

Tangeretin Protects Mice from Alcohol-Induced Fatty Liver by Activating Mitophagy through the AMPK–ULK1 Pathway

Jianjin Guo, Yuan Chen, Fang Yuan,* Li Peng,* and Chen Qiu*

Cite This: *J. Agric. Food Chem.* 2022, 70, 11236–11244

Read Online

ACCESS |



Metrics & More



Article Recommendations



Supporting Information

ABSTRACT: Alcoholic beverages are widely consumed all over the world, but continuous ethanol exposure leads to hepatic steatosis that, without proper treatment, will later develop into severe liver disorders. In this study, we investigated the potential protective effect of tangeretin, a flavonoid derived from citrus peel, against alcoholic fatty liver. The *in vivo* effects of tangeretin were analyzed by oral intake in a chronic-binge alcohol feeding C57BL/6j mouse model, while the underlying mechanism was explored by *in vitro* studies performed on ethanol-treated hepatic AML-12 cells. Ethanol feeding increased the serum alanine aminotransferase and aspartate aminotransferase levels, the liver weight, and the serum and liver triacylglycerol contents, whereas 20 and 40 mg/kg tangeretin treatment promoted a dose-dependent suppression of these effects. Interestingly, tangeretin prevented increases in the liver oxidative stress level and protected the hepatocyte mitochondria from ethanol-induced morphologic abnormalities. A mechanistic study showed that 20 μ M tangeretin treatment activated mitophagy through an AMP-activated protein kinase (AMPK)–uncoordinated 51-like kinase 1 (Ulk1) pathway, thereby restoring mitochondria respiratory function and suppressing steatosis. By contrast, blocking the AMPK–Ulk1 pathway with compound C reversed the hepatoprotective effect of tangeretin. Overall, tangeretin activated mitophagy and protected against ethanol-induced hepatic steatosis through an AMPK–Ulk1-dependent mechanism.

KEYWORDS: alcoholic fatty liver, tangeretin, AMPK, mitophagy, chronic-binge ethanol model

INTRODUCTION

Alcoholic liver disease (ALD) is a common health crisis among consumers of alcoholic beverages worldwide and shows a spectrum ranging from simple steatosis to severe forms of liver injury, such as steatohepatitis, necrosis, fibrosis, and hepatocellular carcinoma.¹ Alcoholic fatty liver, the initial stage of ALD, is defined as an excessive lipid accumulation in hepatocytes. Ethanol-induced hepatic steatosis can be asymptomatic and reversible; however, multiple reports have indicated that it has a potentially pathologic character and can lead to a poor prognosis without proper intervention.² Alleviating ethanol-induced hepatic lipid accumulation may therefore ameliorate the progression of ALD and serve as an effective therapeutic strategy.

Treatment of ethanol-induced liver disorders can involve natural products, as these represent important resources for developing new lead drug compounds. Accumulating evidence now supports a role for flavonoids with variable phenolic structures in alleviating the progression of ALD. These include naturally occurring substances, such as wogonin,³ baicalin,⁴ quercetin,⁵ oroxylin A,⁶ apigenin,⁷ puerarin,⁸ and naringenin.⁹ Our previous work suggested that tangeretin, a polymethoxylated flavone derived from citrus peels, can enhance liver insulin sensitivity and reduce hepatic steatosis in the *db/db* mouse model of diabetes.¹⁰ Since nonalcoholic fatty liver disease and alcohol-induced fatty liver disease share similarities (although they differ in their molecular mechanisms), the aim of the present study was to determine whether tangeretin

would also have a beneficial effect in regulating steatosis in ethanol-treated mice.

In the present study, we analyzed the effects of tangeretin on alcoholic fatty liver disease using the chronic-binge ethanol feeding model, a widely used ALD mouse model that mimics alcoholic liver injury in human patients.¹¹ Additional hepatic cell studies indicated that tangeretin protects the liver from ethanol-induced lipid accumulation by activating mitophagy through an AMPK–Ulk1 pathway.

METHODS AND MATERIALS

Chemicals. Tangeretin ($\geq 99.5\%$, # HY-N0133) was provided by MedChemExpress Company (Monmouth Junction, NJ). For *in vitro* studies, tangeretin was prepared as a 10 mM stock in dimethyl sulfoxide (DMSO) and used at a concentration of 20 μ M to treat AML-12 cells. For *in vivo* studies, tangeretin was mixed into the alcoholic diet to prepare a porridge-like suspension (0.05 and 0.1 mg/mL). The average dietary intake of each mouse (~ 25 g) was approximately 10 mL; hence, the daily consumption of tangeretin was approximately 20 and 40 mg/kg body weight.

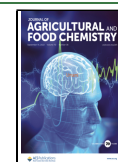
Animal Studies. Male C57/BL6 mice, 8–12 weeks old, were maintained in a 12:12 light/dark cycle at a controlled temperature (21–23 °C) and humidity (55–65%). The mice were randomly

Received: April 27, 2022

Revised: August 16, 2022

Accepted: August 25, 2022

Published: September 5, 2022



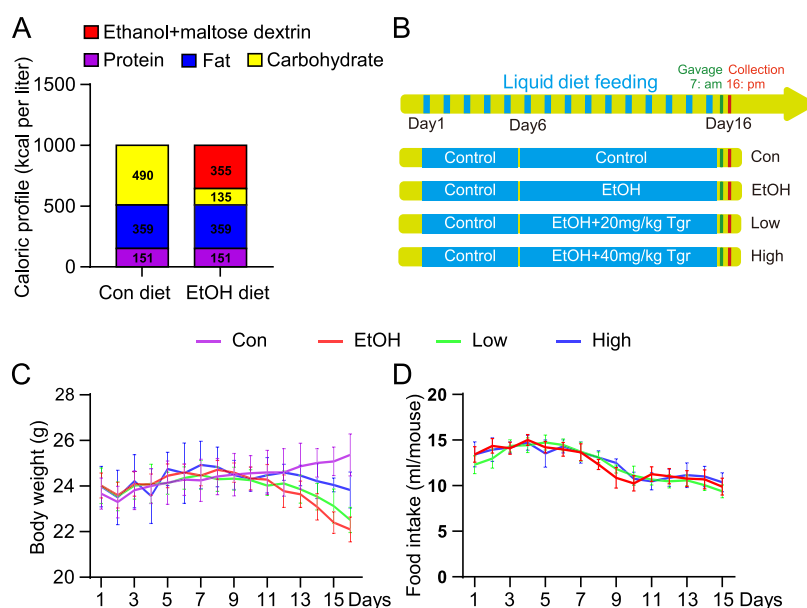


Figure 1. Overview of the experimental schedule. (A) Caloric profile of the control and ethanol liquid diets used in this study. (B) Experimental schedule and grouping information. (C, D) During the feeding period, body weight (C) and food intake (D) were recorded. Curves are mean \pm SEM, $n = 6$ for Con, $n = 8$ for EtOH, Low, and High groups.

