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Effect of *Rubus* extract on TLR4/NF-kappa B signaling pathway in alcoholic liver fibrosis rats

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Abstract: This study aims to explore the effect of the *Rubus* extract on the TLR4/NF-κB signaling pathway in alcoholic liver fibrosis rats. The alcoholic liver rat model was established by continuous ethanol gavage administration. Rats were divided randomly into six groups (i.e., blank control, model, 0.05g/kg *Rubus* extract, 0.125g/kg *Rubus* extract, 0.259 g/kg *Rubus* extract and positive control groups). Liver tissue and blood were collected after treatment for four weeks. The pathological changes in the liver were observed by HE and Masson staining methods. The hyaluronic acid (HA), TNF-α and IL-6 levels were determined by ELISA kits. The TLR4 and p-p65 protein expression levels in liver were detected by Western blot. The liver lesion degree was significantly decreased in the *Rubus* extract group, and a high concentration of the Rubus extract indicated a significant improvement. The TNF-α, HA and IL-6 levels in the *Rubus* extract and positive control groups were significantly lower than those of the model group ($P<0.05$). The TLR4 and p-p65 protein expression levels were also significantly decreased in the *Rubus* extract and positive control groups ($P< 0.05$) with a concentration dependence of *Rubus* extract. The *Rubus* extract could delay the development of alcoholic liver fibrosis through inhibiting the TLR4/NF-κB pathway activity.

Keywords: *Rubus* extract; Alcoholic liver; Liver fibrosis; TLR4; NF-κB

INTRODUCTION

Excessive alcohol consumption is a healthcare problem worldwide. Drinking excessive alcohol for a long time leads to liver injury and then forms alcoholic liver disease (ALD). The liver is the main site of ethanol metabolism. Thus, the tissue injury of the liver is most serious. ALD encompasses a spectrum of injuries, ranging from simple steatosis to more severe stages, such as alcoholic cirrhosis, hepatocellular carcinoma, steatohepatitis and liver failure. Steatosis, the earliest response to alcoholism, is characterized by fat deposition in hepatocytes (Osna *et al.*, 2017; Stickel *et al.*, 2017; Singal *et al.*, 2018).

In clinical practice, the key to the treatment of ALD is to reduce the activation of inflammatory reaction, delay the process of liver fibrosis and prevent the occurrence of liver cirrhosis (Kim *et al.*, 2016). A study suggested that the toll-like receptor 4/nuclear factor kappa-B (TLR4/NF-κB) signaling pathway played a regulatory role in inflammatory response and tissue fibrosis (Strekalova *et al.*, 2016). The activation of this pathway stimulated the nuclear transfer of NF-κB and regulated the expression levels of tumor necrosis factor α (TNF-α), Interleukin 6 (IL-6) and other cytokines. The expression of inflammatory cytokines can activate inflammatory reaction, cause abnormal synthesis and degradation of extra cellular matrix through the activation of hepatic stellate cells and secretion of transforming growth factor-β 1 (TGF-β 1) and then gradually form tissue fibrosis (Strekalova *et al.*, 2016; Lin *et al.*, 2020).

Rubus ideaus L., a member of the Rosaceae family and commonly known as raspberry and marlin, has attracted much attention in recent years. *Rubus ideaus* L. is widely distributed in Europe, Asia and North America. Raspberry is often consumed as fresh fruits, functional beverages and fermented wine because of its attractive color, delicious taste and excellent nutritional characteristics. Raspberry is not only rich in amino acids, vitamins and other nutrients, but also rich in polyphenols, terpenes, sterols and other active substances such as super oxide dismutase (SOD) and raspberry ketone (Kula and Krauze-Baranowska, 2016; Zhou *et al.*, 2018; Wu *et al.*, 2019; Staszowska-Karkut and Materska, 2020; Ispiryan *et al.*, 2021). The *Rubus* extract can prevent hyperlipidemia by regulating lipid metabolism and delay the formation of atherosclerosis by improving arterial endothelial function. The *Rubus* extract has natural anti-inflammatory components and can significantly slow down the release of inflammatory factors and inhibit the inflammatory reaction of arterial plaque to play an anti atherosclerotic role (Jeong *et al.*, 2014; Jeong *et al.*, 2016; Zhou *et al.*, 2018). These studies indicated that the *Rubus* extract could effectively alleviate the damage caused by excessive inflammatory reaction. However, whether the *Rubus* extract can alleviate lipid deposition and excessive inflammation in alcoholic liver has not been reported yet.

