



6,7-Dimethoxycoumarin, Gardenoside and Rhein combination improves non-alcoholic fatty liver disease in rats

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ABSTRACT

Ethnopharmacological relevance: This study explores the potential therapeutic benefits of using a three-component DGR (composed of specific compounds) to target the NLRP3 inflammasome in the context of non-alcoholic fatty liver disease (NAFLD).

Aim of the study: To assess the impact of a three-component DGR on NAFLD, specifically examining its effects on liver lipid accumulation, inflammation, and the diversity of intestinal microbial communities.

Methods: NAFLD was induced in 8-week-old Sprague Dawley rats by feeding them a high-fat emulsion diet every morning for 8 consecutive weeks. Oral administration of DGR or its constituent equivalents in the afternoon. The pharmacological effects of DGR were evaluated using H&E, ORO and ELISA methods to determine the changes in serum and liver tissue indexes of rat-models. Immunohistochemical staining and Western blot were used to assess the interaction between DGR, NLRP3 and IL-1 β .

Results: The induction of NAFLD resulted in elevated hepatic triglycerides (TG), total cholesterol (TC), and free fatty acids (FFA). However, these alterations were ameliorated upon administration of DGR. It is noteworthy that DGR exhibited superior efficacy in comparison to its constituent compounds, manifesting augmented antioxidant activity, diminished hepatic damage, and the attenuation of pro-inflammatory factors. Both DGR and its individual monomeric constituents exhibited the capacity to attenuate the activation of the NLRP3 inflammasome in the liver, leading to an amelioration of the pathological characteristics associated with NAFLD. An analysis of the intestinal flora unveiled an elevated abundance of p_Firmicutes (1.1-fold), p_Cyanobacteria (5.76-fold), and p_Verrucomicrobia (5.2-fold), accompanied by a heightened p_Firmicutes to p_Bacteroidetes ratio (5.49-fold).

Conclusions: In the non-alcoholic fatty liver disease (NAFLD) rat model, the concurrent administration of three-component DGR effectively regulated lipid deposition, suppressed liver inflammation, and restored balance in the intestinal flora, thereby improving NAFLD pathology. These findings propose a promising therapeutic strategy for NAFLD, centered on inhibiting the NLRP3 inflammasome through the use of the three-component DGR.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a major chronic liver disease that affects about 25% of the global adult population (C. Chen et al., 2021; Eslam et al., 2020). This condition poses substantial health and economic challenges (Y. B. Liu and Chen, 2022). NAFLD encompasses a wide range of liver disorders characterized by the excessive accumulation of triglycerides (TG) within liver cells, all occurring in the absence of alcohol abuse (Davis, 2021). Currently, the underlying causes

of NAFLD remain incompletely understood. The "multiple-hit" theory proposes that a combination of various stressors and predisposing factors plays a central role in the development of NAFLD. Both lipid accumulation and inflammation are recognized as crucial elements influencing the progression of this condition (Buzzetti, et al., 2016). Lipid deposits have the potential to trigger inflammation, leading to liver injury, fibrosis, and hepatic steatosis. Notably, the abnormal activation of the NLRP3 inflammasome, a significant contributor to caspase and cytokine release, has been identified as a key factor in the

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pathogenesis of NAFLD (L. Yu et al., 2022). Therefore, the inhibition of the NLRP3 inflammasome may be a promising therapeutic target for NAFLD (T. Liu et al., 2022).

Traditional Chinese Medicine (TCM), renowned globally, advocates the utilization of Chinese herbs and their derivatives in the treatment of various ailments. More recently, TCM herbs have garnered recognition as promising sources of therapeutic interventions for the prevention and treatment of NAFLD (Yan, et al., 2020). Yinchenhao Decoction (YCHD), a remedy documented in the ancient Chinese medical text "Treatise on Febrile Diseases" by Zhang Zhongjing during the Han Dynasty (150–215 A.D.), has been employed for over a millennium in the treatment of jaundice and liver-related disorders (J. Y. Li et al., 2017). Officially included in the Chinese Pharmacopoeia by the Chinese Pharmacopoeia Committee in 2010, YCHD consists of three key components: *Artemisia capillaris* Thunb (Herba Artemisiae Scopariae), *Gardenia jasminoides* J. Ellis (Fructus Gardeniae), and *Rheum palmatum* L. (Radix et Rhizoma Rhei). The plants name has been checked with <http://mpns.kew.org> mentioning the data of accessing that website.

Notably, one of its major constituents is 6, 7-dimethylesculetin (Dim), also known as Scoparone, found in Herba Artemisiae Scopariae. Dim has been shown to enhance the effectiveness of YCHD and exhibits favorable hepatoprotective properties in the treatment of liver diseases (Hui, et al., 2020). Another significant component is Geniposide (Gen), primarily derived from Fructus Gardeniae. Geniposide is notable for its potent antioxidant, anti-inflammatory, and hepatoprotective attributes (Fan, et al., 2020). Additionally, Rhein (Rhe), a major compound in Radix et Rhizoma Rhei, contributes to the enhancement of hepatoprotective effects (Bu, et al., 2018). Dim (D), Gen (G) and Rhe (R) are considered as our quality control markers for the preparation of YCHD (Sup Fig. 1).

In a previous comprehensive meta-analysis, Traditional Chinese Medicine (TCM) treatments were found to effectively reduce liver enzymes like aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as radiological evidence of steatosis, thereby benefiting NAFLD treatment (Shi, et al., 2012). Notably, YCHD treatment has been demonstrated to ameliorate liver injury in NAFLD by inhibiting the excessive accumulation of total cholesterol (TC) and triglycerides (TG) in the liver (Huang, et al., 2022; Shen et al., 2020; Wei et al., 2016). The various pharmacological benefits of YCHD indicate it may become a promising drug for the treatment of liver diseases (J. Y. Li et al., 2017).

The liver maintains a constant physiological connection with the gut, which means it is consistently exposed to pathogenic microorganisms originating from the gut. This exposure triggers immune responses within the liver (Bawa and Saraswat, 2013). Earlier research indicated that the gut microbiome could contribute to the advancement of fatty liver disease (Z. Li et al., 2003). In this regard, gut dysbiosis is considered a feature of NAFLD, and the severity of the disease is reflected in altered bacterial metabolites that promote inflammation. Notably, NAFLD is associated with reduced butyrate production, disturbances in amino acids and choline metabolism, and an excessive production of ethanol, all of which contribute to hepatic or systemic inflammation (J. Chen and Vitetta, 2020). Mouse models of NAFLD have consistently exhibited similarities to human NAFLD patients, including significantly elevated levels of inflammasome (NLRP3) and IL-1 β in the liver (Knorr, et al., 2020).

