Rhoifolin Alleviate Alcoholic Liver Disease by Inhibiting the TLR4 / NF-kB Signaling Pathway and Attenuating Oxidative Stress

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Research Article

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Abstract

Alcoholic liver disease (ALD) is caused by long-term excessive consumption of alcohol, which is affected by TLR4 / NF- κ B mediated inflammatory response and CYP2E1-mediated oxidative stress effects. Rhoifolin (ROF) is a flavonoid compound in *Citrus grandis* 'Tomentosa', with antioxidant and anti-inflammation effect. However, the action of its effect on ALD has been elucidated yet. In the present study, we investigated ROF's anti-inflammatory, antioxidant and antiapoptotic action in the treatment of ALD. In established ALD mice, ROF promoted hepatic function through downgrading the amount of aminotransferase, attenuating oxidative stress, and restoring antioxidant balance in hepatic tissue. Additionally, ROF significantly reduced the expression of inflammatory cytokines, such as NF- κ B, TNF- α , IL-6, and IL-1 β in the mice. In *vitro* experiments indicated that ROF increased the LO2 cells viability and inhibited cells apoptosis. ROF reversed the expression of CYP2E1, NLRP3, p-p65, p-I κ B and TLR4, which were consistent with animal experiment. Overall, ROF can alleviate ethanol-induced liver injury through inhibiting oxidative stress and inflammation, and is a promising compound for ALD treatment.

1. Introduction

Alcoholic liver disease (ALD) is caused by long-term excessive alcohol consumption, which has a series of pathologies starting with fatty liver in the early stage, and then developing into alcoholic hepatitis, liver fibrosis and cirrhosis^[1–2]. It is becoming prevalent in the world and undermine the health of people^[3]. Multiple factors contribute to the development of ALD. First, The occurrence and development of ALD is closely related to inflammatory response^[4]. Ethanol can trigger toll like receptors (TLRs) and activate of nuclear factor-kappa B (NF- κ B), thus promoting the secretion of inflammatory cytokines, such as IL-6 and TNF- α ^[5]. These cytokines can cause liver damage. NOD-like receptor protein 3 (NLRP3) is another key protein in ethanol-induced liver injury^[6], which induces inflammatory response and apoptosis^[7]. Moreover, oxidative stress is also major for ALD^[8]. Alcohol metabolism activate the hepatic cytochrome P450 2E1 (CYP2E1), which leads to the production of reactive oxygen species (ROS) and oxidative stress^[9–10]. The oxidative stress also can trigger the activation of NF- κ B inflammatory pathway in liver^[11]. The elevating reactive ROS triggers oxidative stress, and participates in cell apoptosis via Bcl2-Bax-caspase pathway^[11].

Glucocorticoid and S-adenosylmethionine have been proved by FDA to treat ALD in clinics^[5, 13]. However, the efficacy in liver diseases continues to be debated^[14]. So, it's imperative to develop an effective treatment for ALD with fewer side effects. *Citrus grandis* 'Tomentosa', a traditional Chinese Medicine in Huazhou of Guangdong province^[15], is soaked in water to make the drunk sober up a bit in folk therapy. The total flavonoids (FLA) of *Citrus grandis* 'Tomentosa' has therapeutic effects on ALD^[15]. Naringin, a major effective component in FLA, can treat ALD by reducing lipid accumulation and oxidative stress^[17–18]. ROF is another pharmacodynamic component in FLA. However, little is known about its effects and mechanisms in ALD treatment. Studies have shown that ROF plays a role in anti-inflammation^[19] and

anti-oxidation^[20]. Our previous study has shown that ROF could attenuate inflammation in acute inflammation mouse model and LPS-induced RAW264.7 cells through inhibiting NF- κ B signaling pathway.

In this study, we determine the the hepatoprotective effect of ROF on chronic alcoholic liver damage was investigated from the perspective of anti-inflammatory, antioxidant and antiapoptotic action by establishing ethanol-induced liver injury in vitro and in vivo. The results showed that ROF treatment effectively reduced infiltration of inflammatory cells and oxidative stress, further inhibited hepatocyte apoptosis. It will provide experimental basis for the expanded clinical application and ALD therapy, promote the comprehensive development and utilization of the medicinal resources of *Citrus grandis* 'Tomentosa'.

