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Aerial Parts and Roots of *Pulsatilla koreana* Affect the Viability of HSC-T6 Hepatic Stellate Cells

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Abstract – During liver fibrosis, hepatic stellate cells (HSCs) undergo a complex activation process characterized by increased proliferation and extracellular matrix deposition, which is the major pathological feature of hepatic cirrhosis. Therefore, suppression of HSCs activation has been proposed as therapeutic strategies for hepatic fibrosis. We tried to screen the antifibrotic activity of natural products employing HSC-T6, hepatic stellate cell lines as an *in vitro* assay system. In the present study, we investigated the antiproliferative activity of aerial parts and roots of *Pulsatilla koreana* Nakai (Ranunculaceae). Our present study shows that roots of *P. koreana* exerted more strong inhibitory activity compared to its aerial parts. In addition, among the fractions of the aqueous methanolic extract of *P. koreana* roots, both *n*-hexane and $CHCl_3$ fraction showed the strong inhibitory activity on HSC proliferation. Further study also demonstrated that the *n*-hexane and $CHCl_3$ fraction of *P. koreana* roots significantly inhibited the HSC proliferation in time- and concentration-related manners.
Keywords – *Pulsatilla koreana*, Ranunculaceae, antifibrotic, HSC-T6, hepatic stellate cells, viability

Introduction

Liver fibrosis is a wound-healing response to various chronic liver injuries caused by toxic, infectious or metabolic agents. An early event in the development of hepatic fibrosis is the activation of hepatic stellate cells (HSCs). HSCs play important functions in normal liver, such as retinoid storage, remodeling of ECM (extracellular matrix) and production of growth factors. During liver fibrosis, HSCs undergo a complex activation process characterized by increased proliferation and extracellular matrix deposition, which is the major pathological feature of hepatic cirrhosis (Li and Friedman, 1999; Tsukada *et al*., 2006). Therefore, suppression of HSC activation and proliferation, and induction of apoptosis in activated HSCs have been proposed as therapeutic strategies for the treatment and prevention of hepatic fibrosis (Wu and Zern, 2000; Bataller and Brenner, 2005)

Though severe problems in health, no effective anti-fibrogenic therapy is available for the treatment of fibrosis in chronic liver diseases. Recently, there is a growing interest in searching for anti-fibrotic compounds, especially from natural products. Natural products have been used in the treatment of various diseases for a long time, especially in Asian country. Related to liver fibrosis, diverse skeleton of natural products including flavonoids, lignans, alkaloids and terpenoids have been suggested to have anti-fibrotic activity (Uyama *et al*., 2003; Sakata *et al*., 2004; Park *et al*., 2005; Lee *et al*., 2008, 2009). Therefore, there is a growing interest in searching for antifibrotic compounds from natural products with lower adverse effects.

Pulsatilla koreana Nakai belongs to the family Ranunculaceae and is an endemic species in Korea. Previous phytochemical studies of *Pulsatilla* species have reported the isolation of diverse triterpenoids and saponins, and their antitumor activity (Mimaki *et al*., 1999; Bang *et al*., 2005a, 2005b). Interestingly, various parts of *P. koreana* have been used to treat different diseases in traditional medicine. For example, the roots of *P. koreana* have been used for blood-cooling and detoxifying effects. The flowers of *P. koreana* have been used for the treatment of smallpox and leaves for edema (Bae, 2000). Natural products have been used as different parts considering diverse conditions such as toxicity, efficacy, yield and etc. Recently, the constituents and biological activity of each part of same plants are reported to be somewhat different in many cases (Kim *et al*., 2008). Therefore, we investigated and compared the antifibrotic activity of aerial parts and roots of *P. koreana*, employing

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HSC-T6, a rat hepatic stellate cell line as an *in vitro* assay system. We have also attempted to evaluate antifibrotic activity of *P. koreana*.

