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The Extract of *Limonium tetragonum* Protected Liver against Acute Alcohol Toxicity by Enhancing Ethanol Metabolism and Antioxidant Enzyme Activities

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Abstract – The protective effect of EtOAc fraction of *Limonium tetragonum* extract (EALT) against alcohol-induced hepatotoxicity was assessed following acute ethanol intoxication in Spraque-Dawley rats. EALT (200 mg/kg *p.o.*) was administrated once before alcohol intake (8 g/kg, *p.o.*). Blood ethanol concentration, and the activities of alcohol metabolic enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in the liver were measured. Also, the formation of malondialdehyde (MDA) and the activities of antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GSH-px), catalase were determined after acute alcohol exposure. Pretreatment of rats received ethanol with EALT significantly decreased blood ethanol concentration and elevated the activities of ADH and ALDH in liver. The increased MDA level was decreased, and the reduced activities of SOD, GSH-px and catalase were markedly preserved by the treatment with EALT. This study suggests that EALT prevent hepatic injury induced by acute alcohol which is likely related to its modulation on the alcohol metabolism and antioxidant enzymes activities.
Keywords – *Limonium tetragonum*, halophyte, Alcohol, Alcohol dehydrogenase, Aldehyde dehydrogenase, Antioxidant

Introduction

Alcohol, in moderation, can promote metabolism in the body and help to reduce the risk of heart disease, but, excessive intake of alcohol can cause so-called hangovers and further contributes significantly to the prevalence of liver disease and other health problems. Alcoholic liver disease (ALD), the common consequence of prolonged excessive alcohol consumption is now one of the most common causes of chronic liver disease in the world.[1]

There have been numerous attempts to discover effective substances as preventive agents for disorders induced by alcohol.[2-3] Many natural products have been reported as having inhibitory effect on ethanol absorption, thus being an alternative to synthetic medicines in the prevention of alcohol provoked disorders.[4] In recent years, halophytes, the naturally salt-tolerant plants have become attractive sources of biologically active. They have been received extensive attention for possible medical applications due to the high content of powerful antioxidant components. In a process of continuous adjustment to unfavorable environmental condition, such as high salinity soil, it has been known that halophytes are naturally equipped with powerful antioxidant defense system.[5-6] Some of secondary metabolites including terpenoids (carotenoids, diterpenes, sesquiterpenes and essential oil) and phenolic compounds have been identified to protect cellular molecules and tissue against oxidative stress-induced injuries that lead to the prevention of cell death.[7]

Limonium tetragonum (Thunb.) Bullock (Plumbaginaceae) is a biennial plant growing in mud flat near seashores. The roots and leaves of this plant (known as 'Jin-Chi-Ye-Cao' or 'Bu-Xue-Cao') have been used in folk medicine to treat uterine hemorrhage, oligomenorrhea, dysgalactia and tinnitus. Various biological activities of *limonium*

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species have been reported including antioxidant, antibacterial, antifungal, antiviral and antileishmanial activities[8-10] derived from its active constituents such as myricetin, myricetin glycosides, tannins and caprolactam.[11-13]

Our recent studies showed that the methanolic extract of *L. tetragonum,* exhibited hepatoprotective activities that attenuated hepatic stellate cells proliferation possibly by regulating inflammatory signaling in vitro.[14] In the present study, the protective effects of ethyl acetate (EtOAc) soluble fraction of *L. tetragonum* extract (EALT) on ethanol-induced liver injury have been assessed in vivo. Acute alcohol intoxication model was devised using intragastric administration with a single high dose of ethanol (8 g/kg) in rats. The effects of EALT on alcohol metabolism, the level of malon dealdehyde (MDA), and the activities of antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GSH-px) and catalse in the liver were evaluated following ethanol exposure in experimental rats. The extract of *Hovenia dulcis* fruits, which has long been used in traditional herbal medicine for the treatment of liver diseases and detoxification after alcoholic poisoning in East Asia has been applied as positive control.[15-16]

