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The total saponins from Chinese onion exert pronounced anti-hypercholesterolaemia activity in mice

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ABSTRACT

Chinese onion (*Allium chinense* G. Don) is an edible vegetable as well as a traditional Chinese medicine. It is rich in steroidal saponins and possesses broad health benefits. In this study, the optimal extraction protocol of the total saponins from Chinese onion (ACS) was explored, and the content of the total steroidal saponins in ACS reached 56.62%. Network pharmacology was applied to predict the related signalling pathways and targets between the main phytochemicals in ACS and hypercholesterolaemia. Enrichment analysis showed that ACS might intervene hypercholesterolaemia through the PI3K-Akt signalling pathway. Meanwhile, cholesterol-lowering effects were verified by ACS intervention in high-cholesterol diet-induced hypercholesterolaemia in Kunming mice. Compared with the model group, the TC and LDL-C levels of mice were decreased and the HDL-C level increased significantly after administration of ACS at a dose of 200 mg kg^{-1} day^{-1}. The body weight gain, liver index, and atherosclerosis index all decreased dramatically. ACS could significantly reduce the fat content in the liver and reduce the number of fat droplets from the haematoxylin and eosin (H&E) staining of mouse liver. The immunohistochemical staining indicated that ACS could up-regulate the expression of PI3K protein in the liver, thus playing an anti-hypercholesterolaemic role. This study indicated that ACS exhibited significant therapeutic and preventive effects on hypercholesterolaemia, and exerted anti-hypercholesterolaemia through the PI3K-Akt signalling pathway.

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KEYWORDS

Chinese onion, total saponins, anti-hypercholesterolaemia *in vivo*, network pharmacology, PI3K-Akt signalling pathway

1. INTRODUCTION

Chinese onion (*Allium chinense* G. Don) is a perennial species of the *Allium* genus in the Amaryllidaceae family, which is native to China and widely cultured in East Asia (He et al., 2018). It contains many chemical components with medicinal value, such as steroidal saponins, sulphur-containing compounds, polysaccharides, nitrogen-containing compounds, and so on (Yao et al., 2016). Therefore, besides being eaten as a common vegetable, it is usually used as a traditional herb for preventing and treating cardiovascular diseases in traditional Chinese medicine (Lin et al., 2016). For example, it was made into the Gualou Xiebai Banxia decoction, which is a well-known traditional herbal formula for treating hyperlipidaemia (Luo et al., 2021). Modern pharmacological studies have proved that its active components, especially steroidal saponins, have obvious health benefits in hypercholesterolaemia-related cardiovascular diseases (Xie et al., 2023).

Hypercholesterolaemia leads to atherosclerosis, heart attack, and stroke, which are major health problems in many countries (Śliż et al., 2019). The symptoms of hypercholesterolaemia usually are increased levels of plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), while plasma high-density lipoprotein cholesterol (HDL-C) decreases (Zhao and Chen, 2018). It seems that lipid-lowering foods and phytochemicals have fewer side effects and better compliance than commonly used therapeutic drugs such as statins (Hunter and Hegele, 2017).

Chinese onion and its total saponins exert obvious anti-hypercholesterolaemia-related cardiovascular diseases, but the specific mechanism is not clear. To explore the relationship between the total saponins of Chinese onion and hypercholesterolaemia, a network pharmacology study was carried out to predict the possible signalling pathways (Lin et al., 2022). Based on the previous research of our research group (Wang et al., 2016), the optimal extraction protocol of the total saponins from Chinese onion (ACS) was explored and the cholesterol-lowering effects were investigated in this study.

2. MATERIALS AND METHODS

2.1. Materials

Chinese onion was collected from Jiangxia District, Wuhan, China. Ethanol, *p*-anisaldehyde, ethyl acetate, and sulphuric acid were purchased from Tianjin Zhiyuan Chemical Reagent Co., Ltd. (Tianjin, China) TC, LDL-C and HDL-C assay kits were purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China).

2.2. Extraction and enrichment of the ACS

Single-factor experiments were established to investigate the effects of extraction time, temperature, solid-liquid ratio, and solvent concentration on the yield of ACS. According to the results

of single-factor experiment, the appropriate factors were selected to design the response surface experiment by Box-Behnken design, and the ACS yield was used as the investigation index to select the best conditions for extraction.

