

## Regular Article

Preventive Effect of *Citrus aurantium* Peel Extract on High-Fat Diet-Induced Non-alcoholic Fatty Liver in MiceHyoung-Yun Han,<sup>a,b</sup> Sung-Kwon Lee,<sup>c</sup> Bong-Keun Choi,<sup>c</sup> Dong-Ryung Lee,<sup>c</sup> Hae Jin Lee,<sup>c</sup> and Tae-Won Kim<sup>\*b</sup><sup>a</sup>Korea Institute of Toxicology; 141 Gajeong-ro, Yuseong-gu, Daejeon 34114, Republic of Korea: <sup>b</sup>College of Veterinary Medicine and Institute of Veterinary Science, Chungnam National University; 99 Daehak-ro, Yuseong-gu, Daejeon 34134, Republic of Korea: and <sup>c</sup>Nutrpharm Tec; 302 galmachi-ro, Jungwon-gu, Seongnam, Gyeonggi 13201, Republic of Korea.

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Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic lipid accumulation, which is the most common form of chronic liver disease. Multiple clinical studies using natural compounds such as flavonoids have been conducted to treat NAFLD. In the present study, the pharmacological effect of *Citrus aurantium* L. (Rutaceae) peel extract (CAE), which contains over 27% of polymethoxyflavone nobiletin, on NAFLD was evaluated using a high-fat diet (HFD) animal model susceptible to developing NAFLD. C57BL/6 mice were fed an HFD (60% kcal of energy derived from fat) for 8 weeks to induce obesity. Obese mice were randomly allocated to four groups of eight mice each (HFD alone, HFD with silymarin, HFD with 50 mg/kg CAE, and HFD with 100 mg/kg CAE). After 8 weeks of treatment, all mice were euthanized, and plasma and liver tissues were analyzed biochemically and histopathologically. The results indicate that CAE treatment significantly reduced HFD-induced NAFLD, as shown by decreased serum lipid index and prevented liver histopathology. The expression of genes involved in lipid synthesis including free fatty acid (FFA), peroxisome-proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ), sterol receptor element binding protein 1c (SREBP-1c), and fatty acid synthesis enzyme was suppressed by CAE treatment. Moreover, compared to untreated mice, CAE-treated HFD mice showed decreased pro-inflammatory cytokine expression. These results demonstrated that CAE prevented HFD-induced NAFLD by reducing plasma levels of triglyceride and cholesterol and *de novo* lipid synthesis.

**Key words** cholesterol; polymethoxyflavone; triglyceride

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a type of hepatic steatosis induced by factors not related to alcohol consumption, and its prevalence is increasing worldwide, concomitant with the obesity epidemic.<sup>1)</sup> In recent years, various therapeutic paradigms have reduced severe hepatic complications due to conditions such as viral hepatitis. NAFLD is now being recognized as a major cause of hepatic disorder that could eventually advance to cirrhosis that requires liver transplantation.<sup>2)</sup>

Although NAFLD pathogenesis is a complex serial process, the accumulation of triglycerides (TGs) within the hepatocyte is a classic feature of NAFLD, and the esterification of free fatty acid (FFA) is recognized as a major source for triglyceride production.<sup>3)</sup> Circulating FFAs are derived from the lipolysis of adipose tissues, dietary intake, or *de novo* hepatocyte lipogenesis.<sup>4)</sup> Recently, the mechanism of NAFLD progression has shifted from a traditional two-hit hypothesis, which comprises steatosis and inflammation, to a three-hit hypothesis, which also includes hepatocyte death.<sup>5)</sup> However, the initial stage of fat accumulation remains a priority for therapeutic intervention.

Because of the link to general metabolic disorders, no established NAFLD therapy has been identified to date, and diverse natural compounds including flavonoids have been explored to assess their effects in NAFLD animal models.<sup>6,7)</sup> The consumption of citrus fruits, which contain flavonoids,

was shown to reduce plasma TG as well as liver cholesterol (CHO) levels.<sup>8,9)</sup> Moreover, the broad mechanism of action of flavonoids has drawn attention to their potential usefulness in treating NAFLD that involves multiple pathways.<sup>10)</sup>

There are several subgroups of citrus flavonoids including the polymethoxyflavones (PMFs), nobiletin, and tangeretin, which are abundant in citrus fruit peels.<sup>9)</sup> Many studies have explored the biological properties of PMFs in various disease conditions including inflammation, diabetes, and cancer.<sup>11,12)</sup> In our previous study, *Citrus aurantium* L. (Rutaceae) peel extract (CAE) was found to have various ameliorative effects against chemical-induced liver injury by enhancing antioxidant-related signals.<sup>13)</sup> However, the effect of CAE on hepatic lipid metabolism has not been previously explored. In this study, the effects of nobiletin rich extract, CAE (which contains >50% PMFs), on fatty liver changes were assessed using a high-fat diet (HFD) animal model.

