Protective effects of fermented *Rosa roxburghii Tratt* juice against ethanol-induced hepatocyte injury by regulating the NRF2-AMPK signaling pathway in AML-12 cells

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Abstract. Alcohol-related liver disease (ALD) is a major health concern worldwide. In recent years, there has been growing interest in natural products and functional foods for preventing and treating ALD due to their potential antioxidant and hepatoprotective properties. *Rosa roxburghii Tratt*, known for its rich content of bioactive compounds, has demonstrated promising health benefits, including anti-inflammatory and antioxidant effects. Fermentation has been utilized as a strategy to enhance the bioavailability and efficacy of natural products. In the present study, using a mixture of *Rosa roxburghii Tratt* juice, lotus leaf extract and grape seed proanthocyanidins fermented by *Lactobacillus plantarum* HH-LP56, a novel fermented *Rosa roxburghii Tratt* (FRRT) juice was discovered that can prevent and regulate ethanol-induced liver cell damage. Following fermentation, the pH was significantly decreased, and the content of VC and superoxide dismutase (SOD) were significantly increased, along with a noticeable enhancement in hydroxyl and 2,2-diphenyl-1-picrylhydrazyl free radical scavenging abilities. Alpha Mouse liver 12 cells were exposed to ethanol for 24 h to establish an *in vitro* liver cell injury model. The present study evaluated the effects of FRRT on cell damage, lipid accumulation and oxidative stress markers. The results revealed that FRRT pretreatment (cells were pre-treated with 2.5 and 5 mg/ml FRRT for 2 h) significantly reduced lipid accumulation and oxidative stress in liver cells. Mechanistically, FRRT regulated lipid metabolism by influencing key genes and proteins, such as AMP-activated protein kinase, sterol regulatory element binding transcription factor 1 and Stearyl-CoA desaturase-1. Furthermore, FRRT enhanced antioxidant activity by increasing SOD activity, glutathione and catalase levels, while reducing reactive oxygen species and malondialdehyde levels. It also reversed the expression changes of ethanol-induced oxidative stress-related genes and proteins. In conclusion, a novel functional food ingredient may have been discovered with extensive potential applications. These findings indicated that FRRT has antioxidant properties and potential therapeutic benefits in addressing ethanol-induced liver cell damage through its effects on liver lipid metabolism and oxidative stress.

Introduction

Alcohol-related liver disease (ALD) is a pressing global health issue that leads to a spectrum of liver conditions, from simple steatosis to severe outcomes such as steatohepatitis, cirrhosis and hepatocellular carcinoma (1). The oxidative stress and lipid peroxidation induced by chronic alcohol abuse played a critical role in the pathogenesis of ALD, contributing to hepatocyte damage (2,3). In this context, the body's defense mechanisms against oxidative stress, particularly involving the nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathways and key antioxidant enzymes such as catalase (CAT), glutathione (GSH) and superoxide dismutase (SOD), become essential for cellular protection (4,5).

The disruption of liver lipid metabolism due to long-term alcohol consumption further exacerbates the development of steatosis, highlighting the importance of maintaining cellular energy balance through proteins such as AMP-activated protein kinase (AMPK) (6). Ethanol exposure alters lipid regulators and enzymes, such as sterol regulatory element binding transcription factor 1 (SREBP-1c) and stearyl-coA desaturase-1 (SCD1), leading to triglyceride (TG) accumulation in the liver (7,8). Despite current treatment options for ALD, including alcohol cessation, nutritional support and pharmacological interventions, their efficacy can be limited

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by side effects and costs (9). Therefore, it is urgent to find natural products without toxic side effects for regulating and preventing ALD.

Rosa roxburghii Tratt is a Rosaceae plant species that is widely cultivated in Guizhou, China (10). This plant is widely recognized for its multitude of health benefits, such as its ability to act as an antioxidant, reduce inflammation, lower lipid levels, regulate the immune system and decrease the body's burden of heavy metals (11). The lotus is a plant in the Nymphaeaceae family. Its lotus leaf is a dried leaf that is considered both a medicinal herb and a food source. Research has revealed that lotus leaf has antioxidant, anti-inflammatory and anti-tumor effects (12). Grape seed proanthocyanidins have been reported to have strong antioxidant properties and the ability to eliminate oxygen free radicals (13). Lactic acid fermentation is an ancient and cost-effective method used for food preservation, which is facilitated by lactic acid bacteria (LAB) (14). This method not only enhances the nutritional value and flavor of food but also increases the content of functional components, such as phenolic substances, organic acids and volatile compounds in fruits and vegetables (15). Probiotic fermentation has attracted considerable attention as a convenient and easily prepared functional food (16). Taking into consideration the antioxidant properties of the active substances in the aforementioned plant materials, as well as the safety and functionality of probiotics, the use of *Lactobacillus plantarum* HH-LP56 to ferment these medicinal plants enhances their sensory attributes and antioxidant properties. The aim of the present study was to explore how the fermented *Rosa roxburghii Tratt* (juice (FRRT) impacts ethanol-induced oxidative stress and lipid metabolism in Alpha Mouse Liver 12 (AML-12) cells, focusing on its potential hepatoprotective effects.

By investigating the pharmacological properties of FRRT and its mechanisms of action in ethanol-induced hepatocyte injury, the aim of the present study was to shed light on the therapeutic potential of this fermented juice for liver disease treatment. Emphasizing the application of FRRT in liver health, the present study provided valuable insights into its hepatoprotective effects and potential implications for clinical use.

Materials and methods

Strain, materials and chemicals. Rosa roxburghii tratt juice (freshly squeezed, 100% purity) was obtained from East China Institute of Medicinal Plants (Lishui, China), and stored in -4˚C refrigerator for use. *Lactobacillus plantarum* HH-LP56 was purchased from Xian Miser Biotechnology Co., Ltd. Proanthocyanidins from grape seeds were purchased from Shandong Saint Jia De Biotechnology Co., Ltd. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) and 95% ethanol were purchased from MilliporeSigma. RIPA Lysis Buffer (cat. no. P0013C), Increased Bicinchoninic Acid (BCA) Protein Concentration Kit (cat. no. P0009), Bodipy 500/510 C1, C12 (Fatty Acid Green Fluorescence Probe) (cat. no. C2055), Hoechst 33342 (cat. no. C1022), SOD (cat. no. S0101S), malondialdehyde (MDA) (cat. no. S0131S) and reactive oxygen species (ROS) assay kit (cat. no. S0033S) were obtained from Beyotime Institute of Biotechnology. 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity assay kit (cat. no. BL897A) and Hydroxyl free radical (·OH) scavenging capacity assay kit (cat. no. BL1065A) were purchased from Biosharp, sourced from Langjiako Technology Co., Ltd. Vitamin C (VC; cat. no. A009-1-1), TG (cat. no. A110-2-1) and CAT assay kit (cat. no. A007-1-1) were purchased from Nanjing Jiancheng Bioengineering Institute. Horseradish peroxidase (HRP)-conjugated goat anti-mouse (cat. no. sc-2354) and goat anti-rabbit (cat. no. sc-2357) antibodies were purchased from Santa Cruz Biotechnology, Inc. Primary antibodies against NAD(P)H: quinone oxidoreductase 1 (NQO1) (cat. no. 11451-1-AP), kelch like ECH associated protein 1 (KEAP1) (cat. no. 80744-1-RR) and GAPDH (cat. no. 60004-1-lg) (all 1:1,000 ratio, respectively) were purchased from Proteintech Group, Inc. Primary antibodies against AMPK (cat. no. PAB44300), NRF2 (cat. no. PAB37815), SCD1 (cat. no. RMAB49932), and SREBP-1c (cat. no. PAB39550) (1:1,000, respectively) were purchased from Bioswamp; Wuhan Bienle Biotechnology Co., Ltd.

