Hepatoprotective Effect of *Hovenia dulcis* THUNB. on Experimental Liver Injuries Induced by Carbon Tetrachloride or D-Galactosamine/Lipopolysaccharide

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The hepatoprotective effects of the fruits of *Hovenia dulcis* THUNB. on chemically or immunologically induced experimental liver injury models were examined. The methanol extract showed significant hepatoprotective activity against CCl_4-toxicity in rats and D-galactosamine (D-GalN)/lipopolysaccharide-induced liver injury in mice. The methanol extract also significantly protected against CCl_4-toxicity in primary cultured rat hepatocytes. Hepatoprotective activity-guided fractionation and chemical analysis led to the isolation of an active constituent, (+)-ampelopsin (1) from the methanol extract.

Key words *Hovenia dulcis*; (+)-ampelopsin; hepatoprotective effect; lipopolysaccharide; D-galactosamine; carbon tetrachloride

Various factors have been reported to induce liver injuries. In CCl_4-induced liver injury, free radical-mediated lipid peroxidation of unsaturated fatty acid binding cells and intracellular organelle membranes play important roles.[1,2] On the other hand, D-galactosamine (D-GalN)/lipopolysaccharide (LPS) induce liver injury in mice by an immunological response.[3] This type of hepatitis does not involve direct tissue degradation by chemicals but dependent on the release of potent mediators, such as tumor necrosis factor-α (TNF-α) and superoxide (O_2^-).[4,5]

We have investigated the hepatoprotective activity of crude drugs which have been used as traditional remedies for liver diseases or detoxifying agents for poisoning.[6,7] The fruits of *Hovenia dulcis* THUNB. (Rhamnaceae) is a traditional Chinese medicine used as a detoxifying agent for alcoholic poisoning. Although there are a few reports of the effects of *H. dulcis* on ethanol metabolism,[8,9] none was found concerning its hepatoprotective activity. In this report, we studied the hepatoprotective activity of *H. dulcis* using chemically and immunologically induced liver injury models as well as carrying out the isolation and identification of its active constituent.

MATERIALS AND METHODS

General ^1H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 and Fourier-transform NMR spectrometer with tetramethylsilane (TMS) as an internal standard for ^1H-NMR, and chemical shifts are expressed as δ-values. Optical rotation was measured on a JASCO DIP-4 automatic polarimenter at 25 °C. Column chromatography was performed using Wako gel C-200 (Wako Pure Chemical Industries, Co., Ltd., Japan). Serum aspartate transaminase (AST) and alanine transaminase (ALT) levels were measured by a Refletron S system (Boeringer Mannheim Co., Ltd., Osaka, Japan).

Carbon tetrachloride (CCl_4) and D-GalN were obtained from Wako Pure Chemical Industries, Osaka, Japan. LPS (*Escherichia coli* serotype 055: B5) was purchased from Difco Laboratories, U.S.A. Hanks' balanced salt solution (HBSS), ethylene glycol-*O*,*O*'-bis (2-aminoethyl)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), trypsin inhibitor and collagenase were from Wako Pure Chemical Industries, Osaka, Japan. William's E medium, bovine serum albumin (BSA), insulin, dexamethasone and gentamycin were from Sigma, St. Louis, U.S.A. Collagen type I-coated 24 well plastic plates for hepatocyte culture were from Iwaki Glass, Funabashi, Japan. The fruits of *Hovenia dulcis* THUNB. were obtained from Matsuura Pharmaceutical Co., Ltd., Nagoya, Japan. The voucher sample (TMPW No. 15502) was preserved in the Museum for Materia and Medica, Analytical Research Center for Ethnomedicines, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Extraction and Isolation The pulverized fruits of *H. dulcis* (500 g) were refluxed twice with water or methanol (each 1.5 l × 2) for 3 h. The extracts were filtered and lyophilized to give MeOH (103.2 g) and H_2O (87.6 g) extracts. These extracts were used for the evaluation of hepatoprotective activity and the MeOH extract showed significant activity. To get an active constituent(s), fruits of *H. dulcis* (5 kg) were extracted with MeOH (9 l × 3) to obtain the MeOH extract (730 g). A portion (450 g) of the MeOH extract was suspended in water and partitioned with EtOAc to give EtOAc soluble and insoluble fractions. The EtOAc soluble fraction (55.4 g) showed hepatoprotective activity and was subjected to silica-gel column chromatography (5.4 × 55 cm) and gradient elution with 0% (2 l), 5% (1.2 l), 5% (0.8 l), 10% (2 l), 20% (2 l), 30% (2 l), and 50% (2 l) methanol in $CHCl_3$ to give fraction 1 (7.5 g), fraction 2 (3.2 g), fraction 3 (11.2 g), fraction 4 (10.8 g), fraction 5 (7.7 g), fraction 6 (1.2 g) and fraction 7 (1.0 g), respectively. TLC pattern of fraction 4 showed two major compounds and this was subjected to rechromatography (3 × 18 cm) and eluted with