Macroporous adsorption resin was used to enrich the total saponins. Dynamic adsorption and desorption experiments were conducted on a glass column (17 × 300 mm) packed with the selected resin, and the dynamic adsorption rate, desorption rate, and transfer rate of the resin were calculated (Wu et al., 2012). The type of macroporous adsorption resin was selected, the adsorption conditions such as diameter-height ratio of resin column, concentration, and volume of the sample solution were investigated, and the desorption conditions such as concentration of elution solvent and elution volume were also studied.

2.3. Determination of the total saponins content in ACS by colorimetry

A certain amount of sample solution was accurately transferred to a dry test tube and the solvent was evaporated in a water bath. Then 2 mL of ethyl acetate, 1 mL of 0.5% *p*-anisaldehyde:ethyl acetate (0.5:99.5, v/v), and 1 mL of 50% sulphuric acid:ethyl acetate (50:50, v/v) were added, and the reaction was carried out in a water bath at 60 °C for 20 min, then the reaction was terminated in an ice bath for 10 min. The absorbance at 430 nm was measured by a UV-2550 ultraviolet spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. Qualitative and quantitative analyses of ACS by HPLC-ELSD

The qualitative and quantitative analysis was carried out on a Shimadzu LC-20AR HPLC system (Kyoto, Japan), equipped with an evaporative light scattering detector (SEDEX 75, Sedere, France). A Cosmosil 5C18-MS-II HPLC column (4.6 mm ID × 250 mm) was used for separation at room temperature (flow rate: 1 mL min^{-1}). The mobile phase consisted of acetonitrile (A) – water (B) according to the following gradient program: 0–20 min, 20→26% A; 20–30 min, 26→27% A; 30–40 min, 27→40% A; 40–55 min, 40→60% A; 55–58 min, 60→100% A. The temperature of the drift tube and the pressure of nitrogen gas of ELSD were set at 95 °C and 1.5 bar, respectively. Six independent sample solutions were chosen to determine the repeatability, and the variations were expressed by the relative standard deviation (RSD). The stability was investigated using the sample solution, which was injected onto the column at 0, 2, 4, 8, 16, and 24 h after preparation at room temperature. Six concentrations of reference solutions were injected, and the standard curve was constructed by plotting the peak area with the concentration of each analyte. The content of the total steroidal saponins in ACS was calculated. The accuracy of the quantitative method was evaluated by a recovery test (Sun et al., 2021).

2.5. Network pharmacology analysis

The main sapogenin components in Chinese onion were screened, and the structures and target properties of active ingredients were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The related targets (probability >0) were predicted based on the Swiss Target Prediction database (http://swisstargetprediction.ch/), search for 'hypercholesterolemia' through Genecards database (https://www.genecards.org/), and screen gene symbol with relevance score greater than 0.5. Common gene targets were determined by intersecting the obtained related targets and gene symbols. Using Cytoscape 3.10.0, a drug-target-disease network was

constructed. A protein-protein interaction (PPI) network was built through the STRING database (https://string-db.org/).

Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis were performed on targets of the PPI network; GO enrichment analysis identifies three important functional terms for the targets in terms of biological processes, cellular components, and molecular functions. KEGG enrichment pathway analysis predicted the correlation signalling pathway. Therefore, the mechanistic relationships between the major sapogenin components in ACS and hypercholesterolaemia were analysed through the above pathway (Ma et al., 2022).

2.6. Animal experiment on treatment and prevention of hypercholesterolaemia

Kunming mice (male, 5 weeks old) were purchased from Southern Medical University. All animals were raised in the experimental animal centre of Guangdong Pharmaceutical University under standard conditions (humidity: 55 ± 15%, temperature: 23 ± 3 °C, and 12-hour light and dark period), and the experiment was conducted after one week of quarantine and domestication. Animals had free access to standard water and diet, and weekly weight measurement was performed. This study was conducted in a facility approved by the Association of Institutional Animal Care and Use Committees of Guangdong Pharmaceutical University, and mice were raised according to the Guidelines for the Care and Use of Laboratory Animals.