Preparation of FRRT. A total of 50 ml of *Rosa roxburghii Tratt* juice were enzymatically treated with tannase and pectinase at 45˚C for 100 min, while stirring and filtering to remove precipitates. Prior to preparing the additives, 0.012% (v/v) trichloro-sucrose was dissolved in water at 70˚C. A total of 1 g each of lotus leaf extract and grape seed proanthocyanidin powder were separately dissolved in water at ~45˚C to form colloidal solutions. When mixing, the procedure started by adding the dissolved trichloro-sucrose, followed by the lotus leaf extract and grape seed proanthocyanidin colloids, followed by stirring for 20 min to ensure uniformity. For particle microencapsulation, the mixture was heated to 65˚C, homogenized under a pressure of 200 MPa, then cooled to ~37˚C before inoculating with the plant lactobacillus HH-LP56 for fermentation. After 24 h, the fermentation juice production process was completed with ultrasonic treatment (20-22 kHz).

Determination of active substance concentration and antioxidant activity in FRRT and unfermented Rosa roxburghii Tratt (RRT). The fermented product was chemically characterized by determining the active substance concentration and antioxidant activity in FRRT. The biological activity of the fermented product was assessed by measuring the VC and SOD content. The SOD content in the fruit juice samples was determined using the WST-1 method in an enzyme-linked immunosorbent assay reader, while the VC content was assessed using UV-Visible spectrophotometry (UV-Vis) at a wavelength of 536 nm. The process involved extracting the VC content, leveraging its specific absorption characteristics at a defined wavelength, and measuring the absorbance intensity to quantify its concentration in the fruit juice. While the antioxidant properties were determined by evaluating OH and DPPH radical scavenging ability in FRRT and RRT juice using specific assay kits according to the manufacturer's protocol.

Culture of AML-12 cells. AML-12 cells were obtained from the Cell Bank of the Chinese Academy of Sciences in Shanghai, China. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; HyClone; Cytiva) supplemented with 10% fetal bovine serum (FBS; Hangzhou Dacheng Biotechnology Co., Ltd.), penicillin (100 U/ml), streptomycin (100 μg/ml),

dexamethasone (40 ng/ml), insulin (5 mg/ml), transferrin (5 μg/ml) and sodium selenite (5 ng/ml) at 37˚C under a 95% air and 5% CO_2 atmosphere. Passaging was performed when the cells covered approximately the entire surface of the culture flask, at a ratio of 1:3 to 1:5. Cells from the 3rd to 8th passages in the logarithmic growth phase were selected for subsequent experiments. Before cell treatment, both the FRRT and RRT were filtered using a 0.22-μm membrane.

Cell viability assay. Cell viability was assessed using the MTT assay. Hepatocytes were seeded in a 96-well culture plate at a density of $2x10^4$ cells per well and cultured until reaching 80% confluency. Subsequently, the cells were divided into different groups. The negative control (NC) group received culture medium without FRRT or ethanol, while the ethanol (EtOH) group was exposed to various concentrations of ethanol (25, 50, 100, 200, 400 and 800 mM) for 24, 36 and 48 h. The experimental group was treated with different concentrations of FRRT (2.5, 5, 10, 20, 40, 80 and 160 mg/ml) for 24 h in a 37˚C incubator. The positive control group was treated with different concentrations of RRT (2.5, 5, 10, 20, 40 mg/ml) for 24 h. After the respective incubation periods, MTT solution was added to each well, and the cells were further incubated in darkness at 37˚C for 4 h. Following the dissolution of formazan crystals with DMSO (150 μl/well), the absorbance was measured at 490 nm using a microplate reader. Cell viability was calculated using the following formula:

$$\text{Cell viability}\ (\%) = \frac{A\text{sample}}{A\text{control}} \times 100\% \quad (1)$$

Determination of intracellular TG. AML-12 cells were inoculated on 24-well plates at a density of $5x10^5$ cells per well. After incubation for 24 h in a 37˚C incubator, the cells were divided into different groups: NC group, EtOH group (200 mM ethanol), lower dose FRRT group (2.5 mg/ml), higher-dose FRRT group (5 mg/ml) and RRT positive control group (10 mg/ml). AML-12 cells were pretreated with FRRT (2.5, 5 mg/ml) and RRT (10 mg/ml) for 2 h, respectively, and then stimulated with or without EtOH (200 mM) for 24 h. After discarding the supernatant of the prepared cells, they were washed with PBS and the cell pellet were retained. A total of 0.2 to 0.3 ml of homogenization medium was added for homogenization. Following the manufacturer's protocol, TG assay kit was used to measure the TG content in the homogenate. A BCA protein assay kit was used to determine the protein concentration in the homogenate.

Determination of MDA, CAT, GSH and SOD. After inoculating AML-12 cells on 6-well plates at a density of $2x10^5$ cells per well, they were grouped as per section according to the aforementioned method. Following the cultivation period, the supernatant was discarded and the activity levels of MDA, CAT, GSH and SOD were assessed in the liver cell homogenate using specific assay kits. Additionally, a BCA protein assay kit was used to determine the protein concentration in the liver cell homogenate.

Hoechst 33342 nuclear staining. The following culture methods were performed as aforementioned. After the intervention, cells were stained with Hoechst 33342 staining at 37˚C for 10 min. Following staining, the cells were washed twice with PBS, ensuring that enough PBS was added to evenly cover them. Finally, the results were examined under an inverted fluorescence microscope.

Bodipy dying. Following the intervention, cells were stained with Bodipy dye at 37˚C for 20 min and then examined under an inverted fluorescence microscope.

ROS production. After being cultured, the cell culture medium was treated with the fluorescent dye DCFH-DA at a final concentration of 10 μmol/l. The cells were then cultured in a cell incubator at 37˚C for 20 min. Subsequently, the cells were washed three times with a serum-free cell medium. Finally, images of three different areas of each well were captured using the Olympus IX84 inverted fluorescence microscope.

Reverse transcription-quantitative (RT-qPCR) analysis. After isolating total RNA from AML-12 cells using the TRIzol® method (Invitrogen; Thermo Fisher Scientific, Inc.), a reverse transcription reaction [Accurate Biotechnology (Hunan) Co., Ltd.] was performed following the cDNA synthesis kit protocol. qPCR was performed using the SYBR Green premix Pro Taq HS qPCR Kit (Accurate Biotechnology (Hunan) Co., Ltd.) under the following conditions: Initial denaturation at 95˚C for 30 min, denaturation at 95˚C for 5 sec, annealing/extension at 60˚C for 30 sec and 40 cycles. A periodic threshold (Cq) was determined and the relative expression of the target mRNA was quantified by the $2^{-\Delta\Delta Cq}$ method (17) with *GAPDH* as a normalized reference gene. The qPCR primers used in the present study were synthesized by Hunan Accurate Biotechnology Co., Ltd. and their sequences are listed in Table I.

