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Wild bitter gourd protects against alcoholic fatty liver in mice by attenuating oxidative stress and inflammatory responses

Kuan-Hung Lu^{†a}, Hui-Chun Tseng^{†a}, Chun-Ting Liu^a, Ching-Jang Huang^b, Jong-Ho Chyuan^c and Lee-Yan Sheen^{*ade}

^a *Institute of Food Science and Technology, College of Bio-Resources and Agriculture, National Taiwan University, Taipei, Taiwan*

^b *Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, Taipei, Taiwan*

^c *Hualien District Agricultural Research and Extension Station, Hualien, Taiwan*

^d *Center for Food and Biomolecules, College of Bio-Resources and Agriculture, National Taiwan University, Taipei, Taiwan*

^e *National Center for Food Safety Education and Research, College of Bio-Resources and Agriculture, National Taiwan University, Taipei, Taiwan*

[†]The first two authors contributed equally to the work.

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*** Corresponding author:**

Dr. Lee-Yan Sheen,

Institute of Food Science and Technology, National Taiwan University, No. 1, Sec. 4,

Roosevelt Road, Taipei 10617, Taiwan. Email: lysheen@ntu.edu.tw

Abstract

Bitter melon (*Momordica charantia* L.) is a common vegetable grown widely in Asia that is used as a traditional medicine. The objective of this study was to investigate whether wild bitter melon possessed protective effects against chronic alcohol-induced liver injury in mice. C57BL/6 mice were fed an alcohol-containing liquid diet for 4 weeks to induce alcoholic fatty liver. Meanwhile, mice were treated with ethanol extracts from four different wild bitter melon cultivars: Hualien No.1', Hualien No.2', Hualien No.3' and Hualien No.4'. The results indicated that daily administration of 500 mg/kg body weight of Hualien No.3' extract (H3E) or Hualien No.4' extract (H4E) markedly reduced the steatotic alternation of liver histopathology. In addition, the activation of serum aminotransferases (AST and ALT) and the accumulation of hepatic TG content caused by alcohol were ameliorated. The hepatoprotective effects of H3E and H4E involved the enhancement of the antioxidant defence system (GSH, GPx, GRd, CAT and SOD), inhibition of lipid peroxidation (MDA) and reduction of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) in the liver. Moreover, H3E and H4E supplementation suppressed the alcohol-induced elevation of CYP2E1, SREBP-1, FAS and ACC protein expression. These results demonstrated that ethanol extracts of Hualien No.3' and Hualien No.4' have beneficial effects against alcoholic fatty liver, in which they attenuate oxidative stress and inflammatory responses.

Introduction

The World Health Organisation reports that the hazardous and harmful use of alcohol is one of the world's leading health risks, resulting in approximately 2.5 million deaths every year due to liver injury.¹ The liver plays an essential role in metabolising ingested ethanol; however, chronic or excessive alcohol ingestion may lead to alcoholic liver disease (ALD). ALD is associated with a spectrum of liver injuries ranging from steatosis and steatohepatitis to fibrosis and cirrhosis.² The pathogenesis of ALD involves oxidative stress, gut-derived lipopolysaccharide, pro-inflammatory cytokines and the innate immune system.^{3,4} Alcohol consumption may result in the generation of reactive oxygen species (ROS) through the induction of microsomal protein Cytochrome P450 2E1 (CYP2E1) and the activation of Kupffer cells.^{5,6} Kupffer cells secrete cytokines, such as tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6), and cause liver inflammatory responses.⁷ TNF- α could increase fat deposition in the liver by affecting hepatic lipid metabolism, which involves sterol regulatory element-binding protein-1 (SREBP-1).⁸ In general, fatty liver is considered to be a mild or reversible condition, whereas steatohepatitis can easily lead to more severe and irreversible diseases.⁹

Bitter melon (*Momordica charantia* L.), a tropical and subtropical vine of the family Cucurbitaceae, is grown widely in Asia, Africa and the Caribbean for its edible vegetable and is used in traditional medicines.^{10,11} It exhibits several biological effects, such as regulation of glucose metabolism and blood lipids,^{12,13} in addition to its antioxidant,^{14,15} anti-inflammation,^{16,17} anti-tumour¹⁸ and antimicrobial activities.¹⁹ It has also been demonstrated that the ethanol extract of bitter melon, which contains abundant polyphenols, possesses higher antioxidant capacity compared to extracts from other types of solvent.^{20,21} Because alcohol consumption may result in the generation of reactive oxygen species and liver inflammatory

responses, a reasonable treatment strategy is to attenuate alcohol-induced liver disease using an antioxidative and anti-inflammatory agent such as the ethanol extract of bitter gourd.

In Taiwan, Hualien No.1', Hualien No.2', Hualien No.3' and Hualien No.4' bitter gourds are four new cultivars grown by the Hualien District Agricultural Research and Extension Station. They were bred using a cross breeding approach between wild and native bitter gourds, and they showed excellent antioxidant capacity. For these reasons, we investigated the protective capability of ethanol extracts from the four wild bitter gourds in C57BL/6 mice fed an alcohol-containing liquid diet. The hepatoprotective effects of the wild bitter gourd extracts on serum transaminases, triglyceride accumulation, hepatic antioxidant enzymes and pro-inflammatory cytokines were evaluated in the present study. Furthermore, the potential mechanisms of the hepatoprotective effects were also explored.

Materials and methods

Chemicals

Lieber-DeCarli liquid diet was purchased from Dyets Inc. (Bethlehem, PA, USA). SpotchemTM II reagent strips for measurements of serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum triglyceride (TG) and serum total cholesterol (TC) levels were obtained from Arkray Inc. (Kyoto, Japan). Glutathione (GSH), triglyceride (TG), glutathione peroxidase (GPx), glutathione reductase (GRd), catalase (CAT), superoxide dismutase (SOD) and lipid peroxidation (LPO) assay kits were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). TNF- α , IL-1 β and IL-6 immunoassay kits were purchased from eBioscience (San Diego, CA, USA). The primary antibodies used for Western blotting included CYP2E1, SREBP-1, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (GeneTex, San Antonio, Texas, USA). Methanol, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), gallic acid, aluminum nitrate ($\text{Al}(\text{NO}_3)_3$), potassium acetate (CH_3COOK) and quercetin were purchased from Sigma (St. Louis, MO, USA).

Sample preparation

Four different wild bitter gourd cultivars, Hualien No.1', Hualien No.2', Hualien No.3' and Hualien No.4', were obtained from the Hualien District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, Taiwan. Whole fruits of each cultivar were separately cut into small pieces. The pieces were collected, freeze-dried and ground. The freeze-dried powders were extracted with 80% ethanol (1:20 w/v). The solutions were sonicated for 20 minutes and allowed to stand for 24 h. Then the solutions were centrifuged at $4500\times g$ for 30 minutes and vacuum filtered. The residues were re-extracted by the same extraction process. The filtrates were concentrated by a rotary evaporator to remove ethanol. The concentrates were freeze-dried to remove the remaining water. Finally, the dried ethanol extracts of Hualien No.1' (H1E), Hualien No.2' (H2E), Hualien No.3' (H3E) and Hualien No.4' (H4E) were obtained. The yield of H1E, H2E, H3E and H4E were 24.9%, 26.8%, 20.9% and 23.8% of the dry power weight, respectively. Each ethanol extract was dissolved in carboxymethyl cellulose before oral administration.

Animals and treatments

The use of animals was in compliance with the guidelines established by the Institutional Animal Care and Use Committee of National Taiwan University (approval number: NTU-100-EL-80). A total of 72 male C57BL/6 mice, with body weights of 23-25 g and aged 6 weeks, were obtained from BioLASCO Taiwan Co.,

Ltd (Taipei, Taiwan). The mice were housed 3 mice per cage in the Animal House Facility of the Institute of Food Science and Technology, National Taiwan University. Standard experimental conditions were $22 \pm 3^{\circ}\text{C}$, 50%-70% humidity and 12-h light/dark cycles. The mice were randomly divided into six groups (twelve mice per group). One group received Lieber-DeCarli Regular Liquid Diet Control (#7110027) and the other groups received Lieber-DeCarli Regular Liquid Diet Ethanol (#7110260) (Dyets Inc., Bethlehem, PA, USA). The six groups in this animal study were the control liquid diet-fed group (normal control), ethanol-containing liquid diet-fed group (negative control, AFLD group) and ethanol-containing liquid diet-fed group treated with H1E, H2E, H3E or H4E (AFLD + H1E, AFLD + H2E, AFLD + H3E, AFLD + H4E, respectively). During 2 weeks of acclimatisation, the five alcohol-fed groups were allowed free access to the liquid diet, which was a mixture of the control and the alcohol-containing liquid. The content of the alcohol-containing liquid in the mixture increased gradually from 20% to 100%. After acclimatisation, the mice of four treatment groups were treated daily with 0.1 mL of H1E, H2E, H3E or H4E at a dose of 500 mg/kg body weight by gavage. The normal control and negative control groups were given the same volume of vehicle only. After 4 weeks of treatment, mice were fasted overnight (12 h) and sacrificed by CO_2 asphyxiation.