2. Materials And Methods

2.1 Materials

ROF and FLA were extracted from *Citrus grandis* 'Tomentosa' and purified by HPLC^[21] (the purity of ROF was more than 98%).

2.2 Animals and experimental design

BALB/c male mice (18–20 g) were provided by the experimental Animal center of Guangzhou University of Chinese Medicine (Quality certificate number: SCXK(Yue) 2018–0002). The mice were randomly allocated to seven groups respectively, i.e. control group, ethanol group, bifendatum (BIF) group as the positive control (150 mg·kg⁻¹), FLA groups (100 mg·kg⁻¹) and three ROF treatment groups (40, 20 and 10 mg·kg⁻¹). All mice, except in control group, were fed with gradient ethanol to establish mouse ALD model. The dosage was 6 mL·kg⁻¹ for the first 7 days, and then increased by 1 mL every 7 days (up to 10 mL·kg⁻¹) for a total of 49 days. Meanwhile, oral administration of ROF for 49 days to examine its protect effects of liver. At the end of the experiment, the mice were killed and blood were collected. The livers were promptly removed and the wet weight of the liver was weighed. A part of liver was fixed, and the remaining tissues were stored at -80 °C until use.

2.3 Histological preparation and immunohistochemistry

The hepatic tissues of each group were fixed in 4% buffered paraformaldehyde, and then cut in segments to perform dehydration in graded alcohol series, clearance with xylene, embedding in paraffin, and sectioning at 4 μ m slices. Liver sections were stained with hematoxylin-eosin (H&E). The tissue slices were analyzed through a pathological examination under the optical microscope.

Immunohistochemically stained with p65 and CYP2E1 to evaluate the inflammatory and oxidative injury degree.

2.4 Serum biochemical analysis

Blood samples from mice eyeballs were centrifuged at $3500 \text{ r} \cdot \text{min}^{-1}$ at 4°C for 10 min. The samples were used for biochemical measurements. ALT (alanine aminotransferase), AST (aspartate aminotransferase), were determined using kits from ALT (GPT) Kit (Nanjing Jiancheng Bioengineering Research Institute, batch No: C009-2-1) and AST (GOT) Kit (Nanjing Jiancheng Bioengineering Research Institute, batch No: C010-2-1) following the manufacturer's protocol. The levels of TNF- α (batch No:MM-0132M2), IL-6 (batch No:MM-0163M2) and IL-1 β (batch No:MM-0040M2) in the serum were measured using ELISA kits in accordance with the protocol provided by the manufacturer.

2.5 Triglyceride (TG), Malondialdehyde (MDA), Glutathione (GSH) content and Superoxide dismutase (SOD)activities

Liver tissue was homogenized in PBS to prepare 10% liver homogenate, then centrifugated (4°C , 3000 rpm, 10 min), and the supernatant was collected. The levels of MDA (batch No: A003-1) and GSH (batch No: A006-2-1), the activity of SOD (batch No: A001-3), and the content of TG (batch No: A110-1-1) in the supernatant of liver homogenate were determined according to the requirements of the instructions provided in reagent kits. The protein concentration in the liver was measured with BCA protein assay reagent kit (Beyotime, No: P0010). The values were normalized to total protein in liver tissue.

2.6 Cell culture

LO2 cells were provided by Wuhan Biological Sample Collection Center (BSCC, Wuhan, China). Cells were cultured with DMEM medium (Gibco BRL, Gland Island, NY) which supplemented with 10% fetal bovine serum (Gibco BRL, Gland Island, NY), in a humidified atmosphere of 5% CO_2 at 37°C .

2.7 Cell viability assay

Cell viability was evaluated by a conventional MTT reduction assay. LO2 cells were plated at a density of $5 \times 10^3 \text{ cells} \cdot \text{mL}^{-1}$ in 96-well plates for 24 h. In the experimental group, total 200 μL solution were added simultaneously, which contains $200 \text{ mmol} \cdot \text{L}^{-1}$ ethanol and different concentrations of ROF (25, 50 and $100 \text{ mol} \cdot \text{L}^{-1}$). In the ethanol group, $200 \text{ mmol} \cdot \text{L}^{-1}$ of ethanol was added to stimulate, and the blank well cells were added with the same amount of serum-free medium. After incubation for predetermined times up to 24 h, the cells were incubated in MTT solution ($5 \text{ mg} \cdot \text{mL}^{-1}$) for an additional 4 h at 37°C . After washing each well with PBS, the colored formazan was dissolved in 150 μL of DMSO. The absorption values were read at 490 nm in a microplate reader.

