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Original Article

Extract of *Siraitia grosvenorii* (Luo Han Guo) protects against hepatic fibrosis in mice on a choline-deficient, methionine-lowered, L-amino acid-defined, high-fat diet without trans fatty acids

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ABSTRACT — Nonalcoholic steatohepatitis (NASH) is an aggressive form of nonalcoholic fatty liver disease that presents with steatosis, inflammation, and fibrosis and can progress to cirrhosis and cancer. Thus, methods for controlling this lifestyle-related disease are urgently needed. An extract of *Siraitia grosvenorii* (Luo-Han-Guo) (luohanguo extract (LE)) is widely used as a sweetener; its major bioactive constituents, mogrosides, have shown anti-oxidative and anti-inflammatory properties, exerting multiple pharmacological effects in various disorders. In the present study, we investigated the effects of LE on NASH induced in mice fed a choline-deficient, methionine-lowered, L-amino acid-defined, high-fat diet without *trans* fatty acids (CDAA-HF- T(−)). Mice were fed with CDAA-HF- T(−) and drinking water containing LE at concentrations of 0%, 0.2%, 0.6%, and 2% for 28 weeks. Our results showed that LE was not toxic under the experimental conditions evaluated. In the liver of mice fed CDAA-HF- T(−), LE did not affect steatosis or early phase events from macrophage recruitment to hepatocyte death but inhibited late phase events, the progression of inflammation, and fibrosis (mechanisms independent of transforming growth factor-β signaling). Sweeteners with beneficial biological functions, such as LE, may be useful for controlling lifestyle-related diseases, such as NASH, and promoting human health.

Key words: Nonalcoholic steatohepatitis, Fibrosis, Luohanguo extract

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a common lifestyle-related disease associated with chronic liver damage (Levene and Goldin, 2012). Hepatic phenotypes of NAFLD are diverse, ranging from mild steatosis to various degrees of inflammation and fibrosis. Among them, nonalcoholic fatty liver (NAFL) only presents with steatosis, whereas nonalcoholic steatohepatitis (NASH) presents with steatosis, inflammation, and fibrosis, which can progress to cirrhosis and even malignancy (Angulo, 2002; Farrell and Larter, 2006). Approximately 10–25%

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of patients with NASH eventually develop cirrhosis (Matteoni *et al*., 1999; Adams *et al*., 2005). Although the exact cause of NASH remains unclear, the classic "two-hit hypothesis" proposes that the first hit represents hepatic steatosis development, and the second hit involves oxidative stress and proinflammatory cytokines, inducing further liver injury. This traditional view has been improved to a more complexed “multiple parallel-hit hypothesis,” which suggests that the above and multiple other factors concurrently, rather than sequentially, give rise to steatohepatitis and fibrosis in NASH. Therefore, such diverse factors can be targets for effectively preventing and treating NASH.

Fructus of *Siraitia grosvenorii* (Luo-Han-Guo) is well-known for its sweet taste, and its extract (luohanguo extract (LE)) has been widely used as a sweetener and an edible traditional medicine to treat dry cough, sore throat, lung congestion, and constipation for thousands of years in China (Li *et al*., 2014). Mogrosides are the major bioactive constituents of LE and are cucurbitane-type tetracyclic triterpene glycosides. They are approximately 300-fold sweeter than sucrose but low in calories (Li *et al*., 2014). LE has been approved as a Generally Recognized As Safe substance by the U.S. Food and Drug Administration and as a food additive (sweetener) in China and Japan (Pawar *et al*., 2013). Mogrosides have anti-oxidative and anti-inflammatory properties and thereby exert multiple pharmacological effects on various disorders such as cancer, pulmonary fibrosis, allergic asthma, obesity, and diabetes (Zhang *et al*., 2018; Zhou *et al*., 2009; Takasaki *et al*., 2003; Qi *et al*., 2008; Tao *et al*., 2017; Song *et al*., 2019; Liu *et al*., 2019). In NAFLD, Li *et al*. recently showed that mogroside V, one of the main components of mogroside-rich LE, prevents hepatic steatosis induced in mice fed a high-fat diet by downregulating *de novo* lipogenesis and upregulating lipolysis and fatty acid oxidation via activation of AMP-activated protein kinase (Li *et al*., 2020). However, the effects of LE or mogrosides on NASH have not been investigated.

We established a rat nutritional model of NASH through chronic feeding of a choline-deficient, methionine-lowered, L-amino acid-defined diet (CDAA) (Nakae *et al*., 1992; Nakae *et al*., 1990; Nakae, 1999) and by overcoming resistance to the diet using a mouse model fed a choline-deficient, methionine-lowered, L-amino acid-defined, high-fat diet without trans fatty acids (CDAA-HF- T(−)) (Suzuki-Kemuriyama *et al*., 2020). These models reproduce most of the phenotypic and mechanistic characteristics of human NASH, including rapid induction of fibrosis and proliferation of lesions in the liver. This study was conducted to investigate the effects of LE on NASH development in mice fed CDAA-HF- T(−).