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10—20% MeOH in $CHCl_3$ to obtain two pure compounds. They were identified as (+)-ampelopsin (dihydromyricetin) (**1**) and myricetin (**2**) by the comparison of their ^1H- and ^{13}C-NMR spectroscopic data with the literature.[10] The absolute configuration of **1** was determined by comparison of the $[\alpha]_D$ +45° (c=0.1, Me_2CO) with a previous paper.[11]

Animals Male Sprague-Dawley rats, 6 weeks old, weighing 150—170 g were used for CCl_4-induced liver injury model. Male ddY mice, 6 weeks old, weighing 30—32 g were used for D-GalN/LPS-induced liver injury model. All animals were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan, and maintained under a 12 h light/dark cycle in a temperature and humidity controlled room. The animals were allowed free access to laboratory pellet chow (Clea Japan Inc., Tokyo, Japan; protein 24.0%, lipid 3.5%, carbohydrate 60.5%) and water *ad libitum* before the experiment.

CCl_4-Induced Liver Injury in Rats *In vivo* liver injury in rats induced by CCl_4 was carried out according to a general procedure.[12] In each group 3 or 7 rats were used. After 12 h fasting, rats received a s.c. injection of CCl_4 in olive oil (1 : 1, 6 ml/kg). MeOH or H_2O extract from *H. dulcis* was administered *p.o.* 100 mg/kg, twice a day for 1 week before CCl_4 intoxication. At 24 h after CCl_4 injection, blood samples were collected. Serum was separated by centrifugation and ALT and AST levels were measured to indicate the extent of liver damage.

D-GalN/LPS-Induced Liver Injury in Mice Liver injury was induced by D-GalN/LPS in mice according to the method of Tiegs *et al.*[4] In each group 7 or 11 mice were used. After 12 h fasting, mice were given an i.p. injection of 700 mg/kg D-GalN and 10 μg/kg LPS. The MeOH or H_2O extract from *H. dulcis* was given s.c. 200 mg/kg, twice at 18 and 2 h before D-GalN/LPS challenge. Blood ALT levels were examined 8 h postinjection of D-GalN/LPS to evaluate the extent of liver damage.

Culture of Rat Hepatocytes Rat hepatic parenchymal cells were isolated by the method of Seglen.[13] Simply, the portal vein of rat liver was exposed and cannulated with a teflon catheter. The liver was perfused with Ca^{2+}-free HBSS containing 0.5% BSA and 0.5 mM EGTA aerated with 95% O_2/5% CO_2 at 37 °C. The flow rate of washing buffer was maintained at 30 ml/min. The thoracic portion of the *vena cava* was opened and cannulated. After the liver had been perfused for 10 min, recirculation was started with collagenase solution containing Ca^{2+}-free HBSS, 0.075% collagenase, 4 mM $CaCl_2$ and 0.005% trypsin inhibitor at a flow rate of 15 ml/min. Isolated hepatocytes (2×10^5 cells/ml) were cultured in William's E medium supplemented with 10% calf serum, 50 μg/ml gentamycin, 1 μM dexamethasone and 10 nM insulin under 5% CO_2 in air at 37 °C in a type I collagen-coated 24 well plate.

CCl_4-Induced Hepatocyte Injury *in Vitro* CCl_4-induced hepatocytes injury assay was performed by the procedure of Kiso *et al.*[14] After pre-culture for 24 h, the hepatocytes were exposed to fresh medium containing 10 mM CCl_4 and various concentrations of sample. After CCl_4 exposure for 60 min, the AST concentration in the medium was measured as an indicator of hepatocyte injury.