2.8 Cellular ALT, AST and LDH Assay

After treatment, culture supernatants were harvested for the measurement of ALT (No: C009-2-1), AST (No: C009-2-1) and LDH (No: A020-2) levels by kits according to the supplier's protocols.

2.9 Apoptosis assay

Cell suspension was added to the 6-well plates for 2 ml each well for 12 h at 37°C . After treatment, cells and supernatants were collected respectively, resuspended with 500 μL 1 $\times$ Binding Buffer. Per tube was

added 5μL Annexin V-FITC and 10 μL PI (Sangon Biotech, batch No: 70-AP101-100), incubated for 5 min. Then, the effect of ethanol on LO2 cells apoptosis was observed by flow cytometry.

2.10 Real-time PCR

Total cellular RNA from LO2 cells was extracted using Trizol kit (Cwbio, Beijing, China), quantified and diluted into 1mg·mL⁻¹. cDNA was then synthesized using HiScript RT SuperMix (Vazyme, Nanjing, China) according to the manufacturer’s protocol. Realtime PCR was performed using SYBR qPCR Master Mix (Vazyme, Nanjing, China) with the cycling conditions. The identity and purity of PCR products were assessed by melting curve analysis. The primers were synthesized by (Sangon Biotech, Shanghai, China).

Table 1
Primer sequence of genes

genes	Primer sense(5'-3')	Primer antisense(5'-3')
GAPDH	ACTCCTCCACCTTTGACGCT	GGTCTCTCTCTTCCTCTTGTGC
TNF-α	TGGGATCATTGCCCTGTGAG	GGTGTCTGAAGGAGGGGGTA
IL-6	TAGTGAGGAACAAGCCAGAGC	GTTGGGTCAGGGGTGGTTATT
IL-1b	AGTTGACGGACCCCAAA	TCTTGTTGATGTGCTGCTG
Bax	CCCCGAGAGGTCTTTTCCG	CCGGAGGAAGTCCAATGTCC
Bcl-2	GGTGAACTGGGGGAGGATTG	GTGCCGGTTCAGGTACTCAG
CYP2E1	CCTTTCCCGCTTCCCATCAT	GCTTCCTTCACCGCCTTGTA

2.11 Western Blot

The protein concentration was determined by BCA protein assay reagent kit. The equal amounts of protein were separated on 10% SDS-PAGE gel electrophoresis, then the target protein strip was transferred to the PVDF membrane. The membrane was incubated with primary antibodies anti-CYP2E1, p-IkBα, IkBa, p-p65, p65, TLR4, NLRP3, Caspase-3, Cleaved Caspase3, GAPDH and tubulin-β diluted in 5% BSA in TBST for overnight at 4 °C. The membrane was washed with TBST and incubated with rabbit antibodies for 1h at room temperature. The membrane was washed with TBST again and exposed to ECL solution.

2.13 Statistical analysis

The experimental data was processed by the one-way analysis of variance (ANOVA) in GraphPad Prism 8.0. The test results were expressed as means ± SEM. Comparisons between groups were performed using Student’s t-test method; *P* < 0.05 indicated statistically significant differences.

3. Results

3.1 ROF relieves the liver injury induce by alcohol in vivo

In order to study whether ROF has a protective effect on ALD, we fed the mice with alcohol to establish the alcoholic liver injury models, and then oral administration of FLA ($100 \text{ mg}\cdot\text{kg}^{-1}$) and ROF (10, 20, 40 $\text{mg}\cdot\text{kg}^{-1}$) for 49 days. BIF ($150 \text{ mg}\cdot\text{kg}^{-1}$) was used as positive control to compare the efficacy of ROF against chronic alcohol-induced liver injury.

The liver weight and liver index of the ROF, FLA and BIF group decreased significantly compared with those of the ethanol group (Fig. 1a-b). Mildly hepatocyte edematous, disordered hepatic cord, narrowed hepatic sinusoids, and inflammatory cell infiltration were observed in the alcohol group through the examination of the liver tissue slices. After treatment with ROF, total FLA or BIF, it was found that the cords of hepatocytes tended to be normal, inflammatory infiltration was decreased, and local necrosis was reduced. The pathological degree of the high dose group was significantly improved (Fig. 1c). Our results indicated that ROF relieves the liver injury induced by alcohol in mouse model.

