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Triterpenoids from *Schisandra henryi* with Cytotoxic Effect on Leukemia and Hela Cells *In Vitro*

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Four known lanostane triterpenoids, schiprolactone A (**1**), schisanlactone B (**2**), nigranoic acid (**3**) and schisandronic acid (**4**) were isolated from the stems of *Schisandra henryi* for the first time. Their structures were characterized by IR, MS and NMR techniques. Compounds **1**, **2** and **4** showed moderate cytotoxic activity against Leukemia cells *in vitro*. Cytotoxic activity of compounds **1**-**4** showed IC_{50} of 0.0097, 0.01, 0.097 and 0.0099 μmol/mL respectively toward Leukemia cells and IC_{50} of 0.097, 0.1, 0.097 and 0.099 μmol/mL toward Hela cells respectively. It is the first report that these compounds possess cytotoxic activity on Leukemia and Hela cells.

Key words: *Schisandra henryi*, Triterpenoids, Cytotoxic, Schiprolactone A, Schisanlactone B, Schisandronic acid

INTRODUCTION

Plants of Schisandraceae family are rich sources of dibenzocyclooctadiene lignans and lanostane triterpenoid acids and lactones, and have been found to possess beneficial pharmacological effects including hepatoprotective, antitumor and anti-HIV activities (Chen *et al.*, 1999; Chen *et al.*, 2001; Kuo *et al.*, 1999; Kuo *et al.*, 2001; Liu, 1983; Liu *et al.*, 1984; Liu and Li, 1995). Fruits of *Schisandra henryi* Clarke, which belongs to *Schisandra* genus have been used in folk medicine as a substitute of Wu-Wei-Zi, fruits of *S. chinesis* (Yurcz.) Baill, a famous traditional Chinese tonic and sedative for over 2000 years as. Its stems have been used to promote blood circulation and to treat fracture and irregular menstruation (Yunnan Provincinal Crude Drugs Company, 1993). Literature survey showed that two triterpenoid acids (kadsuric acid and schisanhenric acid) and thirteen lignans (schisanhenrin, schisanhenol, deoxyschizandrin, schisantherin B, epienshicine methyl ether, epischisandrone, epiwulignan A_1, wulignan A_1, A_2, schisandrone, enshicine, henricine and enshizhisu) were isolated from the seeds of *S. henryi* (Li & Xue, 1986; Liu *et al.*, 1980, 1984, 1988b; Tao, *et al.*, 1991). Among them, wulignan A_1, A_2, epiwulignan A_1 and epischisandrone showed inhibitory activity against P-388 cell (Liu *et al.*, 1988b). For the finding of further anticancer principles from *S. henryi*, we describe here the isolation and structure identification of four known triterpenoids, which have not been reported from the title plant.

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MATERIALS AND METHODS

General experimental procedure

IR spectra were recorded as KBr pellets on a Perkin-Elmer 599B spectrophotometer. MS were determined on a Varian Mat-711 mass spectrometer. NMR spectra were measured on Bruker AM-400 and DRx-500 spectrometers with TMS as int. standard and $CDCl_3$ as solvent. Silica gel (200-300 mesh) was used for column chromatography and silica gel GF_{254} for TLC (Qingdao Marine Chemical Co., China). Solvents were of the industrial purity and distilled before using.

Plant materials

The raw materials were collected in December 1999, from Tonghai County of Yunnan province, China and iden-

tified by Mr. Bangtao Yue, a botanist of Tonghai Institute of Drugs Control in Yunnan, China, where a voucher specimen (No. 9909013) was deposited.

Extraction and isolation

The dried powdered stems of *S. henryi* (10.7 Kg) were extracted with 95% EtOH (four times, each 20 L) at room temperature. The EtOH extract was evaporated in vacuo to yield a dark brown residue (700 g). H_2O (2.5 L) was added to the residue, and the resulting solution was extracted with petroleum ether, EtOAc and *n*-BuOH successively (four times, each 1.5 L). The EtOAc extract (200g) was applied to a silica gel column, eluting with petroleum ether containing increasing amounts of EtOAc. The fractions obtained from petroleum ether-EtOAc (4:1) elution were combined and subjected to repeated column chromatography to yield pure compounds **1** (80 mg), **2** (70 mg), **3** (10 g), and **4** (2 g). Their structures were identified by spectral data and by comparison with literature values as the known schiprolactone A (Chen *et al.*, 2001a), schisanlactone B (Chen *et al.*, 2001a; Liu *et al.*, 1983), nigranoic acid (Chen *et al.*, 2001b; Sun *et al.*, 1996) and schisandronic acid (Chen *et al.*, 2001b) respectively.

