Hepatoprotective Effect of *Hovenia dulcis* THUNB. on Experimental Liver Injuries Induced by Carbon Tetrachloride or \( \text{D-Galactosamine/Lipopolysaccharide} \)

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Received November 1, 1996; accepted December 25, 1996

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, \((-\text{-ampelopin})\) from the methanol extract.

Key words *Hovenia dulcis*, \((-\text{-ampelopin})\); 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) induces liver injury in mice by an immunological response.\(^{3,4}\) This type of hepatitis does not involve direct tissue degradation by chemicals but depends on the release of potent mediators, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and superoxide (O\(_2^{-}\)).\(^{5,6}\)

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** \(^{1}\)H- 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 \(^{1}\)H-NMR, and chemical shifts are expressed as \(\delta\)-values. Optical rotation was measured on a JASCO DIP-4 automatic polarimeter 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 ob-

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10—20% MeOH in CHCl₃ to obtain two pure compounds. They were identified as (+)-ampelopsin (dihydropyrimidin) (1) and myricetin (2) by the comparison of their ¹H- and ¹³C-NMR spectroscopic data with the literature. The absolute configuration of 1 was determined by comparison of the [α]D +45° (c=0.1, Me₂CO) with a previous paper.

Animals: Male Sprague-Dawley rats, 6 weeks old, weighing 150—170 g were used for CCl₄-induced liver injury model. Male ddY mice, 6 weeks old, weighing 30—32 g were used for d-GaIN/LPS-induced liver injury model. All animals were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan, and maintained under a 12h 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.

CCl₄-Induced Liver Injury in Rats: In vivo liver injury in rats induced by CCl₄ was carried out according to a general procedure. In each group 3 or 7 rats were used. After 12h fasting, rats received a s.c. injection of CCl₄ in olive oil (1:1, 6 ml/kg). MeOH or H₂O extract from H. dulcis was administered p.o. 100 mg/kg, twice a day for 1 week before CCl₄ intoxication. At 24h after CCl₄ 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-GaIN/LPS-Induced Liver Injury in Mice: Liver injury was induced by d-GaIN/LPS in mice according to the method of Tieg et al. In each group 7 or 11 mice were used. After 12h fasting, mice were given an i.p. injection of 700 mg/kg d-GaIN and 10 μg/kg LPS. The MeOH or H₂O extract from H. dulcis was given s.c. 200 mg/kg, twice at 18 and 2h before d-GaIN/LPS challenge. Blood ALT levels were examined 8h postinjection of d-GaIN/LPS to evaluate the extent of liver damage.

Culture of Rat Hepatocytes: Rat hepatic parenchymal cells were isolated by the method of Seglen. Simply, the portal vein of rat liver was exposed and cannulated with a teflon catheter. The liver was perfused with Ca²⁺-free HBSS containing 0.5% BSA and 0.5 mM EGTA aerated with 95% O₂/5% CO₂ 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 Ca²⁺-free HBSS, 0.075% collagenase, 4 mM CaCl₂ and 0.005% trypsin inhibitor at a flow rate of 15 ml/min. Isolated hepatocytes (2 x 10⁶ 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% CO₂ in air at 37°C in a type I collagen-coated 24 well plate.

CCl₄-Induced Hepatocytie Injury in Vitro: CCl₄-induced hepatocytie injury assay was performed by the procedure of Kiso et al. After pre-culture for 24h, the hepatocytes were exposed to fresh medium containing 10 mM CCl₄ and various concentrations of sample. After CCl₄ exposure for 60 min, the AST concentration in the medium was measured as an indicator of hepatocytie 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 was considered statistically significant.

RESULTS

Effect of H. dulcis Extracts on CCl₄-Induced Liver Injury in Rats: The hepatoprotective effect of the H₂O and MeOH extracts of H. dulcis on chemically induced liver injury in rats is shown in Table 1. In CCl₄-treated controls, serum AST and ALT levels were elevated to 933 ± 144 and 730 ± 212 U/l, respectively, 24h after CCl₄ 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 H₂O extract-pretreated group, no significant decrease was observed. Serum parameters shown in Table 1 suggested that the MeOH extract had a significant protective effect against CCl₄-induced liver injury in rats.

Effect of H. dulcis Extracts on d-GaIN/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-GaIN/LPS-treated control group, the blood ALT was elevated to 2535 ± 497 U/l 18h after d-GaIN/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 H₂O extract treated group, no decrease in blood ALT was observed. With regard to mortality, in the control group, 63.6% mice died within 12h, 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-GaIN/LPS-induced liver injury in mice.

Effect of H. dulcis Extracts on CCl₄-Induced Cultured Hepatocyte Injury and Isolation of the Active Principle: It

