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RESEARCH ARTICLE

Antifibrotic activity of triterpenoids from the aerial parts of *Euscaphis japonica* on hepatic stellate cells

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Abstract

In the course of screening antifibrotic activity of natural products employing HSC-T6, a rat hepatic stellate cell line, as an *in vitro* assay system, the methanolic extract of aerial parts of *Euscaphis japonica* (Tunb.) Kantiz (Staphyleaceae) showed significant inhibitory activity on HSC proliferation. Activity-guided fractionation led to the isolation of four triterpenoids, friedeline (**1**), glut-5-en-ol (**2**), pomolic acid (**3**), and methylrotundate (**4**). Among the triterpenoids isolated, pomolic acid (**3**) significantly inhibited the proliferation of HSCs at concentrations 10 and 100 μM.

Keywords: Euscaphis japonica*; antifibrotic activity; triterpenoid; HSC-T6; hepatic stellate cells; pomolic acid*

Introduction

Hepatic fibrosis occurs as the consequence of a sustained wound-healing response of liver to toxic, infectious, or metabolic agents, and is characterized by excessive accumulation of extracellular matrix (ECM) leading to ultimate liver dysfunction and irreversible cirrhosis[1]. Hepatic stellate cells (HSCs) play important functions in normal liver, such as retinoid storage, remodeling of ECM, and production of growth factors and cytokines. However, in response to liver damage, HSCs undergo a process of activation, developing a myofibroblast-like phenotype associated with increased proliferation, and/or excessive production and deposition of ECM components, which is the major pathological feature of hepatic cirrhosis[2,3]. Therefore, HSCs are considered to play a key role in the pathogenesis of liver fibrosis, and suppression of HSC activation has been proposed as a therapeutic target against hepatic fibrosis[4,5].

In the course of screening antifibrotic activity of natural products employing HSC-T6, a rat hepatic stellate cell line, as an *in vitro* assay system, the methanolic extract of aerial parts of *Euscaphis japonica* (Tunb.) Kantiz (Staphyleaceae) significantly inhibited the proliferation of HSCs. *E. japonica* has been used in the treatment of inflammation in folk medicine[6]. We previously reported anti-inflammatory compounds from *E. japonica*[7]. In a continuation of our work, we have attempted to isolate the antifibrotic constituents from *E. japonica*. In the present study, we isolated four triterpenoids from *E. japonica*: friedeline (**1**), glut-5-en-ol (**2**), pomolic acid (**3**), and methylrotundate (**4**). Among the triterpenoids isolated, pomolic acid (**3**) significantly inhibited the proliferation of HSCs at concentrations 10 and 100 μM.

Methods and materials

General experimental procedures

^{1}H and ^{13}C nuclear magnetic resonance (NMR) measurements were carried out using a Bruker AMX 400 spectrometer operating at 300 and 100 MHz, respectively. Solvent signals were used as internal standards. ^{1}H–^{1}H COSY (correlation spectroscopy), HMQC (heteronuclear multiple quantum correlation), and HMBC (heteronuclear multiple bond correlation) NMR experiments were performed on the same spectrometer. Electron ionization (EI) mass spectra were obtained on a Jeol JMS 700 spectrometer with a 70 eV ionizing potential. Thin layer chromatography (TLC) and column chromatography were carried out on precoated silica gel F_{254} plates (art. 5715; Merck) and silica gel 60 (230–400 mesh; Merck).