## MATERIALS AND METHODS

**Chemicals and Reagents** The ethanol extract of *C. aurantium* peel was purchased from KPLC group (Batch No. Kca-150925, Paris, France), and the main flavonoid component was confirmed using HPLC according to Choi *et al.*<sup>13)</sup> with a nobiletin standard obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

**Animal Studies** Five-week-old male C57BL/6 mice ( $n = 40$ , Daehan Biolink, Chungbuk, Korea) were housed

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in the animal facility at Myoungji University maintained at  $21 \pm 2^\circ\text{C}$  and  $55 \pm 5\%$  humidity on a 12-h light/dark cycle. Obesity was induced by feeding an HFD (60% kcal from fat, #D12492; Research Diets, New Brunswick, NJ, U.S.A.) for 8 weeks. Obese mice were randomly allocated to four groups of eight animals each (HFD alone, HFD with 200 mg/kg silymarin, HFD with 50 mg/kg CAE, and HFD with 100 mg/kg CAE), and normal chow-treated mice were used as the control group. Distilled water was used as the vehicle control, and all groups were administered the designated compounds for 8 weeks by oral gavage. After 8 weeks of compound treatment, all mice were euthanized by carbon dioxide asphyxiation, and the blood and liver samples were collected. The blood samples were centrifuged ( $800 \times g$  for 20 min) for serum collection, and serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total CHO, and TG levels were measured using a Fuji Dri-Chem NX-500i (FUJIFILM, Tokyo, Japan). The liver tissue was carefully dissected, weighed, and subjected to histopathological and Western blot analyses. Animal experiments were approved by the Ethics Committee of Chungnam National University (CNU-00965) and proceeded under their guidelines.

**Histopathological Analysis** The paraffin-embedded liver tissue was cut into  $4\text{-}\mu\text{m}$ -thick sections that were either stained with hematoxylin and eosin (H&E), or Oil Red O. All stained liver slides were evaluated using a light microscope.

**Quantitative Real-Time PCR Analysis** Total liver RNA was extracted using the RNeasy mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. After extraction, cDNA was synthesized using the Primescript first strand cDNA synthesis kit (TaKaRa, Shiga, Japan), and the mRNA expression levels of inflammation- and lipogenesis-related genes were analyzed using a real-time PCR LightCycler 96 system (Roche, Basel, Switzerland) with a SensiMix Plus SYBR kit (Quantace, London, U.K.) according to the manufacturer's instructions. Primer sequences were as follows: interleukin-6 (IL-6): forward, 5'-AGTTGCCCTTCTTGGGACTGA-3' and reverse, 5'-TCCACGATTTCACAGAGAC-3'; tumor necrosis factor (TNF)- $\alpha$ : forward, 5'-AGCCCCAGTCTGTATCCTT-3' and reverse 5'-CTCCCTTTGCAGAAC TCAGG-3'; interleukin-1 $\alpha$  (IL-1 $\alpha$ ): forward, 5'-CCCGTCCTTAAAGCTGTCTG-3' and reverse, 5'-AATTGGAATCCAGGGGAAAC-3'; fatty acid synthase (FAS): forward, 5'-TGGTGGGTTTGGTGAATTGTC-3' and reverse, 5'-GCTTGTCTGCTCTAAGTGAAGT-3'; peroxisome-proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ): forward, 5'-TCACAAGAGGTGACCAATG-3' and reverse, 5'-CCATCCTTACAAGCATGAA-3'; sterol receptor element binding protein 1c (SREBP-1c): forward, 5'-GGCACTAAGTGCCCTCAACCT-3' and reverse, 5'-GCCACATAGATCTGCGAGTGT-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward, 5'-TGTCATACTTGGCAGGTTTCT-3' and reverse, 5'-CGTGTTCTACCCCCAATGT-3'. The real-time PCR cycling conditions were as follows: 2 min at  $95^\circ\text{C}$ ; 45 cycles of 20 s at  $95^\circ\text{C}$ , 20 s at  $60^\circ\text{C}$ , 40 s at  $72^\circ\text{C}$ , and 30 s at  $72^\circ\text{C}$ ; and a final extension for 5 min at  $72^\circ\text{C}$ . The mRNA levels of the target genes were normalized to the GAPDH expression level, and the results were expressed as the fold change relative to the normal control group.

