

The Standardized Extract of *Limonium tetragonum* Alleviates Chronic Alcoholic Liver Injury in C57BL/6J Mice

Na-Hyun Kim, Jeong-Doo Heo, Jung-Rae Rho¹, Min Hye Yang^{2*}, Eun Ju Jeong^{3*}

Gyeongnam Department of Environment and Toxicology, Korea Institute of Toxicology, Gyeongnam 52834, ¹Department of Oceanography, Kunsan National University, Jeonbuk 54150, ²College of Pharmacy, Pusan National University, Busan 46241, ³Department of Agronomy and Medicinal Plant Resources, Gyeongnam National University of Science and Technology, Jinju 52725, Republic of Korea

*Both authors contributed equally to this work.

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ABSTRACT

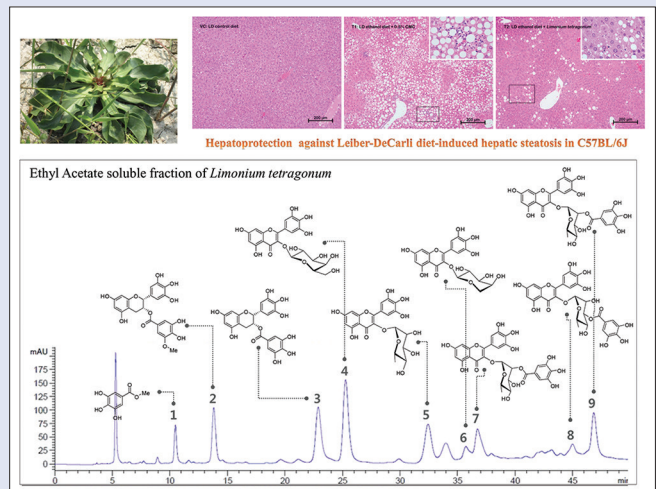
Background: In traditional folk medicine, *Limonium tetragonum* is used in the treatment of uterine hemorrhage, tinnitus, and oligomenorrhea.

Objective: This study aimed to identify the therapeutic effect of *L. tetragonum* EtOAc extract (EALT) on liver of mice with chronic alcohol poisoning. **Materials and Methods:** C57BL/6J mice were administered 100 mg/kg of EALT with a single binge ethanol/Lieber-DeCarli liquid diet for 8 weeks. **Results:** The chronic-binge ethanol diet induced a significant increase in liver marker enzyme activities. Co-administration of EALT reversed the elevation of serum total cholesterol and triglyceride as well as aspartate aminotransferase and alanine aminotransferase due to chronic alcohol consumption. Histologic findings including markedly attenuated fat accumulation in hepatocytes were observed in EALT-treated mice. EALT supplementation prevented alcoholic liver injury through attenuation of inflammatory mediators such as toll-like receptor-4, cytochrome P4502E1, and cyclooxygenase-2, and inflammatory cytokine interleukin-6. **Conclusion:** Results provided direct experimental evidence for the hepatoprotective effect of EALT in the NIAAA mouse model. Therapeutic attempts with the *L. tetragonum* extract might be useful in the management of alcoholic liver disease.

Key words: Alcoholic liver disease, chronic-binge ethanol-fed mice, inflammatory mediators and cytokines, *Limonium tetragonum*, liver marker enzymes

SUMMARY

- Halophyte *Limonium tetragonum* has recently been of interest in Korea for its nutritional value and salty taste which made it an ideal vegetable
- Phytochemical analysis of *L. tetragonum* EtOAc extract (EALT) resulted in nine compounds including catechins and myricetin glycosides as main components
- Administration of EALT for 8 weeks showed hepatoprotective effect on Lieber-DeCarli diet-fed mouse model
- A significant decrease in liver marker enzymes and inflammatory mediators was also detected.



Abbreviations used: EALT: *L. tetragonum* EtOAc extract; TC: Total cholesterol; TG: Triglyceride; ROS: Reactive oxygen species; CYP2E1: Cytochrome P4502E1; TLR-4: Toll-like receptor-4; COX-2: Cyclooxygenase-2.

