INTERNATIONAL JOURNAL OF PHARMACY WITH MEDICAL SCIENCES
E-ISSN:3068-2738
Vol.4, No. 1(2024)
www.ijpams.com

PROTECTIVE ROLE OF SILYMARIN AND ITS SYNERGISTIC COMBINATIONS IN PARACETAMOL-INDUCED HEPATOTOXICITY

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ABSTRACT

Paracetamol overdose is a common cause of drug-induced hepatotoxicity, primarily due to oxidative stress and depletion of hepatic glutathione reserves. Silymarin, a well-known hepatoprotective flavonoid, exerts antioxidant, anti-inflammatory, and membrane-stabilizing effects, while omega-3 fatty acids and coenzyme Q10 are established as potent modulators of oxidative stress and mitochondrial function. The present study aimed to evaluate the hepatoprotective effect of silymarin alone and its combination with omega-3 fatty acids and coenzyme Q10 against paracetamol-induced hepatotoxicity in rats. Experimental animals were divided into control, toxic, and treatment groups receiving silymarin alone or in combination with omega-3 fatty acids and/or coenzyme Q10. Hepatoprotection was assessed using biochemical parameters such as serum ALT, AST, ALP, bilirubin, and total protein, along with antioxidant markers including MDA, SOD, and catalase. Histopathological examination of liver tissue was also performed. Results demonstrated that silymarin alone significantly reduced biochemical markers of liver injury and improved antioxidant status compared to the toxic control. However, the combination of silymarin with omega-3 fatty acids and coenzyme Q10 produced superior hepatoprotective effects, with marked reductions in oxidative stress, normalization of liver function tests, and improved histological architecture. The findings suggest that the synergistic use of natural antioxidants may provide enhanced protection against paracetamol-induced hepatotoxicity compared to monotherapy.

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), considered highly correlated with metabolic syndrome [1,2], is widely prevalent globally [3,4]. Due to the potential of its development into life-threatening chronic liver disease and other metabolic diseases, it has caused a huge economic burden [5–7]. In addition to abnormal liver metabolism promoting fatty liver, intestinal dysbiosis reportedly contributes to the severity of NAFLD, showing associations with altered gut microbiota and microbial metabolome [8–10]. The underlying mechanisms mainly include disruption of tight junctions and heightened gut permeability, translocation of lipopolysaccharide (LPS) and inflammatory mediators, decreased short-chain fatty acids, increased ethanol production, and changes in bile acids (BAs) and amino acid-derived metabolism [11,12].

Numerous phytochemicals in nature have poor bioavailability [13]. These inadequately absorbed constituents undergo substantial interaction with the intestinal microbiota upon ingress into the intestinal tract, which consequently possess the potential to confer health advantages by modulating and restructuring the gut microbiota [14–16]. Our antecedent investigations elucidate that polyphenolic compound resveratrol exhibits the capacity to modulate the intestinal microbiota, and results in the attenuation of microbiotadriven synthesis of secondary BAs within the murine intestinal, which finally mitigates the unwarranted absorption of dietary fats [17]. Furthermore, studies suggests that the

Received: 18-01-2024 | Accepted: 03-03-2024 | Published: 10-03-2024 |

dietary supplementation of plant-derived resistant starch can reshape the gut microbiota, altering the composition of BAs and the biological metabolism of amino acids, with ultimately alleviated hepatic lipid deposition and inflammation [18].

To summarize, modulating the intestinal microbiota and its metabolism through dietary intake of phytochemicals represents a potentially significant and readily accessible approach for treating NAFLD, as it lacks approved clinical therapeutic drugs, with current treatment strategies relying on dietary and lifestyle modifications [19,20].