**Western Blot Analysis** Phosphate buffered saline (PBS)-washed liver tissue (0.03 g) was homogenized in 1 mL radio-

immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.5, 150 mM sodium chloride [NaCl], 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)). After centrifugation (13000 rpm, 15 min,  $4^\circ\text{C}$ ), the supernatant was collected, and the protein contents were evaluated using a BCA Protein assay kit (Sigma-Aldrich). The protein was separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot Turbo (Bio-Rad, CA, U.S.A.) system. For blocking, the membrane was incubated in 15 mL of 5% skim milk for 30 min. After three washes in Tris-buffered saline plus Tween (TBS-T), the primary antibody was adjusted using the Can Get Signal Solution 1 (Toyobo, Osaka, Japan) at  $4^\circ\text{C}$  with the following antibodies: FAS (Cell Signaling Technology, MA, U.S.A.),  $\beta$ -actin (Cell Signaling Technology), nuclear factor-E2-related factor 2 (Nrf2) (Cell Signaling Technology), p-AMP activated protein kinase (AMPK) (Santa Cruz Biotechnology, CA, U.S.A.), and AMPK (Santa Cruz Biotechnology). After overnight incubation, the secondary antibody was adjusted with the Can Get Signal solution 2 (Toyobo) containing goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG (Bio-Rad), and the expression was detected using the Chemiscope 3000 fluorescence/chemiluminescence imaging system.

**Statistical Analysis** All experiments were repeated at least three times, and the results are presented as the mean  $\pm$  standard deviation (S.D.) of three individual experiments. One-way ANOVA with the Tukey's test or Mann-Whitney *U*-test were used to determine the significance of difference between the groups. The  $p < 0.05$  was considered to be statistically significant.

## RESULTS

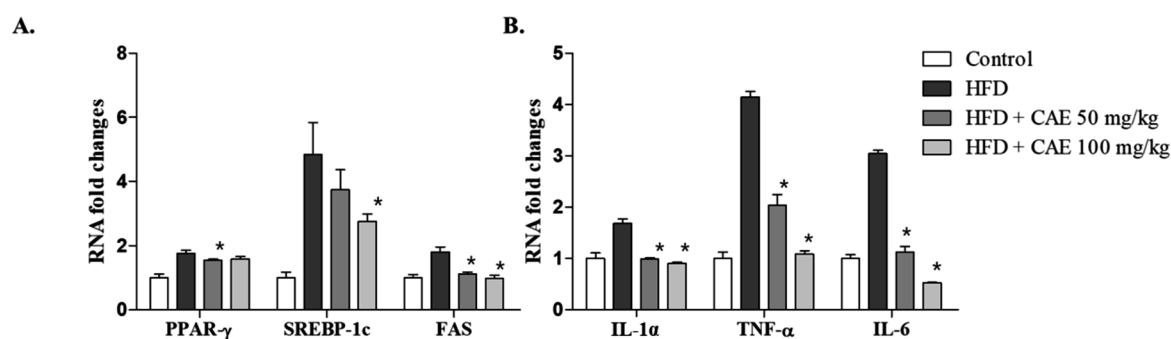
**Effect of CAE on Body Weight Increment and Serum Biochemistry** Average daily HFD intake was about 5 g/group and the difference between groups were without statistical significance. HFD treatment for 8 weeks significantly increased the body weight compared to that of the control group fed a normal diet (Table 1). Although HFD also increased the body weight of CAE-treated mice, the increment was significantly lower than that in the HFD alone group, and the body weight decrement was proportional to the CAE dose (Table 1). HFD increased the liver weight and serum ALT and AST levels. Similar to the effect on body weight, CAE administration prevented the HFD-induced increase in liver weight and enzyme level. Moreover, HFD-induced elevation of serum TG and total CHO levels was decreased by 20–30% after 8 weeks of CAE treatment.