Mice were randomly divided into 8 groups ($n = 6$), which were the control group, model group, prevention low dose group (Pre50, 50 mg kg^{-1} day^{-1}), prevention high dose group (Pre200, 200 mg kg^{-1} day^{-1}), prevention positive drug group (PreP10, 10 mg kg^{-1} day^{-1}), treatment low dose group (Tre50, 50 mg kg^{-1} day^{-1}), treatment high dose group (Tre200, 200 mg kg^{-1} day^{-1}), and treatment positive drug group (TreP10, 10 mg kg^{-1} day^{-1}). All groups, except the control group, were fed high cholesterol diet (HCD), and the positive drug was simvastatin (Liang et al., 2021). Mice feed containing 21% protein, 67% carbohydrate and 12% fat were used as the standard diet, and the mice feed containing 17% protein, 46% carbohydrate and 37% fat were used as high cholesterol diet. The drug intervention in the prevention group lasted for 10 weeks, and the drug intervention in the treatment group started from the fifth week until the end of the tenth week. At the end of the experiment, all mice were fasted for 12 h, blood samples were taken from the eye socket and centrifuged at 4 °C at 3,000 r.p.m. for 5 min after resting at room temperature, and the upper serum was taken to determine the related indices. Subsequently, all mice were sacrificed after anaesthesia, and their livers and other tissues were removed, weighed, and stored in 4% paraformaldehyde.

3. RESULTS AND DISCUSSION

3.1. Optimised extraction factors of ACS by RSM

The results of the single-factor experiment are shown in Fig. 1. According to the experimental results, the appropriate response surface factors were selected. Design-Expert (Stat-Ease Inc., version 10, Minneapolis, MN) was applied to calculate the multiple linear regression equation according to the response value and the experimental conditions. The fitted equation for describing the relationships between various factors and the yield of ACS is given as follows:

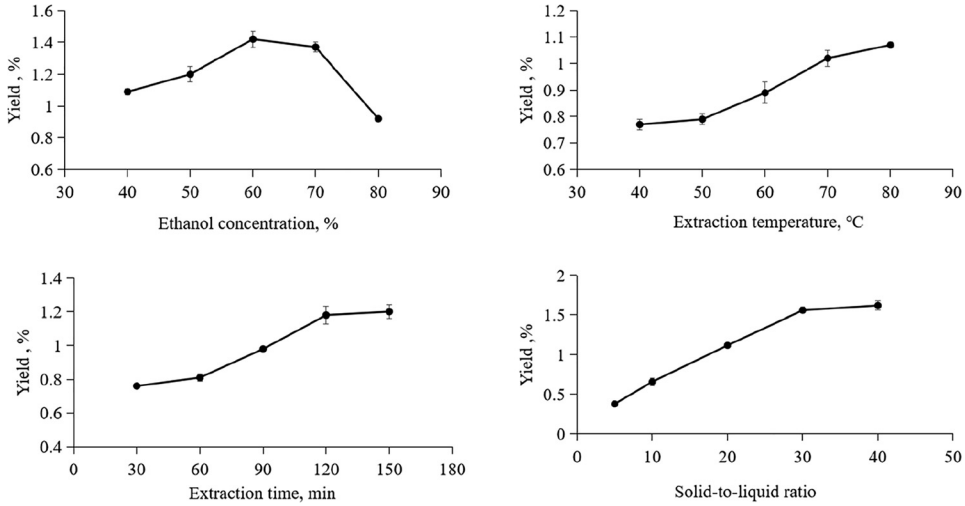

Fig. 1. The results of single factor experiment

$$Y = 1.14 - 0.21 * A + 0.045 * B + 0.00875 * C + 0.017 * AB - 0.01 * AC - 0.047 * BC - 0.21 * A^2 - 0.05 * B^2 + 0.007 * C^2$$

The results of ANOVA showed that according to the Model F-value of 42.36, the model was significant. There was only a 0.01% chance that an F-value this large could occur due to noise. The "Lack of Fit F-value" of 0.42 implied the Lack of Fit was not significant relative to the pure error. There was a 75.10% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit was good, the model needs to fit (Supplementary material, Table S1). The 3D graphs of the effects of ethanol concentration, solid-liquid ratio, and extraction time on the extraction rate of total saponins are shown in Fig. 2.

