TRITERPENOID AND STEROL COMPOUNDS ISOLATED FROM ANODENDRON PANICULATUM (ROXB.) A. DC.

Hoang Thi Nhu Hanh¹, Vu Duc Canh², Hoang Thi Dieu Huong³, Ho Viet Duc⁴, Nguyen Thi Hoai ¹,*

¹ HU – University of Sciences, 77 Nguyen Hue St., Hue, Vietnam
² Agency of Drug Administration – Ministry of Health, 138A Giang Vo St., Ha Noi, Vietnam
³ National Institute of Medicinal Materials, 3B Quang Trung St., Ha Noi, Vietnam
* HU – University of Medicine and Pharmacy, 6 Ngo Quyen St., Hue, Vietnam

Abstract: This phytochemical study on the aerial parts of Anodendron paniculatum led to the isolation of three triterpenes, namely ursolic acid (1), esculentic acid (2), cycloartenol (3), and one sterol: desmosterol (4). Their chemical structures were elucidated on the basis of spectroscopic analyses. The cytotoxicity of the isolated compounds against the growth of human cancer cell lines was evaluated using the sulforhodamine B bioassay. Compounds 1 exhibited a moderate cytotoxicity against the LU–1 and MKN–7 cell lines with IC₅₀ values of 44.37 ± 5.40 and 30.89 ± 3.60 μg/mL, respectively. Meanwhile, compound 4 possessed moderate cytotoxic effects toward the LU–1, KB, Hep–G2, MKN–7 and SW–480 cell lines with IC₅₀ values ranging from 28.11 ± 1.95 to 41.41 ± 2.31 μg/mL.

Keywords: Anodendron paniculatum, ursolic acid, esculentic acid, cycloartenol, desmosterol, cytotoxicity

1 Introduction

Anodendron paniculatum (Roxb.) A. DC. is the climbing species of the Apocynaceae family and broadly distributed in Sri Lanka, India, Bangladesh, Burma, and Southeast Asia [1]. The roots of this plant have been used in traditional folk medicine as a remedy for vomiting and cough in India [2]. In addition, its latex is used to cure snake poisoning and centipede bites [3]. Several cardenolides have been isolated from A. paniculatum in the previous chemical studies [4, 5]. However, the knowledge about the chemical composition of this plant is rather limited. Previously, two flavonoid glycosides (kaempferol-3-O-rutinoside and rutin), and one phenylpropanoid glycoside (sargentol) were isolated from the aerial parts of A. paniculatum by Hanh et al. [6]. In our ongoing search for bioactive components from Vietnamese natural resources, three triterpenoids, namely ursolic acid (1), esculentic acid (2), and cycloartenol (3), and one sterol: desmosterol (4) were isolated. In this paper, we present the isolation, structure elucidation, and cytotoxic activities of these compounds.

* Corresponding: hoai77@gmail.com

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2 Material and methods

2.1 Plant material

The aerial parts of *A. paniculatum* (Roxb.) A. DC. were collected in the Dakrong district, Quang Tri province 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, University of Medicine and Pharmacy, Hue University.

2.2 General experiment procedures

Thin layer chromatography (TLC) was carried out on pre-coated silica gel DC-Alufolien 60 F254 (Merck) or RP18 F254 (Merck) plates. The compounds were detected under UV 254 nm and/or by spraying with 10% H2SO4 reagent, followed by heating for 1–2 min. Column chromatography was performed using silica gel (240–430 mesh, Merck, Germany) or YMC RP18 resin (ODS–60–14/63, Fujisilisa, Japan).

Melting points (mp) were determined on a Buchi Melting Point B–545 apparatus (Sigma–Aldrich, Missouri, USA). Infrared spectra were recorded on an IR Prestige–21 spectrometer with KBr discs (Shimadzu, Kyoto, Japan). Electrospray ionization mass spectrometry (ESIMS) was studied on an Agilent 6310 Ion Trap and Shimadzu LCMS–IT–TOF mass spectrometers. Nuclear magnetic resonance (NMR) spectra were measured on a Bruker AM500 FT–NMR spectrometer using tetramethylsilane as internal standard.