Table 1. Effect of Extracts of Hovenia dulcis on CCl₄-Induced Liver Injury in Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>sALT level (U/l)</th>
<th>sAST level (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>3</td>
<td>37 ± 4.9</td>
<td>74 ± 2.5</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>7</td>
<td>730 ± 212</td>
<td>933 ± 144</td>
</tr>
<tr>
<td>Hovenia dulcis</td>
<td>H₂O extract</td>
<td>100</td>
<td>438 ± 136</td>
<td>761 ± 161</td>
</tr>
<tr>
<td>MeOH extract</td>
<td>—</td>
<td>7</td>
<td>175 ± 65*</td>
<td>311 ± 94*</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± S.D. Significant difference from control, * p<0.05. Liver injury was induced by injecting CCl₄ (3ml/kg) s.c. into 12h fasted rats. Each extract of Hovenia dulcis or vehicle was administered p.o. twice a day (AM 9:00, PM 9:00) for 7d before CCl₄ challenge and blood samples were collected 24h after CCl₄ challenge.
Table 2. Effect of Extracts of *Hovenia dulcis* on α-GalN/LPS-Induced Liver Injury in Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>ALT level (U/l)</th>
<th>ALT decrease (%)</th>
<th>Mortality within 12h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>66±17</td>
<td>—</td>
<td>0/7</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>2535±7497</td>
<td>—</td>
<td>7/11</td>
</tr>
<tr>
<td><em>Hovenia dulcis</em> H₂O extract</td>
<td>200</td>
<td>2701±557</td>
<td>&lt;0</td>
<td>4/11</td>
</tr>
<tr>
<td>MeOH extract</td>
<td>200</td>
<td>661±251*</td>
<td>75.9</td>
<td>2/11</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± S.E. Significant difference from control, * p<0.05. Liver injury was induced by injecting α-GalN (700 mg/kg) LPS (10 μg/ml) i.p. into 12h fasted mice. a) Each extract of *Hovenia dulcis* or vehicle was administered s.c. twice at 18 and 3h before α-GalN/LPS challenge and blood samples were collected 8 h after α-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 CCl₄-Induced Cultured Hepatocytes Injury

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (μg/ml)</th>
<th>n</th>
<th>AST level (U/l)</th>
<th>AST decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>4</td>
<td>14.5±1</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>4</td>
<td>165±28</td>
<td>—</td>
</tr>
<tr>
<td>Glycyrrhizin</td>
<td>—</td>
<td>4</td>
<td>103±25**</td>
<td>39.9</td>
</tr>
<tr>
<td><em>Hovenia dulcis</em> H₂O extract</td>
<td>10</td>
<td>4</td>
<td>116±10*</td>
<td>32.6</td>
</tr>
<tr>
<td>MeOH extract</td>
<td>300</td>
<td>4</td>
<td>104±24*</td>
<td>40.5</td>
</tr>
<tr>
<td>EtOAc sol. portion</td>
<td>500</td>
<td>4</td>
<td>86±6.4***</td>
<td>25.2</td>
</tr>
<tr>
<td>Aqueous sol. portion</td>
<td>200</td>
<td>4</td>
<td>140±17*</td>
<td>16.6</td>
</tr>
</tbody>
</table>

Rat hepatocytes were isolated from rat liver by the collagenase perfusion method. After preincubation for 24h, hepatocytes were exposed to the medium (1 ml) containing 10mM CCl₄ and/or test sample. After 1h of CCl₄ 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.

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 CCl₄. The AST level in the control group was 165±28 U/l 1h after CCl₄ exposure, while the AST level in the MeOH or H₂O 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 H₂O 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). Fracation 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 1H-NMR spectrum of 1 was 11.5 Hz suggesting that these protons are in an anti-configuration. In addition, the [α]D value was +45° which coincided with data from

![Chart 1](image-url)
the literature. Thus, the absolute configuration of I was determined as 2R, 3R, as shown in Chart 1.

The results of the in vitro hepatoprotective effect of I and 2 are shown in the Fig. 1. Compound I showed a significant hepatoprotective effect at a concentration of 10 μg/ml against CCl₄-induced hepatocyte injury and the effect was dose-dependent at concentrations from 1 to 100 μg/ml. The activity of I was comparable with that of silymarin, used as a positive control, although weaker than that of glycerrhizin. 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. CCl₄ is first metabolized to ·CCl₃ 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. D-GaIN is also a hepatotoxin which inhibits protein biosynthesis by uridine trapping specifically in the liver lesion. Moreover, D-GaIN greatly enhances the sensitivity of hepatocytes to LPS because of inhibition of acute protein induction which is a biological mechanism to resist against hepatotoxicity. Hence, co-administration of D-GaIN and a very small, normally subtoxic amount of LPS can induce fulminant hepatitis in mice through an immunological pathway terminated by TNF-α release. 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 CCl₄-toxicity in rats but also blood ALT elevation in D-GaIN/LPS-induced liver injury in mice. In the D-GaIN/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 CCl₄-induced injury, antioxidants are widely known to be able to protect against hepatocyte necrosis because they intercept the CCl₄-induced oxidative stress in hepatocytes by scavenging ·CCl₃ and lipid peroxyl 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-GaIN/LPS-treated mice.

Therefore, pretreatment with radical scavengers can protect against D-GaIN/LPS-induced liver injury, too. We are still unable to identify the 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. Here, we confirmed the hepatoprotective activity of the MeOH extract against CCl₄ or D-GaIN/LPS-induced liver injury. Furthermore, the hepatoprotective activity-guided fractionation of the MeOH extract gave us an active constituent, I, the yield of which was 2% in the EtOAc soluble fraction. Recently, Yoshikawa et al. also isolated I as an alcohol-induced muscle relaxation inhibitory constituent from the seeds and fruits of H. dulcis. Our observation showed that the hepatoprotective effect of I was more potent than that of silymarin which has been used clinically to treat various liver diseases in Europe. Interestingly, (2), which was isolated together with I from the same active fraction, did not exhibit any hepatoprotective activity, despite having a very similar chemical structure. Compound I shows a typical stereochemistry at the C-ring which is lacking in 2 because of a double bond (Δ²). The results of the present experiment clearly indicate that the stereochemistry at the C-ring of I plays an important role in its hepatoprotective activity.

REFERENCES