3.2 ROF ameliorates the production of aminotransferase, oxidative stress factor and inflammatory cytokines

The levels of serum AST and ALT (Fig. 2a-b) significantly increased in the ethanol group compared with the normal group. Treatment with ROF significantly reversed the ethanol-induced increase of serum AST and ALT levels. The contents of TG (Fig. 2c) in liver tissues significantly increased in the ethanol group compared with the normal group. Treatment with FLA, ROF and BIF reversed the ethanol-induced increase of TG content and inhibited hepatic lipid accumulation.

MDA level was significantly increased, and GSH level and SOD activity were significantly decreased in the liver tissues of the ethanol group compared with the normal group (Fig. 2d-f). FLA, ROF and BIF significantly reduced MDA content and suppressed ethanol-induced reduction of GSH activity. ROF at $40 \text{ mg}\cdot\text{kg}^{-1}$ significantly suppressed the ethanol-induced decrease of SOD level. The secretions of TNF- α , IL-6, and IL-1 β in serum were significantly increased after modeling with ethanol in comparison with the control group but decreased after the intervention of FLA, ROF, and BIF (Fig. 2g-i). In short, our results showed that ROF regulates the production of aminotransferase, oxidative stress factor and inflammatory cytokines.

3.3 ROF inhibits the TLR4 / NF- κ B signaling pathways and CYP2E1-mediated oxidative stress in mice

It was reported that NF- κ B signaling pathway plays a role in alcohol-induced liver injury, we wonder whether ROF relieves the liver injury through regulating NF- κ B signaling pathway. Immunohistochemistry results showed that alcohol resulted in clearly increased inflammatory markers and hepatic cytochrome enzyme in the liver. The immunohistochemical results also showed that the ethanol group significantly expressed NF- κ B and CYP2E1 compared with the control group (Fig. 3a-b). The treatment of ROF, FLA and BIF could persuasively downregulate the expression of NF- κ B and CYP2E1. The protein levels of NLRP3

and TLR4 were detected through Western blot to further determine whether the inflammation induced by alcohol was related to the activation of the inflammatory and oxidative pathway (Fig. 3c). The expression levels of these proteins in the ethanol group increased compared with those in the control group. By contrast, ROF, FLA and BIF decreased the expression levels of them. Our results suggested that ROF protects against liver injury through the protective effect of ROF on ALD mice may be through inhibited NF- κ B and CYP2E1 expression.

3.4 ROF increases the viability of ethanol-treated LO2 cells

According to reports in relevant studies, we used different concentrations of ethanol to treat LO2 cells to induce the pathogenesis of alcoholic liver injury as much as possible, meanwhile reduce interference factors such as high nutrition^[22–23]. Ethanol administration resulted in a reduction of cell viability and ROF increases the viability of ethanol-treated LO2 cells (Fig. 4a-b). LDH leakage is another classical indicator of cytotoxicity. Ethanol treatment induced LDH release into the culture medium. Treatment with ROF greatly reduced the level of LDH in the culture medium (Fig. 4c). Administration of ethanol caused a massive release of aminotransferases from LO2 cells. Compared to control cells, the levels of ALT and AST increased in the supernatant of cells treated with ethanol, respectively. ROF at 25, 50 and 100 μ M prevented the elevation of ALT and AST in the culture supernatant (Fig. 4d-e).

3.5 ROF inhibits apoptosis and oxidative stress in LO2 cells

Ethanol consumption generates oxidative stress by CYP2E1 and this oxidative damage induces cell apoptosis, which in turn facilitates the advancement of ALD. We wonder whether ROF relieve the liver injury through inhibiting apoptosis and oxidative stress. Ethanol-induced apoptosis was evaluated by Annexin V-FITC/PI double staining and flow cytometry (Fig. 5a). The expression levels of apoptosis-related genes were analyzed by real-time PCR (Fig. 5b-c). After ethanol treatment, the mRNA expression levels of Bax increased, while that of Bcl-2, an antiapoptotic gene, decreased. ROF down-regulated the levels of Bax, increased the level of Bcl-2, and reduced the ratio of Bax/Bcl-2. To further investigate its apoptotic effect, the expressions of Caspase-3 and Cleaved Caspase-3 were shown (Fig. 5f). The ethanol-treated levels of protein expression increased in cells compared to control cells. Compared with ethanol groups, ROF significantly down-regulated the expressions of Caspase-3 and Cleaved Caspase-3 in LO2 cells.