Serum and liver collection

The blood of mice was collected by cardiac puncture, and serum samples were obtained by centrifugation and stored at -80°C for biochemical analysis. The liver was excised and weighed. A piece of the liver (0.5 cm^3) was fixed in 10% (v/v) neutral phosphate-buffered formalin for histological examination. The other part of the liver was immediately frozen in liquid nitrogen and stored at -80°C for the analysis of GSH levels, antioxidant enzymes activities, lipid peroxidation, TG content,

pro-inflammatory cytokine levels and Western blotting.

Histological examination

Liver histology was assessed by haematoxylin and eosin (H&E) staining of mouse liver sections. The livers were fixed in formalin and embedded in paraffin. Stained sections were observed at a magnification of 400x.

Serum biochemistry

To assess the liver damage, serum AST and ALT levels were measured. Meanwhile, serum TG and TC levels were also determined. The AST, ALT, TG and TC levels were measured by Spotchem™ EZ dry chemistry automated analyser (Arkray Inc., Kyoto, Japan).

Estimations of GSH levels, antioxidant enzymes activities and lipid peroxidation

The frozen liver tissue was homogenised (1:10 w/v) in phosphate buffer (8 mM KH₂PO₄, 12 mM K₂HPO₄, 1.5% KCl, pH 7.4) and centrifuged at 1000×g for 30 minutes at 4°C. The resulting supernatant was collected for the following analyses. The level of total GSH including GSSG was measured using an ELISA kit (Cayman, MI, USA). The activity of GPx, GRd, CAT and SOD were determined using a Cayman assay kit (Cayman, MI, USA). To assess the hepatic lipid peroxidation level, the thiobarbituric acid reactive substance (TBARS) level²² was analysed by Cayman TBARS assay kit (Cayman, MI, USA). Malondialdehyde (MDA), a naturally occurring product of lipid peroxidation, reacts with thiobarbituric acid (TBA) under high temperature (90-100°C) and acidic conditions to form the TBARS. The TBARS was measured colourimetrically at 530-540 nm.

Hepatic TG content and pro-inflammatory cytokine level determinations

The frozen liver tissue was homogenised and centrifuged as described above, and the resulting supernatant was used to determine the content of TG in the liver by ELISA using a Triglyceride colourimetric assay kit (Cayman, MI, USA). The levels of pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 were analysed in the liver using a commercial ELISA kit (eBioscience, San Diego, CA, USA).

Western blot analysis

The frozen liver tissues were homogenised in lysis buffer (7 M Urea, 2 M Thiourea, 2% SDS, 4 ppm bromophenol blue, proteinase inhibitor, phosphatase inhibitor and dithiothreitol). Protein concentrations were quantified with a Bradford assay kit (Bio-Rad, CA, USA). Lysates containing equal amounts of protein were analysed by Western blot as previously described with slight modifications.²³ In brief, samples were subjected to electrophoretic separation in 6% SDS-polyacrylamide gels, blotted onto polyvinylidene fluoride (PVDF) membranes, and probed with primary antibodies against CYP2E1, SREBP-1, FAS, ACC and GAPDH. Following wash cycles with TBST buffer (Tris-Buffered Saline containing 0.05% Tween 20), membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Membranes were then immersed in ECL (enhanced chemiluminescence) detection reagent, and immunoreactive bands were visualised using the UVP Auto ChemiTM system. Finally, the signal intensity was quantified using ImageJ 1.45 software (National Institutes of Health, USA).

Estimation of total phenolic and flavonoid contents

Phytochemical components of the four wild bitter gourd extracts were analyzed by determinations of the total phenolic and total flavonoid contents. Total phenolic

contents of the extracts were determined using the Folin-Ciocalteu method.²⁴ Briefly, 100 mg of each extract was vortexed with 10 mL of methanol to make 1% of extract solution. Then, 0.2 mL of the solution was added with 1.0 mL of 0.2 N Folin-Ciocalteu reagent and incubated for 5 min. After 2 h incubation with 1 mL of 0.5 N Na₂CO₃, absorbance of the solution was taken at room temperature using a spectrophotometer at 750 nm. Gallic acid was chosen as a standard. Using a seven point standard curve (0–250 mg/L), the levels of total phenolic content in H1E, H2E H3E and H4E were determined in triplicate, respectively. The data of total phenolic content were expressed as mg gallic acid equivalents (GAE)/g extract.

The total flavonoid content was determined according as the method described by Lin and Tang²⁵ with some modifications. Briefly, 100 mg of each extract was vortexed with 10 mL of methanol to make 1% of extract solution. Then, 0.5 mL of the solution was mixed with 1.5 mL of deionized water, 0.1 mL of 10% Al(NO₃)₃ and 0.1 mL of 1 M CH₃COOK. After incubation at room temperature for 40 min, absorbance of the reaction mixture was measured at 415 nm on a spectrophotometer. Quercetin was chosen as a standard. Using a seven point standard curve (0–80 mg/L), the levels of total flavonoid content in H1E, H2E H3E and H4E were determined in triplicate, respectively. The data of total flavonoid content were expressed as milligram quercetin equivalents (QE)/g extract.

Statistical analysis

All values were represented as the mean ± SD. Significant differences were analysed by one-way ANOVA coupled with the Duncan's multiple test using SAS 9.0 software (Cary, NC, USA). $p < 0.05$ was considered statistically significant.

Results

Effect of wild bitter gourd extracts on liquid diet intake and body weight

Liquid diet intakes were not significantly different among the 6 groups except during the first week (Table 1). Body weight was monitored every week, as shown in Table 2. The body weight of the AFLD group was lower than that of the control group during the 4 weeks of feeding ($p<0.05$). In contrast, the final body weights of the AFLD + H1E, AFLD + H2E, AFLD + H3E and AFLD + H4E groups were higher than the AFLD group ($p<0.05$). Thus, 4 weeks of treatment with wild bitter gourd extracts ameliorated the inhibition of body weight gain of dietary alcohol.

Effects of wild bitter gourd extracts on liver weight change

After 4 weeks of feeding, the relative liver weight (ratio of liver weight to 100 g body weight) of the AFLD group was significantly higher than that of the control group ($p<0.05$) (Fig. 1). However, the relative liver weights of the AFLD + H3E and AFLD + H4E groups were significantly lower than that of the AFLD group ($p<0.05$). Thus, the changes in relative liver weight in alcohol-fed mice were improved by treatment with H3E or H4E.

Effects of wild bitter gourd extracts on liver histopathology

H&E staining was performed to observe alcohol-induced hepatic lipid accumulation and physiological changes in the mouse liver. Histological observations are shown in Fig. 2. There was slight fatty infiltration with micro- and macro-vesicles of hepatocytes in the portal area of normal control mice (Fig. 2A). In contrast, chronic alcohol feeding for 4 weeks resulted in extensive micro- and macro-vesicular fat droplets in the liver of the AFLD mice (Fig. 2B). However, the alcohol-induced hepatic pathological changes were improved in wild bitter gourd extract-treated mice (Fig. 2C, 2D, 2E and 2F). In particular, administration of H3E and H4E visibly

reduced the steatotic alterations caused by alcohol (Fig. 2E and 2F).

On the basis of morphometric observations of hepatic tissues, the degree of fatty liver from H1E- and H2E-treated mice showed no significant differences compared to the AFLD group (AFLD + H1E 3.8 ± 1.5 , AFLD + H2E 3.3 ± 1.5 , AFLD 3.6 ± 0.9). However, the histopathologic score of the liver from both the H3E- and H4E-treated groups was significantly reduced (AFLD + H3E 2.0 ± 0.6 , AFLD + H4E 2.3 ± 0.9) ($p < 0.05$). Noticeably, the differences in the degree of fatty liver between AFLD + H3E, AFLD + H4E and the normal control groups (1.8 ± 0.4) were not statistically significant ($p > 0.05$).

Effect of wild bitter gourd extracts on serum AST and ALT levels

To assess alcohol-induced liver damage, the levels of serum AST and ALT were analysed as markers for liver injury (Table 3). In the control group, serum AST and ALT levels were 109.7 ± 42.3 and 24.0 ± 6.3 IU/L, respectively. These parameters of the AFLD group were significantly higher than the control group ($p < 0.05$), indicating that liver damage was induced by chronic alcohol consumption. Nevertheless, all 4 groups that received bitter gourd extracts for 4 weeks had comparable levels of serum AST and ALT to those of control mice. These data suggested that wild bitter gourd extracts were hepatoprotective against chronic alcohol-induced liver injury.

Effect of wild bitter gourd extracts on serum lipid levels

Serum TG levels in the AFLD mice were not significantly different from control mice (Fig. 3A). However, serum TG levels of H3E- and H4E-treated mice were significantly lower than the AFLD group (AFLD 128.4 ± 9.0 mg/dL, AFLD + H3E 108.0 ± 19.5 mg/dL, AFLD + H4E 107.7 ± 20.6 mg/dL) ($p < 0.05$). In addition, there was no difference in serum TC levels among the 6 groups of mice ($p > 0.05$) (Fig. 3B).

Effect of wild bitter gourd extracts on hepatic TG content

Fig. 4 illustrates the effect of chronic alcohol consumption on hepatic triglycerides in the AFLD group. This group of mice had higher hepatic TG concentrations (52.20 ± 13.36 mg/g liver) than the control group (36.96 ± 4.32 mg/g liver) ($p < 0.05$). The hepatic TG concentration in all 4 bitter gourd extract-treated groups was significantly lower than in the AFLD group (AFLD + H1E 39.42 ± 3.83 mg/g liver, AFLD + H2E 37.67 ± 4.40 mg/g liver, AFLD + H3E 36.66 ± 4.30 mg/g liver, AFLD + H4E 36.15 ± 2.47 mg/g liver) ($p < 0.05$) and was comparable to the control group.

