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Viral protein R inhibitors from *Swertia chirata* of Myanmar

So-Yeun Woo,[1] Nwet Nwet Win,[1] Wyine Myat Noe Oo,[2] Hla Ngwe,[3] Takuya Ito,[4] Ikuro Abe,[5,6] and Hiroyuki Morita[1,*]

Institute of Natural Medicine, University of Toyama, 2630-Sugitani, Toyama 930-0194, Japan,[1] Department of Chemistry, Taungoo University, 08105 Taungoo, Myanmar,[2] Department of Chemistry, University of Yangon, 11041 Yangon, Myanmar,[3] Faculty of Pharmacy, Osaka Ohtani University, 3-11-1 Nisikiori-kita, Tondabayashi, Osaka 584-8540, Japan,[4] Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan,[5] and Collaborative Research Institute for Innovative Microbiology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan[6]

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Viral protein R (Vpr) is a small, basic accessory protein (14 kDa) that is well conserved in Human immunodeficiency virus-1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV). Numerous investigations over the past 2 decades have suggested that Vpr would be an attractive target for HIV disease treatment. Small molecules, including fumagillin, damnacanthal, quercetin, vipirinin, isopimarane diterpenoids, picrasane quassinoids, iridoids, and bis-iridoid glycosides, have been reported as potent Vpr inhibitors. These compounds may not only represent HIV drug seeds, but also could be new target compounds for biochemical synthesis such as current synthetic biology and enzyme bioengineering approaches, due to their anti-Vpr activities. In our investigations of different types of compounds with Vpr inhibitory activity, we found that the $CHCl_3$ soluble, crude extract of the whole *Swertia chirata* plant inhibited the expression of Vpr in Hela cells harboring the TREx plasmid encoding full-length Vpr (TREx-HeLa-Vpr cells). The purification and isolation of the active $CHCl_3$ soluble portion afforded six secondary metabolites, including four xanthone derivatives, decussatine (1), methylswertianin (2), 1-hydroxy-3,5-dimethoxyxanthone (3), and bellidifolin (4), and two triterpenoids, oleanolic acid (5) and 12-hydroxyoleanolic lactone (6). The evaluation of the anti-Vpr activities of 1, 2, and 4–6 against TREx-HeLa-Vpr cells revealed that 4 and 5 are potent Vpr inhibitors with an effective dose of 10 μM, and are chemically and structurally distinct from previously reported inhibitors.

[**Key words:** *Swertia chirata*; TREx-HeLa-Vpr cells; Vpr inhibitors; Xanthone derivatives; Bellidifolin; Oleanolic acid]

Human immunodeficiency virus-1 (HIV-1) evolved numerous accessary proteins that serve as multifunctional tools to promote the virus life cycle in the host (1). The viral protein R (Vpr) is an HIV accessory protein consisting of 96 amino acids (14 kD) and plays an important role in promoting HIV-1 pathogenesis (2–4). Vpr potentially functions in the initiation step of the reverse transcription process, since this protein is present in the reverse transcription complex, along with two copies of RNA and other viral proteins, such as reverse transcriptase (RT), integrase (IN), and nucleocapsid protein 7 (NcP7) (5,6). Thus, Vpr could be an attractive target for therapeutic intervention. Several groups have reported small molecule Vpr inhibitors, including fumagillin, damnacanthal, quercetin, and vipirinin (7–10). In previous studies, our group has also reported isopimarane diterpenoids, picrasane quassinoids, iridoids, and bis-iridoid glycosides as potent Vpr inhibitors (11–14). Due to their anti-Vpr activities associated with new HIV drugs, these compounds may represent new targets for biochemical synthesis such as current synthetic biology and enzyme bioengineering approaches. As part of our continuing search for new, naturally occurring potent Vpr inhibitors, we found that the $CHCl_3$ soluble extract of *Swertia chirata*, grown in Myanmar, inhibited the expression of Vpr in Hela cells harboring the TREx plasmid encoding full-length Vpr (TREx-HeLa-Vpr cells). *S. chirata* (Gentianaceae) is an annual/biennial medicinal herb that is widely distributed in the Himalayan mountains, Pakistan, India, Nepal, Bhutan, Tibet, and Myanmar (15). In Myanmar, this plant is locally known as Pan-khar or Thinbaw-sega-gyi and is especially distributed in the Taungoo Township of the Bago Region. It has been used in traditional medicine for the treatment of liver disorders, malaria, chronic fever, anemia, bronchial asthma, hepatitis, diabetes, cancer, and AIDS (15–17). Previous phytochemical studies have reported the presence of xanthones, flavonoids, terpenoids, alkaloids, iridoids, secoiridoids, steroids, and phenolic compounds in the whole plant of *S. chirata*. These secondary metabolites showed antitumor, antiviral, anti-diabetic, anti-HIV, and anti-hepatitis bioactivities (16–19). Our detection of the anti-Vpr activity in this plant is in good agreement with the reports of the anti-HIV, antiviral, and anti-hepatitis activities, which are regulated by viral infections (17,20). Herein, we report the isolation and identification of the active constituents from the $CHCl_3$ soluble, crude extracts of *S. chirata* and the evaluation of their anti-Vpr activities.