Correspondence:

Prof. Min Hye Yang,
College of Pharmacy, Pusan National University,
Busan 46241, Republic of Korea.
E-mail: mhyang@pusan.ac.kr
Prof. Eun Ju Jeong,
Department of Agronomy and Medicinal Plant
Resources, Gyeongnam National University of
Science and Technology, Jinju 52725, Republic of
Korea.
E-mail: ejjeong@gntech.ac.kr
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INTRODUCTION

Alcoholic liver disease has been recognized as a major public health hazard without reliable therapeutic approach.^[1] Alcohol-induced liver injury is featured by alcoholic hepatitis, liver steatosis, and other histological findings.^[2] Patients with alcoholism are much more likely to have alcohol-induced liver cell damage. Consumption of heavy alcohol causes progressive hepatic damage such as alcoholic steatohepatitis, fibrosis, and cirrhosis.^[3,4]

Herbal preparations have attracted more attention as sources of medicinal materials to treat liver disease due to multitargeted action and less toxic adverse effects.^[5,6] Multiple mechanisms including excess inflammation and lipid synthesis contribute to the increase in alcoholic

liver damage.^[7,8] Plant extracts or phytochemicals are known to alleviate alcoholic liver disease through various defense mechanisms.^[6] Despite numerous trials to find plant materials against alcoholic liver disease, attention continuously focused on new herbal drugs since there is still

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no clear-cut proof of the therapeutic effect of natural medicines in the treatment of alcoholic liver injuries.^[9,10]

Limonium tetragonum, growing in a harsh environment of high salinity, has been used as edible vegetables in Korea.^[11,12] The *L. tetragonum* is known to have antioxidant and anticancer activities due to the presence of bioactive flavonoids.^[13,14] Our previous researches mainly focused on the liver protection of *L. tetragonum* by suppression of hepatic satellite cells-T6 proliferation,^[15] liver injury following acute alcohol excess,^[16] and diethylnitrosamine-induced liver fibrosis.^[17] This study was undertaken to verify the hepatoprotective effect of *L. tetragonum* in the NIAAA model. Liver function markers, inflammatory mediators, and cytokines were measured in serum and liver tissue to understand the inhibition mechanism.

MATERIALS AND METHODS

Plant material and sample preparation

The *L. tetragonum* sample was collected in Sinan-gun, Korea and a voucher specimen (GNP-70) was deposited in the Laboratory of Pharmacognosy, Gyeongnam National University. The method for extraction of *L. tetragonum* was assessed as previously described by Kim *et al.*^[16] For high-performance liquid chromatography (HPLC) analysis, *L. tetragonum* EtOAc extract (EALT) was freeze-dried and weighed accurately. The dried powder was dissolved with 50% ethanol and directly injected to HPLC.

Chromatographic conditions

The EALT sample was analyzed using an Agilent 1100 series HPLC system for phytochemical characterization. A Phenomenex hyrdo-RP18 (5 μ m, 4.6 mm \times 250 mm) and flow rate of 0.8 mL/min were used for analysis. The solvent gradient conditions (methanol–water with 0.1% acetic acid) are shown in Table 1.

Animals and treatment

Seven-week-old male C57BL/6J mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea) and housed in a controlled room (20°C \pm 2°C, 50% \pm 5% humidity, and 12 h light/dark cycle) with *ad libitum* access to water and standard laboratory diet. After an acclimation period (1 week), mice were randomly divided into four groups: (i) Lieber-DeCarli (LD)-normal group was given 0.5% carboxymethyl cellulose (CMC) orally and was fed Lieber-Decali control diet; (ii) LD-ethanol group received 0.5% CMC orally and was fed Lieber-Decali ethanol diet; (iii) sample group received EALT (100 mg/kg b.w.) and was fed Lieber-Decali ethanol diet; (iv) positive group received silymarin (100 mg/kg b.w.) and was fed Lieber-Decali ethanol diet. The CMC solution, EALT, or silymarin was administered once a day, 7 days a week for 8 weeks. All animals had constant access to water and food (Liber-Decali control diet or Lieber-Decali ethanol diet). Animal experiments were carried out with the permission of Gyeongnam National University of Science and Technology according to the guidelines for animal experiment.