Effect of wild bitter gourd extracts on antioxidant defence systems

Table 4 shows the effects of wild bitter gourd extracts on GSH levels in AFLD mice. Treatment with H2E, H3E or H4E resulted in significantly higher GSH levels in the liver of alcohol-fed mice, whereas H1E treatment did not. As shown in Table 5, the activities of antioxidant enzymes GPx, GRd, SOD and CAT in the livers of AFLD mice were significantly lower than in the control group ($p < 0.05$). However, the activities of GPx, SOD and CAT in all the wild bitter gourd extract-treated groups were comparable to the activities in control mice. The GRd activity was higher in all the bitter gourd extract-treated groups than in the control group ($p < 0.05$). Notably, liver GPx activity in the H3E- and H4E-treated groups was significantly higher than in the control group ($p < 0.05$). Moreover, liver CAT activity in the H3E-treated mice was also significantly higher than in the control mice ($p < 0.05$). As a marker of lipid peroxidation, TBARS levels were used to detect the production of MDA (Table 4). The livers of the AFLD group had the highest TBARS level of the six groups. Moreover, the TBARS level of the AFLD group was 2.4-fold greater than the control group ($p < 0.05$). However, mice in the H3E and H4E groups had significantly lower

TBARS levels than mice in the AFLD group ($p<0.05$).

Effect of wild bitter gourd extracts on pro-inflammatory cytokines.

To determine the effects of wild bitter gourd extracts on the alcohol-induced inflammatory response in the liver, the hepatic levels of TNF- α , IL-1 β and IL-6 were analysed. As shown in Fig. 5, hepatic IL-6, IL-1 β and TNF- α production in the AFLD group significantly surpassed that of the control group ($p<0.05$). This indicated that an inflammatory response was induced by chronic alcohol consumption. However, the response was significantly attenuated in all four groups treated with wild bitter gourd extracts.

Effects of wild bitter gourd extracts on hepatic CYP2E1 protein expression

The effects of H3E or H4E on hepatic CYP2E1 protein expression in AFLD mice were examined by Western blot (Fig. 6). Administration of alcohol for 4 weeks induced a higher hepatic CYP2E1 expression in the AFLD mice. However, the alcohol-mediated CYP2E1 expression was significantly inhibited by H3E or H4E treatment. These findings suggested that the suppression of hepatic CYP2E1 protein expression by treatment with H3E or H4E in AFLD mice may play an important role in the antioxidant effects of H3E and H4E.

Effects of wild bitter gourd extracts on SREBP-1 protein expression

Chronic alcohol feeding for 4 weeks notably enhanced the levels of SREBP-1 protein expression in the livers of AFLD mice (Fig. 7). However, treatment with H3E or H4E reduced SREBP-1 protein expression in the livers of alcohol-fed mice ($p<0.05$).

Effects of wild bitter gourd extracts on FAS protein expression

FAS is a multi-enzyme protein that catalyses fatty acid synthesis. As shown in Fig. 8, FAS protein expression was remarkably higher in the livers of the AFLD group compared to the control group. The higher FAS protein expression in the alcohol-treated mice was ameliorated by the administration of H3E or H4E.

Effects of wild bitter gourd extracts on ACC protein expression

Fig. 9 illustrates the effect of chronic alcohol consumption on the protein level of hepatic ACC, a rate-limiting enzyme in the fatty acid synthesis pathway. The expression of ACC protein was higher in the livers of AFLD mice ($p < 0.05$). However, treatment with H3E or H4E completely reversed the alcohol-induced effect.

Total phenolic and flavonoid contents in wild bitter gourd extracts

As shown in Fig. 10A, total phenolic contents in H1E, H2E, H3E and H4E were 10.24 ± 0.83 , 11.69 ± 0.29 , 13.46 ± 0.72 and 12.83 ± 0.72 mg GAE/g extract, respectively, using the standard curve of gallic acid ($R^2 = 0.9992$). Total phenol contents in H3E and H4E were significantly higher than that in H1E and H2E ($p < 0.05$). Using the standard curve generated by quercetin ($R^2 = 0.9999$), Fig. 10B illustrates that the total flavonoid content in H1E, H2E, H3E and H4E were 0.62 ± 0.07 , 0.80 ± 0.07 , 0.92 ± 0.06 and 0.94 ± 0.14 mg QE/g extract, respectively. Total flavonoid contents in H3E and H4E were significantly higher than that in H1E and H2E ($p < 0.05$).

Discussion

Chronic or excessive alcohol consumption can lead to ALD, initiating from alcoholic fatty liver and developing to steatohepatitis, fibrosis and cirrhosis. The pathogenesis of alcohol-induced liver injury is complex, and may be related to oxidative stress and inflammatory responses.²⁶ Interestingly, recent reports have demonstrated that natural

products can exert protective effects in animal models of alcohol-induced liver injury by reduction of hepatic oxidative stress and/or amelioration of enhanced inflammation. These natural products are from foods including aged black garlic, black rice (*Oryza sativa* L. Japonica) and Chinese raisin tree (*Hovenia dulcis*) peduncles²⁷⁻²⁹. They can also be found in Chinese herbal remedies such as *Antrodia cinnamomea*, *Lycii Fructus*, *Gentianae Radix*, *Platycodonis Radix* and *Magnoliae Officinalis Cortex*.^{30, 31} Because bitter melon is reported to have antioxidative and anti-inflammatory effects, the aim of this study is to investigate the liver protective effects of bitter melon.^{15, 16} Additionally, the ethanol extract of bitter melon, which contains abundant polyphenols, provides outstanding antioxidant capacity compared to other solvent extracts.^{20, 21} Therefore, four ethanol extracts from different cultivars of wild bitter melon, H1E, H2E, H3E and H4E, are used in this study to elucidate their protective capacity in an animal model of chronic alcohol feeding.

Fatty liver, a disorder in which hepatocytes contain macro-vesicular droplets of triglycerides, is the earliest pathology of alcoholic liver disease.³² It is believed that alcoholic fatty liver is a result of an imbalanced fat metabolism, such as enhanced synthesis of triglycerides and decreased mitochondrial lipid oxidation. In the present study, increased levels of serum AST and ALT, which are conventional indicators of liver injury, were observed in the AFLD mice (Table 3). In addition, there was more severe fatty infiltration of the liver and histopathological alterations compared to the control group (Fig. 2). However, treatment of alcohol-fed mice with H1E, H2E, H3E or H4E markedly ameliorated the increased AST and ALT levels to those of control mice. There was also improvement of the histopathological alterations. This indicated that wild bitter melon extracts, especially H3E or H4E, exerted hepatoprotective effects against alcohol-induced liver injury. In addition, the results of histopathological observations clearly demonstrated that extensive micro- and

macro-vesicular steatosis was attenuated, and the degree of fatty liver in the H3E- and H4E-treated groups was similar to the control mice. Moreover, chronic alcohol consumption may cause lipid metabolism imbalances, including decreased lipid oxidation and increased TG synthesis, resulting in hyperlipidemia and fatty liver.³³ The hepatic TG content in all the extract-treated groups was reduced compared to the AFLD group (Fig. 4); however, there was no significant difference in serum TG and TC levels compared to normal control mice (Fig. 3A and 3B). Particularly, relative to the AFLD group, the serum TG levels in H3E- and H4E-treated mice were significantly declined (Fig. 3A). Again, these results demonstrated the liver protective effects of wild bitter gourd ethanol extracts against alcoholic fatty liver in mice.

Alcohol-induced oxidative stress results from the production of excess ROS in the process of alcohol metabolism. Oxidative stress disturbs the capacity of the antioxidant system and leads to an imbalance between free radical production and antioxidant potential, eventually causing liver injury.³⁴ CYP2E1 is an alcohol-inducible enzyme in microsomes that is utilised for alcohol metabolism; it is suggested to be a major contributor to alcohol-induced liver injury.⁵ The level of CYP2E1 is elevated by chronic alcohol feeding and results in alcohol-mediated generation of oxidative stress. In addition, previous study indicates that α -tocopherol, GSH concentrations and GPx activity are significantly decreased in alcohol-fed rats, which results in an increase in lipid peroxidation.³⁵ GPx, GRd, CAT and SOD are also believed to play important roles in the maintenance of redox balance.³⁶ In the present study, hepatic CYP2E1 protein expression is markedly enhanced in the AFLD group compared to normal control mice; H3E or H4E supplementation reduces the alcohol-induced increase of CYP2E1 (Fig. 6). In addition, we observe that the alcohol-induced decline in GPx, GRd, CAT and SOD activities is significantly restored by all of the wild bitter gourd extracts (Table 5). Treatment with

H2E, H3E or H4E further restores the alcohol-induced decrease of GSH concentration (Table 4). Meanwhile, the GRd activity in all of the extract-treated groups is markedly higher than in the normal control group. Furthermore, there is a significant elevation of hepatic GPx activity in the H3E- and H4E-treated groups compared to normal control mice. Specifically, when the mice are co-treated with H3E and alcohol, CAT activity and GSH concentrations are even higher than those in the control mice. Moreover, treatment with H3E or H4E alleviates the increase in MDA levels in AFLD mice (Table 4), implying the inhibition of lipid oxidation by wild bitter gourd extracts in the liver. The above results indicate that the hepatoprotective effects of wild bitter gourd extracts may be exerted by reducing ROS generation through down-regulation of CYP2E1 expression.

