

Dioscorea bulbifera L. induces liver injury in mice partly through bile acids metabolic disorders and gut microbiota dysbiosis mediated by the YY1-FXR/Nrf2 signaling pathway

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Author contributions

Zhao BS contributed to project administration, data curation, and writing the original draft. Xie YD, Zhang YH, and Shi LL were responsible for methodology, data curation, and investigation. Wang X handled project administration, data curation, and investigation. Zhu HZ and Shangguan HZ focused on data curation and investigation. Ding L and Zhu SH contributed to methodology and investigation. Liu JP was involved in writing the review and editing. Ren CC also participated in writing the review and editing.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

DBL, *Dioscorea bulbifera* L.; AKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, Aspartate aminotransferase; BAs, bile acids; BSEP, bile salt export pump; Bax, bcl2-associated x; Bcl-2, B-cell lymphoma-2; CYP7A1, cholesterol 7- α hydroxylase; DILI, drug-induced liver injury; ELISA, enzyme-linked immunosorbent assay; FXR, farnesoid X receptor; GSH, glutathione; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; MDA, Malondialdehyde; SOD, Superoxide dismutase; GSH, glutathione; MRP2, Multi-drug resistance protein 2; Nrf2, Nuclear factor-erythroid 2-related factor 2; NTCP, sodium taurocholate cotransport polypeptide; P-gp, P-glycoprotein; SCFAs, short-chain fatty acids; TCM, traditional Chinese medicine.

Citation

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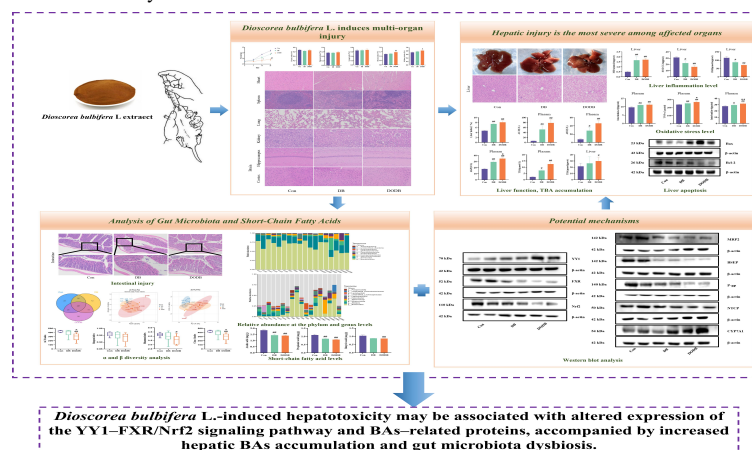
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Abstract

Background: This study aimed to comprehensively elucidate the hepatotoxic effects of *Dioscorea bulbifera* L. (DBL) in mice and to explore the underlying molecular mechanisms. **Methods:** Mice were administered different doses of DBL via intragastric gavage for 21 days. Organ indices of multiple organs were then measured, and histopathological changes were systematically evaluated. Particular attention was paid to biochemical markers and hepatic histopathological alterations associated with liver injury. Subsequently, the composition of the gut microbiota in colonic contents was analyzed using 16S rDNA sequencing, and the levels of short-chain fatty acids (SCFAs) were determined by gas chromatography (GC). Furthermore, protein expression levels of YinYang 1 (YY1), Farnesoid X Receptor (FXR), Nuclear factor erythroid 2-related factor 2 (Nrf2), and bile acids (BAs)-related proteins in the liver were assessed by Western blot analysis. **Results:** Our findings revealed that DBL induced varying degrees of multi-organ toxicity in mice, with the most pronounced hepatotoxicity observed. Liver injury was induced in a dose-dependent manner, accompanied by increased hepatic inflammation and oxidative stress, as well as enhanced hepatocyte apoptosis. Furthermore, DBL may modulate the YY1-FXR/Nrf2 signaling pathway, leading to upregulation of BAs-related proteins such as cholesterol 7 α -hydroxylase (CYP7A1), while downregulating other BAs-related proteins, including sodium taurocholate cotransporting polypeptide (NTCP), bile salt export pump (BSEP), multidrug resistance protein 2 (MRP2), and P-glycoprotein (P-gp). These changes promoted excessive BAs synthesis and impaired the excretion and reabsorption of BAs, ultimately disturbing BAs homeostasis. Additionally, DBL induces gut microbiota dysbiosis and reduces SCFAs production, leading to intestinal damage and disruption of intestinal barrier integrity. **Conclusion:** DBL-induced hepatotoxicity may be associated with altered expression of the YY1-FXR/Nrf2 signaling pathway and BAs-related proteins, accompanied by increased hepatic BAs accumulation and gut microbiota dysbiosis.

Keywords: *Dioscorea bulbifera* L., liver injury, bile acids metabolism, gut microbiota, short-chain fatty acids



Highlights

1. *Dioscorea bulbifera* L. induces dose-dependent multi-organ toxicity, with liver injury as the predominant manifestation.
2. *Dioscorea bulbifera* L. disrupts bile-acid homeostasis via the YY1–FXR/Nrf2 signaling pathway, leading to cholestasis-related abnormalities.
3. *Dioscorea bulbifera* L. induces gut–liver axis dysfunction, including intestinal injury, gut microbiota dysbiosis, and reduced SCFAs, which synergistically aggravates liver injury.

Medical history of objective

Dioscorea bulbifera L. is a species of the genus *Dioscorea*. Its earliest record of internal use as a medicine can be traced to *Qianjin Yaofang*, compiled by the Tang Dynasty physician Sun Simiao, where it was prescribed for “ying,” a traditional term generally interpreted as goiter and related thyroid enlargement associated disorders in modern medicine. The Ming Dynasty classic *Bencao Gangmu* further described it as having the traditional functions of clearing heat and cooling the blood, resolving goiter, and detoxifying. Modern studies have additionally demonstrated that *Dioscorea bulbifera* L. exhibits a broad range of pharmacological activities, including antioxidant, anti-inflammatory, anticancer, and immunomodulatory effects.

Introduction

Dioscorea bulbifera L. (DBL), the dried tuber of the plant *Dioscorea bulbifera* L. in the family Dioscoreaceae, was a traditional Chinese medicinal (TCM) herb characterized by its bitter taste and cold nature, with a long history of clinical use. It was first documented in the “*Qian Jin Yue Ling*” by the renowned Tang Dynasty physician Sun Simiao, and has been traditionally used for cooling blood, reducing goiter, detoxifying, alleviating swelling, and resolving phlegm and nodules. Recent pharmaceutical research has demonstrated that DBL possesses diverse biological activities, including anti-tumor, antibacterial, anti-inflammatory, antioxidant effects, and therapeutic potential in thyroid disorders [1]. However, long-term or excessive use of DBL may lead to a series of adverse reactions, including hepatotoxicity and nephrotoxicity, among which hepatotoxicity is the most prominent [2]. Moreover, the issue of hepatotoxicity in TCM has become the main obstacle restricting its clinical application [3]. Numerous case reports of liver injury have indicated the hepatotoxic potential of DBL and its formulations, with several cases resulting in fatalities [4]. However, in compound formulations, the concurrent use with other herbs often complicates the establishment of a clear causal relationship. Therefore, careful assessment of the hepatotoxicity risk associated with DBL is warranted. The pharmacological and toxicological effects of this herb are primarily attributed to diterpene lactones, which serve not only as its major bioactive constituents but also as the key agents responsible for its toxicity. To date, numerous diterpene lactone compounds, including Diosbulbin A–M and 8-epi-Diosbulbin B acetate, have been isolated and characterized from DBL. Among these, Diosbulbin B (DIOB) and 8-epi-Diosbulbin B acetate are present in relatively high concentrations and exhibit significant biological activities [5]. Although extensive research has been conducted on its toxic manifestations, the precise mechanism underlying DBL-induced hepatotoxicity remains incompletely understood, primarily due to the complexity of its toxicity pathways.