In this study, we assessed the pharmacological effects of DGR (combination of 6,7-Dimethoxycoumarin, Gardenoside and Rhein) and its three individual natural monomer products using rat models of NAFLD. Ursodeoxycholic acid (UDCA), a widely recognized treatment for chronic liver conditions and primary biliary cirrhosis (Oyama, et al., 2002), served as a positive control. Our investigation focused on evaluating DGR as a potential therapeutic target for NAFLD. We examined its impact on lipid metabolism, changes in proteins related to the NLRP3 inflammasome signaling pathway, and its relationship with the gut microbiota. We hypothesized that the DGR combination could regulate the NLRP3 inflammasome signaling pathway, a critical factor in the

development of NAFLD. Given the significant health risks associated with NAFLD and the absence of a definitive cure, this study aims to contribute to the alleviation of this burden by exploring new therapeutic approaches.

2. Material and methods

2.1. Plant material

Artemisia capillaris Thunb, the traditional Chinese name for Yinchenhao, The English name is also known as Herba Artemisiae Scopariae. Distributed most of China (<http://www.theplantlist.org/tpl1.1/record/gcc-76502>). The main composition includes 6,7-Dimethoxycoumarin(D), as known as Scoparone. *Gardenia jasminoides* J. Ellis, the traditional Chinese name for Zhizi, The English name is also known as Fructus Gardeniae. Distributed in southern China. (<http://www.theplantlist.org/tpl1.1/record/kew-88270>). The main composition includes Gardenoside(G). *Rheum palmatum* L., the traditional Chinese name for Dahuang, The English name is also known as Radix et Rhizoma Rhei. Distributed in southern China (<http://www.theplantlist.org/tpl1.1/record/kew-2425567>). The main composition includes Rhein(R). We combined the 6,7-Dimethoxycoumarin (Scoparone, D), the major component of *Artemisia capillaris* Thunb, the Gardenoside (G), the major component of *Gardenia jasminoides* J. Ellis and the Rhein (R), the major component of *Rheum palmatum* L. to form DGR.

2.2. Drugs and reagents

Scoparone (6,7-Dimethoxycoumarin, D) from *Artemisia capillaris* Thunb extract (HPLC $\geq$ 98%, SKU-SS8250), Geniposide (G) from *Gardenia jasminoides* J. Ellis extract (HPLC $\geq$ 98%, SKU-SG8100), and Rhein (R) from *Rheum palmatum* L. extract (HPLC $\geq$ 98%, SKU-SR8100) were acquired from Solarbio (Beijing Solarbio Sci & Tech Group, China). Supplementary Fig. S1 displays the High-Performance Liquid Chromatography (HPLC) analysis of these three products, conducted using the methods described previously. (Yi, et al., 2016). The DGR preparation contained Scoparone (D), Geniposide (G), and Rhein (R) in a ratio of 1.34:19.32:1. Subsequent detection and analysis were performed using the Agilent 1200 HPLC system from Agilent Technologies. In this analysis, a mixture of methanol and water served as the mobile phase within an Edipse XDB-C18 column (4.6 $\times$ 250 mm, 5 μ m). The gas flow rate was set at 1.8 L/min, and a combination of various stressors and predisposing the column temperature was maintained at 30 $^{\circ}$ C. Detection occurred at wavelengths of 210 nm, 238 nm, and 254 nm.

2.3. Animal experiments

This investigation was approved by the Animal experimental Ethics Committee of Changchun University of TCM (NO.2022067). Seventy 8-week SD rats (male) were acquired from the Sipeifu, Beijing, Biotechnology Factory; Laboratory animal quality certificate: 110324200103257923; Production license of Laboratory animal SCXK-(Beijing): 2019-0010. The animal use license was No. SYXK (Ji) 2018-0014. The rats were accommodated in a controlled environment within the Animal Center of Changchun University of Chinese Medicine. They were provided with a morning diet consisting of a fat emulsion and received drug administration (DGR) in the afternoon of the same day (further specifics provided below). The animal room was maintained at a temperature range of 19–23 $^{\circ}$ C, with a relative humidity level of 40–65%. The lighting conditions followed a 12-h cycle of alternating light and darkness. After a 3-day period during which the animals acclimated to their housing conditions, they were randomly divided into seven groups as follows: (1) Scoparone (Dim) group receiving 4.74 mg/kg, (2) Geniposide (Gen) group receiving 68.59 mg/kg, (3) Rhein (Rhe) group receiving 3.55 mg/kg, (4) DGR group receiving a combination of Dim (4.74 mg/kg), Gen (68.59 mg/kg), and Rhe (3.55 mg/kg), (5)

Ursodeoxycholic Acid (UDCA) group serving as a positive control with a dosage of 50 mg/kg, (6) NAFLD group, and (7) a control group utilized to verify the successful induction of the model. With the exception of the control group, all animals received an oral gavage of a prepared fat emulsion (consisting of 20% lard, 5% cholesterol, 1% sodium cholate, 5% sucrose, 5% Tween-80, and 5% 1,2-propylene glycol) every morning, with a respective oral medication volume of 10 mL/kg administered via a standard oral gavage needle. Additionally, all rats received an afternoon oral drug gavage with 0.5% CMC-Na (10 mL/kg) as the solvent for eight consecutive weeks. One hour after the final oral gavage administration, rats were anesthetized with a 3 mL/kg injection of 2% sodium pentobarbital.

Blood samples were collected from the abdominal aorta, allowed to coagulate at room temperature for 2 h, and then the serum fraction was obtained through centrifugation for subsequent use. The liver, cecum, and feces from the cecum were harvested, weighed, and stored in liquid nitrogen for future analysis (Mu, et al., 2021; Xu et al., 2011). Successful modeling of NAFLD rats was determined based on diagnostic criteria involving serum TC levels, standard assessment of liver cell steatosis using oil red O staining, and the liver-to-mass ratio. In each group, the number of successfully modeled NAFLD rats was at least six, with an average weight of approximately 2200 ± 20 g, after eight weeks.

2.4. Enzyme-linked immunosorbent assays (ELISA)

To prepare a 10% liver homogenate, we took 100 mg of fresh liver tissue and homogenized it at 4 °C using nine times the tissue volume of 0.9% normal saline. Subsequently, this preparation underwent centrifugation at 3000 rpm/min at 4 °C for 10 min to collect the resulting supernatant. We quantified the levels of triglycerides (TG; NO.A11011), total cholesterol (TC; NO.A11111), glutamic pyruvic transaminase (ALT; NO.C00921), glutamic oxaloacetic transaminase (AST; NO.C01021), superoxide dismutase (SOD; NO.A00132), catalase (CAT; NO.A00721), tumor necrosis factor- α (TNF- α ; NO.H05212), and interleukin-6 (IL-6; NO.H00712) using enzyme-linked immunosorbent assays (ELISA) from Nanjing Jiancheng Groups, China, following previously described methods (Zhao, et al., 2020).