2.3 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 extract was then suspended in water and successively partitioned with chloroform (CHCl3) and ethyl acetate (EtOAc) (3 times, 2.0 L each) to obtain the CHCl3 fraction (AC, 50.7 g), the EtOAc fraction (AE, 10.2 g), respectively, and the water layer (AW, 27.5 g) after removing the solvents in vacuum.

The AC extract was chromatographed on a silica-gel column and eluted with the gradient of CHCl3–MeOH solvent systems (100:0 → 0:100, v/v) to obtain eight fractions, AC1–AC8. 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. Sub-fraction AC2.3 (0.75 g) was chromatographed on a YMC RP–18 column eluted with MeOH–acetone–water (10:5:1, v/v) to obtain compounds 3 (7.5 mg) and 4 (45.7 mg). Sub-fraction AC2.4 (1.32 g) was chromatographed on a silica gel column eluted with n-hexane–acetone (5:1, v/v) to give compound 1 (156.5 mg). Compound 2 (27.0 mg) was obtained from sub-fraction AC2.5 (0.64 g) in a silica gel column using CHCl3–MeOH (20:1, v/v) as mobile phase.

2.4 Sulforhodamine B assay for evaluating cytotoxic activity

The cytotoxic activity of isolated compounds was tested using the sulforhodamine B assay against the growth of five monolayer human cancer cell lines, namely LU–1 (lung adenocarcinoma), KB...
(epidermoid carcinoma), HepG2 (hepatoma cancer), MKN–7 (stomach cancer), and SW–480 (colon adenocarcinoma). 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-hour 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 the humidified atmosphere with 5 % carbon dioxide. Tumor cells were cultivated in the humidified atmosphere with 5 % CO₂ at 37 °C for 48 hours. Cell viability was examined with the sulforhodamine B (SRB) method for cell density determination, based on the measurement of cellular protein content [7].

Viable cells were seeded in the growth medium (180 µL) into 96-well microplates (4 × 10⁴ cells per well) and allowed to attach overnight. The tested samples were added carefully into each well of the 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 cold 20 % (w/v) trichloroacetic acid for 1 h at 4 °C and stained using 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 was dissolved in a 10-mM Tris base solution for optical density determination at 515 nm on an ELISA Plate Reader (Bio–Rad). Dimethylsulfoxide (DMSO) 10 % was used as a blank sample while ellipticine was used as a positive control. The cytotoxicity was measured at doses of 100 µg/mL, 20 µg/mL, 4 µg/mL, and 0.8 µg/mL and estimated as a half maximal inhibitory concentration (IC₅₀), which was calculated using the program TableCurve Version 4.0. All experiments were performed in triplicates. The inhibition rate (IR) of cells was calculated according to the following formula:

\[
IR \% = \frac{100 \% - [(A_t - A_0)/(A_c - A_0)]}{100}
\]

Where,

Aₜ is the average optical density value at day 3

A₀ is the average optical density value at time-zero

Aₖ is the average optical density value of the blank DMSO control sample

3 Results and discussion

Compound 1 was obtained as a white powder, mp 284–286 °C. The ESIMS analysis of compound 1 showed ion peaks at m/z 479 [M+Na]⁺ and 455 [M–H]⁻. In combination with ¹³C–NMR and DEPT spectra, the molecular formula of 1 was deduced to be C₃₀H₄₈O₃ (M = 456 g/mol). The IR spectrum suggested the presence of the hydroxy (3433 cm⁻¹) and carbonyl (1690 cm⁻¹) groups, and the double bond (1458 cm⁻¹).