Table 1: Solvent gradient conditions for high-performance liquid chromatography analysis of *Limonium tetragonum* EtOAc extract

Time (min)	Flow rate (mL/min)	MeOH (%)	Water (%)
0	0.8	35	65
20	0.8	40	60
30	0.8	40	60
30.1	0.8	45	55
40	0.8	50	50
50	0.8	50	50

Blood chemistry

Blood samples were harvested from caudal vein of mice and centrifuged to separate serum and plasma cells (3000 rpm, 10 min). The serum was used in the analysis of liver marker enzymes.

Histological analysis

For histology, a sample of liver from each mouse was immersed in buffered 10% formaldehyde immediately after removal. Additional liver samples for Western blot experiment were homogenized with radioimmunoprecipitation assay buffer. Fixed liver samples were stained with hematoxylin–eosin for morphological image analysis.

Western blot

Proteins from liver homogenate (50 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis system, transferred to polyvinylidene difluoride membranes, and placed in blocking buffer for 30 min. Membranes were then incubated with primary antibodies (anti-cyclooxygenase-2 [COX2], anti-toll-like receptor-4 [TLR4], and anti-cytochrome P4502E1 [CYP2E1]) overnight followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) at 25°C (1 h). Resulting immunoblots were visualized using enhanced chemiluminescence (Vilber Fusion Fx).

RESULTS

The standardization of *Limonium tetragonum* EtOAc extracts using high-performance liquid chromatography

According to the chromatographic analysis, the solvent condition of acetonitrile/water with 0.1% acetic acid and the wavelength of 254 nm showed the high absorption of overall peaks detected with the improved S/N for the analysis of EALT. The presence of nine compounds: (1) gallincin, (2) (–)-epigallocatechin-3-(3'-O-methyl) gallate, (3) (–)-epigallocatechin-3-gallate, (4) myricetin-3-O- β -D-galactopyranoside, (5) myricetin-3-O- α -L-rhamnopyranoside, (6) myricetin-3-O- α -L-arabinopyranoside, (7) quercetin-3-O- β -D-galactopyranoside, (8) myricetin-3-O-(2''-O-galloyl)- α -L-rhamnopyranoside, (9) myricetin-3-O-(3''-O-galloyl)- α -L-rhamnopyranoside in EALT was verified by comparing their peaks with those of authentic standard compounds [Figure 1].

Effects of *Limonium tetragonum* EtOAc extract on serum liver enzymes in alcoholic liver steatosis mice

An obvious induction of fatty liver by the treatment of single binge ethanol feeding was observed in LD diet-fed mouse model [Table 2]. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the LD diet group were 3.5-fold and 4.8-fold greater than that of the normal diet group. The serum AST and ALT of mice in administration with the EALT was decreased by 25.5% and 38.0%, respectively. The activities of serum triglyceride (TG) and total cholesterol (TC) in the 100 mg/kg EALT-treated group demonstrated similar trends. Plasma TG was modestly elevated in the ethanol-fed mice but was reduced by oral administration of EALT up to 20% of control. The extract of *Limonium* was also found to decrease serum TC amount by 19.3% of the negative control. The serum level of alkaline phosphatase remained unchanged in all groups. When silymarin was treated as a positive control, the serum levels of enzymes were lower than those of the pair-fed control mice, but differences were not significant in some cases.

Histological changes in liver tissue

When the mice were administered with LD diet alone, the histology exhibited no accumulation of lipid droplets in hepatocytes [Figure 2a].

