Antiproliferative activity of triterpenoids from Eclipta prostrata on hepatic stellate cells

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Abstract

Hepatic stellate cells (HSCs) have been known to play a key role in the pathogenesis of liver fibrosis. 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 Eclipta prostrata L. showed significant inhibitory activity on HSCs proliferation. Activity-guided fractionation led to the isolation of five oleanane-type triterpenoids, echinocystic acid (1), eclalbasaponin II (2), eclalbasaponin V (3), eclalbasaponin I (4) and eclalbasaponin III (5), which are all echinocystic acid derivatives. Among the five echinocystic acid derivatives isolated, echinocystic acid (1) and eclalbasaponin II (2) significantly inhibited the proliferation of HSCs in dose- and time-dependent manners. Our present study also suggests the importance of free carboxylic acid at C-28 position in echinocystic acid derivatives for the antifibrotic activity. Taken together, antifibrotic activity of E. prostrata and its triterpenoids might suggest the therapeutic potentials against liver fibrosis.

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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 (Friedman, 2003). 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 (Li and Friedman, 1999; Tsukada et al., 2006). 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 (Wu and Zern, 2000; Bataller and Brenner, 2005).

Recently, there is a growing interest in searching for antifibrotic compounds from natural products. As a result, diverse skeleton of natural products including...
flavonoids, alkaloids and terpenoids have been suggested to have antifibrotic activity (Chen et al., 2005; Uyama et al., 2003; Chen and Zhang, 2003; Sakata et al., 2004; Lin et al., 2006).

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 *Eclipta prostrata* L. (Compositae) significantly inhibited the proliferation of HSCs (51.2% of the control at 100 μg/ml, p < 0.05). *E. prostrata* has been used in the treatment of hepatic diseases, hyperlipidemia and snake venom poisoning in folk medicine (Bae, 2000; Ma-Ma et al., 1978). Triterpenes, triterpenoids, coumestanes and flavonoids have been reported as constituents of *Eclipta* species (Singh and Bhargava, 1992; Yahara et al., 1997; Wagner et al., 1986). In addition, *Eclipta* species have been reported to exert diverse biological activity including hepatoprotective, anti-inflammatory, antihemorrhagic, antihyperlipidemic and antihyperglycemic activities (Wagner et al., 1986; Saxena et al., 1993; Melo et al., 1994; Kumari et al., 2006). To date, however, there were no previous studies on antifibrotic activity of *E. prostrata*. Thus, in the present study, we have attempted to isolate the antifibrotic constituents from *E. prostrata*.

**Materials and methods**

**General experimental procedures**

The 1H and 13C NMR measurements were carried out in a Bruker AMX 400 spectrometer operating at 300 and 100 MHz, respectively. Solvent signals were used as internal standards. 1H–1H COSY, HMQC, and HMBC NMR experiments were performed on the same spectrometer. EI-mass spectra were obtained on a JEOL JMS 700 spectrometer with a 70 eV ionizing potential. TLC and column chromatography (CC) were carried out on precoated silica gel F254 plates (art. 5715, Merck), RP-18 F254 plates (art. 15423, Merck), silica gel 60 (230–400 mesh, Merck), Sephadex LH 20 (18–110 μm, Pharmacia Co. Ltd.) and Diaion HP-20 (250–850 μm, Mitsubishishi Chemical).

**Plant material**

The aerial parts of *E. prostrata* were purchased from Kyung-dong Market, Seoul, Korea in June 2004, and identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University. A voucher specimen (SNUPH-EP2004-06) has been deposited in the Herbarium of the Medicinal Herb Garden, College of Pharmacy, Seoul National University.