**Effect of CAE on mRNA Expression** The mRNA levels for lipid metabolism-related genes and inflammatory cytokines were assessed in fresh tissue using quantitative real-time RT-PCR analysis (Fig. 1). The expression of the lipid synthesis-related genes *PPAR- $\gamma$* , *SREBP-1c*, and *FAS* increased after HFD treatment. Moreover, the expression of *SREBP-1c* and *FAS* was significantly reduced in the CAE-treated group compared to that in the HFD alone group. In addition, HFD markedly increased inflammatory cytokine expression in the liver tissue with the increase in TNF- $\alpha$  expression being the highest among IL-1 $\alpha$ , TNF- $\alpha$ , and IL-6 expression levels. This

Table 1. Effect of the *Citrus aurantium* Peel Extract (CAE) on Body Weight Increment and Serum Biochemistry

	Normal control	HFD	HFD+Silymarin (200mg/kg)	HFD+CAE (50 mg/kg)	HFD+CAE (100mg/kg)
Weight (g)					
Body weight	30.86 ± 2.00*	50.90 ± 1.25	48.49 ± 1.70*	48.76 ± 1.34*	48.61 ± 1.45*
Liver weight	0.96 ± 0.03*	1.91 ± 0.11	1.24 ± 0.24*	1.29 ± 0.10*	1.23 ± 0.11*
Serum Biochemistry (mg/dL)					
ALT	58.2 ± 46.2*	256.4 ± 110.7	130.5 ± 29.2*	126.8 ± 23.5*	114.6 ± 38.7*
AST	108.2 ± 25.0*	250.0 ± 37.3	172.2 ± 10.2*	182.8 ± 37.7*	170.5 ± 30.6*
TG	144.5 ± 28.0*	198.8 ± 34.0	157.6 ± 31.2	156.0 ± 17.1*	142.8 ± 23.3*
TCHO	112.6 ± 28.0*	384.0 ± 40.1	205.2 ± 59.1*	292.6 ± 45.7*	232.6 ± 19.2*

Values are expressed as mean ± S.D. \* $p < 0.05$ , a significant difference in comparison with the HFD alone group.

Fig. 1. Changes in Lipid Synthesis and Inflammation Related Signals after *Citrus aurantium* Peel Extract (CAE) Treatment

C57BL/6 mice were fed an HFD (60% kcal of energy derived from fat) for 8 weeks to induce obesity except for control and the mice were treated with each compound for 8 weeks. Total RNA was extracted from the liver, and mRNA expression levels were quantified using SYBR Green-based real-time PCR using mouse-specific primers. Values are expressed as means ± S.D. \* $p < 0.05$  indicates a significant difference in comparison with the HFD alone group.

increase in the expression of inflammatory cytokines was also significantly reduced after CAE treatment in a dose-dependent manner.

**Western Blot Analysis** The protein levels of FAS, p-AMPK, AMPK, and Nrf2 were assessed using Western blot analysis (Fig. 2). Similar to the results of the mRNA expression analysis, HFD also elevated FAS protein levels. The HFD alone group showed a 1.2-fold increase in FAS protein levels compared with the control group. In the CAE-treated groups, FAS protein levels were lower than those in the normal control group were. In addition, the levels of p-AMPK protein were lower in the HFD alone group than in the control group, and this decrease was prevented in the CAE-treated groups. Moreover, the Nrf2 protein level was also significantly higher in the high-dose CAE-treated group (up to 2.3-fold) than in the HFD alone group.

**Histopathological Analysis** Liver histology was visualized using H&E and Oil Red O staining with 100× magnification (Fig. 3). The normal control group livers stained with H&E showed intact hepatic structure. Extensive lipid droplet accumulation in hepatocytes together with cytoplasmic ballooning and inflammation were observed in the livers in the HFD alone group. Silymarin- and CAE-treated mice showed reduced HFD-induced liver changes with decreased lipid droplet accumulation. Moreover, liver tissues of the 100 mg/kg CAE-treated mice showed well-preserved liver parenchyma with fewer cytoplasmic lipid vacuoles than HFD alone group. This result was also confirmed by Oil Red O staining.

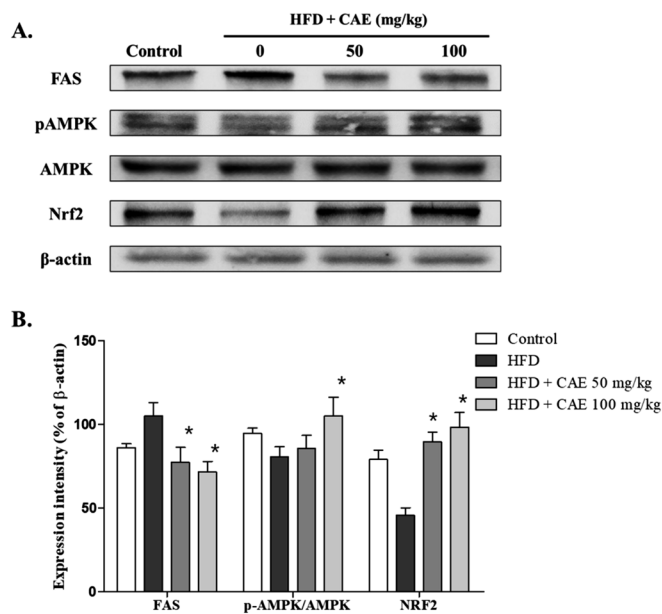


Fig. 2. Changes in FAS, AMP-Activated Protein Kinase (AMPK), and Nuclear Factor, Erythroid 2 Like 2 (Nrf2) Protein Levels after CAE Treatment