* Corresponding author. Tel.: +81 76 434 7625; fax: +81 76 434 5059.
E-mail address: hmorita@inm.u-toyama.ac.jp (H. Morita).

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MATERIALS AND METHODS

General experimental procedures Nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) on a JEOL JNM-ECA500II spectrometer. Chemical shift values were expressed in δ (ppm) downfield from TMS as internal standard. The mass spectra were recorded on an Agilent Technologies 6420 Triple Quadrupole LC-MS. Open-column chromatography was performed with normal-phase silica gel (silica gel 60 N, spherical, neutral, 40–50 μm, Kanto Chemical Co., Inc., Tokyo, Japan) and Cosmosil 75C18-OPN (Nacalai Tesque, Inc., Kyoto, Japan). Medium pressure liquid chromatography (MPLC) was performed with a Büchi Sepacore system (Büchi Labortechnik AG, Flawil, Switzerland). Thin-layer chromatography (TLC) was performed on precoated silica gel 60F_{254} and RP-18 F_{254} plates (Merck, 0.25 or 0.50 mm thickness). The TREx-HeLa-Vpr cell line was available and maintained in our laboratory. Cell culture flasks and 48- and 96-well plates were purchased from Thermo Scientific (Jiangsu, China). An SH-1200 microplate reader (Corona, Hitachinaka, Japan) was used to measure the absorbance to monitor the cell proliferation.

Plant material The whole *S. chirata* plants were collected from Taungoo, Bago Region, Myanmar in July 2013, and were identified by Dr. Khin Cho Cho Oo, Department of Botany, University of Yangon. A voucher specimen (TMPW 28304) was deposited at the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Japan.

Extraction and isolation The dried whole *S. chirata* plants (1.8 kg) were extracted with $CHCl_3$ by sonication (5 L, 90 min, 3×) at 30°C. The $CHCl_3$ extracts were concentrated on a rotary evaporator under reduced pressure at 40°C, to obtain 162.0 g of the $CHCl_3$ extract. A portion of the $CHCl_3$ extract (70.1 g) was chromatographed by MPLC on a silica gel column, eluted with *n*-hexane–EtOAc (9:1, 8:2, 7:3, 6:4, 5:5. 3:7, 1:9 step gradient), to obtain eleven fractions (A–K). Fraction D (5.3 g) was further fractionated by open column chromatography, using Cosmosil 75C18-OPN eluted with MeOH–MeCN–H_2O (2:2:1), to afford eleven sub-fractions (D1 to D11). Sub-fraction D3 (16.8 mg) was purified by normal-phase preparative TLC with 100% CH_2Cl_2 to give compound **3** (1.8 mg). Compound **1** (44.3 mg) was isolated from sub-fraction D4 (138.5 mg) using normal phase preparative TLC with benzene–EtOAc (9:1). Sub-fraction D5 (756.8 mg) was purified by silica gel open column chromatography with 100% CH_2Cl_2, to give compound **2** (10.4 mg). Compound **5** (1.2 g) was obtained from the crystallization of sub-fraction D11 with a combination of $CHCl_3$ and MeOH. Fraction J was rechromatographed on Cosmosil 75C18-OPN, eluted with MeOH–MeCN–H_2O (1:1:1) to give eleven sub-fractions (J1–J11). The purification of sub-fraction J11 (130.3 mg) by silica gel open column chromatography using *n*-hexane–EtOAc (1:1) afforded compounds **4** (5.3 mg) and **6** (6.3 mg).