The gut microbiota—functioning as a highly complex, metabolically active secondary organ—has been increasingly recognized as a key modulator in the pathogenesis and progression of multiple liver diseases [6]. The gut–liver axis serves as a critical interface between the gut microbiota and the liver. The portal venous system and the biliary tract constitute the anatomical foundation for bidirectional crosstalk between these two organs: on one hand, they facilitate the

direct translocation of gut-derived microorganisms and their metabolites to the liver; on the other hand, BAs and other bioactive substances synthesized by the liver are excreted into the intestinal lumen via bile, thereby modulating the composition and structural integrity of the gut microbiota as well as altering the composition and stability of the intestinal microbiome [7]. Emerging evidence indicates that gut microbiota dysbiosis compromises intestinal barrier integrity, allowing microbial components to transfer to the liver. Gut microbiota dysbiosis may impair intestinal barrier function and facilitate the translocation of microbial components to the liver, which has been implicated in the progression of liver injury [8]. However, it remains unclear whether DBL-induced hepatotoxicity is associated with gut microbiota dysbiosis, warranting further investigation.

Bile acids (BAs), as key signaling molecules regulating metabolic processes and energy homeostasis, play a vital role in maintaining systemic health. They are among the most closely interacting metabolites with the gut microbiota. Upon entering the intestine, primary BAs are transformed by gut microbial enzymes into secondary BAs. The intestine reabsorbs approximately 95% of BAs, while the rest is carried to the liver through the portal circulation, thereby completing the enterohepatic circulation of BAs [9]. Accumulating evidence suggests that gut microbiota perturbations are closely linked to bile-acid homeostasis and may be associated with inflammatory and oxidative responses in the liver [10, 11]. Therefore, elucidating the mechanism underlying DBL-induced hepatotoxicity through the regulation of BAs homeostasis is of significant importance.

Evidence indicates that administration of DBL induces oxidative stress and upregulates cytochrome P450 (CYP) expression in both cellular and animal models. Furthermore, DBL disrupts BAs homeostasis by modulating the expression of key regulators including CYP, bile salt export pump (BSEP), and sodium taurocholate cotransporting polypeptide (NTCP), thereby contributing to the development of hepatotoxicity [12]. Total bile acid (TBA) has been recognized as an early and sensitive biomarker of hepatotoxicity induced by DBL [13]. Moreover, cholestasis constitutes a critical pathological mechanism underlying DBL-induced hepatic injury. The preservation of BAs homeostasis depends on the coordinated regulation of various BAs-related transporters and metabolic enzymes. YinYang 1 (YY1) a liver-abundant multifunctional protein, influences the onset of numerous liver disorders by regulating BAs metabolism [14]. Farnesoid X Receptor (FXR), a pivotal nuclear transcription factor highly expressed in hepatic tissue, is essential for maintaining bile acid homeostasis. It regulates the activity of transporters and enzymes crucial for the synthesis, transport, metabolism, and reabsorption of BAs, thereby alleviating hepatotoxicity caused by excessive BAs accumulation [15]. Moreover, Nuclear factor erythroid 2-related factor 2 (Nrf2) serves as a pivotal transcription factor that not only constitutes a central defense mechanism against oxidative stress but also modulates efflux transporters to influence the disposition of BAs [16].

Although the existing evidence suggests that DBL-induced hepatotoxicity may be associated with alterations in bile-acid homeostasis, the relationship between its toxic mechanism, BAs homeostasis disturbance, and gut–liver axis-related changes remains unclear [17]. In particular, it has not been systematically investigated whether DBL-induced liver injury is accompanied by alterations in the YY1–FXR/Nrf2 signaling pathway and BAs-related proteins, as well as gut microbiota dysbiosis. Notably, the properties of TCM do not necessarily reflect their biosafety profiles, underscoring the need for toxicological investigations to more accurately evaluate their potential risks [18]. Therefore, we propose that DBL-induced liver injury may be associated with changes in the YY1–FXR/Nrf2 signaling pathway and BAs-related proteins, accompanied by shifts in gut microbiota composition, which together may contribute to the liver-injury process.

To test this hypothesis, 75% ethanol-extracted DBL was administered to healthy male mice to systematically evaluate multi-organ toxicity, with a focus on bile acid-related indices and gut microbial composition. The aim was to explore its key role in

DBL-induced liver injury and provide mechanistic clues for future functional validation. Elucidating the in vivo toxic effects of DBL will provide valuable insights for toxicological risk assessment and support its safer clinical application.

Materials and methods

Materials and reagents

DBL was provided by Shaanxi Xingsheng Pharmaceutica Co., Ltd (Xi'an, China), Lot No. 20220901, and authenticated by Yan Yonggang from the Department of Traditional Chinese Medicine, Shaanxi University of Chinese Medicine. Alanine aminotransferase (ALT) (Lot:20230227), aspartate aminotransferase (AST) (Lot:20230228), alkaline phosphatase (AKP) (Lot:20230224), glutathione (GSH) (Lot:20230227), malondialdehyde (MDA) (Lot:20230227), superoxide dismutase (SOD) (Lot: 20230520) were measured using kits Nanjing Jianjian Bio-Technology Co. (Nanjing, China), TBA Colorimetric Assay kit (Lot:WW03ZBBP5452) and BCA protein concentration kit (Lot: WW05X82R4960) were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Enzyme-linked immune-sorbent assay (ELISA) kits for mouse interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) with lot numbers 03/2023 were purchased from Shanghai Enzyme Linked Biotechnology Co. (Shanghai, China), Nrf2 (Lot: BC05319455), P-glycoprotein (P-gp) (Lot: BA05105288), FXR (Lot: BB04194866), Bcl-2-associated X protein (Bax) (Lot: BB01286971), B-cell lymphoma-2 (Bcl-2) (Lot: BB01286971), and cholesterol 7 α -hydroxylase (CYP7A1) (Lot: BB10218190) were purchased from Bios (Beijing, China). Multidrug resistance protein 2 (MRP2) (Lot:386-C9A09) and NTCP (Lot:3AA49A46) were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Beta-actin (Lot:10028230) and YY1 (Lot: 10005134) were sourced from Proteintech (Wuhan, China). The bile salt export pump (BSEP) (Lot:1077903-3) was purchased from Abcam (Cambridge, UK). Acetic acid (Lot: MKCP2820), propionic acid (Lot: MKCJ6587), and butyric acid (Lot: STBJ4764) were purchased from Sigma-Aldrich (Darmstadt, Germany).

Preparation of the extract of DBL

To prepare the DBL extract, coarse DBL powder was soaked in water for 30 minutes and then subjected to three extraction cycles using eight volumes of 75% ethanol under reflux conditions (2 hours per cycle) and subsequently concentrated under reduced pressure. Chemical profiling of the DBL extract by LC-MS has been previously reported in the literature [19].

Animals and treatments

Healthy male ICR mice weighing 18–22 g (Approval number: SCXK (CHUAN) 2020–0030) were purchased from Chengdu Dossy Experimental Animals Co., Ltd (Chengdu, China). The animals were housed at 23 °C $\pm$ 2 °C and 65% $\pm$ 5% relative humidity, with a standard diet and ad libitum water access. The experimental protocol was approved by the Ethics Committee of Shaanxi University of Chinese Medicine (Approval number: SUCMDL20230505001). All animal experiments were performed in accordance with the guidelines for animal experiments of the Shaanxi University of Chinese Medicine and the National Guide for the Health and Use of Laboratory Animals revised in 1996 (NIH Publication 80–23).

