

# Optimization of Extraction Conditions of Pomegranate Peel Polyphenols and Its Protective Effect on Acute Alcoholic Liver Injury in Mice

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**Abstract** [Objectives] This study was conducted to explore the optimization of ultrasonic-assisted organic solvent extraction of pomegranate peel polyphenols (PPPs), and to study the protective effect of PPPs on acute alcoholic liver injury in mice. [Methods] The optimal extraction conditions of PPPs were determined by single factor and orthogonal experiments, and an acute alcoholic liver injury model in mice was established. Bifendate was used as the positive control group to investigate the protective effect of low, medium and high doses of PPPs on acute alcoholic liver injury. [Results] The optimum extraction process parameters were followed as 60% ethanol concentration, solid-liquid ratio of 1 : 40 (*w/v*), extraction temperature of 50°C, and extraction time of 1.5 h, and the yield was 1.42%. The results of animal experiments showed that PPPs could effectively reduce the degree of alcoholic liver injury in mice, reduce the levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and reduce the inflammation and necrosis of liver tissue in mice. Meanwhile, the total polyphenols from pomegranate peel also significantly reduced the expression levels of malondialdehyde (MDA), tumor necrosis factor (TNF- $\alpha$ ) and interleukin-6 (IL-6) in mice, and increased the levels of superoxide dismutase (SOD) and reduced glutathione (GSH) in liver tissue of mice, indicating its antioxidant and anti-inflammatory effects, further illustrating its protective effect on alcoholic liver injury. [Conclusions] PPPs could reduce the expression levels of TNF- $\alpha$ , IL-6 and MDA in mice, and increase the expression levels of SOD and GSH to achieve the protective effect on acute alcoholic liver injury in mice. This study will provide new ideas for the development of new anti-alcoholic liver injury drug resources.

**Key words** Pomegranate peel polyphenols; Extraction condition optimization; Alcoholic liver injury; Oxidative stress; Inflammatory factor

**DOI:**10.19759/j.cnki.2164–4993.2023.04.004

Alcoholic liver injury (ALI) is a common disease worldwide, and its incidence and mortality continue to increase. Alcohol intake can lead to liver damage and dysfunction, which may eventually lead to serious consequences such as cirrhosis and liver cancer.

As the largest digestive gland of the human body, the liver is mainly responsible for metabolism, detoxification and immunity, and maintains the normal operation of the body. About 80% of alcohol is rapidly absorbed after drinking, and up to 90% of alcohol is metabolized in the liver. ALI refers to liver damage caused by improper alcohol intake. The proportion of liver cirrhosis caused by alcohol in China has increased by 13% compared with 20 years ago<sup>[1]</sup>. The incidence of liver disease caused by drinking has increased year by year, and ALI has become the second largest liver injury disease in China<sup>[2]</sup>. ALI threatens human life and health, and its pathogenesis is complex, involving the direct and indirect damage of ethanol and its metabolites to the liver, as well as the interaction of various pathogenic factors.

Pomegranate peel, a Chinese medicine, refers to the dried peel of the fruit of pomegranate plant *Punica granatum* L. Pomegranate is cultivated in north and south of China, especially in Jiangsu and Henan. The medicinal use of pomegranate peel was

first recorded in ‘Lei Gong’s theory of fire-blasting’, which originated in the Western Regions (referring to the countries west of Dunhuang). It was introduced into China in the Han Dynasty, and there were many records of herbs in the past dynasties. It has the effects of antidiarrheal, hemostasis and deworming. It is often used for long diarrhea, long dysentery, hematochezia, anal prolapse, metrorrhagia, vaginal discharge, abdominal pain and other symptoms.

Pomegranate peel is rich in amino acids, proteins, sugars and their glycosides, phenols and tannins, organic acids, flavonoids, alkaloids, volatile oils and other chemical components<sup>[3]</sup>. Studies have shown that pomegranate peel has anti-inflammatory, anti-infection, lipid-lowering, hypoglycemic, anti-arteriosclerosis, prevention of liver fibrosis and other pharmacological activity<sup>[4–5]</sup>. Pomegranate is rich in polyphenols, and its content and composition are mainly concentrated in pomegranate peel. Pomegranate peel polyphenols (PPPs) is a general term for polyphenolic hydroxyl compounds in pomegranate peel, accounting for 10%–20% of the dry weight of pomegranate peel. Its main components are punicalagin, gallic acid, epicatechin, chlorogenic acid, etc. As an important physiologically active ingredient in pomegranate peel, PPPs have great application value in food, medicine and other fields<sup>[6]</sup>.