separated into four groups: (1) control group (Con) that was pair-fed with the control diet ($n = 6$); (2) ethanol diet group (EtOH) ($n = 8$); (3) ethanol diet with 20 mg/kg tangeretin group (Low) ($n = 8$); and (4) ethanol diet with 40 mg/kg tangeretin group (High) ($n = 8$). Chronic-binge ethanol feeding was conducted as described by Bertola.¹¹ Briefly, the powder-like diet (Bio-Serv, Flemington, NJ) was mixed with either water or water plus ethanol according to the instruction, and the old diet was replaced daily at approximately 18:00 PM. The composition of the diet is shown in Figure 1A, and the feeding schedule is shown in Figure 1B. All mice were allowed to acclimatize to the control diet for 5 days to adapt to a liquid diet. For the next 10 days, the control group received no intervention, while the ethanol-fed mouse groups were changed to the isocaloric alcoholic diet containing 5% ethanol. The Low and High groups also consumed added tangeretin. For this modeling period, the control group was pair-fed with weight-matched EtOH-treated mice throughout so that these two groups consumed equal amounts of diet and ingested the same amounts of energy. No additional tapping water is needed during the feeding period. In the early morning of day 16, the ethanol-fed experimental mice underwent an acute ethanol administration by gavage with 31.5% (v/v) ethanol (2 mL/100 g body weight). The pair-fed control mice were gavaged with 45% (w/v) maltodextrin solution (2 mL/100 g body weight). At 9 h after gavage, all mice were sacrificed and samples were collected.

All experiments were conducted in accordance with the guidelines of the Research Animal Care Committee of Nanjing Medical University, China (IACUC No.: 1601081-4).

Cell Culture and Treatment. The mouse AML-12 normal hepatocyte line was obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured as described previously.¹² Briefly, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) at 37 °C with 5% CO₂ in a cell incubator. When the cells reached a confluence of 60–70%, they were pretreated with 20 μ M tangeretin for 24 h, followed by cotreatment with 100 mmol/L ethanol for another 24 h. During the ethanol exposure period, water containing the same concentration of ethanol was added to the incubator, as described previously, to prevent ethanol volatilization.¹¹

Biochemical Analysis. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), and total cholesterol (TC) were measured with a Hitachi 7100 analyzer

(Hitachi High-Tech Co., Tokyo, Japan). Total hepatic and cellular lipids were purified using a chloroform/methanol-based method,^{13,14} and measured with a TG kit (BHKT Clinical Reagents, Beijing, China) and a TC kit (SSUF-C, Shanghai, China), respectively. All assays were performed according to the manufacturer's instructions.

Measure of Oxidative Stress. ROS accumulation and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were detected by homogenizing fresh liver tissue in ice-cold normal saline and centrifuging at 1500g for 15 min at 4 °C. The supernatant was analyzed with an appropriate kit (ROS: #E004-1-1; SOD: #A001-3-2; GSH-Px: #A005-1-2; MDA: #A005-1-2; purchased from Nanjing Jiancheng Bioengineering Institute). As for catalase enzyme activity detection, fresh liver tissue was homogenized in RIPA lysis buffer and analyzed using a commercial kit (#S0051, Beyotime), according to the instruction. Levels of SOD, GSH-Px, MDA, and catalase were normalized to the protein content.

Histopathological Analysis. Liver sections were fixed overnight in 10% neutral buffered formaldehyde. The fixed tissues were embedded in paraffin, cut into 5 μ m thick sections, and stained with hematoxylin and eosin (H&E). Lipids were stained with Oil Red O (Sigma) in frozen liver sections (5 μ m).

Transmission Electron Microscopy. Liver tissues were quickly dissected from the mice and immersed in 2.5% glutaraldehyde (Sigma-Aldrich, G7651) overnight. The basilar membranes were fixed with 1% osmium tetroxide for 2 h at room temperature, followed by dehydration through a graded ethanol series (50, 70, 90, 95, and 100% (4 \times), each for 15 min). Samples were gradually embedded in Epon-812 (Sigma-Aldrich, 45345), sectioned with a diamond knife on a PowerTome-PC ultramicrotome (RMC), and placed on copper wire mesh. The ultrathin sections were sequentially poststained with uranyl acetate and Reynold's lead citrate and imaged with a Hitachi H500 transmission electron microscope. The magnification of observation was 4,800 \times .

Fluorescence and Confocal Imaging. AML12 cells were plated on glass-bottomed imaging dishes and stained with each dye, as indicated. Nuclei were marked with 500 nM Hoechst 33342, and lysosomes were labeled with LysoTracker Red (100 nM); mitochondria were marked with 100 nM MitoTracker Green, according to the manufacturer's instructions. Cells were imaged using a Zeiss LSM710 confocal microscope.

