

Original Research

Gentiana Scabra Bge Extract (GSE) Protects Against Alcoholic Liver Disease by Regulating the TLR4/NF- κ B Pathway in Mice

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Abstract

Background: Alcohol abuse leads to alcoholic liver disease (ALD), for which no effective treatment is yet known. *Gentiana Scabra* Bge is a traditional Chinese medicine; its extract has a significant liver protection effect, but its effects on the mechanism of improving alcohol-induced toxicity remain unclear. Therefore, this study used cell and mouse models to investigate how *Gentiana Scabra* Bge extract (GSE) might affect the TLR4/NF- κ B inflammation pathway in ALD. **Methods:** In mice, we induced the alcoholic liver injury model by applying alcohol and induced the inflammatory cell model by lipopolysaccharide (LPS)-induced macrophages. Using an enzyme-linked immunosorbent assay (ELISA) kit, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and tumor necrosis factor- α (TNF- α) levels were measured in liver tissue; we also performed histological analysis of liver tissue sections to assess the hepatoprotective effect of GSE on alcohol. Using real-time fluorescence quantification, we determined the expression of toll-like receptor 4 (TLR4) and nuclear factor κ B (NF- κ B) mRNA levels; we used Western blotting to detect the expression of TLR4/NF- κ B signaling pathway-related proteins. **Results:** We demonstrate that GSE decreased AST and ALT activity, ameliorated liver dysfunction, decreased cytokine levels, and reduced LPS-induced cellular inflammation. In addition, GSE protected mouse liver cells from the inflammatory response by reducing alcohol-induced liver pathological damage and downregulating genes and proteins such as nuclear factors. **Conclusions:** GSE can attenuate liver injury in mice through the TLR4/NF- κ B pathway by inhibiting the activation of nuclear factors.

Keywords: *Gentiana Scabra* Bge extract; alcoholic liver injury; lipopolysaccharide; alcohol; inflammation

1. Introduction

Alcoholic liver disease (ALD) refers to liver diseases caused by long-term or heavy drinking. ALD is the leading cause of liver cirrhosis and liver-related death worldwide, accounting for 4% of global mortality [1–3]. Alcoholic fatty liver often manifests in the initial stage when it develops into alcoholic hepatitis (ASH), liver fibrosis and cirrhosis, and finally, hepatocellular carcinoma [4,5]. Therefore, controlling ALD at an early stage, such as ALD be controlled before the occurrence of ASH [6], may be of great significance for preventing the development of ALD.

The potential mechanisms of acute alcoholic liver injury relate to oxidative stress, steatosis, endotoxin, immune disorders, and inflammation. However, recent research has focused on the inflammatory pathway of ALD. Increasing evidence shows that ASH is caused by the activation pathway of nuclear factor κ B (NF- κ B) induced by the binding of lipopolysaccharides (LPS) and toll-like receptors (TLRs) [7]. TLRs are pattern-recognition receptors that enable the innate immune system to respond immediately to infection by recognizing bacterial and viral components. Of TLRs, toll-like receptor 4 (TLR4) can initiate the activation and cascade of NF- κ B, further leading to the accumulation of pro-inflammatory cytokines and, eventually, the aggrava-

tion of inflammatory progression [8,9]. A relative lack of drugs can treat alcoholic liver injury in clinics [10,11]. Silymarin, a commonly used anti-inflammatory drug for liver protection, can somewhat alleviate the disease's development, but its effect of protecting liver function is limited because of its poor oral bioavailability [12,13]. Therefore, finding well-evidenced and effective drugs with fewer side effects—without altering the therapeutic effect—remains an important goal.

Gentiana Scabra Bge is a Gentianaceae medicinal plant with dried roots and rhizomes. It has the traditional medicinal effects of clearing away heat and dampness and purging liver and gallbladder fire. The chemical components of *Gentiana Scabra* extract (GSE) are mostly iridoid glycosides, such as gentiopicroside, Swertiamarin, and loganin, which have anti-inflammatory, liver-protecting, and hypolipidemic effects. It has been reported that it can protect the damaged liver by improving mitochondrial dysfunction, reducing P2X7 receptor dependence, resisting oxidative stress, and inhibiting inflammation [14–17]. However, although GSE is a commonly used medicine that was put into research earlier and widely used in clinics, the academic literature on the pharmacodynamics of its liver-protecting effective substances primarily focuses on the



liver-protecting effect of its monomer compound, gentiopicroside. In addition, few studies examine the effect and mechanism of GSE active components in treating ALD. The main active components have not been systematically screened, and the related mechanism remains unclear.

Studying the function and mechanism of active components of traditional Chinese medicine is thus a practical, novel approach to the modern development of ethnic medicine. In this study, the active components of traditional Chinese medicine are used to intervene and prevent non-alcoholic fatty liver disease (NAFLD), providing research ideas for ALD drug development. In addition, GSE is reportedly safe for humans: no apparent side effects have been observed after several months of continuous administration [18]. Therefore, Gentiana's anti-inflammatory effect may have a broad application prospect in ALD treatment.