Studies have shown that PPPs can be used as a natural antioxidant raw material, but there are few reports on the protective effect of PPPs on alcoholic liver injury. Therefore, in this study, with pomegranate peel as the material, its protective effect on alcoholic liver injury mice was examined by optimizing the extraction of PPPs, and a preliminary mechanism study was made, which provides experimental basis for further research and in-depth development of pomegranate peel. The results will

Received: May 7, 2023 Accepted: July 11, 2023

Supported by Provincial Key College Students Innovation and Entrepreneurship Training Program Project (202211834033).

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certainly promote the high-value utilization of pomegranate processing industry and local economic development.

## Materials and Methods

### Materials and reagents

Pomegranate peel powder was purchased from Hangzhou Yami Agricultural Technology Co. Ltd. Allic acid was purchased from Shanghai Aladdin Biochemical Technology Co. Ltd. Folin-phenol reagent (FC 1 ml/L) was purchased from Sinopharm Chemical Reagent Co. Ltd. Bifendate dropping pills were purchased from Wanbangle Pharmaceutical Group Co. Ltd. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits, MDA detection kit, SOD detection kit, reduced glutathione (GSH) detection kit, IL-6 ELISA kit, TNF- $\alpha$  ELISA kits were all purchased from Nanjing Jiancheng Biotechnology Co. Ltd. 4% of paraformaldehyde fixative was purchased from Shenzhen Xijing Biotechnology Co. Ltd. Anhydrous ethanol was purchased from Tianjin Fuyu Fine Chemical Co. Ltd. Sodium chloride injection was purchased from Henan Kelun Pharmaceutical Co. Ltd.

### Experimental animals

60 Kunming mice (male and female, 6 weeks old,  $20 \pm 2$  g) were purchased from Henan Experimental Animal Center. The animals were raised under pathogen-free condition (12 h/12 h light-dark cycle,  $22 \pm 2$  °C) for adaptive fed with water and commercial diet for 7 d. All experimental procedures were approved by Animal Ethics Committee of Huanghe Science and Technology University (certificate No. 2023004).

### Determination of PPPs content

The content of PPPs was determined by Folin-Ciocalteu (FC) method. Based on the research of Wu *et al.* [7], a slight improvement was made. In specific, 1 ml of extract was mixed with 1 ml of FC reagent, 2 ml of 12%  $\text{Na}_2\text{CO}_3$  solution and ultrapure water and diluted to 10 ml. The absorbance wavelength was set at 765 nm<sup>[8-9]</sup>. The absorbance was measured after 1 h of color development, and the total polyphenol content was calculated.

PPPs extraction rate (%) = Pomegranate peel total polyphenol mass (g)/Pomegranate peel mass (g).

### Production of PPPs standard curve

Firstly, 0.1 g of gallic acid was resolved by 40% ethanol to 100 ml as V1, and 1 ml of V1 solution was diluted 10 times by 40% ethanol as V2. Next, 0 (blank), 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml of V2 standard liquid were diluted with ultrapure water into 10 times volume, and 1 ml was taken for absorbance determination by FC method. The absorbance wavelength was set at 765 nm. A standard curve was drawn and the regression equation was obtained.

### Extraction and optimization of PPPs

The ultrasonic power was set at 100 W. The ultrasonic group and the constant-temperature water bath group were extracted with 60% of ethanol at a solid-liquid ratio of 1 : 15 (*w/v*) and at 20 °C for 1 h. The extraction rate of PPPs was compared between the two groups.

The basic conditions of single-factor experiments were as followed: solid-liquid ratio of 1 : 15 (*w/v*), reaction at 40 °C for 1 h in 60% ethanol. The ultrasonic power was set to 100 W, and assisted extraction was performed at a temperature of 20 °C for 20 min. After assisted extraction, single-factor experiments were carried out according to ethanol concentration (40%, 50%, 60%, 70%, 80%), temperature (30, 40, 50, 60, 70 °C), solid-liquid ratio (1 : 10, 1 : 20, 1 : 30, 1 : 40, 1 : 50 *w/v*), extraction time (0.5, 1.0, 1.5, 2.0, 2.5 h), respectively. The optimal extraction single-factor of PPPs extraction was investigated, and then the optimal conditions of PPPs extraction were obtained according to the four-factor three-level orthogonal experiment. Polyphenol extraction solution was obtained by filtration, and then the total polyphenols of pomegranate peel were obtained by rotary evaporation and freeze-drying.