After a 7-day adaptation phase, mice were randomly allocated to three groups. and received daily oral gavage administrations for 21 consecutive days. The control (Con) group received the vehicle (0.5% CMC-Na solution), whereas the DB (low-dose DBL extract) and DODB (high-dose DBL extract) groups were administered 1 g/kg and 2 g/kg of the extract, equivalent to 13.5 g/kg and 27 g/kg of crude herb, respectively. All formulations were prepared by dissolving the test substances in the vehicle immediately prior to administration.

After the final treatment, the mice were fasted for 12 hours prior to anesthesia. All mice were anesthetized with 3% pentobarbital intraperitoneally injected. Blood was drawn into heparinized

centrifuge tubes and kept at 4 °C for 1 hour. Then, the samples were centrifuged at 3,000 rpm for 15 min, and the supernatant was transferred to new centrifuge tubes. The plasma sample was preserved at –80 °C pending further analysis.

Following blood collection, the heart, liver, spleen, lung, kidney, brain, and colon were harvested from the mice and thoroughly rinsed with physiological saline. The weight of each organ was measured to calculate the organ index, which serves as an indicator of potential systemic toxicity. Liver morphology was documented by photography, and representative tissue samples were fixed in 4% paraformaldehyde. The colonic contents and liver tissues were rapidly frozen in liquid nitrogen and subsequently stored at –80 °C for further analysis. Organ-to-body weight ratios were calculated using the following formula: Organ index = [organ mass (g) / body weight (g)] $\times$ 100%.

Histological analysis

The heart, liver, spleen, lung, kidney, brain, and colon were examined using hematoxylin-eosin (H&E) staining. Tissue specimens were preserved in 4% paraformaldehyde, dehydrated progressively in ethanol solutions, and impregnated with paraffin. All experimental steps were carried out strictly according to standardized procedures. The prepared sections were meticulously observed under a light microscope for histological assessment.

Apoptosis in liver tissue was assessed using the TUNEL staining method. Paraffin-embedded mouse liver sections were deparaffinized, rehydrated, and fixed, followed by treatment with TUNEL reaction solution. Following PBS washing, the tissue sections were incubated with DAPI for 5 min and subsequently mounted using an anti-fade mounting reagent. Fluorescent images were then captured and analyzed with a fluorescence microscope.

Biochemical index analysis

The plasma concentrations of ALT, AST, AKP, and TBA were quantified using commercially available biochemical assay kits. Additionally, the levels of IL-1 β , IL-6, and TNF- α in plasma were determined by ELISA. Liver tissues were homogenized and then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatants were collected, and the activities or concentrations of SOD, MDA, GSH, and TBA were measured using corresponding biochemical kits. Experimental results were calculated according to the manufacturer's instructions.

Western blotting analysis

Liver tissue samples were homogenized in lysis buffer, and the supernatant was collected following centrifugation at 12,000 rpm for 15 min at 4 °C. The protein concentration was determined using a BCA kit. SDS-PAGE electrophoresis was conducted to transfer the proteins onto a PVDF membrane. After sealing the membrane with 5% skimmed milk powder, it was incubated overnight at 4 °C with the appropriate primary antibody. After washing with TBST, the membranes were incubated at room temperature for 1 h with the secondary antibody. Protein bands were visualized using enhanced chemiluminescence (ECL) and subsequently subjected to optical density analysis with ImageJ software.

16S rDNA sequencing

Genomic DNA was extracted from mouse colonic contents using a commercial kit. The V4 region of the bacterial 16S rRNA gene was amplified using primers 515F/806R. Amplicons were sequenced on the MGI platform (Maiwei Biotechnology Co., Ltd., Suzhou China). Raw reads were quality-filtered, and high-quality sequences were denoised into amplicon sequence variants (ASVs) using Deblur in QIIME 2. Taxonomy was assigned against the SILVA v138.1 database. Alpha and beta diversity were computed, and intergroup differences in community structure were assessed by PERMANOVA.

Measurement of short-chain fatty acids (SCFAs)

The levels of SCFAs in the colonic contents of mice were determined using a 7890A GC produced by Agilent Technologies, Inc. (Santa

Clara, CA, USA). Standard curves were made with standard solutions of acetic acid, propionic acid and butyric acid at different concentrations for quantitative analysis. 200 mg of feces was accurately weighed and mixed with 1 mL of 5% phosphoric acid solution, and then ground three times in a freezing mill at 50 Hz. The mixture was ultrasonicated in an ice water bath for 30 min, and then left to stand at 4 °C for 30 min. After centrifugation at 4 °C and 13,000 rpm for 15 min, 800 μ L of ethyl acetate was added to the supernatant for extraction. After ultrasonication in an ice water bath for 10 min, the mixture was centrifuged at 4 °C and 13,000 rpm for 10 min. The supernatant was taken and added to the injection bottle. The chromatographic column was WM-FFAP polyethylene glycol (30 m $\times$ 0.32 mm $\times$ 0.5 μ m) from YueXu Technology Co., Ltd. (Shanghai, China), the carrier gas was high-purity nitrogen, and the flow rate was 1.0 mL/min. The injection volume was 1 μ L, and the split ratio was 10:1. The initial temperature of the chromatographic column was 60 °C, which was raised to 140 °C at a rate of 25 °C per minute and maintained for 5 min, and then raised to 220 °C at a rate of 8 °C per minute and maintained for 2 min.

Statistics analysis

SPSS 26 was used for statistical analysis and GraphPad Prism 9.0 software was used for Visualization. Results were presented as mean $\pm$ SD. For data that showed normal distribution and homoscedasticity, the student's t-test was used for comparisons between two groups, while one-way ANOVA was applied for multiple-group comparisons, followed by Tukey's post hoc test. In cases where the assumptions of normality or homogeneity of variance were not met, the Kruskal-Wallis test was employed, followed by Dunn's post hoc test. $P < 0.05$ was considered statistically significant.

Results

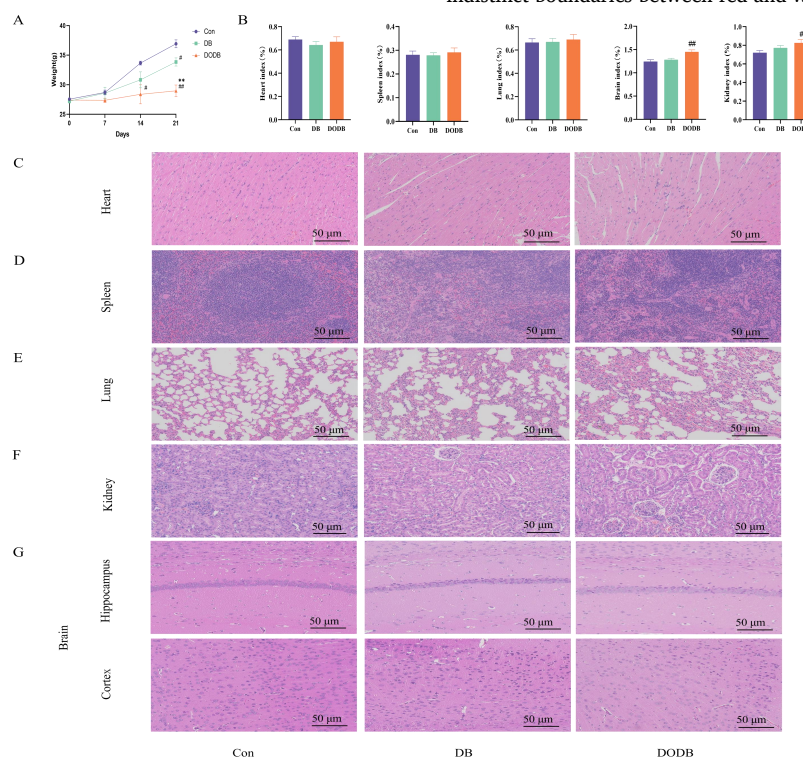


Figure 1 The impact of DBL on body weight, heart, spleen, lung, kidney and brain of mice (n = 8).