Silymarin, a flavonolignan compound derived from the herbal plant Silybum marianum, demonstrates diverse hepatoprotective properties, encompassing antioxidative and hypolipidemic effects, and is characterized by low bioavailability [21–23]. Silybin is identified as its principal active constituent [22,23]. Accumulating evidence suggests that silymarin ameliorates the progression of NAFLD in both patients and experimental animals [24–27], and can reshape the composition of gut microbiota [28–31]. Research reported that silybin intervention reshaped the microbial community, along with an enrichment of short-chain fatty acids (SCFAs) and a decrease in secondary BAs in the gut [29]. Another study found significant changes in the microbiota and bacterial Vitamin B12 production of rats along with NAFLD amelioration following silymarin intervention [30].

Salvianolic acid B (Sal B) and puerarin, also as the active components extracted from traditional herbal medicines in Asia, have been extensively studied for their protective effects on metabolic homeostasis. Studies observed that Sal B could improve liver enzyme levels and regulate hepatic lipid metabolism in NAFLD mice [32]. Puerarin was observed to alleviate hepatic steatosis and metabolic disorders in rats [33]. It is noteworthy that silymarin, in combination with certain herbal ingredients, can significantly ameliorate the clinical symptoms of patients with NAFLD, while also improving lipid levels and liver function [34]. However, it is still unclear whether silymarin, in combination with these two active components, can collaboratively ameliorate NAFLD by modulating the gut microbiota.

Due to the limited insight into the mechanistic actions of silymarin on the functionality of the gut microbiota and its generated metabolites, we conducted a Silybum marianum extract (silymarin) and polyherbal extract (silymarin, Sal B, puerarin) intervention experiment using a high-fat-diet-induced NAFLD mouse model. The intervention increased probiotics such as Akkermansia and Blautia and suppressed the genera related to secondary BAs biosynthesis, along with enriching SCFAs and inhibiting secondary BAs in the gut. Results from fecal microbiome transplantation (FMT) confirmed that the alteration of microbiota and its metabolites was a crucial link in the effect that silymarin and polyherbal extract had in reducing hepatic lipid accumulation, enhancing liver function, and improving NAFLD.

2. Materials and Methods

2.1. Materials

Silybum marianum extract contained silybin and polyherbal extract (Silybum marianum extract, Pueraria root extract, Salvia miltiorrhiza extract, and schisandra extract) contained silybin, Sal B, and puerarin were provided by BYHEALTH Co., Ltd. (Guangzhou, China), and were added to a high-fat diet (HFD; 45% energy from fat, 20% from protein and 35% from carbohydrate; MD12032, Medicience, Yangzhou, Jiangsu, China) for intervention. Specifically, under the sterile laminar flow hood, 45% high-fat powdered feed was incorporated with an intervention substance or left without an intervention substance, followed by thorough homogenization through stirring. Subsequently, the diet was pelletized through compression

molding and stored at −20 ◦C until utilization. Table 1 presents the specific substance content in intervention feeds.

Table 1. The substance content of 100.3 g intervention feeds.

Ingredient	HF	MSL	HSL	PD
Silybin (g)	–	0.101	0.202	0.101
Sal B (g)	–	–	–	0.046
Puerarin (g)	–	–	–	0.042
Fat (g)	24.000	24.000	24.000	24.000
Protein (g)	24.000	24.000	24.000	24.000
Carbohydrate (g)	41.000	41.000	41.000	41.000
Microelement (g)	11.000	11.000	11.000	11.000
Sterile water (g)	0.3	0.199	0.098	0.111

2.2. Animal Models and Experiment Design

Seven-week-old male C57BL/6J mice were purchased from the Experimental Animal Center of Guangdong Province (Guangzhou, China) and maintained in a specific pathogenfree facility under a 12 h dark/light circle at 25 ± 0.5 ◦C and 50–60% humidity (five mice per cage). After a one-week acclimatization period, mice were randomly divided into two groups. The mice in the model group were fed with 45% HFD to induce NAFLD while the normal control mice (NC group, n = 10) were fed with a normal chow diet (4.2% crude fat, MD17121, Jiangsu Medicience, Yangchow, China). After the successful eight-week modeling, NAFLD mice were randomly divided into four groups: (1) The HF mice continued to be fed 45% HFD as previously. (2) The MSL mice were fed an HFD supplemented with a medium dose of silymarin. (3) The HSL mice were fed an HFD supplemented with a high dose of silymarin. (4) The PD mice received HFD supplemented with polyherbal extract. These groups, along with the NC group, participated in the subsequent silymarin intervention experiment for 16 weeks (n = 10 mice/group).