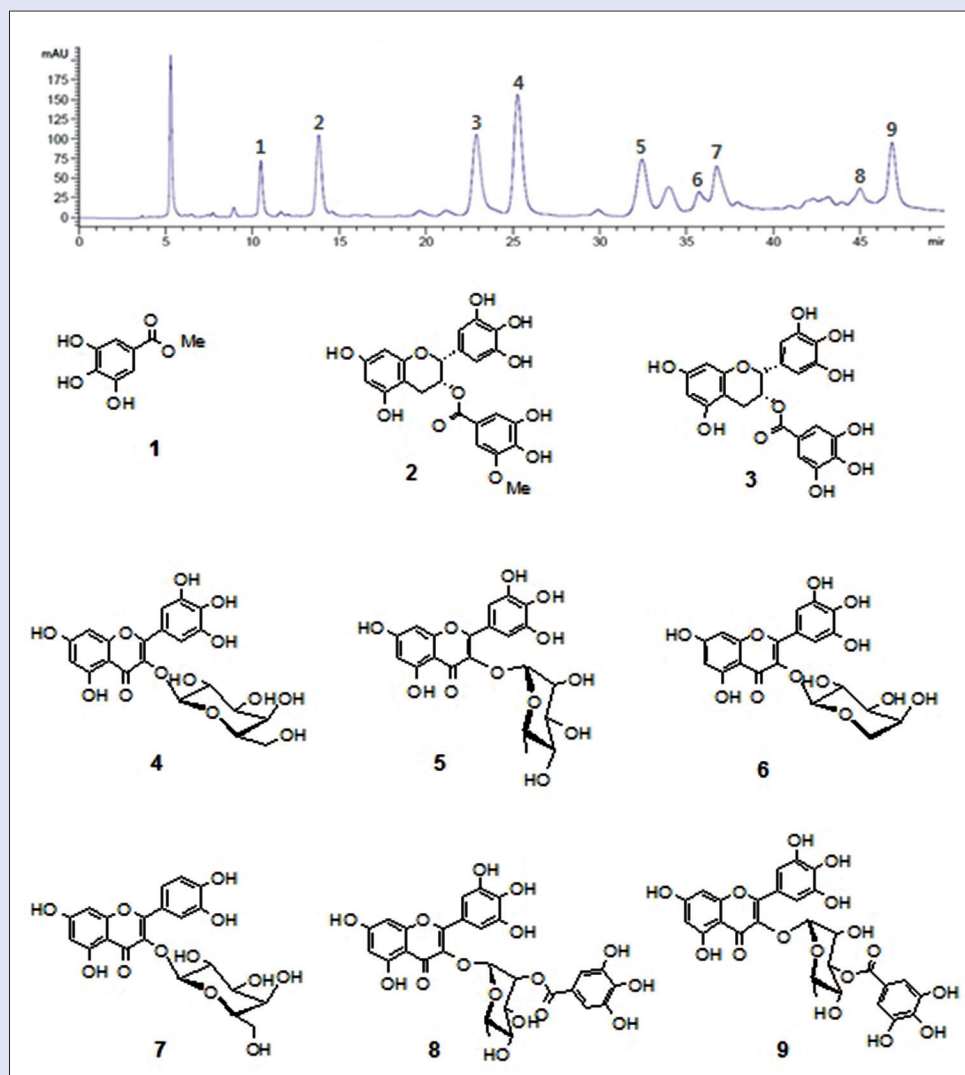


Figure 1: High-performance liquid chromatography chromatogram of *L. tetragonum* EtOAc extract at the wavelength of 254 nm. 1: Gallinacin, 2: (-)-epigallocatechin-3-(3''-O-methyl) gallate, 3: (-)-epigallocatechin-3-gallate, 4: Myricetin-3-O-β-D-galactopyranoside, 5: Myricetin-3-O-α-L-rhamnopyranoside, 6: Myricetin-3-O-α-L-arabinopyranoside, 7: Quercetin-3-O-β-D-galactopyranoside, 8: Myricetin-3-O-(2''-O-galloyl)-α-L-rhamnopyranoside, 9: Myricetin-3-O-(3''-O-galloyl)-α-L-rhamnopyranoside

Table 2: Fasting plasma aspartate aminotransferase (A), alanine aminotransferase (B) and alkaline phosphatase (C), total cholesterol (D), and triglyceride (E) levels in Lieber-DeCarli ethanol diet-fed mice

Treatment groups	AST (U/L)	ALT (U/L)	ALP (U/L)	TC (mg/L)	TG (mg/L)
LD-normal	53.5±7.0	31.3±11.7	335.4±22.4	159.9±12.0	55.3±9.6
LD-ethanol	189.5±22.0	149.6±26.0	314.1±43.1	145.0±11.7	61.7±6.0
Sylimarin	160.0±23.0*	138.3±34.0	330.2±44.3	147.0±8.0	58.3±7.0
EALT	141.3±25.0*	92.7±24.0*	310.5±35.3	117.0±9.2*	49.4±5.0*

Values are expressed as the means±SD ($n=10$). * $P<0.01$ compared with the LD ethanol diet group. LD-normal group: LD normal diet mice; LD-ethanol group: LD ethanol diet mice; Sylimarin group: LD ethanol diet mice administered 100 mg/kg body weight/day of sylimarin; EALT group: LD ethanol diet mice administered 100 mg/kg body weight/day of EALT; EALT: *Limonium tetragonum* EtOAc extract; LD: Lieber-DeCarli; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; TC: Total cholesterol; TG: Triglyceride; SD: Standard deviation