Based on previous research, we aim to obtain the active anti-inflammatory components of Gentiana by extraction and separation; based on the *in vitro* and *in vivo* models of acute alcoholic liver injury, we detect the protein expression levels of lipid synthesis genes and critical targets of the NF- κ B pathway in the model of alcoholic liver injury using qPCR and Western blotting. In doing so, we aim to preliminarily explore the material pharmacodynamic basis of Gentiana active components in alleviating the inflammatory reaction caused by alcohol, to clarify the potential protective mechanism of Gentiana active components on alcoholic liver injury, and to provide the theoretical basis for future research and drug development in liver protection using Gentiana.

2. Materials and Methods

2.1 Materials

BI fetal bovine serum was obtained from Israel Biological Industries (catalog No. 2120140, Beit Haemek, Israel); DMEM culture medium was from Gibco BRL (catalog No. 8121342, Grand Island, NY, USA); trypsin and penicillin-streptomycin double antibodies were sourced from HyClone, USA (catalog Nos. J210033 and J200012, Logan, UT, USA). MycoLumi™ Mycoplasma Detection Kit (# C0298M, Biyuntian Biotechnology Co., LTD, Shanghai, China). Aspartate aminotransferase (AST) (catalog No. MM-44115M1), alanine aminotransferase (ALT) (catalog No. MM-44625M1), leukocyte interleukin 1 β (IL-1 β) (catalog No. MM-0039M1), leukocyte interleukin 6 (IL-6) (catalog No. MM-0163M1), tumor necrosis factor α (TNF- α) (catalog No. MM-0132M1), and enzyme-linked immunosorbent assay (ELISA) kits were purchased from Jiangsu Enzyme Immunoassay Industrial Co., Ltd., Jiangsu, China. RNA extraction, reverse transcription, and cDNA amplification kits are from TaKaRa Bio (catalog Nos. AL40413A, AL50948A, and AL61810A, Shiba, Japan). Rabbit I κ B α polyclonal antibody, rabbit NF- κ B polyclonal antibody, rabbit TLR4 polyclonal antibody,

and horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) secondary antibody were obtained from Wuhan Proteintech (catalog Nos. 00095768, 00086772, 110521211223, and 20000311, Chicago, IL, USA). Rabbit phosphorylated nuclear factor κ B (Phospho-NF κ B p65, p-NF- κ B) polyclonal antibody was purchased from Cell Signaling Technology (catalog No. 17, Danvers, MA, USA). Gentian medicinal materials were purchased from Jilin Beiyao Medicinal Materials Processing Co., Ltd. (catalog No. Idzc3-20171017-2, Jilin, China) and identified as the dried roots and rhizomes of GSE by Professor Weng Lili of Changchun University of Traditional Chinese Medicine.

2.2 Preparation of GSE

Using 3.0 kg of Radix Gentianae, we added water and decocted twice (the ratio of water to medicinal materials was 10:1 and 8:1, respectively), and each time was 1.5 h. The two filtrates were combined and concentrated into a solution with a mass concentration of 1.2 g/mL (based on the crude drug). We diluted the above solution with water into a sample solution with a mass concentration of 0.1 g/mL, added it to D101 macroporous resin column, eluted and purified it with 30% ethanol, and collected eluent. The eluent was distilled and concentrated under reduced pressure at 60 °C and evaporated to dryness to obtain GSE (the yield of GSE was 7%, and the gentiopicroside content was 54.6 mg/g).

2.3 Cell Culture and Therapy

Mouse Mononuclear Macrophages Cells (RAW264.7) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and retained by the laboratory for preservation. The study was carried out in accordance with the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Changchun University of Traditional Chinese Medicine (Protocol No. 2021220). All cell lines were verified by STR analysis and tested negative for mycoplasma. RAW264.7 cells were inoculated in DMEM medium (hereafter "DMEM") containing 10% fetal bovine serum and 1% penicillin/streptomycin and cultured in a cell incubator with 37 °C and 5% CO₂ and were tested for mycoplasma-free using MycoLumi™ Mycoplasma Detection Kit. When the cell fusion degree reached 80%, the culture was digested with 0.25% trypsin. Then it was subcultured and followed up in subsequent experiments. The experimental groups included a control, a model, and three GSE treatment groups (of different concentrations). According to the method of reference [19], the control group was added to a complete medium, the model group was added to a medium containing 1 μ g/mL LPS solution, and each treatment group was added to a medium containing the corresponding concentration of drugs and 1 μ g/mL LPS for 24 h.