Our intervention doses were determined based on reported animal experiments and converted according to the dietary intake of 0.1 g/g·bw·day. Mice were given free access to food and water, and their body weight was recorded weekly. In the last week of the experiment, an adequate amount of feces was collected from the HF mice, HSL mice, and PD mice, frozen in liquid nitrogen, and stored at −80 ◦C for fecal microbiota transplantation (FMT) experiments. After 16 weeks of dietary intervention, all mice were subjected to an overnight fast. Subsequently, they were anesthetized (1% pentobarbital, 0.01 mL/g) for blood collection from the eye sockets and euthanized by cervical dislocation. Serum samples were obtained by centrifuging blood at 3000 rpm for 10 min and stored at −80 ◦C. Additionally, part of their liver samples were fixed in 4% paraformaldehyde (F8775, SigmaAldrich, Hartford, CT, USA) for histological analysis, while the remaining samples were immediately frozen in liquid nitrogen and stored at −80 ◦C. All procedures were approved and permitted by the Institutional Review Boards and Animal Care and Use Committees of Sun Yat-Sen University.

2.3. Fecal Microbiome Transplantation (FMT)

The FMT experiment was executed adhering to a well-established protocol, with slight modifications incorporated for optimization [35]. Fecal samples (100 mg) were resuscitated in a water bath at 37 ◦C for 20 min. Afterward, the samples were re-suspended in 1 mL PBS, thoroughly mixed, and then centrifuged at 1000 rpm for 5 min. Filtered through the 100 µm filter, the transplantation material was obtained. The above preparation was conducted within 30 min before each FMT experiment.

C57BL/6J mice were randomly assigned to the control group (ONC, n = 8) and the model group. After inducing NAFLD for 8 weeks using the 45% HFD, NAFLD mice were further randomized into OHF, OHSL, and OPD groups (n = 8 mice/group). All mice received an oral gavage of broad-spectrum antibiotics for 4 weeks (every three days) to establish a pseudo-germ-free model, according to a validated experimental methodology. Starting from the 12th week, OHF, OHSL, and OPD groups received transplant materials from HF, HSL, and PD groups, respectively, while the ONC group

underwent 10% PBS. FMT experiments were performed every three days, totaling 12 weeks. At the end of the experiment, mice were anesthetized (1% pentobarbital, 0.01 mL/g) for blood collection from the eye sockets and sacrificed by cervical dislocation. Serum samples were obtained by centrifuging blood at 3000 rpm for 10 min, and stored at −80 ◦C. Part of their liver samples were fixed in 4% paraformaldehyde (F8775, Sigma-Aldrich, USA) for subsequent histological analysis, while the remaining samples were immediately frozen in liquid nitrogen and stored at −80 ◦C. All efforts were made to minimize animal suffering and reduce the number of animals used.