### Grouping and administration of animals

60 Kunming mice were randomly divided into 6 groups, which were blank group (N), model group (M), Bifendate Pills (BP, positive control group), and low-dose, medium-dose and high-dose groups of PPPs (LP, MP and HP). After 1 week of adaptive feeding, the administration group was given BP(150 mg/kg), LP(100 mg/kg), MP (200 mg/kg) and HP (400 mg/kg) by gavage every day from the 8<sup>th</sup> day, respectively. And the N group and the M group were given equal volume of normal saline by gavage every day for 1 week.

### Establishment of ALI model

One hour after the last administration, other groups were given 50% ethanol solution 14 ml/kg by gavage except for the blank group which was given equal volume of distilled water by gavage.

### Determination of liver index

After fasting for 12 h, orbital blood was taken, and then the mice were sacrificed. The liver was taken out, and the liver index was analyzed.

Liver index = [Liver mass (mg)/Mouse mass (g)]  $\times$  100%.

### Detection of biochemical indicators

The orbital blood was stood for 2 h, and then centrifuged at 3 500 r/min for 10 min. The levels of ALT, AST, SOD, MDA, GSH, TNF- $\alpha$  and IL-6 in serum were measured according to the instructions, respectively.

### Statistical analysis

SPSS 26.0 was used as statistical software to process the data. The data were expressed as mean  $\pm$  SEM. One-way ANOVA was used for comparison between multiple groups. LSD method was used for pairwise comparison of homogeneity of variance.  $P < 0.05$  was considered statistically significant.

## Results

### Standard curve of PPPs

The standard curve of gallic acid was drawn (Fig. 1), and the regression equation was  $y = 152.1x + 0.0062$ ,  $R^2 = 0.9995$ .

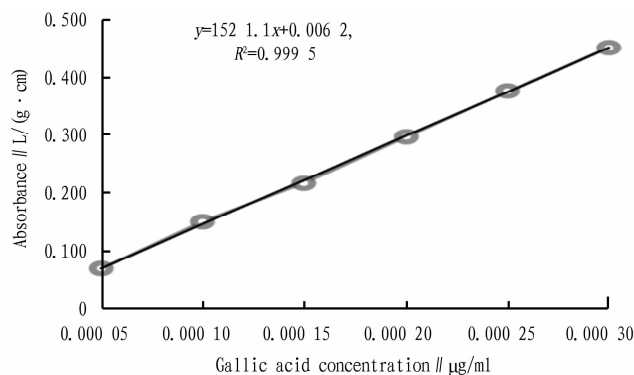


Fig. 1 Standard curve of gallic acid

### Single-factor experiments of PPPs extraction

The extraction rate of ultrasonic extraction was 1.23%, and the extraction rate of constant temperature water bath extraction was 1.07%. The ultrasonic extraction method had higher extraction rate than the traditional constant temperature water bath extraction method.

#### Effect of ethanol concentration on extraction rate of PPPs

The effects of ethanol concentration on the extraction rate of PPPs were tested under the conditions of solid-liquid ratio of 1 : 15, 50 °C and 1 h by only changing ethanol concentration. As shown in Fig. 2, with the increase of ethanol concentration, PPPs extraction rate increased first and then decreased. When the ethanol concentration was 60%, PPPs extraction rate was the highest, at 1.21%.

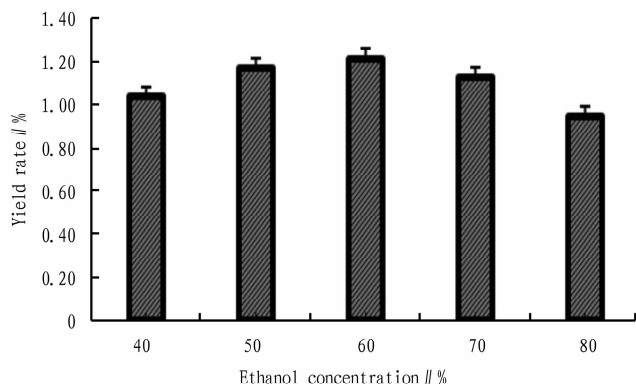


Fig. 2 Effect of ethanol concentration on PPPs extraction rate

#### Effect of time on extraction rate of PPPs

Under the conditions of solid-liquid ratio of 1 : 15, 50 °C and 60% ethanol, and only changing the time, the effect of time on the extraction rate of PPPs was tested. As shown in Fig. 3, with the increase of time, the extraction rate of PPPs increased first and then decreased, and the extraction rate was the highest at 1.5 h, which was 1.26%.

