

Mechanism of the Effect of Compound *Anoectochilus roxburghii* (Wall.) Lindl. Oral Liquid in Treating Alcoholic Rat Liver Injury by Metabolomics

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Purpose: Compound *Anoectochilus roxburghii* (Wall.) Lindl oral liquid (CAROL) is often as a hepatoprotective agent. The present study aimed to elucidate the protective mechanism of CAROL against alcoholic liver injury in rats by untargeted metabolomics combined with multivariate statistical analysis.

Methods: An alcoholic liver disease model was established in sprague-dawley (SD) rats by gavage of alcohol, and CAROL treatment was administered. The hepatoprotective effect of CAROL was evaluated by examining liver tissues changes and detecting biochemical index activities and cytokines in serum and liver homogenates. The metabolites in serum samples were examined using ultrahigh-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC–QTOF/MS) and multivariate statistical analysis to screen for differentially expressed metabolites and Kyoto Encyclopedia of Genes and Genomes (KEGG) to assess potential metabolic pathways.

Results: CAROL has the potential to downregulate inflammation levels and alleviate oxidative stress. The differential metabolites are mainly engaged in riboflavin metabolism, arginine and proline metabolism, phenylalanine, tyrosine and tryptophan biosynthesis metabolism, phenylalanine metabolism, pyrimidine metabolism, and vitamin B6 metabolism to achieve hepatoprotective effects.

Conclusion: CAROL may exhibit beneficial hepatoprotective effects by reducing inflammation, mitigating oxidative stress, and modulating metabolites and their metabolic pathways. This study has important implications for advancing the clinical application of CAROL.

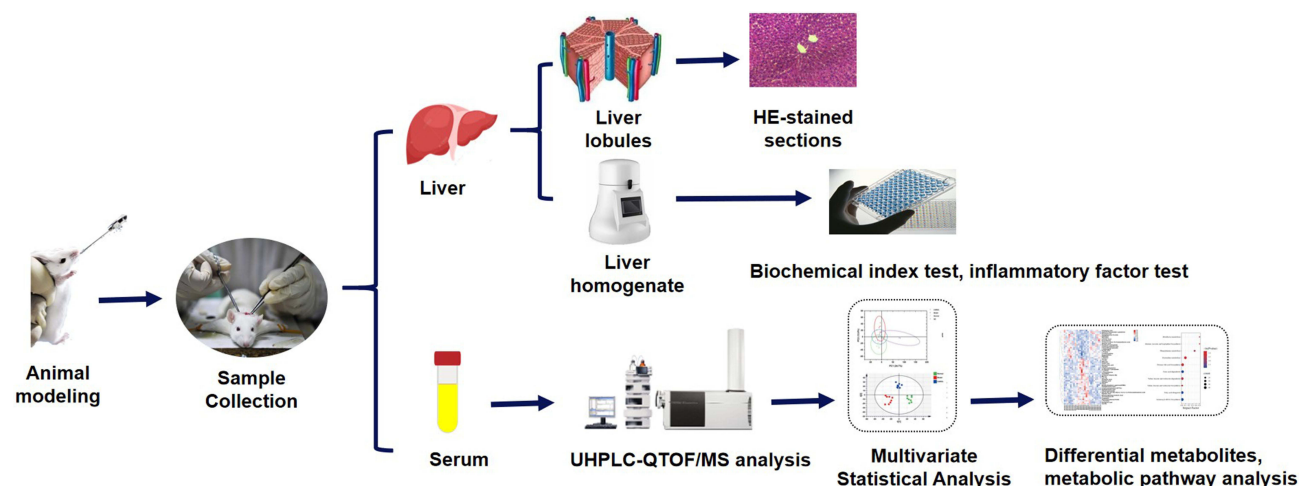
Keywords: compound *Anoectochilus roxburghii* (Wall.) Lindl. oral liquid, alcoholic liver injury, untargeted metabolomics, UHPLC–QTOF/MS, metabolic pathway analysis

Introduction

Alcoholic liver disease (ALD) is the most common disease associated with long-term alcohol abuse. ALD is a spectrum of disease, from hepatic steatosis to alcoholic hepatitis, liver fibrosis to cirrhosis, and even to liver cancer, and it has become a public health problem by posing serious threat to people's health.^{1,2} However, the precise pathogenesis of ALD remain unclear. Cell damage, mitochondrial dysfunction, and oxidative stress may affect the occurrence and development of ALD.³ No drugs have been approved for the treatment of ALD. The only effective treatment is supportive care and abstinence from alcohol.⁴ Therefore, developing or obtaining effective and safe drugs to treat ALD is needed. Traditional Chinese medicine (TCM) is now widely used for liver protection.^{5,6} Owing to their characteristics of multiple targets and small side effects, many TCM preparations have been used to treat ALD, including silymarin, glycyrrhizic acid preparations, and Yigan mingmu oral liquid.^{7,8} However, the efficacy of these drugs for ALD is not known. Therefore, obtaining new TCM preparations to treat ALD is necessary.