Decussatine (**1**) was obtained as yellowish powder and its ^{1}H NMR, ^{13}C NMR and ESIMS data were as follows. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 3.87 (3H, s, 3-OCH_3), 3.92 (3H, s, 8-OCH_3), 3.99 (3H, s, 7-OCH_3), 6.29 (1H, d, J = 2.4 Hz, H-2), 6.31 (1H, d, J = 2.4 Hz, H-4), 7.14 (1H, d, J = 9.3 Hz, H-5), 7.32 (1H, d, J = 9.3 Hz, H-6), 13.25 (1H, s, 1-OH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 55.7 (3-OCH_3), 57.0 (8-OCH_3), 61.7 (7-OCH_3), 91.9 (C-4), 96.8 (C-2), 103.9 (C-8b), 112.7 (C-5), 115.6 (C-8a), 120.3 (C-6), 148.7 (C-8), 149.2 (C-7), 150.9 (C-4b), 157.0 (C-4a), 163.7 (C-1), 166.3 (C-3), 181.1 (C=O). ESIMS: *m/z* 303 $[M+H]^+$.

Methylswertianin (**2**) was obtained as yellow needles and its ^{1}H NMR, ^{13}C NMR and ESIMS data were as follows. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 3.89 (3H, s, 5-OCH_3), 3.95 (3H, s, 3-OCH_3), 6.34 (1H, d, J = 1.7 Hz, H-2), 6.52 (1H, d, J = 1.7 Hz, H-4), 6.70 (1H, d, J = 9.4 Hz, H-7), 7.22 (1H, d, J = 8.8 Hz, H-6), 11.38 (1H, s, 8-OH), 11.97 (1H, s, 1-OH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 56.0 (3-OCH_3), 57.3 (5-OCH_3), 93.1 (C-4), 97.9 (C-2), 102.8 (C-8b), 108.1 (C-8a), 109.3 (C-7), 120.2 (C-6), 139.9 (C-5), 145.3 (C-4b), 154.1 (C-8), 157.7 (C-4a), 162.8 (C-1), 167.4 (C-3), 184.5 (C=O). ESIMS: *m/z* 289 $[M+H]^+$.

1-Hydroxy-3,5-dimethoxyxanthone (**3**) was obtained as **y**ellowish powder and its ^{1}H NMR, ^{13}C NMR and ESIMS data were as follows. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 3.90 (3H, s, 3-OCH_3), 4.04 (3H, s, 5-OCH_3), 6.37 (1H, brs, H-2), 6.57 (1H, brs, H-4), 7.26 (1H, d, J = 7.7 Hz, H-6), 7.31 (1H, m, H-7), 7.83 (1H, d, J = 7.7 Hz, H-8), 12.84 (1H, s, 1-OH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 56.4 (3-OCH_3), 56.5 (5-OCH_3), 92.8 (C-4), 97.5 (C-2), 103.9 (C-8b), 115.6 (C-6), 116.7 (C-8), 121.5 (C-8a), 123.5 (C-7), 146.3 (C-4b), 148.2 (C-5), 157.6 (C-4a), 163.3 (C-1), 166.7 (C-3), 180.8 (C=O). ESIMS: *m/z* 273 $[M+H]^+$.