Kupffer cells play a key role in alcohol-induced inflammatory responses associated with alcoholic liver injury.³⁷ Chronic alcohol consumption increases the passage of gut-derived endotoxins into the portal circulation⁶; subsequently, Kupffer cells are activated and release ROS and pro-inflammatory mediators such as TNF- α , IL-1 and IL-6.^{38,39} Increased levels of circulating endotoxins are observed in alcoholic patients.⁴⁰ Alcohol-induced liver injury in rats is ameliorated by antibiotic or probiotic treatments that reduce bacterial endotoxin production.^{41, 42} In addition, pro-inflammatory mediators, produced by Kupffer cells, further enhance abnormal stellate cell activity and hepatic fibrogenesis by propagating the inflammatory response.⁴³ In this study, the levels of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 are significantly reduced in the extract-treated groups compared to the untreated AFLD group (Fig. 5). Therefore, these results suggest that wild bitter gourd extracts have the anti-inflammatory capacity to protect mice from alcohol-induced liver injury.

TNF- α is produced by Kupffer cells in response to alcohol exposure. TNF- α may

cause fatty liver by increasing the expression of SREBP-1 mRNA; SREBP-1 is a potent transcription factor of lipogenesis.^{8,39} SREBP-1 can activate various enzymes in the lipid synthesis pathway, including ATP citrate lyase, FAS and ACC.⁴⁴ The increase in lipogenesis can eventually result in alcoholic fatty liver. In the present study, co-treatment of H3E or H4E with alcohol reduces the alcohol-induced elevation of SREBP-1 protein expression (Fig. 7). Similarly, a Chinese herbal medicine *Magnoliae Officinalis Cortex* is reported to ameliorate alcohol-induced fatty liver by suppressing TNF- α production and inhibiting SREBP-1 maturation.⁴⁵ The levels of ACC and FAS protein expression in alcohol-fed mice liver are also attenuated by H3E or H4E treatment (Fig. 8 and 9). Accordingly, these data suggest that H3E and H4E may ameliorate alcoholic fatty liver by inhibiting SREBP-1 protein expression.

To explore the difference between the active components in the four wild bitter gourd extracts, we performed chemical analyses to determine the total phenol and total flavonoid contents in these extracts (Fig. 10). Contents of total phenols as well as total flavonoids in H3E and H4E were significantly higher than that in H1E and H2E ($p < 0.05$). Since using an antioxidative agent to attenuate alcohol-induced liver disease is a reasonable treatment strategy, this implied that the hepatoprotective properties of H3E and H4E may have resulted from their higher contents of phenols and flavonoids. Moreover, a previous study has reported that main phenolic constituents in bitter gourd extracts were catechin, gallic acid, gentisic acid, chlorogenic acid and epicatechin.²⁰ To further confirm the major active components in the four wild bitter gourd extracts, we have performed a high-performance liquid chromatography analysis with a UV detector (HPLC-UV). The HPLC chromatographic conditions were according to a previous work⁴⁶ with some modifications to determine phenolic constituents. Two major peaks shown in chromatograms of all four extracts were identified as caffeic acid and epicatechin according to the retention time of peaks

shown in a standard mixture's chromatogram (data not shown). Although ratios of these two peaks in the four extracts were not equal, they might be used as marker components for the standardization of phenol ingredients of wild bitter gourd extracts by HPLC chromatograms. However, the limitation of this HPLC-UV method is that compounds known to be important to bitter gourds such as saponins and triterpenes may be poorly absorbed in the UV detection. Hence, using a mass detector to further study the qualification as well as quantitation of active components in wild bitter gourds will be a worthwhile task to provide more detail information supporting the research outcome in the future.

Conclusions

This study demonstrates that extracts of wild bitter gourd, H3E and H4E, could ameliorate alcohol-induced liver injury in mice by decreasing hepatic steatosis and cellular damage. The hepatoprotective effects of H3E and H4E are exerted by attenuating oxidative stress and inflammatory responses. These findings suggest that the natural resources H3E and H4E may be developed from food to prevent alcoholic fatty liver disease.

Acknowledgements

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Table 1 Effects of wild bitter gourd extracts on daily food intake in alcohol-induced fatty liver in mice.¹

group ²	food intake (mL)			
	week 1	week 2	week 3	week 4
Control	11.92 ± 1.59 ^a	10.02 ± 0.57 ^a	11.09 ± 0.58 ^a	11.10 ± 0.65 ^a
AFLD	10.31 ± 1.08 ^b	10.57 ± 1.55 ^a	11.74 ± 1.05 ^a	12.10 ± 0.65 ^a
AFLD + H1E	9.97 ± 1.86 ^b	10.51 ± 1.75 ^a	11.51 ± 1.95 ^a	11.35 ± 2.31 ^a
AFLD + H2E	9.49 ± 1.38 ^b	10.56 ± 1.48 ^a	11.38 ± 1.37 ^a	11.65 ± 2.31 ^a
AFLD + H3E	9.79 ± 2.42 ^b	10.68 ± 1.68 ^a	12.07 ± 0.66 ^a	12.12 ± 0.92 ^a
AFLD + H4E	9.24 ± 1.13 ^b	9.51 ± 1.37 ^a	11.80 ± 0.95 ^a	12.39 ± 1.12 ^a

¹ Values are represented as the mean ± S.D. (n = 12). ^{a, b} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

² Control, normal Lieber-DeCarli liquid diet; AFLD, alcohol Lieber-DeCarli liquid diet; H1E, Hualien No.1' bitter gourd extract treatment; H2E, Hualien No.2' bitter gourd extract treatment; H3E, Hualien No.3' bitter gourd extract treatment; H4E, Hualien No.4' bitter gourd extract treatment.

Table 2 Effects of wild bitter gourd extracts on body weight in alcohol-induced fatty liver in mice.¹

group ²	body weight (g)				
	initial weight	week 1	week 2	week 3	final weight
Control	24.8 ± 1.0 ^a	25.5 ± 1.0 ^a	25.6 ± 1.1 ^a	25.8 ± 1.2 ^a	26.1 ± 1.7 ^a
AFLD	24.3 ± 1.8 ^a	23.5 ± 3.0 ^b	23.0 ± 2.3 ^b	23.8 ± 1.5 ^b	23.2 ± 1.4 ^d
AFLD + H1E	24.4 ± 1.8 ^a	24.2 ± 1.8 ^{ab}	23.9 ± 2.0 ^b	24.4 ± 1.1 ^b	24.3 ± 1.2 ^c
AFLD + H2E	25.3 ± 0.9 ^a	25.4 ± 1.7 ^a	25.4 ± 1.5 ^a	25.7 ± 1.1 ^a	25.6 ± 1.3 ^{ab}
AFLD + H3E	25.3 ± 0.6 ^a	25.7 ± 2.4 ^a	25.4 ± 1.9 ^a	24.7 ± 1.5 ^{ab}	24.9 ± 0.8 ^{bc}
AFLD + H4E	25.1 ± 0.7 ^a	25.4 ± 1.6 ^a	25.5 ± 1.4 ^a	24.5 ± 0.9 ^b	25.2 ± 1.0 ^{abc}

¹ Values are represented as the mean ± S.D. (n = 12). ^{a, b, c, d} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

² Abbreviations as in Table 1.

Table 3 Effects of wild bitter gourd extracts on serum AST and ALT in alcohol-induced fatty liver in mice.¹

group ²	AST (IU/L)	ALT (IU/L)
Control	109.7 ± 42.3 ^b	24.0 ± 6.3 ^b
AFLD	253.9 ± 88.3 ^a	83.5 ± 32.7 ^a
AFLD + H1E	132.1 ± 104.2 ^b	28.9 ± 10.4 ^b
AFLD + H2E	128.7 ± 68.5 ^b	35.3 ± 18.2 ^b
AFLD + H3E	163.1 ± 55.3 ^b	33.7 ± 9.8 ^b
AFLD + H4E	131.0 ± 52.4 ^b	30.0 ± 12.4 ^b

¹ Values are represented as the mean ± S.D. (n = 12). AST, aspartate transferase; ALT, alanine aminotransferase. ^{a, b}

Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

² Abbreviations as in Table 1.

Table 4 Effects of wild bitter gourd extracts on liver GSH and TBARS in alcohol-induced fatty liver in mice.¹

group ²	GSH	TBARS
	(nmol/mg protein)	(nmol/mg protein)
Control	68.06 ± 9.25 ^{bc}	0.89 ± 0.31 ^c
AFLD	59.46 ± 7.18 ^d	2.16 ± 1.43 ^a
AFLD + H1E	63.33 ± 6.79 ^{cd}	1.94 ± 1.20 ^{ab}
AFLD + H2E	72.86 ± 4.64 ^{ab}	1.87 ± 1.30 ^{ab}
AFLD + H3E	76.19 ± 6.25 ^a	1.21 ± 0.48 ^{bc}
AFLD + H4E	74.13 ± 9.26 ^{ab}	1.23 ± 0.69 ^{bc}

¹ Values are represented as the mean ± S.D. (n = 12). GSH, glutathione; TBARS, thiobarbituric acid reactive substances. ^{a, b, c, d} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

² Abbreviations as in Table 1.