2.4 Animal Treatment

Forty male C57BL/6J mice weighing 20 ± 2 g were randomly divided into groups of 8 mice, resulting in five treatment groups: control, model (50% ethanol, 10 mL/kg, i.g.), positive (tiopronin, 50 mg/kg, i.g.), low-dose GSE, and high-dose GSE (GSE, 200, 400 mg/kg, i.g.). Each received intragastric administration of 10 mL/kg of the respective solution once daily for 1 week. The control and model groups received the same volume of distilled water via intragastric administration. Finally, the mice in each group other than the control were given 10 mL/kg alcohol by intragastric administration 12 h after the final administration and once again at an interval of 12 h, resulting in 3 total administrations [20].

2.5 Cell Viability

The CCK-8 assay was used for testing. First, the range of GSE toxicity to cells was investigated. RAW264.7 cells in the logarithmic growth phase were inoculated into 96-well plates at 1×10^4 cells/well for 12 h, then we set up the control group and different concentrations of GSE treatment groups (0.125, 0.25, 0.5, 1, 2, 4 mg/mL). The control group was added with a complete medium. The treatment group was added with drugs containing the corresponding concentrations. The cell viability was measured after 24 h of culture. Secondly, the effect of GSE on cellular inflammation was investigated. According to the above GSE toxic concentration range and pre-experimental activity results, the appropriate GSE therapeutic concentration was set and divided into control, model, and GSE different concentration treatment groups. A microplate reader measured the absorbance values of each well to calculate the cell viability.

2.6 The Effect of GSE on Biological Parameters in Cells and Mice Liver

RAW264.7 cells in the logarithmic growth phase were inoculated, grouped, modeled, and administered according to the above-described method and cultured for 24 h. The culture medium was collected and centrifuged at 3000 RPM for 15 min, and the supernatant was taken. Following the last gavage, blood was collected from the orbit of the mice, left undisturbed for 30 min, and centrifuged at 3000 RPM for 30 min to separate the serum. The microplate method and ELISA kit instructions directed measurements of AST, ALT, IL-1 β , IL-6, and TNF- α in the serum.

2.7 Pathological Sections of Liver Tissues

The examination was performed using the hematoxylin and eosin (H&E) staining. The liver tissues of mice were cut at the same site of the left hepatic lobe and fixed in 4% paraformaldehyde for 24 h. Routine dehydration, paraffin embedding, histological sectioning, H&E staining, and pathological changes in the liver tissues were observed under a light microscope. Mouse primer sequences used for RT-q-PCR experiments in Table 1.

Table 1. Mouse primer sequences used for RT-qPCR experiments.

Primer name	Primer sequence
β -actin	F-5'/CCACCATGTACCCTGGCATT-3'
	R-5'/CGGACTCGTCATACTCCTGC-3'
TLR4	F-5'/AGGCAGCAGGTGGAATTGTA-3'
	R-5'/GGTCCAAGTTGCCGTTTCTT-3'
NF- κ B	F-5'/CACCGGATTGAAGAGAAGCG-3'
	R-5'/AAGTTGATGGTGCTGAGGGA-3'

TLR4, toll-like receptor 4; NF- κ B, nuclear factor κ B.

2.8 The Effect of GSE on TLR4 and NF- κ B Gene Expression in Cells and Mice Liver

Real-time quantitative PCR (RT-qPCR) was used for detection. Total RNA was obtained from cell samples and mouse liver tissues using an RNA extraction kit, reverse-transcribed into cDNA according to the transcription kit method, and amplified by PCR. PCR reaction conditions were pre-denaturation at 95 °C for 30 s for 1 cycle, denaturation at 95 °C for 3 s, and annealing at 60 °C for 30 s for 40 cycles. According to the relative quantification method, β -actin was used as an internal reference, and the target gene's relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method; the experiment was repeated three times.

2.9 Effect of GSE on TLR4, I κ B α , NF- κ B and p-NF- κ B Protein Expression in Mice Liver

Detection was performed by Western blotting. Total protein was extracted from 100 mg of liver tissue from RIPA lysate at 1:9, which was lysed thoroughly on ice for 30 min and centrifuged at 12,000 r/min for 15 min at 4 °C. The supernatant was then assayed for protein concentration by BCA. After boiling and denaturing, the protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the membrane was transferred and blocked with 5% skimmed milk powder for 1.5 h. TLR4, I κ B α , NF- κ B, p-NF- κ B, and β -actin primary antibodies (dilutions of 1:1000) were added and incubated at room temperature for 2.5 h. TBST was used to wash the membrane three times for 10 min each, and secondary antibodies (dilutions of 1:6000) were added and incubated at room temperature for 1 h. TBST was then used to wash the membrane thrice for 10 min each. Finally, ECL chemiluminescence reagents were added. Imaging was performed using an automatic chemiluminescence imaging analyzer, and Image J 6.0 software (National Institute of Health, Bethesda, MD, USA) was used for analysis. The ratio of gray values of internal reference β -actin was used to express the protein's expression level.