However, histopathologic features of the liver tissues revealed apparent lipid droplet accumulation in the mice fed an ethanol-containing low-fat

diet. After chronic alcohol intake for 8 weeks, the marked pathological changes of hepatic steatosis containing numerous varying-size lipid vacuoles were detected in the centrilobular part of the hepatic lobule. Quantification of hepatic lipid contents resulted that ethanol treatment causes a significant accumulation of lipid (>13 fold) in mouse livers [Figure 2b]. The consumption of the EtOAc extract of *L. tetragonum* and silymarin attenuated the formation of lipids in liver tissues [Figure 2c and 2d]. Treatment with the EALT before ethanol ingestion significantly attenuated alcohol-induced fatty liver by percentage. Lipid droplets were rare in the mice livers administered with EALT compared to the mice fed a LD ethanol diet alone [Figure 2d]. Administration of *Limonium* showed better protective effect on the chronic alcoholic liver injury than silymarin, a potent hepatoprotective agent.

Effects of *Limonium tetragonum* EtOAc extract on the protein expression in liver homogenates

To find out whether the *Limonium* extract affect hepatic inflammatory reactions induced by alcohol, the expression of TLR4, CYP2E1, and

COX-2 was detected by Western blot analysis. Ethanol feeding with liquid diet based on the LD formulation increased the TLR4, CYP2E1, and COX-2 contents by 23.5-fold, 11.4-fold, and 23-fold, respectively, compared to control [Figure 3]. The elevation was reversed by

co-incubation with EALT. Protein level of TLR4 was suppressed by 68.2% in EALT-treated mice, compared with control group. The ethanol metabolizing CYP2E1 and COX-2 were also significantly decreased by treatment of EALT (95.3% and 65.2% decrease, respectively).

Effects of *Limonium tetragonum* EtOAc extract on inflammatory cytokine levels in liver homogenates

Alcoholic liver disease is characterized by dysregulation of cytokine metabolism such as enhanced tumor necrosis factor- α (TNF- α) and TNF- α -inducible cytokines/chemokines production.^[18] The anti-inflammatory property of EALT in LD diet-induced liver injury was evaluated by measuring both TNF- α and interleukin-6 (IL-6) levels. The alcoholic liver damage-induced group showed increased activity of IL-6 (43.8%), which was reduced significantly (39.5%) with *Limonium* extract supplementation [Figure 4]. A positive material attenuated the increase of IL-6 by 30% of control. Hepatic TNF- α activity also increased after ethanol liquid diet intake (49.75% compared with normal diet group). However, recent data have failed to show any change in TNF- α level between EALT-treated and nontreated group.

DISCUSSION

Excess alcohol exposure usually causes liver abnormalities such as fatty liver, liver inflammation, structural damage in the liver, and even liver cancer.^[19-21] Treating liver diseases with herbal drugs such as *Silybum marianum* (milk thistle) and its derivatives, *Phyllanthus* species, and glycyrrhizin (licorice root extract) has a long tradition.^[6] Although these botanical medicines appear to be effective in treating chronic liver failure, data are currently insufficient to recommend their clinical use for patients with alcoholic liver disease.^[9,22] Therefore, searching for