**Extraction and isolation**

The aerial parts of *E. prostrata* (9 kg) were extracted 3 times with 80% MeOH, which yielded the methanolic extract (831 g). The methanolic extract was suspended in H2O and partitioned successively with n-hexane, CHC13, EtOAc and n-BuOH. The CHC13 fraction (77 g) was subjected to CC over silica gel eluted with n-hexane–EtOAc step gradient to give 11 fractions (C1–C11). C9 was subjected to CC over Sephadex LH-20 eluted with CH2Cl2–MeOH (1:1) mixture to afford 3 fractions (C9-1 to C9-3). Fr. 9-1 was applied on CC over Sephadex LH-20 eluted with n-hexane–CH2Cl2–MeOH (5:5:1) mixture to give 4 fractions (C9-1-1 to C9-1-4). Compounds 1 (18.2 mg) was obtained from C9-1-4 in crystallized form. The n-BuOH fraction (138 g) was subjected to CC over HP-20 eluted with 0%, 20%, 40%, 60%, 80% and 100% MeOH to give 6 fractions (B1–B6). B5 was subjected to CC over silica gel eluted with CHC13–MeOH–H2O step gradient to give 10 fractions (B5-1 to B5-10). Compound 2 (403.2 mg) was obtained from B5-4 in crystallized form. B4 was subjected to CC over silica gel eluted with CHCl3–MeOH–H2O step gradient to give 11 fractions (B4-1 to B4-11). B4-11 was rechromatographed over silica gel eluted with CHCl3–MeOH–H2O step gradient to give 11 fractions (B4-11-1 to B4-11-11). Compounds 3 (49.6 mg) and 5 (214.6 mg) were obtained from B4-11-8 and B4-11-10, respectively. B4-9 was subjected to CC over silica gel eluted with CHCl3–MeOH–H2O step gradient to give 8 fractions (B4-9-1 to B4-9-8). B4-9-3 was rechromatographed over Sephadex LH-20 CC eluted with MeOH to give 5 fractions (B4-9-3-1 to B4-9-3-5). Compound 4 (140.8 mg) was obtained from B4-9-3-1.

**Culture of HSC-T6 hepatic stellate cells**

An immortalized rat hepatic stellate cell line, HSC-T6 was kindly provided by Prof. SL Friedman (Columbia University, New York). HSC-T6 cells were maintained in 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% CO2.

**Measurement of cell viability**

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 or as indicated. 18β-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 in an ELISA plate reader.

**Statistical analysis**

The evaluation of statistical significance was determined by the Student’s t-test with a value of \( p < 0.05 \) or less considered to be statistically significant.

**Results**

**Isolation of triterpenoids from *E. prostrata***

The methanolic extract of the aerial parts of *E. prostrata* was further fractionated into \( n \)-hexane, CHCl\(_3\), EtOAc and \( n \)-BuOH fractions. Among them, CHCl\(_3\) and \( n \)-BuOH fraction significantly reduced cell viability at a concentration of 100\( \mu \)g/ml (50.4% and 40.1% of the control, respectively, \( p < 0.01 \)). Therefore, activity-guided fractionation of CHCl\(_3\) and \( n \)-BuOH fractions was carried out for the isolation of active constituents. Further fractionation and separation by several chromatographic methods yielded one triterpene (1) from CHCl\(_3\) fraction and four triterpene glycosides (2-5) from \( n \)-BuOH fraction. The structures of five triterpenoids were identified as echinocystic acid (1), eclalbasaponin II (2), eclalbasaponin V (3), eclalbasaponin I (4) and eclalbasaponin III (5) (Fig. 1), by the direct comparison of their physicochemical and spectroscopic data with those of previously reported (El-Seedi, 2005; Yahara et al., 1994, 1997). All the five triterpenoids isolated from *E. prostrata* have echinocystic acid moiety, an oleanane-type pentacyclic triterpenoid, as an aglycone and only differ in the substitutions at C-3 and C-28 positions. Compound 1 is an aglycone form and compounds 2, 4 and 5 have one, two and three glucose moieties, respectively. Compound 3, interestingly, has a sulfate group, which is a characteristic of *Eclipta* species. All these five compounds are first reported from this plant.

