Scutellarin Ameliorated Carbon Tetrachloride-Induced Chronic Liver Injury in Mice

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Research

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Abstract

Background: Erigeron breviscapus (Vant.) Hand. -Mazz. is an edible and traditional medical herb and its extract scutellarin (SCU) is a widely used flavonoid showing anti-oxidant and anti-inflammatory activities. The purpose of this study was to evaluate the hepatoprotective effect of SCU on carbon tetrachloride (CCl_4)-induced chronic liver injury in mice and reveal the underlying mechanisms.

Methods: Chronic liver injury in mice was induced by intraperitoneal injection of 1 ml/kg CCl_4 every three days. SCU (15 mg/kg, 30 mg/kg and 60 mg/kg) was administered through gavage every day. Bifendate (120 mg/kg) serves as a positive drug to validate the effectiveness of SCU.

Results: The hepatoprotective effect of SCU was confirmed by liver function analysis, histological analysis and TUNEL assay. Administration of SCU recovered the activities of superoxide dismutase (SOD) and reduced the production of malondialdehyde (MDA). Additionally, treatment with SCU significantly decreased the mRNA levels of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α. Moreover, SCU treatment suppressed the activation of NF-κB by decreasing the degradation of IκBα and inhibited the expression of CYP2E1. The 16S rRNA sequencing demonstrated that intake of SCU significantly remodeled gut microbiota, especially enriching the following: *Lactobacillus*, *Coprobacillus*, *Akkermansia*, *Bifidobacterium*, *Parabacteroides*.

Conclusion: Our findings showed that SCU effectively ameliorated CCl_4-induced chronic liver injury. This hepatoprotective effects might be attributed to inhibition of CCl_4-induced NF-κB and CYP2E1 activation and enrichment of beneficial microbial community.

Background

The liver is a central organ and performs numerous vital functions including regulation of glycogen storage and detoxification of metabolites to maintain homeostasis of the body. Due to its multiple metabolic effects, the liver is vulnerable to get injured by long-term drug therapy (such as rifampicin, isoniazid and phenytoin), virus and alcohol, and eventually form chronic liver injury. Repeated hepatic damage leads to the detriment of the hepatic architecture, resulting in functional impairments and jaundice of hepatocytes[1]. As of now, the clinical application of hepatoprotective drugs is limited due to various side effects. Thus, it is of critical importance to develop a hepatoprotective drug with high efficacy and few side effects.

Chronic liver injury is defined by the chronic inflammatory insult to the hepatic parenchyma which results in aggravating fibrosis with ultimate progression to end-stage liver disease over time. The cycle of hepatocyte damage and regeneration not only leads to persistent inflammation but also disrupts the regulation of an intricately balanced relationship between the gut and the liver[2]. The commensal bacteria such as *Lactobacillus*[3], *Bifidobacterium*[4] that have been widely reported to have anti-inflammatory properties and produce beneficial metabolites (such as indoles, propionic acid and

secondary bile acid), and play a vital role in hepatic disease and in preserving the balance of the gut-liver axis[5, 6]. Thus, the focus on them as a therapeutic target to treat chronic liver injury has been growing.

Erigeron breviscapus (Vant.) Hand. -Mazz., a well-known ethnomedicine, has been traditionally used to treat cerebral thrombosis, cerebral hemorrhage and cerebral embolism in Yunnan. Its main extract is scutellarin (SCU), an herbal flavonoid. In recent years, bioactive phytonutrients such as flavonoids and polyphenols derived from plants and foods have attracted considerable attention for hepatoprotection due to their unique bioavailability, multiple targets and low adverse effects[7, 8]. SCU (Fig. 1) possesses multiple beneficial effects including anti-inflammation and anti-oxidation. It has been indicated that SCU administration is safe to various normal cell types[9–11]. Recently, mounting evidence suggests a potential hepatoprotective effect of SCU but the critical mechanism for SCU that protects against liver injury remains elusive[9, 10]. Carbon tetrachloride-induced liver injury in rodent models is widely used to clarify potential for natural compounds to protect the liver against damage due to its similarity with drug-induced liver injury in humans[12]. CCl_4 is a well-known hepatotoxin, mainly metabolized by the hepatic CYP2E1 to yield toxic trichloromethyl-free radical and peroxyl radical. The CCl_4-derived free radicals could attack hepatocyte membranes, resulting in lipid peroxidation, oxidative stress and inflammatory response[13]. NF-κB corresponded to the regulation of pro-inflammatory mediators expression and could significantly induce the expression of various pro-inflammatory genes after translation to the nucleus, thus resulting in adverse consequences[14, 15]. Therefore, CYP2E1 and NF-κB may be potential therapeutic targets of SCU.

In the present study, the therapeutic effect of SCU on CCl_4-induced chronic liver injury was investigated and the underlying mechanisms were explored to develop a potential candidate for chronic liver injury treatment.

Materials And Methods

Chemicals and reagents

Scutellarin (SCU, purity > 97%) and bifendate were purchased from Yunnan Plant Pharmaceutical Co., Ltd (Kunming, Yunnan, China) and dissolved in 0.5% sodium carboxymethyl cellulose (CMC-Na). CCl_4 was purchased from Sinoreagent (Shanghai, China), diluted 1:9 in olive oil. The assay kits for Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin (ALB), malondialdehyde (MDA) and superoxide dismutase (SOD) were purchased from Nanjing jiancheng bioengineering institute (Nanjing, Jiangsu, China). DeadEndTM Fluorometric TUNEL System was purchased from Promega (Wisconsin, USA). TRIzol reagent and RevertAid First Strand cDNA Synthesis Kit were purchased from Thermo Fisher Scientific (NY, USA). TB Green® Premix Ex Taq™ II was purchased from Takara Bio, Inc., (Shiga, Japan). BCA assay kit, RIPA lysis buffer, ECL regents were purchased from Solarbio (Beijing, China). Rabbit anti-NF-κB antibody (#8242, 1:1000), rabbit anti-IκBα antibody (#4812, 1:1000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-CYP2E1 antibody (ab28146, 1:3000), rabbit anti-GAPDH antibody (ab9484, 1:2500) and goat anti-rabbit IgG H&L

(HRP) (ab205718, 1:5000) were purchased from Abcam (Cambridge, UK). All other chemicals and regents were purchased from local firms.

Animal Model

Male BALB/c mice (6–8 week and 18–22 g) were obtained from Tianqin biotechnology Co., Ltd with a certificate of quality No.SCXK-2019-0004 (Changsha, Hunan, China) and acclimatized for 7 days. All animal experiments were performed in accordance with the guidelines of the Care and Use of Laboratory Animals of the Laboratory Animal Ethical Commission of Dali University and all efforts were taken to minimize animals suffering. Chronic liver injury in mice was induced by intraperitoneal injection of 1 ml/kg CCl_4 (diluted 1:9 in olive oil, Sinoreagent, Shanghai, China) every three days. SCU (**PubChem ID 185617**) (15 mg/kg, 30 mg/kg, 60 mg/kg, Yunnan Plant Pharmaceutical Co., Ltd, Kunming, Yunnan, China) was given by gavage every day. The groups were treated as follows (n = 10 each group): (1) Normal group (N) was given an equal volume of solvent (0.5% CMC-Na) and an equal volume of olive oil. (2) Model group (M) was given an equal volume of solvent and 1 ml/kg CCl_4. (3) Low SCU treatment group (ML) was given 15 mg/kg SCU and 1 ml/kg CCl_4. (4) Middle SCU treatment group (MM) was given 30 mg/kg SCU and 1 ml/kg CCl_4. (5) High SCU treatment group (MH) was given 60 mg/kg SCU and 1 ml/kg CCl_4. (6) Positive drug group (BIF, Bifendate served as a positive drug) was given 120 mg/kg Bifendate and 1 ml/kg CCl_4. All animals were treated for an additional 5 weeks. Mice were euthanized and blood samples along with stools and liver tissues were collected 12 h after the injection of CCl_4.

Liver Function and Oxidative Stress Assessment

Blood samples were centrifuged at 2000 rpm and 4℃ for 7 min to obtain serum. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL) and albumin (ALB) were measured according to the manufacture's instructions (Nanjing jiancheng bioengineering institute, Nanjing, Jiangsu, China). Liver tissues were homogenized for 1 min in physiological saline, the homogenates were centrifuged at 12000 rpm and 4℃ for 30 min and the supernatant was collected. Commercial kits were used to determine the superoxide dismutase (SOD) and malondialdehyde (MDA) levels (Nanjing jiancheng bioengineering institute, Nanjing, Jiangsu, China).