2.10 Statistical Analysis

Statistical analyses were performed with SPSS 22.0 software (IBM SPSS statistics, Chicago, IL, USA). The results were expressed as $\bar{x} \pm s$. For comparisons between

multiple groups, one-way ANOVA was used, and pairwise comparisons were performed using the least significant difference method. Differences were considered statistically significant at $p < 0.05$. Graphing was performed using GraphPad Prism 9.5 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1 Effect of GSE on the Viability of RAW264.7 Cells in Response to LPS

The effect of different GSE concentrations on the viability of RAW264.7 cells was determined, with the results shown in Fig. 1A. Except for the 4 mg/mL group, the cell survival rate of each treatment group was more than 90%, indicating that the cell viability was unaffected and there was no significant toxicity in the concentration range below 2 mg/mL. We also determined GSE's effect on the viability of RAW264.7 cells exposed to LPS, which Fig. 1B shows. Compared with the control group, the viability of RAW264.7 cells in the model group was significantly reduced ($p < 0.01$). Compared with the model group, the cell viability was significantly increased in the 0.5 and 1 mg/mL groups of gentian fraction ($p < 0.01$).

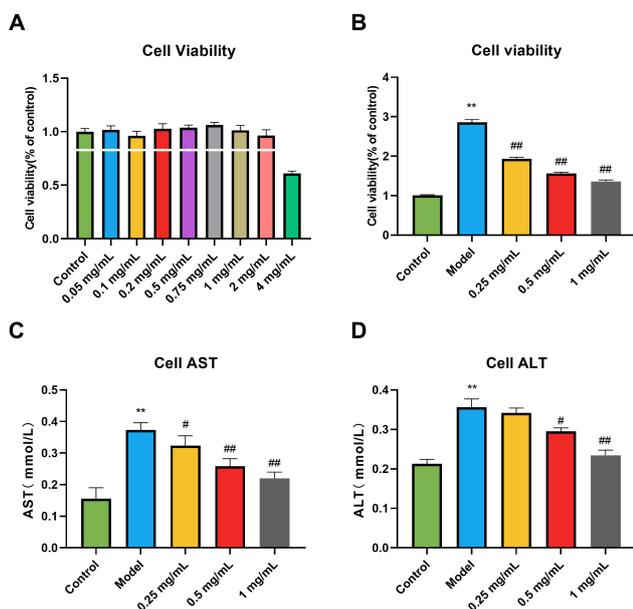


Fig. 1. Effects of *Gentiana Scabra* Bge Extract (GSE) on the cell viability and liver function of RAW264.7 cells induced by lipopolysaccharide (LPS). (A) The range of GSE toxicity. (B) Activity screening of GSE. (C) Alanine aminotransferase (AST) in cell supernatant. (D) Aspartate aminotransferase (ALT) in cell supernatant. All data are presented as the means \pm SDs from three independent experiments performed in triplicate. Note: ** $p < 0.01$ versus the control group; # $p < 0.05$, ## $p < 0.01$ versus the model group.

3.2 Effect of GSE on Liver Function in RAW264.7 Cells Exposed to LPS

We investigated the effect on liver function in RAW264.7 cells exposed to LPS. As shown in the results (Fig. 1C,D), compared with the control group, the contents of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the cells of the model group were significantly increased ($p < 0.01$); compared with the model group, the contents of AST and ALT in the cells of the 0.5 mg/mL and 1 mg/mL groups were significantly decreased ($p < 0.05$ or $p < 0.01$).

3.3 Effect of GSE on Inflammatory Factors in RAW264.7 Cells Exposed to LPS

As shown in the results (Fig. 2), compared with the control group, the contents of interleukin-1 (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor (TNF- α) in the cells of the model group were significantly increased ($p < 0.01$); compared with the model group, the contents of IL-1 β , IL-6, and TNF- α in the cells of the 0.5 mg/mL and 1 mg/mL groups were significantly decreased ($p < 0.05$ or $p < 0.01$).

