# Effect of *Hovenia dulcis* on lipopolysaccharide-induced liver injury in chronic alcohol-fed rats

Koji HASE[a,b] Purusotam BASNET[a] Shigetoshi KADOTA*[a] and Tsuneo NAMBA[a]

[a] *Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines), Toyama Medical and Pharmaceutical University,* [b] *Health Science Laboratories, Yamanouchi Pharmaceutical Co., Ltd.*

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## Abstract

The hepatoprotective effect of the $H_2O$ and MeOH extracts from the fruits of *Hovenia dulcis* THUNB. was examined on endotoxin (lipopolysaccharide; LPS)-induced hepatotoxicity in chronic ethanol-fed rat. Rats were injected with LPS after feeding ethanol (36 % of total calories) diet for 4 weeks. The $H_2O$ extract of *H. dulcis* significantly inhibited the elevation of serum ALT and AST levels by LPS challenge after chronic ethanol consumption. In addition, the $H_2O$ extract suppressed the accumulation of triglyceride (TG), total cholesterol (t-CHOL) and malondialdehyde (MDA) in rat liver.

**Key words** *Hovenia dulcis,* hepatoprotective effect, alcoholic hepatitis, ethanol, lipopolysaccharide.

## Introduction

Chronic ethanol consumption is widely recognized to alter hepatocyte metabolisms and finally induce liver injury. Since endotoxemia is commonly seen in patients with alcoholic liver disease (ALD) and some experimental liver injuries,[1-5] endotoxin is thought to play an etiologic role in several liver diseases. Moreover, some investigators recently reported that alcohol and endotoxin synergically induced liver injury,[6] whose hepatotoxic effects closely relate to the secretion of a harmful cytokine, tumor necrosis factor (TNF).[7,8]

In the courses of finding out hepatoprotective crude drugs,[9,10] we attended to the fruits of *Hovenia dulcis* THUNB. (Rhamnaceae) which has been believed to be effective against alcoholic toxicity. Recently, it was found that this crude drug accelerated the alcohol metabolism in human as well as rat.[11] However, no report on alcoholism such as alcoholic hepatitis and fatty liver was found. In the present study, we evaluated the hepatoprotective effect of *H. dulcis* on LPS-induced liver injury in chronic ethanol-fed rats.

## Materials and Methods

*General*: Serum AST, ALT levels were measured by a Refletron S system (Boeringer Mannheim Co. Ltd., Osaka, Japan). Lipopolysaccharide (LPS ; *Escherichia coli* serotype 055 : B5) was purchased from Difco Laboratories, USA. 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. The Pulverized fruits of *H. dulcis* (500 g) were refluxed twice with water or methanol (1.5 L×2) for 3 h. The extracts was filtered and lyophilized to give MeOH (103.2 g) and $H_2O$ (87.6 g) extracts. These extracts were used for the evaluation of their hepatoprotective activity.

*Animals*: Male Sprague-Dawley rats, 6 or 5 weeks old, weighing 150-170 g were used for liver injury model or ethanol metabolism experiment. The

*〒930-01 富山市杉谷2630番地
富山医科薬科大学和漢薬研究所化学応用部門 門田重利
2630 Sugitani, Toyama 930-01, Japan

animals were purchased from Shizuoka Laboratory Animal Center, Japan, and maintained under 12 h light/dark cycle in a temperature and humidity controlled room. The animals were allowed free access to a laboratory pellet chow (CLEA Japan Inc., Tokyo, Japan ; protein 24.0 %, lipid 3.5 %, carbohydrate 60.5 %) and water *ad libitum* before experiment.

*LPS-induced liver injury in chronic ethanol-ingested rats*: Rats were divided into 5 groups (each $n$= 6). In the first group (Control I), rats were fed with ethanol free liquid diet in which ethanol was substituted with isocaloric sucrose. In other groups, rats were fed with Lieber Decali liquid diet in which 36 % of total calories were from ethanol. The MeOH or $H_2O$ extracts from *H. dulcis* was administered *p.o.*, each 100 mg / kg twice a day (AM 9 : 00 and PM 9 : 00) during ethanol diet. Rats of control groups (Control I, II and III) were administered the same volume of saline for same period. After feeding with liquid diet for 4 weeks, rats of Control III and sample treated groups were challenged with intravenous LPS (1 mg/kg) injection. Blood was collected from the tail vein for the measurement of serum ALT level at 8 h postinjection. All animals were sacrificed at 24 h postinjection and liver perfused with cold saline was removed for the measurement of MDA, TG and t-CHOL.