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Plant materials

The aerial parts of *E. japonica* were collected from Mt. Baekwoon (Kwangyang, Korea) in August 2004 and identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University (Busan, Korea). A voucher specimen (SNU-2004-08) has been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Extraction and isolation

The aerial parts of *E. japonica* (8.1 kg) were extracted three times with 80% MeOH in an ultrasonic apparatus. Upon removal of the solvent *in vacuo*, the methanolic extract yielded 1.7 kg. The methanolic extract was suspended in H_2O and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. The *n*-hexane and EtOAc fractions, which significantly inhibited HSC proliferation, were subjected to column chromatography (CC) to isolate active compounds. The *n*-hexane fraction (77 g) was subjected to CC over silica gel eluted with *n*-hexane–EtOAc–MeOH mixture to afford 14 fractions (H1–H14). Compound **1** (137 mg) was obtained from H2 by recrystallization using MeOH. The residual H2 was subjected to silica gel CC with *n*-hexane–EtOAc–MeOH mixture to yield eight fractions (H2-1–H2-8). Compound **2** (1270 mg) was isolated from H2-7 by recrystallization using MeOH. The EtOAc fraction (349 g) was subjected to CC over silica gel eluted with *n*-hexane–EtOAc–MeOH mixture to afford 17 fractions (E1–E17). E7 was subjected to CC over silica gel eluted with *n*-hexane–EtOAc–MeOH mixture to afford seven fractions (E7-1–E7-7). Compound **3** (8470 mg) was obtained from H7-5 by recrystallization using *n*-hexane. E10 was subjected to CC over silica gel eluted with *n*-hexane–EtOAc–MeOH mixture to afford eight fractions (E10-1–E10-8). Compound **4** (844 mg) was obtained from E10-7 by recrystallization using *n*-hexane.

Friedeline (**1**). White amorphous powder. Positive FAB MS *m/z*: 426 $[M]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.20–2.40 (3H, *m*, H-2, 4), 1.11 (3H, *s*, H-28), 0.98 (3H, *s*, H-27), 0.94 (3H, *s*, H-30), 0.88 (3H, *s*, H-29), 0.82 (3H, *d*, *J* = 6.4 Hz, H-23), 0.80 (3H, *s*, H-25), 0.66 (3H, *s*, H-24) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ 213.3 (C-3), 59.4 (C-10), 58.2 (C-4), 53.1 (C-8), 42.8 (C-18), 42.1 (C-5), 41.5 (C-2), 41.3 (C-6), 39.7 (C-13), 35.3 (C-19), 35.0 (C-29), 32.7 (C-21), 32.4 (C-15), 32.1 (C-28), 31.8 (C-30), 30.5 (C-12), 30.0 (C-17), 28.2 (C-20), 22.3 (C-1), 20.2 (C-26), 18.7 (C-27), 18.2 (C-7), 18.0 (C-25), 14.6 (C-24), 6.8 (C-23) ppm.

Glut-5-en-ol (**2**). White amorphous powder. IR ν_{max} (KBr): 3425 (OH), 1635 (C=C-H), 1380, 1360 (geminal dimethyl) cm^{-1}. EIMS *m/z* (rel. int.): 426 $[M]^+$ (14), 411 (10), 408 (10), 274 (100), 205 (65). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.61 (1H, *d*, *J* = 6.3 Hz, H-6), 3.44 (1H, *t*, *J* = 2.8 Hz, H-3), 1.14 (3H, *s*, H-30), 1.12 (3H, *s*, H-24), 1.07 (3H, *s*, H-27), 1.02 (3H, *s*, H-23), 0.98 (3H, *s*, H-28), 0.97 (3H, *s*, H-26), 0.93 (3H, *s*, H-29), 0.83 (3H, *s*, H-30) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 141.6 (C-5), 122.0 (C-6), 76.3 (C-3), 49.7 (C-10), 47.4 (C-18), 43.0 (C-8), 40.7 (C-14), 39.3 (C-4), 38.9 (C-22), 37.8 (C-13), 36.0 (C-16), 35.0 (C-19), 34.8 (C-9), 34.6 (C-29), 34.5 (C-11), 33.1 (C-21), 32.4 (C-28), 32.0 (C-15), 27.7 (C-7), 25.5 (C-24), 23.6 (C-1), 19.6 (C-27), 18.4 (C-27), 18.2 (C-2), 16.2 (C-25) ppm.