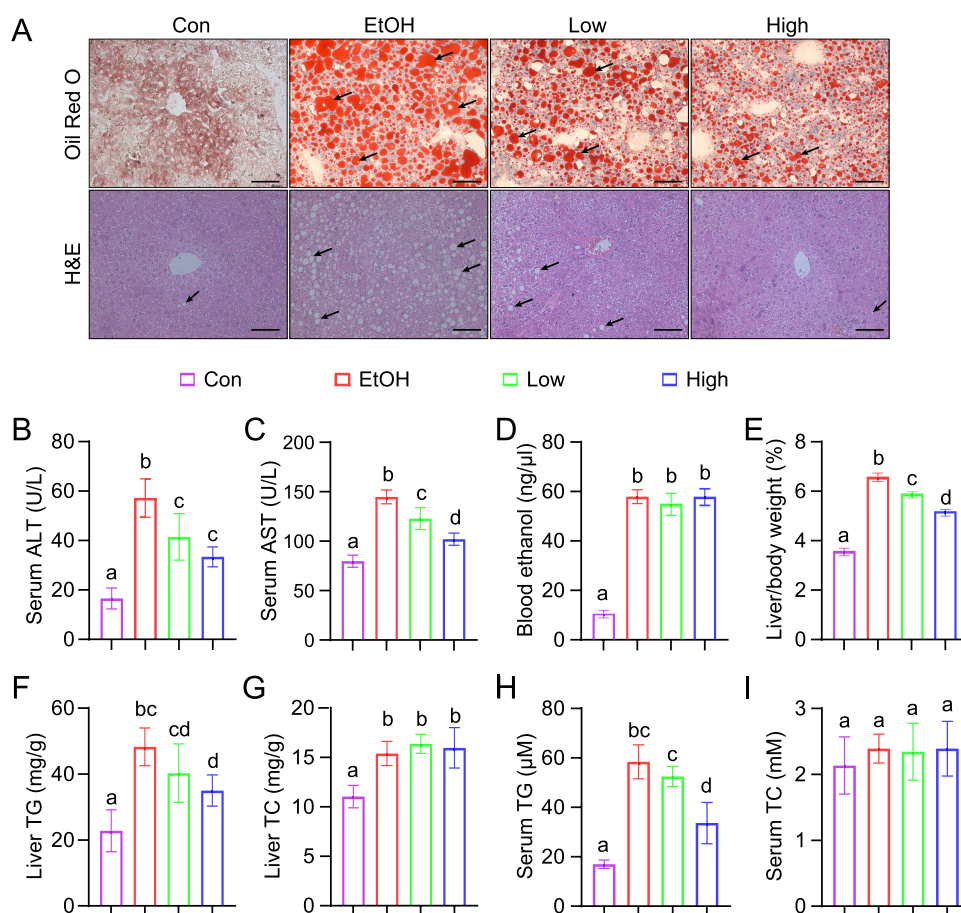


Figure 2. Tangeretin alleviates alcoholic fatty liver *in vivo*. (A–I) Wild-type C57/BL6j mice subjected to a control diet (Con), ethanol diet (EtOH), or ethanol diet supplemented with a low dose (Low) or high dose (High) of tangeretin. Liver samples were collected. Oil red O staining (up) and H&E staining (low) of liver sections (A). Arrow pointed to parts of the lipid droplet. Scale bar: 100 μ m. Serum ALT (B), serum AST (C), blood ethanol concentration (D), liver/body weight ratio (E), liver triglyceride (TG) content (F), liver total cholesterol (G), serum TG (H), and serum total cholesterol (I) were determined. Columns are means \pm SEM, $n = 6$ for Con, $n = 8$ for EtOH, Low, and High groups. Bars with no letters in common are significantly different.

Mitochondrial Respiratory Function Assay. A Seahorse XF96e Analyzer (Seahorse Bioscience—Agilent, Santa Clara, CA) was used to measure mitochondrial respiratory function. In this study, 5×10^4 AML12 cells were plated in each well and subjected to the indicated treatment. Before the assay, the growth medium was replaced with the Seahorse XF assay media (Eagle's modified Dulbecco's medium, w/o glucose, pH = 7.4; Agilent Seahorse) supplemented with 1 mM pyruvate, 10 mM glucose, and 2 mM L-glutamine. The cells were incubated without CO₂ for 1 h, and then the baseline and stimulated oxygen consumption rates (OCRs) (pmol/min) were measured. Three reagents were used during the sequential OCR measurements: 1.0 μ M oligomycin (an inhibitor of the ATP synthase of the electron transport system respiratory chain complex V), 1.5 μ M carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (an oxidative phosphorylation uncoupler), and 0.5 μ M rotenone with antimycin A (an inhibitor of electron transport system respiratory complex I/III).

RNA Isolation and Quantitative RT-PCR. Total RNA from frozen mouse liver was extracted with TRIzol (Takara, Japan), followed by precipitation with isopropanol and ethanol. The cDNA was then prepared using a reverse transcription kit (Takara, Japan), according to the manufacturer's instructions. Quantitative real-time PCR analysis was performed using SYBR Green Master Mix (Vazyme, China) and analyzed using a Roche Real-Time PCR System. The relative abundances of hepatic mRNA species were measured against GAPDH as an internal control using commercial primer sets (Table S1).

Western Blotting. Western blotting was performed as described previously.¹⁰ The primary antibodies were as follows (dilution

1:1000): mouse anti-Lc3B (ab192890, Abcam), rabbit anti-P62 (ab109012, Abcam), rabbit anti-phospho-AMPK α (#50081, Cell Signaling), rabbit anti-phospho-Ulk1 (#14202, Cell Signaling), rabbit anti-AMPK α (#2532, Cell Signaling), rabbit anti-Ulk1 (#8054, Cell Signaling), and mouse anti- β -actin (#3700, Cell Signaling).

Statistics. All data are expressed as mean \pm S.E. For comparison of two groups, a multiple t-test was used; for comparison of multiple groups, one-way ANOVA with Tukey's test were used. Significant differences were assessed using Graphpad PRISM (Graph Pad Software), and a value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Tangeretin Alleviates Alcoholic Fatty Liver *In Vivo*.

We examined the effect of tangeretin on alcoholic liver steatosis by treating wild-type C57/BL6j mice with ethanol following the chronic-binge protocol,¹¹ with or without supplementation with tangeretin. Ethanol administration reduced the body weight compared to the control maltodextrin treatment, and tangeretin had no additional effect on body weight, although the high-dose group showed a slight elevation from day 12 to day 16 (Figure 1C). The daily food intake was comparable between the ethanol group and the tangeretin-treated groups (Figure 1D).

Morphological staining using oil red O and H&E revealed severe hepatic lipid accumulation in response to ethanol

feeding, whereas tangeretin treatment showed a dose-dependent suppression of this ethanol effect (Figure 2A). The liver damage due to ethanol exposure was reflected in an upregulation of serum transaminase levels (Figure 2B,C). The elevation in serum transaminase levels was significantly suppressed by tangeretin treatment (Figure 2B,C). Ethanol exposure increased the blood ethanol concentration (Figure 2D) while also significantly increasing the liver weight (Figure 2E), liver TG (Figure 2F), liver TC (Figure 2G), and serum TG (Figure 2H). Tangeretin treatment dose-dependently suppressed the increases in liver weight, liver TG, and serum TG but had a negligible effect on liver TC and serum TC levels (Figure 2I).

Tangeretin Suppresses the Activation of Lipid Metabolism Genes Induced by Ethanol Feeding. The main trigger of liver steatosis is the dysregulation of hepatic lipid homeostasis.¹⁵ We sought to understand the mechanism by which tangeretin reduces liver steatosis by profiling the expression of key regulators that govern hepatic lipid homeostasis. Key enzymes that regulate lipid synthesis, as well as their transcription factors or cofactors, were analyzed. These included fatty acid synthase (*Fasn*), acetyl-CoA carboxylase (*Acc*), stearoyl CoA desaturase (*Scd1*), and sterol regulatory element-binding protein 1c (*Srebp1c*). We also examined key regulators that govern the fatty acid oxidation rate, including peroxisome proliferator-activated receptor α (*Ppara*) and carnitine palmitoyltransferase 1 α (*Cpt1a*), as well as genes involved in TG secretion (*ApoB* and *ApoE*) or fatty acid uptake (*Cd36* and *Fabp1*). The qPCR results showed opposite changes in *Acc*, *Fasn*, *Ppara*, and *Cpt1a* expression, indicating activation of lipid synthesis and blocking of β -oxidation in the livers of EtOH mice, whereas these ethanol-induced changes were suppressed in the tangeretin-treated groups (Figure 3A). Other genes showed no significant differences in the livers of the tangeretin-treated groups compared to the EtOH-treated group (Figure 3A).