Bellidifolin (**4**) was obtained as yellowish powder and its ^{1}H NMR, ^{13}C NMR and ESIMS data were as follows. ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 3.88 (3H, s, 3-OCH_3), 6.35 (1H, d, J = 1.7 Hz, H-2), 6.56 (1H, brs, H-4), 6.62 (1H, d, J = 8.8 Hz, H-7), 7.24 (1H, d, J = 8.8 Hz, H-6), 11.05 (1H, s, 8-OH), 11.87 (1H, s, 1-OH), 9.68 (1H, s, 5-OH). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 56.2 (3-OCH_3), 92.9 (C-4), 97.4 (C-2), 102.1 (C-8b), 107.4 (C-8a), 109.4 (C-7), 123.7 (C-6), 137.3 (C-5), 143.5 (C-4b), 151.7 (C-8), 157.3 (C-4a), 161.9 (C-1), 167.0 (C-3), 184.0 (C=O). ESIMS: *m/z* 273 $[M-H]^-$.

Oleanolic acid (**5**) was obtained as white amorphous powder and its ^{1}H NMR, ^{13}C NMR and ESIMS data were as follows. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.72 (6H, s, Me-24, Me-26), 0.86 (6H, s, Me-25, Me-30), 0.88 (3H, s, Me-29), 0.93 (3H, s, Me-23), 1.09 (3H, s, Me-27), 2.78 (1H, dd, J = 3.1, 13.4 Hz, H-18), 3.16 (1H, dd, J = 5.5, 10.4 Hz, H-3), 5.23 (1H, brs, H-12). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 15.2 (C-25), 15.4 (C-24), 16.8 (C-26), 18.2 (C-6), 22.9 (C-16), 23.3(C-11), 23.4 (C-29), 25.8 (C-27), 26.7 (C-2), 27.6 (C-15), 27.9 (C-23), 30.5 (C-20), 32.4 (C-22), 32.6 (C-7), 32.9 (C-30), 33.7 (C-21), 36.9 (C-10), 38.3 (C-1), 38.6 (C-4), 39.1 (C-8), 41.0 (C-18), 41.6 (C-14), 45.8 (C-19), 46.3 (C-17), 47.5 (C-9), 55.1 (C-5), 78.8 (C-3), 122.2 (C-12), 143.7 (C-13), 181.6 (C-28). ESIMS: *m/z* 479 $[M+Na]^+$.

12-Hydroxyoleanolic lactone (**6**) was obtained as pale yellowish powder and its ^{1}H NMR, ^{13}C NMR and ESIMS data were as follows. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.79 (3H, s, Me-24), 0.89 (3H, s, Me-25), 0.91 (3H, s, Me-29), 0.99 (3H, s, Me-30), 1.00 (3H, s, Me-23), 1.15 (3H, s, Me-26), 1.31 (3H, s, Me-27), 3.23 (1H, dd, J = 4.6, 11.5 Hz, H-3), 3.90 (1H, brs, H-12). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 15.3 (C-24), 16.3 (C-25), 17.7 (C-6), 18.5 (C-27), 18.6 (C-26), 21.2 (C-16), 23.9 (C-29), 27.2 (C-2), 27.4 (C-23), 27.9 (C-22), 28.0 (C-15), 28.8 (C-11), 31.6 (C-20), 33.3 (C-30), 33.9 (C-7), 34.1 (C-21), 36.4 (C-10), 38.8 (C-1), 38.9 (C-4), 39.4 (C-19), 42.0 (C-8), 42.3 (C-14), 44.6 (C-9), 44.7 (C-17), 51.1 (C-18), 55.2 (C-5), 76.4 (C-12), 78.8 (C-3), 90.5 (C-13), 179.9 (C-28). ESIMS: *m/z* 495 $[M+Na]^+$.