Pomolic acid (**3**). White amorphous powder. IR ν_{max} (KBr): 3400 (OH), 1690 (C=O), 1045, 1025 cm^{-1}. EIMS *m/z* (rel. int.): 472 $[M]^+$ (13), 427 (22), 302 (100), 286 (20), 273 (10), 121 (10). ^{1}H-NMR (300 MHz, pyridine-d_5): δ 5.61 (1H, *t*, *J* = 3.6 Hz, H-12), 3.60 (1H, *s*, H-3), 3.06 (1H, *s*, H-18), 1.73 (3H, *s*, H-29), 1.45 (3H, *s*, H-27), 1.23 (3H, *s*, H-25), 1.12 (3H, *s*, H-23), 1.11 (3H, *d*, *J* = 7.2 Hz, H-30), 1.02 (3H, *s*, H-24), 0.91 (3H, *s*, H-26) ppm. ^{13}C-NMR (75 MHz, pyridine-d_5): δ 180.6 (C-28), 139.9 (C-13), 128.0 (C-12), 78.1 (C-3), 72.6 (C-19), 55.8 (C-5), 54.6 (C-18), 48.2 (C-17), 47.7 (C-9), 42.3 (C-20), 42.0 (C-14), 40.3 (C-8), 39.3 (C-4), 38.9 (C-1), 38.4 (C-22), 37.3 (C-10), 33.5 (C-7), 29.2 (C-15), 28.7 (C-23), 28.0 (C-2), 27.1 (C-21), 26.9 (C-29), 26.3 (C-16), 24.6 (C-27), 24.0 (C-11), 18.9 (C-6), 17.1 (C-26), 16.7 (C-24), 16.5 (C-30), 15.5 (C-25) ppm.

Methylrotundate (**4**). White amorphous powder. IR ν_{max} (KBr): 3429 (OH), 2935 (OH), 1692 (C=O), 1236 (COO-), 1160, 1043 (CO), 534 cm^{-1}. Positive FAB MS *m/z*: 525 $[M + Na]^+$, 503 $[M + H]^+$, 471 $[M\text{-}OCH_3]^+$, 453, 395. ^{1}H-NMR (300 MHz, pyridine-d_5): δ 3.87 (1H, *d*, *J* = 10.1, H-23a), 3.16 (1H, *d*, *J* = 10.1, H-23b), 3.97 (3H, *s*, OCH_3), 1.38 (3H, *s*, H-27), 1.09 (3H, *s*, H-29), 0.83 (3H, *s*, H-24), 0.80 (3H, *d*, *J* = 7.2 Hz, H-30), 0.75 (3H, *s*, H-25), 0.68 (3H, *s*, H-26) ppm. ^{13}C-NMR (75 MHz, pyridine-d_5): δ 180.6 (C-28), 140.0 (C-13), 128.0 (C-12), 73.5 (C-3), 72.7 (C-19), 68.0 (C-23), 54.6 (C-18), 52.3 (OCH_3), 48.6 (C-5), 48.6 (C-17), 48.2 (C-9), 42.8 (C-4), 42.3 (C-20), 42.1 (C-14), 40.3 (C-8), 38.8 (C-1), 38.4 (C-22), 37.1 (C-10), 33.2 (C-7), 30.8 (C-15), 27.6 (C-2), 27.0 (C-29), 26.9 (C-21), 26.3 (C-16), 24.6 (C-27), 24.0 (C-11), 18.7 (C-6), 17.2 (C-25), 16.7 (C-26), 15.9 (C-30), 13.0 (C-24) ppm.

Culture of HSC-T6 hepatic stellate cells

An immortalized rat hepatic stellate cell line, "HSC-T6" instead of HSC-T68, was kindly provided by Professor S. L. Friedman (Columbia University, New York). HSC-T6 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 95% air–5% CO_2[9].