The ¹H–NMR spectrum of 1 exhibited signals of five tertiary methyl groups at δ_H 0.78, 0.81, 0.93, 0.98, and 1.09, and two secondary methyl groups at δ_H 0.86 (d, J = 6.5 Hz) and 0.95 (d, J = 6.0 Hz). Furthermore, the typical signals of one carbinol proton at δ_H 3.20 (dd, J = 10.0, 6.0 Hz) and one olefinic proton at δ_H 5.24 (br s) were observed. The α-axial orientation of H–3 was deduced from the coupling constant values of 10.0 and 6.0 Hz between H–2 and H–3.
The $^{13}$C–NMR and DEPT spectra showed the signals of thirty carbons belonging to seven methyl, nine methylene, seven methine, and seven quaternary carbons. Among those, the signal at $\delta$ 180.8 (C–28) was assigned to the carbonyl carbon. The signals of two olefinic carbons at $\delta$ 138.3 (C–13) and 125.6 (C–12), and one oxygenated carbon at $\delta$ 79.0 (C–3) confirmed the presence of one tri-substituted double bond and one carbinol group, respectively. On the basis of the aforementioned observations, compound 1 was determined to be 3β-hydroxy-12-ursen-28-oic acid (known as ursolic acid) (Fig. 1) [8].

![Chemical structure of isolated compounds 1–4](image)

**Fig. 1.** Chemical structure of isolated compounds 1–4

Compound 2 was isolated as a white powder, mp 267–268 °C. Its molecular formula was determined to be C$_{30}$H$_{46}$O$_5$ using HRESIMS at $m/z$ 511.3390 [M + Na]$^+$ in conjunction with NMR data analysis, corresponding to seven degrees of unsaturation. The IR spectrum of 2 revealed strong absorption bands for the hydroxy (3425 cm$^{-1}$) and carbonyl (1690 cm$^{-1}$) groups, and double bond (1458 cm$^{-1}$). The $^1$H–NMR spectrum of 2 showed characteristic signals of one olefinic proton [$\delta$H 5.47 (H–12)], two carbinol groups [$\delta$H 4.28 (br d, $J$ = 11.0 Hz, H–2), 4.16 (br s, H–3)], and one oxymethylene group [$\delta$H 3.76 (d, $J$ = 10.5 Hz, H–23a) and 3.93 (d, $J$ = 10.5 Hz, H–23b)]. Furthermore, the signals of four tertiary methyl (δH 0.87, 1.00, 1.07, 1.14) and two secondary methyl groups (δH 0.93, 0.96) were observed (Table 1).

The analysis of the $^{13}$C–NMR and HMOC spectra of 2 revealed thirty signals, namely six methyl, nine methylene, eight methine, and seven quaternary carbons. The $^{13}$C–NMR spectrum also showed characteristic signals of one carboxyl carbon at $\delta$C 180.5 (C–28), two olefinic carbons at $\delta$C 126.0 (C–12) and 139.8 (C–13), and three oxygenated sp$^3$ carbons at $\delta$C 66.7 (C–2), 79.3 (C–3), and 71.7 (C–23). The $^1$H– and $^{13}$C–NMR spectroscopic data of 2 were indicative of an ursenoic acid. The planar structure of 2 was similar to that of 1, except for the addition of two hydroxy groups at C–2 and C–23. All proton and carbon signals of 2 were assigned using 2D–NMR spectra, including HMOC, HMBC, and $^1$H–$^1$H COSY (Fig. 2).

The HMBC correlations of H–18 (δH 2.61) and H–27 (δH 1.14) to C–13, of H–12 (δH 5.47) to C–14 (δC 43.1) and C–18 (δC 54.0) confirmed the double bond position at Δ–12. In addition, the attachment of a carboxylic group at C–17 was confirmed by the HMBC correlations from H–16 (δH 1.96, 2.08)/H–22 (δH 1.98)/H–18 to C–28. The position of the hydroxy groups at C–3 and C–23 was assigned based on the HMBC correlations of H–23 (δH 3.76, 3.93)/H–24 (δH 0.87) and C–3/C–4 (δC 42.4)/C–5 (δC 44.0). The remaining hydroxy group was located at C–2 by using the COSY correlation between H–2 and H–3.
The stereochemistry of 2 was determined on the basis of the NOESY experiment (Fig. 3). The NOESY correlations between H–2 and H–24/H–25 confirmed the β-axial orientation of H–2, Me–24, and Me–25 in the chair conformation of the A ring. In a similar fashion, H–3 was shown to correlate with H–24 suggesting the β-equatorial configuration of H–3. The cross-peak of H–18 and H–29 in the NOESY spectrum and the lack of correlation between H–18 and H–27 revealed the β-configuration of H–18 [9]. Thus, compound 2 was identified to be 2α,3α,23-trihydroxyurs-12-en-28-oic acid and was given a trivial name as esculentic acid [10].