Histological Analysis

Liver samples were fixed in 4% paraformaldehyde and embedded in paraffin wax. Embedded tissues were cut into 4 μm thick sections and stained with hematoxylin–eosin (H&E) for the histological analyses. The histological score of sections was assessed by two pathology experts (Table S3).

TUNEL Assay

Cell apoptosis in the liver tissue was detected by DeadEndTM Fluorometric TUNEL System according to the manufacture's protocol (Promega, Wisconsin, USA). Paraffin sections were briefly digested by 20 μg/mL of proteinase K solution for 7 min and then equilibrated with equilibration buffer for 10 min at room temperature. The sections were incubated with TdT reaction mix for 60 min at 37℃ in a humidified chamber before being transferred to 2 × SSC buffer for 15 min to stop the reaction. After immersing in propidium iodide solution (PI) for 15 min at room temperature in the dark, the sections were analyzed under a fluorescence microscope. Areas of apoptosis were quantified by Image J (National Institute of Health, Bethesda, MA, USA).

Immunohistochemistry (IHC)

Immunohistochemistry for CYP2E1 was performed with the paraffin-embeded liver sections. Antigen retrieval was carried out by incubation in EDTA buffer after the slides were deparaffinized. Endogenous peroxidase in the section was blocked with 0.3% hydrogen peroxide solution in the dark for 10 min. The sections were incubated with primary antibody against mouse CYP2E1 (1:500, Abcam, Cambridge, UK) at 4℃ for 60 min and then with HRP-conjugated secondary antibodies at room temperature for 15 min. Finally, the sections were stained with DAB substrate and counterstained with hematoxylin. Areas of CYP2E1 expression were quantified by Image J (National Institute of Health, Bethesda, MA, USA).

Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted from liver tissues with TRIzol reagent (Invitrogen, Thermo Fisher Scientific, NY, USA). 3 μg of total RNA was reverse-transcribed into complementary DNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, NY, USA) following the supplier's protocol. The PCR primer sequences are listed in Table 1. RT-qPCR was performed in a StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific, NY, USA) in combination with TB Green® Premix Ex Taq™ II (Takara Bio, Inc., Shiga, Japan). The reaction was as follows: a precycling stage at 95℃ for 30 s, 40 cycles of denaturation at 95℃ for 5 s and annealing at 60℃ for 30 s. The expression levels, which were normalized to GAPDH, were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primers for real-time PCR

Genes	Forward primers (5'-3')	Reverse primers (5'-3')
IL−6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL−1β	TGTGAAATGCCACCTTTTGA	GGTCAAAGGTTTGGAAGCAG
TNF-α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
CYP2E1	TTTCCCTAAGTATCCTCCGTGAC	CTTAATCGAAGCGTTTGTTGA
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC

Western Blot

The total protein from liver tissues was extracted by RIPA lysis buffer (Solarbio, Beijing, China) on ice and then quantified using BCA protein assay kit (Solarbio, Beijing, China). The protein was resolved through SDS-PAGE gel separation with a Bio-Rad electrophoresis system and transferred to polyvinylidene difluoride membranes (Millipore, MA, USA). The membranes were blocked with 5% skim milk (BD, USA) for 1.5 h. And then incubated with the following antibodies overnight at 4°C: anti-CYP2E1 (Abcam, Cambridge, UK, 1:2500), anti-IκBα (Cell Signaling, MA, USA, 1:1000), anti-NF-κB P65 (Cell Signaling, MA, USA, 1:1000) and anti-GAPDH (Abcam, Cambridge, UK, 1:2500) antibodies. The blots were incubated with HRP-conjugated secondary antibody (Abcam, Cambridge, UK, 1:8000) at room temperature for 1 h and then quantified by G:BOX gel imaging system (Syngene, Cambridge, UK).

16S rRNA gene sequence analysis

The sequencing service was provided by Personal Biotechnology Co., Ltd. (Shanghai, China). The fecal DNA from each group was briefly extracted by QIAamp DNA Stool Kit (Qiagen, Valencia, USA) and quantified by Nanodrop (Thermo Fisher Scientific, NY, USA). The bacterial 16S rRNA gene V3–4 region was amplified by PCR using the forward primer (338F: 5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer (806R: 5'-GGACTACHVGGGTWTCTAAT – 3'). The PCR products were separated and purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Shanghai, China) according to manufacturer's recommended instructions. The sequencing library was built up with obtained products and then sequenced on a MiSeq sequencing platform (Illumina, USA). The sequencing data analysis was carried out as previously described[16]. The Chao1, Shannon and Pielou indices were calculated for α-diversity evaluation. UniFrac-based principal coordinates analysis (PCoA) was employed to assess β-diversity. The different abundant bacteria among all groups were performed by hierarchical clustering heatmap. The correlation between gut microbiota and liver injury indicators was analyzed using spearman index. All figures were performed by Personalbio genescloud (https://www.genescloud.cn/chart/). The raw data were deposited into NCBI Sequence Read Archive (SRA) database and accession numbers are SRR12278403-SRR12278407.

Statistical analysis

Statistical analysis was performed with SPSS 22.0 (IBM Corp., NY, USA). All data are presented as the mean ± SD (n = 5). Comparisons between groups were assessed by a one-way analysis of variance (AVONA), and Dunnett's test was employed as a post hoc test. Statistical significance was set at $P < 0.05$.

Results

Effects of SCU on serum parameters

In order to assess the degree of liver injury, we detected the levels of ALT, AST, TBIL and ALB in serum. We also used bifendate (BIF) as a positive control, which is widely prescribed to treat hepatic injury. As presented in Fig. 2, the serum ALT, AST, TBIL levels in the M group were elevated obviously compared to those in the N group. In contrast, SCU treatment significantly blunted the serum ALT, AST, TBIL levels in the CCl_4-exposed mice. As for ALB, no significant difference was observed among the five groups. These results suggest that SCU harbors hepatoprotective properties against CCl_4-induced chronic liver injury.

Effects of SCU on Histopathologic Changes

H&E staining was performed to evaluate the protective effects of SCU on CCl_4-induced chronic liver injury. As shown in Fig. 3, in the N group, the hepatic lobule structure was clear, the hepatic cells were arranged in neat row and there were no hepatocyte necrosis, hepatocyte swelling and inflammatory cell infiltration. After repeated injection of CCl_4, large areas of necrosis accompanied with inflammatory cell infiltration were apparent in the central parts of the hepatic lobule portal area. Hepatocyte necrosis areas and inflammatory cell infiltration were reduced in the SCU treatment group in a dose-dependent manner. The histopathological results demonstrate that SCU treatment effectively alleviates CCl_4-induced hepatocyte necrosis and inflammatory cell infiltration.

Effects of SCU on CCl4-Induced Hepatocyte Apoptosis

As shown in Fig. 4, TUNEL assay demonstrated that compared with the N group, there was a large amount of hepatocyte apoptosis that occurred in the liver tissue of the M group, while in SCU-treatment groups, hepatocyte apoptosis was significantly decreased in a dose-dependent fashion. These results imply that SCU treatment effectively attenuates CCl_4-induced hepatocyte apoptosis.

Effects of SCU on Liver Oxidative Stress

In order to quantify oxidative liver injury, we measured the levels of SOD and MDA in the liver. As shown in Fig. 5, compared with the N group, SOD activity was decreased significantly, and MDA level was increased significantly in the M group. SCU treatment significantly inhibited MDA level and increased SOD activity. These data demonstrate that SCU exerts hepatoprotective effects by alleviating oxidative stress.

Effects of SCU on the Pro-inflammatory Cytokines Production

Inflammatory response is an important factor that leads to liver injury, we then assessed the IL-6, IL-1β and TNF-α mRNA levels that are the key inflammatory cytokines in the liver tissues by RT-qPCR. In the M

group, the IL-6, IL-1β and TNF-α mRNA levels were rapidly increased but oral administration of SCU resulted in significant attenuation of these pro-inflammatory cytokines in the liver (Fig. 6). These data hint that the hepatoprotective effects of SCU are achieved by suppressing inflammatory response.

Effects of SCU on NF-κB Activation

We further explored the underlying anti-inflammatory mechanism of SCU against CCl_4-induced chronic liver injury. IκBα and NF-κB play a critical role in the production of inflammatory cytokines such as IL-6, IL-1β and TNF-α, thus we detected the changes in the protein levels of IκBα and NF-κB. As shown in Fig. 7, after CCl_4 intoxication, the protein level of IκBα was decreased significantly and the protein level of NF-κB was increased significantly. Compared with the M group, the protein levels of IκBα were increased and the protein levels of NF-κB were decreased in the SCU treatment group. These data suggest that SCU suppresses inflammatory response through inhibiting the activation of NF-κB by decreasing the degradation of IκBα.

