Antibacterial activities of chemical constituents from the aerial parts of Hedyotis pilulifera

Hoai Thi Nguyen, Duc Viet Ho, Hung Quoc Vo, Anh Tuan Le, Hien Minh Nguyen, Takeshi Kodama, Takuya Ito, Hiroyuki Morita and Ain Raal

Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Hue University, Hue City, Vietnam; Quang Tri Center of Science and Technology, Mientrung Institute for Scientific Research, Quang Tri, Vietnam; Institute of Natural Medicine, University of Toyama, Toyama, Japan; Institute of Pharmacy, University of Tartu, Tartu, Estonia

ABSTRACT

Context: Hedyotis pilulifera (Pit.) T.N. Ninh (Rubiaceae) has been used in Vietnamese ethnomedicine; the methanol extract exhibited antibacterial activity in our preliminary screening.

Objectives: In this study, compounds from H. pilulifera were isolated and their antibacterial activity in vitro was evaluated.

Materials and methods: The aerial parts of H. pilulifera (1.4 kg) were extracted with MeOH, suspended in water and ethyl acetate extract was chromatographed on a silica gel column. The structures of isolated compounds were elucidated by the combination analyses of spectroscopy including 1D-, 2D-NMR, HRMS and in comparison with the reported NMR data in the literature. All isolated compounds were evaluated for inhibitory effect using the microdilution method toward Staphylococcus aureus, Bacillus subtilis and Mycobacterium smegmatis, and MIC values were determined.

Results: Twenty compounds were isolated, including five triterpenoids, two steroids, two aromatic compounds, three fatty acids, one quinone derivative, one lignan glycoside, one ceramide and five glycolipids. Among these, oleanolic acid showed significant antibacterial activity against M. smegmatis with the MIC value of 2.5 µg/mL. Remarkably, rotungenic acid showed strong activity against S. aureus, B. subtilis, M. smegmatis with MIC values of 2.5, 2.5 and 1.25 µg/mL, respectively. Rotundic acid exhibited significant antibacterial activity against B. subtilis with the MIC value of 5 µg/mL. To the best of our knowledge, the antibacterial activity of rotungenic acid, stigmaster-4-en-3,6-dione and (25,35,4R,2′R)-2′-(2′-hydroxytetracosenoylamino) octadecane-1,3,4-triol was reported for the first time.

Conclusions: Oleanolic acid, rotungenic acid, and rotundic acid were considered to be useful for developing new antimicrobial therapeutic agents for human.

Introduction

Hedyotis pilulifera (Pit.) T.N. Ninh (Rubiaceae), an herbal plant, is known to be distributed in Laos and Vietnam (Ho 2003; Newman et al. 2007). It has been used as the remedy for abdominal pain and osteoarthritis (Bosseré et al. 2006). The valuable experience of ethnomedicine should not be underestimated: for example, according to our studies, the needles of Pinus sylvestris L. (Pinaceae) showed cytotoxic effect against breast cancer cells (Hoai et al. 2015), they have been used against cancer in Estonian ethnomedicine (Sak et al. 2014).

In our previous report, one new iridoid aglycone, 10-O-acetylborriariagenin, and five known iridoidal glycosides were isolated from the aerial parts of H. pilulifera (Hoai et al. 2016). Generally, the chemical composition of genus Hedyotis, including H. pilulifera, has been investigated just to a small extent. In our preliminary unpublished data, the methanol extract of aerial parts of H. pilulifera showed significant antimicrobial activity. Therefore, the current study was conducted to isolate those compounds and to evaluate their antibacterial activity in vitro.