![Fig. 2. Key HMBC and COSY correlations of compounds 2, 4](image1)

![Fig. 3. Key NOESY correlations of compound 2](image2)

Compound 3 was collected as a white amorphous powder. The strong absorption bands at ν 3448 cm⁻¹ and 1635 cm⁻¹ in the IR spectrum of 3 suggesting the existence of the hydroxy group and double bond, respectively. The ¹H–NMR spectrum showed typical signals of one olefinic proton (δH 5.10), one oxymethine group (δH 3.38), and seven methyl groups [δH 0.81 (3H, s, H–29), 0.88 (3H, d, J = 6.5 Hz, H–21), 0.89 (3H, s, H–30), 0.96 (6H, s, H–18 & H–28), 1.60 (3H, s, H–27) and 1.68 (3H, s, H–26)]. In addition, the presence of a three-member ring was derived from two methylene protons at δH 0.33 and 0.55 (each, 1H, br d, J = 3.5 Hz, H–19). The ¹³C–NMR and HMOC spectra revealed that compound 3 possessed cycloartane-type skeleton consisting of seven methyl, eleven methylene, six methine, and six quaternary carbons. The ¹³C–NMR data also supported the presence of one tri-substituted double bond [δC 125.4 (C–24), 131.1 (C–25)], and one oxygenated methine carbon [δC 79.0]. Remarkably, the orientation of H–3 was determined to be α on the basis of comparison of the chemical shifts of C–3 [δC 79.0], C–5 (δC 47.3) with those in two diastereoisomers [3α–OH: δC ~ 77 (C–3), ~ 41 (C–5), 3β–OH: δC ~ 79 (C–3), ~ 47 (C–5)] [11, 12]. Consequently, compound 3 was verified as (3β,20R)-cycloart-24-en-3-ol (known as cycloartenol) [13].
Compound 4 was obtained as a white amorphous powder with the melting point 122–124 °C. Its IR spectrum revealed absorption bands for the hydroxy group (3445 cm⁻¹) and double bond (1635, 1443 cm⁻¹). Its molecular formula was established to be C₂₇H₄₄O from the pseudo-molecular ion peak at m/z 385.3513 [M + H]⁺ (calculated for C₂₇H₄₅O, 385.3470) in HR–ESI–MS. The ¹H–NMR spectrum of 4 showed the signals of two olefinic protons at δH 5.06, 5.32, one oxymethine proton at δH 3.49, and five methyl groups at δH 0.65, 0.98, 1.57, 1.65 (each 3H, s), and 0.91 (3H, d, J = 6.5 Hz). The ¹³C–NMR and HMQC spectra revealed five methyl, ten methylene, eight methine, and four quaternary carbons. The signals of four olefinic carbons with δC values ranging from 121.7 to 140.9 suggesting the existence of two double bonds. Furthermore, the appearance of one carbinol group was derived from the signal at δC 71.8.