Effects of SCU on CYP2E1 Expression

CYP2E1 have been deemed as a marker of chemical liver injury, playing a pivotal role in CCl_4-induced liver injury. We then examined the expression of CYP2E1 mRNA and protein in the liver tissue through IHC, RT-qPCR and western blot, respectively. As shown in Fig. 8, the expression of CYP2E1 mRNA and protein were dramatically up-regulated in the M group, which were then dose-dependently inhibited by SCU treatment. These data suggest that the hepatoprotective effects of SCU are achieved by suppressing CYP2E1 expression.

Effects of SCU on gut microbiota diversity and composition

Gut microbiota plays a pathogenic role in the development of many disease and modulation of the microbiome is a potential therapeutic approach for prevention of liver injury[16, 17]. Moreover, in our previous work, we proved that SCU markedly modulated the gut microbiota (Table S1, Figure S1). Therefore, we hypothesized the SCU exert its hepatoprotective effect through modulation of the gut microbiota. In order to verify the conjecture, we analyzed the effects of SCU on the diversity and composition of gut microbial community in CCl_4-exposed mice by 16S rRNA sequencing. Repeated injection of CCl_4 significantly raised the α-diversity of the gut microbiota as indicated by the Chao1, Shannon and Pielou indices that represent the richness, diversity and uniformity of gut microbiota, respectively. Treatment of SCU significantly reduced richness, diversity and uniformity with lower Chao1, Shannon and Pielou indices compared with those of the M group (Fig. 9A). β-diversity was used to compare the similarity of the overall community structure, which employed an unsupervised multivariate statistical assessment such as PCoA. PCoA displayed a marked structure shift in the M group in contrast to that of N group, while after daily SCU treatment, gut microbiota was restored to be similar with that of

N group (Fig. 9B). Furthermore, a taxon-based analysis was used to clarify the alteration of gut microbiota in each group at phylum level. Overall, a total of twenty phyla were shared from all five groups (Fig. 9C). Bacteroidetes and Firmicutes were selected due to their quantitative dominance in the microbial community. CCl_4 injection significantly raised the relative abundance of Bacteroidetes and reduced the relative abundance of Firmicutes compared to the N group. SCU treatment significantly raised the relative abundance of Bacteroidetes compared to the M group (Fig. 9D). Altogether, SCU administration had a substantial effect on remodeling the gut microbiota in response to CCl_4.

Effect of SCU on key phylotypes of gut microbiota

To further investigate the differences of various bacteria in five groups, we used a hierarchical clustering heatmap based on genus level, and the major genera ranking the top forty from each group were selected (Fig. 10A, Table S2). Although LefSe analysis indicated that no genus is specific for any group, there were five genus exhibited obvious differences. As shown in Fig. 10B, *Lactobacillus*, *Coprobacillus*, *Akkermansia*, *Bifidobacterium*, *Parabacteroides* were evidently higher in SCU treatment groups relative to the M group. To investigate the relationship between gut microbiota and liver injury indicators, the top forty genera in all samples and AST, ALT, TBIL, ALB, SOD, MDA, IL-6, TNF-α, IκBα, NF-κB, CYP2E1 were analyzed using the spearman index. As shown in Fig. 10C, *Lactobacillus*, *Coprobacillus*, *Akkermansia*, *Bifidobacterium*, *Parabacteroides* had beneficial effects on the liver because they are negatively related with AST, ALT, TBIL, MDA, IL-6, TNF-α, NF-κB, CYP2E1 and positively related with ALB, SOD, IκBα. These data suggest *Lactobacillus*, *Coprobacillus*, *Akkermansia*, *Bifidobacterium*, *Parabacteroides* may be the specific genera that are responsible for the hepatoprotective effects of SCU.

Discussion

Chronic liver injury induced by long-term drug therapy, virus and alcohol occurs frequently in clinic, which brings a considerable burden on the healthcare system and the pharmaceutical industry. Natural products such as silymarin and oleanolic acid act as hepatoprotective drugs due to high efficiency and few adverse effects, making the development of hepatoprotective agents based on natural products popular worldwide[18, 19]. SCU is an herbal flavonoid, possessing various beneficial effects and widely used to treat cardiovascular and cerebrovascular diseases in China. Recently, emerging studies have suggested that SCU has the potential for liver protection but its mechanisms of hepatoprotective effect are still unclear. In this study, we investigated the impacts of SCU on CCl_4-induced chronic liver injury as well as revealed potential mechanisms underlying the hepatoprotective effect of SCU.

CCl_4-induced hepatocellular damage and the subsequent rupture of the plasma membrane cause the release of intracellular enzymes such as ALT and AST into the extracellular space. The released enzymes eventually enter the circulation, thereby increasing serum ALT and AST levels. Serum ALT and AST levels reflect hepatocyte damage and are considered to be a quite specific clinical biomarker of hepatotoxicity[20]. In our study, we clearly observed that CCl_4 intraperitoneal injection dramatically

increased the levels of serum ALT and AST compared to the N group. Most importantly, our results demonstrated that serum ALT and AST levels were alleviated by SCU in a dose-dependent manner. The concentration of bilirubin in the serum is determined by the balance between bilirubin production and clearance by hepatocytes. Elevated serum bilirubin levels may be caused by release of unconjugated or conjugated bilirubin from injured hepatocytes. TBIL is another specific biomarker of liver injury. The results showed that SCU treatment dose-dependently reduced serum TBIL level. The liver is the exclusive site of synthesis of ALB. Thus, serum ALB serves as true test of hepatic synthetic function. However, serum ALB has a very long half-life, which may not be affected in the short-term experimental liver injury model and low serum albumin may be a sign of advanced liver disease[21]. Our results confirmed that Both CCl_4 intoxication or SCU treatment did not produce any apparent effects to ALB. A great many studies demonstrate that lipid peroxidation and oxidative stress are vital factors for hepatic dysfunction in the CCl_4-induced mice[22]. CCl_4-induced lipid peroxidation and oxidative stress can bring injury to the lipids, proteins and nucleic acids, ultimately inducing liver injury. MDA, the final product of lipid peroxidation, can be detected to estimate the severity of CCl_4-induced lipid peroxidation. SOD, an antioxidant enzyme with high endogenous expression in the liver, is the major defense mechanism against reactive oxygen species[23]. In our study, ingestion of SCU significantly repressed the increase of MDA in the liver induced by CCl_4 and markedly enhanced SOD activity. An excessive inflammatory response can worsen injured hepatic tissue and hinder repair processes[24]. Inflammatory response can be activated at the local sites of injury after hepatic damage, followed by elevated expression of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α, which can subsequently damage endothelial cells to aggravate liver injury[14]. Inflammatory systems operate through various signaling pathways, among which the most important pathway, NF-κB has been long studied as a major target for anti-inflammatory treatments[25]. CCl_4 stimulation leads to the degradation of IκBα, resulting in the free pass and translocation of NF-κB from cytoplasm to nucleus[26]. The activation of NF-κB leads to release of pro-inflammatory cytokines and aggravation of liver injury[27]. Therefore, NF-κB might be a potential target for the treatment of chronic liver injury. The results demonstrated that SCU treatment significantly reduced IL-6, IL-1β and TNF-α mRNA levels, and restored IκBα degradation as well as suppressed NF-κB activation. Histological analysis can directly reflect the physiological and pathological process of tissues; the results showed that intake of SCU significantly reduced necrosis and inflammatory cell infiltration induced by CCl_4. After TUNEL assay examination, it was confirmed that CCl_4-induced hepatocyte apoptosis was weakened by SCU treatment. Taken together, SCU shows the hepatoprotective effect on CCl_4-induced chronic liver injury and the hepatoprotective properties of SCU may be associated with its anti-oxidative and anti-inflammatory capacities.

It is well-documented that CCl_4 mediated by CYP2E1 to generate trichloromethyl free radical that could attack hepatocyte membranes, causing lipid peroxidation, oxidative stress and inflammatory response[28]. At the same time, highly destructive reactive radicals can also significantly elevate the activity of CYP2E1, concentrating the activation of the positive feedback and finally causing severe hepatic damage[29]. Our results showed that SCU significantly down-regulated the CYP2E1 expression

levels in the liver of mice exposure to CCl_4, indicating the beneficial roles of SCU on CCl_4-induced chronic liver injury may be mediated by down-regulating the expression of CYP2E1.