Methods and materials

Plant material – The aerial parts and roots of *P. koreana* were collected from Gyunggi province, Korea in April 2008. They were identified by the herbarium of College of Pharmacy at Chungbuk National University, where a voucher specimen was deposited (CBNU200804-PK)

Extraction and isolation – The dried roots of *P. koreana* (200 g) were extracted 3 times with 80% MeOH, which yielded the methanolic extract (46.1 g). The methanolic extract was then suspended in H_2O and partitioned successively with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH to yield *n*-hexane (4.6 g), $CHCl_3$ (0.7 g), EtOAc (1.2 g), *n*-BuOH (18.0 g) and aqueous fraction, respectively. The dried aerial parts of *P. koreana* (150 g) were extracted and partitioned as described above, which yielded the methanolic extract (15.9 g), *n*-hexane (0.8 g), $CHCl_3$ (0.3 g), EtOAc (0.4 g), *n*-BuOH (1.5 g) and aqueous fraction, respectively.

Culture of HSC-T6 hepatic stellate cells – An immortalized rat hepatic stellate cell line, HSC-T6 was kindly provided by Prof. SL Freidman (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% CO_2.

Measurement of cell viability – Samples 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 samples to be tested for 48 hr or as indicated. 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 hr 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 and discussion

Although liver fibrosis has been regarded as irreversible, recent studies in animal models and patients have suggested that hepatic fibrosis is, at least to some degree, a reversible process (Iredale *et al*., 1998; Issa *et al*., 2004). During fibrosis, HSCs undergo a complex activation process characterized by increased proliferation and extracellular matrix deposition, which is the major pathological feature of hepatic cirrhosis (Li and Friedman, 1999; Tsukada *et al*., 2006). Elimination of the activated HSCs has been linked to the reversal of liver fibrosis and treatments that induced HSC apoptosis and/or reduced proliferation are currently under investigation as the potential treatment for liver fibrosis. Recently, there is a growing interest in searching for antifibrotic compounds, especially from natural products. We have tried to search for antifibrotic compounds from natural resources

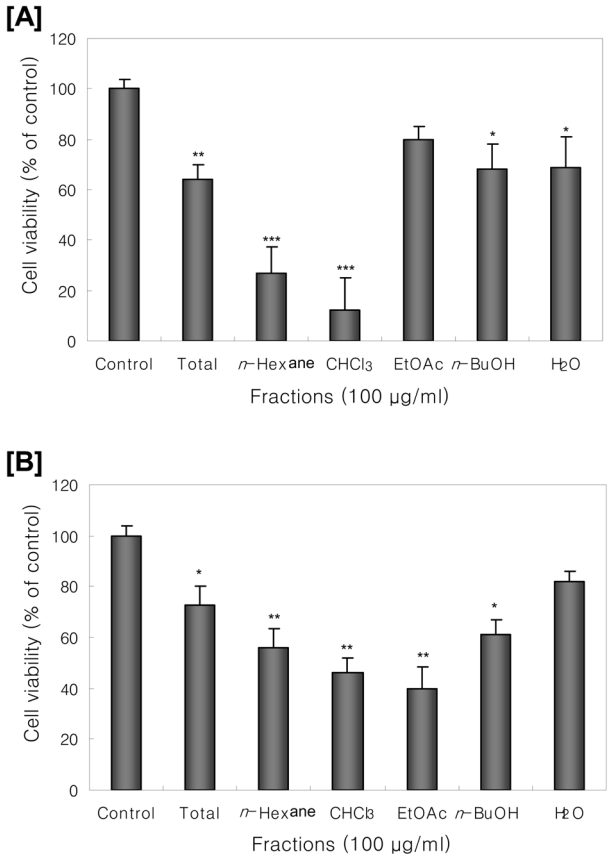

Fig. 1. Effects of total methanolic extract and each fraction of roots (A) and aerial parts (B) of *P. koreana* on cell viability in HSC-T6 cells.

HSC-T6 cells were incubated with 100 μg/ml each fraction for 48 hr. Cell viability was measured by the MTT assay. The percent of cell viability (%) was calculated as 100 × (absorbance of sample-treated / absorbance of control). Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control.