MATERIALS AND METHODS

Test materials

LE (SAN-EI Sweetener™ M50) was generously supplied by San-Ei Gen F.F.I., Inc. (Osaka, Japan). SAN-EI Sweetener™ M50, the composition of which is shown in Table 1, is comprise of more than 50% of mogroside V, a natural sweetened substance. The total amount of sweet-tasting mogroside compounds combined with mogroside V, 11-oxo mogroside V, mogroside IV, and Siamenoside I was 69%. LE was dissolved in drinking water at concentrations of 0.2%, 0.6%, and 2% and administered to the mice *ad libitum*.

Diets

The control diet of CE-2 was composed of 58% carbohydrate, 13% fat, and 29% protein on a caloric basis and contained 0.21% choline, and 0.44% methionine (CLEA Japan, Inc., Tokyo, Japan). The experimental diet of CDAA-HF-T(−) (fat content of 45 kcal% by shortening without trans fatty acids (TFAs), Primex Z®, and methionine content of 0.1%) was a made-to-order product (ID: A16032902) from Research Diet, Inc. (New Brunswick, NJ, USA). The components of these diets have been described previously (Suzuki-Kemuriyama *et al*., 2020). CDAA-HF-T(−) was frozen before use and changed every 2 days to prevent oxidation. The diets were administered *ad libitum* to the mice.

Animals

Five-week-old male C57BL/6J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and acclimated for one week before the study. The mice were kept under temperature-controlled conditions (22°C on average) in colony cages with a 12-hr light/12-hr dark cycle and given free access to food and water during the acclimation and experimental periods.

Experimental protocol

At 6 weeks of age, the mice were randomly assigned to six groups (n = 6): control diet/water, control diet/LE 2% CDAA-HF-T(−)/water, CDAA-HF-T(−)/LE 0.2%, CDAA-HF-T(−)/LE 0.6%, and CDAA-HF-T(−)/LE 2% for 28 weeks. The body weight, food consumption, and water consumption of the mice were monitored weekly. At the end of the experimental period, blood samples were collected from the tail veins of all mice. The mice were euthanized by exsanguination under isoflurane anes-

Table 1. Compositions of LE used in this study.

Nutrient (%)	SAN-EI Sweetener™ M50
Moisture	4.7
Protein	8.2
Fat	0.0
Carbohydrate	86.9
Dietary fiber	0.0
Ash	0.2
Total	100.0
Mogroside V	58.4
Sweet-tastingmogroside compounds	68.6

Sweet-tasting mogroside compounds are composed of mogroside V, 11-oxo mogroside V, mogroside IV and Siamenoside I.

thesia. All organs were carefully studied during autopsy. The liver and other lesion-bearing organs were excised and weighed if necessary.

Histological analysis

Liver samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, and cut into 4-μm-thick sections for hematoxylin–eosin and Sirius red staining. Using Sirius red-stained specimens, the fibrosis areas were measured using CellSens Dimension software (Olympus, Tokyo, Japan). Immunohistochemical analyses were conducted as previously described (Suzuki *et al*., 2007) using the following primary antibodies: rat anti-mouse monoclonal antibody for the cluster of differentiation 68 (CD68) as a marker of macrophages (1:200; Abcam, Cambridge, UK) and rabbit anti-human polyclonal antibody for α-smooth muscle actin as a marker of activated hepatic stellate cells (1:200; Abcam). Antibody binding was visualized using a Histofine Simple Stain Kit (Nichirei Corp., Tokyo, Japan). All immunohistochemically stained sections were counterstained with hematoxylin.

Histopathological and immunohistochemical examinations were conducted in an unaware of manner. The findings were graded as normal (−), minimal (1+), moderate (2+), and severe (3+), and assigned scores of 1, 2, 3, and 4, respectively.

Plasma and hepatic chemistry

Plasma was obtained from the blood samples to measure triglyceride (TG) and total cholesterol (TCHO) levels and aspartate (AST) and alanine aminotransferase (ALT) activities using an automatic analyzer (DRI-CHEM; Fujifilm, Tokyo, Japan). Hepatic TG and TCHO levels were measured as previously described (Matsuzaka *et al*., 2007).

RNA extraction and analysis

Total RNA was extracted from the liver using a Sepasol reagent (Nacalai Tesque, Kyoto, Japan) and reverse-transcribed using a PrimeScript RT Master Kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was conducted using SYBR Premix Ex Taq (Takara Bio Inc.) and specific primer sets using a Thermal Cycler Dice Real-Time System Single (Takara Bio Inc.). The primer sequences used for qPCR are listed in Table 2. mRNA expression levels were normalized to those of cyclophilin mRNA.

Statistical analysis

Numerical values are expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test was used to assess differences among groups. Differences were considered significant at $p < 0.05$.