Table 5 Effects of wild bitter gourd extracts on antioxidant enzyme activities in alcohol-induced fatty liver in mice.¹

group ²	GPx (nmol/min/mg protein)	GRd (nmol/min/mg protein)	CAT (nmol/min/mg protein)	SOD (U/mg protein)
Control	623.5 ± 35.8 ^b	76.10 ± 5.74 ^c	300.51 ± 42.02 ^b	68.50 ± 8.15 ^a
AFLD	545.9 ± 37.4 ^c	65.89 ± 4.40 ^d	238.15 ± 30.68 ^c	55.52 ± 11.62 ^b
AFLD + H1E	644.2 ± 69.7 ^b	87.68 ± 7.00 ^{ab}	304.57 ± 89.06 ^b	72.80 ± 7.28 ^a
AFLD + H2E	661.7 ± 69.2 ^b	83.06 ± 11.36 ^b	320.54 ± 72.53 ^b	74.92 ± 7.24 ^a
AFLD + H3E	728.5 ± 63.0 ^a	93.68 ± 8.34 ^a	375.58 ± 70.35 ^a	71.80 ± 9.09 ^a
AFLD + H4E	707.6 ± 33.8 ^a	85.63 ± 9.20 ^b	311.00 ± 47.94 ^b	68.37 ± 4.33 ^a

¹ Values are represented as the mean ± S.D. (n = 12). GPx, glutathione peroxidase; GRd, glutathione reductase; CAT, catalase; SOD, superoxide dismutase. ^{a, b, c} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

² Abbreviations as in Table 1.

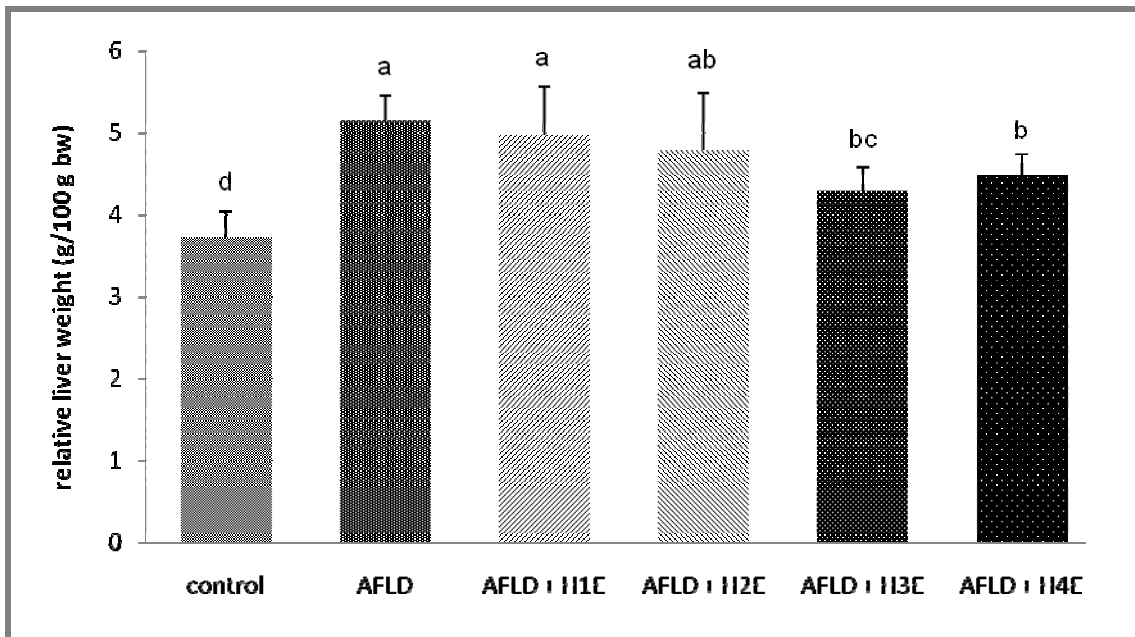


Fig. 1 Effect of wild bitter gourd extracts on relative liver weight in alcohol-induced fatty liver in mice. Values are represented as the mean \pm S.D. ($n = 12$). ^{a, b, c, d} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$). Abbreviations of groups as in Table 1.

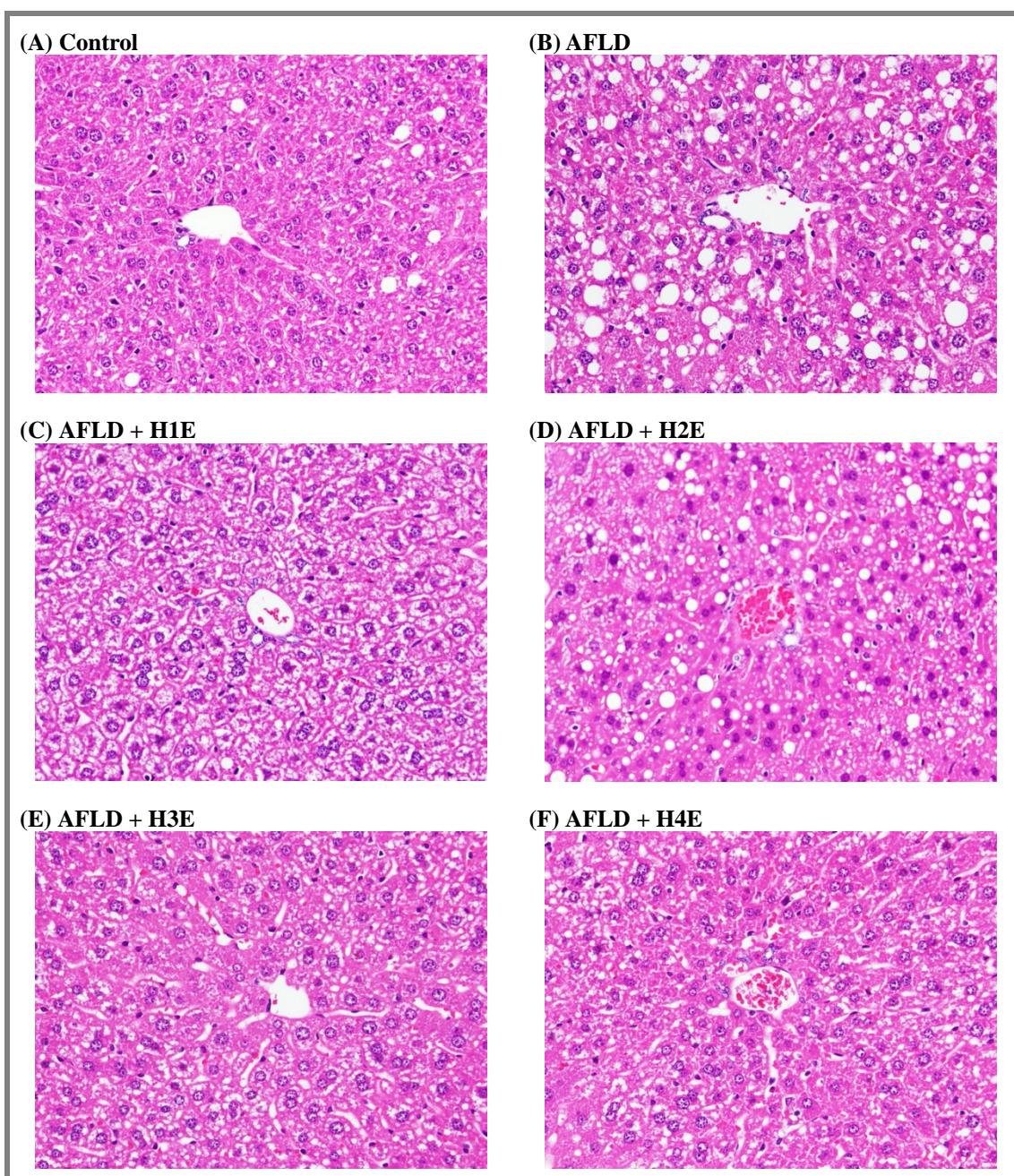


Fig. 2 Effects of wild bitter melon extracts on liver histopathological alterations in alcohol-induced fatty liver in mice. Livers were stained with H&E and visualised at 400x. (A) Control, normal Lieber-DeCarli liquid diet; (B) AFLD, alcohol Lieber-DeCarli liquid diet; (C) AFLD + H1E, Hualien No.1' bitter melon extract treatment; (D) AFLD + H2E, Hualien No.2' bitter melon extract treatment; (E) AFLD + H3E, Hualien No.3' bitter melon extract treatment; (F) AFLD + H4E, Hualien No.4' bitter melon extract treatment. Abbreviations of groups as in Table 1.