3. RESULTS

3.1. Silymarin and Polyherbal Extract Attenuate HFD-Induced Steatohepatitis

NAFLD mice were induced by an HFD for 8 weeks, after which the mice were treated with silymarin or polyherbal extract for 16 weeks (Figure 1a). The body weight of mice with an HFD increased significantly compared to the control group, which showed no significant difference by silymarin or polyherbal extract intervention (Figure A1a). To evaluate the effect of the indicated treatment on liver function, we examined whether an HFD caused severe liver function injury, as indicated by an increase in the serum ALT and a decrease in the serum AST/ALT ratio. Treatment with silymarin, especially for the polyherbal extract intervention, restored liver function injury (Figure 1b,c) and this hepatoprotective effect of silymarin was further tested by histologic evaluations. Dietary silymarin and polyherbal extract supplement alleviated the hypertrophy and graying of liver morphology in contrast with the NAFLD mice. H&E and oil red O staining of livers showed that silymarin could effectively reduce the serious accumulation of liver lipid droplet and the extent of hepatocyte ballooning degeneration of the liver (Figure 1d). Additionally, results from hepatic TG, hepatic TC (Figure 1e,f), serum TC, HDL, and LDL (Figure 1g–i) showed that silymarin and the polyherbal extract had restored lipid metabolism disorders in the HF group. Further, the fasting glucose was significantly increased in the HF group and was rescued by silymarin and polyherbal extract (Figure 1j). Moreover, the silymarin-treated group had an improved glucose tolerance (Figure 1k) and insulin tolerance (Figure 1l). The pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-17 (IL17) in the liver were also decreased after silymarin intervention (Figure A1b,c). These observations suggested comparable improvement in HFD-induced liver damages upon silymarin and polyherbal extract supplement.

3.2. Silymarin and Polyherbal Extract Modulated-Flora Are Associated with Improvement in Steatohepatitis

In light of the fact that silymarin is characterized by notably low bioavailability, which suggests its effective interaction with the intestinal microbiota, and with the prevailing recognition in the association between gut microbiota and NAFLD, it could be suggested that silymarin intervention induced benefits that might be derived from the alteration of intestinal microbiota. We thus employed 16S rRNA gene sequencing on fecal samples to examine whether silymarin and polyherbal extract intervention might result in microbiota that potentially exert the effects of ameliorating NAFLD.

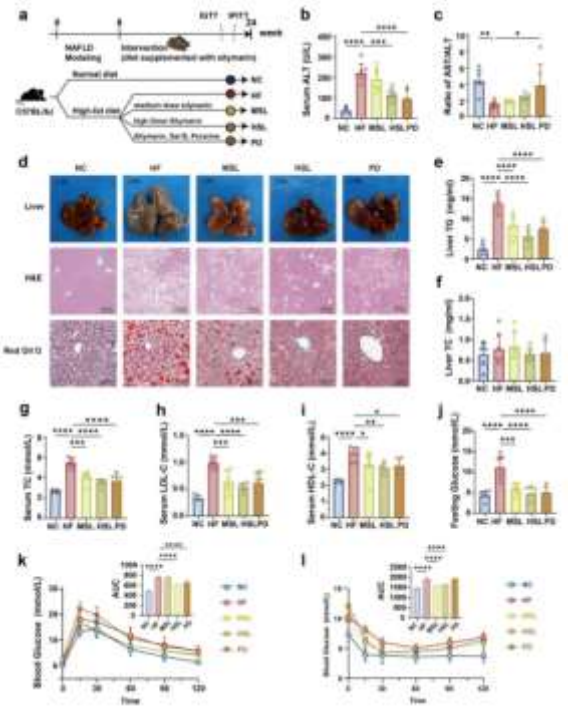


Figure 1. Effect of silymarin and polyherbal extract on the pathological and biochemical indexes of NAFLD in mice. (a) Experiment design of silymarin and polyherbal extract intervention. (b,c) Levels of serum ALT (b) and the ratio of serum AST to ALT (c). (d) Representative morphology (Scale bars, 1 cm), representative microphotograph of hematoxylin and eosin (H&E) staining (Scale bars, 200 μm) and Oil Red O (ORO) staining (Scale bars, 100 μm) of livers. (e,f) Levels of liver TG (e) and liver TC (f); n = 7. (g–j) Levels of serum TC (g), LDL-C (h), HDL-C (i) and fasting blood glucose (j); n = 6 for the NC, MSL and PD group and n = 7 for the HF and HSL group. (k,l) Blood glucose level and area under the curve (AUC) during IGTT (k) and IPITT (l); n = 8. Values were shown as mean ± SD. Statistical significance was evaluated by two-sided one-way ANOVA with Dunnett's post hoc test (compared with HF group); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ALT alanine transaminase, AST aspartate aminotransferase, TG triglycerides, TC total cholesterol, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, IGTT intraperitoneal glucose tolerance test, IPITT intraperitoneal insulin tolerance test.