2.5. Analysis of serum biochemical parameters

We used chemiluminescent immunoassay was performed with the absorbance read on the ELISA Reader (Thermo Fisher Scientific, USA) to measure various parameters of the serum fraction, including nonesterified fatty acids (FFA; NO.A04221), high-density lipoprotein cholesterol (HDL-C; NO.A11211), low-density lipoprotein cholesterol (LDL-C; NO.A11311), the low-density lipoprotein receptor (LDLR; NO.H645), lipoprotein lipase (LPL; NO.A06712), apolipoprotein-B (ApoB; NO.H12412), and glycated hemoglobin (GHb; NO.A05611). To perform these measurements, we employed commercial kits sourced from Nanjing Jiangguang Biological Technology Research Institute, Nanjing, China, and adhered to the manufacturer's instructions (Zhao et al., 2020).

2.6. Tissue histological examination

To calculate the somatohepatic ratio (body weight to liver weight), rats were weighed 1 h after the drug gavage, followed by pentobarbital injection (2%) and cervical dislocation for euthanasia. After sacrifice, the liver was collected and weighed. For histology analyses, fresh liver tissue was immersed in a fixative solution for more than 24 h at room temperature, dehydrated, and cleared through a standard ethanol and xylene series. It was then embedded in paraffin and sectioned into 4 μ m thick slices using a microtome (RM2016, LEICA, Germany). The sections were rehydrated, cleared using standard ethanol and xylene procedures, and stained with H&E dye. Frozen sections underwent dehydration through a two-step sucrose incubation at 4 °C, followed by embedding in

OCT and sectioning into 5 μ m thick slices using a CRYOSTAR NX50 cryostat (Thermo Scientific, USA). These sections were stained with oil red O (ORO) staining solution (Servicebio G1015). Optical microscopy (Nikon E100, Japan) and an imaging system (Nikon DS-U3, Japan) were employed to capture images of the sections for subsequent pathological analysis. In brief, we defined the NAFLD activity score (NAS) as the sum of scores for steatosis, lobular inflammation, and ballooning (B. Liu et al., 2019).

2.7. Immunohistochemical staining

Following the sacrifice of the rats, the livers were subjected to paraffin embedding. After dewaxing the sections, antigen retrieval was carried out using citric acid buffer (pH 6.0) and Tris-EDTA buffers (pH 8.0 and pH 9.0). Peroxidase activity was blocked by incubating the samples with a 3% hydrogen peroxide solution at 25 °C for 30 min. Subsequently, the sections were blocked with 3% BSA at 25 °C for 30 min and then incubated overnight with IL-1 β primary antibodies (dilution 1:1000; Abcam ab254360) at 4 °C. Tissues were thoroughly washed with PBS and incubated with HRP secondary antibodies at 25 °C for 1 h. Finally, the sections were developed with a DAB color development solution (Servicebio G1212), and images were captured using an optical microscope (Nikon Eclipse E100, Japan) (Jiang, et al., 2022).

2.8. Western blot

For the extraction of proteins intended for Western Blot (WB) analysis, we adhered to established procedures (Zhao et al., 2020). Briefly, the liver samples were washed three times utilizing 4 °C PBS, cut into sections, and then lysed in cold RIPA buffer (G2002, Servicebio) for half an hour. The supernatant was centrifuged at 12,000RCF at 4 °C for 10min. The total protein concentration was detected using a BCA kit.

The target proteins were separated through SDS gel electrophoresis (SDS-PAGE) using a 10% separation gel and a 5% stacking gel. Subsequently, they were transferred to PVDF membranes employing a wet transfer method conducted on ice. The incubation was conducted on a shaker with anti-NLRP3 (1:1000; Abcam ab270449), anti-ASC (1:1000; Abcam ab307560), anti-caspase-1 (1:1000; Abcam ab286125), anti-IL-1 β (1:1000; ab254360), and GAPDH (Abcam ab181602; reference standard) antibodies at 4 °C for 30min. The PVDF membranes were subjected to three washes with TBST for a duration of 10 min each and then incubated with horseradish-coupled secondary antibodies. Detection was carried out utilizing an ECL substrate (Clarity Western ECL Substrate, 1705061, Bio-Rad). Band images were captured using a gel imaging system (Gel XR+, Bio-Rad, USA) and subsequently analyzed using Photoshop software (Adobe Systems, USA).

2.9. 16S sequencing

Ileal fecal samples were collected and directly stored in liquid nitrogen. To profile the gut microbiota composition, high-throughput sequencing (HTS) was performed on the V3–V4 region of the 16S ribosomal RNA (rRNA) gene, following established protocols (X. Zhang et al., 2021). The genomic DNA of each sample was extracted using the Magnetic Soil And Stool DNA (Tiangen-DP712, Beijing, China), and then quantified using the Qubit 2.0 Fluorometer.

Afterward, the V3–V4 region of the gene was amplified by PCR using custom general-purpose bacterial primers (Primer 341F-806R amplifying regions V3–V4; Fwd: CCTAYGGGRBGCASCAG; Rev: GGAC-TACNNGGGTATCTAAT). The thermal cycling included initial denaturation at 98 °C for 1 min, 30 cycles of denaturation at 98 °C for 30 s, annealing at 50 °C for 30 s, elongation at 72 °C for 30 s, and 72 °C for 5 min.

Sequencing was conducted using Illumina Miseq platforms at both McGill University and the Quebec Center for Genome Innovation. Electrophoresis was performed on 2% agarose gels, and samples

displaying a prominent main strip at 400–450 bp were chosen for further analysis (C. Chen et al., 2021). The 16s rRNA amplicon was sequenced on Illumina sequencing platforms (NovaSeq6000) and annotated using the Silva 132 (<https://www.arb-silva.de/>) database.

2.10. Statistical analysis

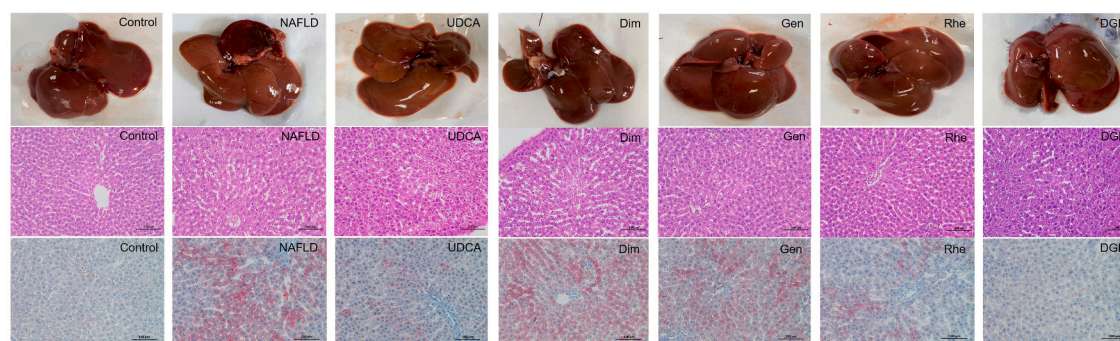
Data were presented as mean $\pm$ SD. Group differences were evaluated using t-tests or one-way ANOVA, followed by minimum significance difference tests where applicable. Significance was determined when $p < 0.05$. All statistical analyses were performed using GraphPad Prism version 5.0.