Materials and methods

General

Melting points were determined on a Yanaco Micro MP apparatus. Optical rotations were measured on a JASCO P-2100 polarimeter (Hachioji, Tokyo, Japan). Infrared spectra were recorded on JASCO FT/IR-460 Plus spectrometer. 1D and 2D NMR were carried out using Bruker Avance 500 spectrometer (Bruker, Mass., Billerica, MA) with tetramethylsilane as an internal standard. ESI-MS data including high-resolution mass spectrum were measured on Shimadzu LCMS-IT-TOF spectrometer (Kyoto, Japan) HREIMS was recorded on a JEOL MStation JMS-700 spectrometer (JEOL Ltd., Tokyo, Japan). Column chromatography was performed using silica gel (Kanto, 40–50 µm, Tokyo, Japan), Cosmosil 75C18-OPN (Nacalai Tesque Inc., Kyoto, Japan) and Sephadex LH-20 (Dowex® 50WX2-100,Sigma-Aldrich, Tokyo, Japan). Analytical TLC was performed on precoated silica gel 60F254 and RP-18 F254 plates (0.25 or 0.50 mm thickness, Merck KGaA, Darmstadt, Germany). Cosmosil 5C18-
AR-II (Nacalai Tesque Inc., Kyoto, Japan) was used for analytical and semi-preparative HPLC (250 × 4.6 mm for analytical HPLC, and 250 × 10.0 mm for semi-preparative HPLC).

**Plant material**

The aerial parts of *H. pilulifera* were collected in the Quang Tri province (17°02′15.2″N 107°03′55.9″E), Vietnam, in August 2014 and were identified by Dr Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST, Vietnam. A voucher specimen (VLO1) was deposited at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam.

**Extraction and isolation of constituents**

The aerial parts of *H. pilulifera* (1.4 kg) were extracted by soaking with hot MeOH (3 × 3 L, 3 h each, 60 °C) to yield 38.0 g of a dark solid extract. This extract was suspended in water and successively partitioned with chloroform (CHCl₃) and ethyl acetate (EtOAc) to obtain the CHCl₃ (HC, 13.0 g), the EtOAc (HE, 11.0 g) and the water (HW, 14.0 g) extracts after the removal of solvents in vacuo.

The HC extract was chromatographed on a silica gel column eluting with *n*-hexane:acetone gradient system (100:0, 95:5, 90:10, 50:10, 10:10, 0:100 v/v, each 1.0 L) to obtain 6 corresponding fractions, HC1–HC6. Fraction HC3 (1.9 g) was subjected to a silica gel column eluting with *n*-hexane:EtOAc (6:1, v/v) to afford 3 smaller fractions, HC3A–HC3E. Fraction HC3B (210 mg) was then purified by silica gel column chromatography eluting with CHCl₃:acetone (15:1, v/v) to give 2 (11.0 mg), 6 (5.5 mg) and 10 (12.0 mg). Fraction HC3C (180 mg) was loaded onto an open silica gel column eluting with *n*-hexane:CHCl₃:acetone (6:5:1, v/v) to obtain 5 (8.0 mg) and 12 (7.5 mg). Fraction HC3E (2.1 g) was chromatographed over silica gel column using chloroform:EtOAc (5:1, v/v) as eluent to obtain 6 sub-fractions (HC3E1–HC3E6). Fraction HC3E4 (125 mg) was chromatographed on a silica gel column eluting with *n*-hexane:EtOAc:acetone (5:5:1, v/v) to afford 3 (2.6 mg), 7 (4.9 mg) and 13 (9.0 mg). Fraction HC6 (1.5 g) was subjected to silica gel column eluting with *n*-hexane:CHCl₃:MeOH (1:3:1, v/v) to give 6 smaller fractions (HC6A–HC6F). Fraction HC6E (360 mg) was further purified by using reverse-phase RP-18 silica gel column eluting with MeOH:water (30:1, v/v) to yield 1 (8.1 mg) and 4 (4.5 mg).

