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Anti-hepatotoxic Activity of Icariside II, a Constituent of *Epimedium koreanum*

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Icariside II, a flavonol glycoside, was isolated from the aerial part of *Epimedium koreanum* Nakai by the anti-hepatotoxic activity guided fractionation technique employing CCl_4-intoxicated primary cultured rat hepatocytes as an assay system. Its anti-hepatotoxic activity was evaluated by measuring activity of glutamic pyruvic transaminase released from the CCl_4-intoxicated primary cultured rat hepatocytes. Icariside II significantly reduced the activity of glutamic pyruvic transaminase released from the CCl_4-intoxicated primary cultured rat hepatocytes and resulted in 78% recovery of the toxicity at the concentration of 200 μM. The anti-hepatotoxic activity of icariside II on the CCl_4-intoxicated primary cultured rat hepatocytes was as potent as that of silybin.

Key words : Anti-hepatotoxic activity, Icariside II, CCl_4, *Epimedium koreanum*, Glutamic pyruvic transaminase, Primary cultured rat hepatocytes.

INTRODUCTION

We have been employing primary cultures of rat hepatocytes acutely intoxicated by CCl_4 as a system to screen natural products for potential hepato-protective agents. In searching for such products, we found that a methanolic extract of *Epimedium koreanum* Nakai (Berberidaceae) exhibited a significant anti-hepatotoxic activity against CCl_4-induced cytotoxicity. The aerial part of *E. koreanum* has been used as a tonic as well as a treatment for impotence and forgetfulness in Oriental medicine (Tang and Eisenbrand, 1992).

A number of studies have been conducted to elucidate chemical constituents (Mizuno *et al.*, 1987; Ito *et al.*, 1988; Kang *et al.*, 1990; Mizuno *et al.*, 1990) and pharmacological activities (Munekazu *et al.*, 1990) of the aerial part of this plant. However, no precise correlation between particular components of the plant extract and pharmacological activities has been elucidated. Moreover, heretofore, no note has been made of any anti-hepatotoxic activity of constituents of this plant. The present work was initiated to isolate and identify specific anti-hepatotoxic constituents from the aerial part of *E. koreanum* using cultured rat hepatocytes intoxicated by CCl_4 as a screening system.

In the present communication, we report that fractionation of *E. koreanum* coupled with screening by *in vitro* anti-hepatotoxic assay resulted in the isolation of a flavonol glycoside, icariside II. Icariside II was found to significantly reduce the level of glutamic pyruvic transaminase (GPT) released from primary cultured rat hepatocytes acutely intoxicated by CCl_4.