Statistical Analysis All values were expressed as means ± S.D. or S.E. for n experiments. Student's t-test for unpaired observations between control and tested samples was carried out to identify statistically differences; a p value of 0.05 or less as considered statistically significant.

RESULTS

Effect of *H. dulcis* Extracts on CCl_4-Induced Liver Injury in Rats The hepatoprotective effect of the H_2O and MeOH extracts of *H. dulcis* on chemically induced liver injury in rats is shown in Table 1. In CCl_4-treated controls, serum AST and ALT levels were elevated to 933 ± 144 and 730 ± 212 U/l, respectively, 24 h after CCl_4 administration. In contrast, in the MeOH extract-pretreated group, serum AST and ALT levels were 311 ± 94 and 175 ± 65 U/l, respectively. However, in H_2O extract-treated group, no significant decrease was observed. Serum parameters shown in Table 1 suggested that the MeOH extract had a significant protective effect against CCl_4-induced liver injury in rats.

Effect of *H. dulcis* Extracts on D-GalN/LPS-Induced Liver Injury in Mice The hepatoprotective effect of *H. dulcis* on immunologically induced liver injury in mice is shown in Table 2. In the D-GalN/LPS-treated control group, the blood ALT was elevated to 2535 ± 497 U/l 8 h after D-GalN/LPS challenge, while in the MeOH extract pretreated group, the blood ALT was 661 ± 251 U/l, much lower than that of the controls. In the H_2O extract treated group, no decrease in blood ALT was observed. With regard to mortality, in the control group, 63.6% mice died within 12 h, while, in the MeOH extract-pretreated group, only 27.2% mice died. These results demonstrated that the MeOH extract had a significant protective effect against D-GalN/LPS-induced liver injury in mice.

Effect of *H. dulcis* Extracts on CCl_4-Induced Cultured Hepatocyte Injury and Isolation of the Active Principle It

Table 1. Effect of Extracts of *Hovenia dulcis* on CCl_4-Induced Liver Injury in Rats

Group	Dose (mg/kg)	n	sALT level (U/l)	sAST level (U/l)
Normal	—	3	37 ± 4.9	74 ± 2.5
Control	—	7	730 ± 212	933 ± 144
Hovenia dulcis H_2O extract	100	7	438 ± 136	761 ± 161
MeOH extract	100	7	175 ± 65*	311 ± 94*

The results are expressed as mean ± S.E. Significant difference from control, * $p<0.05$. Liver injury was induced by injecting CCl_4 (3 ml/kg) s.c. into 12 h fasted rats. Each extract of *Hovenia dulcis* or vehicle was administered *p.o.* twice a day (AM 9:00, PM 9:00) for 7 d before CCl_4 challenge and blood samples were collected 24 h after CCl_4 challenge.

Table 2. Effect of Extracts of *Hovenia dulcis* on D-GalN/LPS-Induced Liver Injury in Mice

Group	Dose[a] (mg/kg)	ALT level (U/l)	ALT decrease[b] (%)	Mortality within 12 h
Normal	—	66± 17	—	0/ 7
Control	—	2535±497	—	7/11
Hovenia dulcis H_2O extract	200	2701±557	<0	4/11
MeOH extract	200	661±251*	75.9	3/11

The results are expressed as mean±S.E. Significant difference from control, * $p<0.05$. Liver injury was induced by injecting D-GalN (700 mg/kg)/LPS (10 μg/ml) i.p. into 12 h fasted mice. *a*) Each extract of *Hovenia dulcis* or vehicle was administered s.c. twice at 18 and 2 h before D-GalN/LPS challenge and blood samples were collected 8 h after D-GalN/LPS challenge. *b*) ALT decrease (%) is calculated from the ALT level of controls.