Schiprolactone A (1)

Amorphous powder, mp: 215-217°C. ^{1}H-NMR (500 MHz): δ = 6.68 (1H, d, *J* = 12.2 Hz, H-1), 6.23 (1H, s, H-19), 5.81 (1H, d, *J* = 12.2 Hz, H-2), 4.57 (1H, s, H-22), 2.95 (1H, t like, H-23), 2.13 (3H, s, -$COCH_3$), 1.72 (3H, s, H-21), 1.53 (3H, s, H-28), 1.40 (3H, s, H-29), 1.06 (3H, s, H-30), 0.76 (3H, s, H-18). ^{13}C-NMR (100 MHz): δ = 143.2 (C-1), 117.3 (C-2), 167.4 (C-3), 80.2 (C-4), 49.0 (C-5), 33.5(C-6), 27.4 (C-7), 150.4 (C-8), 128.0 (C-9), 139.3 (C-10), 26.3 (C-11), 30.0 (C-12), 44.7 (C-13), 51.3 (C-14), 31.0 (C-15), 27.3 (C-16), 48.2 (C-17), 17.2 (C-18), 143.5 (C-19), 82.0 (C-20), 25.1 (C-21), 84.5 (C-22), 45.2 (C-23), 32.2 (C-24), 48.3 (C-25), 170.4 (C-26), 32.5 (C-27), 26.4 (C-28), 29.1 (C-29), 27.4 (C-30), 169.9, 21.8 (CH_3COO-). HREIMS: *m/z* 522.2972 (calcd. 522.2981). EIMS: *m/z* 522[M]$^+$ ($C_{32}H_{42}O_6$), 504, 489, 464, 231, 197, 173 (100), 155, 111. All data were identical to that of schiprolactone A (Chen *et al.*, 2001a).

Schisanlactone B (2)

Amorphous powder, mp 204-206°C (lit. mp 205-207°C) (Liu *et al.*, 1983). ^{1}H-NMR (500 MHz): δ = 6.57 (1H, br d, *J* = 6.5 Hz, H-24), 6.09 (1H, d, *J* = 12.7 Hz, H-1), 5.90 (1H, d, *J* = 12.7 Hz, H-2), 4.43 (1H, dt, *J* = 13.4, 3.5 Hz, H-22), 1.88 (3H, s, H-27), 1.36 (3H, s, H-28), 1.34 (3H, s, H-29), 1.19, 1.00 (each 1H, d, *J* = 5.0 Hz, H-19), 0.95 (3H, s, H-18), 0.94 (1H, d, *J* = 6.0 Hz, H-21), 0.86 (3H, s, H-30). ^{13}C-NMR (100 MHz): δ = 150.9 (C-1), 120.1 (C-2), 167.6 (C-3), 84.6 (C-4), 44.9 (C-5), 26.8(C-6), 24.3 (C-7), 48.3 (C-8), 28.7 (C-9), 33.4 (C-10), 34.9 (C-11), 32.3 (C-12), 46.2 (C-13), 48.5 (C-14), 32.1 (C-15), 28.8 (C-16), 47.9 (C-17), 16.9 (C-18), 23.9 (C-19), 39.0 (C-20), 13.1 (C-21), 80.4 (C-22), 23.4 (C-23), 139.4 (C-24), 128.2 (C-25), 165.6 (C-26), 17.1 (C-27), 29.0 (C-28), 22.0 (C-29), 19.8 (C-30). HREIMS: *m/z* 466.3094 (calcd. 466.3082). EIMS: *m/z* 466[M]$^+$ ($C_{30}H_{42}O_4$), 448, 423, 405, 367, 327, 283, 233, 215, 191, 139, 95 (100). All data were identical to that of schisanlactone B (Chen *et al.*, 2001a; Liu *et al.*, 1983).