Tangeretin Protects Mitochondria from Ethanol-Induced Morphologic Abnormalities. Alcohol induces ROS production and damages mitochondrial function. Based on transmission electron microscopy, we found that the mitochondrial structures in the livers of ethanol-fed mice were severely changed: the cristae disappeared. Both doses of tangeretin treatment prevented these morphological abnormalities in mitochondria (Figure 4A). We then analyzed the oxidative stress level, which plays a preeminent role in the clinical and pathological spectrum of ALD. The levels of liver ROS (Figure 4B) and MDA (Figure 4C) were markedly increased by ethanol feeding, while the levels of antioxidative GSH-Px (Figure 4D), SOD (Figure 4E), and catalase (Figure 4F) were reduced. Tangeretin dose-dependently suppressed the aggravated oxidative damage induced by ethanol (Figure 4B–F).

Tangeretin Activates the AMPK–Ulk1 Pathway and Promotes Mitophagy. The molecular pathways regulating alcoholic fatty liver appear to involve AMPK, a reported target of tangeretin,¹⁶ that acts as a master regulator of autophagy/mitophagy. Once activated, AMPK directly phosphorylates downstream targets, such as Ulk1¹⁷ and ubiquinol-cytochrome c reductase core protein 2 (Uqcrc2),¹⁸ thus activating mitophagy and protecting against alcohol-induced liver injury. Our immunoblotting results showed that Lc3B II, phospho-AMPK, and phospho-Ulk1 were downregulated and P62 was

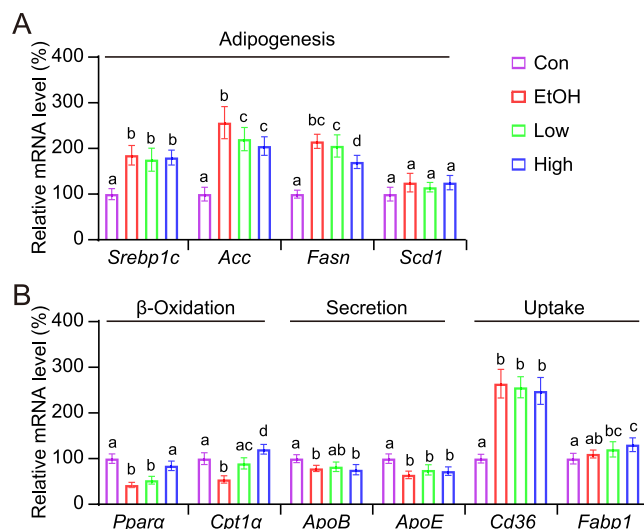


Figure 3. Tangeretin enhances hepatic lipid β -oxidation. (A, B) Wild-type C57/BL6j mice fed a control diet (Con), ethanol diet (EtOH), or ethanol diet supplemented with a low dose (Low) or high dose (High) of tangeretin. Liver tissue lysates were collected. QPCR profiling the expression of genes that govern hepatic adipogenesis (A), β -oxidation, lipid secretion and uptake (B). Values are mean \pm SEM, $n = 6$ for Con, $n = 8$ for EtOH, Low, and High groups. Within each gene, bars with no letters in common are significantly different.

upregulated in the livers of the EtOH-treated mice and that tangeretin treatments suppressed these effects (Figure 5A).

Using hepatic AML12 cells, we then analyzed whether tangeretin activates mitophagy and restores mitochondrial function. The fluorescent staining of mitochondria and lysosomes showed that 20 μ M tangeretin treatment significantly enhanced the costaining, which reflected mitophagy (Figure 5B) when compared to the ethanol-exposure group. We also tested the OCR, which reflects the oxidative respiratory function of the mitochondria, using the Seahorse assay. Tangeretin treatment raised both the baseline and maximal respiration in ethanol-exposed AML12 cells (Figure 5C).

Tangeretin Suppressed Ethanol-Induced Steatosis through an AMPK-Dependent Pathway. We next examined whether the protective effect of tangeretin against alcoholic fatty liver disease was dependent on the activation of AMPK by blocking AMPK with Compound C (Figure 6A) in the AML-12 mouse hepatic cell line and exposing the cells to 100 mM ethanol to mimic alcoholic fatty liver *in vitro*.¹² Both the cellular TG content (Figure 6B) and morphological staining by oil red O (Figure 6C) revealed suppression of steatosis by tangeretin, whereas blocking AMPK completely prevented this protective effect.

DISCUSSION

Tangeretin is a key flavonoid that possesses many positive biological activities¹⁹ that are beneficial to the neurologic system,²⁰ liver,²¹ skeletal muscle,²² and gut microbiota.²³ We recently discovered that tangeretin exerts a beneficial effect on hepatocyte insulin sensitivity by blocking the MEK-ERK1/2 pathway.¹⁰ Interestingly, our previous data showed that tangeretin treatment also ameliorated the fatty liver condition in *db/db* mice.¹⁰ A new study has also shown that tangeretin reduced hepatic lipid accumulation in the nonalcoholic steatohepatitis (NASH) mouse model.²⁴ This evidence for a

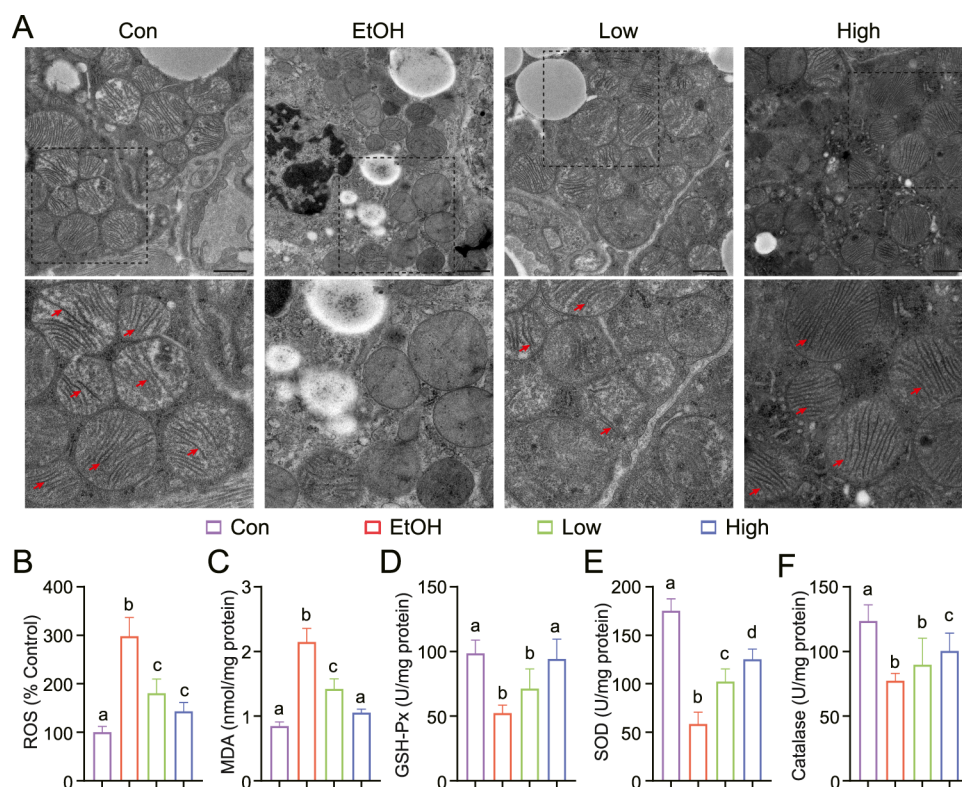


Figure 4. Tangeretin ameliorates alcoholic-induced mitochondrial abnormality and ROS accumulation. (A–F) Wild-type CS7/BL6j mice fed a control diet (Con), ethanol diet (EtOH), or ethanol diet supplemented with a low dose (Low) or high dose (High) of tangeretin. Liver tissue sections or lysates were collected. (A) Transmission electron microscope-based determination of mitochondria morphology. Arrow: cristae. Scale bar: 1 μ m. (B–F) Biochemical measurements of ROS (B), MDA (C), GSH-Px (D), SOD (E), and catalase (F) in liver tissue. Values are mean \pm SEM, n = 6 for Con, n = 8 for EtOH, Low, and High groups. Bars with no letters in common are significantly different.