*Measurement of hepatic MDA, TG and t-CHOL*: The hepatic MDA content was measured as an indicator of lipid peroxidation in the liver. A lobe of liver was homogenized in cold 1.15 % KCl solution (4 ml/g wet tissue) and MDA content in the homogenate was measured by TBA reaction.[12] The protein concentration of the liver homogenate was determined by Lowry method[13] using bovine serum albumin as the standard.

Hepatic TG and t-CHOL contents were measured according to the method of Haug, *et al.*[14] Simply, for each rat, a 10 % (wt/vol) homogenate of hepatic tissue was prepared in isopropanol. The homogenate was kept at 4°C for 2 d to extract TG and t-CHOL, and then centrifuged for 10 min at 1000×g. Aliquots of the supernatants were measured enzymatically for TG and t-CHOL with commercial kits (Boeringer Mannheim Co. Ltd., Osaka, Japan).

*Ethanol metabolism experiment*: Rats were fasted for 12 h prior to experiment, but water was given *ad libitum*. Rats were administered ethanol (2 g/kg) as 50 % aqueous solution with gastric catheter. The $H_2O$ extract of *H. dulcis* (100 mg/kg) or saline was administered *p.o.* 1 h before ethanol. Then, blood was collected from the tail vein at just before and 1, 3, 6 h after ethanol administration. Eight volume of ice cold perchloric acid (0.33 mol/L) was added to each blood sample to exclude proteins. The solution was shaken with vortex mixer and centrifuged at 3000 × rpm. Aliquots of the supernatant was used to measure ethanol concentration enzymatically using a commercial kit (Boeringer Mannheim Co. Ltd., Osaka, Japan).

*Statistical analysis*: All value expressed as mean± S.E. obtained from $n$ number of experiments. The Studen's $t$ - test for unpaired observation between control and experimental samples was carried out for statistical evaluation of a difference ; $p$ value of 0.05 or less were considered as statistically significant.

## Results

Nutritional data evaluation showed that ethanol free diet-fed rats (Control I) consumed more calories than did rats in the ethanol diet-fed group (Control II and III). Rats ingested with ethanol free diet gained more weight than rats fed with ethanol. However, no difference of caloric consumption and body weight between ethanol fed control groups (Control II or III) and sample ($H_2O$ or MeOH extract) treated groups during ethanol diet-fed periods was observed (Table I). As for the serum ALT and AST levels, both parameters in control II (ethanol diet) were a little higher than that of control I (ethanol free diet). In control III (ethanol diet+LPS), serum ALT and AST levels were two or three times elevated at 8 h after LPS challenge. On the other hand, administration of the $H_2O$ extract obviously suppressed the ALT and AST levels (Table II). In the case of MeOH extract treated group, no significant decrease of serum parameters was observed.

Then, the hepatic MDA was measured as an indicator of lipid peroxides generation. In control III, a pronounced elevation of hepatic MDA was observed, whereas in the $H_2O$ extract treated group, the produc-

Table I Nutritional data after 4 week ingestion of ethanol diet in rats.

| Group | Treatment | Dose (mg/kg) | EtOH[a] Diet | LPS[b] challenge | Body weight[c] (g) | Consumption[d] (kcal/kg/day) |
|---|---|---|---|---|---|---|
| Control I | Saline | —— | − | − | 223.5±10.9 | 551.1±53.7 |
| Control II | Saline | —— | + | − | 186.2±20.1 | 388.1±69.2 |
| Control III | Saline | —— | + | + | 185.8±3.0 | 381.4±66.7 |
| *Hovenia dulcis* | $H_2O$ ext. | 100 | + | + | 188.0±8.0 | 369.9±46.5 |
| | MeOH ext. | 100 | + | + | 195.7±9.4 | 382.8±81.4 |

The result was expressed as mean±S.E., $n$=6. Test sample or saline were administered twice a day in the whole period of ethanol diet. a) Lieber diet, which replaced 36 % of total calories with ethanol, was given rats for 4 weeks. Liquid diet, which was substituted carbohydrate for ethanol, was used as ethanol free diet (control I).
b) Lipopolysaccharide (LPS; 1 mg/kg) was *i.v.* injected through tail vein in rat for enhancement of liver injury after ingesting ethanol diet for 4 weeks.
c) The body weight at 4 weeks after ingesting ethanol diet.
d) Mean of diet consumption per day was expressed in calories.