Experimental

Plant material and preparation – The whole plants of *L. tetragonum* were collected from the foreshore in Sinan-gun, Korea in July 2013. A voucher specimen (GNP-70) has been deposited in the laboratory of pharmacognosy, Gyeongnam National University of Science and Technology. The aerial parts of *L. tetragonum* were dried using freezing-dryer, and then extracted three times with methanol. Removal of the solvent in vacuo yielded a methanolic extract, which was further suspended in distilled water and partitioned successively with $CHCl_3$, EtOAc, and *n*-BuOH. EtOAc soluble fraction of *L. tetragonum* extract (EALT) was filtered and evaporated in vacuum and then suspended in 0.5% carboxy methyl cellulose (CMC). The dried fruits of *Hovenia dulcis* were purchased from Kyung Dong market (Seoul, Korea). The fruits were extracted with distilled water for 3 h at 100 °C, using reflux. After filtration, the extract of *H. dulcis* (HDE) was concentrated and then suspended in 0.5%-CMC.

Experimental animals and treatment – Seven-week-old male SD rat was purchased from Orient Bio (Seongnam, Korea). They were acclimatized for 1 week before the experiment, and were housed in plastic cages in a room maintained at 23 ± 3 °C under a 12 h day/night cycle throughout the experiment. The rats were randomly assigned to four groups (n = 8 for each group) based on sample treatment. Thirty minutes after the treatment of CMC or sample, rats were received water or intragastric alcohol, as follow.

I: normal control group, 0.5%-CMC + distilled water (10 mg/kg, *p.o.*)
II: negative control group, 0.5%-CMC + 40%-ethanol (10 ml/kg, *p.o.*)
III: sample group, EALT (200 mg/kg, *p.o.*) + 40%-ethanol (10 ml/kg, *p.o.*)
IV: positive control group, HDE (200 mg/kg, *p.o.*) + 40%-ethanol (10 ml/kg, *p.o.*)

Analysis of serum alcohol concentration – About 1 ml of whole blood was collected from the ophthalmic vein of experimental mice in a serum separation tube at 1, 2 and 4 h after the ethanol challenge. After leaving a serum separation tube in room temperature for 30 min, blood samples were centrifuged at 3,000 rpm for 10 min. The supernatant consisted of blood serum was stored at – 70 °C before use, and was used in the analysis of blood ethanol levels. Ethanol was quantified using an ethanol assay kit (Bio-vision, Cat# K620-100) following to the manufacturer's instructions.

Alcohol metabolism enzyme activities – Following blood sample collection, rats were euthanized. The liver was dissected rapidly under standard conditions at 4 °C and homogenated in four volume of 0.25 M-sucrose solution. The supernatant obtained by sequential centrifugation at 600 × g for 10 min → 1,000 × g for 20 min → 15,000 × g for 1 h was applied for assays. The activities of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) were determined colorimetically by quantifying the amount of NADH produced using assay kit (Biovision Cat. #787-100 for ADH assay, Biovision Cat. #731-100 for ALDH assay). The detailed procedures followed the manufacturer's instructions.

TBARS assay – The liver was homogenized in 0.02 M phosphate buffer (pH 7.4) at a concentration of 10% (w/v). The homogenate was diluted to 5% (w/v) and resuspended with a hand homogenizer. The homogenate was incubated at 37 °C. Four-ml of homogenate was taken for MDA measurement by the thiobarbituric acid reaction (Buege and Aust, 1978; Stocks *et al*., 1974). A 2 ml of trichloroacetic acid (28% w/v in 0.25 N HCl) was added to 4 ml of the homogenate followed by centrifugation. Then, the 4ml of supernatant was combined with 1 ml of thiobarbituric acid (1% w/v in 0.25 N HCl) and boiled for 15 min to allow for chromophore development. The absorbance was read at 535 nm using a spectrophotometer. The MDA content was calculated using a molar extinction coefficient

of 1.56 × 105 M^{-1} cm^{-1}.[17]

Hepatic antioxidant enzyme activities – The liver was homogenated in 0.1 M phosphate buffer (pH 7.4) under standard conditions at 4 °C, and centrifuged at 3000 × g for 30 min and the supernatant (cytosolic and mitochondrial fractions) was collected for assessing antioxidative enzyme activity and GSH content. The activity of SOD was determined according to the method of McCord and Fridovich (1969)[18] by the xanthine-xanthine oxidase reaction. The activity of glutathione peroxidase (GSH-px) was determined by quantifying the rate of oxidation of GSH to GSSG by cumene hydroperoxide.[19] Values shown are the mean ± S.D. Protein concentrations were determined using a bicinchoninic acid (BCA) kit with bovine serum albumin as a standard.[20] Total GSH in the supernatant was determined spectrophotometrically using the enzymatic cycling method.[21]

Statistical analysis – Data for blood ethanol concentration and the activities of hepatic enzymes were expressed as the mean ± S.D. The values were analyzed by one-way ANOVA. The data was considered to be statistically significant if the probability had a value of 0.05 or less.