Compound *Anoectochilus roxburghii* (Wall.) Lindl oral liquid (CAROL) is a hospital preparation formulated by Mengchao Hepatobiliary Hospital of Fujian Medical University. It is commonly prescribed for the treatment of liver

Graphical Abstract



disorders and possesses several beneficial properties, including heat removal, detoxification, and blood cooling. It is also an excellent hepatoprotective drug. CAROL is a preparation made from two valuable herbs, *Anoctochilus Roxburghii* (Wall.) Lindl. (*A. roxborhii*) and *Ganoderma lucidum* (*G. lucidum*) extracted with water.⁹ *A. roxborhii* and *G. lucidum* are valuable traditional medicinal herbs containing various active ingredients with significant pharmacological activity in liver protection and the treatment of alcoholic liver injury.^{8,10} Published studies have reported that CAROL contains hepatoprotective active ingredients, such as nucleosides, flavonoids, and triterpenoids.^{9,11} More active substances in CAROL and the protective effect on alcoholic liver injury need to be further investigated.

Metabolomics is the qualitative and quantitative analysis of all endogenous low-molecular-weight metabolites (< 1.5 kDa) in the biological organism at a given time. By studying changes in metabolites in the body and their relationship with physiological and pathological changes, it is possible to identify biomarkers associated with diseases and explore the mechanisms from the metabolic pathway that are relevant to the pathogenesis of the organism or to the treatment of the drug. Metabolomics offers promising opportunities for the development of new diagnostic and prognostic biomarkers, as well as a deeper understanding of the underlying causes of various diseases and the processes involved in pharmacological treatment.¹² Several research techniques are commonly employed in metabolomics, including nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS) et al.¹³ LC-MS is one of the most used technologies in metabolomics because of its high sensitivity, high selectivity, easy sample handling, high throughput analysis, and ease of operation. The metabolomic study of liver-protective effects of Chinese herbal medicines is one of the hot spots in clinical research.^{14,15} Liu Fang et al found that *Panax ginseng* was effective in restoring metabolic disorders caused by ethanol and identified 12 potential biomarkers for alcoholic fatty liver in a liver-protective metabolomics study.¹⁶ The metabolomic approach employed by Lian et al allowed for a comprehensive assessment of the metabolic changes induced by Zhi-Zi-Da-Huang decoction, shedding light on its potential therapeutic benefits for alcoholic liver damage.¹⁷

This study utilized ultra-high-performance liquid chromatography quadrupole time-of-flight/mass spectrometry (UHPLC-QTOF/MS) to perform qualitative analysis of the active ingredients in CAROL and evaluate its protective effect against alcohol-induced liver injury in rats by metabolomics. A rat model of alcohol-induced liver injury was established. Inflammatory cytokine levels were measured using enzyme-linked immunosorbent assay (ELISA) to assess the therapeutic impact of CAROL. Serum non-targeted metabolomics based on UHPLC-QTOF/MS and multivariate analysis were utilized to reveal the hepatoprotective mechanisms and metabolic pathways altered upon CAROL

treatment. This study establishes a theoretical foundation for the clinical application of CAROL in the treatment of alcohol-induced liver injury and provides guidance for the clinical application of herbal formulations.

Materials and Methods

Materials and Reagents

AR224CN electronic balance (Orhaus Instrument Co., Ltd., China), R404A centrifuge (Eppendorf Company, Germany), KQ2200E type ultrasonic cleaner (Kunshan Ultrasonic Instruments Co., Ltd., China), net letter JXFSTPRP-24 homogenizer (Shanghai Industrial Development Co., Ltd., China), 1510 enzyme labeler (Thermo Company, USA), VG3S25-type vortex mixer (IKA Instrument Co., Ltd., Germany), DW-86L338J 86°C ultralow-temperature refrigerator and BCD-530WGPZV refrigerator (Haier Company, China), SZ-93 automatic double distill water (Shanghai Yarong Biochemical Instrument Co., Ltd., China), UltiMate 3000 UHPLC system (Thermo Co., Ltd., Germany), AB Sciex Triple TOF X500R high-resolution mass spectrometer (AB Sciex, USA), Agilent 1290 ultrahigh-performance liquid chromatograph (Agilent Co., Ltd., USA), and AB Sciex Triple TOF 6600 high-resolution mass spectrometer (AB Sciex, USA).