Compelling evidence supports that gut microbiota is related to various liver diseases, such as non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease (AFLD) and hepatocellular carcinoma (HCC)[2, 30, 31]. In this study, the results of the 16S rRNA sequencing demonstrated that parenterally administered (intraperitoneal injection) CCl_4 significantly modulated the composition of gut flora, as shown by the result of α-, β-diversity indices. Oral administered SCU significantly remodeled the gut microbiota, making it similar to the N group. At the genus level, intake of SCU had a selective increase in the enrichment of *Lactobacillus*, *Coprobacillus*, *Akkermansia*, *Bifidobacterium*, *Parabacteroides*. *Lactobacillus* and *Bifidobacterium* are widely known as probiotics for their potential pathogens inhibition, useful metabolites production (butyrate, succinic acid) and intestinal barrier protection[3, 4]. *Coprobacillus* has been found to play beneficial role in maintaining intestinal stability and liver function[32]. *Akkermansia* is also a butyrate-producing genus and has been confirmed to improve immunological disorders and the gut barrier to protect against immune-mediated liver injury[33]. *Parabacteroides* can produce succinic acid, secondary cholic acid and activate different signaling pathways and has been deemed as a potential and new type of probiotics against metabolic syndrome and liver injury[34]. Moreover, their metabolites such as short chain fatty acids can maintain gut mucosal permeability and function, preventing the translocation of microbiota-derived toxin (such as LPS) to the liver to induce inflammation[35]. Short chain fatty acids can also activate hepatic GPCRs via the portal vein and/or systemic circulation, leading to the suppression of pro-inflammatory mediators such as IL-6, IL-1β and TNF-α[4].Correlation analysis also demonstrated that *Lactobacillus*, *Coprobacillus*, *Akkermansia*, *Bifidobacterium*, *Parabacteroides* showed beneficial effect to the liver. Therefore, the beneficial roles of SCU on CCl_4-induced chronic liver injury may be through raising the abundance of *Lactobacillus*, *Coprobacillus*, *Akkermansia*, *Bifidobacterium*, *Parabacteroides*.

Conclusion

In conclusion, SCU (60 mg/kg) can effectively protect against CCl_4-induced chronic liver injury, which was closely similar to efficacy of bifendate (120 mg/kg) and its mechanism for hepatic protection is through inhibiting NF-κB, CYP2E1 activation and remodeling gut microbiota (Fig. 11).

Abbreviations

SCU, scutellarin; CCl_4, carbon tetrachloride; CMC-Na, sodium carboxymethyl cellulose; ALT, aminotransferase ; AST, aspartate aminotransferase ; TBIL, total bilirubin; ALB, albumin; MDA, malondialdehyde; SOD, superoxide dismutase; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α. H&E, hematoxylin–eosin; IHC, Immunohistochemistry; PCoA, UniFrac-based principal coordinates analysis; ANOVA, analysis of variance.

Declarations

Acknowledgements:

Not applicable.

Author's Contributions:

Z.M. and Y.Z. conceived and designed the research; Z.M. and Y.Z. and C.L. performed the experiments; L.M. and J.Z. performed the statistical analysis; Z.M. and Y.Z. wrote the draft and prepared figures. Y.L. and H.L. reviewed and revised the manuscript.

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Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approved and consent to participate:

All animal experiments were performed in accordance with the guidelines of the Care and Use of Laboratory Animals of the Laboratory Animal Ethical Commission of Dali University (Number: 2017-1201).

Consent for publication:

All of authors consent to publication of this study in Journal of *Chinese Medicine*.

Conflict of Interest:

There are no conflicts of interest in this work.

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Figures

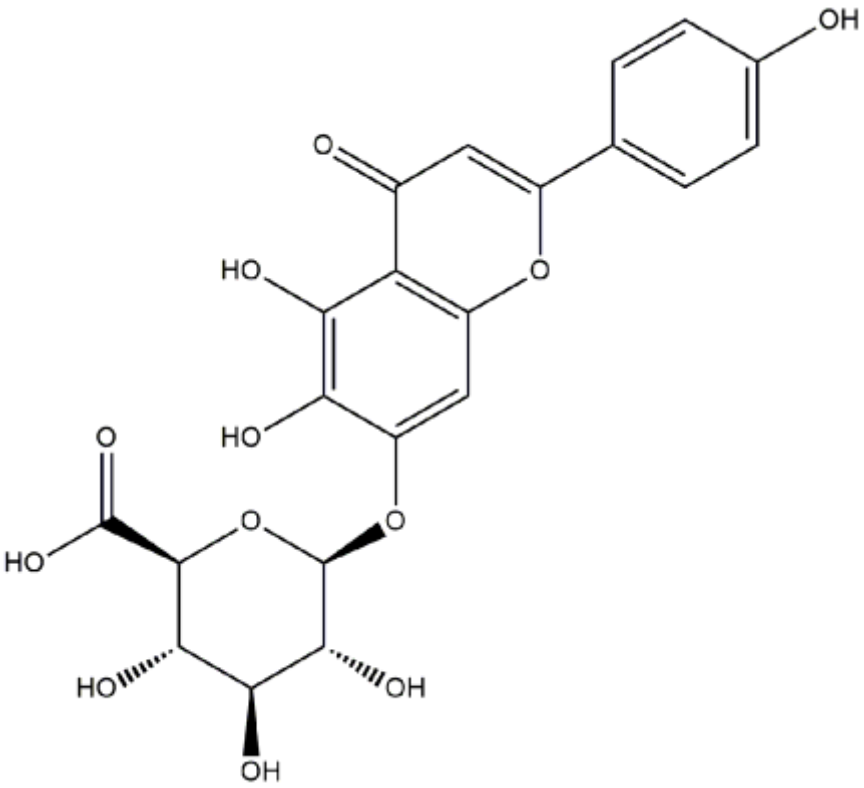

Figure 1

Structure of Scutellarin (SCU)

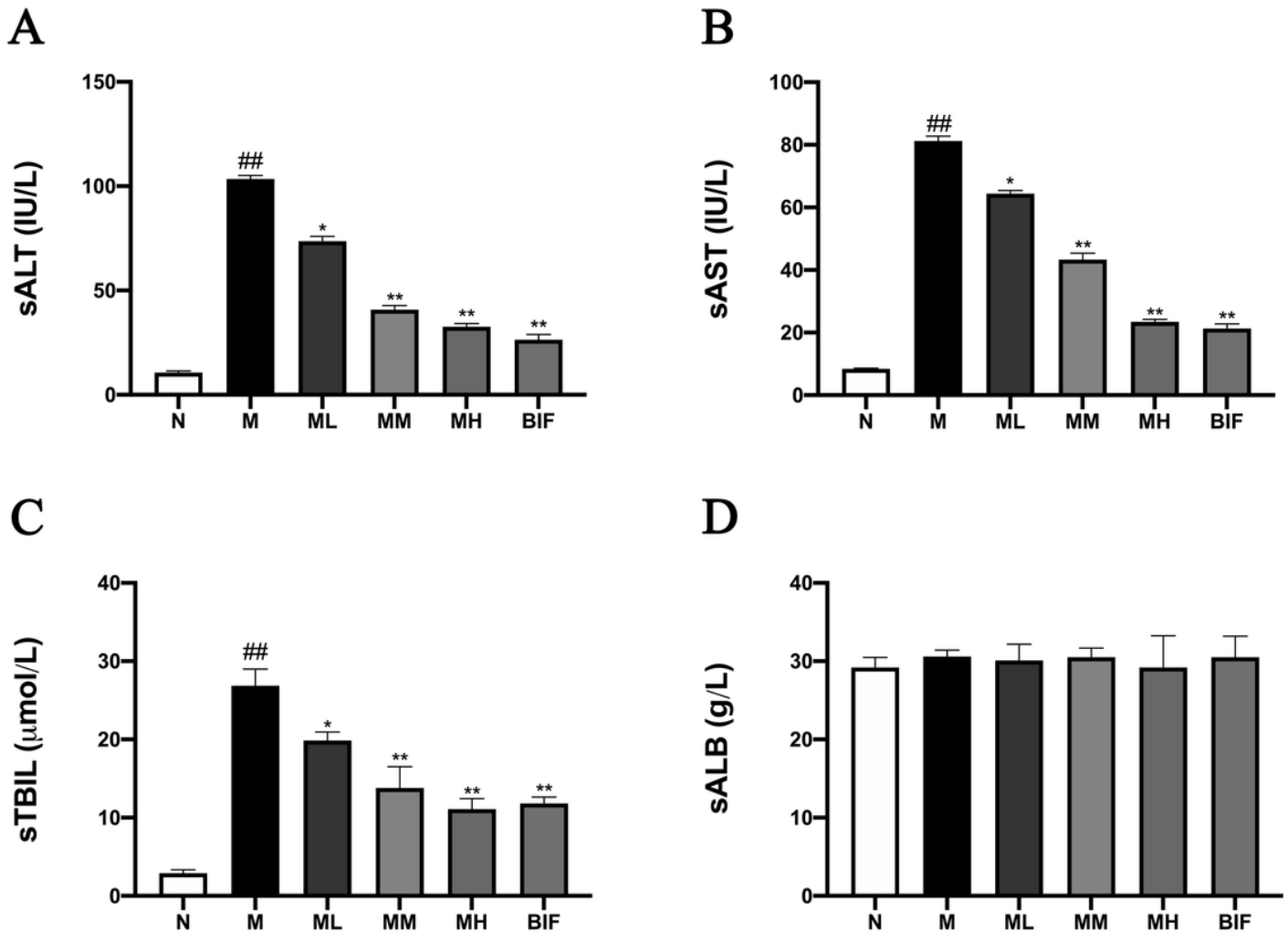

Figure 2

Effects of SCU on CCl4-Induced chronic liver injury. (A) serum alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST), (C) total bilirubin (TBIL) and (D) albumin (ALB) levels. The data and error bars are presented as mean ± SD (n = 5). ##P < 0.01 vs. N group. *P < 0.05, **P < 0.01 vs. M group.