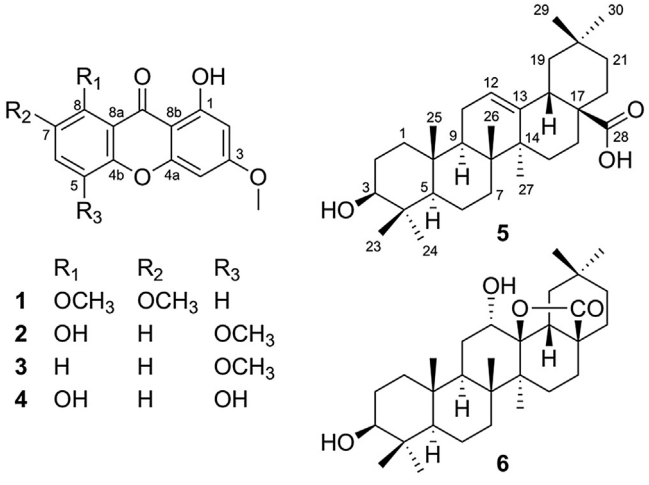
***In vitro* Vpr inhibitory activities** The *in vitro* Vpr inhibitory activities of the crude extracts and compounds were evaluated, according to the procedure reported by Win et al. (11) with a slight modification. Briefly, the TREx-HeLa-Vpr strain (12,000 cells/well, 150 μL) we previously established was seeded in 48-well plates and incubated in α-minimal essential medium (α-MEM, Wako Pure Chemical Industries, Osaka, Japan), supplemented with 10% fetal bovine serum (FBS, Nichirei Bioscience), 1% antibiotic antimycotic solution (Sigma–Aldrich), 5 μg/mL blasticidin (Invitrogen), and 50 μg/mL zeocin, at 37°C under a 5% CO_2 and 95% air atmosphere, for 24 h. To induce the Vpr expression, 50 μL of tetracycline (15 μg/mL) was added to each well, and after a 24 h incubation, 50 μL portions of the samples were added to the tetracycline-treated cells at different concentrations (5 and 10 μg/mL for the crude extracts and μM for the compounds). The wells without samples were used as controls. After a 48 h incubation, 50 μL of MTT solution (0.5 mg/mL) was added to the wells. After a 3 h incubation, the medium was discarded and 200 μL of DMSO was added to dissolve the MTT formazan crystals. The absorbance at 570 nm was measured after 15 min of shaking, and the cell proliferation was calculated from the mean values of data from three wells, by the following equation:

$$\text{Cell proliferation (\%)} = 100 \times [\{\text{Abs}_{(\text{test samples})} - \text{Abs}_{(\text{blank})}\}/\{\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})}\}] \quad (1)$$

The inhibitory activity of the tested sample was obtained by comparing the number of viable cells treated with both tetracycline and sample to the number of viable cells treated with tetracycline without sample.

RESULTS AND DISCUSSION

Isolation and identification of compounds from *S. chirata* The $CHCl_3$ extract of the whole *S. chirata* plants showed anti-Vpr activity, as discussed later. Thus, the extract was subjected to silica gel MPLC, and further separations by a series of chromatographic methods, including reversed phase and normal phase silica gel column chromatography and preparative TLC, and crystallization, which afforded four xanthones, decussatine (**1**) (21), methylswertianin (**2**) (22), 1-hydroxy-3,5-dimethoxyxanthone (**3**) (23), and bellidifolin (**4**) (24), and two terpenoids, oleanolic acid (**5**) (25) and 12-hydroxyoleanolic lactone (**6**) (26) (Fig. 1).



	R_1	R_2	R_3
1	OCH_3	OCH_3	H
2	OH	H	OCH_3
3	H	H	OCH_3
4	OH	H	OH

5 **6**

FIG. 1. Structures of compounds (**1**–**6**) isolated from whole *S. chirata* plants.