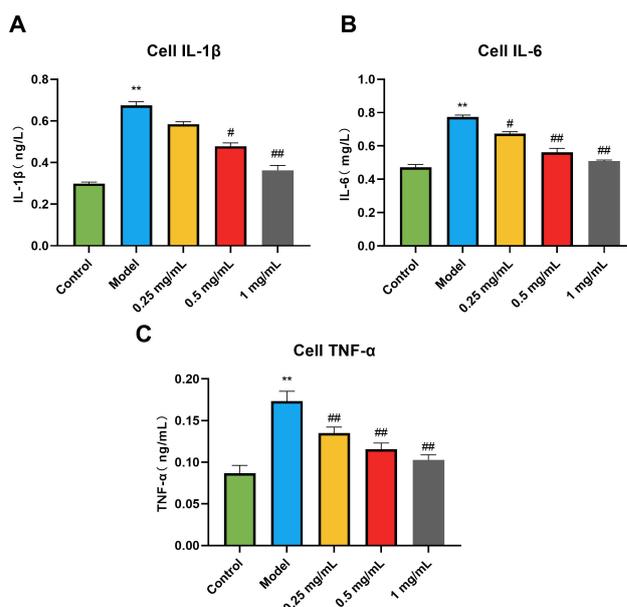


Fig. 2. Effects of GSE on the levels of inflammatory factors of RAW264.7 cells induced by LPS. (A) IL-1 β in cell supernatant. (B) IL-6 in cell supernatant. (C) TNF- α in cell supernatant. All data are presented as the means \pm SDs from three independent experiments performed in triplicate. Note: ** $p < 0.01$ versus the Control group; # $p < 0.05$, ## $p < 0.01$, versus the Model group.

3.4 Effect of GSE on TLR4 and NF- κ B mRNA Expression in RAW264.7 Cells Exposed to LPS

As shown in the results (Fig. 3), compared with the control group, the TLR4 and NF- κ B mRNA contents in the

cells of the model group were significantly increased ($p < 0.01$). Compared with the model group, the TLR4 and NF- κ B mRNA contents in the cells of the 0.5 mg/mL and 1 mg/mL groups were significantly decreased ($p < 0.05$ or $p < 0.01$).

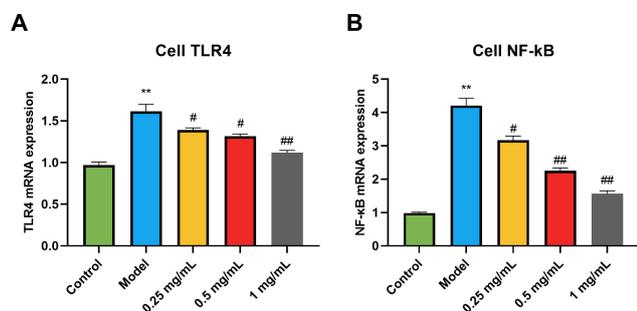


Fig. 3. Effects of GSE on the levels of TLR4, NF- κ B mRNA RAW264.7 cells induced by LPS. (A) The expression of TLR4 mRNA. (B) The expression of NF- κ B mRNA. All data are presented as the means \pm SDs from three independent experiments performed in triplicate. Note: ** $p < 0.01$ versus the Control group; # $p < 0.05$, ### $p < 0.01$, versus the Model group.

3.5 General Examination

Observation of ALD mice from appearance and morphology revealed that mice in the control group had a smooth coat color and animated activity levels. Compared with the control group, the mice in the model group had slow movement, unkempt hair, poor gloss, and murmuring in the lungs. The treatment group's mice were observed with slightly smoother coats, higher activity levels, and improved mental status compared to the model group. Based solely on an isolated comparison of the mice's body weight (Fig. 4A), no significant difference in body weight was found between the groups. However, over time, the body weight of mice in the model group tended to be lower than that in the control group, and the body weight of mice in the treatment group tended to increase. As the changes in the liver index (Fig. 4B) show, compared with the control group, the liver index of mice in the model group became larger, reflecting the liver swelling to a certain extent; compared with the model group, the liver index of mice in the GSE treatment group and the positive group was significantly reduced ($p < 0.05$, $p < 0.01$), improving the liver index.

3.6 GSE Impacts Liver Function Levels in ALD Mice

Changes in liver function parameters were investigated to determine the effects of AST and ALT levels on liver function in the serum of mice with alcoholic liver injury (Fig. 4C,D). Compared with the control group, the enzyme activities of ALT and AST in the serum of mice in the model group were significantly increased ($p < 0.01$);

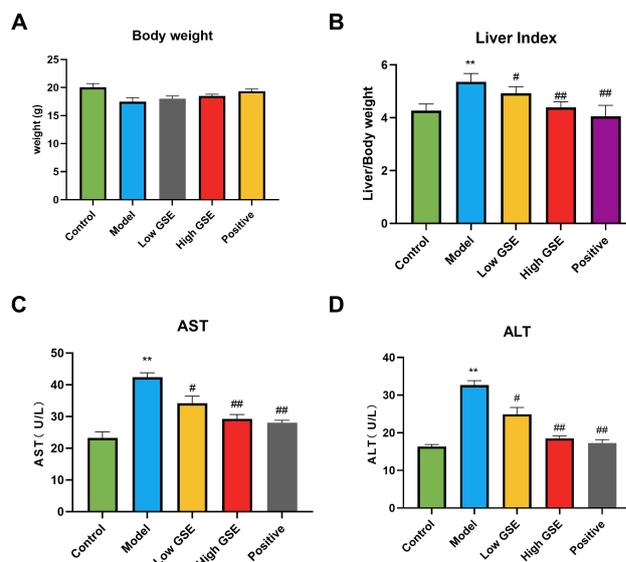


Fig. 4. Effect of GSE on general inspection and liver function in ALD mice. (A) Body weight. (B) Liver index. (C) AST in serum. (D) ALT in serum. All data are presented as the means \pm SDs from three independent experiments performed in triplicate. Note: ** $p < 0.01$ versus the control group, # $p < 0.05$, ### $p < 0.01$ versus the model group.

compared with the model group, the enzyme activities of ALT and AST in the serum of mice in the treatment group were significantly decreased ($p < 0.01$), indicating that GSE could significantly improve alcohol-induced abnormal AST and ALT levels in liver function.