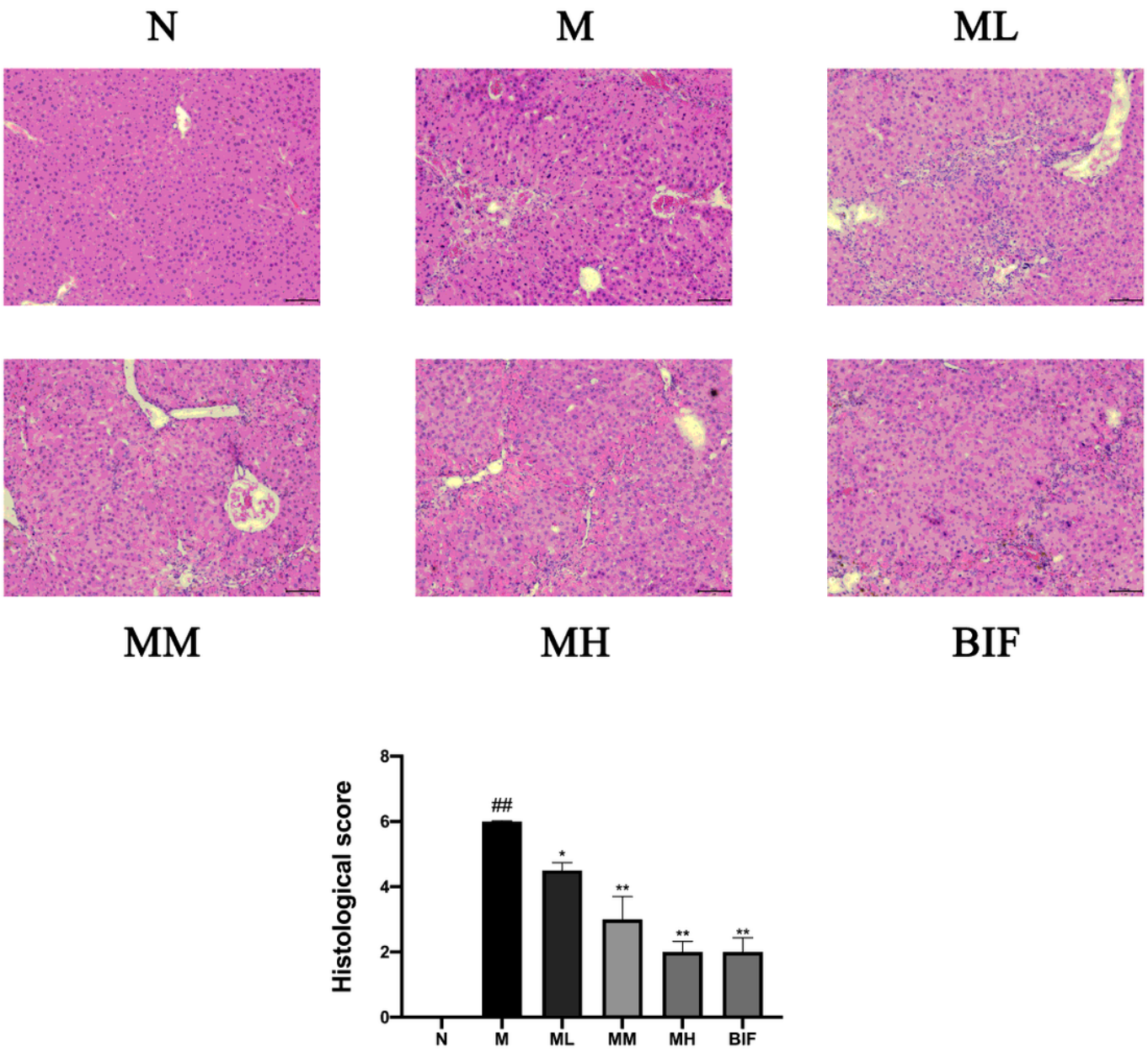

Figure 3

Effects of SCU on Histopathologic Changes. Liver histology (hematoxylin and eosin, H&E) of each group, scale bar 100 μm, magnification 200×. The data and error bars are presented as mean ± SD (n = 5). ##P < 0.01 vs. N group. *P < 0.05, **P < 0.01 vs. M group.

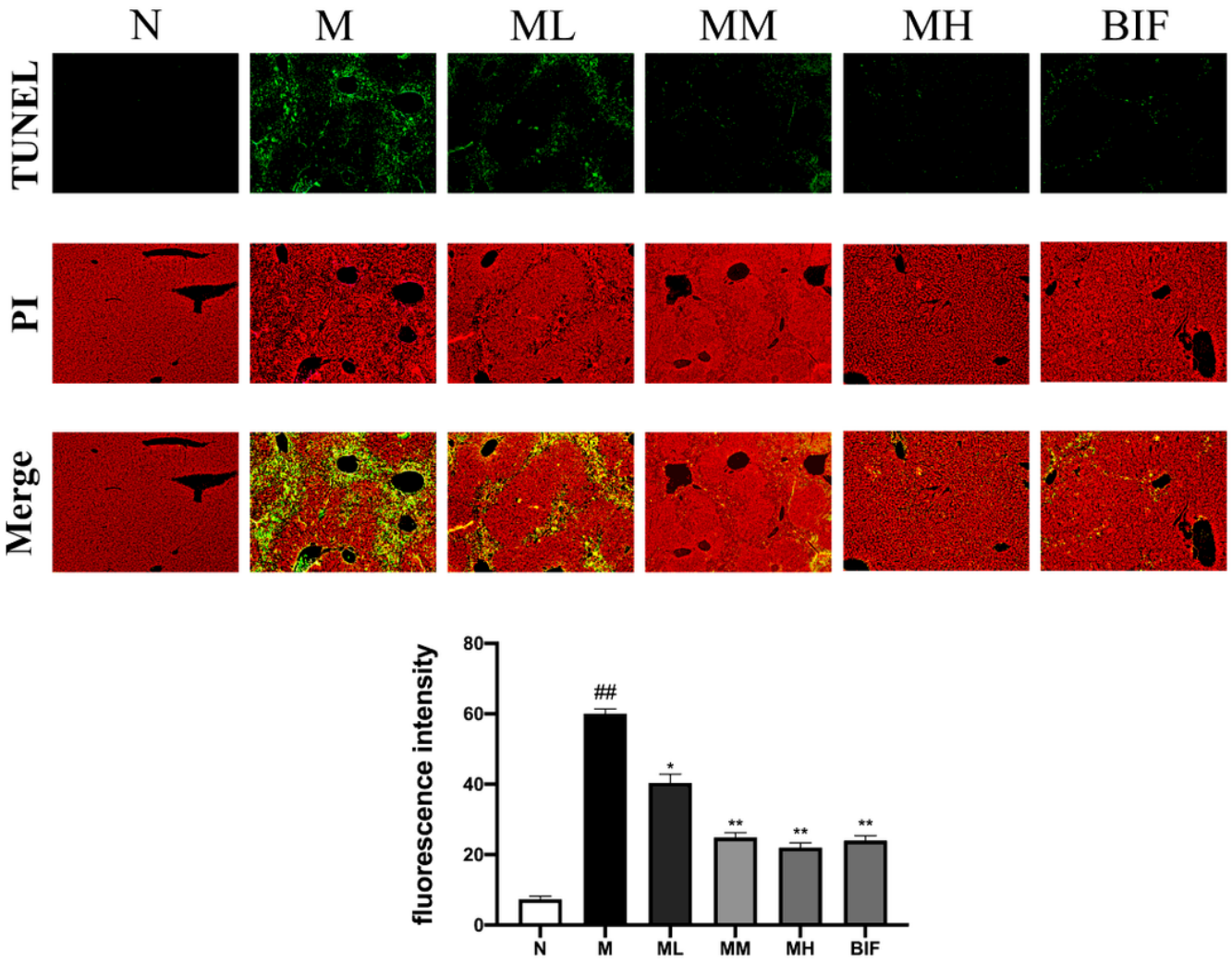

Figure 4

Effects of SCU on CCl4-Induced Hepatocyte Apoptosis. TUNEL assay in the livers from different treatment groups scale bar 100 μm, magnification 200×. The data and error bars are presented as mean ± SD (n = 5). ##P < 0.01 vs. N group. *P < 0.05, **P < 0.01 vs. M group.