3. Results

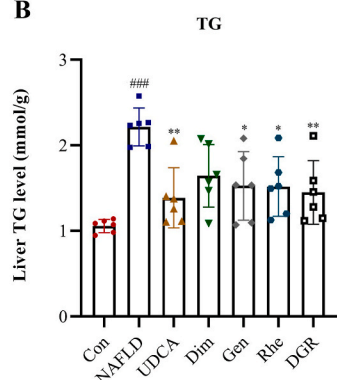
3.1. DGR effects on TG and TC in injured non-alcoholic fatty liver (NAFL)

Liver biochemical parameters were assessed to evaluate the therapeutic impact of DGR on NAFLD. Our findings indicated that DGR effectively reduced the accumulation of TC and TG in the livers of NAFLD model rats and mitigated organ injuries. In the control group, after 8 weeks of consuming a high-fat diet, liver histology revealed hepatocyte expansion and inflammatory cell infiltration in the liver lobules, as demonstrated by hematoxylin and eosin (H&E) staining. Additionally, ORO staining highlighted the presence of typical ballooned steatosis at the center of the lobule, accompanied by a significant increase in lipid deposition within the majority of hepatocyte

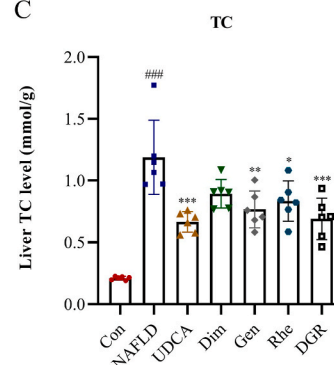
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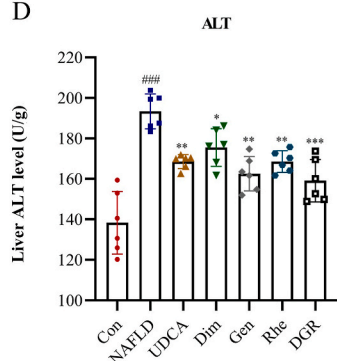
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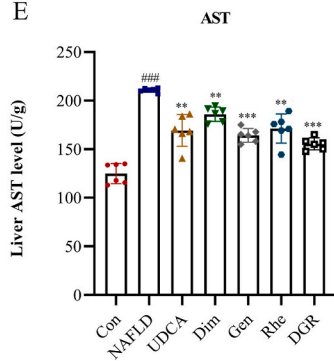


Fig. 1. The DGR combination improved liver fat deposition induced by lipid emulsion in NAFLD rat. (A) H&E(200x) staining and Oil Red O staining (200x) for liver samples. TG(B), TC(C), ALT(D) and AST(E) content of liver in groups (n = 6). Data are displayed as the mean $\pm$ SD, [#]p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001 vs con group; ^{*}p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001 vs NAFLD group. DGR: combination of dim, geniposide, and rhein. NAFLD: non-alcoholic fatty liver disease. H&E: hematoxylin and eosin. TG: triglycerides. TC: total cholesterol. ALT: glutamic pyruvic transaminase. AST: glutamic oxaloacetic transaminase. SD: standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cytoplasm.

Following 8 weeks of a diet consisting of high-fat milk in combination with DGR treatment, examination of liver tissue using H&E and ORO stains demonstrated a significant reduction in severe hepatic steatosis and inflammation-driven balloon-like degeneration compared to the control group (see Fig. 1A). Notably, NAS was significantly reduced in various treatment groups as compared to the NAFLD group, with a 50% reduction in UDCA, 25% reduction in D, 42% reduction in G, 33% reduction in R, and a 50% reduction in DGR ($p < 0.05$). DGR significantly reduced liver TG (Fig. 1B) and TC levels (Fig. 1C) while also attenuating the elevation of ALT (Fig. 1D) and AST (Fig. 1E) caused by NAFL, consequently mitigating liver injury. We similarly observed

improvements in liver pathology and biochemistry markers following monomer interventions, including D (resulting in a 26% reduction in TG, a 25% reduction in TC, a 9% reduction in ALT, and a 12% reduction in AST), G (resulting in a 31% reduction in TG, a 35% reduction in TC, a 16% reduction in ALT, and a 22% reduction in AST), and R (resulting in a 31% reduction in TG, a 30% reduction in TC, a 13% reduction in ALT, and a 19% reduction in AST). However, it is noteworthy that these individual interventions were significantly less effective compared to DGR, which led to a substantial reduction of TG by 35%, TC by 42%, ALT by 18%, and AST by 26%.