Results and Discussion

ALD is primarily driven by alcohol metabolism by products. Almost 90% of alcohol is absorbed from the gastrointestinal tract. Followed by alcohol absorption, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) act as primary alcohol metabolizing enzymes in the liver. Alcohol is rapidly oxidized to acetaldehyde by ADH and then is further metabolized to acetic acid by ALDH.[22] Since the polymorphism in the ADH pathway that is related to the production of acetaldehyde and to first-pass elimination of alcohol has been revealed,[23] the ADH pathway has been considered as a major pathway of alcohol metabolism.

On the basis of hepatoprotective effects of *L. tetragonum* extract that attenuated the hepatic stellate cells activation without cytotoxicity in hepatocytes in vitro,[14] in the present study, we attempted to determine whether *L. tetragonum* acts on the alcohol metabolism pathway to reduce liver damage in vivo. The extract of *L. tetragonum* was successively fractionated into $CHCl_3$, EtOAc, *n*-BuOH, and H_2O fractions. The EtOAc fraction of *L. tetragonum* extract (EALT), which showed significant antiproliferative activity in HSC-T6 cells (data not shown), was further subjected to evaluation of any effects on the blood alcohol concentration and the activities of hepatic ADH and ALDH following acute alcohol exposure in rats.

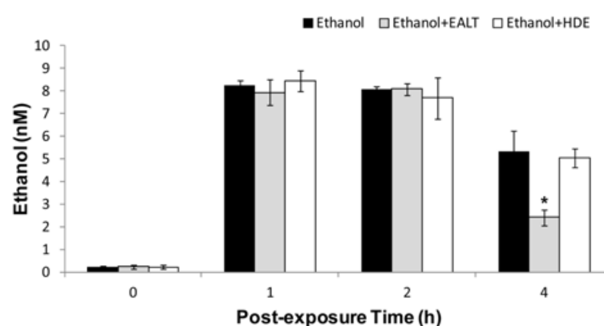
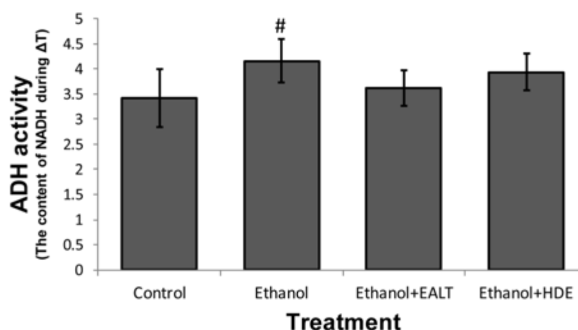


Fig. 1. Effects of EALT on blood alcohol concentration following acute ethanol exposure in rats. EALT or HDE (200 mg/kg body weight) was orally administrated once 1 h before ethanol intake (8 g/kg body weight, *p.o.*) in rats. The values shown are the mean ± S.D.

#Results significantly differ from the values non-treated control (group I): $p < 0.01$

*Results significantly differ from the values ethanol-only treated (group II): $p < 0.01$

(A)



(B)

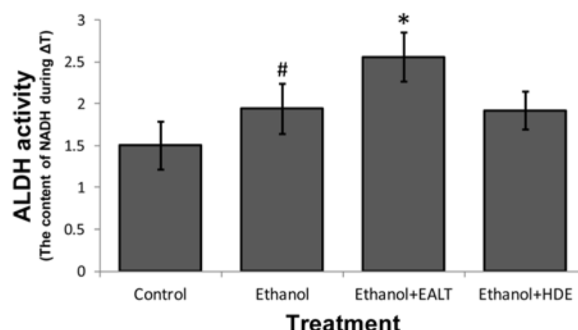


Fig. 2. Effects of LTE on the activities of ADH (A) and ALDH (B) in the liver following acute ethanol exposure in rats. LTE or HDE (200 mg/kg body weight) was orally administrated once 1 h before ethanol intake (8 g/kg body weight, *p.o.*) in rats. The values shown are the mean ± S.D.