Sodium chloride (Xilong Scientific Co., Ltd., China), ethyl alcohol (Sinopharm Chemical Reagent Co., Ltd., China), 4% paraformaldehyde tissue fixative (Meilun Biotechnology Co., Ltd., China), methanol, acetonitrile, and ammonium acetate with tedia were purchased from CNW Technologies GmbH (Germany). Ammonium hydroxide was purchased from Fisher Chemical (USA). ALT kit, AST kit, SOD kit, GSH kit, MDA kit, and TG kit were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). TNF- α kit, IL-6, and NF- κ B were purchased from Fujian Misco Biotechnology Co., Ltd (Fujian, China).

CAROL (batch no. 20220104) was obtained from the Mengchao Hepatobiliary Hospital of Fujian Medical University. Silymarin (batch no. 210810) was purchased from Jiangsu Zhongxing Pharmaceutical Co., Ltd.

Identification of Active Substances in UHPLC–QTOF/MS of CAROL

Sample Pretreatment

A CAROL sample (500 μ L) was placed in a centrifuge tube and mixed with three times the volume of methanol using a vortex mixer for 1 min. The mixture was then centrifuged at 17,000 rpm for 15 min at 4 °C. The supernatant was subjected to vacuum freeze-drying until nearly dry, followed by reconstitution in 50% methanolic water. The resulting solution was filtered through a 0.22 μ m organic filter membrane to obtain the test solution.

UHPLC–QTOF/MS Conditions

The UltiMate 3000 UHPLC system coupled with a Q-TOF X500R was used to collect the active substances' information of CAROL. Chromatographic separation was carried out on an ACQUITY UPLC HSS T3 (2.1 $\times$ 100 mm, 1.8 μ m, Waters) equipped with a binary solvent system.

The mobile phases of the electrospray ionization source in positive ion mode (ESI+) were 0.1% formic acid water (A) and 0.1% formic acid acetonitrile (B). The mobile phases in negative ion mode (ESI–) were 2 mM ammonium acetate water (A) and acetonitrile (B). The elution-gradient program was as follows: 0–3.0 min, 5% B; 3.0–5.0 min, 5%–10% B; 5.0–40.0 min, 10%–40% B; 40.0–50.0 min, 95% B; 50.0–56.0 min, 95%–95% B; and 56.0–60.0 min, 95%–5%. The flow rate was 0.4 mL/min, the injection volume was 5 μ L, and the column temperature was maintained at 40 °C.

The AB X500R Triple TOF mass spectrometer was used on the basis of information dependent acquisition (IDA) function for primary and secondary mass spectral data acquisition. In each data collection cycle, molecular ions with the strongest signal intensity and m/z greater than 100 were selected for the corresponding secondary mass spectral data acquisition by using the following parameters: first-level acquisition range of m/z 50–1200, bombardment energy of 30 eV, and 10 second-level spectrograms per 50 ms. The ESI ion-source parameters were set as follows: atomization pressure (GS1) of 60 psi, auxiliary pressure of 60 psi, curtain pressure of 35 psi, temperature of 650 °C, and spray voltage of 5000 V (ESI+) or –4000 V (ESI–).

Data Processing

First, the data were formatted. The MSDIAL version 4.6 software was used to process the data. The databases of Metlin, MassBank, MoNA, and HMDB that come with the system were applied to identify and analyze the components in CAROL on the basis of the information of primary mass spectra, secondary cleavage characteristic fragment ions, and isotopic distribution matched with the compounds in the spectral library. The setting identification parameters of MSDIAL were as follows: accurate mass tolerance (MS1) of 0.01 Da, MS2 of 0.05 Da, and identification score cutoff of 90 points.

Experimental Animals

SPF-grade 2-month-old SD male rats weighing 200 ± 20 g were purchased from Fujian Medical University Laboratory Animal Center (SCXK2017-0005, Fujian, China). The rats were housed in a room where the temperature and humidity were maintained at $25 \text{ }^{\circ}\text{C} \pm 0.5 \text{ }^{\circ}\text{C}$ and $55\% \pm 5\%$, respectively, while alternating periods of light and darkness were maintained at 12/12 h. The animal experiment was approved by the Professional Committee of Animal Protection and Use of Fujian Medical University (no. FJMU IACUC 2021-NSFC-0010) in accordance with the Guidelines for Animal Experimentation.