In this study, we established an alcoholic liver model in

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rats and explored the effect of *Rubus* extract on alcoholic liver fibrosis rats and the mechanism, providing certain theoretical and experimental bases for the follow-up study on alcoholic liver treatment.

MATERIALS AND METHODS

Animals

Thirty-six healthy male Sprague-Dawley (SD) rats of Specific Pathogen Free (SPF) grade (6-8 weeks old, 180-220 g) were purchased from Guizhou Medical University Animal Center. The alcoholic liver fibrosis rat model was established through the intragastric administration of 5% ethanol (8 g/kg) for 12 weeks. After successful modeling, rats were treated with distilled water, *Rubus* extract and compound embryonic bovine liver extract tablets and randomly divided into model, *Rubus* extract low concentration (0.05g/kg), *Rubus* extract medium concentration (0.125g/kg), *Rubus* extract high concentration (0.259g/kg), positive control (compound embryonic bovine liver extract tablets, 21.6mg/kg) group, and control (distilled water) groups (six rats in each group). Four weeks later, the rats were sacrificed and the liver tissue and blood were collected.

Ethics approval

All experiments were approved by the Animals Committee of Guizhou Provincial People's Hospital.

Hematoxylin-eosin (HE) staining

The left lobe of liver was collected and fixed with 4% paraformaldehyde for 12h. Then, samples were embedded in paraffin, and sliced to obtain a thickness of 15-25 mm. Routine HE staining was conducted, and the pathological features of liver tissue were observed using a biomicroscope.

Masson staining

Sample slices were prepared with the same method as HE staining. The Masson staining was conducted using the Masson trichromatic staining kit following the manual. Slices were dehydrated by ethanol (95% and absolute). Xylene was changed twice and the slices were covered using a coverslip. The pathological features of the liver tissue were observed using a biomicroscope.

Detection of serum hyaluronic acid (HA), TNF-α and IL-6 expression levels

The serum from different groups was collected by centrifugation. The expression levels of serum HA, IL-6 and TNF-α were determined using enzyme linked immunosorbent assay (ELISA) kits following the instructions.

Western blotting detection

Liver tissues were homogenized with 1 ml RIPA lysis buffer and centrifuged (12000 rpm, 4°C) for 10min, and the supernatant was collected. Total proteins were extracted and their concentration was determined using the Bicinchoninic Acid Assay (BCA) method. Proteins (50μg/lane) were separated with 12% sodium dodecyl sulfate-polyacrylamide gel electro-phoresis (SDS-PAGE) and electro transferred onto a Polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was rinsed using TBS for 15 min and blocked with blocking buffer. They were washed and added with appropriate dilutions of primary antibodies (1:2000, TLR4; 1:2000, p-p65; 1:5000, GAPDH; Abcam, Cambridge, UK), and incubated at 4°C overnight. The membrane was rinsed with TBST thrice, added with the goat anti-mouse IgG secondary antibody (1:50000, Abcam, Cambridge, UK), and incubated at room temperature for 1 h. Protein bands were detected using an enhanced chemiluminescence kit (Perkin-Elmer Inc., Waltham, MA, USA) and quantified as the ratio to GAPDH. Quantification was performed with Image Lab™ Software.

STATISTICAL ANALYSIS

Statistical analysis was conducted by the SPSS 21.0 software. The data were expressed as mean ± standard deviation (SD). The difference was compared by t test. $P<0.05$ was considered significant.

RESULTS

Morphological effects of the Rubus extract on liver fibrosis

In the blank control group, HE staining results showed a clear structure of hepatic lobule, normal shape of hepatocytes, uniform size, neat arrangement, complete hepatic cord structure, and no fibrous connective tissue proliferation and inflammatory infiltration in the portal area. However, in the model group, destroyed structure of hepatic lobule, steatosis in hepatocytes, disordered the arrangement of hepatic cords, fibrous connective tissue hyperplasia and infiltration of inflammatory cells in the portal area and central venous areas were observed. Compared with the model group, the *Rubus* extract and positive control groups had decreased degree of liver tissue destruction, degeneration of hepatocytes, the connective tissue proliferation and inflammatory cell infiltration in the confluence area. A high concentration of the *Rubus* extract resulted in significant improvement (fig. 1).