The alpha diversities of Chao1, Richness, and Shannon_2 (Figure 2a,b) were comparable with intra-individual variance in the HF group and treated groups. However, the Bray–Curtis principal coordinate analysis (Figure 2c) manifested that the operational taxonomic units (OTUs) were clearly separated into four isolated groups, confirming an altered gut microbiota composition upon silymarin and polyherbal extract intervention. The relative abundance analysis revealed that, at the phylum level (Figure 2d), the treated group had a decreased Firmicutes along with an increased Verrucomicrobia and Bacteroidetes, and the HFD-induced high ratio of Firmicutes/Bacteroidetes was decreased in silymarin supplemented groups (Figure 2e). In other studies, these alterations were considered related to NAFLD improvement [43,44]. As Figure 2f displays, at the genus level, the abundance of Costridium_sensu_stricto_1, Ileibacterium, and Lactobacillus, which were encouraged by an HFD, reduced significantly in the HSL group, while the abundance of Desulfovibrio, Blutia, and Akkermansia increased compared with the HF group. In addition, this tendency of variation had a greater magnitude upon polyherbal extract intervention (Figure 2g–l). To sum up, silymarin and polyherbal extract treatment repressed HFD-induced microbiota, especially Clostridium and Ileibacterium, and benefited some probiotics such as Akkermansia

3.3. Transplantation of Altered-Microflora Ameliorate NAFLD

To verify the participation of intestinal microbiota and its metabolites in the amelioration of NAFLD upon silymarin and polyherbal extract treatment, we conducted fecal microbiota transplantation (FMT) experiments.

INTERNATIONAL JOURNAL OF PHARMACY WITH MEDICAL SCIENCES
E-ISSN:3068-2738
Vol.4, No. 1(2024)
www.ijpams.com

Fecal microbiota from HF, HSL and PD mice were transferred into three additional groups of HFD-induced NAFLD mice, respectively (Figure 4a). After 12 weeks of FMT, we observed a reduced level of serum ALT and an increased ratio of AST to ALT in HSL-FMT and PD-FMT mice (Figure 4b,c), consistent with the liver function recovery in intervention experiments, paralleling with significantly reduced extent of obviously visible lipid droplets accumulation and hepatocyte ballooning degeneration in the liver tissue of H&E and ORO staining (Figure 4d). Further, silymarin and polyherbal extract FMT likewise led to lower levels of serum TG, TC, LDL, HDL (Figure 4e–h). In addition, an improved glucose tolerance was observed in HSL-FMT mice (Figure 4i).

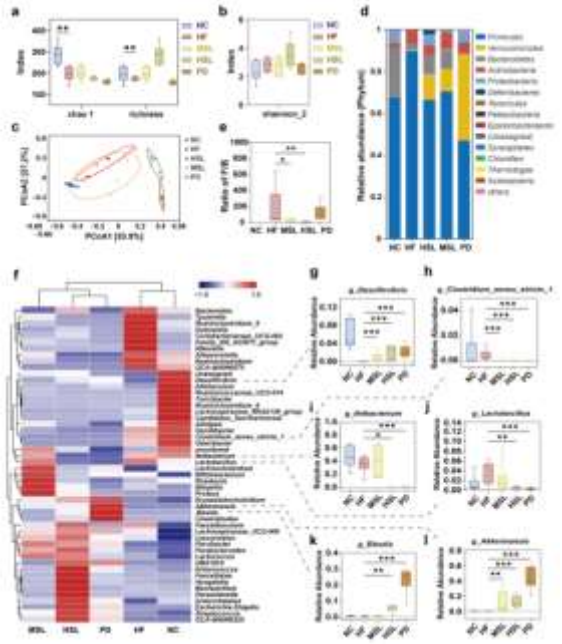
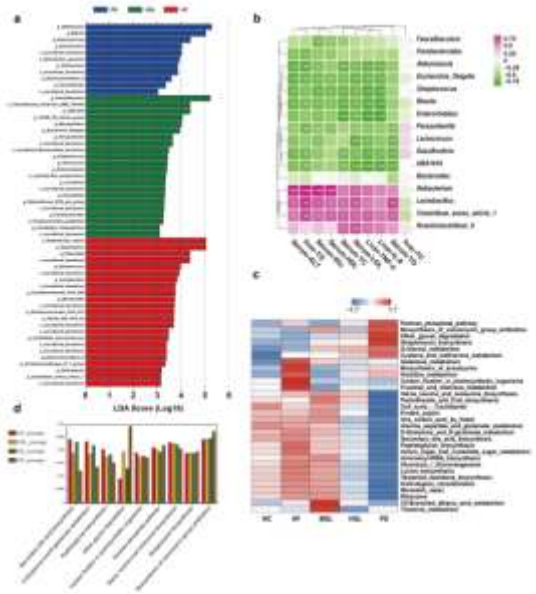