Nigranoic acid (3)

Colorless crystals, mp 123-124°C (lit. mp 128-130°C) (Sun *et al.*, 1996; Chen *et al.*, 2001b). IR (KBr) cm^{-1}: 2870~3050 (carboxylic acid), 2600, 1700, 1638, 1458, 1415, 1376, 1254, 1218, 1165, 1077, 936, 890. ^{1}H-NMR (400 MHz): δ = 6.08 (1H, t, *J* = 7.2 Hz, H-24), 4.82, 4.73 (each 1H, br s, H-28), 1.90 (3H, s, H-27), 1.68 (3H, s, H-29), 0.97 (3H, s, H-18), 0.95 (3H, s, H-30), 0.89 (3H, d, *J*= 6.4 Hz, H-21), 0.75, 0.40 (each 1H, d, *J* = 4.6 Hz, H-19). ^{13}C-NMR (100 MHz): δ = 31.3 (C-1), 32.9 (C-2), 177.8 (C-3), 149.4 (C-4), 45.7 (C-5), 27.6 (C-6), 24.9 (C-7), 47.6 (C-8), 20.5 (C-9), 27.9 (C-10), 36.0 (C-11), 35.9 (C-12), 45.9 (C-13), 49.3 (C-14), 33.2 (C-15), 28.8 (C-16), 52.0 (C-17), 18.3 (C-18), 30.4 (C-19), 35.7 (C-20), 19.1 (C-21), 27.7 (C-22), 26.9 (C-23), 145.5 (C-24), 126.1 (C-25), 171.6 (C-26), 21.2 (C-27), 111.3 (C-28), 20.9 (C-29), 19.8 (C-30). HREIMS: *m/z* 470.3380 (calcd. 470.3395). EIMS: *m/z* 470[M]$^+$ ($C_{30}H_{46}O_4$), 455, 406, 397, 371, 329, 235, 135, 121, 107, 95 (100). All data were identical to that of nigranoic acid (Chen *et al.*, 2001b; Sun *et al.*, 1996).

Schisandronic acid (4)

Colorless crystals, mp 167-168°C (lit. mp 165-166°C) (Takahashi & Takani, 1975; Chen *et al.*, 2001b). ^{1}H-NMR (500 MHz): δ = 6.07 (1H, t, *J* = 7.2 Hz, H-24), 1.90 (3H, s, H-27), 1.08 (3H, s, H-28), 1.02 (3H, s, H-30), 0.97 (3H, s, H-29), 0.89 (3H, d, *J* = 5.9 Hz, H-21), 0.88 (3H, s, H-18), 0.76, 0.55 (each 1H, d, *J* = 4.6 Hz, H-19). ^{13}C-NMR (100 MHz): δ = 36.1 (C-1), 34.7 (C-2), 218.1 (C-3), 47.6 (C-4), 52.5 (C-5), 19.7 (C-6), 26.6 (C-7), 41.9 (C-8), 37.1 (C-9), 37.2 (C-10), 21.3 (C-11), 31.2 (C-12), 44.8 (C-13), 50.2 (C-14), 31.2 (C-15), 28.1 (C-16), 50.6 (C-17), 16.1 (C-18), 21.3 (C-19), 36.6 (C-20), 18.8 (C-21), 36.3 (C-22), 27.0 (C-23), 147.4 (C-24), 126.2 (C-25), 173.8 (C-26), 20.7 (C-27), 24.5 (C-28), 23.5 (C-29), 21.5 (C-30). HREIMS: *m/z* 454.3447 (calcd. 454.3435). EIMS: *m/z* 454[M]$^+$ ($C_{30}H_{46}O_3$), 439, 421, 381, 355, 342, 313, 301, 235, 217, 175, 149, 135, 109, 95 (100). All data were identical to that of schisandronic acid (Chen *et al.*, 2001b).

Cytotoxicity assay

All compounds were solublized in DMSO (Sigma, St. Louis, MO, USA), and stored at -20°C. Cytotoxicity assays (IC_{50}, μg/mL) were carried out against the cancer cells

murine leukemia (ATCC: CCRF-CEM) and Hela (ATCC-17). Methodology of the *in vitro* cytotoxicity screening was conducted by measuring toxicity against cancer cells using NIH-NCI protocol (Grever *et al.*, 1992; Alley *et al.*, 1988). Cytotoxicity was measured by measuring cell viability.