Ethical considerations

All animal husbandry and experiments were conducted in compliance with the guiding principle of the Tokyo University of Agriculture and approved by the Animal Experiment Committee of the university. This study complied with all related domestic and international laws, regulations, and guidelines. Animal experiments complied with the ARRIVE guidelines and were conducted in accordance with the U.K. Animals (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). This study used only male mice because our previous studies clearly demonstrated that female rodents are resistant to CDAA (Nakae *et al*., 1995).

RESULTS

Physiologic and hematologic chemical changes

The mouse body weight tended to increase in the CDAA-HF-T(−) groups compared to in the control diet groups from around 20 weeks. Effect of the LE co-administration was not seen in the control diet groups, but when 2% tended to increase in the CDAA-HF-T(-) groups. (Fig. 1). Despite the differences in body weight, food consumption was similar among groups (Table 3). Water consumption tended to decrease in the CDAA-HF-T(−)/LE 0.6% and 2% groups (Table 3). LE intake was estimated, and the results are shown in Table 3. There was no alter-

Table 2. Sequence information of primers for the qPCR analyses.

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
CD68	ACCGCCATGTAGTCCAGGTA	ATCCCCACCTGTCTCTCTCA
CCR-2	AGCACATGTGGTGAATCCAA	TGCCATCATAAAGGAGCCA
Ly6c	GCAGTGCTACGAGTGCTATGG	ACTGACGGGTCTTTAGTTTCCTT
TNF-α	AGGGTCTGGGCCATAGAACT	CCACCACGCTCTTCTGTCTAC
Col1a1	TAGGCCATTGTGTATGCAGC	ACATGTTCAGCTTTGTGGACC
Col4a1	CACATTTTCCACAGCCAGAG	GTCTGGCTTCTGCTGCTCTT
TIMP1	AGGTGGTCTCGTTGATTTCT	GTAAGGCCTGTAGCTGTGCC
TGFβ1	GCAACATGTGGAACTCTACCAGA	GACGTCAAAAGACAGCCACTCA
TGFβ2	TACTGCAGGAGAAGGCAAGC	AACTGGGCAGACAGTTTCGG
TGFβ3	ATCTGTTCCGGGCAGAGTTC	ATGTGCTCATCCGGTCGAAG
TGFβr1	AGCTCCTCATCGTGTTGGTG	TGCCCAAGGGCAGTATTCAA
TGFβr2	TCCCAAGTCGGTTAACAGTG	TGTCGCAAGTGGACAGTCTC
TGFβr3	TCCAGAGTCGGAACCTGAGT	GGGGCTCTCTAGGCTTCTCT

ation in the general condition of the mice in any group, including those administered LE, during the experimental period. The final body weight, organ weights, and plasma chemistry at the end of week 28 are shown in Table 4. Compared to the control diet groups, the absolute and relative weights of the liver, epididymal white adipose tissue, and spleen were increased in the CDAA-HF-T(−) groups. The absolute liver weight in the CDAA-HF-T(−)/LE 2% group was significantly higher than that in the CDAA-HF-T(−)/water group. The heart, lung, and kidney weights were not altered among groups (data not shown). Plasma ALT and AST activities were markedly elevated in the CDAA-HF-T(−) groups, and ALT activity in the CDAA-HF-T(−)/LE 2% group was elevated compared to that in the CDAA-HF-T(−)/water group. Plasma TCHO and TG levels tended to be increased in the CDAA-HF-T(−) groups, and TCHO level in the CDAA-HF-T(−)/LE 2% group tended to be increased further compared to that in the CDAA-HF-T(−)/water group.

Effects of LE on liver steatosis and hepatic lipid accumulation in CDAA-HF-T(−)-fed mice

Representative microscopic features of the liver tissues are shown in Fig. 2A. At the end of week 28, no specific histological changes were observed in the livers of the control diet groups with or without LE administration.

In the livers of the CDAA-HF-T(−) groups, macrovesicular steatosis was observed in almost all hepatocytes, which were characterized by single large cytoplasmic vacuoles. This lesion tended to be enhanced in the CDAA-HF-T(−)/LE 2% group compared to in the CDAA-HF-T(−)/water group.

Hepatic TG and TCHO levels were markedly elevated in the CDAA-HF-T(−) groups and tended to be further increased in the CDAA-HF-T(−)/LE 2% group (Fig. 2B).