hepatoprotective effect of tangeretin prompted the present study to determine whether tangeretin might also prevent alcoholic fatty liver, the precursor of many severe forms of liver damage.

In this study, the chronic-binge alcohol feeding protocol was used to induce alcoholic fatty liver in mice. We demonstrated that alcohol-induced liver injuries were suppressed by daily oral administration of tangeretin at 40 mg/kg. Tangeretin significantly prevented the ethanol-induced increases in ALT, AST, and liver/serum TG levels and the accumulation of hepatic lipids. The cell model study of the mechanism of tangeretin effects on suppressing ethanol-induced disruption of lipid metabolism indicated that tangeretin enhanced mitophagy through an AMPK-dependent pathway. Taken together, the findings of our study demonstrated that tangeretin effectively ameliorates alcoholic fatty liver disorders, both *in vivo* and *in vitro*.

Recent studies have found that tangeretin can be metabolized into a variety of substances *in vivo*, such as 4'-demethyltangeretin,^{21,25,26} 6'-demethyltangeretin, 3'-hydroxy-4'-demethyltangeretin, and many di- or even tri-demethyltangeretin.²⁷ Some of these metabolites may possess even stronger biological activities. A recent study showed that compared to tangeretin, 4'-demethyltangeretin, the main metabolites of this flavone, produced stronger inhibition of LPS-stimulated inflammatory response in macrophages.²⁵ Little is known whether ethanol ingestion affects the metabolism of tangeretin, and further study is needed to fully clarify the precise contribution of tangeretin and its metabolite on alleviating alcoholic fatty liver.

Once ingested, alcohol is converted to acetaldehyde in the cytoplasm and then diffuses into the mitochondria, where it is further metabolized into acetic acid by acetaldehyde dehydrogenase. Thus, mitochondria are highly sensitive to ethanol toxicity, as damaged mitochondria, together with inhibition of respiratory function, dysregulated fatty acid metabolism, and irreversibly oxidized mitochondrial proteins, interact to promote the progression of ALD. Mitophagy, the selective autophagic clearance of damaged or unneeded mitochondria, promotes the scavenging of overproduced ROS and acts as a fully fledged antioxidative process.²⁸ In our study, ethanol stimulated the ROS level and blocked mitophagy activity, while tangeretin treatment significantly suppressed this effect by activating AMPK. Besides regulating mitophagy, AMPK also acts as an energy switch that governs the delicate balance of lipid synthesis and oxidation. Once activated, AMPK directly phosphorylates downstream targets and reduces hepatic steatosis by activating β -oxidation and by blocking adipogenesis.^{12,29} Our qPCR results revealed that tangeretin enhanced the β -oxidation of fatty acids. In other words, the protective effect of tangeretin against ALD may involve activation of both mitophagy and fatty acid degradation.

Interestingly, the total effect of ethanol on hepatic mitophagy could be intricate. Reports show that within 6 h of a single ethanol intraperitoneal injection to rats, the mitochondrial protein ubiquitin ligase parkin was translocated to mitophagosomes and mitophagy was enhanced in rat liver.³⁰ However, a newly published paper has revealed an opposite phenomenon in separated mice primary hepatocytes, as a 24 h

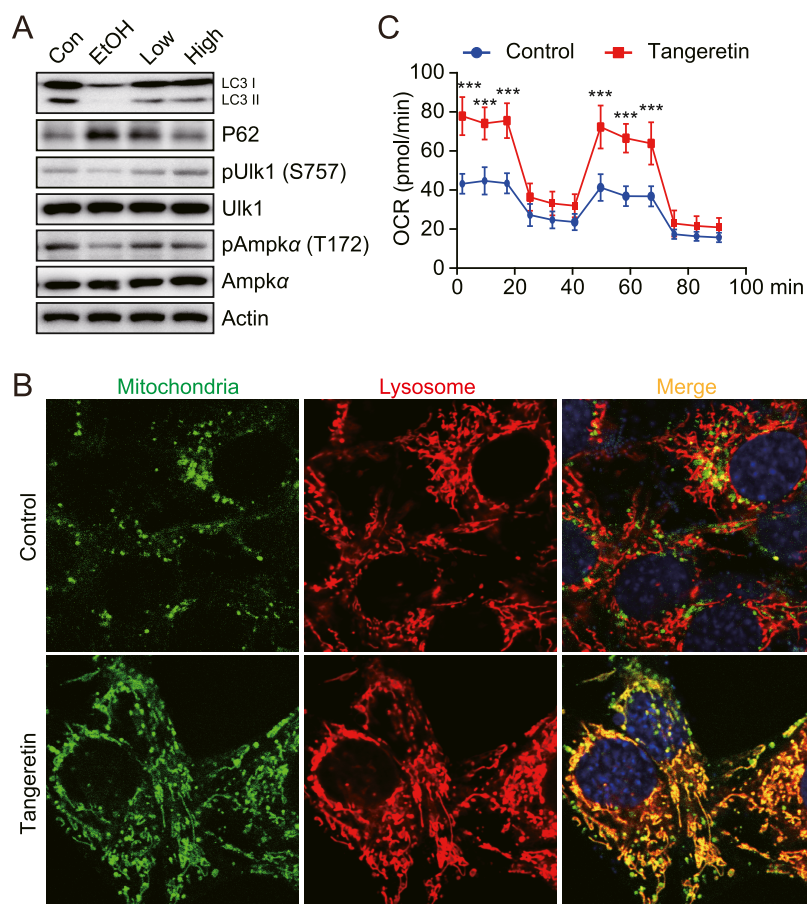


Figure 5. Tangeretin activates mitophagy and restores oxidative respiration. (A) Wild-type C57/BL6j mice fed a control diet (Con), ethanol diet (EtOH), or ethanol diet supplemented with a low dose (Low) or high dose (High) of tangeretin. Liver tissue sections or lysates were collected. (B, C) AML12 cells were pretreated with 20 μ M tangeretin for 24 h, followed by 100 mM ethanol exposure for 24 h. (A) Immunoblotting results for liver Lc3b, P62, phospho-/total-Ulk1, and Ampk α . Actin served as an internal control. (B, C) AML12 cells were pretreated by 20 μ M tangeretin for 24 h, followed by 100 mM ethanol treatment for 24 h. (B) Fluorescent staining of mitochondria (MitoTracker, green) and lysosome (LysoTracker, red). (C) Analysis of mitochondrial respiration by OCR analysis. Values are mean \pm SEM, $n = 6$ for each group. *** $P < 0.001$, control vs tangeretin.