Hela and Leukemia cell cultures ware grown in MEM containing 10% FBS and 1 mL of an anti-bacterial solution for every 400 mL medium. The culture medium was changed every 3-4 days. A response curve for compounds **1-4** was determined individually through several dilutions (100, 80, 60, 40, 20, 1 and 0.5 μg/mL respectively) of each in a 96-well plate. Four replicates were prepared. Compound solutions were prepared at concentrations of 100, 80, 60, 40, 20, 1 and 0.5 μg/mL in DMSO respectively. In a 96-well plate, 180 μL of cell suspension were added (final count of 10,000 cells/well) and 20 μL of the appropriate compound concentration in DMSO. The cells were incubated with compounds for 24 h, washed with GKN, stained with crystal violet, incubated for ten minutes and immersed in tap water to remove excess crystal violet. The stained proteins were solublized in 1% SDS and analyzed on a 96-well plate reader at 577 nm.

Negative control of the cells alone was also prepared to compare with compounds under investigation. To eliminate the cytotoxicity provided by the solvent (DMSO) used to dissolve the compound, 20 μL of DMSO was added to the negative control. The cells were incubated with the compound for 24 h and stained in the same manner as above.

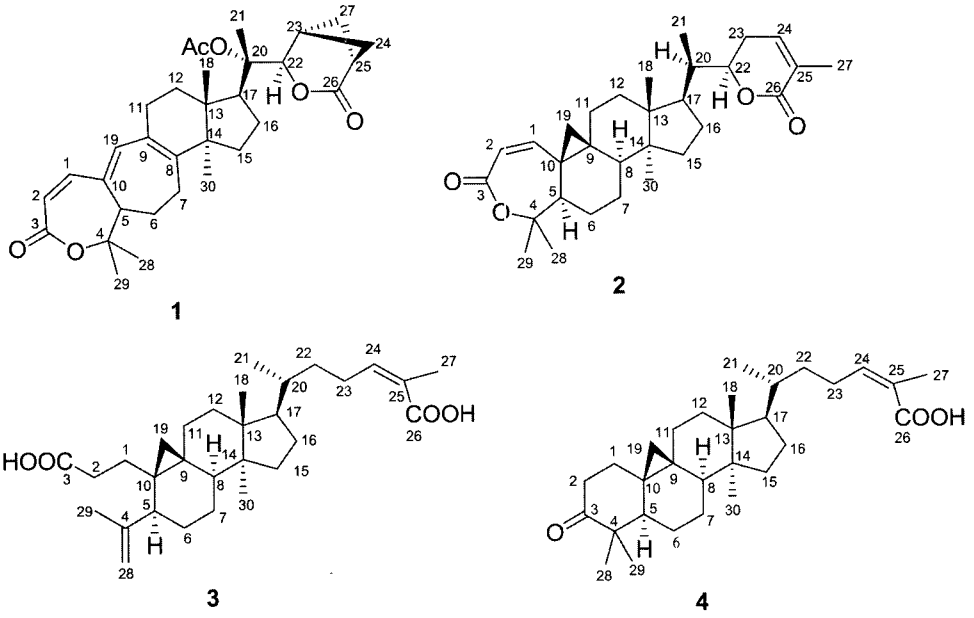
The percent growth of cells from their initial concentration, which is proportional to stained protein, was calculated through the equations shown below; these values were calculated relative to the negative control (cells alone). The equation related each absorbance to the control set run at each concentration to factor out cytotoxicity from solvent effects and compound(s).

$$CG = 100 - \left(\frac{(A_{control} - A_{Ech.})}{A_{control}} \times 100 \right)$$

Where CG is the cell growth values relative to the negative controls (the overall cell growth between compounds and the controls, A_{Ech} is the absorbance of the wells containing compound (s) at the desired concentration, and $A_{control}$ is the absorbances of the cells alone as negative control. These equations directly correlated the absorbance measured of each well to the cell growth during the incubation period.