Western blot analysis. To quantify protein expression in cells, RIPA lysis buffer was used to lyse cells (contains 1% phosphatase inhibitor and 1% protease inhibitor), and a BCA assay kit was used to determine protein concentration. Each experimental group was adjusted for protein concentration. A total of 20 μg/lane cell lysates were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Subsequently, 10% SDS-PAGE gels were electrophoretically separated: 80 V for 30 min and 120 V for 60-90 min. Isolated proteins were transferred to a PVDF membrane at low temperature using a transfer device (200 mA, 120 min). After blocking with 5% skim milk at room temperature for 1 h, the membrane was incubated overnight at 4˚C with the primary antibody against the target protein. TBST (0.1% Tween-20 solution was used to prepare) was used for washing membranes to remove unbound antibodies, and secondary antibodies (goat anti-rabbit or anti-mouse IgG-horseradish peroxidase; cat. nos. sc-2357 and sc-2354 respectively; Santa Cruz Biotechnology, Inc.) were applied (1:50,000 dilution;.) at room temperature for 1 h. TBST was used to remove unbound antibodies after incubation with the secondary antibody. SuperPico ECL Chemiluminescence Kit (Vazyme Biotech Co., Ltd.) was used to visualize protein bands, and digital gel imaging systems to capture images. Gray-level analysis of the images was performed using ImageJ software (version 1.53e; National Institutes of Health), and the relative expression of the target protein was normalized to the expression of the valet protein GAPDH.

Table I. Primer sequence for reverse transcription-quantitative PCR.

Target genes	Primer sequences (5'-3')
NQO1	Forward: CAGCCAATCAGCGTTCGGTA
	Reverse: CTTCATGGCGTAGTTGAATGAT GTC
NRF2	Forward: TCTTGGAGTAAGTCGAGAAG TGT
	Reverse: GTTGAAACTGAGCGAAAAAGGC
GAPDH	Forward: TGTGTCCGTCGTGGATCTGA
	Reverse: TTGCTGTTGAAGTCGCAGGAG
SCD1	Forward: ACCCGGCTGTCAAAGAGAAG
	Reverse: CGCAAGAAGGTGCTAACGAAC
SREBP-1c	Forward: TCAGAGCCGTGGTGAGAAG
	Reverse: GCAAGAAGCGGATGTAGTCG
KEAP1	Forward: GTCGCCCTGTGCCTCTATG
	Reverse: CGCCAATCCTCCGTGTCAA
HO-1	Forward: AAGCCGAGAATGCTGAGTTCA
	Reverse: GCCGTGTAGATATGGTACAAGGA
AMPK	Forward: CTACTTGTCTGGGTCCTTCAACA
	Reverse: GCTGGTTACTATTGGCTCAGAAG

NQO1, quinone oxidoreductase 1; NRF2, nuclear factor erythroid 2-related factor 2; SCD1, stearyl-coA desaturase-1; SREBP-1c, sterol regulatory element binding transcription factor 1; KEAP1, kelch like ECH associated protein 1; HO-1, heme oxygenase 1; AMPK, AMP-activated protein kinase.

Statistical analysis. All experiments were performed in triplicate. The experimental data were analyzed using GraphPad Prism 9.5.1 software (Dotmatics), and the results are presented as the mean ± standard deviation (SD) of ≥3 independent experiments performed under identical conditions. For comparisons involving three or more groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to determine differences. In cases where there were two groups being compared, unpaired Student's t-test was conducted. Furthermore, ImageJ software was utilized to quantify the grayscale values of protein bands. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Changes in active substance content and antioxidant activity during fermentation. Changes in pH, VC content, SOD activity, ·OH and DPPH radical scavenging rate during the fermentation process of FRRT are demonstrated in Fig. 1. The results demonstrated that the color of the fermented juice significantly lightens (Fig. 1A), and the production of LAB during fermentation led to a decrease in pH of the FRRT compared with RRT (Fig. 1B). Furthermore, there was a significant increase in VC content and SOD activity (Fig. 1C and D). The ·OH and DPPH free radical scavenging abilities of FRRT were enhanced compared with RRT (Fig. 1E and F), which indicated an improved antioxidant capacity post-fermentation ($P<0.05$).

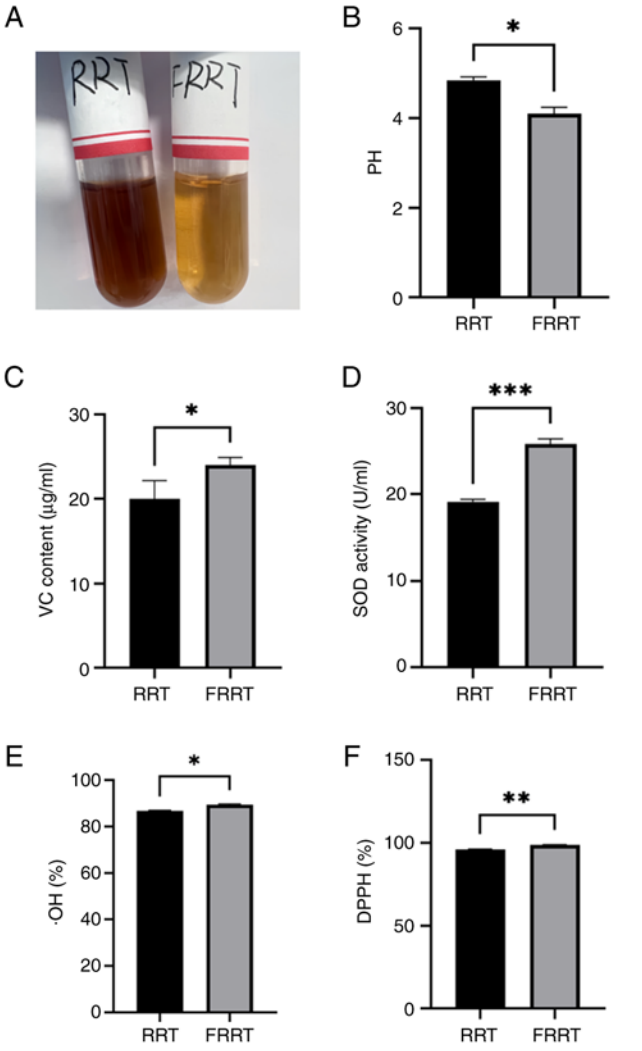

Figure 1. Changes in active substance content and antioxidant activity during fermentation. (A) Color changes of FRRT and RRT. (B) PH variation. (C and D) VC content and SOD activity were measured using corresponding assay kits. (E and F) ·OH and DPPH radical scavenging rate (%) were also measured using corresponding assay kits. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the RRT group. FRRT, fermented *Rosa roxburghii Tratt*; RRT, unfermented *Rosa roxburghii Tratt;* VC, vitamin C; SOD, superoxide dismutase; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

Effect of ethanol and FRRT on AML-12 cell viability. The results revealed that, as compared with the control group (cells not exposed to ethanol), cell viability decreased with the increase of ethanol concentration. This trend was consistent across all three exposure periods (24, 36 and 48 h; Fig. 2A-C). Specifically, after a 24 h exposure period, cell viability significantly decreased from 100±1.5% in the control group to 86.4±2.9% in cells exposed to 200 mM ethanol ($P<0.001$). These findings suggested that ethanol hampers cell viability and inhibits the proliferation of AML-12 cells in a concentration-dependent manner. No significant changes were observed in the data at 36 and 48 h when compared with the 24 h data. This could potentially be attributed to the volatility of ethanol. Therefore, the 24 h exposure period and a concentration of 200 mM ethanol were selected to establish an ethanol-induced hepatocyte injury model. Treatment with lower concentrations of FRRT (FRRT-L) (2.5-40 mg/ml) significantly increased cell viability, while higher concentrations (80-160 mg/ml)