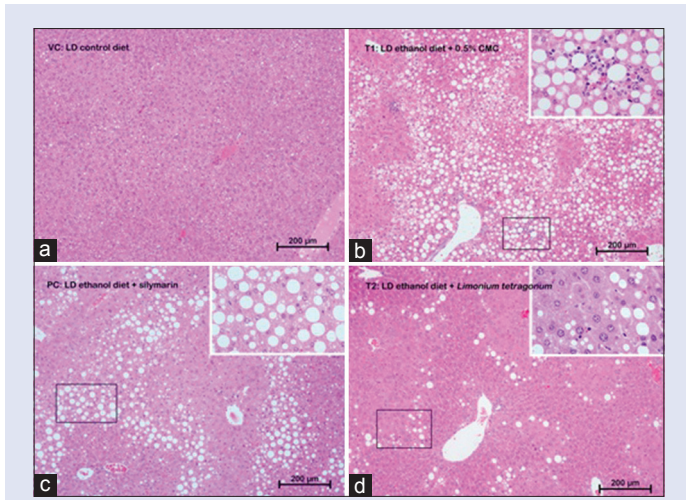


Figure 2: Effect of *Limonium tetragonum* on the histological changes of Lieber-DeCarli diet-induced alcoholic fatty liver in C57BL/6J mice. Hepatocytes staining was carried out with the hematoxylin and eosin staining method. (a) LD-normal diet group, (b) LD-ethanol diet group, (c) LD ethanol diet group administered 100 mg/kg body weight/day of silymarin, (d) LD ethanol diet group administered 100 mg/kg body weight/day of EALT; EALT: *Limonium tetragonum* EtOAc extract; LD: Lieber-DeCarli

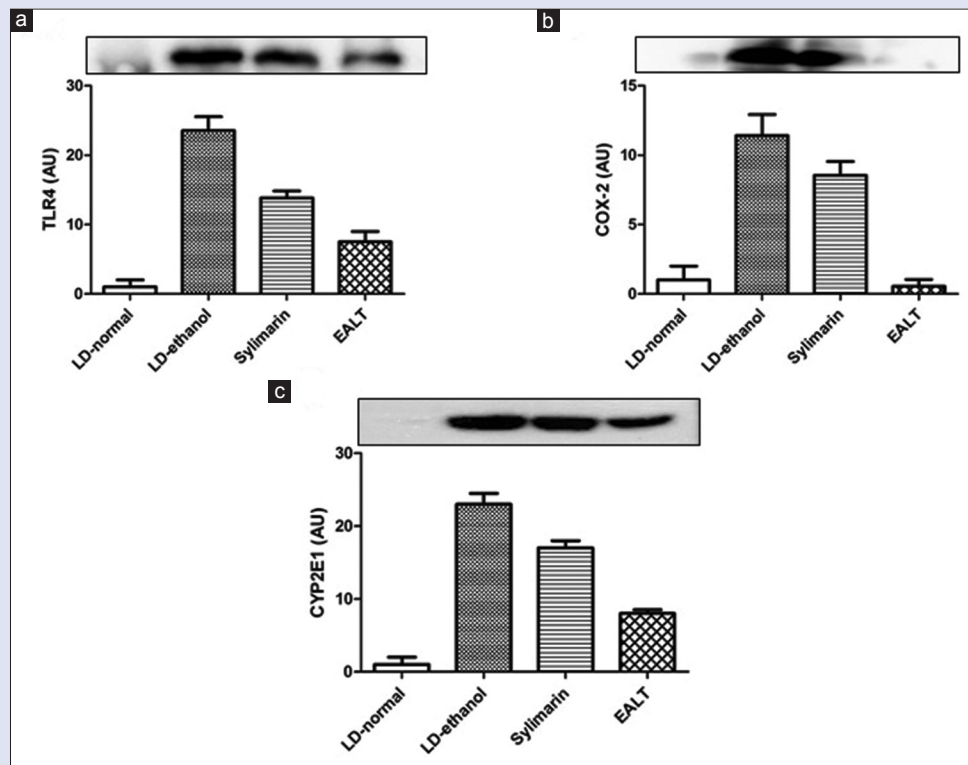


Figure 3: Effects of *Limonium tetragonum* on the protein expression of toll-like receptor-4 (a), cyclooxygenase-2 (b), and cytochrome P4502E1 (c) in Lieber-DeCarli diet-induced alcoholic fatty liver. Values are the mean \pm standard deviation of 10 mice. # $P < 0.01$ compared to Group I; * $P < 0.1$ compared to Group II; ** $P < 0.01$ compared to Group II