Effects of LE on liver inflammation in CDAA-HF-T(−)-fed mice

Representative CD68 immunohistochemistry results of the liver and the CD 68 grading scores are shown in Figs. 3A and 3B. Inflammatory clusters consisting of markedly accumulated hypertrophied macrophages were visualized using CD68 immunohistochemistry. The magnitude of CD68 reactivity was greater in the CDAA-HF-T(−) groups than in the control diet groups; this reactivity was not influenced by LE administration. Similarly, the mRNA expression of *CD68* was strongly upregulated in the CDAA-HF-T(−) groups and not affected by LE administration (Fig. 3C). In addition, the mRNA expression of markers of infiltrating macrophages, *CCR2* and *Ly6c*, was strongly upregulated in the CDAA-HF-T(−)

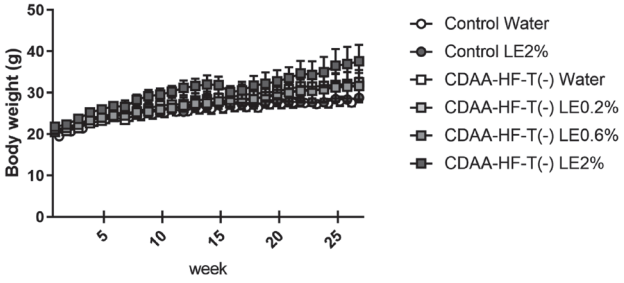

Fig. 1. Body weight changes. Body weight changes of C57BL/6J mice in the control diet/water, control diet/LE 2%, CDAA-HF-T(−)/water, CDAA-HF-T(−)/LE 0.2%, CDAA-HF-T(−)/LE 0.6%, and CDAA-HF-T(−)/LE 2% groups for 28 weeks. The values are presented as the means ± standard deviations (SDs).

Table 3. Food intake, water intake and estimated LE intake.

Group	Control Water	Control LE 2%	CDAAHF-T(-) Water	CDAAHF-T(-) LE 0.2%	CDAAHF-T(-) LE 0.6%	CDAAHF-T(-) LE 2%
Food intake (g/day/mouse)	2.8 ± 1.5	2.9 ± 1.3	2.8 ± 0.4	3.0 ± 0.3	2.9 ± 0.4	2.9 ± 0.3
Water intake (g/day/mouse)	4.6 ± 1.3	5.9 ± 1.6	6.4 ± 2.0	5.8 ± 2.0	3.9 ± 1.3	3.7 ± 2.0
Estimated LE intake (mg/kg/day/mouse)	0	4068 ± 1079	0	353 ± 120	735 ± 251	1964 ± 1047

Values are means ± SDs.

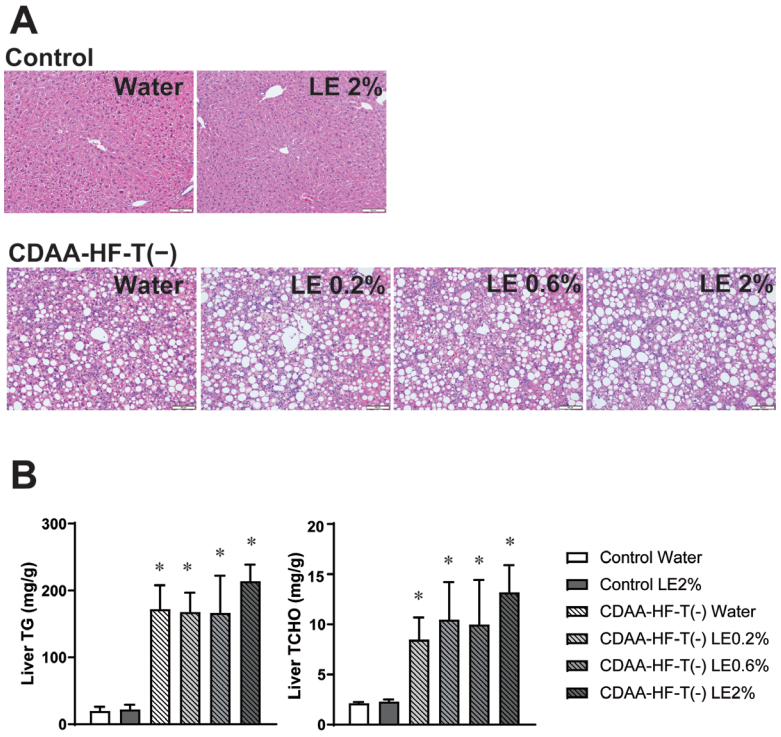

Fig. 2. Effects of LE on liver steatosis and hepatic lipid accumulation in CDAA-HF-T(−)-fed mice. Representative liver histopathology (hematoxylin and eosin staining) (A), and hepatic TG and TCHO levels (B). Values are presented as the means + standard deviations (SDs). Difference between the means was considered as significant when $p < 0.05$, using one-way ANOVA followed by Tukey–Kramer multiple comparisons test. *Significantly different from the control diet/water group value.

groups, with LE administration exerting only equivocal effects (Fig. 3C).

The mRNA expression of an inflammation-related cytokine, tumor necrosis factor-α (*TNF -α*), was extensively elevated in the CDAA-HF-T(−) groups, and LE administration reduced the RNA levels (significant difference was observed between the CDAA-HF-T(−)/water and CDAA-HF-T(−)/LE 0.6% group values) (Fig. 3D).

Effects of LE on liver fibrosis in CDAA-HF-T(−)-fed mice

The profile of hepatic fibrosis analyses is summarized in Fig. 4. The number of activated liver stellate cells and Sirius red-positive areas was strongly elevated in the CDAA-HF-T(−)/water groups. In comparison, LE administration inhibited both fibrosis and liver stellate cell activation in a relatively dose-dependent manner (Figs. 4A–C).