Figure 2. Silymarin and polyherbal extract modulates the composition of gut microbiota. 16S rRNA gene sequencing analysis in fecal bacterial DNA from NC, HF, MSL, HSL, and PD mice was performed; n = 7 individuals/group. (a,b) Alpha diversity was assessed by chao 1, observed richness (a) and Shannon_2 diversity index (b), respectively. (c) Bray–Curtis beta diversity was visualized with the principal coordinate analysis (PCoA). (d,e) The stacking histogram showing the taxonomic summary of phyla composition in feces from all groups (d) and the boxplots showing the ratio of fecal Firmicutes to Bacteroidetes in relative abundance (e). (f) The heatmap shows the relative abundance clustering (average) of microbial communities at the genus level in all groups. (g–l) The boxplots show statistical differences of selected differentially abundant genus between groups. Twosided Mann–Whitney nonparametric test were conducted for comparisons; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The horizontal line in each box represents the median, the top and the bottom of the box the 25th and 75th percentiles, and the whiskers the min to max.



gure 3. Silymarin and polyherbal extract modulated microbiota are related to NAFLD improvement and secondary bile acid biosynthesis. (a) Histogram of LDA score generated by LEfSe depicting the taxonomic contribution between microbiome communities from HF, HSL and PD mice (LDA > 3.0); n = 7. (b) Heatmap shows the correlations between the selected differentially abundant genus and various indicators related to NAFLD in HF and HSL groups; n = 6 and the correlations were

analyzed using two-sided Spearman's correlation, FDR-adjusted $p < 0.05$ (*, **, and *** indicate adjusted $p < 0.05$, 0.01, and 0.001, respectively) was shown. (c) Heatmap of KEGG functional pathway clustering (average) analysis reflects the functional composition between five groups based on OTU abundance. (d) The KEGG pathway with significant differences (adjusted $p < 0.05$) analyzed by Kruskal-Wallis H test among NC, HF, HSL and PD mice was shown; n = 7. LDA linear discriminant analysis, FDR false discovery rate, KEGG Kyoto Encyclopedia of Genes and Genomes.

4. DISCUSSION

Natural plant extracts have been a focal point of research for their potential as viable healthy food agents to ameliorate NAFLD. The present study demonstrated that silymarin or silymarin with salvianolic acids B and puerarin formula-improved HFD-induced hepatic steatosis. Furthermore, the beneficial alterations might be linked with gut microbiota and their metabolites, especially BAs as a crucial one for the amelioration of NAFLD (Figure 8).