Evaluation of antifibrotic activity

Compounds to be tested were dissolved in dimethylsulfoxide (DMSO). Our preliminary study showed that DMSO at a final concentration of 0.1% in media did not affect the cell viability. HSC-T6 cells were treated with vehicle or compounds to be tested for 48 h. β-Glycyrrhetinic acid (Sigma-Aldrich Co.) was used as a positive control. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay. HSC-T6 cells were incubated with 0.5 mg/mL of MTT in the last 2 h of the culture period tested. Reduction of MTT to formazan was assessed using an enzyme-linked immunosorbent assay (ELISA) plate reader.

Statistical analysis

The evaluation of statistical significance was determined by Student's *t*-test with a value of $p < 0.05$ or less considered to be statistically significant.

Results and discussion

Hepatic stellate cells (HSCs) are considered to play a key role in the pathogenesis of liver fibrosis. During liver fibrogenesis, HSCs are activated and acquire a myofibroblast-like phenotype which is accompanied by increased proliferation and ECM synthesis[2,3]. HSC activation *in vivo* can be induced by many factors, such as cytokines and soluble factors derived from damaged hepatocytes and Kupffer cells. HSC activation can also be induced in various conditions *in vitro*. Culturing HSCs on uncoated plastic plates is known to cause spontaneous activation, mimicking the process seen *in vivo*. The HSC-T6 cell line comprises immortalized rat hepatic stellate cells, and has been known to retain all features of activated stellate cells, including expression of desmin, α-smooth muscle actin, and glial fibrillary acidic protein, and it can esterify retinol into retinyl esters[8]. Thus, we evaluated antifibrotic activity employing HSC-T6 cells by assessment of cell viability using the MTT assay.

The methanolic extract of the aerial parts of *E. japonica* was further fractionated into *n*-hexane, EtOAc, and *n*-BuOH fractions. Among them, the *n*-hexane and EtOAc fractions significantly reduced cell viability at the concentration of 100 μg/mL (Figure 1). Therefore, activity-guided fractionation of *n*-hexane and EtOAc fractions was carried out for the isolation of active constituents. Further fractionation and separation by several chromatographic methods yielded two triterpenoids (**1–2**) from the *n*-hexane fraction and two triterpenoids (**3–4**) from the EtOAc fraction. The structures of the triterpenoids were identified as friedeline (**1**), glut-5-en-ol (**2**), pomolic acid (**3**), and methylrotundate (**4**) (Figure 2), by direct comparison of their physicochemical and spectroscopic data with those previously reported[10–14]. Among the four triterpenoids isolated, friedeline (**1**), glut-5-en-ol (**2**), and methylrotundate (**4**) are first reported from this plant.

We further investigated the antiproliferative activity of these triterpenoids in HSC-T6 cells by assessing cell viability using the MTT assay. Among the compounds tested, compound **3** showed the most potent inhibitory activity on HSC cell viability at concentrations of 10 and 100 μM for 48 h incubation (Figure 3). At the concentration of 100 μM, compound **3** inhibited cell viability up to 21%, which is comparable to that of β-glycyrrhetinic acid (GA), a positive control. Compound **4** showed weak activity; however, compounds **1** and **2** showed little activity (Figure 3).

Recently, there has been growing interest in the search for antifibrotic compounds from natural products. As a result, a diverse skeleton of natural products including flavonoids, alkaloids, and terpenoids have been suggested to have antifibrotic activity[15–19]. We previously reported the antifibrotic activity of triterpenoids from *Eclipta prostrata*[9]. In addition, many other triterpenoids have been reported to have antifibrotic activity. Glycyrrhetinic acid and carbenoxolone, a