| Table 1. ¹H (500 MHz) and ¹³C (125 MHz) NMR data of compounds 1–4 [δ (ppm), J (Hz)] |
| C | ¹H | ¹³C |
|---|---|---|
| | δC | δH | δC | δH | δC | δH | δC | δH |
| 1 | 38.8 | – | 43.2 | 1.81 | 12.0 | 1.95 | 32.1 | 1.24 | 1.60 | 37.4 | 1.04 | 1.82 |
| 2 | 27.0 | – | 66.7 | 4.28 | 11.0 | 4.01 | 32.6 | 3.18 | 1.55 | 1.76 | 31.7 | 1.47 | 1.81 |
| 3 | 79.0 | 3.20 | 79.3 | 4.16 | 6.0 | 4.28 | 79.0 | 3.38 | – | 71.8 | 3.49 | m |
| 4 | 38.7 | – | 42.4 | – | – | 40.6 | – | – | 42.3 | 2.20 | 2.26 |
| 5 | 55.3 | – | 44.0 | 2.05 | – | 47.3 | 1.30 | – | 140.9 | – |
| 6 | 18.4 | – | 18.8 | 1.37 | 1.59 | 21.3 | 0.79 | 1.57 | 121.7 | 5.32 |
| 7 | 33.1 | – | 33.7 | 1.35 | 1.73 | 26.2 | 1.08 | 1.32 | 32.0 | 1.92 | 1.98 |
| 8 | 39.6 | – | 39.9 | – | – | 48.1 | 1.50 | dd | 12.5 | 4.5 | 32.0 | 1.49 |
| 9 | 47.7 | – | 48.5 | 1.95 | – | 20.1 | – | – | 50.2 | 0.90 |
| 10 | 37.0 | – | 38.9 | – | – | 26.2 | – | – | 36.6 | – |
| 11 | 23.4 | – | 24.4 | 1.96 | 2.07 | 26.6 | 1.06 | 1.98 | 21.2 | 1.41 | 1.48 |
| 12 | 125.6 | 5.24 | 126.0 | 5.47 | br s | 33.0 | 1.61 | – | 39.9 | 1.13 | 1.98 |
| 13 | 138.3 | – | 139.8 | – | – | 45.4 | – | – | 40.4 | – |
| 14 | 42.1 | – | 43.1 | – | – | 48.9 | – | – | 56.8 | 0.97 |
| 15 | 28.1 | – | 29.1 | 1.18 | 2.33 | 35.7 | 1.28 | – | 24.3 | 0.98 | 1.55 |
| 16 | 24.3 | – | 25.4 | 1.96 | 2.08 | 28.3 | 1.28 | 1.90 | 28.3 | 1.24 | 1.83 |
| 17 | 47.9 | – | 48.5 | – | – | 52.4 | 1.58 | – | 56.2 | 1.06 |
| 18 | 52.9 | 2.19 | 54.0 | 2.61 | br | 18.2 | 0.96 | s | 12.0 | 0.65 | s |
Measure in \(^{13}\)CDCl\(_3\), \(^{1}\)pyridine–\(^{5}\), \(^{*}\)overlapped signals

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

| Compounds          | \(IC_{50}\) (μg/mL) | LU-1 | MKN-7 | HepG2 | KB | SW-480 |
|--------------------|----------------------|------|-------|-------|----|--------|
| Ursolic acid (1)   | 44.37 ± 5.40         | 30.89 ± 3.60 | NT\(^{b}\) | NT | NT |
| Esculenic acid (2) | > 100                | > 100 | NT    | NT   | NT |
| Cycloartenol (3)   | > 100                | > 100 | NT    | NT   | NT |
| Desmosterol (4)    | 41.41 ± 2.31         | 38.06 ± 1.18 | 30.01 ± 2.92 | 28.11 ± 1.95 | 40.10 ± 2.39 |
| Ellipticine\(^{*}\) | 0.31 ± 0.07          | 0.39 ± 0.02 | 0.39 ± 0.02 | 0.44 ± 0.07 | 0.50 ± 0.04 |