Masson staining results showed that only a spot of collagen was deposited in the portal area in the blank group. A lot of collagen deposition in the portal area, central venous area and hepatocytes were observed in the model group. The collagen deposition in the *Rubus* extract and positive control groups was significantly reduced compared with that in the model group and a high concentration of *Rubus* extract resulted in minimal collagen deposition (fig. 2).

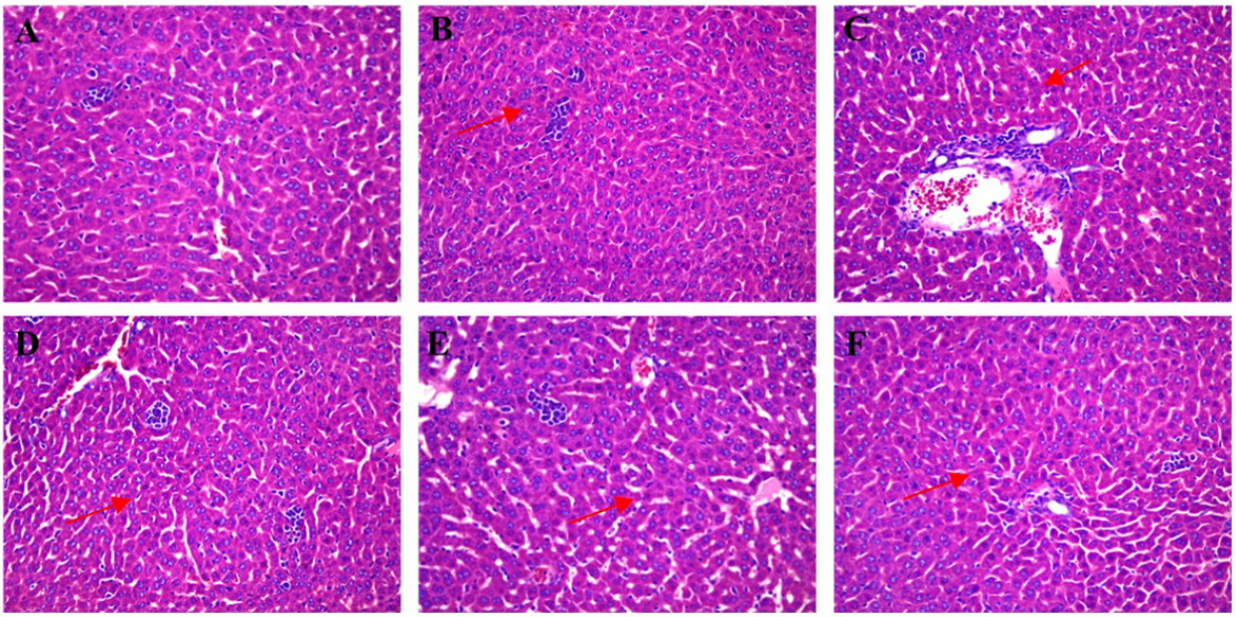
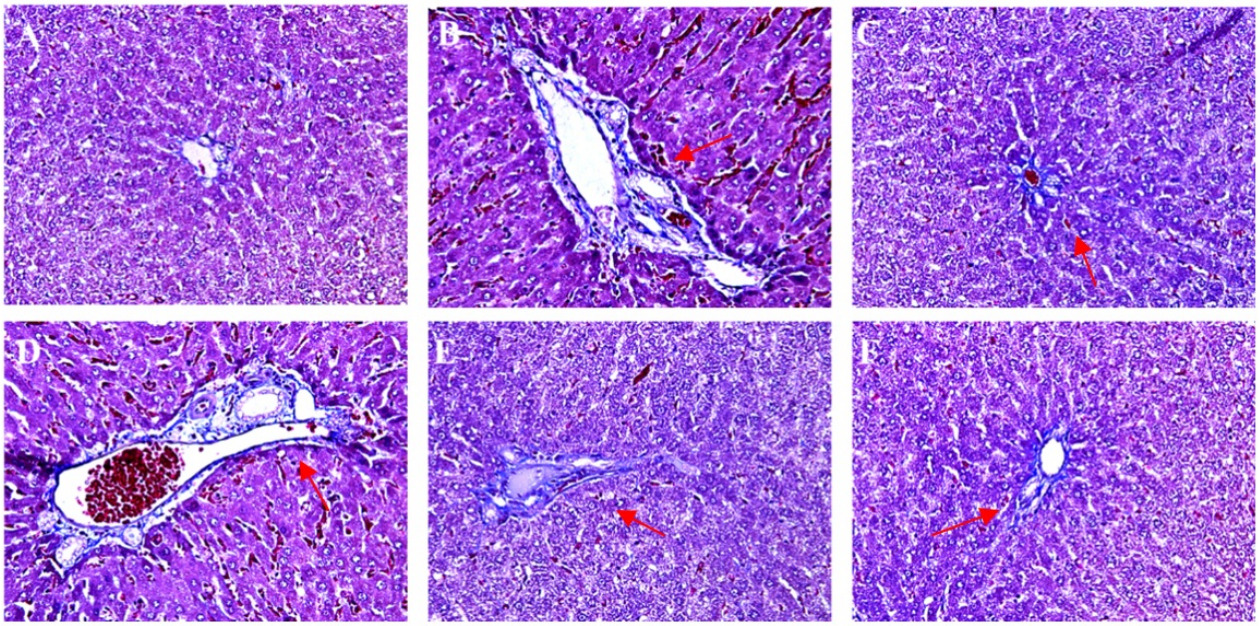