(A) Body weight changes in different experimental groups. (B) Organ indices of the heart, liver, spleen, lung, and kidney for each group of mice. (C–G) Histopathological evaluation of mouse heart, spleen, lung, kidney, and brain (H&E, 200 $\times$). Values expressed as mean $\pm$ SEM (n = 8). $^{\#}P < 0.05$, $^{##}P < 0.01$, compared with the Con group values, $^*P < 0.05$, $^{**}P < 0.01$, compared with the DB group values. DBL, *Dioscorea bulbifera* L.; DB, low-dose DBL extract; DODB, high-dose DBL extract.

displayed narrowed and irregular alveolar spaces, accompanied by variable inflammatory cell infiltration and alveolar wall thickening. Renal tubules were dilated, with evidence of epithelial vacuolar degeneration and necrosis, abundant intraluminal casts, and glomerular swelling. In the hippocampal CA1 and prefrontal cortical regions, neuronal arrangement was disorganized, and nuclei exhibited pyknosis, hyperchromasia, and an increased number of necrotic cells (Figure 1C–G).

DBL-induced liver injury and functional abnormalities in mouse

The hepatotoxic effects of different doses of DBL were evaluated through macroscopic inspection and H&E staining. Visual inspection of the livers revealed that mice in the Con group exhibited healthy organs with smooth surfaces, a rich dark coloration, and no visible signs of pathological changes, such as nodules, bruising, or fatty infiltration. In contrast, the livers of mice treated with DB and DODB exhibited clear signs of damage, characterized by rough surfaces, noticeable nodular formations, and dark areas indicative of internal hemorrhage (Figure 2A). Histopathological analysis revealed that hepatocytes in the Con group were uniformly arranged, with intact hepatic cords, well-preserved lobular architecture, and no cytoplasmic vacuolization or inflammatory cell infiltration. By comparison, mice treated with DBL exhibited dose-dependent histological abnormalities, including disorganized hepatic architecture, hepatocyte necrosis, and marked inflammatory infiltration. Notably, the liver demonstrated the most severe and pronounced pathological damage compared to other organs examined (Figure 2B).

To evaluate DBL's hepatotoxic potential, we measured the liver index and relevant plasma biochemical indicators for liver function in mice from various experimental groups. The results demonstrated that, relative to the Con group, the DB and DODB groups exhibited significantly elevated liver indices and increased activities of plasma

AST, ALT, and AKP ($P < 0.05$, $P < 0.01$). When comparing the DODB group to the DB group, liver index and plasma AST and ALT activities showed an increasing trend, although these differences did not reach statistical significance; however, AKP activity was significantly elevated in the DODB group ($P < 0.05$). Furthermore, we measured TBA concentrations in plasma and liver tissues to investigate the effects of DBL on BAs metabolism. The results demonstrated that TBA concentrations in both plasma and liver tissues were significantly elevated in the DB and DODB groups compared to the Con group ($P < 0.05$). Moreover, TBA concentrations in both compartments were higher in the DODB group than in the DB group. Although these differences did not reach statistical significance, they exhibited a pronounced dose-dependent increasing trend. These results collectively indicate that DBL induces hepatic dysfunction and disrupts BAs homeostasis in mice, with evidence of a dose-responsive effect (Figure 2C–H).

DBL-induced inflammatory and oxidative stress responses in mouse liver

To further evaluate the extent of hepatotoxicity induced by DBL, we measured hepatic levels of MDA, SOD, and GSH—key markers associated with oxidative stress. A significant, dose-dependent increase in MDA levels was observed in comparison to the Con group ($P < 0.01$) (Figure 3A). In contrast, SOD and GSH levels decreased with increasing dosage. Both SOD and GSH were significantly reduced in the DB group relative to the Con group ($P < 0.05$), while the DODB group exhibited even greater reductions, with highly significant differences ($P < 0.01$) (Figure 3B, 3C). These findings indicate that DBL promotes the accumulation of oxidative metabolites in the liver while impairing antioxidant defenses, leading to an imbalance between pro-oxidant and antioxidant systems and ultimately contributing to hepatic injury.

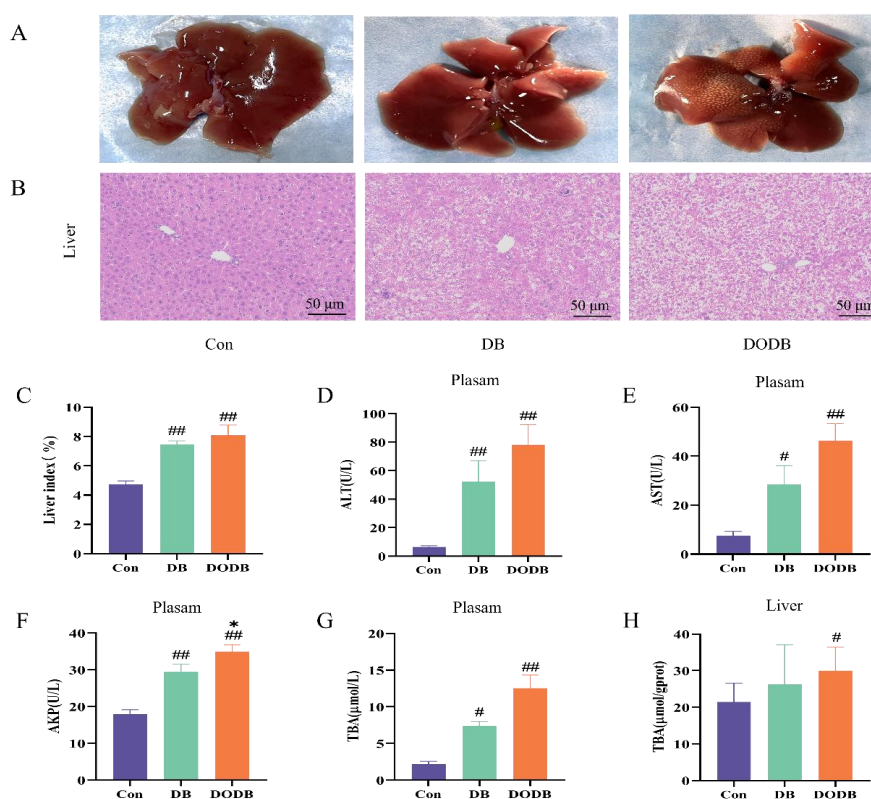


Figure 2 The impact of DBL on liver and function in mice.

(A) Representative images of liver tissues from each experimental groups. (B) Histopathological evaluation of the mouse liver (H&E, 200×). (C) Liver index of mice in each group. (D–F) Plasma activities of ALT, AST, and AKP. (G–H) Plasma and liver concentrations of TBA. Values expressed as mean ± SEM (n = 8). # $P < 0.05$, ## $P < 0.01$, compared with the Con group values, * $P < 0.05$, ** $P < 0.01$, compared with the DB group values. DBL, *Dioscorea bulbifera* L.; DB, low-dose DBL extract; DODB, high-dose DBL extract; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AKP, alkaline phosphatase; TBA, total bile acid.