To understand the mechanism underlying the protective effects of ROF against oxidative stress induced by ethanol, we evaluated the mRNA (Fig. 5d) and protein expression (Fig. 5e) of CYP2E1. Ethanol treatment increased the protein and mRNA expressions of CYP2E1 genes in LO2 cells while ROF inhibited. Our data suggested that the protective effect of ROF against ethanol-induced oxidative stress is associated with down-regulation of CYP2E1.

3.6 ROF inhibites inflammatory cytokines production via the TLR4/NF- κ B signaling pathway in LO2 cells

To test whether ROF possesses anti-inflammatory activity, the mRNA expression levels of TNF- α , IL-6 and IL-1 β were measured (Fig. 6a-c). The expression of both TNF- α , IL-6 and IL-1 β were significantly increased in LO2 cells of ethanol group while ROF reduced the secretion of inflammatory cytokines. In order to evaluate the effects of ROF in ethanol-induced LO2 oxidative damage, the expressions of TLR4, NLRP3, p65 and I κ B α are shown in Fig. 6d-e. Compared to control cells, the levels of protein expression increased in the supernatant of cells treated with ethanol, respectively. Compared with ethanol groups, ROF (50 $\mu\text{mol}\cdot\text{L}^{-1}$ and 100 $\mu\text{mol}\cdot\text{L}^{-1}$) significantly down-regulated the expressions of them in LO2 cells. The data suggest that the protective effect of ROF on LO2 cells closely related to TLR4 / NF- κ B targeting to induce liver inflammation.

4. Discussion

Citrus grandis 'Tomentosa' is a commonly used Chinese medicine for resolving phlegm and cough with a long history of application. Modern pharmacological research show that the main active site are flavonoids ingredients, with antioxidant anti-inflammation antipyretic analgesic effect^[24]. ROF is one of the main flavonoids. In previous reports indicated, the protective effects of ROF against alcohol-induced liver injury had not been studied in depth. Therefore, this study is mainly to clarify the protective effect of ROF and its mechanism.

In this study, the hepatoprotective effect of ROF from *Citrus grandis* 'Tomentosa' on the ethanol-induced liver injury model in *vivo* and in *vitro* were investigated. Results suggested that ROF reduced the liver injury induced by ethanol through its anti-inflammatory and antioxidant effects, and the underlying mechanism may be associated with the reduced release of inflammatory cytokines and apoptosis-related genes by inhibiting the TLR4 / NF- κ B signaling pathway.

There were many pathogenic factors in the development of ALD. Among them, long term excessive consumption of alcohol was a key factor in ALD, which lead to chronic liver damage. Mice chronic alcoholic liver model was used to simulate the long-term drinking of human beings. Liver index refers to the percentage of liver and body weight, which is one of the indicators to judge whether the liver is healthy. When alcoholic liver injury occurs, the liver pathology may show hepatomegaly, unclear boundary, watery degeneration and inflammatory cell infiltration, which eventually leads to weight increase and liver index increase^[25]. ALT and AST play important roles in the synthesis and decomposition of amino acids in the body, and are important indicators reflecting liver function in clinic. When the liver is gradually damaged, ALT and AST will continue to release into the blood, so that the level of them in blood can reflect the degree of liver damage. The expression of AST and ALT in the serum of alcohol induced mice increased significantly, while the expression of them decreased significantly after administration of ROF, total FLA and BIF.

Metabolized alcohol will produce a large number of oxidative free radicals and consume a large number of enzymes. SOD and GSH are important antioxidant enzymes in the body^[26]. The activity level of these enzymes directly reflects the body's antioxidant capacity and plays an important role in eliminating

excessive free radicals. Compared with the control group, GSH and SOD in the ethanol group were significantly decreased, while the expression levels of them were increased after administration. The destruction of the body's antioxidant system leads to lipid peroxidation of the liver cell membrane and the increase of MDA level. When the antioxidant enzymes in the human body decrease, it will inhibit the metabolism of acetaldehyde, resulting in the output of triglyceride disorder, and then a large amount of deposition in the liver^[26]. The content of TG and MDA in the therapeutic group was decreased compare with the ethanol group. Hepatic CYP2E1 thought to be a major source of ROS production and could be induced by alcohol^[28]. The results showed the hepatic CYP2E1 protein expression were reduced after ROF treatment, suggesting that ROF might exert a beneficial effect on CYP2E1-mediated oxidative stress.