Fig. 1: HE staining results of liver tissue in each group (× 200)



A: blank control group; B: model group; C: 0.05 g/kg *Rubus* extract group; D: 0.125 g/kg *Rubus* extract group; E: 0.259 g/kg *Rubus* extract group; F: positive control group.

Fig. 2: Masson staining results of liver tissue in each group (×200)

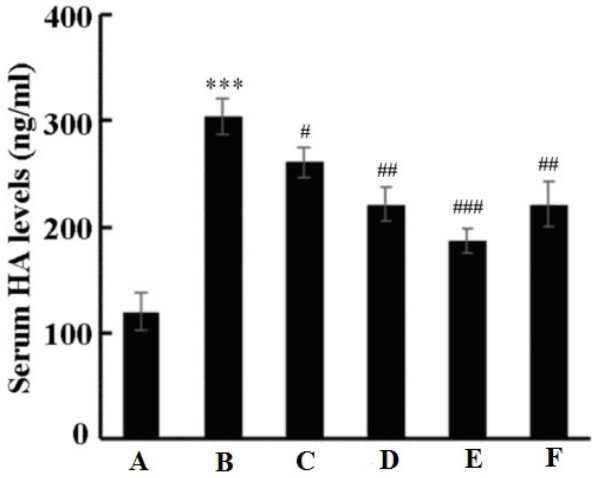
Effect of the Rubus extract on serum HA in rats

As shown in fig. 3, the serum HA level in the model group was significantly increased compared with that in the blank group (P<0.05). The levels of HA in the *Rubus* extract group were lower than those in the model group with dose dependence (P<0.05). The HA level in the positive control group was also significantly decreased than that in the model group (P<0.05). No significant difference was found between the *Rubus* extract group and positive control group (P>0.05).

Effect of the Rubus extract on the serum TNF-α and IL-6 levels of rats

Serum IL-6 and TNF-α levels in the model group were significantly higher than those in the blank control group (P<0.05). The IL-6 and TNF-α levels in *Rubus* extract group were significantly lower than those in the model group with dose dependence (P<0.05). The IL-6 and TNF-α levels in the positive control group were also significantly decreased than those in the model group (P< 0.05). No significant difference was found between the

Rubus extract and positive control groups (P>0.05) (fig. 4).



A: blank control group; B: model group; C: 0.05g/kg *Rubus* extract group; D: 0.125 g/kg *Rubus* extract group; E: 0.259 g/kg *Rubus* extract group; F: positive control group.
*** p<0.001 vs. blank control group; $^{\#}p$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. model group.

