3 (C-20). Therefore, the structure of compound **1** was suggested as a

lupane-type triterpene with a hydroxy group at C-20 position. This is further confirmed by HMBC correlations of H-29 (δ_H 1.25) and H-30 (δ_H 1.14) to C-20 (δ_C 73.3) (Figure 2). The coupling constants of H-3 ($J = 4.5$; 11.5 Hz) suggested that the hydroxy group was at β position. Moreover, the cross-peaks of H-3 (δ_H 3.19) and H-5 (δ_H 0.67), H-3 and H-23 (δ_H 0.96) were observed in the NOESY spectrum (Figure 2). On the basis of the above spectral evidence, **1** is determined as lupan-3β,20-dihydroxy-28-carbaldehyde, a new natural compound.

Table 1. ^{1}H and ^{13}C-NMR data of new compound **1** (^{a} 500 MHz, ^{b}125MHz)

C	^{1}H^a	^{13}C^b	DEPT
1	1.70 (m, 1H) 0.88 (m, 1H)	38.7	CH$_2$
2	1.59 (m, 2H)	27.3	CH$_2$
3	3.19 (1H, dd, $J = 4.5$ Hz; 11.5 Hz)	79.0	CH
4	-	38.8	C
5	0.67 (br d, $J = 11.0$ Hz)	55.2	CH
6	1.51 (m, 1H) 1.37 (m, 1H)	18.2	CH$_2$
7	1.37 (m, 2H)	34.6	CH$_2$
8	-	41.2	C
9	1.25 (m, 1H)	50.3	CH
10	-	37.1	C
11	1.48 (m, 1H) 1.25 (m, 1H)	21.2	CH$_2$
12	1.68 (m, 1H) 1.45 (m, 1H)	28.1	CH$_2$
13	2.04 (dt, $J = 3.5$ Hz; 11.5 Hz, 1H)	38.2	CH
14	-	43.5	C
15	1.79 (m, 1H) 1.26 (m, 1H)	29.2	CH$_2$
16	2.13 (m, 1H) 1.44 (m, 1H)	29.5	CH$_2$
17	-	61.5	C
18	1.70 (m, 1H)	46.9	CH
19	2.18 (m, 1H)	49.4	CH
20	-	73.3	C
21	1.44 (m, 1H) 1.18 (m, 1H)	29.0	CH$_2$
22	1.60 (m, 1H) 1.29 (m, 1H)	33.0	CH$_2$
23	0.96 (s)	27.9	CH$_3$
24	0.75 (s)	15.3	CH$_3$
25	0.90 (s)	16.1	CH$_3$
26	0.82 (s)	16.1	CH$_3$
27	0.99 (s)	14.5	CH$_3$
28	9.60 (1H, s)	206.7	CH
29	1.25 (s)	31.2	CH$_3$
30	1.14 (s)	25.0	CH$_3$

Known compounds including stigmas-3-one (**2**) [12], lupeol (**3**) [13], betulinal (**4**) [14], acetyl ursolic acid (**5**) [15], vanillin (**6**), betulin (**7**) [16], ursolic acid (**8**) [17], betulinic acid (**9**) [16], scopoletin (**10**) [18], 3,4-dihydroxybenzaldehyde (**11**) and 4-hydroxybenzoic acid (**12**) were elucidated by comparing their NMR data to the previous literature (Figure S1). All these compounds were reported for the first time from *Adinandra* genus. Our phytochemical study showed that *A. hainanensis* is rich in lupane-type triterpenes, among which betulinic acid is the most abundant one (around 0.45% w/w). Notably, the chemical composition of *A. hainanensis* in Vietnam is quite different from Chinese *A. nitida* plant with flavonoid camellinin A as the major component [4].