The protective effects of ROF against ALD was studied using ethanol-induced LO2 cells in *vitro*. MTT experiments found that $100\ \mu\text{mol}\cdot\text{L}^{-1}$ ROF could significantly enhance cell viability. Therefore, the concentration of ROF was selected as 25, 50 and $100\ \mu\text{mol}\cdot\text{L}^{-1}$ in the follow-up experimental study. When the concentration of ethanol was selected by MTT, the cell survival rate could reach 36% when the concentration of ethanol was $200\ \text{mmol}\cdot\text{L}^{-1}$. Annexin V could bind phosphatidylserine (PS) specifically, which indicated that cells were apoptotic. Annexin V-FITC/PI double staining and flow cytometry were used to evaluate the inhibitory effect of ROF on ethanol induced apoptosis of LO2 cells^[29]. The results showed that the early and late apoptosis rates in the ethanol group were significantly increased, $50\ \mu\text{mol}\cdot\text{L}^{-1}$ and $100\ \mu\text{mol}\cdot\text{L}^{-1}$ ROF treatment were significantly decreased.

Bax and Bcl-2 are two important regulatory factors of apoptosis. They have opposite effects on cell apoptosis. The increase of Bax expression level leads to cell apoptosis^[30]. On the contrary, when the level of Bcl-2 is higher, it inhibits cell apoptosis. Caspase-3 as the executive factor of apoptosis, its significantly increased expression level can promote cell apoptosis^[31]. Studies have shown that excessive alcohol concentration in LO2 cells will promote the overexpression of Caspase-3 factor and the imbalance of Bax and Bcl-2 factor expression, thus promoting a large number of cell apoptosis.

Compared with the blank group, the expression levels of Bax and caspase-3 mRNA were increased and the expression levels of Bcl-2 mRNA were decreased in LO2 cells treated with ethanol alone for 24 h. Compared with the ethanol group, the expression level of Bax mRNA decreased, while the expression level of Bcl-2 mRNA increased. In order to further study its apoptotic effect, we detected the expression of Caspase-3 and Cleaved Caspase-3 by Western Blot. The protein expression level of ethanol treated cells was increased. However, ROF significantly down regulated the expression of Caspase-3 and Cleaved Caspase-3 in LO2 cells. It is suggested that ROF can promote the proliferation of LO2 cells induced by ethanol and inhibit their apoptosis.

Inflammatory response injury plays a key role in the occurrence of ALD. Endotoxin activates Kupffer cells that release some inflammatory factors, including TNF- α , IL-1 β , and IL-6, which can injure the normal cells of the human body and then induce liver injury^[5]. In our experiment, ELISA was used to detect the mice serum and PCR was used to detect the expression of inflammatory factors in the supernatant of LO2

cells. The results show that ROF can effectively reduce the production of TNF- α , IL-6 and IL-1 β , thus reducing the inflammatory reaction in *vivo* and in *vitro*.

TLR4 is closely related to inflammation of liver injury induced by alcohol. It can activate NF-kB pathway, regulate the expression of inflammatory factors, and promote the production of inflammatory cytokines, such as TNF- α and IL-6. Our study shows that ROF can significantly reduce the expression of TLR4 and NF-kB, thereby inhibiting the production of TNF- α and IL-6. In addition, TLR4 / NF-kB can also target the NLRP3 to induce liver inflammation. Activation of NLRP3 can transform IL-1 β secretion. The results clarified that ethanol could increase the inflammatory level of NLRP3 and promote the secretion of IL-1 β . ROF treatment significantly reduced the production of these inflammatory factors, suggesting that ROF treatment may play an anti-inflammatory role by inhibiting the nuclear translocation of NF-kB and affecting the activation of NLRP3 by reducing the expression of TLR4.

In conclusion, ROF had protected effects on ethanol-induced mouse ALD model and ethanol-induced LO2 cells. This study suggests that ROF may be used as a potential therapeutic for ALD, and provided research ideas for the development and utilization of *Citrus grandis* 'Tomentosa' resources. We suggest that ROF be considered as candidate potential anti-ALD option and it is worth further exploring clinical trials in future research.