Animal and Experimental Protocol

Fifty male rats were divided into five groups, including normal group, model group, positive control group, and high-dose and low-dose administration groups of CAROL (10 rats per group), and fed adaptively for 7 days before the experiment. The model group was gavaged with 5 g/kg (10 mL/kg) of alcohol solution once a day. The alcohol concentration was gradually increased from 40% to 45%, 50%, 55%, and 60%, of which 40%, 45%, 50%, and 55% were separately gavaged for 3 days, and finally maintained at 60% for 8 days. The positive control group was gavaged with alcohol in the same manner as the model group, and 100 mg/kg silymarin was gavaged once a day 30 min after gavage with alcohol. The method of alcohol gavage in the CAROL administration group was the same as that in the model group. After 30 min of alcohol gavage, 5.4 mL/kg (low-dose) and 10.8 mL/kg (high-dose) of CAROL were gavaged once a day. The normal group was given a similar dose of saline in the same manner.

Sample Collection and Processing

The rats were weighed after 12 h of fasting and deprived of water after the last dose. They were euthanized, and then liver and blood samples were taken. The blood samples were allowed to stand at room temperature for 1 h and then centrifuged at 3000 rpm for 10 min at $4 \text{ }^{\circ}\text{C}$ to isolate serum. The liver was weighed, and the liver lobules were taken at room temperature, fixed with 4% paraformaldehyde, dehydrated with ethanol, and embedded in paraffin. The liver tissue was cut into 5 μm slices, infiltrated with xylene, and stained with hematoxylin–eosin (H&E). The tissue sections were observed and evaluated under a light microscope. Another 0.2 g of liver was taken, and 10% liver homogenate was prepared by adding physiological saline. The rest of the liver tissues were frozen at $-80 \text{ }^{\circ}\text{C}$ for storage.

Calculation of Liver Index (LI)

The weight of the rat's liver was recorded. LI was calculated using the following formula to assess the extent of liver damage: $\text{LI} = \text{liver weight/body weight}$.

Detection of Biochemical Indicators and Cytokines

The alanine transaminase (ALT) and aspartate transaminase (AST) in serum and the superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA), and triglyceride (TG) in the homogenate of liver tissue were measured in accordance with the instructions provided by the kits' supplier. The ELISA kit was used to detect cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, and nuclear regulatory factor (NF)- κB , in the homogenate of liver tissue in accordance with the kit's instructions.

Metabolomics Analysis

Serum Sample Preparation

The serum samples were prepared as follows: first, 100 μ L of the sample was collected and mixed with four times the volume of extraction solvent (acetonitrile: methanol = 1:1, v/v) containing the isotopically labelled internal standard mixture was added. After vortex mixing, the samples were sonicated in an ice water bath for 15 min, incubated at -40°C for 1 h, and then centrifuged at 4°C and 12,000 rpm for 15 min. The supernatant (400 μ L) was concentrated to dryness under vacuum at 37°C . Subsequently, 200 μ L of 50% acetonitrile was added, and the samples were ultrasonicated in ice water bath for 10 min. The samples were then centrifuged at 11,000 rpm for 20 min at 4°C , and the obtained supernatant was utilized for UHPLC–QTOF/MS analysis. Quality control (QC) samples were obtained by mixing the same amount of supernatant from all serum samples to evaluate the reliability and stability of the analytical system. The QC samples were injected after each sample analysis.

UHPLC–QTOF/MS Conditions

The UHPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with a 6600 Q-TOF was used to collect the metabolite profile information of serum samples. Chromatographic separation was carried out on a BEH C18 column (2.1 $\times$ 100 mm, 1.7 μ m, Waters) equipped with a binary solvent system. The mobile phase was composed of an aqueous buffer (containing 25 mmol/L NH_4Ac and 25 mmol/L ammonia, pH 9.75, A) and acetonitrile (B). The elution-gradient program was as follows: 0–0.5 min, 5% B; 0.5–7.0 min, 5%–35% B; 7.0–8.0 min, 35%–60% B; 8.0–9.0 min, 60% B; and 9.0–12.0 min, 60%–5% B. The flow rate was 0.5 mL/min, the injection volume was 2 μ L, and the column temperature was maintained at 25°C .