Based on the actual situation, the ethanol concentration was set to 55%, the liquid-solid ratio was set to 20, and the extraction time was set to 120 min. The experiment was repeated three times to verify the conditions. The average extraction yield of ACS was 1.15% ± 0.0011 by colorimetry, which was consistent with the values predicted using the constructed model.

3.2. Optimal enrichment factors of ACS by macroporous adsorption resin

The adsorption rate, desorption rate, and transfer rate of five different types of macroporous adsorption resins were determined. The D-101 was finally selected according to the experimental results and the principle of economy and practicality. According to the experimental results, the sampling conditions with the highest transfer rate and the most economical and suitable elution conditions were selected (Supplementary material, Table S2-S4). Finally, it was determined that the concentration of Chinese onion extract was 2 g mL^{-1}, the diameter-height ratio of D-101 macroporous adsorption resin column was 1:15, the sample volume was 2 body volume (BV), and 3 BV was desorbed with 70% ethanol (Fig. 3). The desorption solution was evaporated, concentrated, and freeze-dried into the powder of the total saponins of Chinese onion (ACS). The average content of ACS was 56.62 ± 1.03% measured by colorimetry.

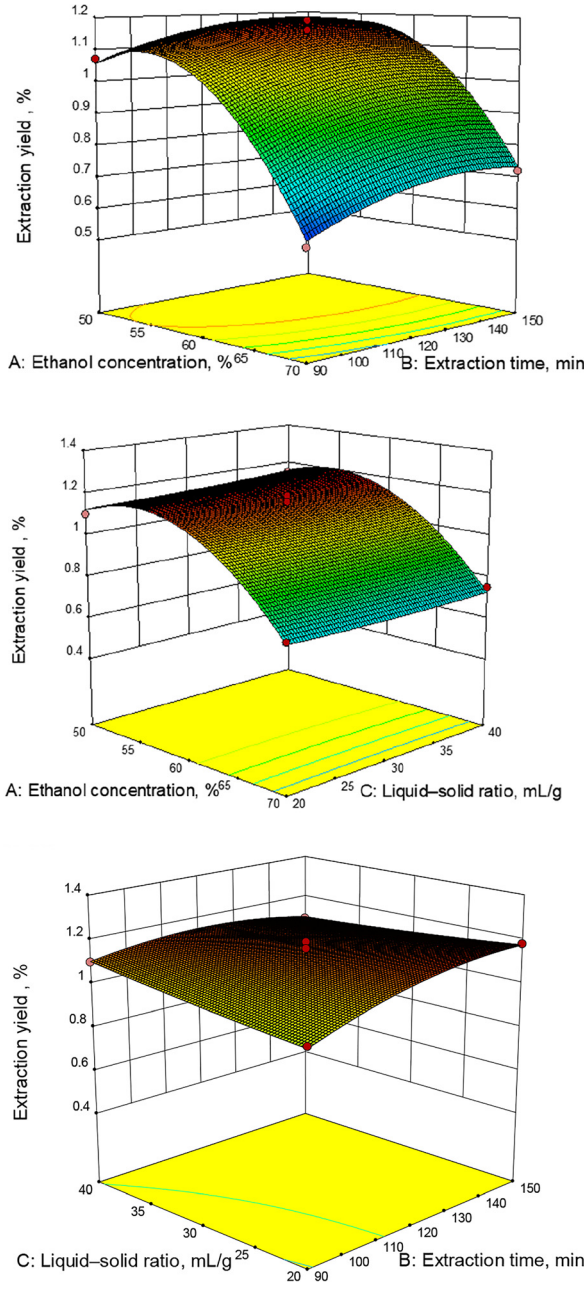

Fig. 2. 3D response surface diagram of response surface methodology (RSM)