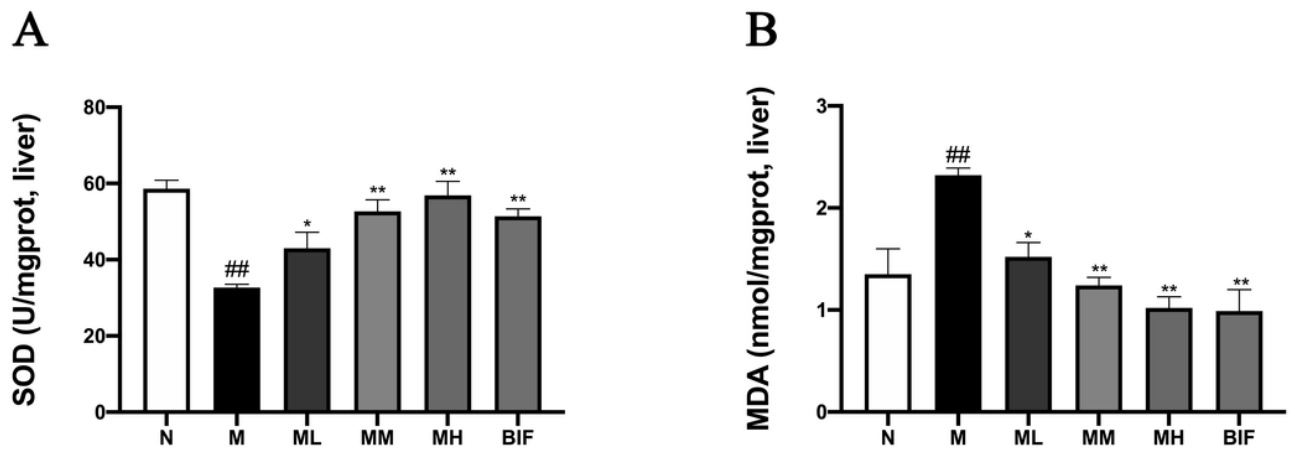

Figure 5

Effects of SCU on Liver Oxidative Stress. (A) superoxide dismutase (SOD) activity and (B)malondialdehyde (MDA) level. The data and error bars are presented as mean ± SD (n = 5). ##P < 0.01 vs. N group. *P < 0.05, **P < 0.01 vs. M group.

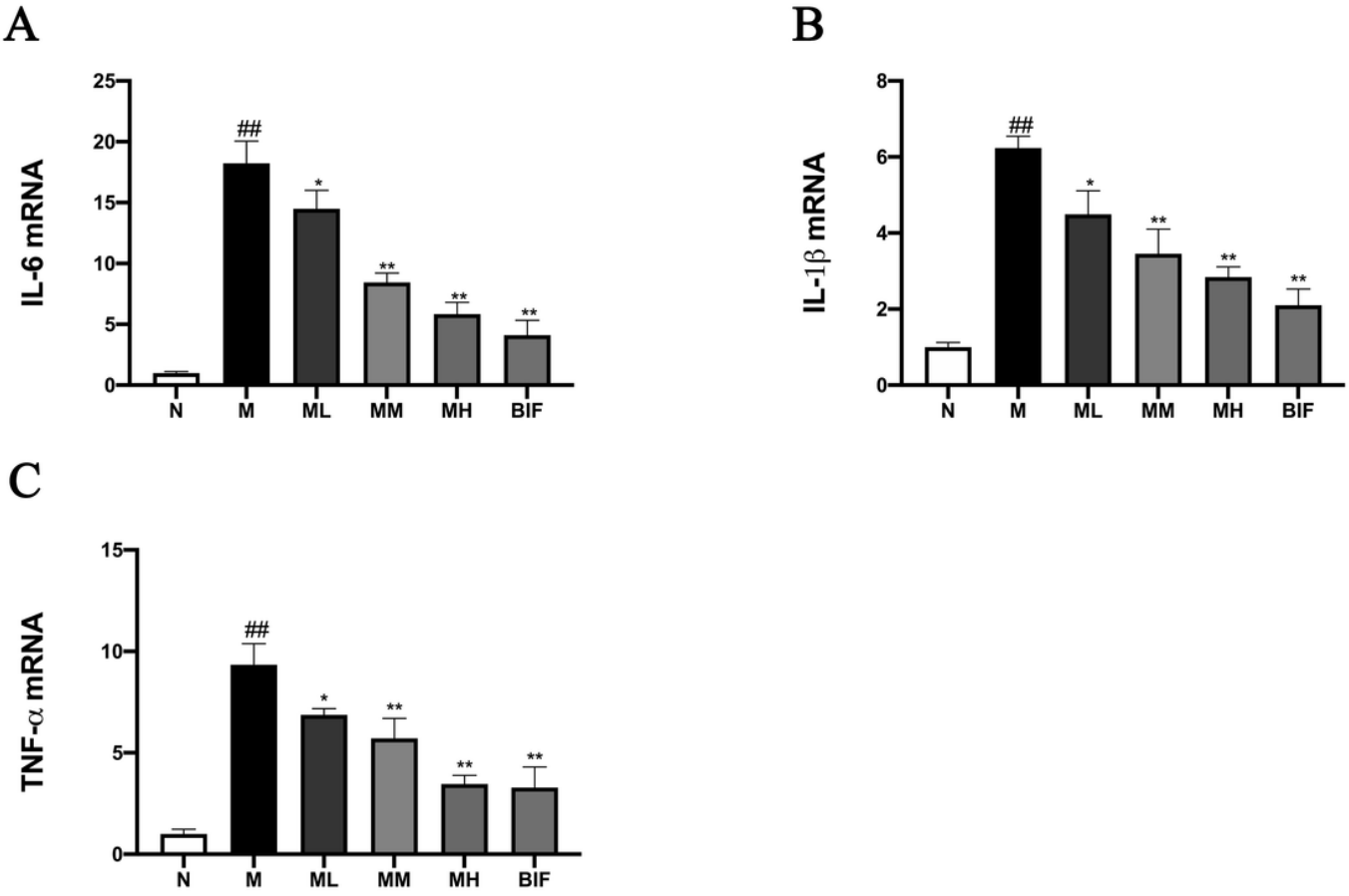

Figure 6

Effects of SCU on the Pro-inflammatory Cytokines Production. Relative expression of (A) IL-6, (B) IL-1β and (C) TNF-α mRNA in the liver tissues. The data and error bars are presented as mean ± SD (n = 5). ##P < 0.01 vs. N group. *P < 0.05, **P < 0.01 vs. M group.

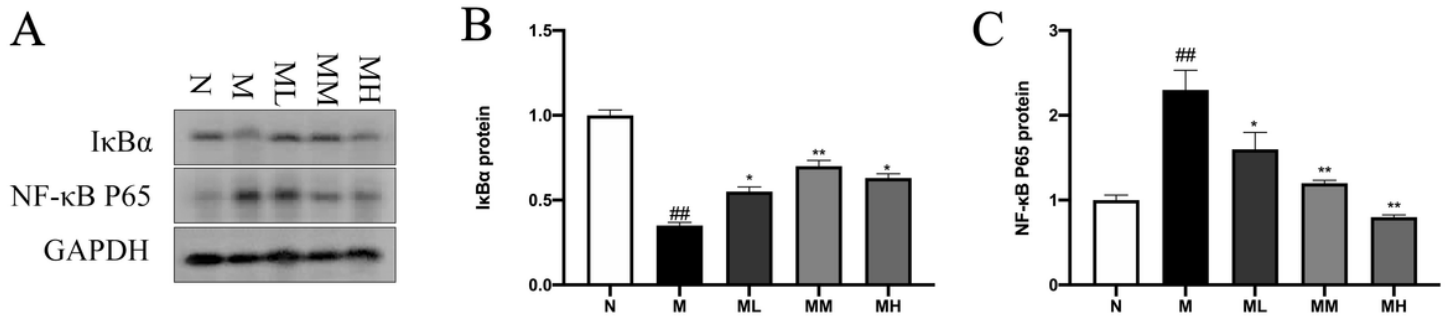

Figure 7

Effects of SCU on NF-κB Activation. (A) Western blotting analysis of IκBα and NF-κB. GAPDH was used to as the loading control. Quantification of the relative protein levels of (B) IκBα and (C) NF-κB. The data and error bars are presented as mean ± SD (n = 5). ##P < 0.01 vs. N group. *P < 0.05, **P < 0.01 vs. M group.