Fraction HE was crudely separated into 4 smaller fractions (HE1–HE4) by silica gel column using CHCl₃:EtOAc:MeOH (15:15:1, v/v) as eluent. Fraction HE3 (1.8 g) was chromatographed on a silica gel column eluting with CHCl₃:acetone (10:1, v/v) to yield 11 (4.7 mg) and 3 sub-fractions (HE3A–HE3D). Fraction HE3C was then partitioned on a sephadex LH-20 column eluting with MeOH to furnish 8 (20.5 mg). Compound 9 (3.4 mg) was purified from sub-fraction HE3D by preparative TLC using CHCl₃:MeOH:water (15:1, v/v) as a mobile phase. Fraction HE4 (1.1 g) was further subjected to reverse-phase RP-18 silica gel column eluting with MeOH:water (15:1, v/v) to afford 14 (4.5 mg) and 3 smaller fractions (HE4A–HE4C). HE4A was purified by preparative reversed phase HPLC using MeOH:water (95:5, flow rate 3 mL/min) as eluent to afford 17 (6.8 mg), 18 (4.5 mg), 19 (11.0 mg) and 20 (6.0 mg). Finally, HE4B was purified by repeated reverse-phase RP-18 silica gel column using acetone:MeOH:water (3:5:1, v/v) as eluent to give 15 (4.6 mg) and 16 (5.2 mg).

**Bioassay**

Antimicrobial activity was performed using the dilution method to a published procedure (Eloff 1998) with slight modifications.

*Staphylococcus aureus* (NBRC 100910), *Bacillus subtilis* (NBRC 13719), *Mycobacterium smegmatis* (NBRC 13167) were used for this assay. These strains were tested by using microdilution assays, and MIC values were determined (Eloff 1998). Bacterial strains were inoculated on YP agar plates [1% peptone (Nihon Pharmaceutical, Tokyo, Japan), 0.2% yeast extract (Difco, Mich., Detroit, MI), 0.1% MgSO₄-7H₂O, and 2% agar (Nacalai Tesque Inc., Kyoto, Japan)] and incubated at 37 °C for 12 h. A stock solution of samples was prepared at 1 mg/mL in DMSO and further diluted to varying concentrations in 96-well plates that contained microbial strains incubated in YP medium for the bacterial strains. The plate was further incubated at 37°C for 12 h. Ampicillin (Nacalai Tesque Inc., Kyoto, Japan) were used as the reference reagents for bacterial strains (Eloff 1998).

**Results**

The chemical investigations led to the isolation and the structural elucidation of 20 known compounds including five triterpenoids, two steroids, two aromatic compounds, three fatty acids, one quinone derivative, one lignan glycoside, one ceramide and five glycolipids.

Compound 1 was obtained as a white amorphous powder. The HREIMS of 1 showed a molecular ion peak at m/z 456.3611 [M]+. Its molecular formula was thus determined to be C₃₀H₄₀O₅ by HREIMS in conjunction with NMR data analysis. The ¹H-NMR of 1 showed the presence of seven quaternary methyl groups at δH 0.92 (3H, s), 0.98 (3H, s), 1.05 (3H, s), 1.05 (6H, s), 1.27 (3H, s), 1.31 (3H, s) and one olefinic proton at δH 5.53 (1H, br.s, H-12). The ¹³C-NMR spectrum revealed 30 signals. The presence of one carboxylic group [δC 180.3 (C-28)], one tri-substituted double bond [δC 122.7 (C-12) and 144.9 (C-13)], one oxygenated carbon [δC 78.2 (C-3)] were observed. Based on the above MS and NMR data (Table 1), compound 1 was determined to be oleanolic acid, consistent with reported data (Mahato & Kundu 1994).