Flavonoid compound silymarin has long been utilized in traditional medicine for treating liver and bile diseases [45]. Numerous recent studies have observed the positive effects of silymarin in improving NAFLD [46,47]. Several clinical randomized controlled trials indicate that silymarin contributes significantly to ameliorating the liver of patients with NAFLD. This improvement includes reductions in liver fat deposition, hepatocellular ballooning, and liver fibrosis, along with decreases in liver transaminase levels [26,48–50]. Similarly, several studies suggest that Sal B, a natural polyphenol compound derived from Radix Salvia Miltiorrhiza, exhibits protective effects against hepatic fat deposition and inflammation induced by an HFD [32,51,52]. Additionally, as a type of flavonoid compound, puerarin has also been extensively researched, demonstrating its potential to treat various chronic diseases, including NAFLD [33,53,54]. The present study provides robust evidence supporting the notion mentioned above. In lieu of the oral gavage method, we incorporated silymarin or polyherbal extract (silymarin in combination with Sal B and puerarin) into an HFD for the treatment of NAFLD in mice. We observed that both silymarin and polyherbal extract significantly improved NAFLD, as manifested by reduced hepatic lipid droplet accumulation, enhanced liver function, decreased levels of hepatic TG and serum TC, and the restoration of glucose tolerance. Additionally, insulin resistance, a factor associated with NAFLD, and levels of liver inflammatory cytokines TNF-α and IL-6, were also ameliorated with supplementation of silymarin.

Generally, insulin resistance is associated with lower HDL-C levels [55]. In this study, both the MSL and HSL groups showed improvements in insulin levels, yet the HDL-C levels in mice from these groups, as well as the PD group, decreased, which seems inconsistent with the aforementioned research. However, some studies have found that increases in HDL-C levels may also be accompanied by hepatic lipid accumulation and worsening insulin resistance [56,57]. Interestingly, following intervention with certain phytochemicals in mice, both increased HDL-C levels and improvements in insulin resistance have been observed [58,59]. The significant improvement in hepatic steatosis in this study may be related to more cholesterol being metabolized into bile acids in the liver. Therefore, a comprehensive consideration of hepatic lipid metabolism, serum HDL levels, and insulin resistance is needed to interpret the results. In addition, it is noteworthy that the weight change in treated mice was observed without significant differences compared to HFD mice, suggesting that the beneficial effects of silymarin might not be related to reduced energy intake.

Given the widely acknowledged dysregulation of the gut microbiota in the pathogenesis and progression of NAFLD, we examined whether the dysbiosis could be improved following silymarin and polyherbal extract intervention. At the genus level, supplementation with high doses of silymarin and polyherbal extract increased the abundance of probiotics like Akkermansia and Blautia. Moreover, silymarin and polyherbal extract resulted in the significant suppression of HFD-induced genera such as Lactobacillus, Bacteroides, Clostridium, and Ileibacterium. These bacteria above are reported to exhibit bile salt hydrolase activity and are primary contributors to the secondary BA synthesis [60,61]. Further, we observed that the transplantation of silymarin and polyherbal extract adapted feces likewise improved NAFLD, and changes in Clostridium and Blautia in the FMT experiment aligned with the results of the intervention experiment. The present findings validated that silymarin and polyherbal extract could enhance liver function and ameliorate NAFLD by modulating the gut microbiota. The alteration of gut microbiota composition usually generated the different microbiota metabolites which are directly linked with the changes in hepatic pathogenesis [9,11]. We employed untargeted metabolomics to further explore the impact of gut microbiota on metabolite levels. As anticipated, the differential metabolites changed by silymarin and polyherbal extract intervention primarily include BAs and derivatives, carboxylic acids and their derivatives, glycerophospholipids, medium and long-chain fatty acids, glutathione and its derivatives, and branched-chain amino acids (BCAAs) and their derivatives. **5. CONCLUSIONS**

This study confirms the hepatoprotective potential of silymarin in paracetamol-induced liver injury and highlights the superior efficacy of its combination with omega-3 fatty acids and coenzyme Q10. The synergistic effect was evident in the significant restoration of biochemical, antioxidant, and histopathological parameters, suggesting that combination therapy offers a more comprehensive protective mechanism against oxidative stress and cellular damage. These findings support the therapeutic promise of multi-antioxidant strategies in preventing drug-induced hepatotoxicity and may have implications for clinical management of paracetamol overdose and other oxidative stress–mediated hepatic disorders. Further studies, including molecular investigations and clinical evaluations, are warranted to validate these experimental outcomes and translate them into effective therapeutic interventions.

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