Table 3. Effect of Extracts and Its Fractions from *Hovenia dulcis* on CCl_4-Induced Cultured Hepatocytes Injury

Group	Concentration (μg/ml)	*n*	AST level (U/l)	AST decrease[a] (%)
Normal	—	4	14.5± 1	—
Control	—	4	165 ±28	—
Glycyrrhizin	10	4	103 ±25**	39.9
Hovenia dulcis H_2O extract	500	4	116 ±10*	32.6
MeOH extract	500	4	104 ±24*	40.5
EtOAc sol. portion	500	4	86 ± 6.4***	52.5
Aqueous sol. portion	500	4	140 ±17*	16.6

Rat hepatocytes were isolated from rat liver by the collagenase perfusion method. After preincubation for 24 h, hepatocytes were exposed to the medium (1 ml) containing 10 mM CCl_4 and/or test sample. After 1 h of CCl_4 exposure, AST concentration in the medium was measured. Results are expressed as mean±S.D., $n=4$. Significant difference, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control. *a*) AST decrease (%) is calculated from the AST level of controls.

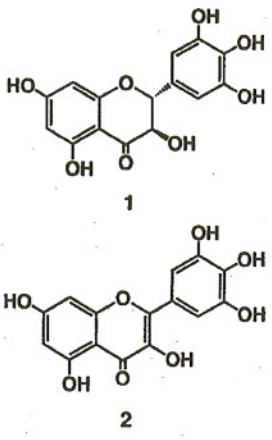

Chart 1

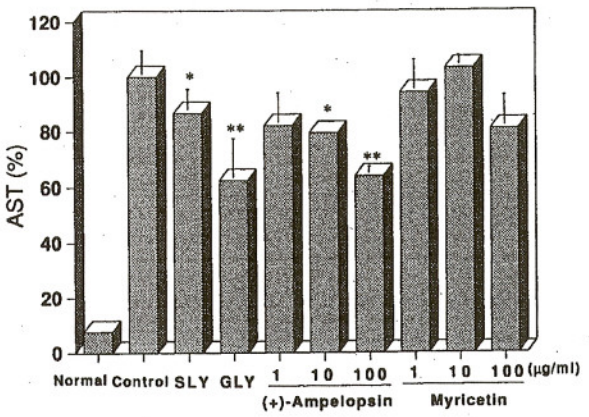

Fig. 1. Effect of **1** and **2** on CCl_4-Induced Cultured Hepatocytes Injury

Data are expressed as a percentage against control. Significantly different from control, * $p<0.05$, ** $p<0.01$. Mean AST concentration of control was 221±44 U/l. Silymarin (SLY; 100 μg/ml) and glycyrrhizin (GLY; 10 μg/ml) were used as positive controls.

was concluded from the above results that the MeOH extract had strong hepatoprotective effects in chemically or immunologically induced liver injury models. To identify the active constituents, we performed an *in vitro* assay and the results are shown in Table 3. The extent of hepatocyte injury was expressed in terms of AST released into the medium after treatment with CCl_4. The AST level in the control group was 165±28 U/l 1 h after CCl_4 exposure, while the AST level in the MeOH or H_2O extract-treated group was 104±24 or 116±10 U/l, respectively. These data indicate that the effect of the MeOH extract was significantly different from that of the control, which was more effective than the H_2O extract.

The MeOH extract which showed significant hepatoprotective activity in the *in vitro* as well as *in vivo* experiments, was fractionated into EtOAc soluble and insoluble fractions. The EtOAc soluble fraction which was more active than the insoluble one, was subjected to silica gel column chromatography to obtain 7 fractions. The activity of each fraction was tested and fr. 4 was found to be the most active (data not shown here). Fraction 4, at a concentration of 100 μg/ml, reduced the AST release into the medium by 28.5% compared with that of the control. Two major compounds were isolated from fr. 4 and identified as (+)-ampelopsin (**1**) and myricetin (**2**) (Chart 1). The coupling constant value between H-2 and H-3 in the ^{1}H-NMR spectrum of **1** was 11.5 Hz suggesting that these protons are in an anti-configuration. In addition, the $[\alpha]_D$ value was +45° which coincided with data from

the literature.[11] Thus, the absolute configuration of **1** was determined as 2*R*, 3*R*, as shown in Chart 1.