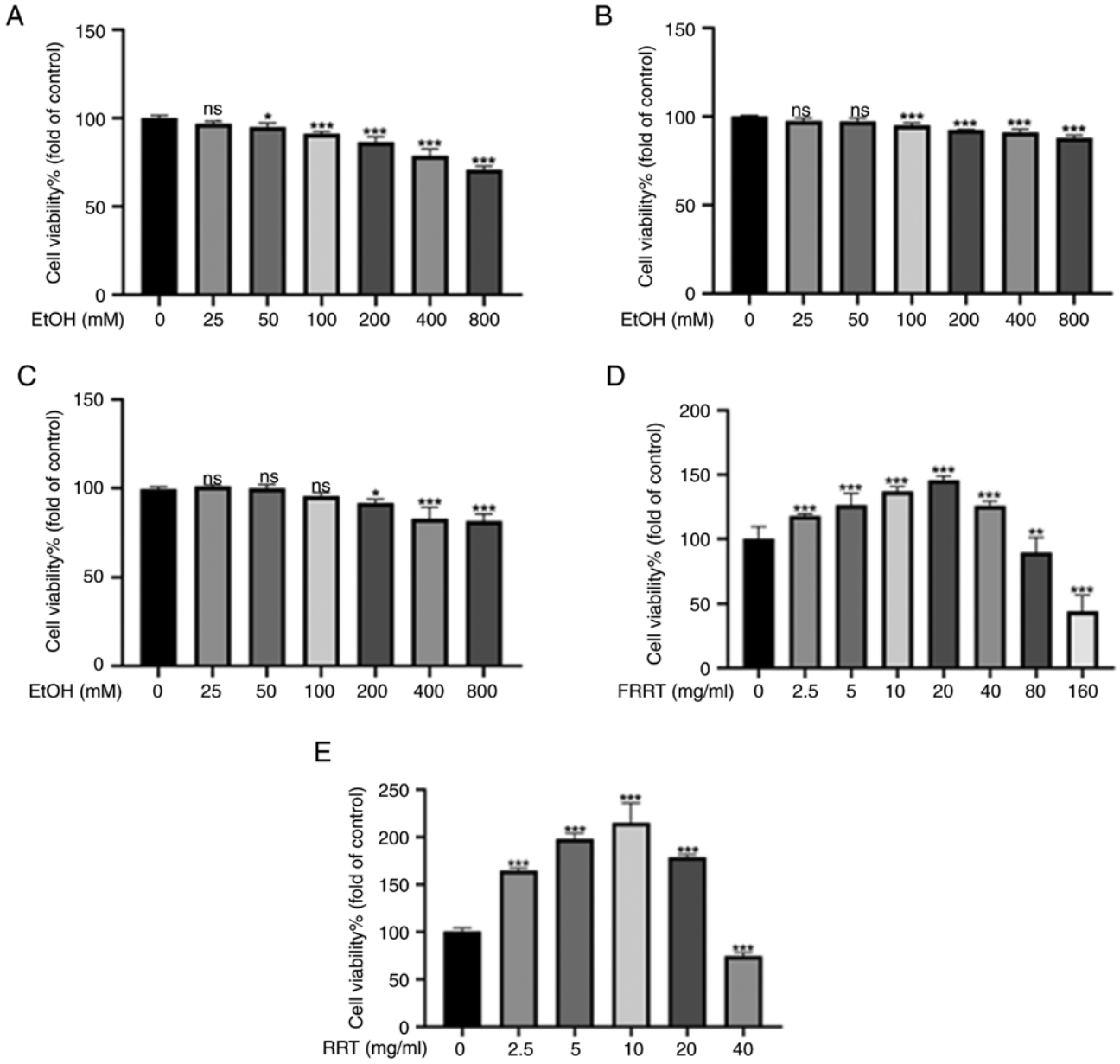

Figure 2. Effect of ethanol or FRRT on AML-12 cell viability. (A-C) Comparison of the effects of different concentrations of ethanol on AML-12 cell viability following 24, 36 and 48 h exposure periods. (D and E) The effect of FRRT and RRT on AML-12 cell viability. Statistical analysis was performed using one-way ANOVA. *P<0.05, **P<0.01 and ***P<0.001 compared with the control group. FRRT, fermented *Rosa roxburghii Tratt*; RRT, unfermented *Rosa roxburghii Tratt*; ns, no significance.

led to a decrease in cell viability (P<0.001; Fig. 2D). The results of the preliminary research revealed that the overall antioxidant effects of 2.5 and 5 mg/ml were greater than those of ≥10 mg/ml (Fig. S1). Therefore, in subsequent studies, a total of 2.5 and 5 mg/ml as the intervention concentrations were chosen. Additionally, the results demonstrated that treatment with low concentrations of RRT (~2.5-20 mg/ml) significantly increased cell viability, while high concentration of RRT treatment (40 mg/ml) resulted in a decrease in cell viability (P<0.001; Fig. 2E). Therefore, the concentration of 10 mg/ml was selected, which exhibited the most significant enhancement in cell viability, as the positive control group.

FRRT improves ethanol-induced hepatic lipid metabolism in AML-12 cells. The results revealed that the EtOH group exhibited a significant increase in TG content compared with the NC group (P<0.001; Fig. 3A). However, pretreatment with FRRT at concentrations of 2.5 and 5 mg/ml significantly reduced the ethanol-induced increase in TG content in the AML-12 cells when compared with the EtOH group. This reduction occurred in a dose-dependent manner (P<0.001). To further investigate the impact of FRRT on lipid accumulation, BODIPY™ staining was performed. The images obtained from BODIPY staining revealed a substantial accumulation of lipid droplets in the EtOH group (Fig. 3B). However, in the FRRT pretreatment group, both the density and staining intensity of lipid droplets in the cells were significantly reduced compared with the ethanol-treated group. These reductions also exhibited a dose-dependent relationship. These findings suggested that pretreatment with FRRT at concentrations of