Compound 4 was obtained as a white amorphous powder. The ¹H-NMR spectrum showed the typical signals of one olefinic proton (δH 5.62), two protons of oxymethylene group [δH 3.68 (d, δ = 10.5 Hz), 4.51 (d, δ = 10.5 Hz)], one proton of carbonyl group [δH 3.64 (dd, δ = 11.5, 3.5 Hz)], and seven methyl groups [δH 0.90 (s), 1.09 (s), 1.14 (d, δ = 6.5 Hz), 1.47 (s), 1.56 (s), and 1.74 (s) (each, 3H)]. The coupling constant value of H-3 (11.5 and 3.5 Hz) is typical for its α,α-orientation in chair conformation of A ring. The ¹³C-NMR spectrum indicated 30 signals. Some characteristic signals were found, including one carbonyl carbon (δC 181.2), two olefinic carbons (δC 128.4 and 140.4), one oxygenated methine carbon (δC 80.7), one oxygenated quaternary carbon (δC 73.2) and one oxygenated methylene carbon (δC 65.1). The complete assignment of all protons and carbons of 4 was conducted by the analysis of HMQC and HMBE spectra (Table 1). The HMBC correlations from methyl protons (δH 1.56) and oxymethylene protons (δH 3.68 and 4.51) to C-3 (δC 80.7)/C-4 (δC 43.6) confirmed that the position of oxygenated methine carbon (C-3) in the A ring. Similarly, the HMBC correlations from H-18 (δH 3.07) and C-12 (δC 128.4)/C-13 (δC 140.4)/C-28 (δC 181.2) confirmed the position of carboxyl group at C-17 as well as the tri-substituted double bond at C-12/C-13. In addition, the HMBC correlation between H3-29 (δH 1.47) and C-18 (δC 55.1)/C-19 (δC 73.2)/C-20 (δC 42.9) led us to locate the hydroxyl group at C-19. Next, the remaining hydroxyl group was linked to C-24 by comparison of the chemical shifts of methyl carbon (δC 24.1) and oxymethylene carbon (δC 65.1) with those...
of the two stereoisomers [24-OH: δC 23.5 (C-23)/64.5 (C-24); 23-OH: δC 67.9 (C-23)/12.8 (C-24)] (Zhang & Yang 1994). Consequently, compound 4 was concluded to be rotundic acid (Nakatani et al. 1989).

Compound 5 was obtained as a white amorphous powder. The 1H and 13C NMR chemical shifts of 5 were almost the same as those of 4 (Table 1), suggesting that these two compounds possessed the analogous structures. The only difference is configuration of chiral centre at C-4. Similar to 4, the hydroxyl group was located at C-23 due to the chemical shifts of methyl carbon (δC 12.7) and oxymethylene carbon (δC 67.5) at C-4 (δC 43.3) were similar to those of 23-OH form [δC 67.9 (C-23)/12.8 (C-24)], but quite different from those of 24-OH form [δC 23.5 (C-23)/64.5 (C-24)] (Zhang & Yang 1994). Thus, compound 5 was identified to be rotundic acid (He et al. 2012).

The remaining compounds were identified as betulinic acid (2) (Hess & Monache 1999), pomoic acid 3β-acetate (3) (Neto et al. 2000), stigmast-4-ene-3,6-dione (6) (Saca et al. 2000), daucosterol (7) (Yang et al. 2013), benzyl hydroperoxide (8) (Kyasa et al. 2013), 2,3-di-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-0ne (9) (Baderschneider & Winterhalter 2001), octadeca-9Z,12Z-dienoic acid (10) (Butovich & Lukyanavaa 2008), 9,12,13-trihydoxyoctadeca-10E,14E-dienoic acid (11) (Oueslati et al. 2006), 9,12,13-trihydroyoctadeca-10E,15Z-dienoic acid (12) (Oueslati et al. 2006), α-tocopherolquinone (13) (Sung et al. 1999), (7S,8R,7’.R,8’S)-icaril A2-9-O-β-xylopyranoside (14) (Chung et al. 2011), (2S,3S,4R,2’R)-2-(2’-hydroxytetraosanolamino)octadecane-1,3,4-trioli (15) (Gao et al. 2001), (2S)-1-O-linolenoyle-2-O-linolenoyle-3-O-β-D-galactopyranosyl-sn-glycerol (16) (Kim et al. 2012), (2S)-1-O-linolenoyle-2-O-linolenoyle-3-O-β-D-galactopyranosyl-sn-glycerol (17) (Ibrahim et al. 2011), (2S)-1-O-linoleoyl-2-O-linoleoyl-3-O-β-D-galactopyranosyl-sn-glycerol (18) (Janwityayanuchit et al. 2003), (2S)-1-O-linolenoyle-2-O-palmitoyl-3-O-β-D-galactopyranosyl-sn-glycerol (19) (Murakami et al. 1991) and (2S)-1-O-linolenoyle-2-O-palmitoyl-3-O-β-D-galactopyranosyl-sn-glycerol (20) (Murakami et al. 1991) by comparing their spectroscopic data with those reported in the literature. The chemical structures of all isolated compounds are shown in Figure 1.