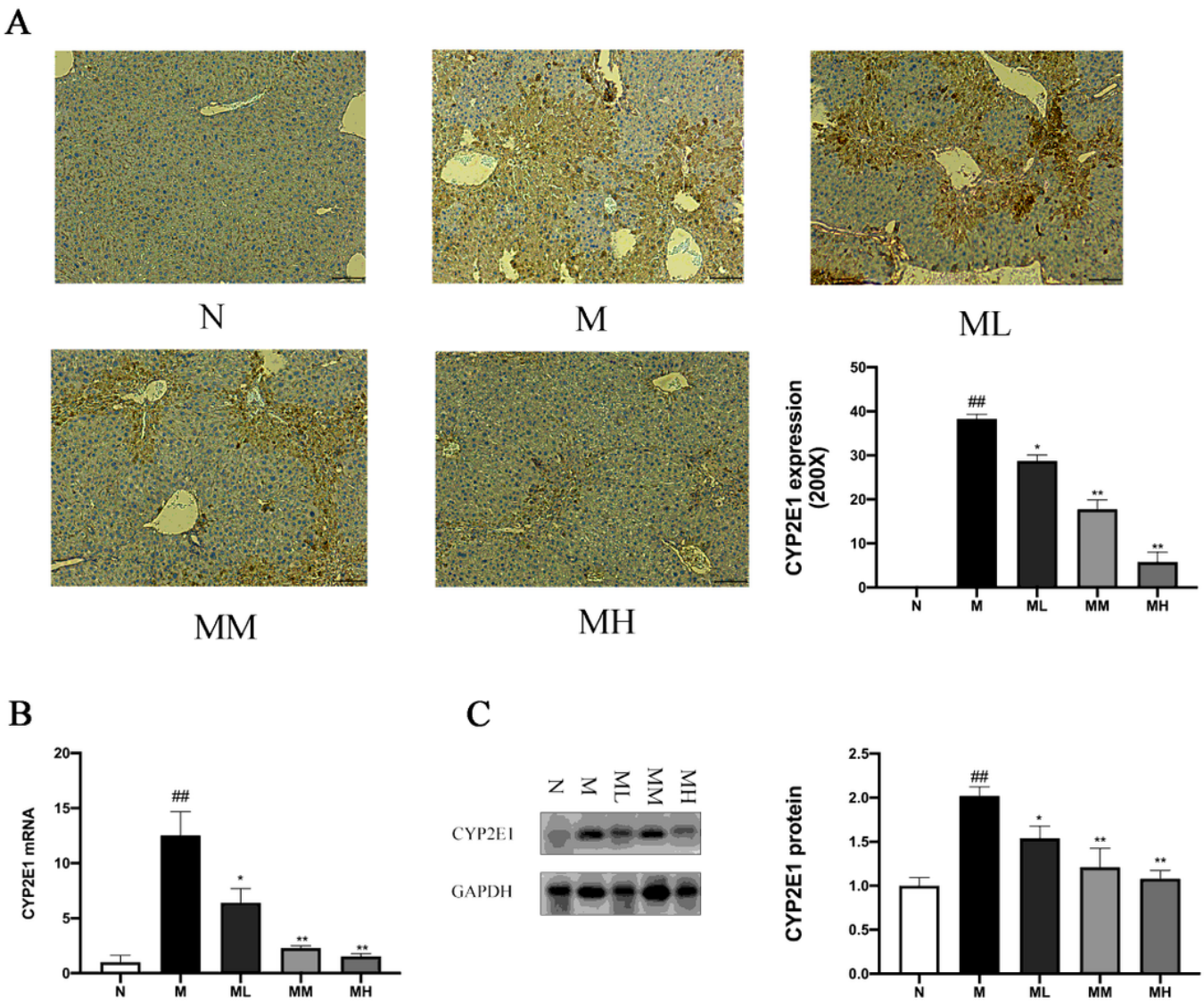

Figure 8

Effects of SCU on CYP2E1 Expression. (A) Immunohistochemistry analysis of CYP2E1. (B) Relative expression of CYP2E1 mRNA in the liver tissues. (C) Western blotting analysis of CYP2E1. GAPDH was used to as the loading control. Quantification of the relative protein levels of CYP2E1. The data and error bars are presented as mean ± SD (n = 5). ##P < 0.01 vs. N group. *P < 0.05, **P < 0.01 vs. M group.

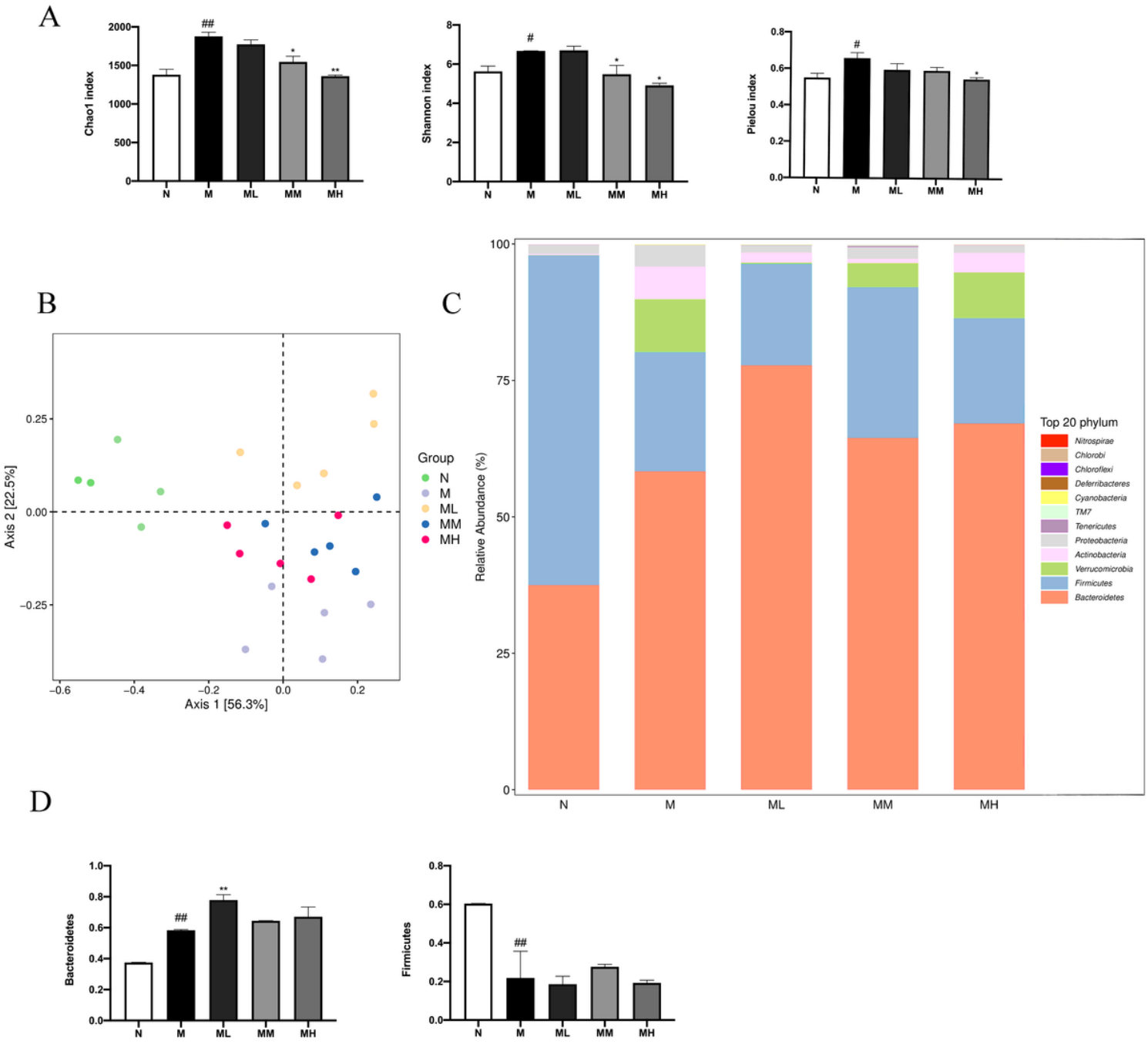

Figure 9

Effects of SCU on gut microbiota diversity and composition. (A) α-diversity of the gut microbiota. (B) β-diversity of the gut microbiota. (C) Relative abundance of different phyla in the gut microbiota of mice. (D) The top two gut microbial communities at phylum level. The data and error bars are presented as mean ± SD (n = 5). #P < 0.05, ##P < 0.01 vs. N group. *P < 0.05, **P < 0.01 vs. M group.