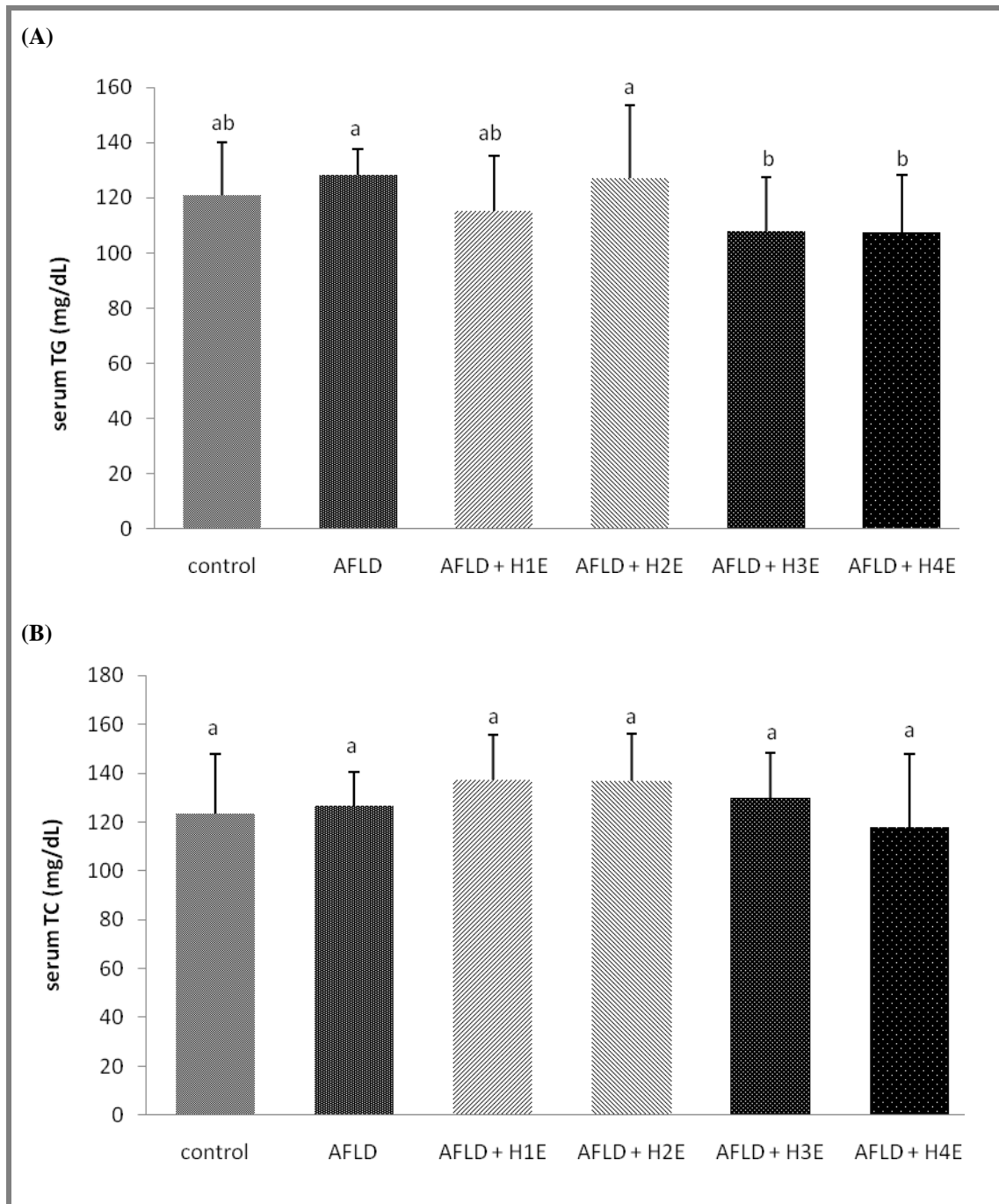


Fig. 3 Effects of wild bitter gourd extracts on serum TG and TC levels in alcohol-induced fatty liver in mice. Values are represented as the mean \pm S.D. (n = 12). TG, triglyceride; TC, total cholesterol. ^{a, b} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$). Abbreviations of groups as in Table 1.

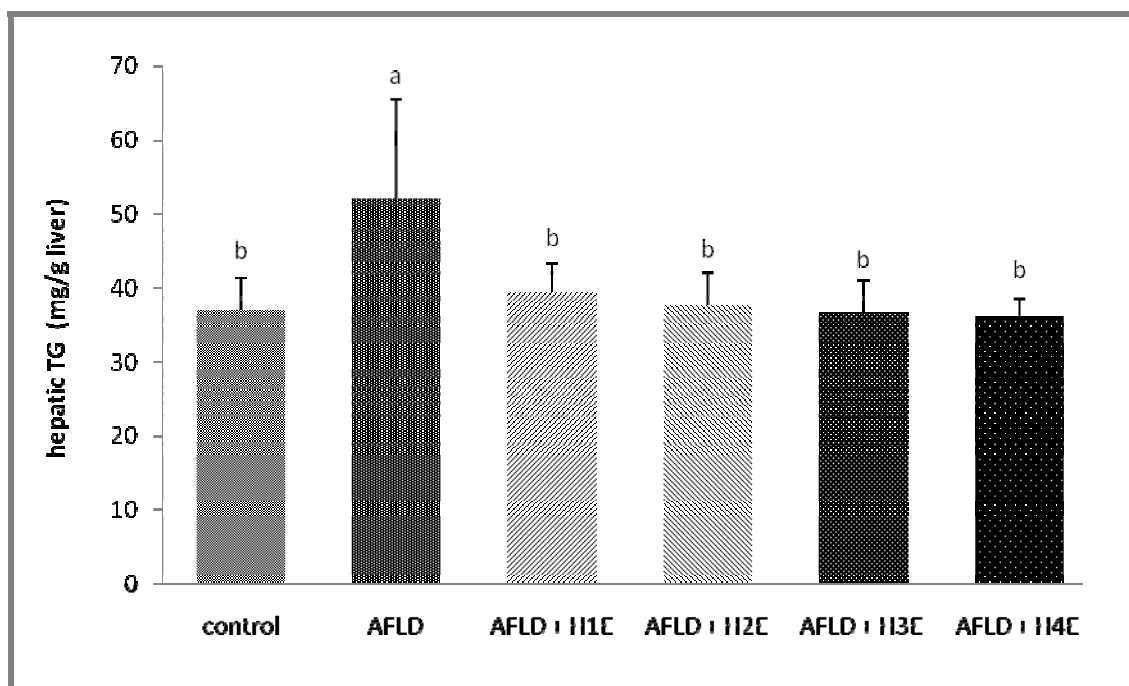


Fig. 4 Effects of wild bitter gourd extracts on hepatic TG levels in alcohol-induced fatty liver in mice. Values are represented as the mean \pm S.D. ($n = 12$). TG, triglyceride. ^{a, b} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$). Abbreviations of groups as in Table 1.

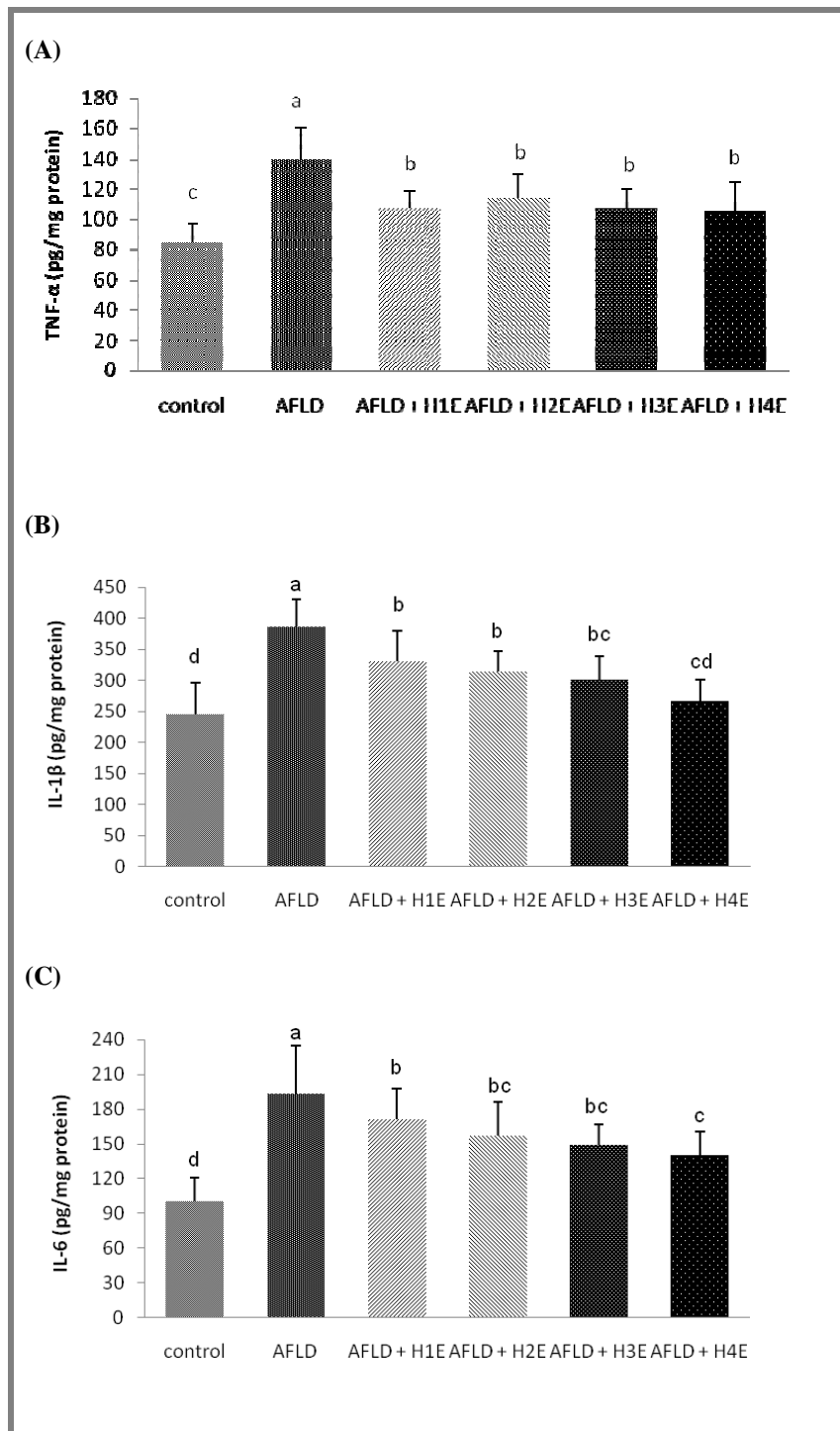
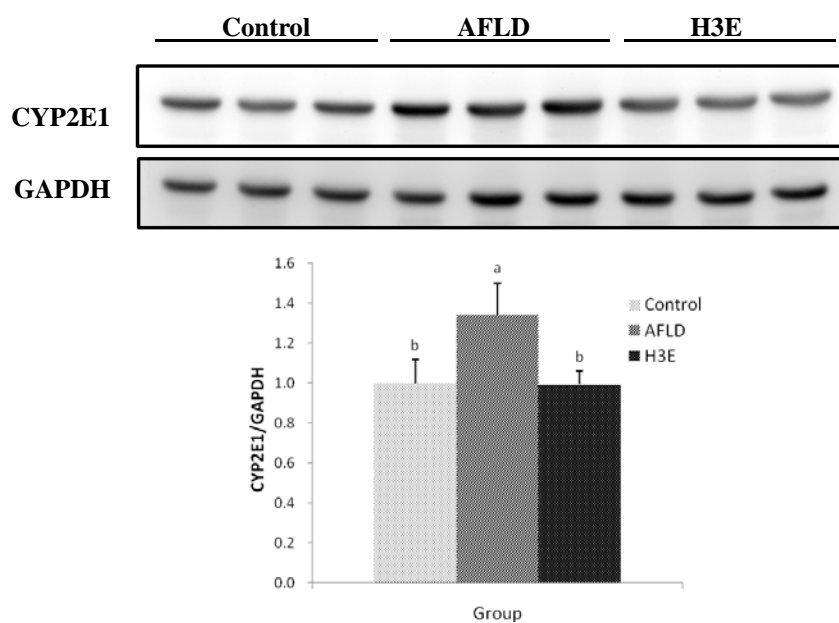