RESULTS AND DISCUSSION

The stems of *S. henryi* was extracted with 95% EtOH, and then fractionated into petroleum ether, ethyl acetate and *n*-butanol soluble fractions. The ethyl acetate fraction was subjected to repeated column chromatography on silica gel to yield compounds **1-4**. Their structures including stereochemistry were identified by spectral data (^{1}H-NMR, ^{13}C-NMR, MS and IR) and by comparison with literature values as the known schiprolactone A (Chen *et al.*, 2001a), schisanlactone B (Chen *et al.*, 2001a; Liu *et al.*, 1983), nigranoic acid (Chen *et al.*, 2001b; Sun *et al.*, 1996) and schisandronic acid (Chen *et al.*, 2001b) respectively. Compounds **1-3** belongs to 3, 4-seco



Scheme 1. Structure of compounds **1-4** isolated from *Schisandra henryi*

lanostanetriterpenoids. It was the first time that these compounds were isolated from the plant.

It was interesting to note that stems of Schisandrace plants usually contain more lanostane tritepenoid acids and lactones, whereas seeds provide rich sources of lignans especially dibenzocyclooctadiene lignans (Chen *et al.*, 2001; Ikeya *et al.*, 1990; Takahashi and Takani, 1975).

As several lignans from the seeds of *S. henryi* and triterpenoid acids from Schisandrace plants were reported to show anticancer activities (Liu & Pan, 1991; Liu & Huang 1991; Liu *et al.*, 1988a), cytotoxic activity of compounds **1-4** were examined by measuring toxicity against cancer cells *in vitro*. They showed IC_{50} of 5.06, 4.66, 45.6 and 4.50 μg/mL (or 0.0097, 0.01, 0.097 and 0.0099 μmol/mL) against leukemia cells respectively (Fig. 1). Also, they showed IC_{50} of 50.6, 46.6, 45.6 and 45.0 μg/mL (or 0.097, 0.1, 0.097 and 0.099 μmol/mL) against Hela cells, respectively (Fig. 2). Compounds **1**, **2** and **4** were more cytotoxic to Leukemia cells than Hela cells. It is the first report that these compounds possess cytotoxic activity on Leukemia and Hela cells.

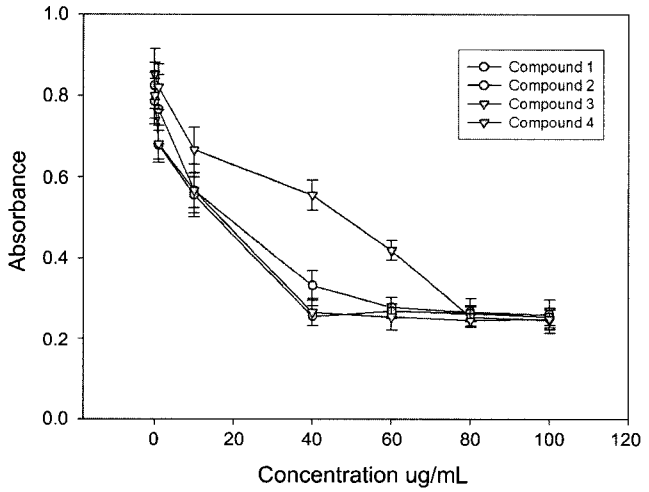


Fig. 1. Effect of compounds **1-4** isolated from *Schisandra henryi* on Leukemia cell culture growth

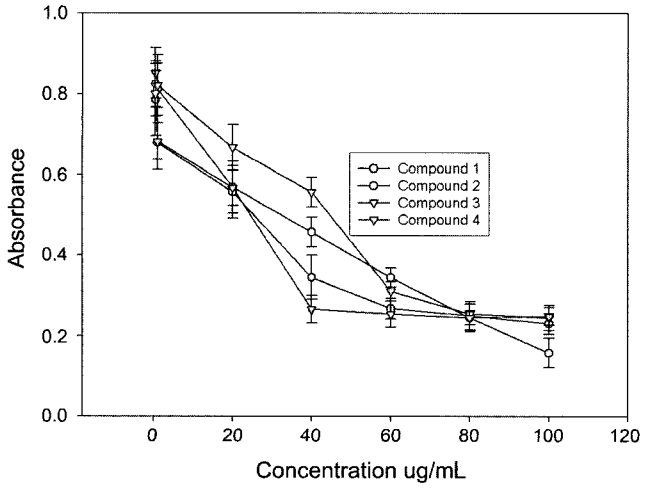


Fig. 2. Effect of compounds **1-4** isolated from *Schisandra henryi* on Hela cell culture growth

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