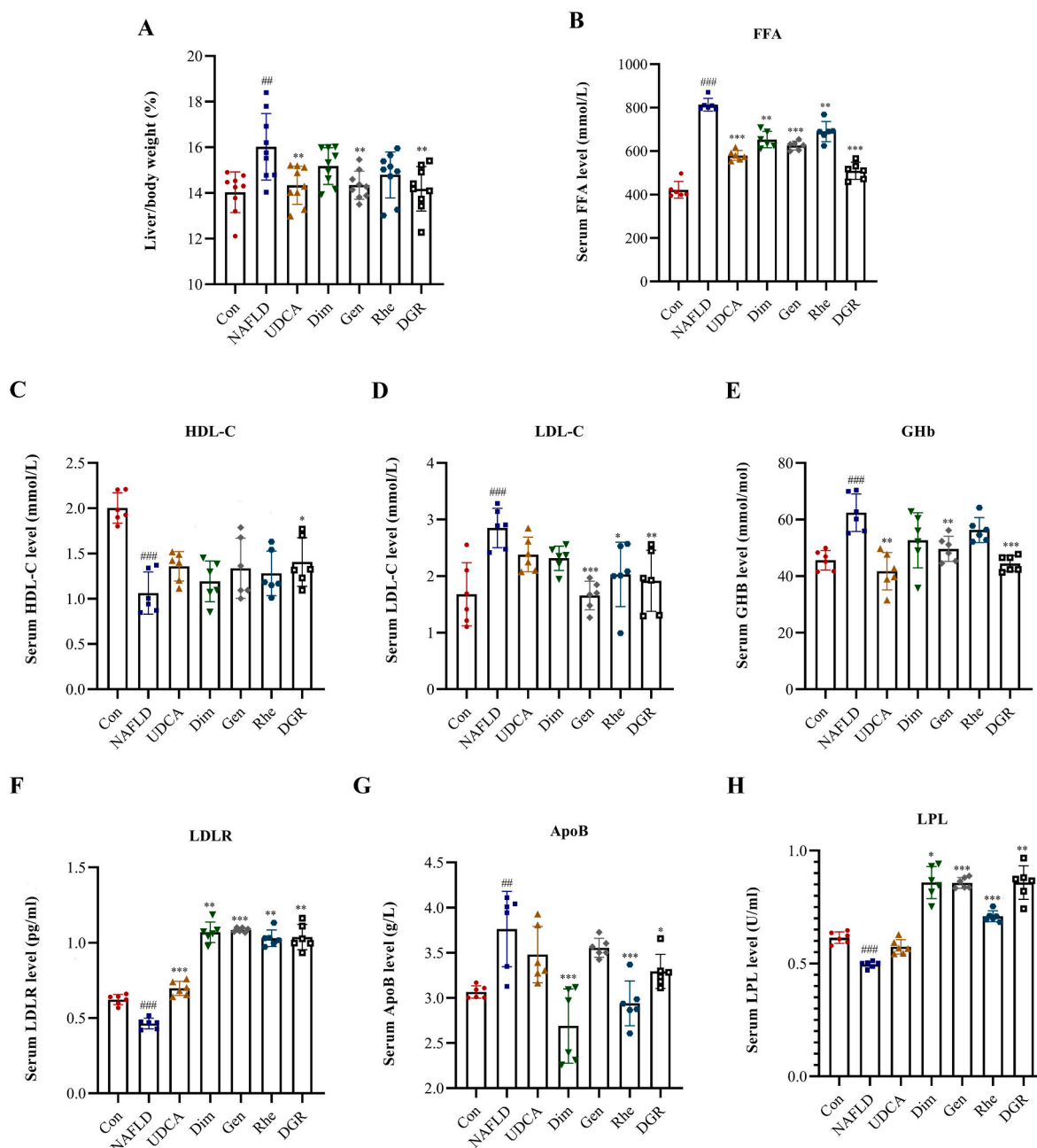


Fig. 2. Serum biochemical parameters of NAFLD rats were reversed by DGR combination. (A) Rat liver/body weight. The level of serum FFA(B), HDL-C(C), LDL-C(D), Ghb(E), LDLR(F), ApoB(G) and LPL (H) in groups ($n = 6$). Data are displayed as the mean $\pm$ SD, [#] $p < 0.05$, ^{##} $p < 0.01$, ^{###} $p < 0.001$ vs con group; ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs NAFLD group. NAFLD: non-alcoholic fatty liver disease. DGR: combination of dim, geniposide, and rhein. FFA: nonesterified fatty acids. HDL: high-density lipoprotein cholesterol. LDL: low-density lipoprotein cholesterol. Ghb: glycated hemoglobin. LDLR: low-density lipoprotein receptor. ApoB: apolipoprotein B. LPL: lipoprotein lipase. SD: standard deviation.

3.2. DGR effect on serum lipid index in NAFLD

To test if DGR could treat fatty liver induced by fatty milk, we analyzed the body weights and liver weights of each group. Moreover, we conducted the analysis of serum fractions using commercial kits to detect and quantify FFA, HDL-C, LDL-C, LDLR, LPL, ApoB, and GHb. Additionally, liver homogenates were subjected to ELISA for the measurement of TG, TC, ALT, AST, SOD, CAT, TNF- α , and IL-6. We found that the hepatosomatic ratio in all drug groups was significantly lower compared to the NAFLD group (Fig. 2A). However, there were no evident differences among the treatment groups. The DGR group demonstrated significantly lower levels of HDL-C, LDL-C, and FFA compared to the other groups (Fig. 2B-D). Moreover, the DGR group exhibited a reduction in the elevated levels of GHb levels typically associated with NAFLD (Fig. 2E). Additionally, the serum levels of LDLR, ApoB, and LPL in NAFLD rats were also reversed to varying degrees by DGR (Fig. 2F-H). Lastly, the DGR combination showed a greater attenuation of blood lipid levels and other NAFLD indicators compared to D, G, or R alone.

3.3. DGR effect on oxidative stress and inflammatory factors in NAFLD

To test the influence of DGR on cytokines and oxidative stress factors in NAFLD rats, we quantified the oxidative stress factors CAT (Fig. 3A) and SOD (Fig. 3B), as well as the cell factor TNF- α (Fig. 3C), and IL-6 (Fig. 3D) in a rat liver tissue homogenate. Given the close association of TNF- α and IL-1 β with the presence and progression of NAFLD, their response to DGR serves as an indicator of the drug's efficacy. Notably, DGR exhibited the most significant impact on TNF- α levels, resulting in a 35% reduction compared to the NAFLD group, and on IL-6 levels, with a reduction of 46% compared to NAFLD ($p < 0.05$). Furthermore, in concordance with these findings, immunohistochemical staining revealed an elevated presence of IL-1 β in the livers of NAFLD rats, primarily localized around hepatic cells (Fig. 3E). DGR effectively mitigated this increase in IL-1 β by approximately 90% ($p < 0.05$).