The expression of fibrosis-related genes reflected morphological changes. The mRNA expression of collagen type 1 α1 (*Col1a1*), collagen type 4 α1 (*Col4a1*), and tissue inhibitor of metalloproteinase 1 (*TIMP1*) were markedly increased in the CDAA-HF-T(−)/water group (Fig. 4D). In comparison, LE administration relatively dose-dependently inhibited the expression of these genes (Fig. 4D). The mRNA expression of transforming growth

Table 4. Organ weights and plasma chemistries at the end of week 28.

Group	Control Water	Control LE 2%	CDAAHF-T(-) Water	CDAAHF-T(-) LE 0.2%	CDAAHF-T(-) LE 0.6%	CDAAHF-T(-) LE 2%
Final body weight (g)	28.6 ± 1.2	29.2 ± 0.9	32.0 ± 3.0	33.0 ± 4.6	31.9 ± 3.9	37.8 ± 4.3*
absolute liver weight (g)	1.2 ± 0.1	1.3 ± 0.1	3.1 ± 0.3*	3.3 ± 0.9*	3.2 ± 0.6*	4.2 ± 0.5*+
Relative liver weight (%BW)	4.3 ± 0.5	4.4 ± 0.3	9.6 ± 0.8*	9.7 ± 1.6*	10.1 ± 0.9*	11.0 ± 0.5*
absolute adipose tissue weight (g)	0.5 ± 0.2	0.4 ± 0.1	1.3 ± 0.3*	1.2 ± 0.6*	1.2 ± 0.4*	1.8 ± 0.5*
Relative adipose tissue weight (%BW)	1.6 ± 0.6	1.4 ± 0.4	4.0 ± 0.8*	3.5 ± 1.5*	3.7 ± 1.0*	4.7 ± 0.9*
absolute spleen weight (g)	0.067 ± 0.01	0.07 ± 0.01	0.2 ± 0.03*	0.2 ± 0.02*	0.19 ± 0.023*	0.2 ± 003*
Relative spleen weight (%BW)	0.24 ± 0.04	0.24 ± 0.03	0.61 ± 0.07*	0.62 ± 0.14*	0.60 ± 0.13*	0.56 ± 0.07*
Plasma ALT (IU/L)	66.3 ± 83.7	49.3 ± 15.4	214 ± 17.3*	227 ± 35.5*	223 ± 52.3*	317 ± 33.5*+
Plasma AST (IU/L)	79.7 ± 50.8	73.1 ± 45.6	230 ± 23.8*	188 ± 41.6*	175 ± 37.6*	193 ± 41.2*
Plasma TG (mg/dL)	56 ± 17.9	51.8 ± 15.6	62.6 ± 6.4	62.5 ± 19.9	63.3 ± 9.11	71.5 ± 21.9
Plasma TCHO (mg/dL)	65.7 ± 8.3	67.7 ± 15.7	86.8 ± 25.6	105 ± 40.2	101 ± 26.5	132 ± 34.8*

Values are means ± SDs, n = 6.
eWAT; epididymal white adipose tissue, TG; triglyceride, TCHO; total cholesterol.
*Significantly different from the control value.
+Significantly different from the CDAA-HF-T(−) value.

factor-β (*TGF-β)1*, *2*, and *3* and their receptors (*TGF-βr)1*, *2*, and *3* was significantly increased in the CDAA-HF-T(−)/water groups, and LE did not alter these expression levels (Fig. 4E).

DISCUSSION

Our results suggest that LE can prevent NASH induced in mice by chronic feeding with CDAA-HF-T(−). We found that LE significantly reduced liver fibrosis without toxic effects. The partial suppression of inflammation, but not hepatic steatosis, may be involved in the preventive effects of LE on NASH.

The final stage of NASH is advanced liver fibrosis, cirrhosis, or hepatic tumors. Since the initial description of the disease, various pathological diagnostic criteria have been proposed, among which liver fibrosis is generally considered as the most important factor. Younossi *et al*. (2011) suggested that NASH-associated fibrosis was the best independent parameter for the association with long-term liver-related mortality. In the present study, LE significantly inhibited CDAA-HF-T(−)-induced hepatic fibrosis. Activated hepatic stellate cells and upregulated collagen expression by CDAA-HF-T(−) were dose-dependently inhibited in the LE treatment groups. LE treatment also reduced the upregulation of factors involved in hepatic fibrosis, such as *TIMP1* (Vizzutti *et al*., 2010; Tomita *et al*., 2006). Hepatic fibrosis occurs when hepatic stellate cells are activated to differentiate into myofibroblast-like cells, leading to an abnormal increase in collagen secretion by various stimuli (Friedman, 2008). Among them, TGF-β is widely known to trigger activation of hepatic stellate cells (Friedman, 2008). In fact, mRNAs of *TGF-β*s and their receptors were upregulated in the CDAA-HF-T(-) groups, and LE did not affect the expression of these genes. Immune cells have also been identified to play key roles in the hepatic fibrotic cascade by exerting injury-inducing or repair-promoting effects (Pellicoro *et al*., 2014). Inflammation-related cytokines are clearly associated with disease severity in patients with NALFD and NASH-associated cirrhosis (du Plessis *et al*., 2016). In the present study, as expected, the plasma ALT and AST activities, number of infiltrated macrophages, and levels of chemokine strongly increased in the CDAA-HF-T(−) groups; however, LE did not affect these changes. In contrast, LE reduced or tended to reduce the mRNA expression of inflammatory cytokine, TNF -α. Thus, LE may not affect early phase events from the recruitment of macrophages to hepatocyte death but may inhibit late phase events, such as the progression of inflammation and fibrosis (mechanisms independent from TGF-β signaling), and in turn exert a preventive effect on NASH.