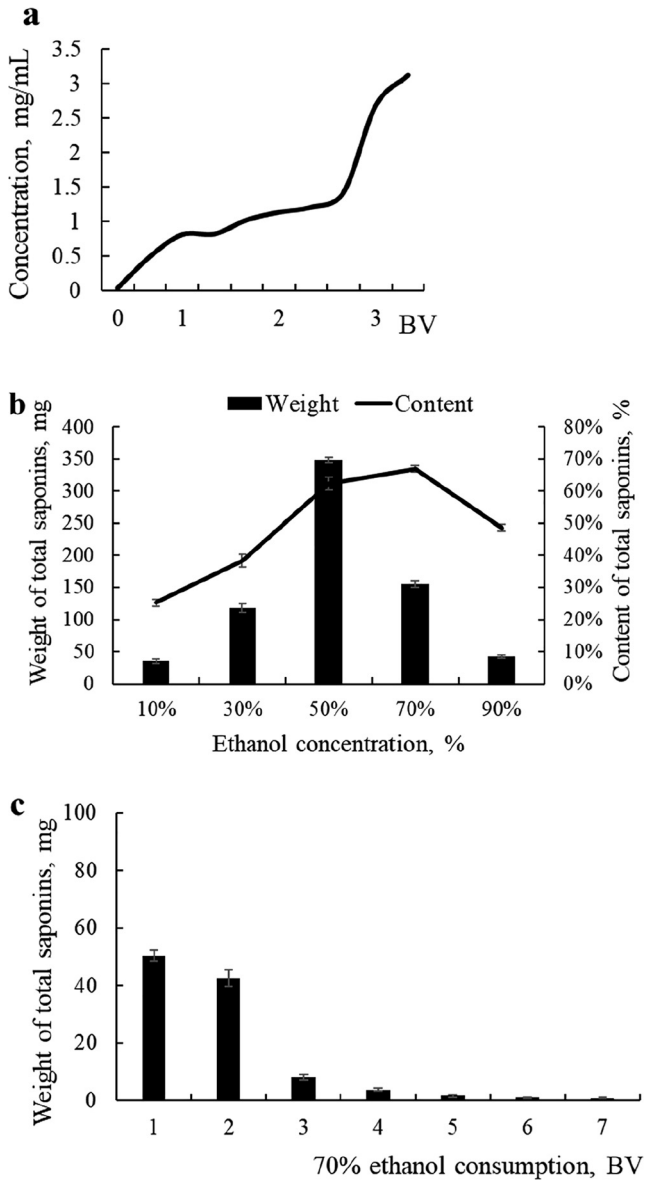

Fig. 3. Adsorption and elution conditions of macroporous adsorption resin. (a: Adsorption capacity investigation; b: Elution solvent concentration investigation; c: Elution volume investigation)

3.3. Qualitative and quantitative analyses of ACS by HPLC-ELSD

The reference saponins were obtained from the monomer separated and purified previously from Chinese onion by our research group, and their structures are shown in Fig. 4. The HPLC-ELSD chromatograms of the sample and the reference substance are shown in Fig. 5.