Table II Effect of *Hovenia dulcis* extracts on serum ALT and AST level on LPS-induced liver injury in chronic ethanol fed-rats.

| Group | | Dose (mg/kg) | sALT (U/L) | sAST (U/L) |
|---|---|---|---|---|
| Control I | | —— | 37.5±3.5 | 64.2±2.6 |
| Control II | | —— | 60.0±8.4 | 134.2±49.4 |
| Control III | | —— | 117.8±32.3 | 235.2±58.3 |
| *Hovenia dulcis* | $H_2O$ extract | 100 | 53.9±12** | 102.6±16.8*** |
| | MeOH extract | 100 | 83.4±5.7 | 259.0±39.2 |

The result was expressed as mean±S.E., $n$=6. The blood sample was collected to measure serum ALT and AST level at 8 h postinjection of LPS following to ethanol diet feeding for 4 weeks. Control I: saline+ethanol free liquid diet, Control II: saline+ethanol diet, Control III: saline+ethanol diet+LPS, Sample treatment group: sample+ethanol diet+LPS. Significant difference, $^{**}p<0.01$ and $^{***}p<0.001$ vs Control III.
Test sample or saline was administered twice a day, *p.o.* in the whole period of ethanol diet.

Table III Effect of *Hovenia dulcis* extracts on accumulation of hepatic triglyceride (TG), total cholesterol (t-CHOL) on LPS-induced liver injury in chronic ethanol-fed rats.

| Group | | Dose (mg/kg) | MDA[a] (nmol/100 mg protein) | TG (mg/g Liver) | t-CHOL (μmol/g Liver) |
|---|---|---|---|---|---|
| Control I | | —— | 87±9.1 | 10.8±0.8 | 17.1±2.8 |
| Control II | | —— | 118±6.0 | 68.8±12.1 | 58.9±6.1 |
| Control III | | —— | 219±2.8 | 45.8±19.6 | 51.9±8.5 |
| *Hovenia dulcis* | $H_2O$ extract | 100 | 151±11.6* | 18.2±3.9* | 36.2±2.6** |
| | MeOH extract | 100 | 209±14.1 | 19.7±2.4** | 40.9±5.2* |

The result was expressed as mean±S.E., $n$=6. The rat liver was taken to measure hepatic TG and t-CHOL levels at 24 h postinjection of LPS following ethanol diet ingesting for 4 weeks. Control I: saline+ethanol free liquid diet, Control II: saline+ethanol diet, Control III: saline+ethanol diet+LPS, Sample treatment group: sample+ethanol diet+LPS. Significant difference, $^{*}p<0.05$, $^{**}p<0.01$ vs Control III.
a) Generation of lipid peroxides was expressed as the amount of malondialdehyde (MDA).

tion of hepatic MDA was suppressed to 50 % of the former (Table III). In the ethanol diet-fed groups (control II and III), regardless of LPS treatment, hepatic TG and t-CHOL were 4-6 fold and 3 fold accumulated than that of control I, respectibely. On the other hand, the treatment of $H_2O$ extract significantly inhibited hepatic TG and t-CHOL accumulation compared with control III (Table III). These data clearly demonstrated that the $H_2O$ extract of *H. dulcis* possessed potential hepatoprotective activity against LPS-induced liver injury in chronic ethanol-fed rats. The MeOH extract also showed hepatoprotective activity such as suppression of the hepatic TG and t-CHOL accumulation, although it did not affect the serum enzymes and hepatic MDA level (Table II and III).

Moreover, we tested the effect of the $H_2O$ extract on metabolism of ethanol. Ethanol was administered orally and the time course of blood ethanol level was examined as shown in Fig. 1. Just before ethanol administration, blood ethanol level in both groups was almost zero. The ethanol level in both groups reached a peak at 1 h after ethanol administration. Then, in the control group, blood ethanol level gradually decreased to 6 h, whereas in the $H_2O$ extract treated group, it rapidly decreased to 6 h. At 3 h and 6 h after ethanol administration, blood ethapol level in the $H_2O$ extract treated group was observed to be pronouncedly lower than that in control group.