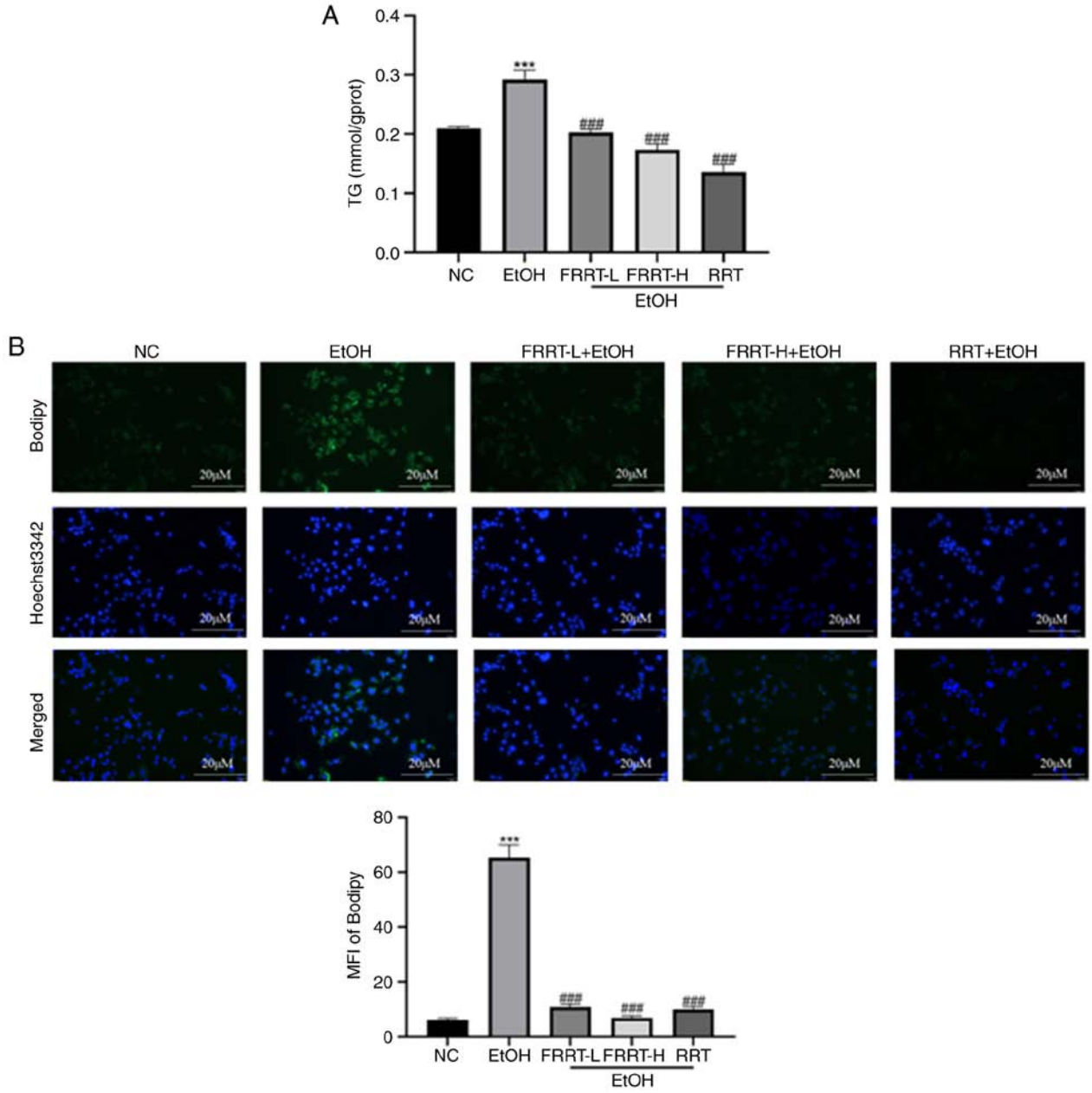

Figure 3. FRRT improves ethanol-induced hepatic lipid metabolism in AML-12 cells. (A) The level of TG in AML-12 cells with ethanol-induced hepatocyte injury was expressed as mmol/g of protein. (B) Hoechst 33342-Bodipy fluorescence staining was used to visualize the hepatocytes. ***P<0.001 compared with the NC group; $^{###}$P<0.001 compared with the EtOH group. FRRT, fermented *Rosa roxburghii Tratt*; TG, triglyceride; NC, negative control; RRT, unfermented *Rosa roxburghii Tratt;* L, low; H, high; ns, no significance.

2.5 and 5 mg/ml can effectively attenuate ethanol-induced TG accumulation and lipid droplet formation in AML-12 cells. The dose-dependent response indicated that a higher concentration of FRRT (FRRT-H) may provide even stronger protection against lipid accumulation.

FRRT enhances hepatic lipid metabolism by activating the AMPK-SREBP-1c-SCD1 pathway. To elucidate the molecular mechanism underlying the regulatory effects of FRRT on intracellular lipid metabolism, the expression levels of pivotal lipogenic genes, namely *AMPK*, *SREBP-1c* and *SCD1* were quantified using qPCR. It was observed that, compared with the NC group, the gene expression of the *SREBP-1c* and *SCD1* gene was significantly increased in the EtOH group, while that of the *AMPK* gene was decreased (P<0.001). Of note, these alterations in gene expression were effectively reversed by treatment with FRRT, as depicted in Fig. 4B. Furthermore, the protein levels of AMPK, SREBP-1c and SCD1 were examined using western blotting. Compared with the NC group, the group exposed to ethanol exhibited a statistically significant decrease in liver AMPK protein levels (P<0.05). In addition, there was a significant trend towards increased SREBP-1c and SCD1 protein levels in the liver; however, this increase did not reach statistical significance. Markedly, pretreatment with FRRT resulted in a significant reversal of these alterations, particularly with FRRT-H exerting pronounced effects on ethanol-induced changes in AMPK, SREBP-1c and SCD1 protein levels (Fig. 4A).