Compound **1** was obtained as a yellowish powder. The ^{1}H NMR spectrum of **1** showed one chelated hydroxy proton (δ_H 13.25), two *meta* coupled aromatic protons (δ_H 7.32, 7.14), and three methoxy protons (δ_H 3.99, 3.92, 3.87). The ^{13}C NMR spectrum of **1** displayed 16 signals, including a carbonyl carbon (δ_C 181.1), twelve aromatic carbons, including six oxygenated aromatic carbons, and three methoxy groups. Through the key HMBC correlations from the chelated hydroxyl proton (δ_H 13.25) to C-1 (δ_C 163.7)/C-8b (δ_C 103.9), from an aromatic proton (δ_H 6.31) to C-2 (δ_C 96.8)/C-8b, and from an aromatic proton (δ_H 7.14) to C-4b (δ_C 150.9)/C-7 (δ_C 149.2)/C-8a (δ_C 115.6), compound **1** was suggested to be a xanthone derivative. These spectroscopic data and analyses of 2D NMR spectroscopic data (Fig. 2) were found to be identical to those of the reported decussatine, and thus the structure of **1** was identified as shown in Fig. 1 (21).

Compound **2** was obtained as yellow needles. Its ^{1}H and ^{13}C NMR spectroscopic data were similar to those of **1**. The significant difference was the presence of one more chelated hydroxy proton at δ_H 11.38. By analyzing the 2D NMR spectroscopic data of **2** (Fig. 2) and by comparing the NMR data with those in the literature, **2** was identified as methylswertianin (22).

Compounds **3** and **4** were obtained as yellowish powders. Their NMR spectroscopic data were similar to those of **2**. The significant differences were the absence of a chelated proton in **3** and the lack of a methoxy group in **4**. Comparisons of the NMR data of **3** and **4** including their 2D NMR spectroscopic data (Fig. 2) with those in the literature revealed that **3** and **4** were 1-hydroxy-3,5-dimethoxyxanthone (23) and bellidifolin (24), respectively.

Compound **5** was obtained as a white amorphous powder. The ^{1}H NMR spectroscopic data of **5** revealed signals including seven methyl singlets (δ_H 1.09, 0.93, 0.88, 0.86, 0.86, 0.72, 0.72), an oxygenated methine proton (δ_H 3.16), and an olefinic methine proton (δ_H 5.23). The ^{13}C NMR data showed 30 signals, including one carbonyl carbon [δ_C 181.6 (C-28)], two olefinic carbons [δ_C 143.7 (C-13), δ_C 122.2 (C-12)], and one oxygenated methane [δ_C 78.8 (C-3)], suggesting that **5** was an oleanolic type of pentacyclic triterpenoid. By comparing the NMR data of **5** with those in the literature as well as analyzing 2D NMR spectroscopic data of **5** (Fig. 2), compound **5** was identified as oleanolic acid (25).

Compound **6** was obtained as a pale yellowish powder. The ^{1}H NMR spectrum indicated signals including seven signals of methyl groups (δ_H 1.31, 1.15, 1.00, 0.99, 0.91, 0.89, 0.79) and two oxygenated methine protons (δ_H 3.23, 3.90). The ^{13}C NMR spectroscopic data revealed the presence of 30 signals, including one carbonyl group carbon [δ_C 179.9 (C-28)] and two oxygenated methanes [δ_C 78.8 (C-3), δ_C 76.4 (C-12)]. The NMR data of **6** were similar to those of **5**, except for the absence of olefinic carbons. Furthermore, the appearance of an oxygenated quaternary carbon signal at δ_C 90.5 (C-13) suggested the presence of an oxygenated linkage between C-13 and the carbonyl carbon of C-17 in **6**. Based on the analyses of the 2D NMR spectroscopic data (Fig. 2) and comparisons of the NMR data of **6** with those in the literature, compound **6** was identified as 12-hydroxyoleanolic lactone (26).

Vpr inhibitory activity The crude extracts and phytoconstituents of *Swertia* species have been studied for various biological activities, including antibacterial, antifungal, antiviral, anticancer, anti-inflammatory, antidiabetic, and antioxidant activities (20, 27–35). However, their anti-Vpr activities related to HIV-1 infection have not been assessed. Hence, the present study is the first evaluation of the anti-Vpr activities of the $CHCl_3$ soluble, crude extract and the isolated compounds (**1**, **2**, **4**, **5**, **6**). Compound **3** was excluded, due to its minute amount.