The results of the *in vitro* hepatoprotective effect of **1** and **2** are shown in the Fig. 1. Compound **1** showed a significant hepatoprotective effect at a concentration of 10 μg/ml against CCl_4-induced hepatocyte injury and the effect was dose-dependent at concentrations from 1 to 100 μg/ml. The activity of **1** was comparable with that of silymarin, used as a positive control, although weaker than that of glycyrrhizin. However, **2** failed to protect, even at concentration of 100 μg/ml.

DISCUSSION

Carbon tetrachloride is widely known to induce liver injury and its mechanism is known to involve a chemical reaction mediated by a free radical oxidative reaction. CCl_4 is first metabolized to $\cdot CCl_3$ by metabolic enzymes such as cytochrome P450 in the hepatocellular microsomes. This highly reactive radical directly injures the hepatocytes and organelles resulting in a series of physicochemical alterations: peroxidation of the membrane lipids, denaturation of proteins, and other chemical changes that lead to distortion or destruction of the liver. These changes are the first stage in the injury process which culminates in necrosis and steatosis.[2,15] D-GalN is also a hepatotoxin which inhibits protein biosynthesis by uridine trapping specifically in the liver lesion.[16] Moreover, D-GalN greatly enhances the sensitivity of hepatocytes to LPS because of inhibition of acute protein induction which is a biological mechanism to resistant against hepatotoxicity.[4,17] Hence, co-administration of D-GalN and a very small, normally subtoxic, amount of LPS can induce fulminant hepatitis in mice through the immunological pathway terminated by TNF-α release.[4,5,18] Whatever the route of liver cell injury, levels of enzymes such as ALT and AST significantly increase and these are regarded as parameters to monitor the extent of liver injury.

In the present experiment, the MeOH extract of *H. dulcis* protected not only against the elevation of serum ALT and AST levels seen in CCl_4-toxicity in rats but also blood ALT elevation in D-GalN/LPS-induced liver injury in mice. In the D-GalN/LPS-induced liver injury model, ALT abruptly increases because of severe liver damage. The animals die from the liver failure. Therefore, mortality is also regarded as a parameter of liver failure. The MeOH extract of *H. dulcis* also improved the mortality. These results obviously indicate that the MeOH extract has pronounced hepatoprotective effect in both chemically and immunologically induced liver injury models. In CCl_4-induced injury, antioxidants are widely known to be able to protect against hepatocyte necrosis because they intercept the CCl_4-induced oxidative stress in hepatocytes by scavenging $\cdot CCl_3$ and lipid peroxy radicals. We recently found that the MeOH extract of *H. dulcis* possesses a potent radical-scavenging activity (unpublished data). On the other hand, the formation of reactive oxygen species is related to the release of TNF-α from macrophages in D-GalN/LPS-treated mice.[19] Therefore, pretreatment with radical scavengers can protect against D-GalN/LPS-induced liver injury, too.[19,20] We are still unable to identify the exact mechanisms of the hepatoprotective effect of *H. dulcis*; however, the radical-scavenging activity of *H. dulcis* is an important factor in its hepatoprotective activity. The relationship between the radical-scavenging activity and the hepatoprotective effect of *H. dulcis* is now being investigated.

The fruit of *H. dulcis* is a Chinese medicine which has been traditionally used for the treatment of alcoholism and as a detoxifying agent. However, only a few chemical and pharmacological reports have been published.[21,22] Here, we confirmed the hepatoprotective activity of the MeOH extract against CCl_4 or D-GalN/LPS-induced liver injury. Furthermore, the hepatoprotective activity-guided fractionation of the MeOH extract gave us an active constituent, **1**, the yield of which was 2% in the EtOAc soluble fraction. Recently, Yoshikawa *et al.* also isolated **1** as an alcohol-induced muscle relaxation inhibitory constituent from the seeds and fruits of *H. dulcis*.[21] Our observation showed that the hepatoprotective effect of **1** was more potent than that of silymarin which has been used clinically to treat various liver diseases in Europe.[23] Interestingly, (**2**), which was isolated together with **1** from the same active fraction, did not exhibit any hepatoprotective activity, despite having a very similar chemical structure. Compound **1** shows a typical stereochemistry at the C-ring which is lacking in **2** because of a double bond ($\Delta^{2,3}$). The results of the present experiment clearly indicate that the stereochemistry at the C-ring of **1** plays an important role in its hepatoprotective activity.

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