#### Effect of temperature on extraction rate of PPPs

Under the conditions of solid-liquid ratio of 1 : 15, 60% ethanol and 1 h, the effect of water bath temperature on the extraction rate of PPPs was tested by only changing the water bath temperature. As shown in Fig. 4, with the increase of water bath temperature, the extraction rate of PPPs increased first and then

decreased. When the water bath temperature was 50 °C, the extraction rate was the highest, which was 1.15%.

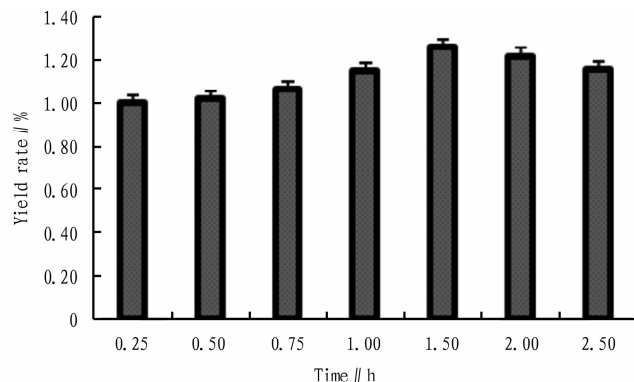


Fig. 3 Effect of time on PPPs extraction rate

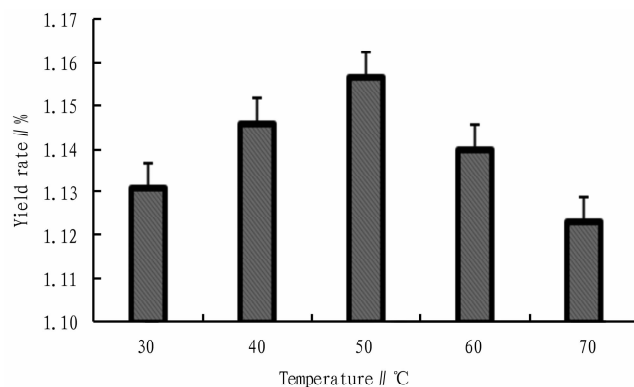


Fig. 4 Effect of temperature on PPPs extraction rate

#### Effect of solid-liquid ratio on extraction rate of PPPs

Under the conditions of ethanol concentration of 60%, 50 °C and 1 h, the effect of solid-liquid ratio on the extraction rate of PPPs was tested by only changing the solid-liquid ratio. As shown in Fig. 5, with the increase of solid-liquid ratio, the extraction rate of PPPs increased first and then decreased. When the solid-liquid ratio was 1 : 40, the extraction rate was the highest, at 1.44%.

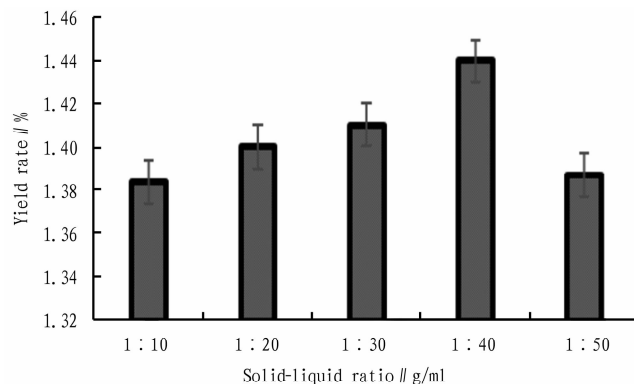


Fig. 5 Effect of solid-liquid ratio on the extraction rate of PPPs

### Orthogonal experimental design and its results

According to the results of single-factor experiments, an  $L_9(3^4)$  orthogonal experiment (Table 2) was carried out with the ratio of material to liquid (A), ethanol concentration (B), time

(C) and temperature (D) as the investigation levels (Table 1) and the extraction rate of PPPs as the index, and the results were verified by experiments. Because the extraction rate of solid-liquid ratio of 1 : 50 was not much different from that of other solid-liquid ratios, considering the cost of raw materials and saving resources, the solid-liquid ratios of 1 : 20, 1 : 30 and 1 : 40 were selected for orthogonal arrangement, and other three groups of single factors were arranged normally, as shown in Table 1.