In contrast to fibrosis, LE did not affect CDAA-HF-T(−)-induced hepatic steatosis. A previous study showed that mogroside V inhibits hepatic steatosis in mice fed a high-fat diet by downregulating *de novo* lipogenesis (Li *et al*., 2020). Unlike a high-fat diet, the cause of fatty liver by CDAA-derived diets does not occur through enhancement of *de novo* lipogenesis, but rather through inhibition of the synthesis and release of very low-density lipoprotein (VLDL) (Yao and Vance, 1990; Ibrahim *et al*., 2016). Because inhibiting the synthesis and release of VLDL from the liver has been indicated as a key factor

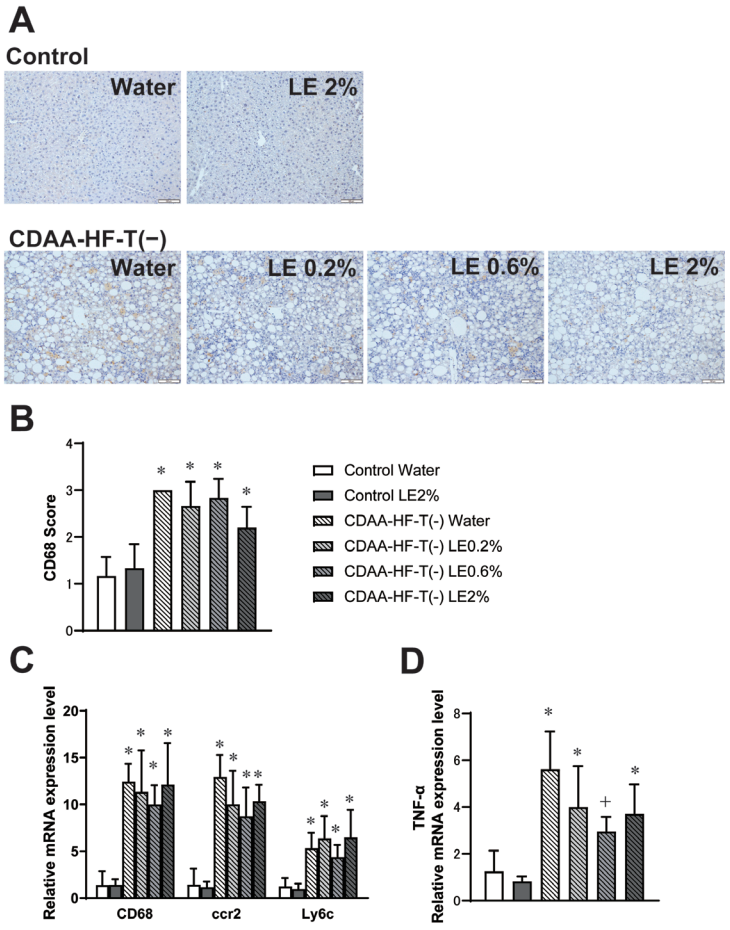

Fig. 3. Effects of LE on liver inflammation in CDAA-HF-T(−)-fed mice. Representative immunohistochemistry for CD68 (A) and scores for CD68 (B) in the liver at the end of week 28. qPCR of genes involved in related macrophage (C) and inflammation cytokine (D). Values are presented as the means + standard deviations (SDs). Difference between the means was considered as significant when $p < 0.05$, using one-way ANOVA followed by Tukey–Kramer multiple comparisons test. *Significantly different from the control diet/water group value. ⁺Significantly different from the CDAA-HF-T(−)/water group value (among CDAA-HF-T(−) groups).

in the pathogenesis of human NASH (Fujita *et al*., 2009), the present model is useful for assessing the mechanisms underlying NASH and exploring evidence-based strategies for controlling the disease. The CDAA-HF-T(−)/LE 2% group showed a tendency to have an increased body weight. As no change in body weight was observed in the control diet/2% LE group and no change in food intake was observed in the CDAA-HF-T(−)/LE 2% group, it is unlikely that LE directly affects appetite and body weight gain. We recently found that when mice were fed with a different CDAA-derived high-fat diet (lard-based) supplemented with 0.6% methionine, the animals became obese, although food intake was not altered, and the liver pathology was very mild, showing the NAFL phenotype rather than NASH, as observed in the livers of mice fed a diet containing only 0.1% methionine (Suzuki-Kemuriyama *et al*., 2021). In such a study, the liver pathology remained as NAFL following methionine supplementation, which is partially attributed to the possible recovery of VLDL release from the liver. Therefore, LE may recover VLDL release from the liver, which requires further analysis.