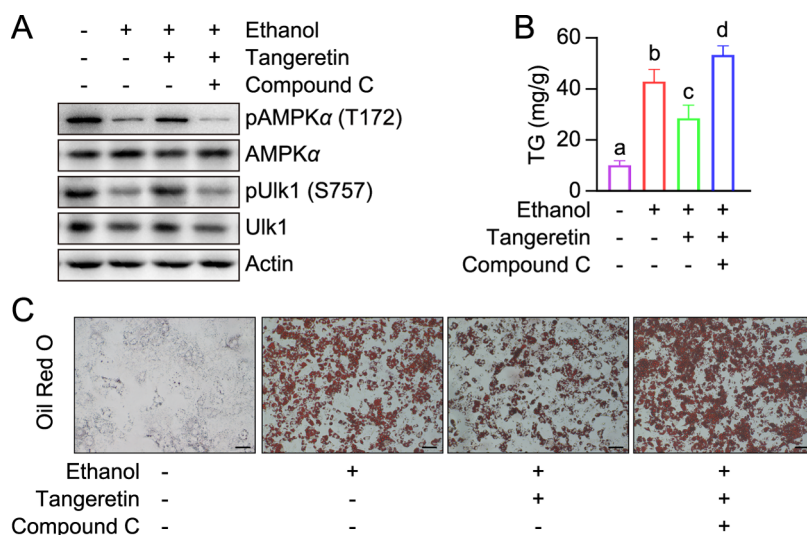


Figure 6. Tangeretin ameliorates alcoholic hepatic lipid accumulation by activating AMPK. (A–C) AML-12 cells were pretreated with 20 μ M tangeretin for 24 h, followed by 100 mM ethanol treatment for 24 h. For Compound C treatment, cells were cotreated with 10 μ M Compound C and 20 μ M tangeretin for the same time period. (A) Protein levels of phospho-/total-AMPK and Ulk1 were determined by immunoblotting. (B) Cellular triglyceride content was determined. (C) Oil red O staining showed lipid accumulation in the chemically treated AML-12 cells. Scale bar: 100 μ m. Bars with no letters in common are significantly different.

alcohol exposure blocked mitophagy.³¹ Our findings show that chronic and binge ethanol treatments reduced mice liver mitophagy through an AMPK–Ulk1-dependent pathway. Given that the biological activity of AMPK is inhibited by ethanol treatment,³² the total effect of ethanol stimulation on mitophagy might rely on the dynamic and delicate regulation of multiple pathways. In the early stage of ethanol exposure, ethanol damages mitochondria and mitophagy is adaptively activated. At the later stages, the AMPK–Ulk1 pathway is blocked and mitophagy is weakened.

Ethanol inhibited the biological activity of AMPK, whereas activation of AMPK by its agonist has shown a protective effect against the progression of ethanol-induced liver steatosis.³³ Hence, compounds that specifically activate the AMPK pathway can provide new insights into potential treatments for alcoholic fatty liver. Our data suggest that tangeretin stimulates an AMPK pathway, thereby pharmacologically reducing alcoholic fatty liver. Tangeretin could also possibly ameliorate ALD through other targets, but this possibility requires further study. Interestingly, our previous study showed that tangeretin enhances insulin sensitivity by suppressing the MEK–ERK1/2 pathway. However, MEK–ERK1/2 is not a likely target for the regulation of ALD by tangeretin, since blocking this pathway leads to serious hepatic steatosis.^{14,34}

Many animal models are available that largely or partly mimic the progression of ALD. These include the chronic ethanol feeding model (the Lieber–DeCarli model), the intragastric gavage model (the Tsukamoto–French model), and the chronic-binge ethanol feeding model (the NIAAA model)³⁵ used here. Among these models, the chronic-binge model produces a more severe hepatic steatosis and liver damage, as reflected by higher serum transaminase levels, and it more strongly recapitulates the alcoholic liver injury observed in human patients.³⁵ Our data showed that the chronic-binge model indeed stimulated hepatic steatosis; however, a point worth noting is that some of the mouse characteristics were not quite the same. When compared with the control group, the originally reported chronic-binge model mice showed only a slight decline in body weight in the first 2 days of alcohol exposure, and this weight was quickly regained.¹¹ However, our data suggested that the body weight of the ethanol-treated mice declined during the last 5 days, while the body weight of the pair-fed control mice slightly increased, in agreement with several other reports.³⁶ This discrepancy arises for many reasons, and even though we maintained a precise energy intake, changes in energy expenditure due to ethanol feeding could lead to differences in body weight. For example, alcohol consumption can activate the hypothalamic neural circuits and dramatically stimulate the thermogenesis of brown fat and upregulate energy expenditure through the expression of uncoupling protein 1.³⁷ Therefore, the calorie-mimic diet could lead to changes in body weight.

In conclusion, using *in vivo* and *in vitro* assays, we have shown that tangeretin, a flavonoid obtained from citrus peels, can protect the liver from alcoholic liver steatosis by activating the AMPK pathway.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.2c02927>.

Sequences of primers used for qPCR (Table S1) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Fang Yuan – Department of Oncology, The Fifth Medical Centre, Chinese PLA General Hospital, Beijing 100071, China; Email: yuanfsc@gmail.com

Li Peng – Department of Endocrinology and Metabolism, the Fourth Affiliated Hospital of Nanjing Medical University, Nanjing 211166, China; Email: penglee@njmu.edu.cn

Chen Qiu – Key Laboratory of the Model Animal Research, Animal Core Facility of Nanjing Medical University, Nanjing 211166, China; orcid.org/0000-0002-4545-2225; Email: chenqiu@njmu.ac.cn

Authors

Jianjin Guo – Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, Taiyuan 030032, China; Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Yuan Chen – Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, Taiyuan 030032, China; Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Complete contact information is available at: <https://pubs.acs.org/10.1021/acs.jafc.2c02927>

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (81900703) to C.Q., by the National Key Research and Development Program of China (2017YFA0106200) to F.Y., and by the Medical Science and Technology Development Foundation of Nanjing Department of Health (YKK21248) to L.P. The following grants also contributed to the current study: Fund Program for the Scientific Activities of Selected Returned Overseas Professionals in Shanxi Province (20200041), Research Project Supported by Shanxi Scholarship Council of China (2020-188), and Senmei China Diabetes Research Fund of China International Medical Foundation (Z-2017-26-1902) to J.G.