**Table1 Factors and levels orthogonal test for PPPs extraction**

Level	A (solid-liquid ratio) //g/ml	B (ethanol concentration) //%	C (time) h	D (temperature) °C
1	1 : 20	50	1.0	40
2	1 : 30	60	1.5	50
3	1 : 40	70	2.0	60

According to the *R* value in Table 2, the order of the factors affecting the extraction rate of total polyphenols was B > A > D > C, that is, ethanol concentration > solid-liquid ratio > temperature > time. The best combination of PPPs extraction was B<sub>1</sub>A<sub>3</sub>D<sub>2</sub>C<sub>1</sub>, i. e., 60% ethanol as solvent, solid-liquid ratio 1 : 50 (*w/v*), extraction temperature 40 °C, and extraction time 1.5 h, with which the extraction rate of PPPs was 1.42%.

**Orthogonal results and range analysis**

**Table 2 Orthogonal experimental design and results of the orthogonal test L<sub>9</sub> (3<sup>4</sup>)**

No.	A	B	C	D	Extract // %
1	1	1	1	1	1.22
2	1	2	2	2	1.30
3	1	3	3	3	1.08
4	2	1	2	3	1.25
5	2	2	3	1	1.21
6	2	3	1	2	1.21
7	3	1	3	2	1.42
8	3	2	1	3	1.34
9	3	3	2	1	1.18
K <sub>1</sub>	1.348	1.460	1.413	1.351	
K <sub>2</sub>	1.378	1.444	1.398	1.478	
K <sub>3</sub>	1.478	1.300	1.393	1.375	
R	0.130	0.160	0.020	0.127	

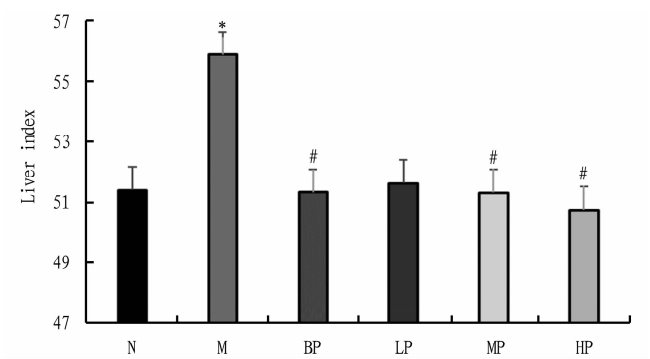
**Effects of PPPs on liver index in mice**

The result of effect of PPPs on liver index was shown in Fig. 6. Compared with the N group, the liver index of the M group was significantly increased (*P* < 0.05). Compared with the M group, the liver index of BP group, MP and HP groups was significantly decreased (*P* < 0.05), and the effect of PPPs MP group was similar to that of BP group. The liver index of the LP group was lower than that of the M group, but there was no significant difference (*P* > 0.05).

**Effects of PPPs on serum ALT and AST levels in mice**

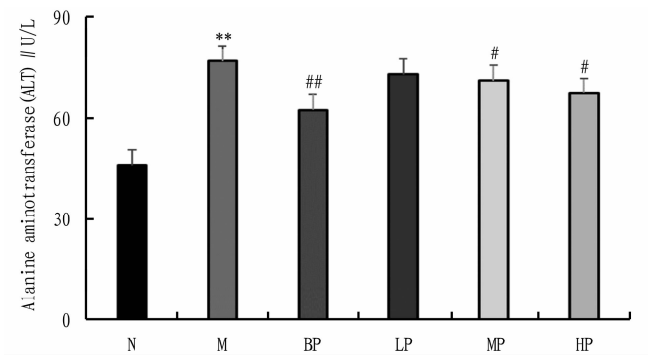
Compared with the N group, the levels of ALT and AST in the M group were significantly increased (*P* < 0.01), which also indicated the success of animal experiment modeling. Compared with the M group, the levels of ALT and AST in the BP group

(*P* < 0.01) and the MP and HP groups of PPPs (*P* < 0.05) were significantly reduced, and the efficacy of the HP group of PPPs was closer to the BP group than the MP group. Although there was a slight decrease in the LP group of PPPs, there was no significant difference (*P* > 0.05), as shown in Fig. 7 and Fig. 8.