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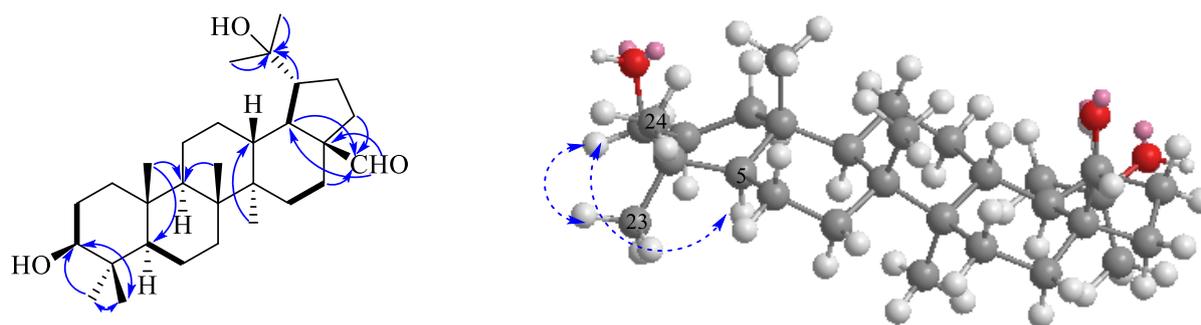
Figure 2. Key COSY (──), HMBC (──▶) and NOESY (◀--▶) correlations of the compound **1**

3.2. Biological Activity

Several isolated compounds were tested for cytotoxicity and α-glucosidase enzyme inhibitory activity. As shown in table 2, all the tested compounds displayed stronger activity than reference compound acarbose (IC_{50} value of 184.0 ± 3.01 µg/mL) in enzyme inhibition assay. New compound **1** also showed good enzyme inhibitory activity with IC_{50} value of 12.25 ± 0.36 µg/mL. The triterpenes **3-5**, **7** and **9** showed strong α-glucosidase inhibitory activities with IC_{50} values ranging from 2.27 ± 0.05 µg/mL to 4.56 ± 0.20 µg/mL while ursolic acid (**8**) exhibited moderate activity. Scopoletin (**10**) had weak α-glucosidase inhibitory activity with IC_{50} of 86.24 ± 1.28 µg/mL.

Table 2. Cytotoxicity and α-glucosidase inhibitory activity of isolated compounds.

Compounds	IC_{50} (µg/mL)				
	KB	Hep-G2	LU	MCF-7	α-glucosidase inhibitory activity
1	76.78 ± 2.12	79.49 ± 2.57	85.61 ± 3.13	>128	12.25 ± 0.36
3	98.16 ± 4.25	88.35 ± 3.43	79.77 ± 2.88	99.37 ± 4.63	3.67 ± 0.10
4	77.53 ± 1.20	50.07 ± 1.12	86.16 ± 2.98	85.63 ± 2.89	2.27 ± 0.05
5	84.53 ± 2.24	72.25 ± 1.57	>128	>128	2.78 ± 0.09
7	63.36 ± 0.88	59.29 ± 0.99	69.43 ± 1.98	70.57 ± 3.63	2.96 ± 0.06
8	>128	>128	>128	>128	30.76 ± 0.79
9	21.71 ± 1.20	8.01 ± 0.51	8.00 ± 0.55	26.54 ± 1.54	4.56 ± 0.20
10	>128	32.00 ± 0.55	>128	43.63 ± 0.90	86.24 ± 1.28
Ellipticine	0.30 ± 0.05	0.33 ± 0.05	0.45 ± 0.05	0.46 ± 0.03	-
Acarbose	-	-	-	-	184.0 ± 3.01

In the cytotoxic assay against different cancer cell lines, compound **1** showed weak cytotoxicity against KB, HepG-2, and LU cancer cell lines with IC_{50} values of 76.78 ± 2.12 µg/mL, 79.49 ± 2.57 µg/mL and 85.61 ± 3.13 µg/mL, respectively. Scopoletin (**10**) displayed selective activity against HepG-2 and MCF-7 cell lines over KB and LU (Table 2). Betulinic acid that was found in high abundance in the stems showed good cytotoxicity to all tested cell lines while other isolates were weakly active or inactive. The anticancer activity of betulinic acid (**9**) has been well reported [16, 19-20]. Our chemical and biological results might give an explanation to the traditional use of *A. hainanensis* for the treatment of throat cancer in Vietnam.

In summary, a new compound, namely lupan-3β,20-dihydroxy-28-carbaldehyde (**1**) together with eleven known compounds were isolated from the stems of *A. hainanensis*. Among them, betulinic acid was isolated as major component. New compound **1** showed good α-glucosidase inhibitory activity with IC_{50} value of 12.25 ± 0.36 µg/mL and the triterpenes **3-5**, **7** and **9** strongly inhibited enzyme activity. In the cytotoxic assay, betulinic acid (**9**) played good cytotoxic activity against all tested cancer cell lines while new compound **1** showed weak cytotoxicity against KB, HepG-2, and LU cancer cell lines.

Acknowledgments

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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