3.7 Histopathological Examination

The results of H&E staining (Fig. 5) showed that the hepatic lobule structure of the control group was intact, the hepatic cords were arranged regularly, and no deformation or necrosis occurred. In addition, the hepatic lobule structure of the alcohol model group was blurred and disorganized, and there was obvious cell nuclear pyknosis and inflammatory cell infiltration of different sizes around the central area. However, in both low and high-dose GSE treatment groups, alcohol-induced pathological changes were alleviated, hepatic cords were arranged neatly, and few inflammatory factors and fat vacuoles were observed. Among them, inflammatory cell infiltration was significantly reduced in the liver group of mice treated with a high dose, and the degree of repair was close to that in the positive group.

3.8 GSE Affects the Serum Levels of IL-1 β , IL-6, and TNF- α in Mice

ELISA measured serum inflammatory factor levels to observe the effect of GSE on liver inflammation in mice with alcoholic liver injury (Fig. 6). Compared with the control group, the levels of IL-1 β , IL-6, and TNF- α in the serum of mice in the model group were significantly in-

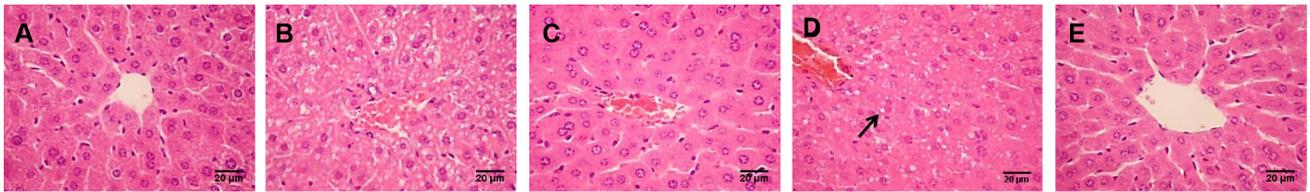


Fig. 5. Pathological sections of liver tissues (H&E staining ×400). (A) Control group. (B) Model group. (C) Low-dose GSE group. (D) High-dose GSE group. (E) Positive group. The scales bar represents 20 μm. The arrows are vacuoles in the liver cells.

creased ($p < 0.01$), and the positive group and the expression of IL-1 β , IL-6, and TNF- α in the GSE-treated group were significantly decreased ($p < 0.01$) compared with the model group, indicating that GSE significantly improved alcohol-induced abnormal levels of inflammatory cytokines IL-1 β , IL-6, and TNF- α .

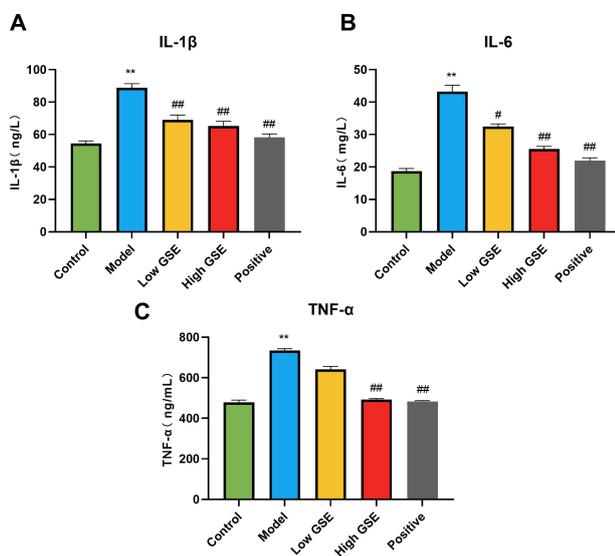


Fig. 6. Effect of GSE on IL-1 β , IL-6, and TNF- α serum levels in ALD mice. (A) IL-1 β level in serum. (B) IL-6 level in serum. (C) TNF- α level in serum. All data are presented as the means \pm SDs from three independent experiments performed in triplicate. Note: ** $p < 0.01$ versus the control group; # $p < 0.05$, ### $p < 0.01$ versus the model group.

3.9 GSE Affects TLR4 and NF- κ B Gene Expression in Mouse Liver

We detected the levels of TLR4 and NF- κ B mRNA in the liver tissue of mice, finding that the mRNA expression levels of TLR4 and NF- κ B in the liver of mice in the model group were significantly increased compared with those in the control group ($p < 0.01$). In addition, the mRNA expression levels of TLR4 and NF- κ B in the liver of mice in the GSE-treated and positive groups were significantly decreased compared with those in the model group ($p < 0.01$), indicating that GSE could significantly improve alcohol-induced abnormal TLR4 and NF- κ B mRNA levels (Fig. 7).