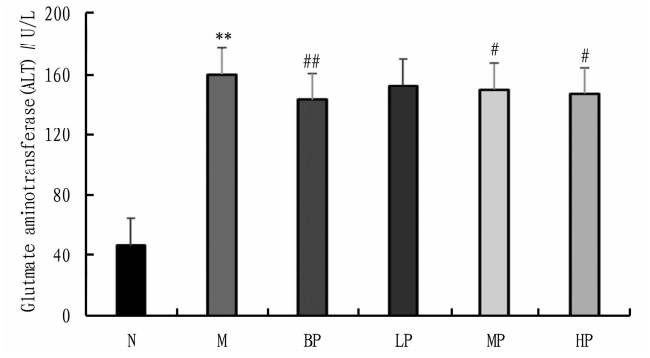
Compared with the N group: \* *P* < 0.05; compared with the M group: # *P* < 0.05 vs M group.

**Fig. 6 Comparison of liver index of mice in various groups**



Compared with the N group: \*\* *P* < 0.01; compared with the M group: # *P* < 0.05, ## *P* < 0.01.

**Fig.7 Comparison of ALT levels in mice of various groups**



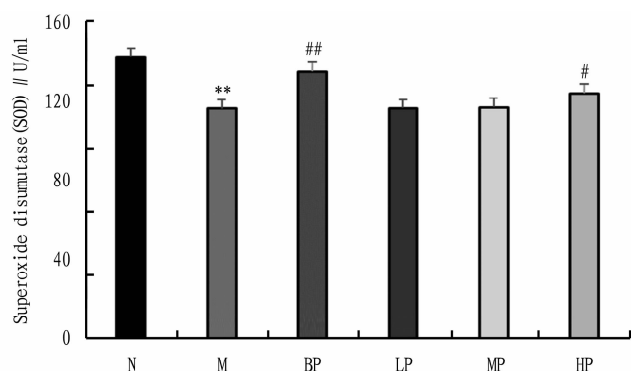
Compared with the N group: \* \* *P* < 0.01 ; compared with the M group: # *P* < 0.05, ## *P* < 0.01.

**Fig. 8 Comparison of AST levels in mice of various groups**

**Effects of PPPs on the levels of SOD and GSH in serum of mice**

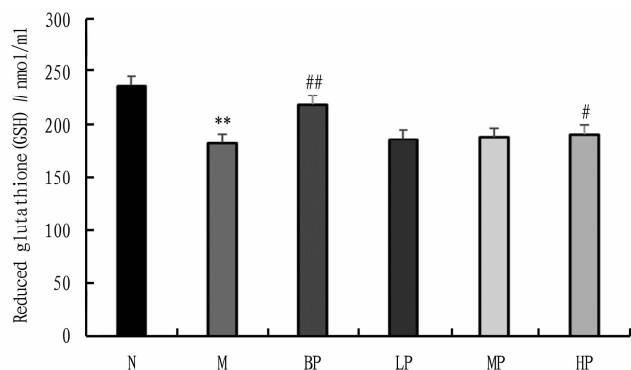
The effects of PPPs on levels of SOD and GSH in serum are shown in Fig. 9 and Fig. 10. Compared with the N group, the levels of SOD and GSH in the M group were significantly decreased (*P* < 0.01). Compared with the M group, the levels of SOD and GSH in the BP group (*P* < 0.01) and HP group of PPPs

( $P < 0.05$ ) were significantly increased. Compared with the M group, SOD and GSH in the LP and MP groups of PPPs increased slightly, but there was no significant difference ( $P > 0.05$ ).



Compared with the N group: \*\*  $P < 0.01$ ; compared with the M group: #  $P < 0.05$ , ##  $P < 0.01$ .

**Fig. 9** Comparison of SOD levels in mice of various groups

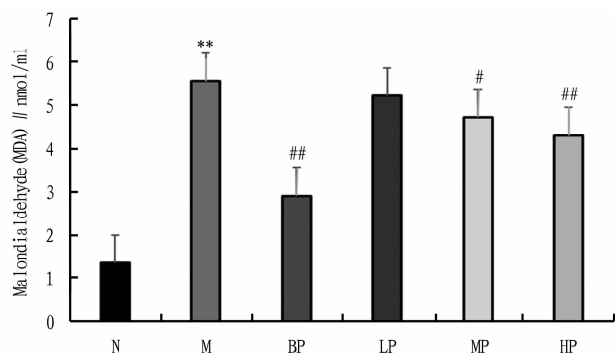


Compared with the N group: \*\*  $P < 0.01$ ; compared with the M group: #  $P < 0.05$ , ##  $P < 0.01$ .