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MATERIALS AND METHODS

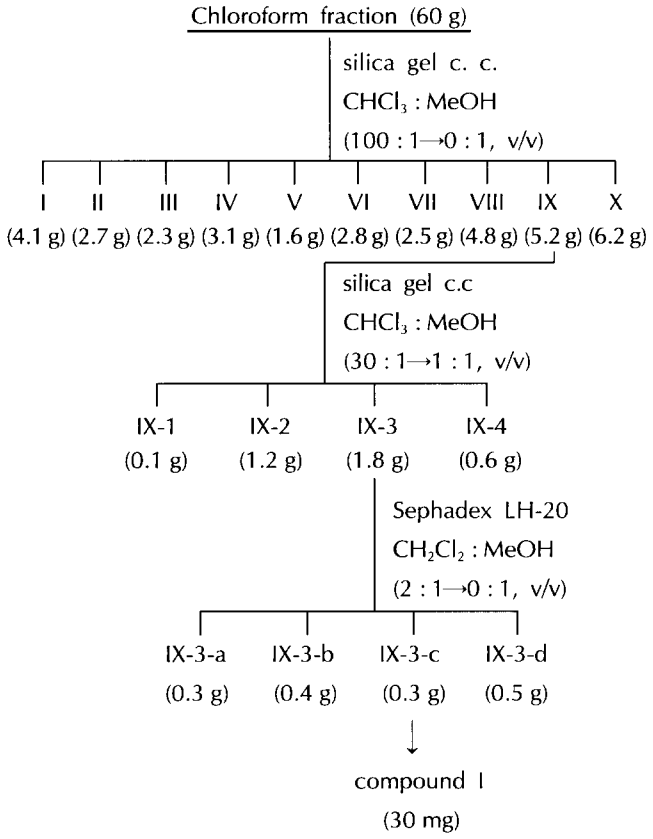
Plant material

Aerial part of *E. koreanum* was collected from Kyounggi province of Korea and authenticated by Dr. Dae S. Han, *Professor Emeritus*, College of Pharmacy, Seoul National University. Voucher specimen documenting these collections has been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Isolation of compound I

Dried plant materials (3.0 kg) were extracted 5 times for 1 hr with 80% MeOH in an ultrasonic apparatus. The resulting methanol extract was concentrated by vacuum at 40°C to obtain residue (202 g), which then was suspended in water and partitioned with *n*-hexane, $CHCl_3$ and *n*-BuOH successively to yield a *n*-hexane fraction (21 g), a $CHCl_3$ fraction (60 g) and a *n*-BuOH fraction (73.7 g) (Scheme 1).

The $CHCl_3$ fraction which showed a significant anti-



Scheme 1. Chromatographic separation of chloroform fraction of *E. koreanum*.

hepatotoxic activity was chosen to isolate active components. The $CHCl_3$ fraction was subjected to silica gel column chromatography and eluted using mixture of $CHCl_3$: MeOH increasing polarity (100 : 1→0 : 1) to give 10 fractions (Fr. 1 to 10). Fr. 9 (5.2 g) was rechromatographed over silica gel column and eluted with the mixture of $CHCl_3$: MeOH (30 : 1→1 : 1) and followed by Sephadex LH-20 column chromatography eluted with the mixture of CH_2Cl_2 : MeOH (2 : 1→0 : 1) to yield compound I (30 mg). The physical properties of compound I are indicated below.

Compound I: Yellow needle from MeOH : m.p. 205-206°C, $[\alpha]_D^{16}$-121° (C, 0.09, MeOH), UV λ_{max} (MeOH) (log ε) : 272 (4.33), 298 (4.30), 350 (3.90) IR ν_{max} (KBr) 3200 (O-H), 2850 (C=O), 1650 cm^{-1} (chelated carbonyl group); ^{1}H-NMR (400 MHz, CD_3OD) 0.93 (3H, d, rhamnosyl -CH_3), 1.69 and 1.74 (6H, br.s, 14, 15-CH_3), 3.90 (3H, s, OCH_3), 3.78-5.40 (m, sugar protons), 6.32 (1H, s, H-6), 7.05 (2H, d, J=9Hz, H-2', 5'), 7.87 (2H, J=9Hz, H-3', 6'), 12.52 (1H, s, C-5 OH) ppm; ^{13}C-NMR (125 MHz, CD_3OD) δ 17.7 (C-6"), 18.1 (C-15), 22.4 (C-11), 25.8 (C-14), 56.0 (OMe), 71.9 (C-5"), 72.1 (C-2"), 72.2 (C-3"), 73.5 (C-4"), 99.4 (C-6), 103.5 (C-1"), 106.0 (C-8), 107.9 (C-10), 115.1 (C-3', 5'), 123.7 (C-12, 1'), 132.4 (C-13), 132.5 (C-2', 6'), 136.3 (C-3), 155.8 (C-2), 160.8 (C-4'), 163.2 (C-7), 163.3 (C-5), 179.9 (C-4), FAB-MS 515 ([M+H], 15.0) 369 (100.0) 313 (60.1) 135 (41.8).