Fig. 5 Effects of wild bitter gourd extracts on pro-inflammatory cytokines in alcohol-induced fatty liver in mice. Values are represented as the mean \pm S.D. ($n = 12$). TNF- α , tumour necrosis factor; IL-1 β , interleukin 1 β ; IL-6, interleukin 6. ^{a, b, c, d} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$). Abbreviations of groups as in Table 1.

(A)



(B)

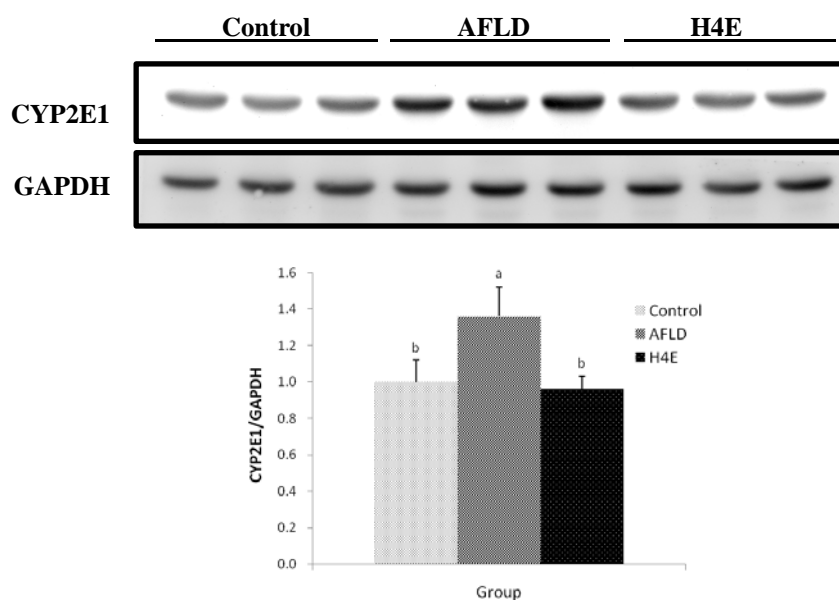
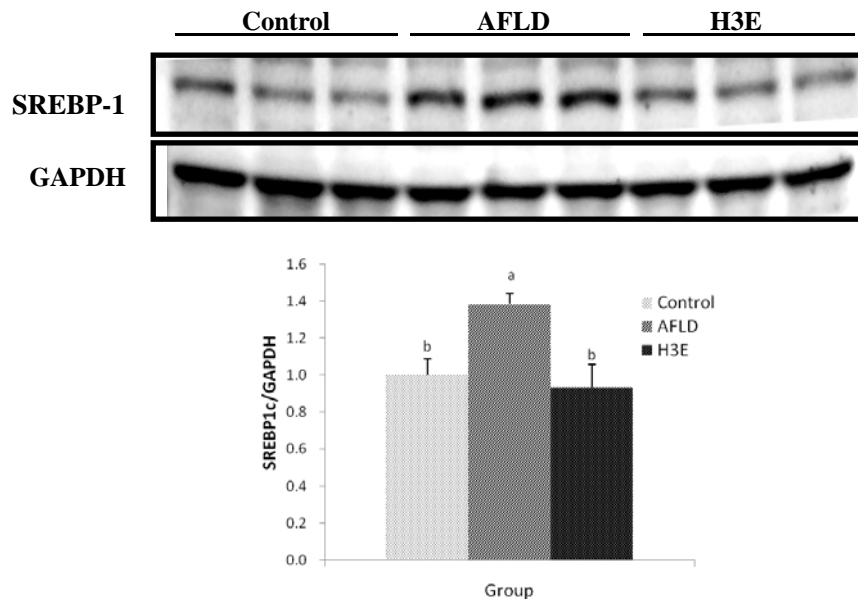


Fig. 6 Hepatic CYP2E1 protein expression in alcohol-induced fatty liver in mice. CYP2E1 expression in (A) H3E-treated and (B) H4E-treated AFLD mice livers. Values are represented as the mean \pm S.D. ($n = 3$). ^{a, b} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

(A)



(B)

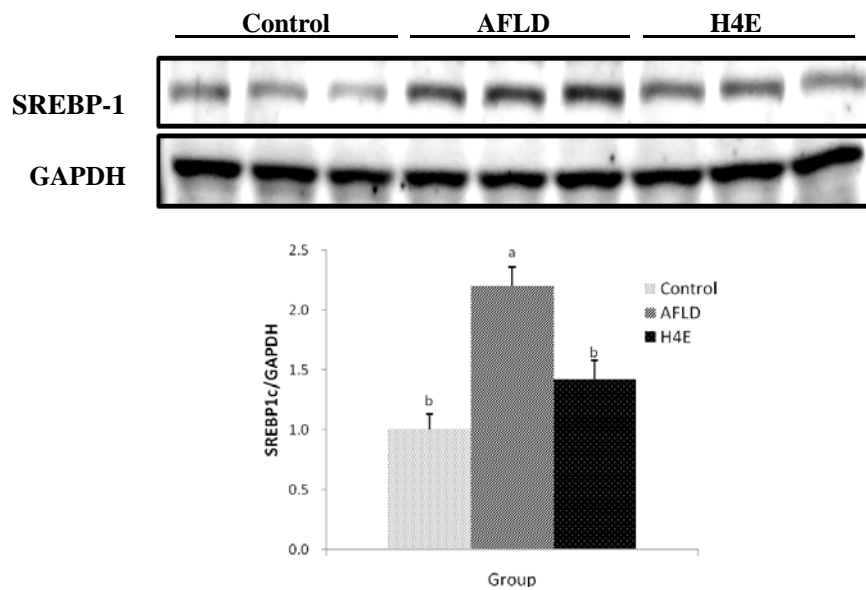
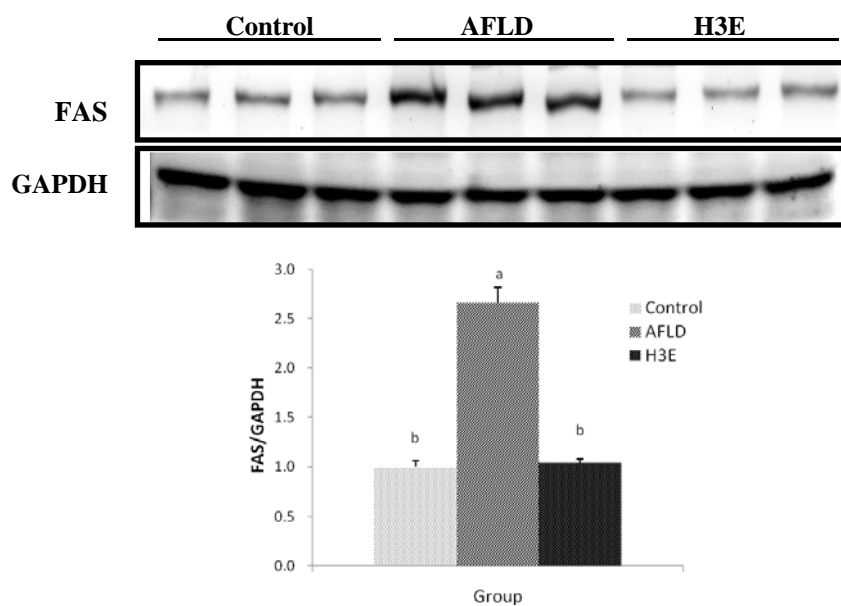