**Fig. 10** Comparison of GSH levels in mice of various groups

### Effect of PPPs on serum MDA level in mice

Compared with the N group, the MDA level of the M group increased ( $P < 0.01$ ) (Fig. 11). Compared with the M group, the levels of MDA in the BP group ( $P < 0.01$ ), MP group ( $P < 0.05$ ) and HP group of PPPs ( $P < 0.01$ ) decreased. Compared with the M group, the MDA in the LP group of PPPs decreased slightly, but there was no significant difference ( $P > 0.05$ ).

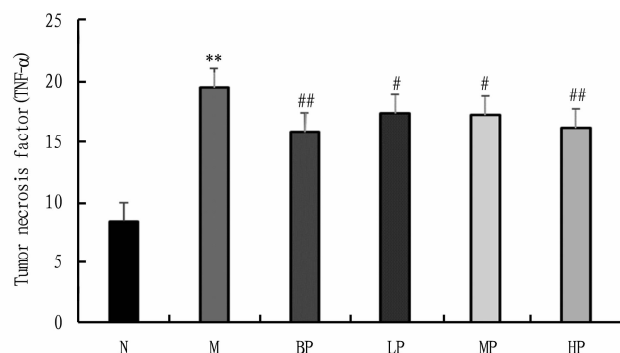


Compared with the N group: \*\*  $P < 0.01$ ; compared with the M group: #  $P < 0.05$ , ##  $P < 0.01$ .

**Fig. 11** Comparison of MDA levels in mice of various groups

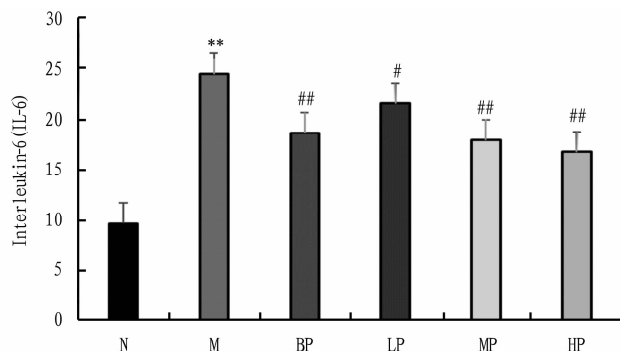
### Effects of PPPs on serum TNF- $\alpha$ and IL-6 levels in mice

Compared with the N group, the levels of TNF- $\alpha$  and IL-6 in the M group increased ( $P < 0.01$ ) (Fig. 12 and Fig. 13). Compared with the M group, the levels of TNF- $\alpha$  and IL-6 in the BP group ( $P < 0.01$ ) and LP group ( $P < 0.05$ ), MP group ( $P < 0.05$ ) and HP group ( $P < 0.01$ ) of PPPs were significantly decreased.



Compared with the N group: \*\*  $P < 0.01$ ; compared with the M group: #  $P < 0.05$ , ##  $P < 0.01$ .

**Fig. 12** Comparison of TNF- $\alpha$  levels in mice of various groups



Compared with the N group: \*\*  $P < 0.01$ ; compared with the M group: #  $P < 0.05$ , ##  $P < 0.01$ .

**Fig. 13** comparison of IL-6 levels in mice of various groups

The results of this study showed that PPPs could alleviate or treat inflammation in mice, and the efficacy of HP group and BP group was the most significant. Although there was a certain gap between the overall efficacy of PPPs and the efficacy of BP group, with the increase of the LP, MP and HP gradients of PPPs, the efficacy was also significantly increased. It could be seen that there was a positive correlation between PPPs dose gradient and the therapeutic effect of ALI in mice.