Culture of hepatocytes

Isolated hepatocytes were prepared from male Wistar rats by the collagenase perfusion technique of Berry and Friend with minor modification (Berry and Friend, 1969; Berry *et al.*, 1991)

Culture condition

The cell suspension was diluted to 5.0×10^5 cells/ml in the culture medium consisted of Waymouth MB 752/1 medium supplemented with 5% fetal bovine serum, 2.0 mg/ml bovine serum albumin (fraction V), 10^{-6}M dexamethasone, 10^{-7}M insulin, 5.32×10^{-2} M L-serine, 4.09×10^{-2} M L-alanine, 2.67×10^{-2} M $NaHCO_3$, 10,000 IU/100 ml penicillin, 10,000 IU/100 ml streptomycin and 500 μg/100 ml amphotericin B. Cells were inoculated onto collagen-precoated culture dishes and were incubated at 37°C in a humidified incubator gassed with 5% CO_2/95% air.

Carbon tetrachloride exposure

One day after the isolated rat hepatocytes were plated, the cultured hepatocytes were exposed to a medium (1.0 ml) containing 0.01 ml of 1 M ethanolic CCl_4 for 1.5 hr to induce cytotoxicity (Kiso *et al.*, 1983).

Screening anti-hepatotoxic activity

In order to screen the anti-hepatotoxic activity of each fraction of *E. koreanum*, aliquots of each fraction were lyophilized and dissolved in dimethylsulfoxides (DMSO, final concentration of 0.1%). Icariside II was also dissolved in DMSO (final concentration of 0.1%). Anti-hepatotoxicity was determined by measuring activity of GPT in the culture medium.

Measurement of glutamic pyruvic transaminase activity

The activity of GPT in the culture medium was determined by the method of Reitman-Frankel (Reitman and Frankel, 1957) using an assay kit.

Statistical analysis

The evaluation of statistical significances was determined by "ANOVA" test.

RESULTS AND DISCUSSION

The methanolic extract of *E. koreanum* which showed intense anti-hepatotoxic activity was fractionated by monitoring its activity in CCl_4-intoxicated hepa-

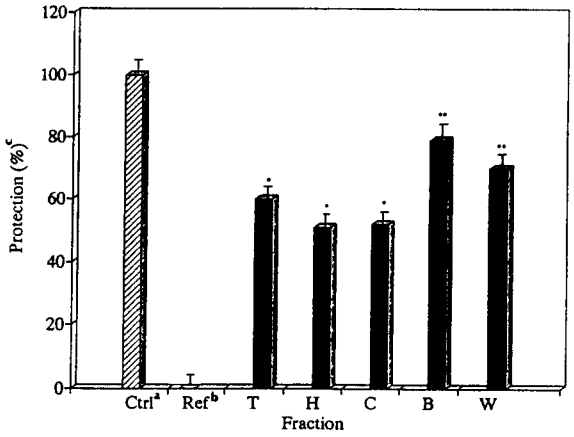


Fig. 1. Effects of each fraction of *E. koreanum* on CCl_4-intoxicated primary cultured rat hepatocytes. (Ctrl: control, Ref: reference, T: total MeOH extract, H: *n*-hexane fraction, C: $CHCl_3$ fraction, B: *n*-BuOH fraction, W: aqueous fraction)
[a]Control is the value of hepatocytes which were not challenged with CCl_4. The control value was 25.3±1.84 IU/l.
[b]Reference is the value of hepatocytes which were challenged with CCl_4. The reference value was 108.3±1.92 IU/l.
[c]The % of protection is calculated as 100× (GPT value of reference-GPT value of sample)/(GPT value of reference-GPT value of control). Significantly different from the control, effective *p<0.05, **p<0.01

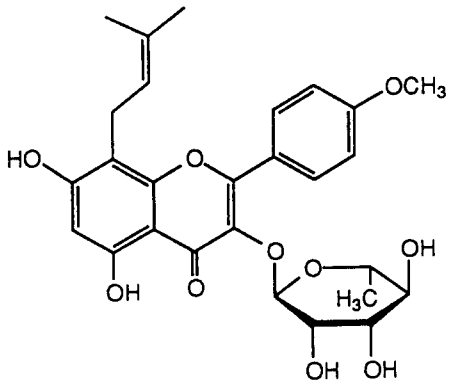


Fig. 2. The structure of icariside II.