3.4. DGR and the regulation of the NLRP3 inflammasome in NAFLD

In the intestines, the NLRP3 inflammasome/IL-1 β axis is a key sensor

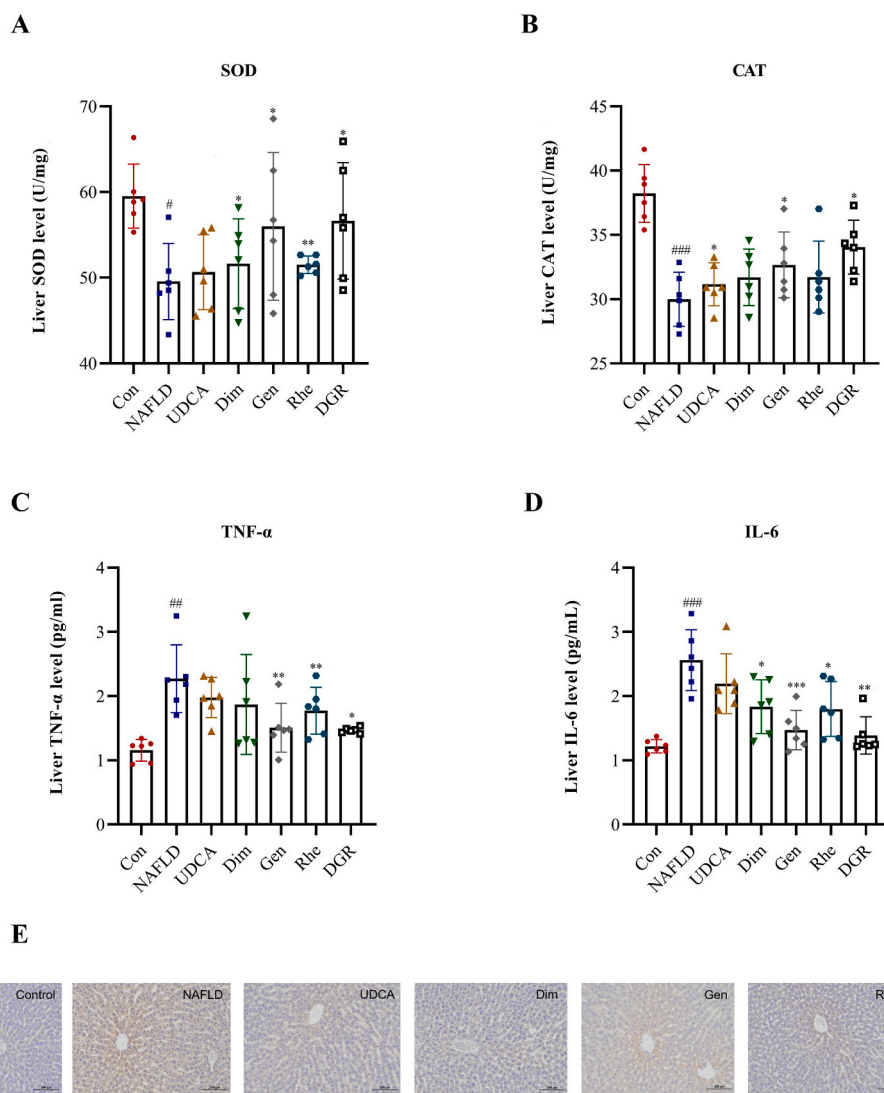


Fig. 3. DGR impact on the regulation of oxidative stress and inflammatory factors in the liver of NAFLD rats. Oxidative stress indices SOD(A) and CAT(B), and inflammatory cytokines TNF- α (C) and IL-6(D) in the liver of rats in groups ($n = 6$). (E) The expression of IL-1 β (Brown area, 200x) in liver tissue was detected by immunohistochemistry. Data are displayed as the mean $\pm$ SD, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs con group; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs NAFLD group. DGR: combination of dim, geniposide, and rhein. NAFLD: non-alcoholic fatty liver disease. SOD: superoxide dismutase. CAT: catalase. TNF- α : tumor necrosis factor-alpha. IL-6: interleukin 6. IL-1 β : interleukin 1 β . SD: standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

that protects the body from exogenous pathogens by triggering the release of inflammatory cytokines. Therefore, our objective was to assess whether DGR attenuates NAFLD by regulating the activation of the NLRP3 inflammasome upstream of IL-1 β . For this purpose, we employed Western blot analysis to examine alterations in the levels of key proteins within the NLRP3/caspase-1/IL-1 β axis. DGR exhibited significant inhibition of the NLRP3 inflammasome (by 50%), as did the Gen (G) monomer (by 22%). Both interventions also resulted in reduced levels of caspase-1 and IL-1 β (by 53% and 28%, respectively) (Fig. 4). Given the critical role of the NLRP3 inflammasome and IL-1 β , the effect of DGR on their levels could indicate the degree of attenuation of the inflammatory reaction in NAFLD.

3.5. DGR restored intestinal flora homeostasis in NAFLD rats

To assess the impact of NAFLD and DGR on the gut microbiome, we obtained a total of 4,928,662 reads from 42 fecal samples, with an average of 117,349 sequences per sample. Subsequently, we quantified the microbial diversity (alpha diversity) within each group. The results showed that the NAFLD model was associated with a disturbed intestinal flora balance. This was, fatty milk-fed NAFLD rats had a lower richness of microbiota community compared to the controls (Fig. 5 A and B). We observed that DGR notably restored the composition of the microbiota, indicating its potential to enhance intestinal flora diversity in NAFLD rats.

The microbial populations were subsequently subjected to analysis using UniFrac unweighted principal coordinate analysis (PCoA). As depicted in Fig. 5F, the control and NAFLD groups exhibited significant separation along the PC2 axis, which accounted for 11.58% of the total variance. Following treatment with UDCA, Dim, Gen, Rhe, and DGR, the intestinal flora displayed a distinct separation from the NAFLD group along the PC2 axis. Notably, the most substantial difference along the PC2 axis was observed between the DGR and NAFLD groups, signifying that DGR exerted the most significant impact on NAFLD. This shift in the community structure tended to restore it closer to that of the control group.

Through hierarchical cluster analysis of operational taxonomic units (OTUs) in each group, the differences among the groups were revealed, and 2934 OTUs were identified as seven groups in total (similarity about 24%) (Fig. 5E and D). Taxonomic analyses revealed that p_Fusobacteria, p_Cyanobacteria, p_Deferribacteres, p_Acidobacteria, and p_Firmicutes were the most abundant phyla in the intestinal tracts of fat emulsion-fed rats. After DGR treatment, we noted a significant recovery of p_Patescibacteria, p_Actinobacteria, p_Chloroflexi, p_Proteobacteria, p_Epsilonbacteraeota, and p_Spirochaete compared to the model group (Fig. 5D).

As shown in Fig. 5E, the abundance of 43 bacteria genera was clearly higher in the NAFLD group versus the control group. These included Anaerofustis, Erysipelotrichaceae UCG-003, Ruminococcaceae UCG-005, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Ruminiclostridium 6, Alistipes, Enterorhabdus, Firmicutes bacterium CAG:822, Flavobacterium, Anaeroplasm, Rodentibacter, Fournierella, Negativibacillus, Adlercreutzia, Romboutsia, Ruminococcaceae UCG-011, Saccharofermentans, Acetobacter, Psychrobacter, Dorea, Faecalibacterium, Aerococcus, Odoribacter, uncultured Lachnospiraceae bacterium, IS-44, Faecalitalea, Gaiella, Lysinibacillus, Acidovorax, Anaerotruncus, Lactobacillus, Haemophilus, Reyranelia, Clostridium sp. K4410.MGS-306, Fusobacterium, Dubosiella, Allobaculum, [Eubacterium] coprostanoligenes group, UBA1819, Paeniclostridium, Gemella, Hungatella, [Eubacterium] ventriosum group, Subdoligranulum, [Clostridium] innocuum group, Tuncioacier, Lachnospiraceae XPB1014 group, Ruminiclostridium 1, Coriobacteriaceae UCG-002, Christensenellaceae R-7 group, Lachnospiraceae NC2004 group, Anaerospiraceae, Synergistes, Candidatus Soleasterrea, Lachnospiraceae FCS020 group, Lachnospiraceae UCG-010, [Ruminococcus] gauvreau group, Anaerostipes, Candidatus Stoquefichus, Marvinbryantia, Peptoniohilus,

Paucibacter, Phocaea, Blautia, Lachnospiraceae UCG-008, [Eubacterium] hallii group, Holdemania, [Ruminococcus] torques group, Erysipelatoclostridium, Sutterella, Family XIII AD3011 group, Globicatella, Klebsiella, Moryella, Solibacillus, Oscillospira, Eggerthella, and Mucispirillum. However, this increased relative abundance returned to normal levels after DGR treatment, suggesting that DGR has the capacity to alleviate the alterations in intestinal flora induced by g_Mucispirillum in NAFLD rats.