3.10 GSE Affects TLR4, I κ B α , NF- κ B and p-NF- κ B Protein Expression in Mouse Liver

We also investigated the levels of TLR4, I κ B α , NF- κ B, and p-NF- κ B protein in mouse liver tissues, finding the following: compared with the control group, the I κ B α protein content in hepatocytes of the model group was significantly reduced ($p < 0.01$); compared with the model group, the I κ B α protein content in the treatment group was significantly increased ($p < 0.01$); compared with the normal value, the p-NF- κ B protein content in the model group was significantly increased compared with the NF- κ B protein content ($p < 0.01$); and compared with the model group, the p-NF- κ B protein content in the GSE-treated and positive groups was significantly reduced compared with the NF- κ B protein content ($p < 0.01$) (Fig. 8). Thus, GSE alleviates alcohol-induced inflammation by regulating the TLR4/NF- κ B signaling pathway.

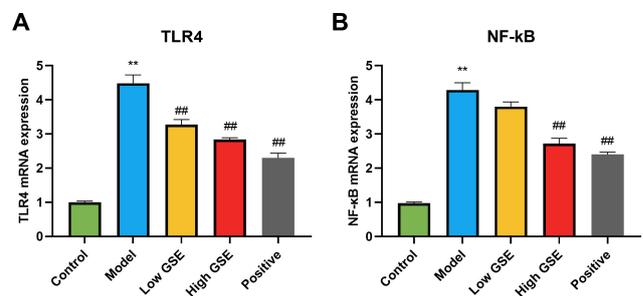


Fig. 7. Effect of GSE on TLR4 and NF- κ B mRNA expression levels in the liver of ALD mice. (A) The relative mRNA expression levels of TLR4. (B) The relative mRNA expression levels of NF- κ B. All data are presented as the means \pm SDs from three independent experiments performed in triplicate. Note: ** $p < 0.01$ versus the Control group; ### $p < 0.01$ versus the Model group.

4. Discussion

GSE acts in various biological functions, including hepatoprotective, cholagogic, and anti-inflammatory, and it has long been studied as therapeutic for chemical and drug-induced liver injuries [21,22]. However, studies on the mechanism of regulating ALD are rare and remain to be clarified. Accordingly, this study established a macrophage inflammation model by LPS induction in mice to explore

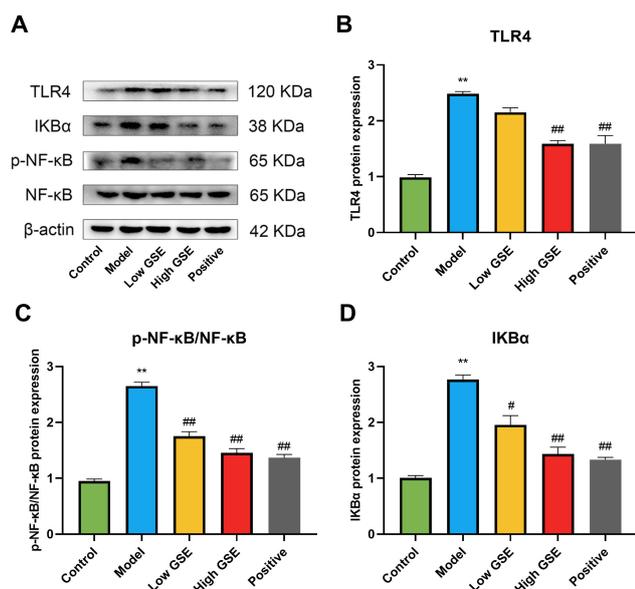


Fig. 8. Effect of GSE on the expression of TLR4, I κ B α , p-NF- κ B/NF- κ B proteins in the liver of ALD mice. (A) The expression level of TLR4, I κ B α , p-NF- κ B/NF- κ B. (B) The relative expression of TLR4. (C) The relative expression of p-NF- κ B/NF- κ B. (D) The relative expression of I κ B α . All data are presented as the means \pm SDs from three independent experiments performed in triplicate. Note: ** p < 0.01 versus the Control group; # p < 0.05, ### p < 0.01 versus the Model group.

the mechanism of action of GSE in ameliorating alcohol-induced inflammatory response. Determination of cell viability revealed that GSE at concentrations below 4 mg/mL was non-significantly toxic, while concentrations of 0.25–1 mg/mL GSE had good anti-inflammatory and hepatoprotective activity. We also measured biological parameters, with results showing that liver function and inflammatory factor levels were abnormally increased in the cell supernatant of the LPS model group.