Several compounds such as triterpenoids, flavonoids, and amino acids have been isolated from *Siraitia grosvenorii*, and their active constituents possess broad pharmacological properties, such as antioxidative, hypoglycemic, immunologic, anti-tussive, sputum-reducing, hepatoprotective, antimicrobial, and other effects (Gong *et al*., 2019). In the liver, LE exerts anti-hepatocarcino-

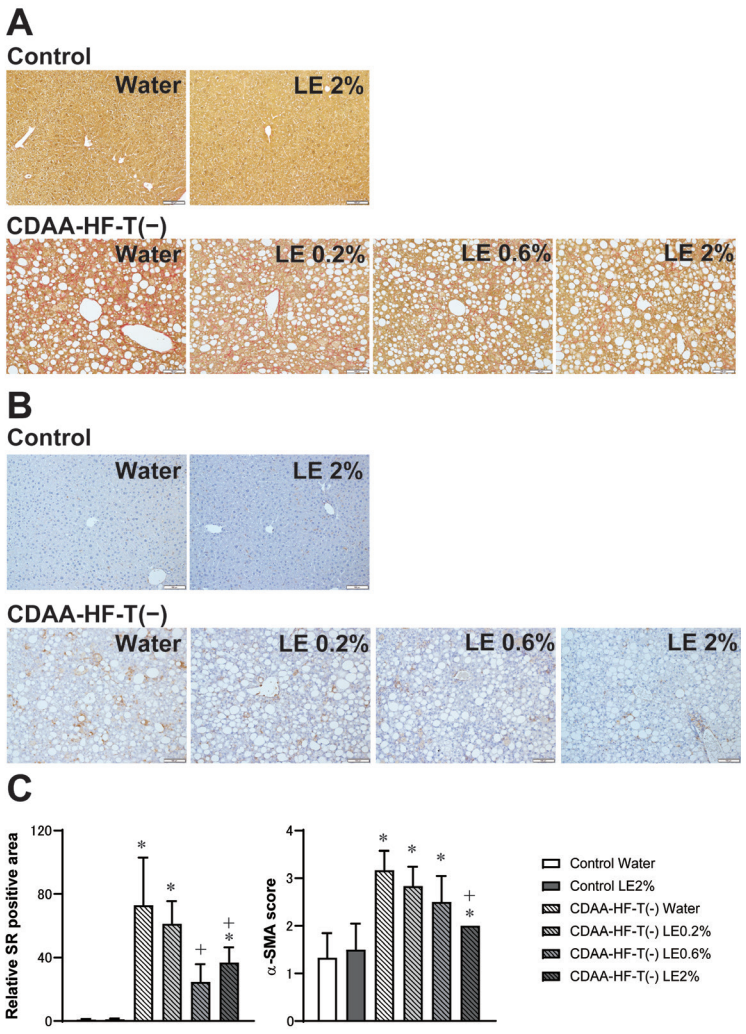

Fig. 4. Effects of LE on liver fibrosis in CDAA-HF-T(−)-fed mice. Representative immunohistochemistry for Sirius red (A) and α-smooth muscle actin (B) and scores for Sirius red and α-smooth muscle actin (C) in the liver at the end of week 28. qPCR of genes involved in related collagen (D) and TGF-βs and these receptors (E). Values are presented as the means + standard deviations (SDs). Difference between the means was considered as significant when $p < 0.05$, using one-way ANOVA followed by Tukey–Kramer multiple comparisons test. *Significantly different from the control diet/water group value. ⁺Significantly different from the CDAA-HF-T(−)/water group value (among CDAA-HF-T(−) groups).

genic effects by inhibiting the generation of reactive oxygen species via suppression of CYP1a1 (Matsumoto *et al*., 2009). The LE used in this study contained mogroside V at a concentration of 58%. Mogroside V and 11-oxo-mogroside V have remarkable reactive oxygen species scavenging abilities (Chen *et al*., 2007). Mogroside V has a therapeutic effect on lung inflammation by reducing ovalbumin-induced activation of nuclear factor-κB (Song *et al*., 2019). Oxidative stress and signaling alteration due to nuclear factor-κB activation are important causes of rodent NASH induced by CDAA-derived diets (Nakae *et al*., 1992, 1990; Nakae, 1999) and human NASH (Videla *et al*., 2009; Ucar *et al*., 2013). Mogroside V is considered to play a role in the preventive effects of LE on NASH, although other constituents in LE may also be involved.