**Antiproliferative activity of triterpenoids isolated from *E. prostrata***

We investigated the antiproliferative activity of these triterpenoids in HSC-T6 cells by assessing the cell viability using MTT assay. Among the compounds tested, compound 2 showed most potent inhibitory activity on HSC cell viability at a concentration of 100\( \mu \)M for 48 h incubation, which followed by compound 1 (Fig. 2). Other three triterpenoids (3-5), however, showed weak activity. Our present study also showed that compounds 1 and 2 decreased the HSC proliferation in dose- and time-dependent manners (Fig. 3).

Since compounds 1 and 2 exerted potent inhibitory activity on HSC proliferation, we further observed the cell morphology under a phase-contrast microscope. As shown in Fig. 4, HSCs cultured in the absence of compounds exhibited flattened and membranous processes, representing myofibroblastic morphology. However, the morphology of HSCs treated with compounds 1 or 2 was changed to slender cell shape at concentrations ranging from 30 to 100\( \mu \)M (Fig. 4).

**Discussion**

Hepatic stellate cells (HSCs) are considered to play a key role in the pathogenesis of liver fibrosis. During liver
Fig. 2. Effect of triterpenoids isolated from *E. prostrata* on cell viability in HSC-T6 Cells. HSC-T6 cells were incubated with compounds at a concentration of 100 µM for 48 h. 18β-Glycyrrhetinic acid (GA) was used as a positive control. Cell viability was measured by the MTT assay. The percent 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 < 0.01, ***p < 0.001 compared with control.

Fig. 3. Concentration- and time-dependent effects of compounds 1 and 2 on cell viability in HSC-T6 cells. HSC-T6 cells were incubated compounds 1 or 2 at 100 µM for indicated time (A), or at the concentrations ranging from 10 to 100 µM for 48 h (B). Cell viability was measured by the MTT assay. The percent 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 < 0.01, ***p < 0.001 compared with control.
A triterpenoid, as an aglycone moiety and only differ in the substitutions in 3 and 28 positions. Compound 1, an aglycone form, showed antiproliferative activity and addition of a glucose moiety to C-3 hydroxyl group increased antiproliferative activity, as shown in compound 2. However, addition of glucose to C-28 carboxylic acid group showed the different pattern on antiproliferative activity. Compounds 2 and 4 have an identical structure except a glucose moiety at C-28 position, but only compound 2 showed potent antiproliferative activity, suggesting that the free carboxylic acid moiety at C-28 position in echinocystic acid skeleton might be important for exerting the antiproliferative activity. Compound 5, which possesses a glucose moiety at C-28 carboxylic acid, also showed weak activity, which supported our postulation. Interestingly, compound 3, which has an identical structure to compound 2 except a sulfate group at C-2 glucose moiety at C-3, showed weak activity. Taken together, although the structure–activity relationship was not conclusively demonstrated with these results, our present study suggested the importance of free carboxylic acid at C-28 in echinocystic acid derivatives in HSC-T6 cells.

In summary, we isolated five triterpene derivatives with echinocystic acid skeleton from *E. prostrata* and evaluated their antiproliferative activity together with their structure–activity relationship using HSC-T6 cells. Among the five echinocystic acid derivatives isolated, echinocystic acid (1) and eclalbasaponin II (2) showed significant antiproliferative activity in dose- and time-dependent manner. Our results also suggest the importance of free carboxylic acid at C-28 position in echinocystic acid derivatives for the antiproliferative activity. Thus, it will be of interest to test further whether these triterpenoids exert antifibrotic effects in vivo, for example, in animal models of liver fibrosis, to explore their therapeutic potentials. This will provide further insight into the design of new approaches to liver fibrosis.

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**References**


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**Fig. 4.** Effects of compounds 1 and 2 on cell morphology in HSC-T6 cells. HSC-T6 cells were incubated with vehicle (control), compound 1 (30 and 100 μM, respectively) and compound 2 (30 and 100 μM, respectively) for 48 h. Cells were observed with phase-contrast microscope (original magnification × 100).