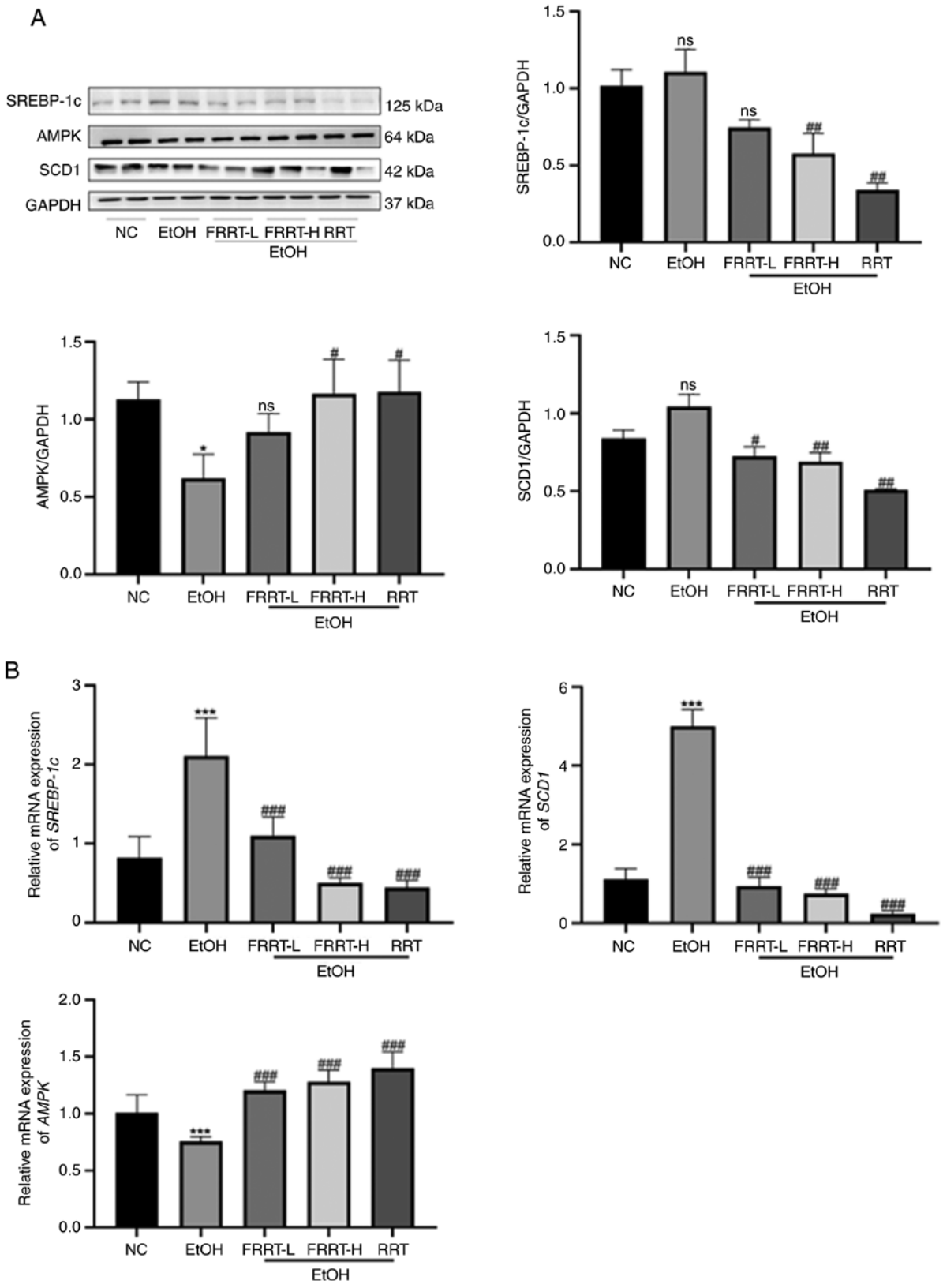

Figure 4. FRRT enhances hepatic lipid metabolism by activating the AMPK-SREBP-1c-SCD1 pathway. (A and B) Western blotting and quantitative PCR were performed to verify the effects of FRRT on the expression of AMPK, SREBP-1c and SCD1 with ethanol treatment. *P<0.05 and ***P<0.001 compared with the NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with the EtOH group. AMPK, AMP-activated protein kinase; SREBP-1c, sterol regulatory element binding transcription factor 1; SCD1, stearyl-coA desaturase-1; FRRT, fermented *Rosa roxburghii Tratt*; NC, negative control; L, low; H, high; RRT, unfermented *Rosa roxburghii Tratt*; ns, no significance.

FRRT alleviates ethanol-induced hepatic oxidative stress in AML-12 cells. Superoxide anion is recognized as one of the primary forms of ROS within mitochondria (18). Increased superoxide anion release leads to oxidative stress and

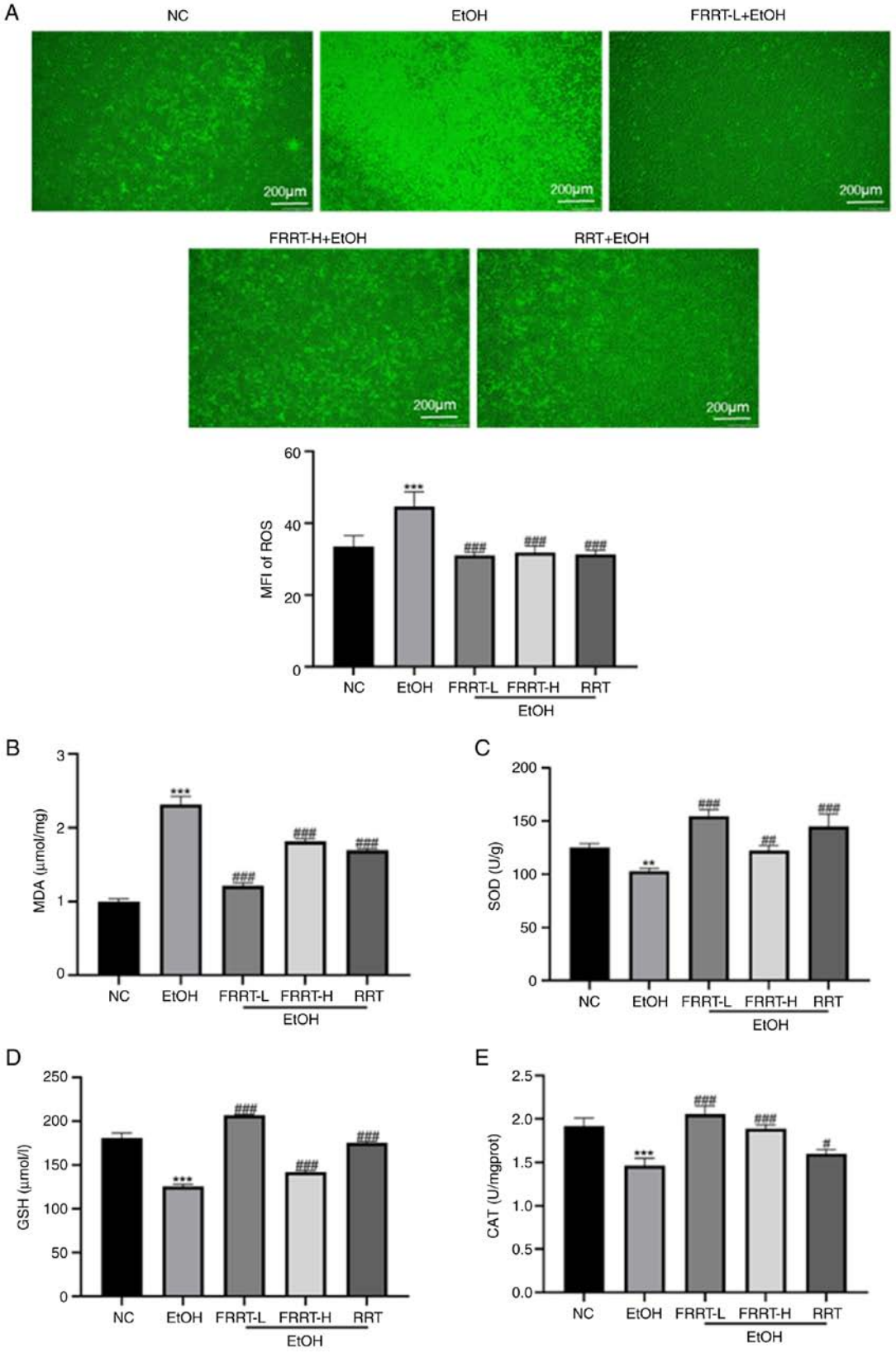

Figure 5. FRRT alleviates ethanol-induced hepatic oxidative stress in AML-12 cells. (A) The ROS level in AML-12 cells. (B) The MDA level in AML-12 cells. (C-E) The activities of SOD, GSH and CAT in AML-12 cells. **P<0.01 and ***P<0.001 compared with the NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with the EtOH group. FRRT, fermented *Rosa roxburghii Tratt*; ROS, reactive oxygen species; MFI, mean fluorescence intensity; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; NC, negative control; L, low; H, high; RRT, unfermented *Rosa roxburghii Tratt*; ns, no significance.

apoptosis (19). To visualize hepatocyte damage, DCFH-DA staining was used to detect the distribution and quantity of superoxide free radicals. Antioxidant levels in the cells were assessed by measuring MDA, SOD, CAT and GSH. The results