Furthermore, plasma concentrations of inflammatory cytokines were quantified. The findings revealed a dose-dependent elevation in TNF- α , IL-1 β , and IL-6 levels ($P < 0.01$) (Figure 3D–F). Notably, the DODB group exhibited markedly higher TNF- α and IL-6 concentrations than the DB group ($P < 0.05$). Taken together, these observations indicate that DBL-induced hepatic injury is closely associated with systemic inflammatory activation.

DBL-induced hepatocyte apoptosis in mice

Growing evidence indicates that DBL enhances inflammatory mediator synthesis and elevates oxidative stress in mice, consequently triggering cellular apoptosis. To evaluate hepatic apoptosis, we performed TUNEL staining and Western blot analysis on liver tissues.

TUNEL staining revealed that, compared with the Con group, the green fluorescent signal in mouse liver tissues increased significantly in a dose-dependent manner with escalating DBL administration (Figure 4A), indicating progressively enhanced hepatocyte apoptosis. Furthermore, relative to the Con group, the DODB group exhibited a significant upregulation of the pro-apoptotic protein Bax ($P < 0.05$), whereas the DB group showed an increasing trend without statistical significance. In contrast, the expression of the anti-apoptotic protein Bcl-2 was markedly downregulated in both DB and DODB groups ($P < 0.01$), with more pronounced suppression observed in the latter (Figure 4B). Collectively, these findings demonstrate that DBL induces hepatocyte apoptosis in mice.

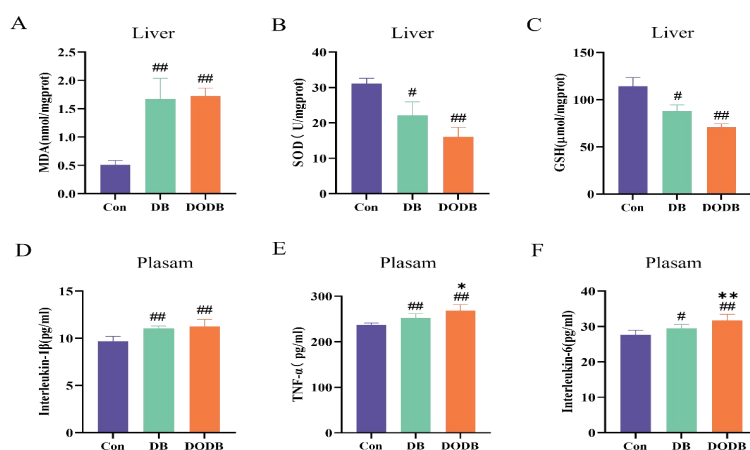


Figure 3 The impact of DBL on plasma inflammation level and liver oxidative stress in mice.

(A–C) Liver oxidative stress levels. (D–F) Plasma levels of inflammatory factors. Values expressed as mean $\pm$ SEM (n = 8). # $P < 0.05$, ## $P < 0.01$, compared with the Con group values, * $P < 0.05$, ** $P < 0.01$, compared with the DB group values. DBL, *Dioscorea bulbifera* L.; DB, low-dose DBL extract; DODB, high-dose DBL extract.

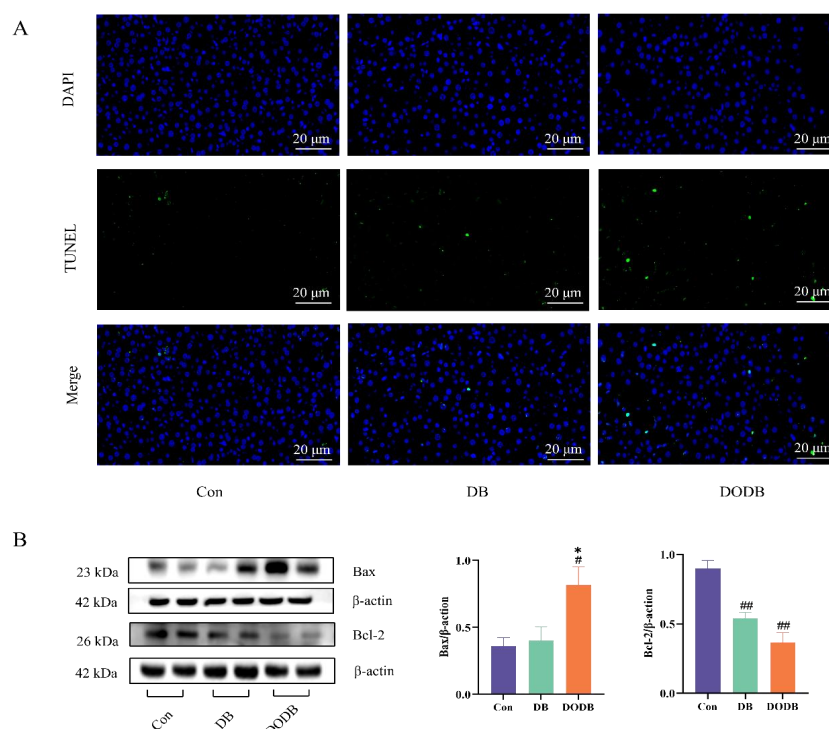


Figure 4 The impact of DBL on hepatocyte apoptosis in mice.

(A) Representative images of TUNEL staining in mouse liver tissue (TUNEL, 400 $\times$) (n = 3) (B) Expression of Bcl-2, Bax protein. Values expressed as mean $\pm$ SEM (n = 3). # $P < 0.05$, ## $P < 0.01$, compared with the Con group values, * $P < 0.05$, ** $P < 0.01$, compared with the DB group values. DBL, *Dioscorea bulbifera* L.; DB, low-dose DBL extract; DODB, high-dose DBL extract.

Effects of DBL on the YY1-FXR/Nrf2 signaling pathway and BAs-related proteins

YY1, FXR, and Nrf2 are pivotal BAs metabolism. Consequently, we investigated the expression levels of proteins essential for the synthesis, excretion, and reabsorption of BAs. Western blot analysis revealed that, compared with the Con group, DBL treatment led to a dose-dependent downregulation of hepatic FXR, Nrf2, MRP2, NTCP, BSEP, and P-gp protein levels ($P < 0.05$, $P < 0.01$). In contrast, YY1 and CYP7A1 protein levels were dose-dependently upregulated ($P < 0.05$, $P < 0.01$). Furthermore, relative to the DB group, the DODB group exhibited significantly lower expression of FXR, Nrf2, MRP2, NTCP, BSEP, and P-gp ($P < 0.01$), along with significantly higher levels of YY1 and CYP7A1 ($P < 0.05$, $P < 0.01$) (Figure 5A, 5B). Collectively, our data indicate that DBL promotes BAs synthesis while suppressing BAs reabsorption and excretion, thereby contributing to intrahepatic cholestasis in mice.