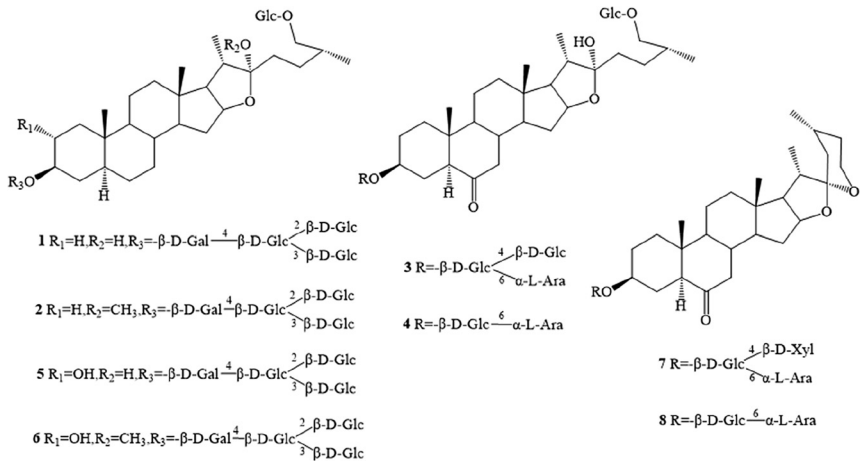

Fig. 4. Chemical structure of reference saponins isolated from Chinese onion

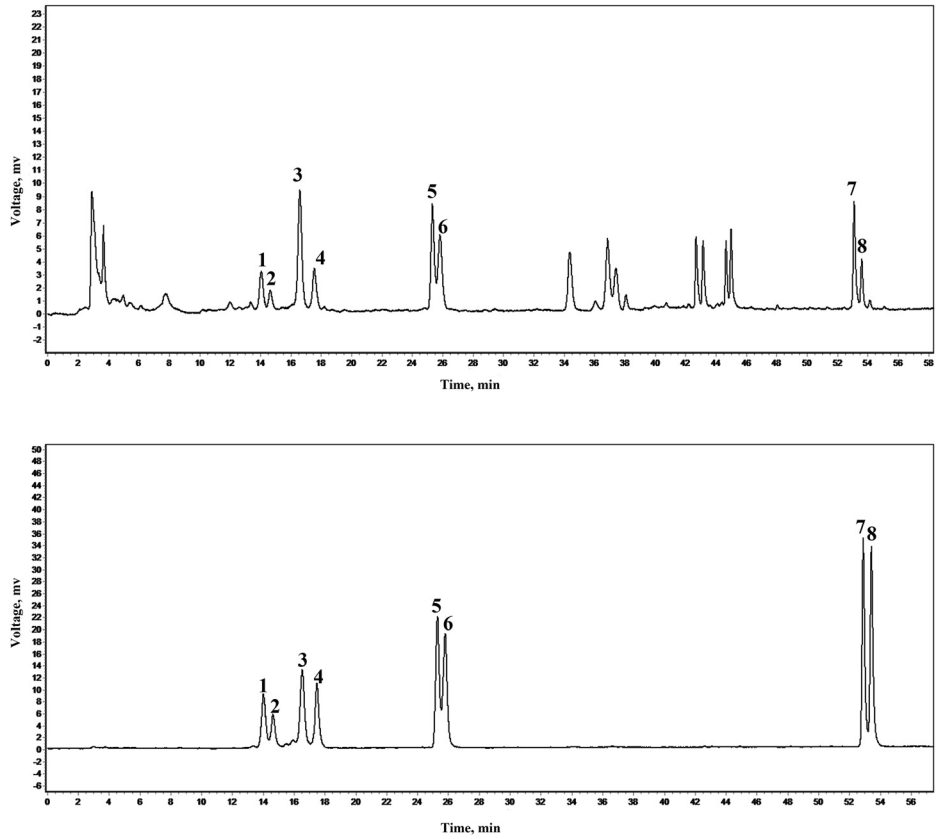

Fig. 5. HPLC-ELSD analysis diagram (a. sample; b. reference)

The standard curve, precision RSD, recovery rate, and recovery rate RSD of the reference substances are shown in Supplementary material, Table S5. The content, repeatability, and stability RSD of the sample are shown in Supplementary material, Table S6. It was calculated that the sum content of the eight saponins in Chinese onion was 46.6%.

3.4. Anti-hypercholesterolaemia signalling pathways prediction by network pharmacology

Based on the previous studies, 9 main sapogenins: tigogenin, neogitogenin, macrostemonoside A, laxogenin, gitogenin, furostan, chinenoside I, chinenoside II, and chinenoside III were screened for subsequent prediction. The related target genes of components and diseases were predicted respectively through the database, and the same common target genes were obtained (Fig. 6a). The drug-component-target network diagram (Fig. 6b) and protein-protein interaction (PPI) network diagram (Fig. 6c) were constructed by common target genes. GO enrichment analysis and KEGG pathway enrichment analysis are shown in Fig. 6d–e. Through the above results, it was speculated that the ACS may regulate hypercholesterolaemia through the PI3K-Akt signalling pathway.