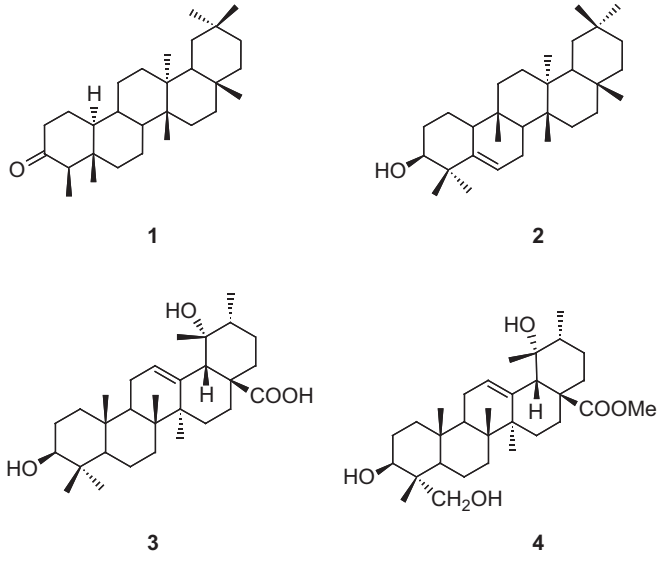

Figure 2. Triterpenoids isolated from the aerial parts of *E. japonica*.

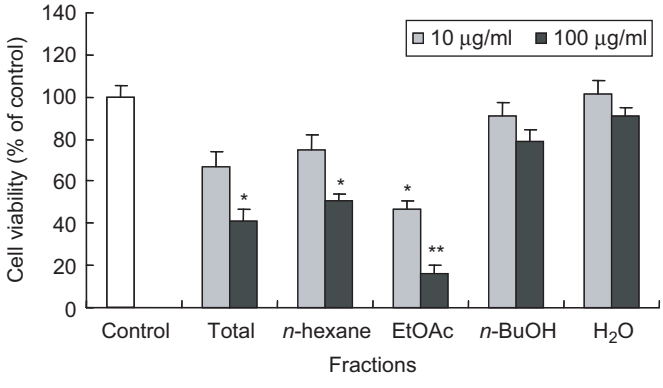

Figure 1. Effect of total methanolic extract and the fractions of *E. japonica* on cell viability of HSC-T6 cells. HSC-T6 cells were incubated with test samples at the concentration of 10 or 100 μg/mL for 48 h. Cell viability was measured by the MTT assay. The percent of cell viability (%) was calculated as 100 × (absorbance of compound-treated/absorbance of control). Results are expressed as the mean ± SD of three independent experiments, each performed using triplicate wells. *$p < 0.05$, **$p < 0.001$ compared with control.

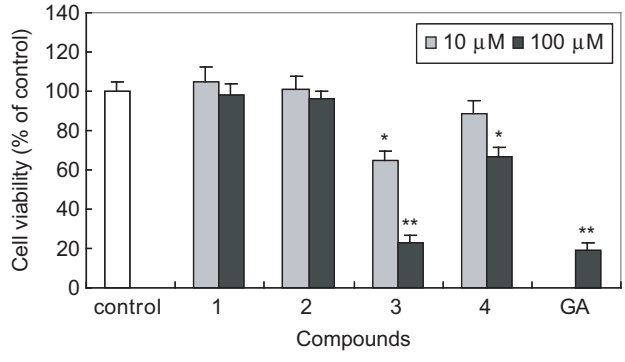

Figure 3. Effect of triterpenoids isolated from *E. japonica* on cell viability of HSC-T6 cells. HSC-T6 cells were incubated with compounds at the concentration of 10 or 100 μM for 48 h. GA (β-glycyrrhetinic acid) was used as a positive control. Cell viability was measured by the MTT assay. The percent of cell viability (%) was calculated as 100 × (absorbance of compound-treated/absorbance of control). Results are expressed as the mean ± SD of three independent experiments, each performed using triplicate wells. *$p < 0.05$, **$p < 0.001$ compared with control.

derivative of glycyrrhetic acid, inhibited HSC proliferation and ECM production[15,20]. Asiatic acid and its derivatives were reported to exert an antifibrotic effect in HSC-T6 cells by reduction of collagen synthesis and cell proliferation[21]. These results together with those from our present study suggest the therapeutic potential of triterpenoids in liver fibrosis.

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