DBL-induced colon injury and gut microbiota dysbiosis in mice

In addition to inducing intestinal tract injury, DBL has been shown to impair intestinal function by disrupting BAs metabolism and altering the composition of the gut microbiota in mice. To evaluate the impact of DBL on intestinal morphology, H&E staining was performed. In the Con group, the intestinal mucosa exhibited an intact architecture, with well-organized and clearly stratified cells in the submucosal layer and uniformly distributed goblet cells. In contrast, DBL treatment disrupted colonic histological structure, characterized by mild edema of the mucosal epithelium, atrophy of glands in the lamina propria, widened interstitial spaces, and focal infiltration of inflammatory cells, with more pronounced damage observed in the DODB group (Figure 6A). Intestinal inflammation is associated with impaired barrier integrity, which may perturb the gut microbial community and subsequently disrupt the enterohepatic circulation of BAs. To evaluate the impact of DBL on the gut microbiota, we subjected fecal samples sourced from the intestines of each experimental group to a 16S rDNA sequencing process. Rarefaction and rank-abundance curves indicated adequate sequencing depth and diverse, evenly distributed microbial communities (Figure 6B). A Venn diagram of amplicon sequence variants (ASVs) revealed distinct microbial compositions among the Con, DB, and DODB groups (Figure 6C). Notably, only 28 ASVs were

shared between the Con and DODB groups, substantially fewer than the 82 ASVs shared between the Con and DB groups and the 41 ASVs shared between the DB and DODB groups, indicating that DBL intervention markedly reshaped the gut microbiota structure, leading to a significant reduction in shared microbial taxa between the treated and Con groups. The Principal Coordinates Analysis (PCoA) and Principal Component Analysis (PCA) indicated that the gut microbiota communities in each mouse group were clearly separated, suggesting that DBL may influence the composition and structure of the intestinal microbiota (Figure 6D). Furthermore, α -diversity index analysis revealed decreased microbial richness and diversity in both the DB and DODB groups compared to the Con group. Specifically, the ACE, Shannon, and Chao1 indices were significantly lower in the DODB group ($P < 0.05$, $P < 0.01$). The ACE and Chao1 indices in the DB group were also significantly reduced relative to the Con group ($P < 0.05$), collectively indicating that DBL negatively impacts complexity and variety of the gut microbiome mice (Figure 6E).

To assess the influence of DBL on gut microbiome structure, we performed a comparative study of gut microbial abundance across various taxonomic tiers within the experimental groups. At the phylum level, *Bacteroidota* and *Firmicutes* were the predominant taxa, with significantly higher relative abundances compared to *Verrucomicrobiota*. Compared with the Con group, the relative abundance of *Bacteroidota* in the DB and DODB groups decreased from 72.29% to 63.46% and 54.71% ($P < 0.05$), respectively, while that of *Firmicutes* increased from 20.92% to 21.51% and 22.92%. This shift was associated with a non-significant increasing trend in the *Firmicutes/Bacteroidota* (F/B) ratio (Figure 7A, 7B). Furthermore, a significant elevation in the relative abundance of *Verrucomicrobiota* was observed in both the DB and DODB groups ($P < 0.05$). At the genus level, the relative abundances of *Alistipes* and *Alloprevotella* were reduced in both treatment groups compared to the Con group, whereas *Akkermansia* exhibited an increasing trend. Unexpectedly, although the relative abundance of *Akkermansia* was similar in the Con and DB groups, it was significantly elevated in the DODB group of mice. We speculate that this observation may be attributable to biological variability among individuals (Figure 7C, 7D). Collectively, these findings demonstrate that DBL induces intestinal damage and disrupts the structural balance of the gut microbiota in mice.

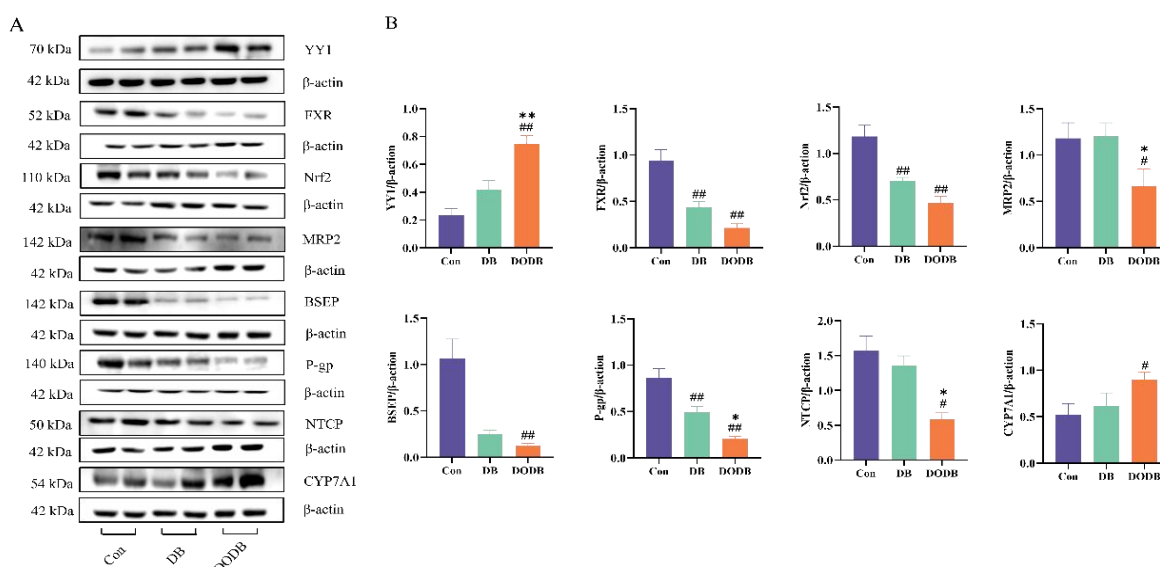


Figure 5 The impact of DBL on YY1-FXR/Nrf2 signaling pathway and BAs-related proteins in mouse liver.

(A) Representative Western blot results for the YY1-FXR/Nrf2 signaling pathway and BAs-related proteins in liver tissue. (B) Expression levels of liver YY1-FXR/Nrf2 signaling pathway and BAs-related proteins in liver tissue. Values expressed as mean $\pm$ SEM ($n = 4$). * $P < 0.05$, ** $P < 0.01$, compared with the Con group values, # $P < 0.05$, ## $P < 0.01$, compared with the DB group values. DBL, *Dioscorea bulbifera* L.; DB, low-dose DBL extract; DODB, high-dose DBL extract. P-gp, P-glycoprotein; CYP7A1, cholesterol 7 α -hydroxylase; MRP2, multidrug resistance protein 2; BSEP, bile salt export pump; NTCP, sodium taurocholate cotransporting polypeptide; FXR, farnesoid X receptor; Nrf2, nuclear factor erythroid 2-related factor 2; YY1, YinYang1.



(A) Histopathological evaluation of the mouse colon (H&E, 200 × and 400 ×). (B) Rarefaction curve and Rank-Abundance curves across groups. (C) Venn diagram of ASVs. (D) PCA and PCoA for each group. (E) α -diversity analysis of the microbiota. Values expressed as mean $\pm$ SEM (n = 6). * P < 0.05, ** P < 0.01, compared with the Con group values, * P < 0.05, ** P < 0.01, compared with the DB group values. DBL, *Dioscorea bulbifera* L.; DB, low-dose DBL extract; DODB, high-dose DBL extract.



(A) Gut microbial composition at the phylum level in different groups of mice. (B) Relative abundance of gut microbiota at the phylum level across groups. (C) Gut microbial composition at the genus level in different groups of mice. (D) Relative abundance of gut microbiota at the genus level across groups. Values expressed as mean $\pm$ SEM (n = 6). * P < 0.05, ** P < 0.01, compared with the Con group values, * P < 0.05, ** P < 0.01, compared with the DB group values. DBL, *Dioscorea bulbifera* L.; DB, low-dose DBL extract; DODB, high-dose DBL extract.