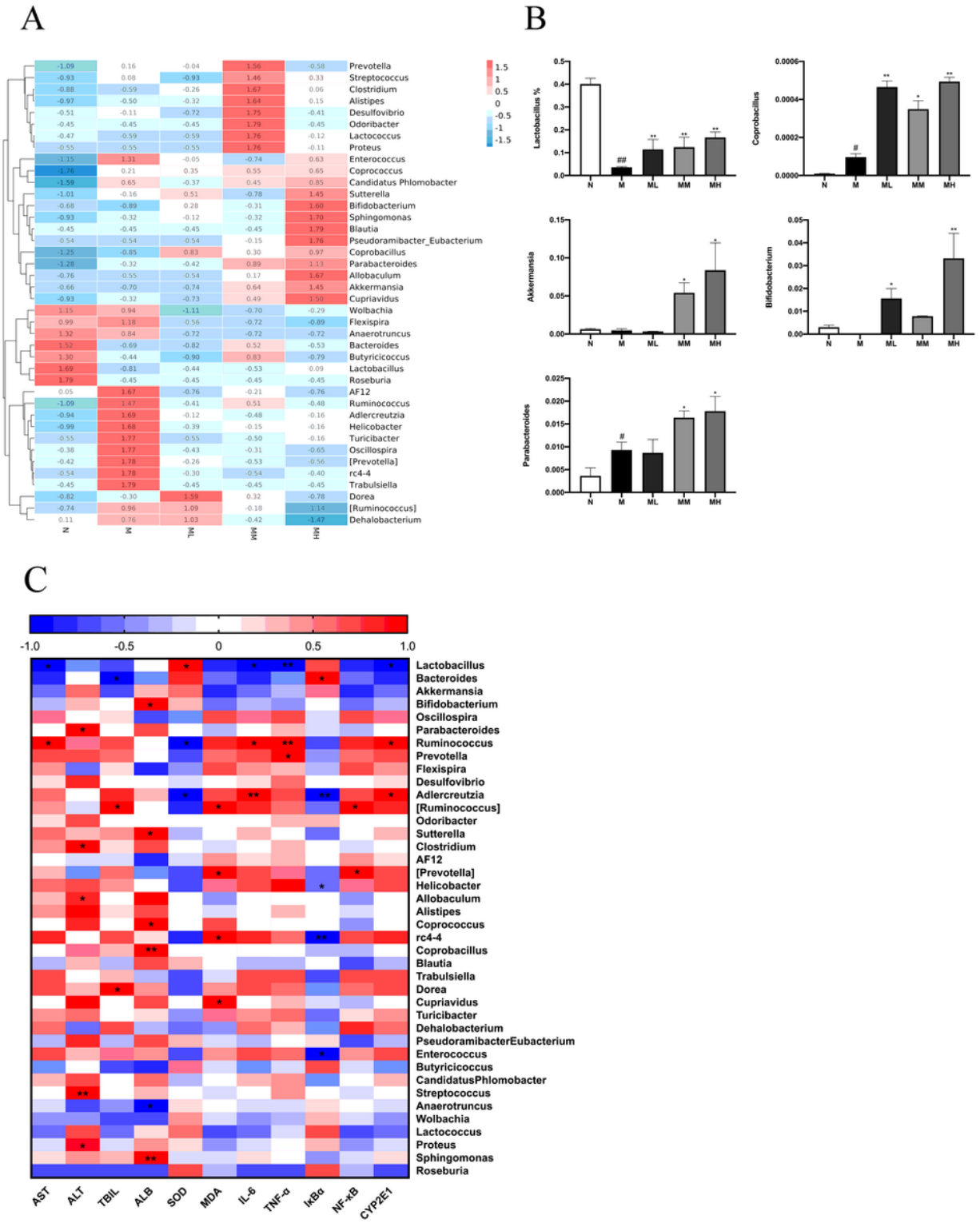

Figure 10

Effect of SCU on key phylotypes of gut microbiota. (A) Heat map showing the relative abundance of major genera ranking top forty. (B) Significant difference was found in ten genera. The data and error bars are presented as mean ± SD (n = 5). #P < 0.05, ##P < 0.01 vs. N group. *P < 0.05, **P < 0.01 vs. M group.

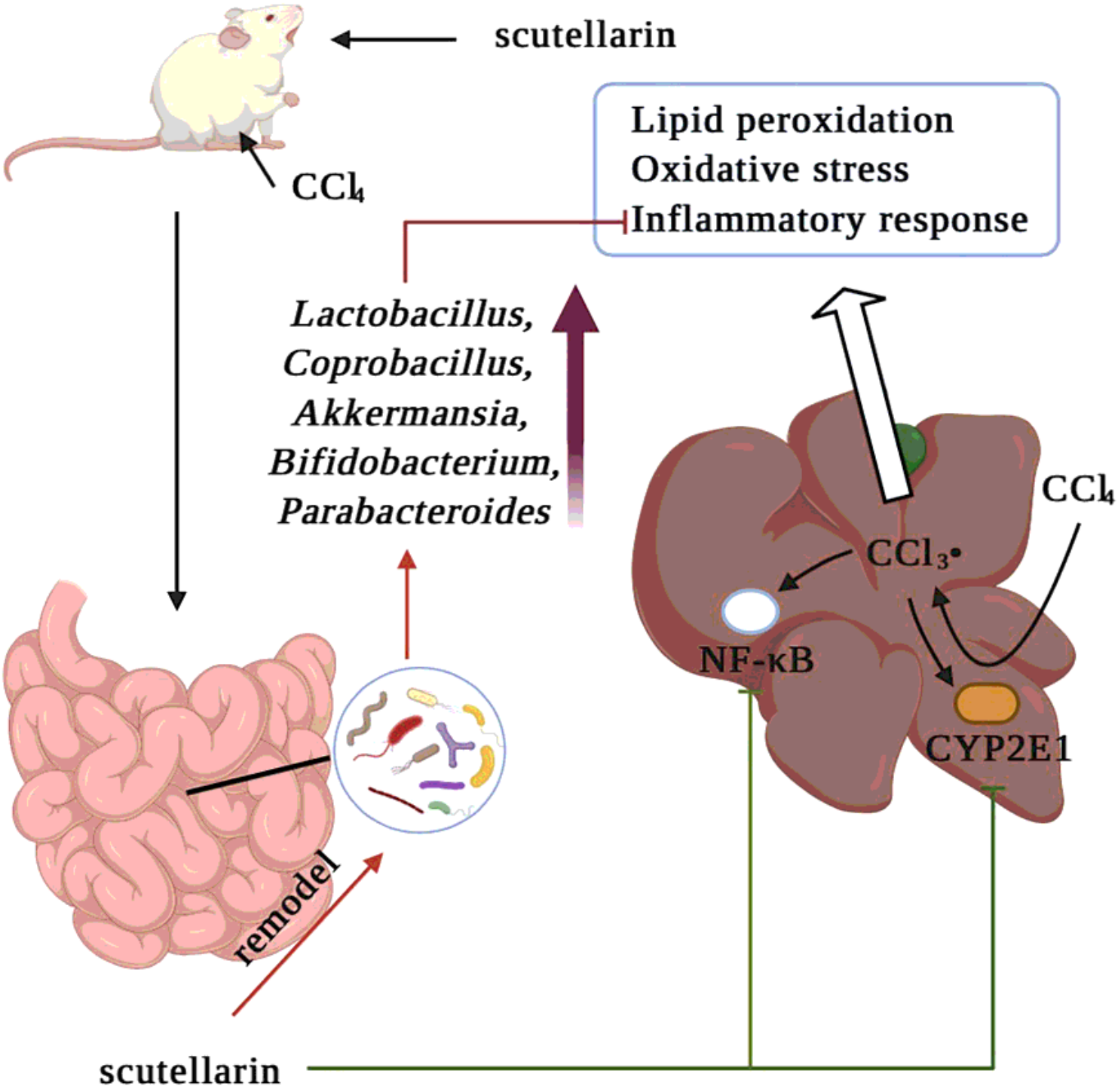

Figure 11

A schematic summary illustrating the mechanism of SCU-mediated liver protection. Oral administration of SCU interacts with the gut microbiota. On one side, SCU exert hepatoprotective effect by inhibiting NF-κB, CYP2E1 activation. On the other side, SCU remodels inflammation-related gut microbiota to exert the hepatoprotective effect indirectly.

Supplementary Files

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