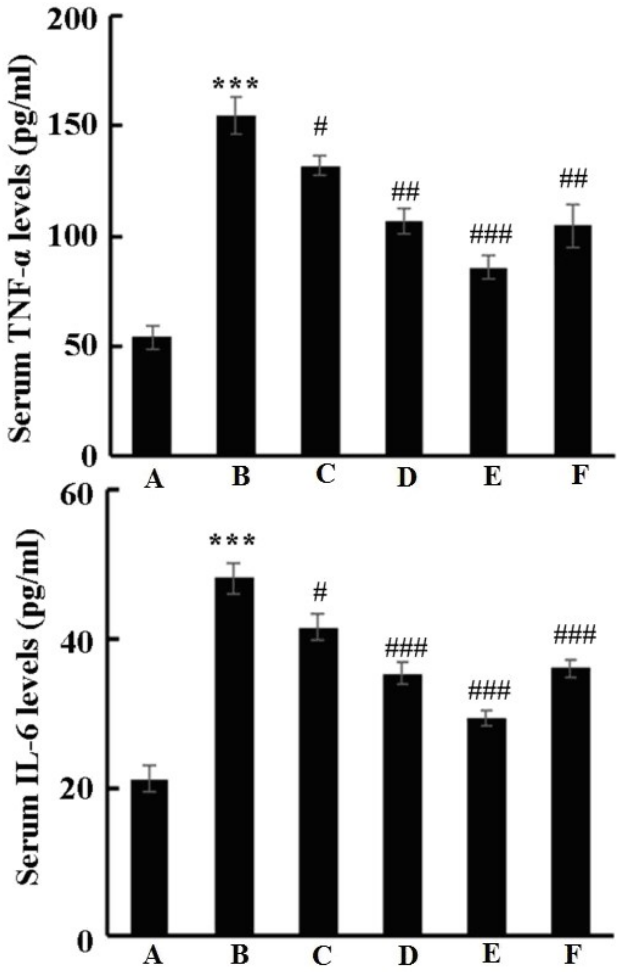
Fig. 3: The serum HA content of experimental rats in each group

Effect of the Rubus extract on TLR4 and p-p65 protein expression levels

Protein expression results were shown in fig. 5. The TLR4 and p-p65 protein levels in the model group were significantly higher than those in the blank control group (P<0.05). The TLR4 and p-p65 protein levels in the *Rubus* extract group were lower than those in the model group with dose dependence (P<0.05). The TLR4 and p-p65 protein expression levels in the positive control group were also significantly decreased than those in the model group (P<0.05). No significant difference was found between the *Rubus* extract and positive control groups (P >0.05).

DISCUSSION

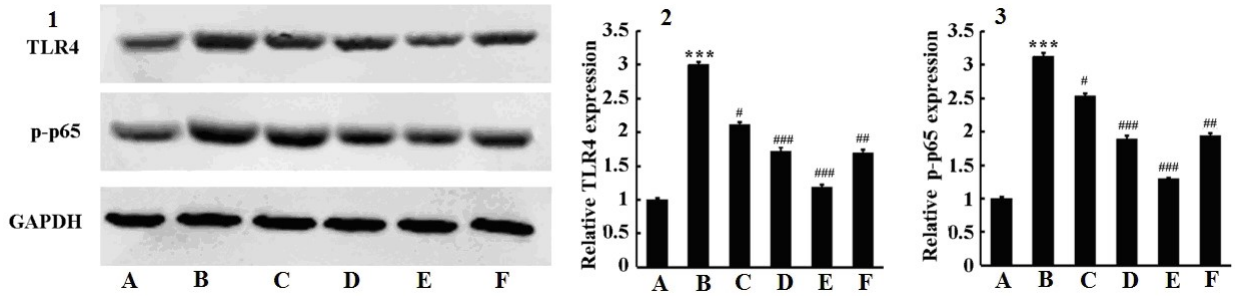
Alcohol use constitutes a large economic and health burden worldwide. Although alcohol is known to have toxic effects on the liver, an effective and safe drug therapy in this field to manage patients with ALD is not available (Singal *et al.*, 2018). The bioactive substances in berries contain phenolic compounds (flavonoids, such as flavonols, anthocyanins and tannins) and ascorbic acid. These compounds are in charge of various health benefits of berries either individually or in combination. These health benefits include prevention of cardiovascular diseases, inflammation disorders prevention and protective effects against various cancers. The phenolic compounds of *Rubus* could inhibit the peroxisome proliferator-activated gene receptor G (PPAR-γ) and CCAAT enhancer-binding protein (C/EBP) and reduce the lipid synthesis efficiency (Namiesnik *et al.*, 2014; Skrovankova *et al.*, 2015; Zhang *et al.*, 2018). In the present study, we found that the degrees of liver tissue damage and liver cell degeneration in the *Rubus* extract group were reduced and that the proliferation of fibrous connective tissue and inflammatory cell infiltration in the portal area were reduced. A high concentration of the *Rubus* extract resulted in significant improvement of liver injury, which suggesting that the *Rubus* extract could effectively slow down the deposition of fat in the liver, and delay the occurrence of fibrosis.