Chemopreventive agents should not be toxic to humans, and thus, the potential risks of the agents should be carefully assessed and managed. A previous study showed that LE is not genotoxic and does not have strong

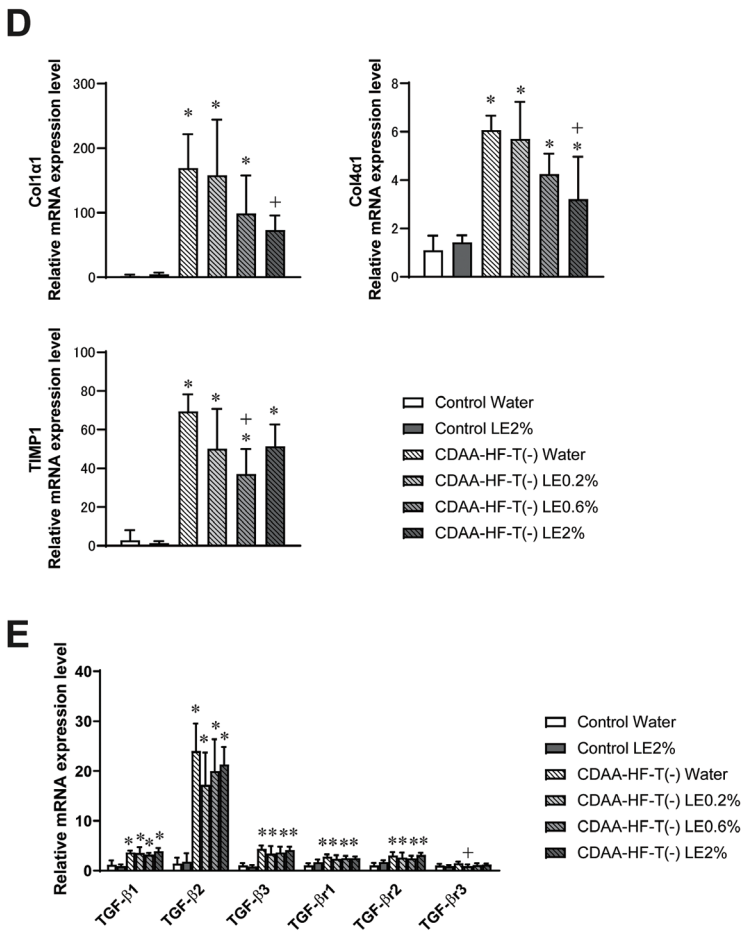

Fig. 4. (Continued).

subacute toxicity in 90-day repeated dose toxicity tests using rodents (Anonymous, 2019). For instance, in these tests, Fischer 344 rats were administered LE at concentrations of 0.25%, 0.5%, 1.0%, and 2.0% in the drinking water as repeated administration for 90 days, which resulted in no toxicity, including no weight loss or death, no lesions in major organs, and no abnormal findings in serum biochemistry. Wistar Hannover (GALAS) rats were administered LE at concentrations of 0.04%, 0.2%, 1%, and 5% in the diets as a repeated administration for 90 days; no deaths were observed in any animals and there were no changes in body weight gain, hematological and serum biochemical tests, organ weights, or histopathological examinations. Qin *et al*. investigated the safety of PureLo, a non-caloric powdered concentrate of *Siraitia grosvenorii*, and found that its sweetening properties were conferred by mogrosides. Male and female dogs were administered 3,000 mg/kg body weight/day PureLo for either 28 or 90 days. The results revealed no changes in body weight, organ weight, or food consumption. There were no significant effects on blood chemistry or urinalysis values. The results indicate that PureLo did not induce organ or systemic toxicity (Qin *et al*., 2006). Marone *et al*. conducted a 28-day dietary toxicity study using Hsd:SD rats and PureLo at dietary concentrations of 10,000, 30,000 and 100,000 ppm, showing that the no observed adverse effect level was 100,000 ppm in the diet, which is equivalent to 7.07 and 7.48 g/kg body weight/day for male and female rats, respectively (Marone *et al*., 2008). In the present study, LE showed no toxicity in mice even after 28-week administration in the drinking water up to a dose of 2.0%. The highest intake of LE was estimated as 4,068 mg/ kg body weight in the control diet/2% LE group, in which LE showed no toxici-

ty. According to the available data, LE is safe even at substantially high doses.

In summary, our results suggest that LE was not toxic under the experimental conditions evaluated and that it prevents NASH in mice fed CDAA-HF-T(−) by inhibiting fibrosis without altering steatosis. The suppression of the progression phase of inflammation may be involved in these preventive effects. Sweeteners with beneficial biological functions, such as LE, may be useful for controlling lifestyle-related diseases, such as NASH, and in turn promoting human health.

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Conflict of interest---- In the present study, LE was supplied by San-Ei Gen F.F.I., but the study was conducted independently from the company.

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