■ REFERENCES

- (1) Stickel, F.; Seitz, H. K. Alcoholic steatohepatitis. *Best. Pract. Res. Clin. Gastroenterol.* **2010**, *24*, 683–693.
- (2) Teschke, R. Alcoholic Liver Disease: Current Mechanistic Aspects with Focus on Their Clinical Relevance. *Biomedicines* **2019**, *7*, 68.
- (3) O'Shea, R. S.; Dasarthy, S.; McCullough, A. J. Alcoholic liver disease. *Hepatology* **2010**, *51*, 307–328.
- (4) Li, H. D.; Chen, X.; Yang, Y.; Huang, H. M.; Zhang, L.; Zhang, X.; Zhang, L.; Huang, C.; Huang, C.; Meng, X. M.; Meng, X. M.; Li, J. Wogonin attenuates inflammation by activating PPAR- γ in alcoholic liver disease. *Int. Immunopharmacol.* **2017**, *50*, 95–106.
- (5) He, P.; Wu, Y.; Shun, J.; Liang, Y.; Cheng, M.; Wang, Y. Baicalin Ameliorates Liver Injury Induced by Chronic plus Binge Ethanol Feeding by Modulating Oxidative Stress and Inflammation via CYP2E1 and NRF2 in Mice. *Oxid. Med. Cell Longev.* **2017**, *2017*, 1.
- (6) Tang, Y.; Li, Y.; Yu, H.; Gao, C.; Liu, L.; Xing, M.; Liu, L.; Yao, P. Quercetin attenuates chronic ethanol hepatotoxicity: implication of "free" iron uptake and release. *Food Chem. Toxicol.* **2014**, *67*, 131–138.