The antibacterial assay of isolated compounds was evaluated against three Gram (+) bacterial strains, including Staphylococcus aureus, Bacillus subtilis and Mycobacterium smegmatis. As shown in Table 2, compound 4 demonstrated potent inhibition with MIC values ranging from 1.25 to 2.5 μg/mL. The inhibitory effect of 4 against the tested bacterial strains was comparable with that of the positive control, ampicillin. Compounds 1 and 5 exhibited significant antibacterial activity against M. smegmatis, B. subtilis with MIC values of 2.5 and 5 μg/mL, respectively. The other compounds (2, 6, and 15) showed moderate inhibitory effect.

Table 1. 1H (500 MHz) and 13C (125 MHz) NMR data of compounds 1, 4, and 5 (δ ppm, J (Hz)).

| Position | δC 1H | δH 1H | δC 1H | δH 1H | δC 1H | δH 1H |
|----------|------|------|------|------|------|------|
| 1 | 39.0 | 39.2 | 0.96 | 1.53 | 39.1 | 1.66 | 1.77 |
| 2 | 28.2 | 28.9 | 1.89 | 2.05 | 27.1 | 1.28 | 1.56 |
| 3 | 78.2 | 80.7 | 3.64 | 2.93 | 13.2 | 1.27 | 1.54 |
| 4 | 39.5 | 43.6 | – | – | 34.3 | 41.0 | – |
| 5 | 55.9 | 56.9 | 1.00 | – | 48.7 | 2.0 | 1.2 |
| 6 | 18.9 | 19.7 | 1.38 | 1.71 | 19.3 | 1.47 | – |
| 7 | 33.4 | 34.4 | 1.38 | 1.62 | 33.7 | 1.32 | 1.68 |
| 8 | 39.9 | 40.8 | – | – | 41.0 | 1.4 | – |
| 9 | 48.2 | 48.3 | 1.84 | – | 48.5 | 1.75 | – |
| 10 | 37.5 | 37.6 | – | – | 37.9 | 1.40 | – |
| 11 | 23.8 | 24.7 | 1.96 | 2.08 | 24.7 | 2.01 | 1.24 |
| 12 | 122.7 | 128.4 | 5.62 | 3.9 | 129.4 | 5.30 | 3.9 |
| 13 | 144.9 | 140.4 | – | – | 140.1 | 1.37 | – |

a Measured in pyridine-d5.
b Measured in CD3OD.
c Overlapped signals.
d Reassigned using 2D-NMR.

Discussion

To the best of our knowledge, compounds 3–6, 8–9 and 11–20 were firstly isolated from the genus Hedysorhis. From our understanding, the antibacterial activity of compounds 4, 6, and 15 was reported for the first time in this study.

The structure–activity relationship of 4 and 5 may be due to the difference in absolute configuration of C-4 under the experimental conditions. The 4S configuration (in 4) was considered to be more active than 4R configuration (in 5). Compounds 1, 4, and 5 represent pentacyclic triterpenoid skeleton which should
be responsible for the bioactivity of the methanol extract of this species.

Previous studies discovered the antibacterial activity of 1 against Enterococcus faecium, Streptococcus pneumoniae, Staphylococcus aureus (Horiuchi et al. 2007; Jo et al. 2014), Listeria monocytogenes (Hee 2015), Streptococcus downei (Park & Kim 2011), Streptococcus mutans and S. sobrinus (Kim et al. 2010) and Propionibacterium acnes (Jo et al. 2014), whereas compound 2 exhibited mild inhibitory effect against B. subtilis (Chandramu et al. 2003). Moreover, compound 5 showed broad antimicrobial activity against bacteria, yeast and filamentous fungi (Haraguchi et al. 1999).

Conclusions

Based on the obtained results, oleanolic acid (1), rotugenic acid (4), and rotundic acid (5) from the aerial parts of H. pilulifera were considered to be useful for developing new antimicrobial therapeutic agents for human.

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Disclosure statement

The authors report no declarations of interests.

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