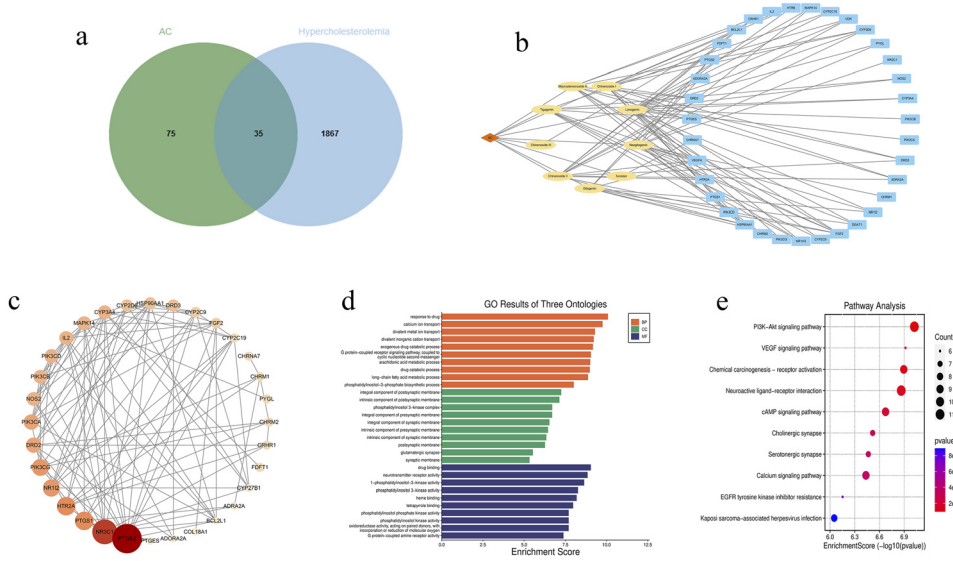

Fig. 6. Network pharmacology analysis for the saponins of Chinese onion (a. Venn diagram of common target, b. drug-component-target network diagram, c. PPI network diagram, d. GO enrichment analysis, e. KEGG enrichment analysis)

3.5. Treatment and prevention of hypercholesterolaemia in Kunming mice by ACS

The results of Fig. 7 show that the body weight gain, atherosclerosis index (AI), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels in the model group were significantly different from the blank group, which