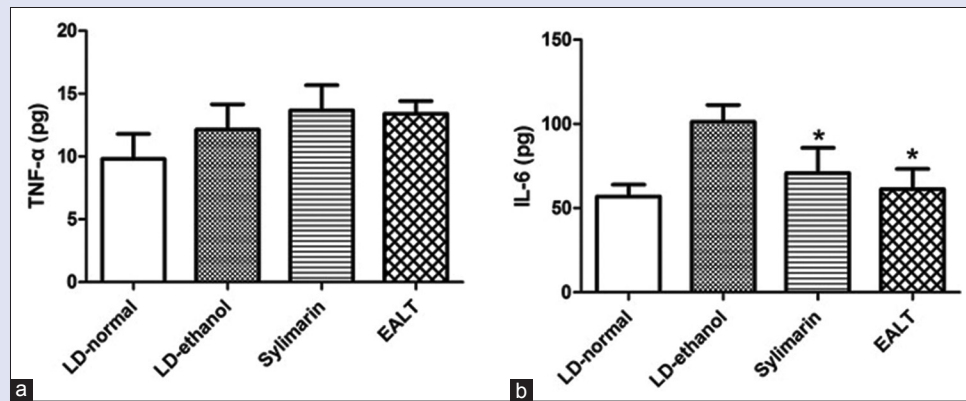


Figure 4: Effects of *Limonium tetragonum* on the production of inflammatory cytokines interleukin-6 (a) and tumor necrosis factor-alpha (b) in Lieber-DeCarli diet-induced alcoholic fatty liver. Values are the mean \pm standard deviation of 10 mice. # $P < 0.01$ compared to Group I; * $P < 0.1$ compared to Group II

new hepatoprotective agents from natural sources might be a promising target for the treatment of alcoholic liver dysfunction.

In our study, an apparent increase in serum AST and ALT was observed in the NIAAA model. Administration of EALT (100 mg/kg b.w.) on the LD diet mice normalized serum TC and TG concentrations as well as AST and ALT, liver function marker enzymes. According to microscopic histological examination for liver tissues, fatty liver could be produced by prolonged ethanol intake in mice. Liver steatosis (fatty liver) is the major pathologic manifestation of alcohol-induced liver injury together with steatohepatitis (formerly alcoholic hepatitis) and cirrhosis.^[23,24] The first change in features of liver histology attributable to alcohol is the increased prevalence of fatty liver.^[25] Alcoholic fatty liver is featured by TG deposition in the liver due to the excessive fatty acids synthesis.^[26] In this study, lipid vacuoles occupied much of hepatocyte in chronic alcohol-fed mice, being significantly reduced by EALT treatment for 8 weeks. Our observations clearly revealed that the EALT possesses therapeutic efficacy against chronic alcoholic liver damage in the NIAAA mice.

The involvement of oxidative damage in alcohol toxicity has emerged from experimental and clinical studies with chronic alcohol-fed animals and alcoholic liver disease patients.^[27] Acute and chronic alcohol consumption promotes the reactive oxygen species generation, and CYP2E1 is a crucial component of the ethanol-induced oxidative stress in the liver.^[28,29] Increasing evidence also indicates an important role for both COX-2 and TLR-4 in alcohol-induced steatosis and liver damage.^[30-33] Our results showed enhanced protein expressions of CYP2E1 and TLR4 in chronic ethanol-fed mice with >23-fold increase in comparison to the vehicle control. The suppression of inflammatory mediators including CYP2E1, TLR4, and COX-2 by EALT administration is a direct evidence of the hepatoprotective effect of *L. tetragonum* against oxidative liver damage by ethanol consumption. Elevated inflammatory cytokines have been consistently detected in alcohol-fed animals and IL-6 is considered as a pro-inflammatory cytokine linked to alcoholic liver disease.^[34,35] Increased inflammatory cytokine IL-6 was apparently decreased by feeding a LD ethanol diet with the *Limonium* extract. Taken together, downregulation of ethanol-induced oxidative stress and inflammation by treatment of EALT might contribute to attenuation of chronic alcoholic liver damage in mice.

CONCLUSION

This study provides a beneficial effect of *L. tetragonum* on chronic ethanol-mediated experimental liver damage in mice. The *Limonium* extract alleviated alcoholic liver steatosis in the NIAAA model. Suppression

of chronic alcoholic liver injury by *Limonium* administration might be partly mediated by downregulation of inflammatory mediators and cytokines release. Nine compounds including catechins and myricetin glycosides were identified as major components of EALT, but the effects of the constituents were not presented in this report, which is a limitation of this study. The positive outcomes of our research could be a strong basis for treating alcohol-induced liver disease relevant to *L. tetragonum* administration, which warrants further investigations of isolates from *L. tetragonum* in animal model.

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Conflicts of interest

There are no conflicts of interest.

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