### Discussion

In previous studies, the extraction of PPPs usually used traditional methods, such as soaking, hot water extraction<sup>[10]</sup> and ethanol solvent extraction<sup>[11]</sup>. These methods are simple, but there are a series of drawbacks such as high energy consumption, high cost and environmental pollution<sup>[12]</sup>. Ultrasonic technology is a new extraction method, which has obvious advantages in extraction efficiency and energy consumption. It can provide scientific basis for the industrial production of PPPs, and also provide reference for ultrasonic technology in the extraction of other natural products. Ethanol is oxidized to acetic acid by alcohol dehydrogenase

(ADH) and aldehyde dehydrogenase (ALDH) in hepatocytes. The normal body has a relatively complete antioxidant system, including SOD and GSH-Px and other enzyme antioxidant systems, as well as glutathione (GSH) and vitamin C and other non-enzymatic antioxidant systems, which can produce reactive oxygen species in the antioxidant process. However, when a large amount of ethanol is ingested, the antioxidant substances in the liver will be rapidly consumed, and the antioxidant system cannot immediately remove excessive free radicals, causing oxidative stress, resulting in a rapid increase in lipid peroxidation products<sup>[13]</sup>. In addition, a large amount of hydrogen ions can also be produced during the conversion of ethanol metabolism to acetic acid. Hydrogen ions can bind to NAD<sup>+</sup> in liver cells to form nicotinamide adenine dinucleotide (NADH), resulting in an increase in the percentage of NADH in liver cells. Excessive NADH in cells can cause 'reductive stress'. By inducing ferritin to release ferrous<sup>[14]</sup> or oxidizing NADH<sup>[15]</sup> by xanthine dehydrogenase to generate a large amount of reactive oxygen species (ROS), liver cell membrane damage is caused. MDA is the main lipid peroxidation end product, and its concentration can reflect the intensity of lipid peroxidation in the body, and to a certain extent, it can indirectly reflect the severity of free radical attack on somatic cells. GSH has antioxidant and integrated detoxification effects. Reduced GSH reacts with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and lipid peroxides to scavenge lipid peroxides and oxygen free radicals in the body. The results of this study showed that AST and ALT in the serum of mice with acute alcoholic liver injury were significantly higher than those in the normal group. Meanwhile, MDA production in liver tissue increased significantly, SOD and GSH decreased significantly. The three dose groups of PPPs could increase the expression levels of SOD and GSH and reduce the production of MDA to varying degrees ( $P < 0.05$ ), indicating that PPPs could enhance the scavenging ability of ethanol-induced liver reactive oxygen species and lipid peroxidation products to protect liver cells.

The NF- $\kappa$ B pathway is a nuclear transcription factor and an essential factor for the transcription of various inflammatory factors. It can initiate and strengthen the inflammatory response. The activation of various inflammatory signaling pathways can eventually cause the activation of the NF- $\kappa$ B pathway signaling pathway. The NF- $\kappa$ B pathway dimer dissociates and activates into the nucleus to regulate the transcription of inflammatory factors, releasing downstream inflammatory factors such as IL-1, IL-6, TNF- $\alpha$  and other damaged organs, further aggravating oxidative stress and triggering cell membrane lipid peroxidation to cause biofilm damage, causing cell necrosis and apoptosis<sup>[16]</sup>. Oxidative stress and inflammatory immunity produced by alcohol metabolism are the main pathogenic factors leading to ALI<sup>[17]</sup>. The results of this study showed that the three dose groups of PPPs could down-regulate the expression levels of TNF- $\alpha$  and IL-6 to varying degrees ( $P < 0.01$ ), indicating that PPPs could protect the liver by reducing ethanol-induced inflammatory response in mice. However, whether PPPs can protect liver by regulating NF- $\kappa$ B pathway remains to be further studied.

In summary, PPPs could reduce the content of serum MDA and increase the expression levels of SOD and GSH to enhance the

antioxidant capacity of mice. Meanwhile, PPPs also down-regulated the expression levels of TNF- $\alpha$  and IL-6 to reduce the inflammatory response in mice. Therefore, PPPs could protect the liver of mice through anti-inflammatory and anti-oxidation.

## Conclusion

The MP and HP of PPPs could significantly reduce the serum ALT and AST levels of ALI mice ( $P < 0.05$ ), and down-regulate the expression levels of TNF- $\alpha$  and IL-6 in ALI mice ( $P < 0.01$ ), increase the expression levels of SOD and GSH ( $P < 0.05$ ), and reduce the content of MDA ( $P < 0.05$ ) to achieve the protective effect on ALI mice. And the LP, MP and HP groups of PPPs had obvious therapeutic gradient on ALI mice. Therefore, PPPs had a significant protective effect on ALI, and its mechanism might be related to reducing liver inflammation, scavenging free radicals and anti-lipid peroxidation in mice. This study provides a basis for the development and utilization of PPPs in the field of anti-alcohol health care.

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