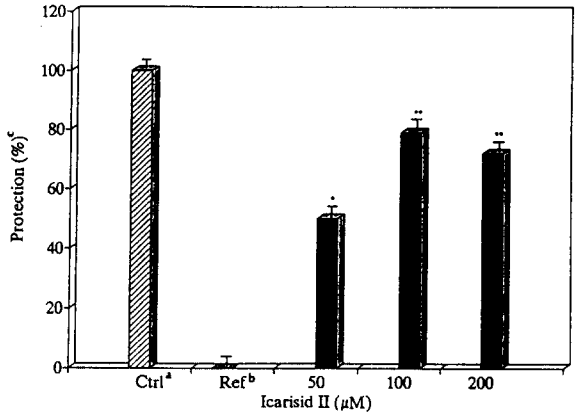


Fig. 3. Effects of icarisid II on GPT on CCl4-intoxicated primary cultured rat hepatocytes. The experimental protocol is same as in Fig. 1. [a,b] and [c] are same as in Fig. 1. Significantly different from the control, effective *p<0.05, **p<0.01

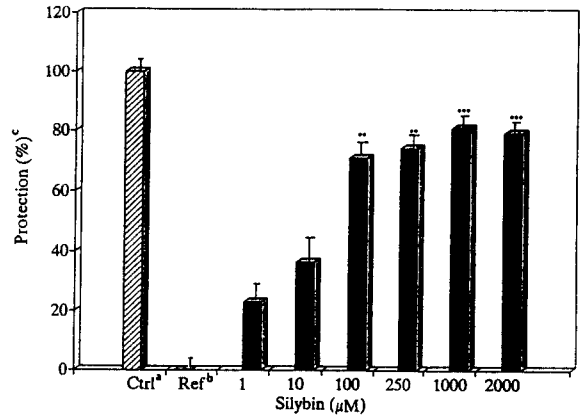


Fig. 4. Effects of silybin on GPT on CCl4-intoxicated primary cultured rat hepatocytes. The experimental protocol is same as in Fig. 1. [a,b] and [c] are same as in Fig. 1. Significantly different from the control, effective **p<0.01, ***p<0.001

tocytes. After the extract was suspended in water, it was successively partitioned with *n*-hexane, $CHCl_3$, *n*-BuOH and each fraction was screened for anti-hepatotoxicity (Fig. 1). In our previous study, we report that icariin, isolated from the BuOH fraction, showed intense anti-hepatotoxic activity (Lee *et al.*, 1995). Thus, we tried to reveal the active components in the $CHCl_3$ fraction which also showed a significant anti-hepatotoxic activity. The $CHCl_3$ fraction was subjected to silica gel column chromatography to yield compound I. Compound I was identified to be icariside II (Fig. 2) from its spectral data. After isolating, icariside II was quantified by measuring its protective effects on the release of GPT into the culture medium of primary cultures of rat hepatocytes intoxicated with CCl_4. Icariside II blocked the release of GPT from the CCl_4-intoxicated rat hepatocytes. Icariside II showed a significant anti-hepatotoxic activity at 200 μM concentration (Fig. 3).

If one compares the anti-hepatotoxic activity of icariside II with that of silybin, a drug which is used as a supplementary treatment for the liver disease, icariside II showed equipotency to silybin (Fig. 4). Thus, from the results, we could conclude that icariside II exhibited intense preventive activity against CCl_4-induced cytotoxicity in primary cultured rat hepatocytes and might hold a significant therapeutic value in the treatment of liver disease.

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