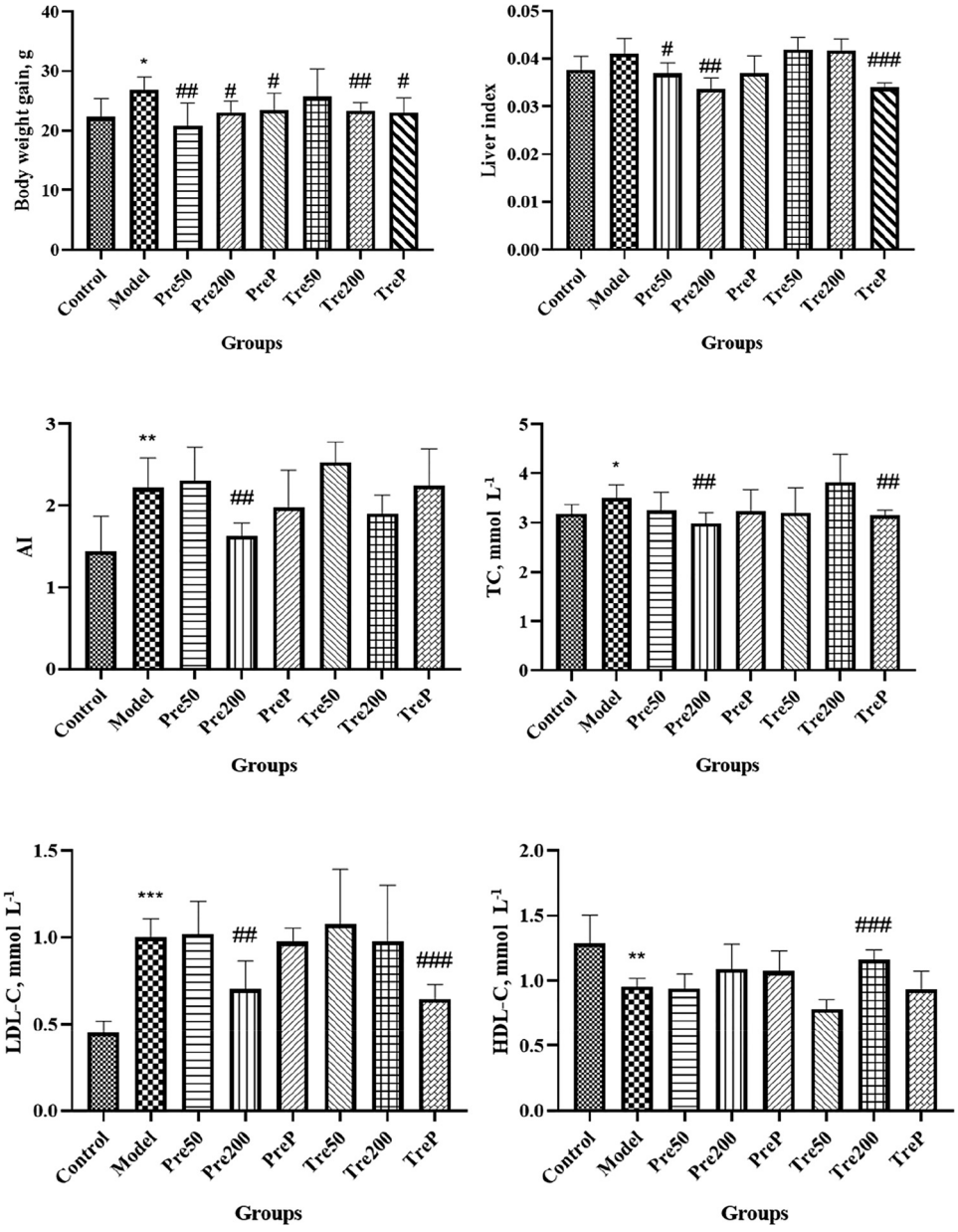

Fig. 7. Body weight gain, Liver index, AI, TC, LDL-C, and HDL-C of mice ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the Control group and both $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$ compared with the Model group)

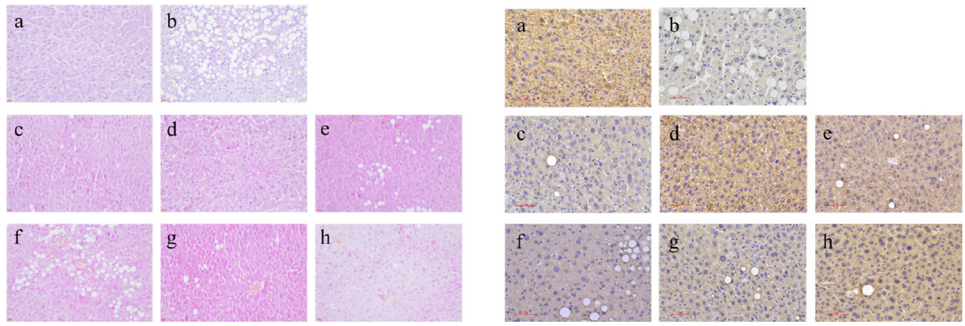

Fig. 8. H&E staining and PI3K antibody immunohistochemical staining of mouse liver (a. Control, b. Model, c. Pre50, d. Pre200, e. PreP, f. Tre50, g. Tre200, h. TreP)

proved that the hypercholesterolaemia model in mice was correct. Compared with the model group, the Pre 200 group significantly reduced the weight gain, liver index, AI, TC, and LDL-C levels of mice, and the HDL-C level was increased. In Fig. 8, H&E staining results of liver tissue sections show that there were more lipid droplets in the model group compared with the blank group, while their number in the drug intervention group decreased, especially in the Pre200 group. This suggested that the daily intake of 200 mg kg^{-1} ACS may play a preventive role in hypercholesterolaemia. Immunohistochemical results showed that the expression of PI3K protein in the model group was lower and the expression of PI3K protein increased after drug intervention compared with the blank group. Combined with the results of network pharmacology, it is speculated that the ACS exerted anti-hypercholesterolaemia by regulating the PI3K protein and thus, the PI3K-Akt signalling pathway.

In addition to the main protein PI3K, other proteins such as Akt and mTOR also play an important role in PI3K-Akt signalling pathway. A study has confirmed their role in intervening cardiovascular diseases (Feng et al., 2023), so further exploration is needed.

4. CONCLUSIONS

We optimised the extraction and enrichment scheme of ACS by RSM, determined the content of ACS by colorimetry and HPLC, and confirmed that ACS can reduce cholesterol by intervening the PI3K protein expression. However, the relationship between specific saponin monomers and hypercholesterolaemia and whether there are other signal pathways to intervene are still unclear, further research is necessary.

Conflicts of interest: The authors declare no competing interest.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIALS

Supplementary data to this article can be found online at https://doi.org/10.1556/066.2024.00025.

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