4. Discussion

This study successfully established a rat NAFLD model through an eight-week high-fat diet, which induced liver lipid deposition, abnormal lipid and liver indices, and pathological liver changes. Our findings indicate that the DGR combination significantly mitigated these pathological features associated with NAFLD, demonstrating anti-lipogenic and anti-liver injury effects. These results align with previous studies highlighting the therapeutic potential of YCHD in addressing liver injuries stemming from NAFLD (Guo, et al., 2003). Regarding the individual constituents of DGR, D has been recognized for its hepatoprotective properties in liver injuries and its effectiveness in treating liver diseases, thereby enhancing the therapeutic effects of YCHD (A. Zhang et al., 2011). G is known to improve liver injury by strengthening the antioxidative defense system and decelerating apoptotic signaling pathways (Kim, et al., 2013). Lastly, R can mend liver function and eradicate hepatic fibrosis through via anti-inflammatory and antioxidant pathways, and prevent TGF- α activity and hepatic stellate cell activation (Guo, et al., 2003).

In comparison to its individual components (D, G, and R monomers), the DGR combination exhibited a synergistic effect, resulting in the most significant overall improvement in the abnormal lipid metabolism and liver indices induced by NAFLD. Notably, unlike the D, G, and R monomers, DGR substantially mitigated liver lipid deposition. This synergistic effect of YCHD has been observed in previous studies, where the therapeutic efficacy of YCHD exceeded that of its individual constituents, including capillaries, gardenia, and rhubarb herbs (Z. G. Yu et al., 2007). Another study conducted an in-depth analysis of D, G, and R when administered alone or in combination, employing immunohistochemistry, biochemistry, metabolomics, and proteomics. The findings revealed that the combined administration of these three components resulted in a more potent therapeutic effect compared to using any one or two of them individually in a rat model of hepatic injury (Wang, et al., 2013).

Furthermore, the combined therapy of DGR significantly ameliorated liver inflammation in NAFLD rats, as evidenced by reduced expression of TNF- α , IL-6, NLRP3 inflammasome, and IL-1 β in the liver. Simultaneously, DGR increased the levels of antioxidants CAT and SOD, indicating that it was involved in the regulation of antioxidant effects. These findings align with previous reports on the positive impact of YCHD in reducing liver cell inflammation, enhancing liver microcirculation, reducing the absorption of intestinal toxins, and facilitating bilirubin excretion (Y. Q. Zhang et al., 2013). Notably, individually, D, G, and R have also demonstrated anti-inflammatory effects, with D exhibiting antioxidant properties as well (J. Y. Li et al., 2017).

A close relationship between liver dysfunction and intestinal flora imbalance has also been reported (Albhaisi, et al., 2020; Milosevic et al., 2019). NLRP3, which contributes to inflammation and influences gut flora, can lead to weight and metabolic changes, ultimately resulting in conditions like metabolic syndrome, obesity, and atherosclerosis. In our investigation, through an analysis of the intestinal flora in NAFLD rats using 16S rRNA sequencing, we observed that the DGR combination effectively restored intestinal flora balance and improved the composition of the intestinal microbial community. This suggests that DGR exerts its attenuating effects on NAFLD pathology through multiple mechanisms.