The liver is the primary organ responsible for metabolism and is subjected to more tissue damage than other organs due to the accumulation of LPS [23]. ALT and AST are common indicators to reflect whether the liver is damaged; ALT is the most abundant in hepatocytes in the body. When hepatocytes undergo inflammation or necrosis, ALT rapidly increases—a sensitive indicator of hepatocyte damage [24,25]. AST is mainly found in the mitochondria of hepatocytes and increases when hepatocytes are destroyed or severely necrotic, while the rise in pro-inflammatory factors also indicates an increase in the inflammatory response [26]. Thus, our results indicate that the study successfully constructed a classical inflammatory cell model, and after GSE treatment, the abnormal increase in liver function and inflammatory factors was significantly reduced. Thus, GSE may be anti-inflammatory and hepatoprotective in mice.

Second, a mouse model of alcoholic liver injury disease was established by alcohol induction. The researchers observed that mice's mental status and body weight changed, and abnormal increases in serum liver function and inflammatory factor levels were detected. However, the AST and ALT liver function parameters and the IL-1 β , IL-6, and TNF- α inflammatory cytokines' levels were significantly decreased by GSE treatment. This finding aligns with reports that alcoholic liver injury can cause abnormal expression of liver function parameters and inflammatory factors in the serum of mice [27,28]. Interleukins and TNF- α play roles in inflammation and can affect immune regulation by activating macrophages. TNF- α is a pro-inflammatory factor produced during TLR4/NF- κ B signaling pathway activation. It acts as the earliest initiator in the inflammatory mediator chain and can regulate the release of other inflammatory factors [29,30].

TLR4 is an innate immune receptor expressed on the surface of macrophages that effectively recognizes pathogen-associated molecular patterns and is the primary receptor for LPS [30–32], which binds to TLR4 and activates nuclear factor- κ B (NF- κ B) through a dependent pathway or interferon regulatory factors [33]. Nuclear factor- κ B (NF- κ B) is a classical nuclear transcription factor that regulates the expression of many genes with essential roles in regulating apoptosis, cell growth, inflammation, and various autoimmune diseases [34,35]. NF- κ B's role in liver-related diseases has been widely reported, including its interactions with liver inflammation, cancer, and injury [36,37]. Therefore, TLR4 is closely related to the NF- κ B pathway during both LPS-induced cellular inflammation and alcohol-induced LPS accumulation-induced inflammation [30,38].

Accordingly, to further clarify its mechanism of action, this study measured TLR4, p-NF- κ B/NF- κ B, and I κ B α 's protein expression and TLR4 and NF- κ B's gene expression, demonstrating that GSE can release NF- κ B by activating the TLR4 receptor and activating I κ B kinase through a complex phosphorylation reaction, which degrades it; NF- κ B also enters the nucleus, binds target gene promoter regions, and regulates the transcription and expression of these genes. Accordingly, the hepatoprotective mechanism of GSE may be due to the activation of TLR4/NF- κ B signaling pathway expression. In addition, nuclear phosphorylated NF- κ B pathway protein p65 expression was significantly increased following TLR4 overexpression in RAW264.7 cells, while nuclear-phosphorylated p65 expression was significantly reduced following decreased TLR4 expression. These experimental results confirm that TLR4 could inhibit macrophage activation and reduce the expression of downstream inflammatory factors may through the NF- κ B signaling pathway, thus reducing the progression of alcoholic liver injury.

In this study, LPS-induced activation of RAW264.7 cells and alcohol-induced TLR4 expression in the liver of

mice were significantly increased. We also find that TLR4 may be involved in macrophage polarization in ALD. Thus, this study reveals that TLR4 regulates the development of ALD by regulating NF- κ B signal transduction, providing theoretical support for further study of ALD. The study of TLR4 expression changes and regulatory mechanisms in ALD is intriguing, and our findings show exciting potential in this field. However, we have only performed simple studies on cells and mice and have not further explored the application of TLR4 in the clinic, and future studies will further explore its exact molecular mechanisms and signaling pathways.

5. Conclusions

In this study, we studied the potential effect of GSE on alcoholic liver injury. First, we found that GPE significantly improved LPS-induced macrophage inflammatory response. Secondly, using the *in vivo* model, the results show that GPE can reduce the infiltration of inflammatory cells in mouse liver caused by alcohol. In addition, the mechanism study shows that GPE down-regulates the levels of inflammatory genes and proteins, which is the potential mechanism of GPE in treating ALD. The current work has laid an important foundation for the relationship between GPE and ALD.

Availability of Data and Materials

Data are available upon reasonable request to the corresponding author.

Author Contributions

LLW and YQ designed the research study; LJW and YXJ conducted the research and wrote the manuscript. CPX advised and guided the research in cell experiments and data interpretation. QY and JS analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All animal procedures were reviewed and approved by Changchun University of Traditional Chinese Medicine's Ethics Committee (Approval No. 2021220).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2811309>.

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