#Results significantly differ from the values non-treated normal control (group I): $p < 0.01$

*Results significantly differ from the values ethanol-only treated (group II): $p < 0.01$

Table 1. Effects of EALT on the level of MDA and the activities of SOD, GSH-px, Catalase in the liver following acute ethanol exposure in rats

Group		MDA (nmol/mg protein)	SOD (nmol/mg protein)	GSH-px (nU/mg protein)	Catalase (U/g protein)
I	Control	23.45 ± 1.39	102.79 ± 12.78	326.80 ± 25.57	204.79 ± 19.89
II	Ethanol	49.89 ± 3.45#	69.22 ± 8.89#	259.61 ± 31.90#	165.77 ± 13.34#
III	Ethanol + EALT	35.44 ± 3.20*	78.34 ± 5.57*	318.99 ± 22.28*	189.57 ± 17.20*
IV	Ethanol + HDE	34.59 ± 2.29*	72.84 ± 6.77	305.09 ± 25.78	169.40 ± 11.64

MDA, malon dealdehyde; SOD, superoxide dismutase; Gpx, glutathione peroxidase
$^{\#}p < 0.01$ versus non-treated control (group I), $^{*}p < 0.01$ versus ethanol only-treated (group II).
The values shown are the mean ± S.D.

As shown in Fig. 1, the blood ethanol concentration reached peak at 1 h after alcohol intake and maintained until 2 h. The blood alcohol concentration was significantly lower at 4 h after alcohol administration in the 200 mg/kg EALT-treated group. In 200 mg/kg HDE-treated group (positive control), the blood ethanol concentration was shown as not statistically different to that of ethanol-only treated control. Following the blood sample collection, the liver was rapidly dissected and the activities of hepatic ADH and ALDH were measured. Acute administration of ethanol in rat induced significant increment of ADH and ALDH activities in the liver. The activity of ADH was virtually unchanged by administration of EALT. On the other hand, pretreatment of rat received ethanol with EALT increased remarkably the activity of ALDH (Fig. 2). It has been reported that acetaldehyde, the primary metabolite of EtOH, is highly toxic to hepatocytes than EtOH itself, and has been known as main cause of hangovers after heavy drinking. These findings led to the notion that the rapid elimination of acetaldehyde from the blood is important following acute alcohol intoxication. The concentration and retention time of acetaldehyde in the blood are determined crucially by the activity of ALDH. The elevated activity of ALDH which converts acetaldehyde to acetic acid resulted in rapid reduction and elimination of acetaldehyde in the blood. No significant changes in the activities of ADH or ALDH were found in HDE-treated positive control.

The excess accumulation of the metabolic end-products of alcohol can also induce the production of reactive oxygen species (ROS),[24] and promote lipid peroxidation and inflammation which are known to be harmful for cell homeostasis. Oxidative stress and inflammation induced during alcohol metabolism cause liver damage,[25-26] and further contribute to the progression of a variety of alcohol-related diseases on different body systems and organs.[27-28] The findings led to the notion that the drugs with antioxidative effects might be beneficial for preserving liver function and promote hepatoprotective effects to prevent alcohol-induced liver injury. Following acute exposure of ethanol to rats, the antioxidant status in the liver was assessed by determining the level of MDA, and the activities of SOD, GSH-px and catalase. As shown in Table 1, pretreatment of rats with EALT significantly abolished MDA increase in the liver after ethanol intake. Moreover, the activities of SOD, GSH-px and catalase in the liver were markedly enhanced in EALT-treated group as compared to non-treated control.

Taken together, in this study, we have demonstrated that pretreatment with EtOAc fraction of *L. tetragonum* extract could significantly enhance alcohol metabolism and prevent hepatic injury after acute alcohol intoxication, which is likely related to its modulation on the alcohol metabolism and antioxidant enzyme activities.

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