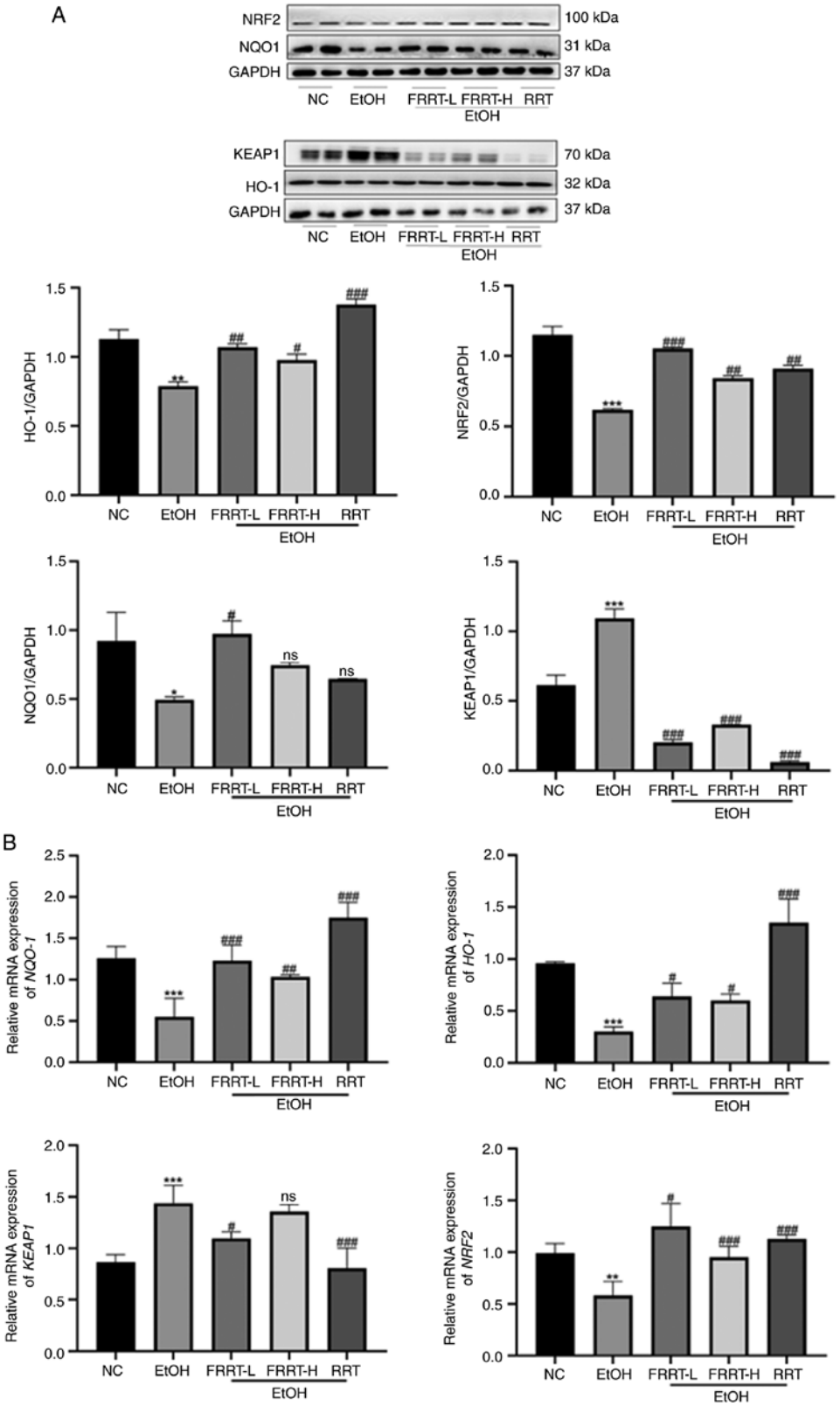

Figure 6. FRRT inhibits hepatic oxidative stress via activating NRF2-KEAP1-NQO1-HO-1 pathway. (A) Western blot analysis verifying the total protein level of NRF2, NQO1, HO-1 and KEAP1. GAPDH was used as an internal control for protein normalization. (B) Reverse transcription-quantitative PCR verifying the expression levels of NRF2-KEAP1-related genes, including *NRF2, KEAP1*, *HO-1* and *NQO1*. *P<0.05, **P<0.01 and ***P<0.001 compared with the NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with the EtOH group. FRRT, fermented *Rosa roxburghii Tratt*; NRF2, nuclear factor erythroid 2-related factor 2; KEAP1, kelch like ECH associated protein 1; NQO1, quinone oxidoreductase 1; HO-1, heme oxygenase 1; NC, negative control; L, low; H, high; RRT, unfermented *Rosa roxburghii Tratt*; ns, no significance.

demonstrated that ROS levels in AML-12 cells were significantly increased following ethanol exposure but were reversed following treatment with FRRT, as indicated by fluorescence intensity results (Fig. 5A). Compared with the NC group, the

EtOH group exhibited a significant reduction in the intracellular activities of SOD, CAT and GSH, along with a significant increase in MDA levels (Fig. 5B-E; P<0.01). These findings suggested an imbalance in cellular oxidative status, indicating the presence of oxidative injury. However, the pretreatment of cells with FRRT at concentrations of 2.5 and 5 mg/ml resulted in significantly lower MDA levels compared with the EtOH group (Fig. 5B; P<0.001), suggesting that FRRT can suppress the ethanol-induced elevation of MDA in a dose-dependent manner within this concentration range. Furthermore, the addition of different concentrations of FRRT led to increased SOD, GSH and CAT levels compared with the EtOH group (Fig. 5C-E; P<0.01). Of note, the lower dose of FRRT intervention demonstrated a more pronounced reduction in MDA levels and greater enhancement of GSH, CAT and SOD activities compared with the RRT group (Fig. 5B-E). Collectively, these experimental findings confirmed the protective effect of FRRT against ethanol-induced liver cell damage through the modulation of intracellular oxidative stress response within a specific concentration range.

FRRT inhibits hepatic oxidative stress by activating the NRF2-KEAP1-NQO1-heme oxygenase 1 (HO-1) pathway. To study the molecular mechanism of the FRRT-induced inhibition of oxidative stress, the total protein levels of NRF2, NQO1, HO-1 and KEAP1 were detected by western blotting. As demonstrated in Fig. 6A, compared with the NC group, the hepatic protein levels of HO-1, NRF2 and NQO1 were decreased, while the protein level of KEAP1 was increased in the EtOH group (P<0.05). However, FRRT pretreatment effectively reversed these changes, leading to the restoration of NRF2, NQO1 and KEAP1 protein levels. In addition, the gene expression levels of *NRF2*, *KEAP1*, *HO-1* and *NQO1* were assessed in AML-12 cells using qPCR. The results included in Fig. 6B revealed that the FRRT-treated groups exhibited a significantly increased mRNA expression of *NRF2*, *HO-1* and *NQO1*, accompanied by a decreased expression of *KEAP1* compared with that in the EtOH group (P<0.05). The dose-dependent response suggested that FRRT-L may potentially exhibit a stronger protective effect against oxidative stress.

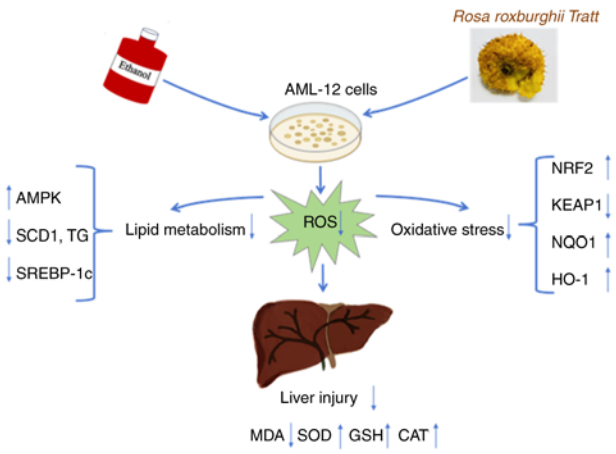

Figure 7. Graphical abstract. The present study evaluated the therapeutic potential of fermented FRRT in the treatment of alcohol-related liver disease. The research utilized an *in vitro* model established with AML-12 cells exposed to ethanol to assess cell damage, lipid accumulation and oxidative stress markers. Pre-treated FRRT significantly reduced the formation of lipid droplets and triglyceride levels in liver cells by regulating genes and proteins involved in lipid metabolism, demonstrating antioxidant properties and reversing the changes in genes and proteins associated with oxidative stress caused by ethanol exposure. These findings provide a new strategy for the management of ALD. FRRT, fermented *Rosa roxburghii Tratt*; ALD, alcohol-related liver disease; AMPK, AMP-activated protein kinase; SCD1, stearyl-coA desaturase-1; TG, triglyceride; SREBP-1c, sterol regulatory element binding transcription factor 1; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; CAT, catalase; NRF2, nuclear factor erythroid 2-related factor 2; KEAP1, kelch like ECH associated protein 1; NQO1, quinone oxidoreductase 1; HO-1, heme oxygenase 1.