\(^{*}\)Positive control, \(^{a}\)Not tested

The HMBC correlations between H–18 (δ\(_{H}\) 0.65) and C–12 (δ\(_{C}\) 39.9)/C–13 (δ\(_{C}\) 42.4)/C–14 (δ\(_{C}\) 56.8)/C–17 (δ\(_{C}\) 56.2), between H–19 (δ\(_{H}\) 0.98) and C–1 (δ\(_{C}\) 37.4)/C–5 (δ\(_{C}\) 140.9)/C–9 (δ\(_{C}\) 50.2)/C–10 (δ\(_{C}\) 36.6), between H–21 (δ\(_{H}\) 0.91) and C–17/C–20 (δ\(_{C}\) 36.2)/C–22 (δ\(_{C}\) 35.7), between H–26 (δ\(_{H}\) 1.65)/H–27 (δ\(_{H}\) 1.57) and C–24 (δ\(_{C}\) 125.3)/C–25 (δ\(_{C}\) 131.0) indicated the position of five methyl groups and two double bonds as shown in Fig. 1. In addition, the COSY correlation H–4 (δ\(_{H}\) 2.20, 2.26)/H–3 (δ\(_{H}\) 3.49) as well as the HMBC from H–4 to C–3 (δ\(_{C}\) 71.8)/C–5 (δ\(_{C}\) 140.9)/C–6 (δ\(_{C}\) 121.7) suggested that the hydroxy group connected to C–3. Compound 4 was thus identified preliminarily as cholesta-5,24-dien-3-ol. The NOESY correlation was found for H–3/H–1a (δ\(_{H}\)
1.04), and this indicated the α-axial orientation of these protons. Meanwhile, the 20R configuration was deduced by comparing the δC values of C–17 (δC 56.2), C–20 (δC 36.2), C–22 (δC 35.7) with those in desmosterol [20R: C–17 (δC 56.01), C–20 (δC 36.06), C–22 (δC 35.57)] and 20-epidesmosterol [20S: C–17 (δC 55.76), C–20 (δC 35.56), C–22 (δC 35.16)] [14]. As a consequence, compound 4 was determined to be (3β,20R)-cholesta-5,24-dien-3-ol (known as desmosterol) [14].

The cytotoxicity of the isolated compounds against the growth of various cancer cell lines was tested using the sulforhodamine B assay, and the results are described in Table 2. The data showed that compounds 1 exhibited a moderate cytotoxicity against the LU–1 and MKN–7 cell lines with IC50 values of 44.37 ± 5.40 and 30.89 ± 3.60 μg/mL, respectively. Compound 4 possessed moderate cytotoxic effects toward the LU–1, KB, HepG2, MKN–7 and SW–480 cell lines with IC50 values ranging from 28.11 ± 1.95 to 41.41 ± 2.31 μg/mL. Meanwhile, compounds 2 and 3 showed no inhibition against two tested cell lines (IC50 values > 100 μg/mL).

Several previous studies indicated that ursolic acid (1) showed the growth inhibitory activity against many human cancer cell lines such as H460 (large cell lung carcinoma), ME–180 (epidermoid cervical carcinoma), DU 145 (prostate metastatic carcinoma), MCF–7 (breast adenocarcinoma), M–14 (melanoma), HT–29 (colon adenocarcinoma), PC–3 (prostate adenocarcinoma), K562 (chronic myelogenous leukemia) [15], HL–60 (leukemia cancer cell), BGC (gastric cancer cell), Bel–7402 (hepatocellular carcinoma cell) and Hela (cervical cell) [16], MGC–803 (gastric cancer cell) and Bcap–37 (breast cancer cell) [17], KB, HepG2, MCF–7, LU [18]. This compound also induced apoptosis of SW–480 cells via p53 gene activation [19]. In addition, desmosterol (4) showed a significant cytotoxicity against NBT–T2 rat bladder epithelial cells [20]. Therefore, ursolic acid (1) and desmosterol (4) might be selected for further studies regarding their anticancer features.

4 Conclusion

Four compounds, namely ursolic acid, esculetin acid, cycloartenol, and desmosterol were isolated from the aerial parts of Anodendron paniculatum for the first time. The structures of all the isolated compounds were identified using extensive spectroscopic methods, including 1D, 2D–NMR, IR, and ESIMS. Ursolic acid and desmosterol showed a moderate inhibition against various cancer cell lines. Based on the obtained results, these two compounds were considered to be useful for developing new anticancer therapeutic agents for the human.

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