Our findings position DGR as a potential therapeutic agent for

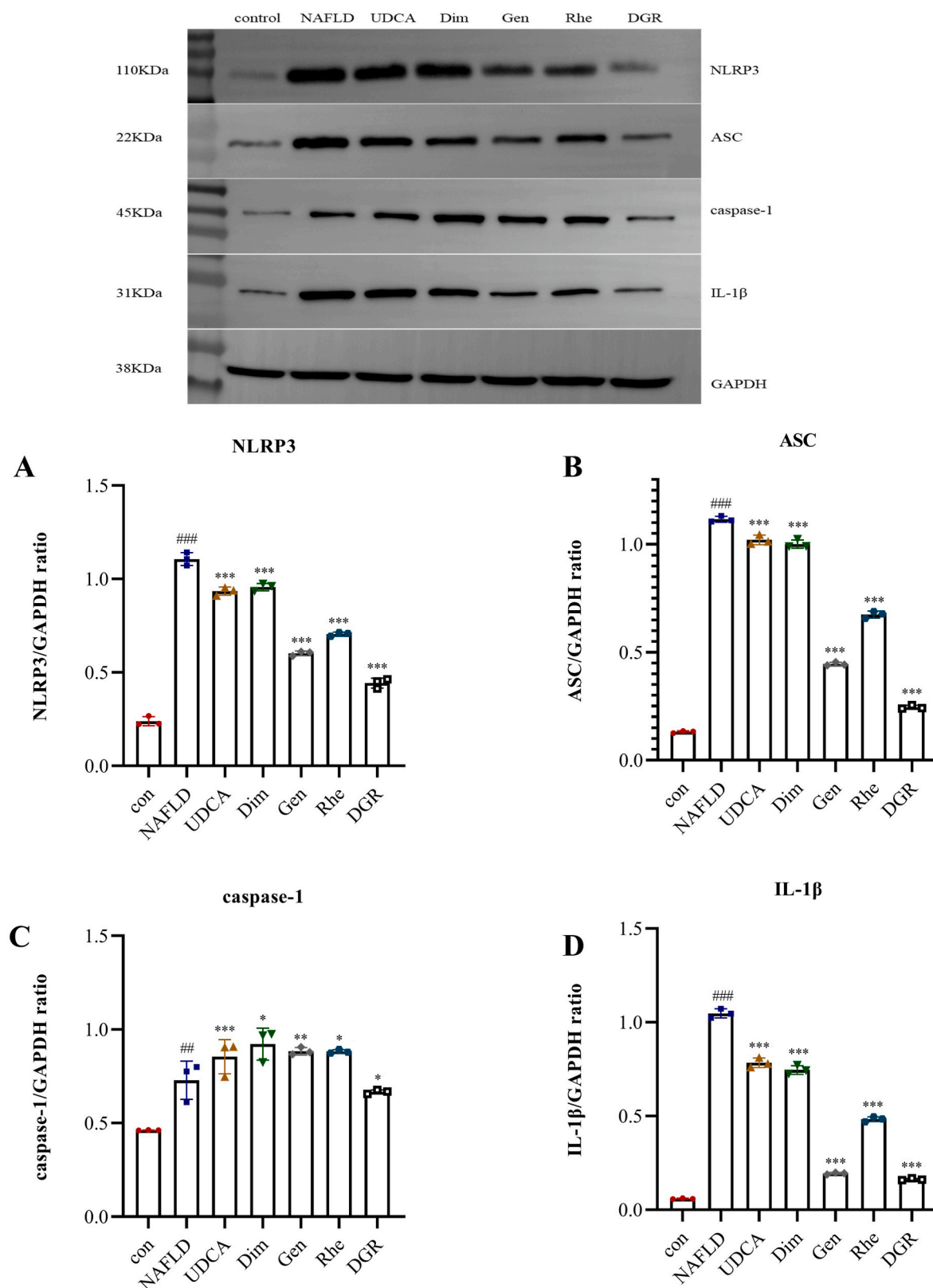


Fig. 4. Influence of DGR combination on the expression of NLRP3/caspase-1/IL-1 β axis of NAFLD rats. NLRP3(A), ASC(B), caspase-1(C) and IL-1 β (D) protein levels in rats liver was measured, and the relative expression levels were corrected based on GAPDH. The liver samples were 3 rats/group. Data are displayed as the mean $\pm$ SD, [#] $p < 0.05$, ^{##} $p < 0.01$, ^{###} $p < 0.001$ vs con group; ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs NAFLD group. DGR: combination of dim, geniposide, and rhein. NLRP3: NLR family pyrin domain containing 3. IL-1 β : interleukin 1 β . NAFLD: non-alcoholic fatty liver disease. ASC: Apoptosis-associated speck-like protein containing a C-terminal caspase activation and recruitment domain. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

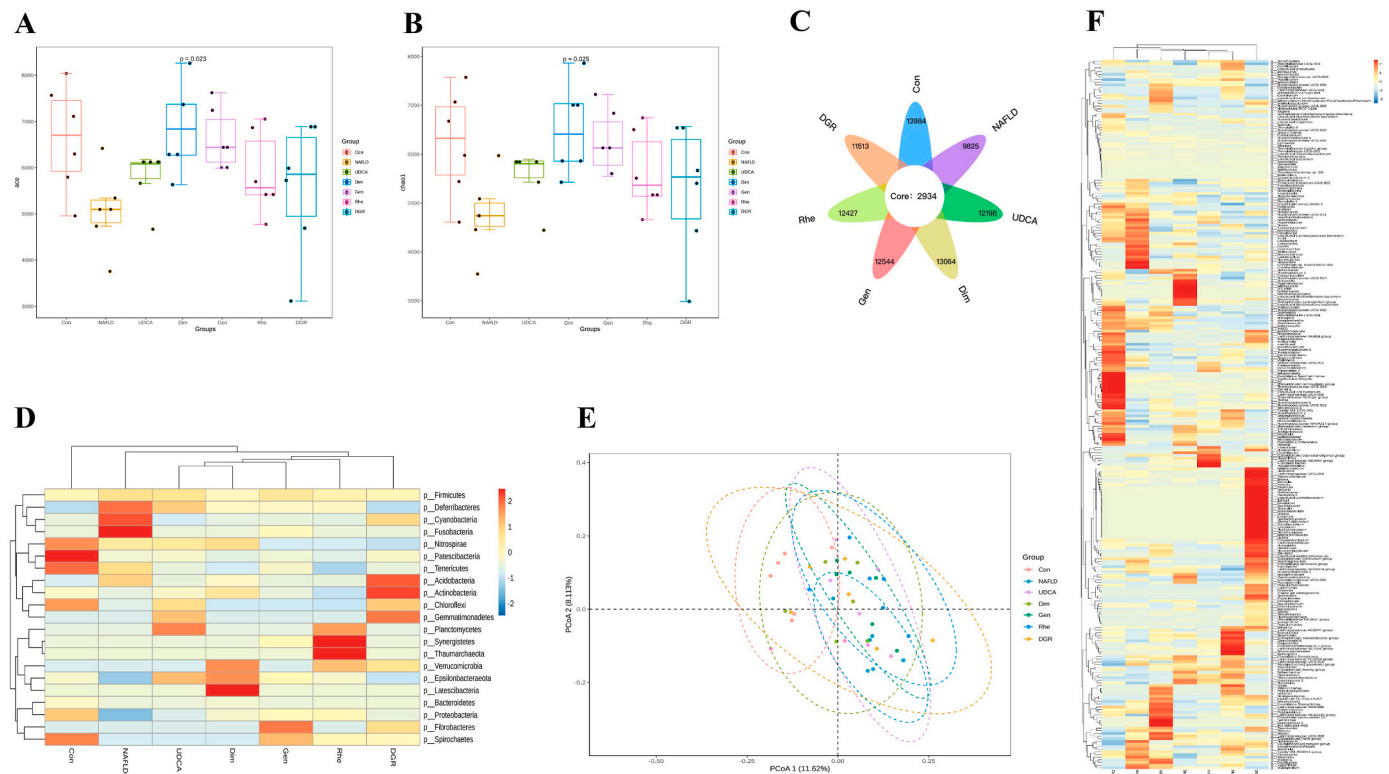


Fig. 5. Effect of NAFLD and DGR on the gut microbiome. (A, B) Alpha diversity comparison between controls and fatty milk-fed NAFLD subjects. The line inside the box represents the median, whereas the whiskers denote the lowest and highest values within the 1.5 interquartile range (IQR). (C) Operational taxonomic units (OTUs). (D, E) Hierarchical and taxonomic analysis of the microbiota in each group. (F) Analysis of microbial populations using UniFrac unweighted principal coordinate analysis (PCoA).

addressing inflammation and dysbiosis, two hallmark features of NAFLD. While research on the gut microbiota and its relation to liver health has made significant progress (Beyoğlu and Idle, 2022; Ganesan et al., 2022), more studies are needed to confirm the association between DGR and intestinal flora balance restoration in liver injury.

5. Conclusions

Our study showed that the combined DGR treatment effectively reduced NLRP3 inflammasome activity, restored intestinal flora balance, and ameliorated NAFLD pathology by regulating liver fat accumulation. This intervention contributed to the mitigation of NAFLD pathophysiology in rats (see Graphical abstract). Our research lays the groundwork for future investigations into the connection between liver detoxification-induced inflammation and the production of intestinal cytotoxins. Furthermore, these findings hold the potential to inform improved therapies for NAFLD and other liver-related diseases.

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Ethics approval

This research was approved by the Animal experimental Ethics Committee of Changchun University of TCM (NO.2022067)

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

CRediT authorship contribution statement

Tianyi Zhao: Writing – original draft, Supervision, Data curation, Conceptualization. Shiyi Lun: Methodology, Data curation. Maoying Yan: Methodology, Investigation. JongPil Park: Software, Conceptualization. Shumin Wang: Writing – review & editing, Resources, Project administration, Funding acquisition, Formal analysis. Changbao Chen: Writing – review & editing, Resources, Project administration, Funding acquisition, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2023.117646>.

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