DBL-induced reduction of SCFAs in colonic contents

SCFAs serve as key mediators in the interaction between the host and gut microbiota and are implicated in the development of liver injury [20]. To evaluate the impact of DBL on SCFA levels, we quantified the levels of acetic acid, propionic acid, and butyric acid in mice colonic contents using gas chromatography. Among the measured SCFAs, acetic acid was the most abundant. Compared with the Con group, mice treated with DBL exhibited significantly reduced levels of acetic acid, propionic acid, and butyric acid in the colonic contents ($P < 0.01$) (Figure 8A–C). These findings suggest that DBL exacerbates hepatic injury in mice by suppressing the production of SCFAs.

Discussion

This study systematically evaluated the multi-organ toxicity of DBL in mice and identified the liver as the primary target organ, showing the most prominent pathological alterations. DBL exposure was accompanied by biochemical evidence of liver injury and cholestatic features, together with increased inflammatory responses, oxidative stress, and hepatocyte apoptosis. To further investigate the underlying mechanisms, we found that DBL activates the YY1-FXR/Nrf2 signaling pathway and causes dysregulation of FXR-related proteins expression. At the same time, DBL induces gut microbiota dysbiosis and reduces the levels of SCFAs. These findings indicate that the disruption of FXR homeostasis and alterations in the gut microbiota, may collectively contribute to DBL-induced hepatotoxicity.

Despite its wide range of therapeutic benefits, DBL—a staple in traditional Chinese medicine—faces significant limitations in clinical application due to its potential hepatotoxicity. In addition to hepatic damage, emerging evidence indicates that DBL exerts toxic effects on multiple organs, including the kidneys and heart [21, 22]. To comprehensively assess its multi-organ toxicity, we evaluated organ indices and performed histopathological analyses. Our results indicate that DBL administration may lead to multi-organ damage, with the most severe injury observed in the liver. Macroscopically, the liver exhibited a rough surface with nodular ecchymoses; histopathological analysis revealed characteristic lesions including hepatocyte vacuolization, inflammatory infiltration, and architectural disarray, thereby confirming the liver as the primary target organ of

DBL-induced toxicity. Biochemical markers such as ALT and AST are well-established indicators of liver injury and their elevated levels reflect the extent of liver damage. Meanwhile, elevated AKP activity and TBA levels are commonly used to assess cholestatic liver conditions [23]. In this study, continuous DBL treatment significantly increased plasma levels of ALT, AST, and AKP, as well as TBA activity in both liver and plasma, collectively indicating that DBL induces hepatocellular damage and cholestasis. Additionally, DBL-induced hepatic injury is closely correlated with inflammatory responses and oxidative stress. DBL was found to increase MDA content, decrease GSH and SOD levels, and upregulate the expression of pro-inflammatory cytokines [24, 25]. Our research indicates, prolonged exposure to DBL led to a notable elevation in plasma levels of inflammatory cytokines, accompanied by decreased levels of SOD and GSH, as well as an increase in MDA content. Moreover, significant alterations in apoptosis-related proteins were observed in liver tissue, and TUNEL staining confirmed the occurrence of hepatocyte apoptosis. Importantly, these changes exhibited a clear dose-dependent pattern, suggesting that the severity of DBL-induced liver injury is directly related to the administered dose. Based on these findings—particularly the marked elevation in TBA levels—we propose that the hepatotoxicity of DBL may be closely associated with dysregulation of FXR metabolism.

BAs are crucial endogenous molecules synthesized by hepatocytes, acting as key regulators of metabolic processes [26, 27]. The homeostasis of BAs is primarily controlled through the coordinated action of hepatic enzymes and transporters [28]. Disruption of this regulatory system can lead to the accumulation of intrahepatic BAs, which exacerbates liver injury. YY1, an essential transcriptional inhibitor, is pivotal in managing cellular processes related to lipid breakdown and is recognized as a central mediator in governing inflammation and the immune response in various pathological conditions [29]. Recent evidence indicates that DIOB induces cholestasis by modulating the YY1-FXR signaling axis, thereby suppressing the expression of FXR-related transporters [30], highlighting its critical involvement in cholestatic liver injury. In contrast, FXR, a nuclear receptor for BAs, serves as a central regulator of BAs homeostasis by controlling the expression of key metabolic enzymes and transporters to maintain BAs balance in the liver and intestine [31, 32]. Upon activation, FXR effectively prevents excessive BAs accumulation and subsequent hepatotoxicity through multiple

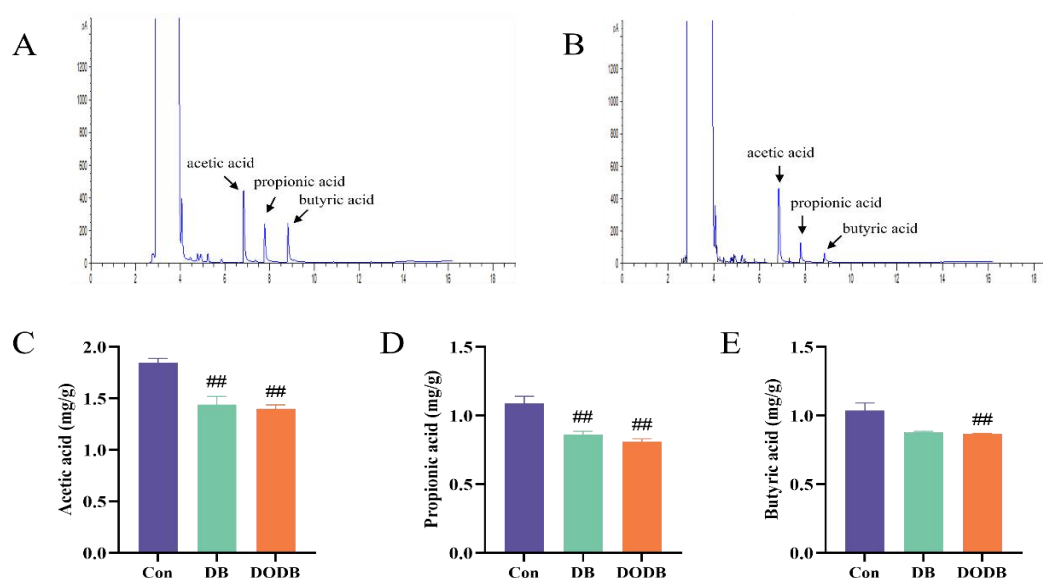


Figure 8 The impact of DBL on the levels of SCFAs in the colonic contents of mice.

(A, B) Representative GC chromatograms of the standard solution and the control blank (from the Con group). (C) Acetic acid level in colonic contents. (D) Propionic acid level in colonic contents. (E) Butyric acid level in colonic contents. Values expressed as mean $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, compared with the Con group values; # $P < 0.05$, ## $P < 0.01$, compared with the DB group values. DBL, *Dioscorea bulbifera* L.; DB, low-dose DBL extract; DODB, high-dose DBL extract.

mechanisms: it downregulates CYP7A1 to reduce hepatic BAs synthesis, upregulates BSEP and MRP2 to promote biliary excretion [33]. Moreover, FXR collaborates with Nrf2—a transcription factor that initiates the expression of antioxidant genes to counteract oxidative stress, but also regulates the expression of genes involved in BAs metabolism and transport. This collaborative interaction contributes to the reduction of intracellular BAs accumulation in hepatocytes through the enhancement of FXR-mediated regulatory pathways [34]. The results of our study demonstrate that DBL activates YY1 expression, which in turn suppresses the FXR/Nrf2 signaling pathway. Specifically, following DBL treatment, YY1 expression was upregulated. At the same time, the FXR/Nrf2 signaling pathway was inhibited, resulting in increased protein levels of CYP7A1 and significantly reduced expression of reabsorption protein NTCP and efflux transporters BSEP, MRP2, and P-gp. Collectively, these findings suggest that DBL-induced liver injury may be associated with the dysregulation of YY1–FXR/Nrf2 signaling pathway and the disruption of intrahepatic BAs homeostasis, including processes involved in BAs synthesis, reabsorption, and efflux, which may contribute to hepatic bile-acid accumulation.