C57BL/6 mice were fed an HFD (60% kcal of energy derived from fat) for 8 weeks to induce obesity except for control and the mice were treated with each compound for 8 weeks. Protein levels of FAS, AMPK, and Nrf2 in the liver tissue were assessed using Western-blot analysis. Values are expressed as means ± S.D. \* $p < 0.05$  indicates a significant difference in comparison with the HFD alone group.



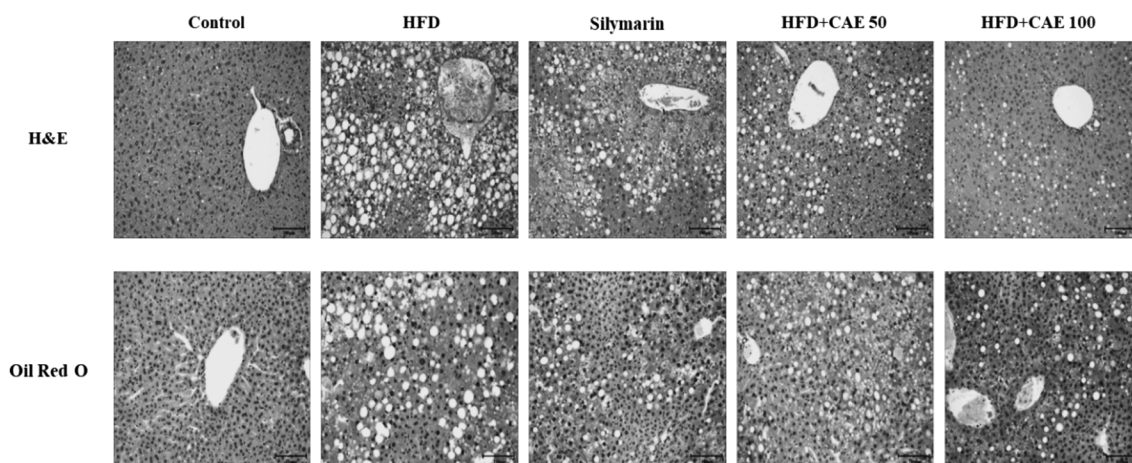


Fig. 3. Effect of CAE on HFD-Induced Histopathologic Alterations in the Liver

C57BL/6 mice were fed an HFD (60% kcal of energy derived from fat) for 8 weeks to induce obesity except for control, and the mice were treated with each compound for 8 weeks. Histopathologic changes and cytoplasmic lipid in the liver tissue were assessed using hematoxylin and eosin (H&E) and Oil red O staining, respectively. Major HFD-induced histopathological changes in the liver were cytoplasmic ballooning, inflammation, and lipid droplet accumulation. Scale bar, 100  $\mu$ m.

## DISCUSSION

The increased incidence of metabolic syndrome-associated NAFLD along with the availability of effective therapies used to treat severe chronic viral hepatitis have changed the clinical approach in the treatment of chronic liver disease. Recently, many studies have shown that some active ingredients found in natural materials extracts have proven efficacious in the prevention and treatment of NAFLD.<sup>10)</sup> In the present study, the hepatic lipid-lowering effect of CAE was assessed using an HFD model. HFD treatment for 8 consecutive weeks increases average body weight by >60% compared to that of the normal chow-fed mice, which indicates obesity induction in mice.<sup>14)</sup> Silymarin (a flavonoid present in milk thistle extract) was used as a positive control due to its widely known its pharmacological effects including lipid-lowering effect.<sup>15)</sup> In this study, nobletin rich extract, CAE (containing approximately 27% nobletin) treatment successfully prevented HFD-induced NAFLD with improved systemic lipid profiles and liver function. The increase in plasma TG and total CHO levels after HFD treatment was markedly reduced in the CAE-treated group in a dose-dependent manner. This was in line with the previous study that reported decreased plasma CHO or TG levels or both after oral PMF administration in both animals and humans.<sup>16–18)</sup> Furthermore, nobletin (0.3%) in fat high diet reported to increase hepatic FA oxidation that leads to evidently decreased hepatic triglyceride.<sup>19)</sup>