Fig. 7 Hepatic SREBP-1 protein expression in alcohol-induced fatty liver in mice. SREBP-1 expression in (A) H3E-treated and (B) H4E-treated AFLD mice livers. Values are represented as the mean \pm S.D. (n = 3). ^{a, b} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

(A)



(B)

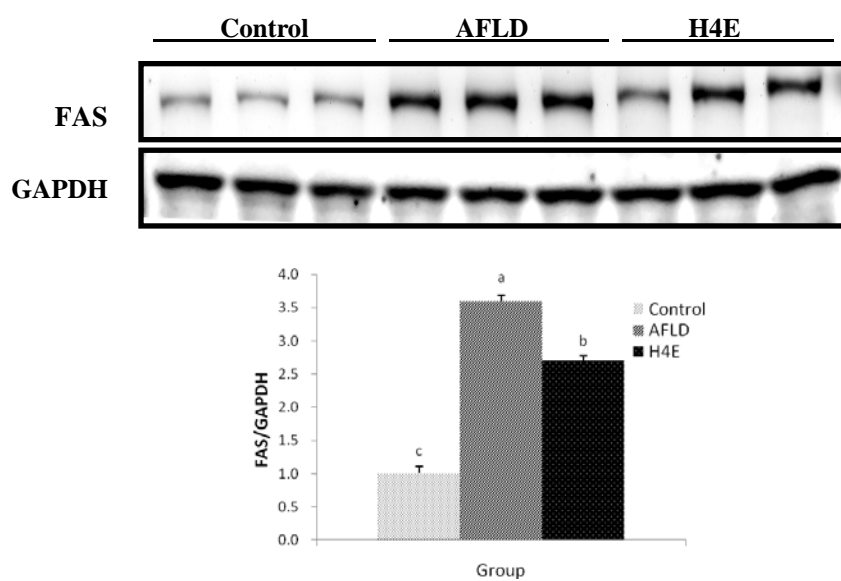
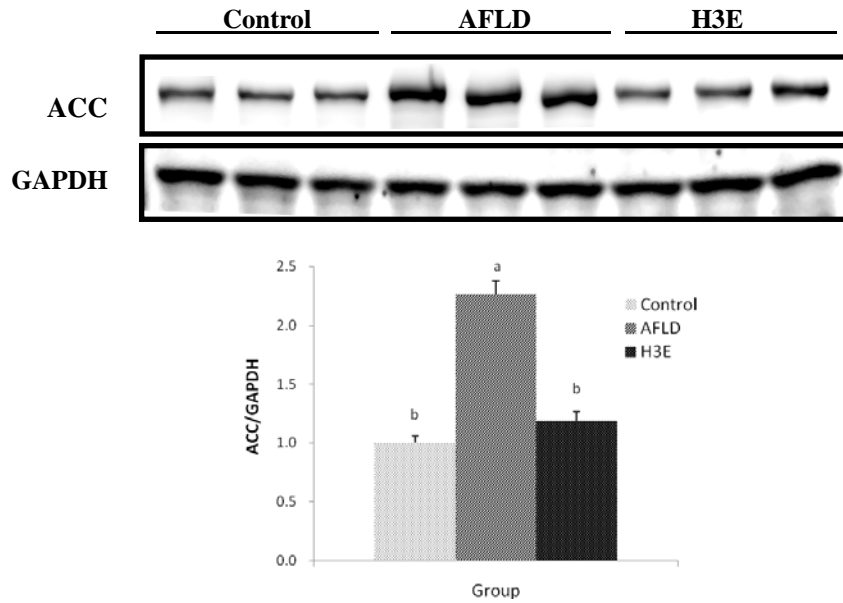


Fig. 8 Hepatic FAS protein expression in alcohol-induced fatty liver in mice. FAS expression in (A) H3E-treated and (B) H4E-treated AFLD mice livers. Values are represented as the mean \pm S.D. ($n = 3$). ^{a, b, c} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

(A)



(B)

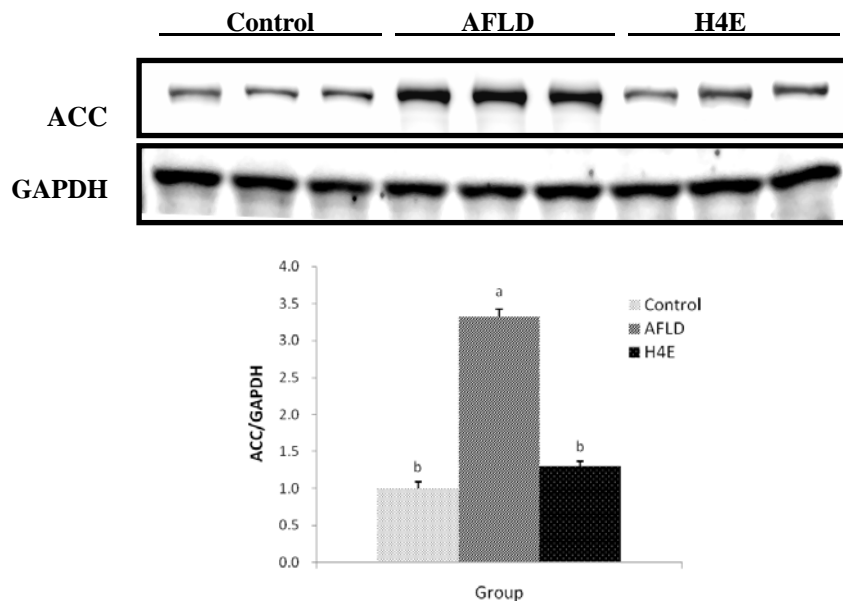


Fig. 9 Hepatic ACC protein expression in alcohol-induced fatty liver in mice. ACC expression in (A) H3E-treated and (B) H4E-treated AFLD mice livers. Values are represented as the mean \pm S.D. ($n = 3$). ^{a, b} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

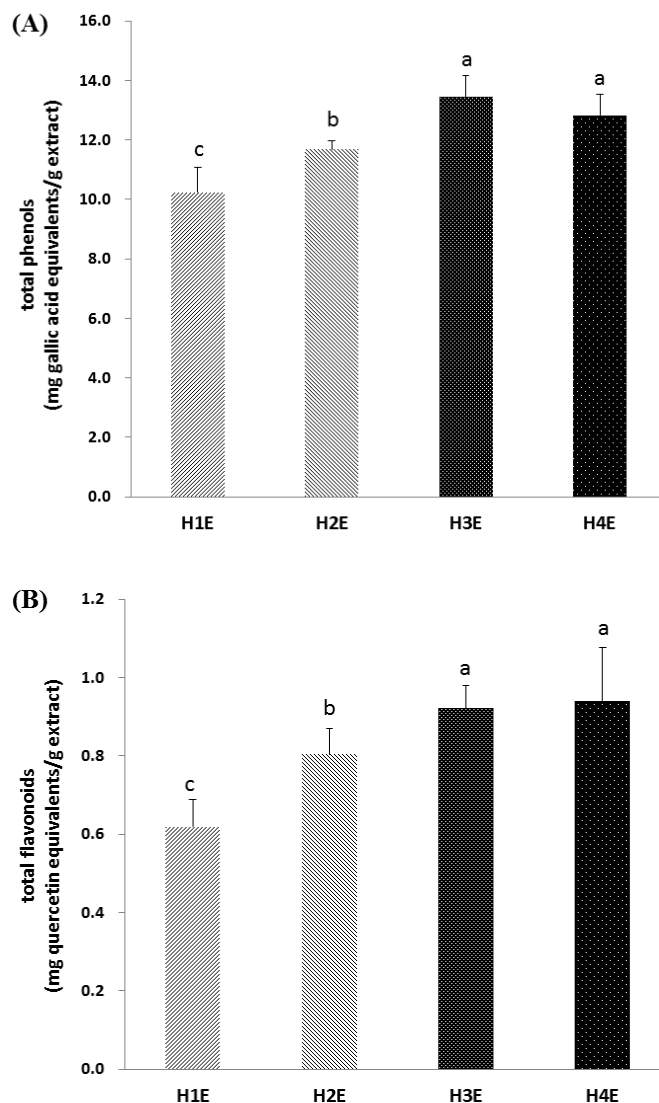


Fig. 10 Contents of (A) total phenols and (B) total flavonoids in the wild bitter gourd extracts. The total phenol content and total flavonoid content were expressed as milligram gallic acid equivalents (GAE) per gram of extract and milligram quercetin equivalents (QE) per gram of extract, respectively. Values are represented as the mean \pm S.D. ($n = 9$). ^{a, b, c} Different letters indicate significantly different values according to a one-way ANOVA with Duncan's multiple test ($p < 0.05$).

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