The gut microbiota is crucial for maintaining the overall balance of the organism. Through the gut–liver axis, it establishes a bidirectional regulatory network with BAs, jointly preserving intestinal barrier integrity and metabolic equilibrium. The gut microbiota not only modulates BAs metabolism but is also shaped by BAs composition and signaling [35]. Furthermore, a healthy intestinal barrier effectively blocks pathogenic elements like bacteria and endotoxins from reaching the liver. Dysbiosis and BAs metabolic disturbances can compromise this barrier, increase intestinal permeability, and thereby exacerbate hepatic injury via the enterohepatic circulation [36, 37]. Given this interplay, we further investigated the effects of DBL on the intestine and gut microbiota based on the enterohepatic circulation framework. Our study demonstrates that DBL damages the intestinal structure and compromises intestinal integrity, reduces microbial diversity, increases the F/B ratio, and decreases the relative abundance of beneficial genera. These effects may result from DBL-induced disruption of the intestinal environment, which alters the availability of nutrients and growth factors, thereby promoting the proliferation of potentially harmful bacteria like *Firmicutes* while suppressing beneficial taxa such as *Alistipes*, ultimately leading to dysbiosis [38]. Previous studies indicate that *Bacteroidota* can activate bile salt hydrolase (BSH)-expressing bacteria, and its depletion impairs BAs hydrolysis and excretion in the gut [39]. Moreover, an elevated F/B ratio further exacerbates impaired BAs excretion, resulting in BAs accumulation and facilitating the translocation of harmful substances across the compromised intestinal barrier, thereby

promoting both local and systemic inflammatory responses [40–42]. In this study, we observed excessive accumulation of plasma TBA following DBL treatment, which is associated with intestinal injury. Collectively, our findings suggest that DBL-induced intestinal damage and gut microbiota dysregulation are likely driven by BAs metabolic imbalance, leading to disruption of the enterohepatic circulation and potentially synergizing with inflammatory mediators to promote liver injury. Unexpectedly, the relative abundance of *Akkermansia* was significantly increased following 2 g/kg DBL extract treatment. *Akkermansia* plays a crucial role in regulating immune and metabolic functions, as well as in maintaining host homeostasis [43]. While previous studies have documented beneficial effects of *Akkermansia* in various disease contexts, including cancer and depression, it may exert adverse effects under certain conditions due to multiple influencing factors [44]. Furthermore, alterations in its abundance are closely associated with disease progression [45]. Studies have demonstrated that under conditions of overall gut microbiota dysbiosis, *Akkermansia* may promote mucus layer degradation, impair intestinal barrier integrity, and thereby potentially increase susceptibility to inflammatory responses [46, 47]. However, the precise mechanisms underlying *Akkermansia*'s role and its abundance dynamics in DBL-induced hepatotoxicity remain to be fully elucidated.

Moreover, SCFAs—essential metabolites generated by the microbial fermentation of dietary fibers in the intestine—serve a pivotal function in preserving intestinal homeostasis and regulating overall metabolic equilibrium [48]. SCFAs not only inhibit inflammation and maintain intestinal barrier integrity, but also indirectly modulate hepatic bile acid homeostasis through receptors such as the FXR and G protein-coupled receptors (GPR41/43), thereby attenuating the progression of liver diseases [49, 50]. Our investigation revealed that DBL substantially decreased the levels of acetic, propionic, and butyric acids in mice colonic contents. The research revealed that the depletion of acetic and propionic acids was closely associated with decreased relative abundance of *Bacteroidota* [51], while reduced butyrate acid levels may exacerbate microbial dysbiosis and compromise epithelial barrier function, thereby promoting inflammatory responses [52]. Recent studies have shown that the reduction of SCFAs can disrupt the structure and function of the gut microbiota, thereby compromising BAs homeostasis and exacerbating cholestatic liver injury [53]. This is consistent with our findings. Collectively, our results suggest that DBL-induced reduction in SCFAs production represents a key mechanism underlying disruption of the gut microbiota–BAs axis and subsequent exacerbation of liver injury. Figure 9 illustrates the proposed mechanism of DBL-induced hepatotoxicity.

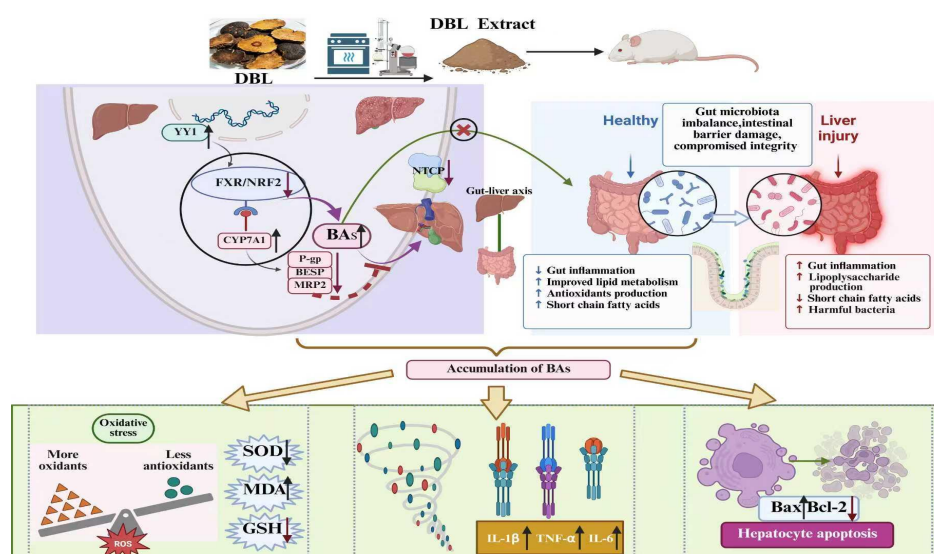


Figure 9 Schematic illustration of the mechanism underlying DBL-induced hepatotoxicity

However, our study has several limitations. First, this study provides preliminary evidence that the YY1-FXR/Nrf2 signaling pathway and BA metabolism-related proteins are involved in DBL-induced liver injury. At present, there is a lack of targeted analysis of BA species and functional validation of this signaling pathway, and the causal relationship cannot be clearly determined. Future studies incorporating targeted BA metabolomics and intervention-based investigation of this pathway, such as YY1 gene knockdown or overexpression, are warranted to test causality. Second, while the gut microbiota is implicated as a potential mediator of hepatotoxicity, our current analysis focuses primarily on taxonomic composition changes without functional characterization of specific bacterial species or causal validation through fecal microbiota transplantation (FMT). Further verification will be pursued through targeted experimental approaches in future studies. Third, the study relies heavily on animal models, which, despite exhibiting consistent overall phenotypes, are subject to considerable inter-individual variability and limited experimental control. Future work will include more precise mechanistic investigations at the cellular level and in vitro validation of key phenotypes and signaling pathways to strengthen the translational relevance of our findings.

Conclusions

To summarize, DBL caused marked liver injury in mice, accompanied by elevated TBA and altered expression of the YY1-FXR/Nrf2 signaling pathway and BAs-related proteins, together with gut microbiota dysbiosis and decreased levels of SCFAs. These findings suggest that disruption of BAs homeostasis and gut microbiota alterations may be involved in DBL-induced hepatotoxicity, providing mechanistic clues for toxicological risk assessment and future mitigation strategies.

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