In the present study, reduced circulating plasma lipid levels were observed following CAE treatment of HFD-fed mice, which could be the result of decreased lipid synthesis. SREBPs are a family of transcription factors that stimulate gene expression involved in lipid biosynthesis, and the overexpression of lipid metabolism-related transcription factors was reported in the liver from obese patients with NAFLD with increased SREBP-1c and FAS levels as the most representative changes.<sup>20,21)</sup> Moreover, SREBP-1c is known to control the expression of the PPAR- $\gamma$  transcription factor and its endogenous ligand.<sup>22)</sup> PPAR- $\gamma$  is known to be involved in lipid metabolism by modulating genes involved in fatty acid uptake, binding, and transport leading to increased *de novo* fatty acid biosynthesis.<sup>23)</sup> In addition, cross-regulation of both pro-lipogenic

factors, PPAR- $\gamma$  and SREBP-1c, are reported to affect the NAFLD liver by altering liver cell signaling leading to hepatic steatosis.<sup>24)</sup> Previously, Miyata *et al.* demonstrated that treatment with prenylated flavonoid from hops suppressed *de novo* lipid synthesis by inhibiting SREBPs.<sup>25)</sup> In the present study, CAE treatment found to suppress SREBP-1c and FAS expression levels, which in turn prevented the accumulation of TGs in the liver as confirmed by Oil Red O staining. Although, the present decrease in PPAR- $\gamma$  levels after CAE administration was not dose-dependent, the nobletin, which is a major component in CAE, was reported to have inhibitory effect against several adipogenic transcription factors including PPAR $\gamma$  in different type of cells.<sup>26)</sup>

As mentioned above, the pathogenesis of NAFLD is closely linked to inflammatory events, which are the so-called second hits. After hepatic TG accumulation, the liver is vulnerable to stress factors including inflammatory mediators and oxidative stress, which lead to further injury.<sup>27)</sup> In addition, steatosis development was accompanied by evidently elevated of IL-1 $\alpha$ , potent inflammatory cytokine initiates inflammation, and IL-1 $\alpha$  deficiency inhibited steatohepatitis pathogenesis in atherogenic high-fat diet treated mice model.<sup>28,29)</sup>

Our results indicate that CAE treatment decreased HFD-induced expression levels of inflammatory cytokines IL-1 $\alpha$ , TNF- $\alpha$ , and IL-6 in a dose-dependent manner. This was in line with well-characterized anti-inflammatory effects of PMFs.<sup>30)</sup> Moreover, combination of PMFs, hesperidin, nobletin, and tangeretin, found to suppress lipopolysaccharide-induced proinflammatory cytokine secretion in BV2 microglial cell.<sup>31)</sup>

Nrf2 is a nuclear transcription factor, which is known to initiate a cytoprotective signal cascade against various oxidative stresses.<sup>32)</sup> It is widely demonstrated that diverse PMFs reported to prevent oxidative stress by activating Nrf2 signal pathway including hesperidin, nobletin, and tangeretin.<sup>33–35)</sup> Furthermore, apart from its anti-oxidant action, Nrf2 also plays a role in hepatic lipid metabolism by suppressing the expression of small heterodimer partner.<sup>36)</sup> In addition, a recent study showed that increased phosphorylation reduced hepatic lipid accumulation by phosphorylating acetyl-CoA carboxylase and inhibiting ATP-consuming processes.<sup>37)</sup> Moreover,

many other recent studies have reported cross-signaling between the AMPK and Nrf2 signaling pathways in cellular homeostasis.<sup>38,39</sup> In the present study, CAE treatment was found to increase Nrf2 expression and AMPK phosphorylation in an HFD mouse model.

In this study, CAE prevented HFD-induced fatty liver changes by lowering plasma TG and CHO levels. Although CAE-induced changes in PPAR- $\gamma$  were not dose-dependent, our study suggests that suppressed SREBP-1c/FAS signaling might contribute, at least in part, to decreased *de novo* lipid synthesis. Moreover, the anti-inflammatory and Nrf2-enhancing effects of CAE were confirmed in the HFD animal model. Additional mechanistic and safety-related studies on CAE are needed to assess its applicability in the treatment of NAFLD.

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**Conflict of Interest** The authors declare no conflict of interest.

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