The $CHCl_3$-soluble, crude extract and the isolated compounds **1**, **2**, and **4**–**6** were examined for their inhibitory effects on the expression of Vpr in TREx-HeLa-Vpr cells, in which Vpr expression was induced by the addition of tetracycline. The anti-Vpr activity is represented by the cell proliferation (%) that occurs due to the inhibitory effect of the compounds on the expression of Vpr (Table 1, Fig. 3). Damnacanthal, a reported potent Vpr inhibitor, was

TABLE 1. Inhibitory effects of the $CHCl_3$ extract and isolated compounds of *S. chirata* and the positive control damnacanthal against the expression of Vpr in TREx-HeLa-Vpr cells.

Sample	Cell proliferation (%) ± SD[a]	
	Tetracycline (+), sample (+)	
	5 μg/mL (for compounds) / 5 μM (for $CHCl_3$ extract)[b,c]	10 μg/mL (for compounds) / 10 μM (for CHCl3 extract)[b,c]
1	96 ± 3	49 ± 4
2	74 ± 0.4	67 ± 4
4	114 ± 8	116 ± 7
5	128 ± 7	168 ± 14
6	88 ± 0.1	85 ± 0.3
$CHCl_3$ extract	109 ± 2	128 ± 2
Damnacanthal[d]	150 ± 2	158 ± 2
Control[e]	100 ± 0	100 ± 0

[a] Data are means ± SD from three different experiments.
[b] Treatment concentrations for compounds (μM).
[c] Treatment concentrations for extract (μg/mL).
[d] Positive control.
[e] Tetracycline (+), sample (−).

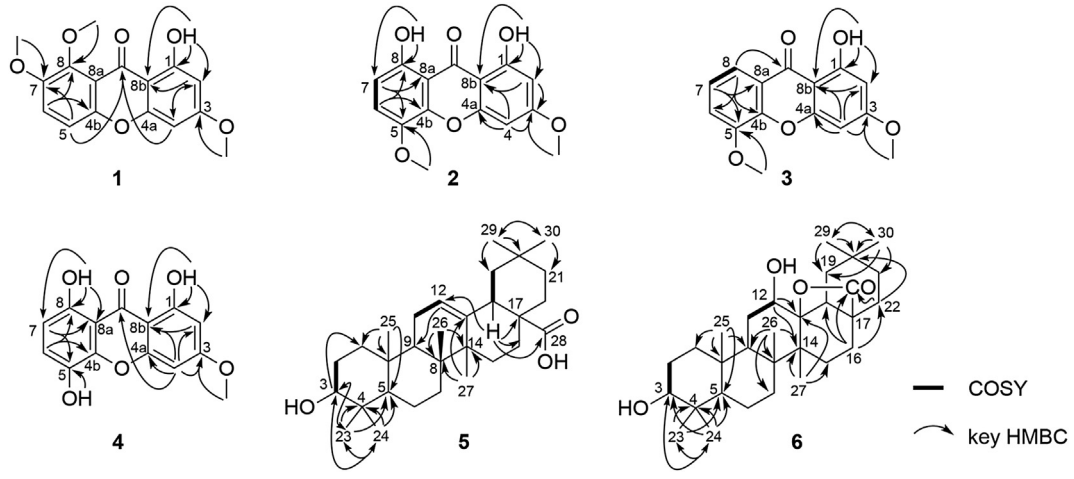


FIG. 2. Key HMBC and COSY correlations of **1**–**6**.