Discussion

ALD remains a prominent global health risk. While currently there is no ideal drug for the treatment of ALD, accumulating evidence reveal that natural products can inhibit the progression of ALD by regulating lipid metabolism, and by inhibiting oxidative stress, apoptosis and programmed cell death, among other ways (20-22). The present study successfully developed a flavorful and refreshing FRRT juice with antioxidant properties. During fermentation, the pH of the juice was significantly decreased, indicating an increase in LAB production, as well as increased SOD and VC activities, leading to a significant improvement in the free radical scavenging ability. Further *in vitro* cell experiments confirmed the protective effect of FRRT against ethanol-induced liver cell damage. Although research has revealed that *Rosa roxburghii Tratt* juice can effectively prevent chronic alcohol liver injury by reducing oxidative stress and improving lipid metabolism through modulating the pathways mediated by nuclear receptor Chimeric Antigen Receptor, peroxisome proliferator-activated receptors and NRF2, there is currently lack of studies investigating the effects of FRRT juice on ALD (23). The present study demonstrated, for the first time to the best of our knowledge, the relevance of the findings to human cell lines by extrapolating from studies such as by Sefried *et al* (24), which investigated the suitability of hepatocyte cell lines HepG2, AML12 and THLE-2 for insulin signaling and hepatokine gene expression (25). The authors acknowledge the limitations of the present study, which only involved preliminary validation of the efficacy of FRRT using AML-12 cells. In order to ensure the scientific validity and applicability of the research findings, future studies will be extended to human cell lines to further validate the biological effects of FRRT.

Oxidative stress in ALD is caused by an imbalance between free radicals and antioxidants, resulting in increased ROS and MDA production and decreased antioxidant activity. This damages cellular components and worsens liver injury, while endogenous antioxidants such as SOD, CAT and GSH protect against oxidative damage (25-27). In the present study, the cellular oxidative stress levels were assessed by measuring the levels of MDA, SOD, GSH, CAT and ROS. The findings confirmed that hepatocytes exposed to ethanol exhibited elevated levels of ROS and MDA, a marker of lipid peroxidation, along with decreased activities of antioxidant enzymes, such as SOD, CAT and GSH. These findings indicated increased oxidative stress, impaired antioxidant defense and increased lipid peroxidation, suggesting cellular toxicity. However, treatment with FRRT reversed these

effects, restoring antioxidant enzyme activities and reducing lipid peroxidation. This suggested that FRRT can mitigate oxidative stress and restore cellular antioxidant capacity in ethanol-induced hepatocyte injury. In addition, excessive hepatic lipid production disrupts the cellular redox state, leading to oxidative damage (28). To further investigate the molecular mechanism of FRRT, the effects of FRRT on the NRF2-KEAP1-NQO1-HO-1 pathway were studied. The NRF2-KEAP1-antioxidant response element (ARE) system is a defense mechanism that helps maintain cellular homeostasis and counteract oxidative stress. KEAP1 acts as a negative regulator of NRF2, but under oxidative stress conditions, KEAP1 undergoes a conformational change and dissociates from NRF2, leading to the activation and nuclear translocation of NRF2 (29,30). Subsequently, NRF2 binds to ARE in the nucleus, initiating the transcriptional activation of several downstream genes. This activation results in the upregulation of protective proteins, including Heme-oxygenase 1 (HO-1), NQO1 and SOD, among others, which play crucial roles in regulating antioxidant responses (31,32). HO-1 is an important antioxidant enzyme regulated by NRF2, playing a crucial role in cellular redox homeostasis and serving as a significant cellular protective enzyme (33). Upon stimulation, NRF2 is activated and translocated to the nucleus, where it binds with ARE elements to upregulate HO-1, thereby reducing oxidative stress (34). The study found that the targeted overexpression of NQO1, specifically in AML-12 cells of mice, effectively mitigated the excessive production of ROS and lipid peroxidation caused by chronic alcohol exposure (35). The present study observed that FRRT reversed the decreased expression of NRF2 and NQO1, as well as the increased ethanol-induced KEAP1 expression in AML-12 cells. This suggested that FRRT may activate the NRF2 pathway and enhance antioxidant defense against alcohol-induced oxidative stress. Due to its antioxidant effects, NRF2 has been widely studied as a potential anti-inflammatory target, as it can reduce ROS levels and restore redox homeostasis, thus protecting cells (36).

Furthermore, ROS produced during ethanol metabolism act as critical regulatory factors that can influence lipid metabolism. One important regulator of cellular metabolism is AMPK. When activated, AMPK can restore impaired fatty acid β-oxidation and influence lipid metabolism. AMPK activation inhibits hepatic fatty acid synthesis by suppressing SREBP-1c, the primary regulator of gene expression related to hepatic lipogenesis (37,38). Another vital protein involved in lipid metabolism is SCD1. It acts as a central lipogenic enzyme that catalyzes the conversion of saturated fatty acids to unsaturated fatty acids. Its preferred substrates include various structural lipids such as TG, cholesterol esters and membrane phospholipids (39). In the present study, it was observed that FRRT reduced the increased levels of ethanol-induced TG in AML-12 cells. It also decreased the expression of SCD1 and SREBP-1c, while increasing the expression of AMPK in AML-12 cells. These findings indicated that FRRT has beneficial effects in mitigating alcohol-induced liver injury by regulating lipid metabolism and reducing lipid accumulation.

There is cross-talk between the AMPK and NRF2 pathways; when AMPK is activated, it phosphorylates NRF2, promoting its translocation into the nucleus. This enhances the cell's antioxidant capacity by activating genes involved in antioxidant defense mechanisms (40-43). The study demonstrated that empagliflozin may mitigate the onset of ferroptosis by facilitating the AMPK-mediated NRF2 activation pathway (44). In xanthine-treated mouse embryonic fibroblasts, NRFR/HO-1 expression was found to be reduced in the context of AMPKα1 deletion (45). These examples appear to show that there is an interdependent signaling relationship between energy and redox homeostasis through AMPK and NRF2. Activated AMPK appears to enhance NRF2 signaling. By contrast, NRF2 responds negatively to this enhancement by restoring the redox and metabolic balance, with some delay, thus limiting the signal to activate AMPK (46).

In conclusion, the present study demonstrated that FRRT significantly rescued ethanol-induced hepatocyte injury by alleviating hepatic oxidative stress and improving lipid metabolism. The antioxidant effects are mainly achieved through the NRF2-KEAP1-NQO1-HO-1 axis, while the lipid-lowering effects are regulated through the AMPK-SREBP-1c-SCD1 pathway (Fig. 7). These discoveries offer new perspectives on the application of natural products for preventing and managing ALD.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LZ, ZY and JH collected the data, performed the data analysis and drafted the manuscript. HZ and DW conceived and designed the study, drafted the manuscript and supervised the study. XW acquired the data and supervised the study. QH and SL made substantial contributions to conception and design, and revised the manuscript. QH, SL and LZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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