- (6) Jin, H.; Lian, N.; Bian, M.; Zhang, C.; Chen, X.; Shao, J.; Wu, L.; Chen, A.; Guo, Q.; Zhang, F.; Zheng, S. Oroxylin A prevents alcohol-induced hepatic steatosis through inhibition of hypoxia inducible factor 1 α . *Chem. Biol. Interact.* **2018**, *285*, 14–20.
- (7) Wang, F.; Liu, J. C.; Zhou, R. J.; Zhao, X.; Liu, M.; Ye, H.; Xie, M. L. Apigenin protects against alcohol-induced liver injury in mice by regulating hepatic CYP2E1-mediated oxidative stress and PPAR α -mediated lipogenic gene expression. *Chem. Biol. Interact.* **2017**, *275*, 171–177.
- (8) Peng, J. H.; Cui, T.; Huang, F.; Chen, L.; Zhao, Y.; Xu, L.; Xu, L. L.; Feng, Q.; Hu, Y. Y. Puerarin ameliorates experimental alcoholic liver injury by inhibition of endotoxin gut leakage, Kupffer cell activation, and endotoxin receptors expression. *J. Pharmacol. Exp. Ther.* **2013**, *344*, 646–654.
- (9) Lin, H.; Zhou, Z.; Zhong, W.; Huang, P.; Ma, N.; Zhang, Y.; Zhou, C.; Lai, Y.; Huang, S.; An, H.; et al. Naringenin inhibits alcoholic injury by improving lipid metabolism and reducing apoptosis in zebrafish larvae. *Oncol. Rep.* **2017**, *38*, 2877–2884.
- Jayaraman, J.; Veerappan, M.; Namasivayam, N. Potential beneficial effect of naringenin on lipid peroxidation and antioxidant status in rats with ethanol-induced hepatotoxicity. *J. Pharm. Pharmacol.* **2010**, *61*, 1383–1390.
- (10) Guo, J.; Chen, J.; Ren, W.; Zhu, Y.; Zhao, Q.; Zhang, K.; Su, D.; Qiu, C.; Zhang, W.; Li, K. Citrus flavone tangeretin is a potential insulin sensitizer targeting hepatocytes through suppressing MEK-ERK1/2 pathway. *Biochem. Biophys. Res. Commun.* **2020**, *529*, 277–282.
- (11) Bertola, A.; Mathews, S.; Ki, S. H.; Wang, H.; Gao, B. Mouse model of chronic and binge ethanol feeding (the NIAAA model). *Nat. Protoc.* **2013**, *8*, 627–637.
- (12) Hu, M.; Wang, F.; Li, X.; Rogers, C. Q.; Liang, X.; Finck, B. N.; Mitra, M. S.; Zhang, R.; Mitchell, D. A.; You, M. Regulation of hepatic lipin-1 by ethanol: role of AMP-activated protein kinase/sterol regulatory element-binding protein 1 signaling in mice. *Hepatology* **2012**, *55*, 437–446.
- (13) Folch, J.; Lees, M.; Sloane Stanley, G. H. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509.
- (14) Xiao, Y.; Liu, H.; Yu, J.; Zhao, Z.; Xiao, F.; Xia, T.; Wang, C.; Li, K.; Deng, J.; Guo, Y.; et al. Activation of ERK1/2 Ameliorates Liver Steatosis in Leptin Receptor-Deficient (db/db) Mice via Stimulating ATG7-Dependent Autophagy. *Diabetes* **2016**, *65*, 393–405.
- (15) Browning, J. D.; Horton, J. D. Molecular mediators of hepatic steatosis and liver injury. *J. Clin. Invest.* **2004**, *114*, 147–152.
- (16) Kou, G.; Li, Z.; Wu, C.; Liu, Y.; Hu, Y.; Guo, L.; Xu, X.; Zhou, Z. Citrus Tangeretin Improves Skeletal Muscle Mitochondrial Biogenesis via Activating the AMPK-PGC1- α Pathway In Vitro and In Vivo: A Possible Mechanism for Its Beneficial Effect on Physical Performance. *J. Agric. Food Chem.* **2018**, *66*, 11917–11925.
- Kim, M. S.; Hur, H. J.; Kwon, D. Y.; Hwang, J. T. Tangeretin stimulates glucose uptake via regulation of AMPK signaling pathways in C2C12 myotubes and improves glucose tolerance in high-fat diet-induced obese mice. *Mol. Cell. Endocrinol.* **2012**, *358*, 127–134.
- (17) Kim, J.; Kundu, M.; Viollet, B.; Guan, K. L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat. Cell Biol.* **2011**, *13*, 132–141.
- (18) Lu, X.; Xuan, W.; Li, J.; Yao, H.; Huang, C.; Li, J. AMPK protects against alcohol-induced liver injury through UQCRC2 to up-regulate mitophagy. *Autophagy* **2021**, *17*, 3622–3643.
- (19) Ashrafizadeh, M.; Ahmadi, Z.; Mohammadinejad, R.; Ghasemipour Afshar, E. Tangeretin: a mechanistic review of its pharmacological and therapeutic effects. *J. Basic Clin. Physiol. Pharmacol.* **2020**, *31*, 2019–0191.
- (20) Braid, N.; Behzad, S.; Habtemariam, S.; Ahmed, T.; Daglia, M.; Nabavi, S. M.; Sobarzo-Sanchez, E.; Nabavi, S. F. Neuroprotective Effects of Citrus Fruit-Derived Flavonoids, Nobiletin and Tangeretin in Alzheimer's and Parkinson's Disease. *CNS Neurol. Disord. Drug Targets* **2017**, *16*, 387–397.
- (21) Cheng, Z.; Surichan, S.; Ruparelia, K.; Arroo, R.; Boarder, M. R. Tangeretin and its metabolite 4'-hydroxytetramethoxyflavone attenuate EGF-stimulated cell cycle progression in hepatocytes; role of inhibition at the level of mTOR/p70S6K. *Br. J. Pharmacol.* **2011**, *162*, 1781–1791.
- (22) Suzuki, T.; Shimizu, M.; Yamauchi, Y.; Sato, R. Polymethoxyflavones in orange peel extract prevent skeletal muscle damage induced by eccentric exercise in rats. *Biosci. Biotechnol. Biochem.* **2021**, *85*, 440–446.
- (23) Chen, J.; Wang, Y.; Zhu, T.; Yang, S.; Cao, J.; Li, X.; Wang, L. S.; Sun, C. Beneficial Regulatory Effects of Polymethoxyflavone-Rich Fraction from Ougan (Citrus reticulata cv. Suavissima) Fruit on Gut Microbiota and Identification of Its Intestinal Metabolites in Mice. *Antioxidants* **2020**, *9*, 831.
- (24) Suguro, R.; Pang, X. C.; Yuan, Z. W.; Chen, S. Y.; Zhu, Y. Z.; Xie, Y. Combinational application of silybin and tangeretin attenuates the progression of non-alcoholic steatohepatitis (NASH) in mice via modulating lipid metabolism. *Pharmacol. Res.* **2020**, *151*, 104519.
- (25) Guo, S.; Wu, X.; Zheng, J.; Smith, S. A.; Dong, P.; Xiao, H. Identification of 4'-Demethyltangeretin as a Major Urinary Metabolite of Tangeretin in Mice and Its Anti-inflammatory Activities. *J. Agric. Food Chem.* **2021**, *69*, 4381–4391.
- (26) Surichan, S.; Arroo, R. R.; Tsatsakis, A. M.; Androustopoulos, V. P. Tangeretin inhibits the proliferation of human breast cancer cells via CYP1A1/CYP1B1 enzyme induction and CYP1A1/CYP1B1-mediated metabolism to the product 4' hydroxy tangeretin. *Toxicol. in Vitro* **2018**, *50*, 274–284.
- (27) Wang, M.; Zhao, H.; Wen, X.; Ho, C. T.; Li, S. Citrus flavonoids and the intestinal barrier: Interactions and effects. *Compr. Rev. Food Sci. Food Saf.* **2021**, *20*, 225–251.
- (28) De Gaetano, A.; Gibellini, L.; Zanini, G.; Nasi, M.; Cossarizza, A.; Pinti, M. Mitophagy and Oxidative Stress: The Role of Aging. *Antioxidants* **2021**, *10*, 794.
- (29) You, M.; Matsumoto, M.; Pacold, C. M.; Cho, W. K.; Crabb, D. W. The role of AMP-activated protein kinase in the action of ethanol in the liver. *Gastroenterology* **2004**, *127*, 1798–1808.
- Shen, Z.; Liang, X.; Rogers, C. Q.; Rideout, D.; You, M. Involvement of adiponectin-SIRT1-AMPK signaling in the protective action of rosiglitazone against alcoholic fatty liver in mice. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **2010**, *298*, G364.
- (30) Eid, N.; Ito, Y.; Otsuki, Y. Triggering of Parkin Mitochondrial Translocation in Mitophagy: Implications for Liver Diseases. *Front. Pharmacol.* **2016**, *7*, 100.
- (31) Zhou, Y.; Wu, R.; Wang, X.; Jiang, Y.; Xu, W.; Shao, Y.; Yue, C.; Shi, W.; Jin, H.; Ge, T.; et al. Activation of UQCRC2-dependent mitophagy by tetramethylpyrazine inhibits MLKL-mediated hepatocyte necroptosis in alcoholic liver disease. *Free Radical Biol. Med.* **2022**, *179*, 301–316.
- (32) You, M.; Matsumoto, M.; Pacold, C. M.; Cho, W. K.; Crabb, D. W. The role of AMP-activated protein kinase in the action of ethanol in the liver. *Gastroenterology* **2004**, *127*, 1798–1808.
- (33) Tomita, K.; Tamiya, G.; Ando, S.; Kitamura, N.; Koizumi, H.; Kato, S.; Horie, Y.; Kaneko, T.; Azuma, T.; Nagata, H.; et al. AICAR, an AMPK activator, has protective effects on alcohol-induced fatty liver in rats. *Alcohol Clin. Exp. Res.* **2005**, *29*, 240S.
- Bergheim, I.; Guo, L.; Davis, M. A.; Lambert, J. C.; Beier, J. I.; Duvell, I.; Luyendyk, J. P.; Roth, R. A.; Arteel, G. E. Metformin prevents alcohol-induced liver injury in the mouse: Critical role of plasminogen activator inhibitor-1. *Gastroenterology* **2006**, *130*, 2099–2112.
- (34) Xiao, Y.; Liu, H.; Yu, J.; Zhao, Z.; Xiao, F.; Xia, T.; Wang, C.; Li, K.; Deng, J.; Guo, Y.; et al. MAPK1/3 regulate hepatic lipid metabolism via ATG7-dependent autophagy. *Autophagy* **2016**, *12*, 592–593.
- (35) Mathews, S.; Xu, M.; Wang, H.; Bertola, A.; Gao, B. Animals models of gastrointestinal and liver diseases. Animal models of alcohol-induced liver disease: pathophysiology, translational relevance, and challenges. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **2014**, *306*, G819.

(36) Liu, G.; Zhang, Y.; Liu, C.; Xu, D.; Zhang, R.; Cheng, Y.; Pan, Y.; Huang, C.; Chen, Y. Luteolin alleviates alcoholic liver disease induced by chronic and binge ethanol feeding in mice. *J. Nutr.* **2014**, *144*, 1009–1015. Grander, C.; Grabherr, F.; Spadoni, L.; Enrich, B.; Oberhuber, G.; Rescigno, M.; Tilg, H. The role of gut vascular barrier in experimental alcoholic liver disease and *A. muciniphila* supplementation. *Gut Microbes* **2020**, *12*, No. 1851986.

(37) Shen, H.; Jiang, L.; Lin, J. D.; Omary, M. B.; Rui, L. Brown fat activation mitigates alcohol-induced liver steatosis and injury in mice. *J. Clin. Invest.* **2019**, *129*, 2305–2317.



CAS INSIGHTS™

EXPLORE THE INNOVATIONS SHAPING TOMORROW

Discover the latest scientific research and trends with CAS Insights. Subscribe for email updates on new articles, reports, and webinars at the intersection of science and innovation.

Subscribe today

CAS
A division of the American Chemical Society