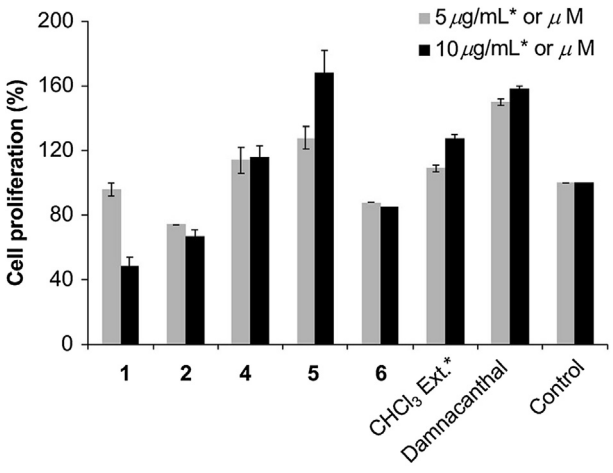


FIG. 3. The inhibitory effects of the $CHCl_3$ extract and isolated compounds of *S. chirata* and the positive control damnacanthal against the expression of Vpr in TREx-HeLa-Vpr cells. Control represents cell viability for tetracycline (+), sample (−) cells.

used as a positive control. The $CHCl_3$ soluble crude extract inhibited the expression of Vpr with an effective dose of 10 μM. Among the tested compounds, only bellidifolin (**4**) and oleanolic acid (**5**) exhibited anti-Vpr activities. Bellidifolin (**4**) and oleanolic acid (**5**) inhibited the expression of Vpr at 5 μM doses [cell proliferation (%): 114 (**4**), 128 (**5**)]. Similar inhibitory effects were observed when the cells were treated with 10 μM doses [cell proliferation (%): 116 (**4**), 168 (**5**)]. In particular, 10 μM oleanolic acid (**5**) exhibited more potent activity, which was comparable to that of the positive control, damnacanthal [cell proliferation (%): 158]. Interestingly, oleanolic acid (**5**) has been reported to be a triterpenoid with antioxidant, antitumor, anti-inflammatory, antidiabetic, and antimicrobial effects (36–40). Furthermore, Kashiwada et al. (41) have reported that **5** inhibited HIV-1 replication in acutely infected H9 cells with an EC_{50} value of 1.7 μg/mL, and inhibited H9 cell growth with an IC_{50} value of 21.8 μg/mL. Our finding of oleanolic acid as a Vpr inhibitor suggested that further studies of the therapeutic applications of **5** and/or its derivatives, as an anti-HIV drug, should be performed.

In addition, naturally occurring xanthones have emerged as an important class of organic compounds, in terms of their remarkable pharmacological and biological activities. In the case of our isolated xanthones, decussatine (**1**), methylswertianin (**2**), and bellidifolin (**4**), only **4** was identified as a Vpr inhibitor with effective doses of 5 and 10 μM. From the standpoint of the structure-activity relationship, the presence of the hydroxy groups at C-5 and C-8 in **4** favored the inhibition of the expression of Vpr, rather than the presence of the methoxy substituents at C-5, C-7, and C-8 in **1** and **2**, which led to a decrease in the activity [cell proliferation (%) at 10 μM dose: 116 (**4**), 49 (**1**), 67 (2)]. Bellidifolin (**4**) is a simple oxygenated xanthone that reportedly possesses strong hypoglycemic activity (42) and monoamine oxidase activity (43). This compound also reportedly inhibits the production of the pro-inflammatory cytokines interleukin-6 (IL-6) and TNF-α and the production of prostaglandin E2 (PGE2), by suppressing the protein expression of cyclooxygenase-2 (COX-2), in LPS-stimulated RAW 264.7 macrophages (44). Treatments with **4** also reportedly suppressed the phosphorylation of the inhibitor κB kinase-β (IKK-β), Akt, and the p65 subunit of nuclear factor-kappaB (NF-κB). Thus, our finding of Vpr inhibition by **4** provides new insight into its bioactivity.

In conclusion, these studies illustrated that a simple oxygenated xanthone, bellidifolin, and a triterpenoid, oleanolic acid, are naturally occurring Vpr inhibitors that are chemically and structurally distinct from the previously reported ones (7–14). In addition to the previously reported naturally occurring Vpr inhibitor, these compounds may not only represent HIV drug seeds, but also could be new target compounds for biotechnological synthesis, due to their anti-Vpr activities.

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