## Discussion

It is widely accepted that chronic ethanol consumption leads to liver injury in human and experimental animals. However, the mechanisms are still controversial, although numerous studies have been performed. It is known that the hepatotoxicity of ethanol results from excessive generation of the reduced nicotinamide adenine dinucleotide (NADH) as well as acetaldehyde. The NADH can disorder the ability of hepatocytes to maintain redox homeostasis and a number of metabolic reactions. Acetaldehyde also induces the alternation of metabolisms and generates harmful free radicals. Also, chronic ethanol consumption - induced malnutrition affects functional impairment of liver. These concepts about the pathology of alcoholic liver diseases have been evolved in the previous reviews.[15, 16] In addition to the metabolic complications, the role of the immunological system on alcoholic liver diseases can not be neglected.[17] Chronic ethanol consumption and alcoholic liver diseases (ALD) frequently result in endotoxemia.[4, 18] On the other hand, endotoxin (LPS) can induce liver injury througt the induction of cytotoxic cytokines such as tumor necrosis factor from macrophage and enhance the injury of other hepatotoxins.[19] Therefore, it seems reasonable to postulate that LPS may play an important role in the ALD. Bhagwandeen *et al.*[6] reported that administration of endotoxin on chronic ethanol - fed rat induced acute alcoholic hepatitis (AAH). This experimental ALD model we used in the present study was patterned after that described by Bhagwandeen.[6] Here, rats were fed a liquid diet with 36 % of total calories supplied as ethanol or carbohydrates (as pair-control). At the end of a constant feeding period of 4 weeks, rats were received LPS challenge. Thus a combination of chronic ethanol ingestion and LPS challenge induce a histopathological alternation which partly resembles human AAH.[6] In the present study, the $H_2O$ extract of *H. dulcis* showed a significant suppression on blood ALT elevation, and hepatic MDA, TG and t-CHOL accumulation. The MeOH extract also significantly decreased TG and t-CHOL accumulation, however it did not affect the other parameters.

The fruits of *H. dulcis* have been traditionally used for the treatment of alcoholism and detoxification in China and believed to be able to abolish alcoholic toxicity. However, only a few chemical and pharmacological reports on this crude drug are seen in literature. Recently, Yoshikawa *et al.* reported that several methylmigrated 16, 17-*seco*-dammarane type triterpene glycosides having histamine release inhibitory activity were isolated from the seeds and fruits of this plant.[20, 21] Moreover, recent reports proved that the $H_2O$ extract of *H. dulcis* accelerate the metabolism of ethanol in rats and human.[11, 22] As shown in Fig. 1, our observation also confirmed the effect of the $H_2O$ extract from *H. dulcis* on ethanol metabolism. Thus, the activity of $H_2O$ extract on ethanol metabolism may display the hepatoprotective effect against LPS-induced liver injury in chronic ethanol-

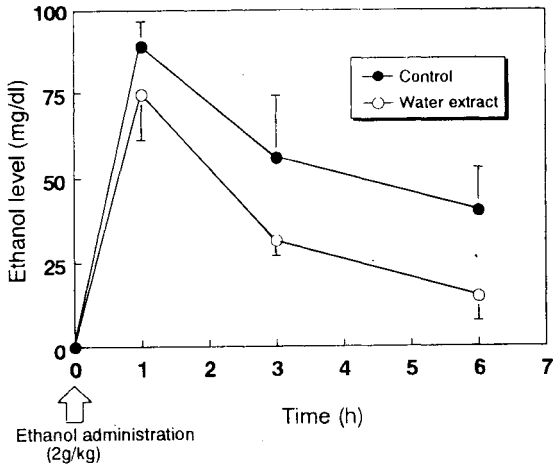


Fig. 1 Effect of water extract from *Hovenia dulcis* on ethanol metabolism in rats.
The result was expressed as mean±S.E., $n$=4. Ethanol (2 g/kg) was administered *p.o.* and blood sample was collected just before and at 1, 3 and 6 h after ethanol administration to measure blood ethanol level. The water extract (100 mg/kg) was administered *p.o.* 1 h prior to ethanol.

fed rats. The $H_2O$ extract of *H. dulcis* could be a possible therapeutic agent to alcohol-related liver diseases.

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## 和文抄録

枳椇子はクロウメモドキ科(Rhamnaceae)ケンポナシ(*Hovenia dulcis* THUNB.)の成熟果実あるいは種子を乾燥したものである。その薬効として酒毒を解すとされており，古来，酒酔いの治療や解毒薬として用いられてきた。今回，長期エタノール摂取ラットにLPSを単回投与することにより肝障害を誘発するモデル動物を用いて，枳椇子水およびメタノールエキスの肝臓保護効果を検討した。枳椇子メタノールエキス投与群においては，肝臓内TG, t-CHOL値の上昇を有意に抑制したものの，血清パラメーターに顕著な変化は認められなかった。一方で，水エキス投与群では血清AST, ALT値および肝内TG, t-CHOL, MDA値の上昇をそれぞれ有意に抑制する効果が認められた。

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