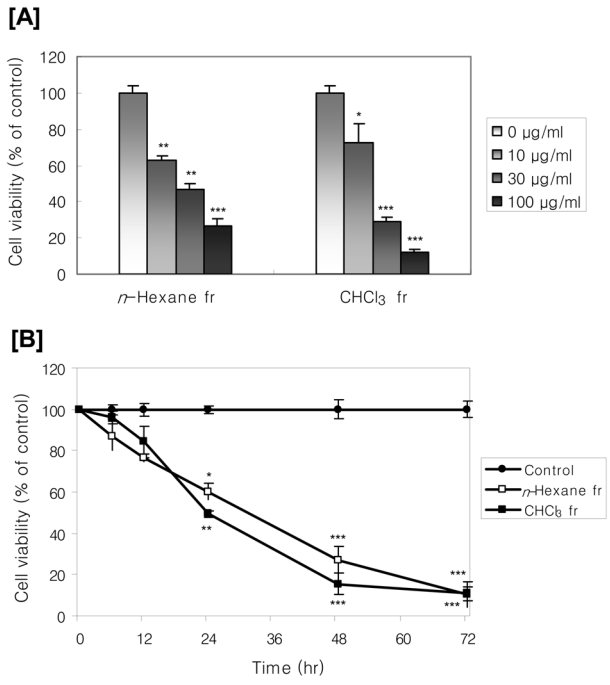

Fig. 2. Concentration- and time-dependent effects of *n*-hexane and $CHCl_3$ fraction of *P. koreana* roots on cell viability in HSC-T6 cells.

HSC-T6 cells were incubated with *n*-hexane and $CHCl_3$ fraction at the concentrations ranging from 10 to 100 μg/ml for 48 hr (A), or 100 μg/ml for indicated time (B). Cell viability was measured by the MTT assay. The percent of cell viability (%) was calculated as 100 × (absorbance of sample-treated / absorbance of control). Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control.

employing HSC-T6, a rat hepatic stellate cell line as an *in vitro* assay system.

Pulsatilla koreana Nakai (Ranunculaceae) is widely distributed in Korea and various parts of *P. koreana,* such as roots, flowers and leaves have been used to treat different diseases (Bae, 2000). Therefore, we attempted to investigate and compare the antifibrotic activity of aerial parts and roots of *P. koreana*, employing HSC-T6, a rat hepatic stellate cell line as an *in vitro* assay system. The aerial parts and roots of *P. koreana* were extracted with 80% MeOH, respectively. The methanolic extract was further fractionated into *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH fractions and tested for their antiproliferative activity. Both methanolic extract of aerial parts and roots of *P. koreana* significantly inhibited HSC proliferation. However, effective fraction of aerial parts and roots of *P. koreana* was different in our study. In case of roots of *P. koreana*, nonpolar fractions such as *n*-hexane and $CHCl_3$ fraction showed strong activity whereas polar fractions such as EtOAc, *n*-BuOH and aqueous fraction showed weak activity (Fig. 1A). In case of aerial parts of *P. koreana*, EtOAc fraction showed strong activity, followed by *n*-hexane and $CHCl_3$ fraction (Fig. 1B). Among them, *n*-hexane and $CHCl_3$ fraction of *P. koreana* roots showed strong inhibitory activity on HSCs proliferation (Fig. 1). Therefore, we further evaluated the antifibrotic activity of *n*-hexane and $CHCl_3$ fraction of *P. koreana* roots. Our present study also showed that both *n*-hexane and $CHCl_3$ fraction of *P. koreana* roots decreased the HSC proliferation in dose- and time-dependent manners (Fig. 2).

Since *n*-hexane and $CHCl_3$ fraction of *P. koreana* roots exerted potent inhibitory activity on HSC proliferation, we further observed the cell morphology under a phase contrast microscope. The time-dependent antiproliferative activity of *n*-hexane or $CHCl_3$ fraction was confirmed by cell morphology (Fig. 3A). Interestingly, *n*-hexane or $CHCl_3$ fraction showed differential effects on cell morphology as concentration increased (Fig. 3B). HSCs cultured in the absence of compounds exhibited flattened and membranous processes, representing myofibroblastic morphology. However, the morphology of HSCs was changed by the treatment with either *n*-hexane or $CHCl_3$ fraction. HSC-T6 cells treated with 10 μg/ml *n*-hexane or $CHCl_3$ fraction showed slender cell shape. However, HSC-T6 cells treated with 100 μg/ml *n*-hexane or $CHCl_3$ fraction showed the morphological feature of necrosis such as membrane breakdown and lysis. Generally, elimination of HSC can be achieved by various pathways, such as inhibition of cell proliferation and/or induction of cell death. Our data about cell morphology under microscope suggests the differential effect of *n*-hexane and $CHCl_3$ fractions as the concentration increased. The *n*-hexane and $CHCl_3$ fraction of *P. koreana* might reduce HSC proliferation by interference in cell proliferation at low concentration, whereas reduce HSC proliferation in part by necrosis. The exact mechanism needs to be clarified by further study.