A: blank control group; B: model group; C: 0.05g/kg *Rubus* extract group; D: 0.125 g/kg *Rubus* extract group; E: 0.259g/kg *Rubus* extract group; F: positive control group.
***p<0.001 vs. blank control group; $^{\#}p$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. model group.

Fig. 4: The serum TNF-α and IL-6 levels of rats in each group.

The inhibition of the persistent activation of inflammatory response in the liver is also a key point in the treatment of ALD (Stickel *et al.*, 2017; Singal *et al.*, 2018). In the present study, we found that the collagen deposition in liver tissue was significantly decreased in the *Rubus* extract group and a high concentration of *Rubus* extract



1: Western blotting image; 2: Quantitative determination of TLR4 protein levels; 3: Quantitative determination of p-p65 protein levels. A: blank control group; B: model group; C: 0.05g/kg *Rubus* extract group; D: 0.125g/kg *Rubus* extract group; E: 0.259g/kg *Rubus* extract group; F: positive control group. ***p<0.001 vs. blank control group; $^{\#}p$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. model group.

Fig. 5: The TLR4 and p-p65 protein levels of rats in each group

resulted in minimal collagen deposition. The *Rubus* extract could reduce the expression levels of TNF-α, HA and IL-6 in the serum of rats with alcoholic liver fibrosis in a concentration-dependent manner. These results indicated that the *Rubus* extract could reduce the excessive inflammatory reaction in the liver and delay the process of alcoholic liver fibrosis.

The TLR4/NF-κB pathway is one of the classic pathways that regulate inflammatory response. When alcoholic liver occurs, the intestinal endotoxin and lipopolysaccharide-binding protein constantly stimulate and activate TLR4 on hepatic stellate cells. Activated TLR4 can phosphorylate NF-κB (p65) into p-p65, and enter the nucleus to stimulate the release of inflammatory factors such as IL-6 and TNF-α continuously, resulting in excessive inflammatory reaction in liver tissue and accelerating liver fibrosis (Tilg *et al.*, 2011; Tang *et al.*, 2017; Wu *et al.*, 2017). The TLR4/NF-κB pathway is a key factor in alcoholic liver fibrosis. The inhibition of the TLR4/NF-κB signaling pathway was an effective way to delay alcoholic liver fibrosis (Suk *et al.*, 2014; Xu *et al.*, 2020; Yang *et al.*, 2020). In the present study, we found that the *Rubus* extract could inhibit the TLR4 and p-p65 expression levels in the liver tissue of rats with alcoholic liver fibrosis. This finding suggested that the *Rubus* extract could inhibit the activity of the TLR4/NF-κB pathway and slow down excessive liver inflammation, thereby delaying alcoholic liver fibrosis.

CONCLUSIONS

In conclusion, we found that the *Rubus* extract could delay the development of alcoholic liver fibrosis by inhibiting the fat deposition in liver tissue, and the activity of the TLR4/NF-κB signaling pathway, and reducing the release of inflammatory factors, such as HA, TNF-α and IL-6. This phenomenon reduced the excessive liver inflammatory reaction, thus delaying the development of alcoholic liver fibrosis. These findings provide new ideas for the prevention of alcoholic liver fibrosis. The relationship between *Rubus* extract and TLR4/NF-κB should be further explored to understand the function of the *Rubus* extract and its application.

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