Declarations

AVAILABILITY OF DATA AND MATERIALS

Data are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

Concept and design: Fengxia Xiao and Baoyu Mai; data collection: Baoyu Mai and Zelin Cao; data analysis and interpretation: Baoyu Mai; generating the manuscript: Baoyu Mai, Ling Han, Zelin Cao, Jiaqi Fang, Xiaoying Zhang, Jiarui Zhong, and Zelin Gao.

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Ethics approval. The study protocols were approved by the Ethical Committee on Animal Research at the School of Guangzhou University of Chinese Medicine. All animal experiments complied with the ethical requirements of the ethical committee.

Consent to Participate. Not applicable.

Consent for Publication. Not applicable.

Competing Interests. The authors declare no competing interests.

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Figures

Figure 1

Effects of ROF on liver index and liver pathological changes in mice with alcoholic liver injury: Mice were pretreated with FLA (100 mg·kg⁻¹), ROF (40,20 and 10 mg·kg⁻¹) and BIF (150 mg·kg⁻¹). Macroscopic morphology of liver tissue (a) and liver index (b) were observed. The liver index was calculated by the following formula: liver index (%) = liver wet weight/mouse body weight*100%. (c) were showed the

histopathological damages of the liver (200× and 400×). Data show mean $\pm$ SEM (n=7). (###) $P < 0.001$ compared with the control group; (*) $P < 0.05$ and (**) $P < 0.01$ compared with the ethanol group.

Figure 2

Effect of ROF on the secretion of inflammatory cytokines and indicators of oxidative stress in ALD mice: The levels of ALT(a), AST(b) in serum, Hepatic TG(c), MDA(d), GSH (e) and SOD(f) were determined according to the requirements of the instructions provided in reagent kits. The levels of TNF- α (g), IL-6 (h) and IL-1 β (i) were determined by ELISA. Data are expressed as mean $\pm$ SEM (n=7). (##) $P < 0.01$ and (###) $P < 0.001$ compared with the control group; (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$ compared with the ethanol group.

Figure 3

Effect of ROF on TLR4 / NF- κ B signaling pathways and CYP2E1-mediated oxidative stress in mice: Immunohistochemistry used to detect the expression level of p65 (a) and CYP2E1 (b) in liver from 7 groups (magnification200 $\times$). The expression levels of NLRP3 and TLR4 proteins were assessed via Western Blot (c). Data express mean $\pm$ SEM(n=3). (###) $P < 0.01$ compared with control group; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ compared with ethanol group.

Figure 4

Effect of ROF on the cell viability(a-b) and production of LDH(c), ALT(d) and AST(e) of ethanol-induced LO2 cells: Cells were treated with Ethanol (200 mM) and Ethanol + ROF for 24h, and viability was measured by MTT. The LO2 cells supernatant were divided into control group, Ethanol ethanol group and ROF group (25, 50, 100 μM) used for kit test. Data express mean $\pm$ SEM (n=3). (###) $P < 0.001$ compared with the control group; (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$ compared with the ethanol group.

Figure 5

Effects of ROF on inhibition of apoptosis and oxidative stress in LO2 cells: LO2 cells were double stained with FITC-labeled annexin-V/PI. The apoptotic rates were determined by flow cytometry (a). Bax (b), Bcl-2 (c) and CYP2E1 (d) mRNA expression in LO2 cells were shown via RT-PCR. The expression levels of CYP2E1, Caspase-3 and Cleaved Caspase-3 proteins were assessed via Western Blot (e-f). Data express

mean $\pm$ SEM(n=3). (##) $P<0.01$, (###) $P<0.01$ compared with control group; (*) $P<0.05$, (**) $P<0.01$, (***) $P<0.001$ compared with ethanol group.

Figure 6

Effect of ROF on TLR4 / NF κ B signaling pathways in LO2 cells: The cells were divided into control group, ethanol group and ROF group (25, 50, 100 μ mol $\cdot$ L⁻¹). TNF- α (a), IL-6 (b) and IL-1 (c) mRNA expression in LO2 cells were shown via RT-PCR. The expression levels of TLR4, NLRP3, I κ B α , p65 and the corresponding

phosphorylated proteins were assessed via Western Blot (d-e). Data express mean $\pm$ SEM(n=3). (#) $P<0.05$, (##) $P<0.01$, (###) $P<0.001$ compared with control group; (*) $P<0.05$, (**) $P<0.01$, (***) $P<0.001$ compared with ethanol group.