In summary, both aerial parts and roots of *P. koreana* showed significant antiproliferative activity, although roots exerted better activity. We also demonstrated that *n*-hexane and $CHCl_3$ fraction of the roots of *P. koreana* inhibited HSC proliferation in dose- and time-dependent manner. In addition, cell morphology under microscope suggests the differential effect of *n*-hexane and $CHCl_3$ fractions as the concentration increased. Liver is composed of several cell types including hepatocytes and HSCs. Therefore, further investigation for the evaluation of the effects on hepatocytes and on animal model of liver fibrosis might be needed for their therapeutic antifibrotic potentials.

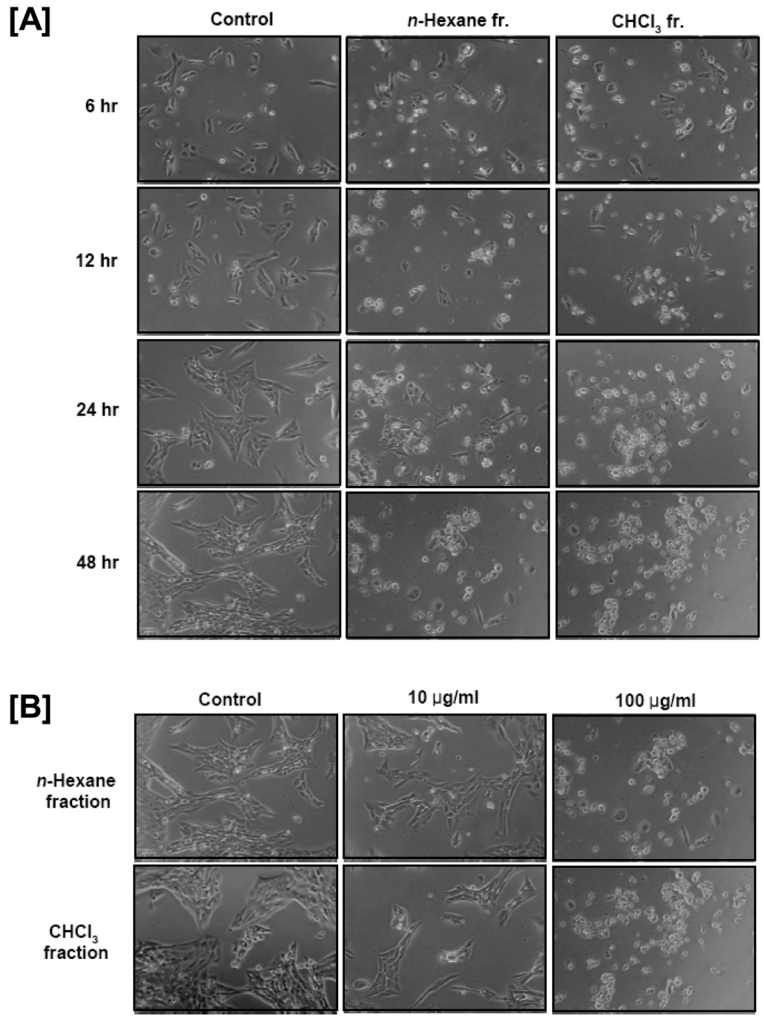

Fig. 3. Effects *n*-hexane and $CHCl_3$ fraction of *P. koreana* roots on cell morphology in HSC-T6 cells.
HSC-T6 cells were incubated with 100 μg/ml *n*-hexane and $CHCl_3$ fraction for indicated time (A) or incubated with 10 or 100 μg/ml *n*-hexane and $CHCl_3$ fraction for 48 hr (B). Cells were observed with phase contrast microscope (original magnification ×100).

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