Data acquisition was performed in full scan/dd-MS2 mode at the scan range of m/z 50–1200, and the ESI source in positive and negative ion modes was used. The mass spectrometric parameters were as follows: atomizer gas and auxiliary gas of 60 psi; curtain gas of 35 psi; ion source temperature of 600°C ; declustering potential of 60 V; and ion-spray floating voltages of 5000 V (ESI+) and -4000 V (ESI $-$) in positive and negative modes, respectively.

Data Processing and Statistical Analysis

One-way analysis of variance (ANOVA) was used when the variation between groups followed a normal distribution; otherwise, the Games–Howell test was used. The results of body weight, LI, biochemical parameters, and cytokines were calculated and expressed as mean $\pm$ standard deviation. Statistical analysis was performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA), with $P < 0.05$ and $P < 0.01$ indicating significant differences and highly significant differences, respectively.

The MS data were processed through R packet. Multivariate statistical analysis containing principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) was performed afterwards. In accordance with the variable importance in projection (VIP), the criteria of $\text{VIP} > 1$ and $P < 0.05$ were selected as differential metabolites. Student's t test was used for statistical analysis by using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA).

The metabolites were identified on the basis of public databases, including KEGG (<http://www.genome.jp/kegg/>) and HMDB (<http://www.hmdb.ca/>). Metabolic pathway analysis (MetPA) was used to explore the most relevant metabolic pathways. The mechanism of the protective effect of CAROL on alcoholic liver injury in rats was explored from the metabolite level. A flowchart of the whole experiment is shown in [Supplementary Figure 1](#).

Results

CAROL as an Hepatoprotective Active Ingredient

A total of 22 kinds of active substances were identified from CAROL by UHPLC–QTOF/MS in accordance with the experimental method in “Identification of active substances in CAROL”. The results are shown in [Table 1](#). These compounds included six flavonoids, five nucleosides, three organic acids, one coumarin, one vitamin, one alkaloid, one steroid, two terpenoids, and two others. Among them, 15 are kinds of liver-protective active substances, including vanillin, rutin, baicalein, hesperidin, uridine, guanosine, adenine, scopoletin, riboflavin, betaine, narcissoside, baicalin, ursolic acid, betulonic acid, and citric acid.

Table I The 22 Active Ingredients in CAROL

No.	RT/min	Compound	Category	Formula	Molecular Mass	Parention m/z	Daughterion m/z	Mode
1	8.52	Vanillin	Other	C ₈ H ₈ O ₃	152.15	153.0534	81.0370 109.0220	[M+H] ⁺
2	9.00	Rutin	Flavonoids	C ₂₇ H ₃₀ O ₁₆	610.50	611.1604	271.0610 465.0999	[M+H] ⁺
3	14.75	Kaempferol-3-O-robinobioside	Flavonoids	C ₂₇ H ₃₀ O ₁₅	594.50	595.1660	287.0575 449.1080	[M+H] ⁺
4	19.90	Baicalein	Flavonoids	C ₁₅ H ₁₀ O ₅	270.24	271.0575	123.0095 86.9509	[M+H] ⁺
5	18.27	Hesperidin	Flavonoids	C ₂₈ H ₃₄ O ₁₅	610.60	611.1944	303.0842 449.1423	[M+H] ⁺
6	1.76	Uridine	Nucleosides	C ₉ H ₁₂ N ₂ O ₆	244.20	245.0771	113.0344 73.0277	[M+H] ⁺
7	1.77	Guanosine	Nucleosides	C ₁₀ H ₁₃ N ₅ O ₅	283.24	284.0971	152.0562 85.0274	[M+H] ⁺
8	1.39	Adenine	Nucleosides	C ₅ H ₅ N ₅	135.13	136.0604	119.1653 64.8279	[M+H] ⁺
9	1.27	2'-Deoxyadenosine	Nucleosides	C ₁₀ H ₁₃ N ₅ O ₃	252.14	252.1091	332.0769 136.0633	[M+H] ⁺
10	9.75	2'-Deoxyuridine	Nucleosides	C ₉ H ₁₂ N ₂ O ₅	228.20	229.0832	136.1999 119.0000	[M+H] ⁺
11	10.14	Scopoletin	Coumarins	C ₁₀ H ₈ O ₄	192.17	193.0479	122.0358 133.0270	[M+H] ⁺
12	1.55	4-Guanidinobutyric acid	organic acid	C ₅ H ₁₁ N ₃ O ₂	145.16	146.0926	45.0381 86.0661	[M+H] ⁺
13	9.04	trans-4-Coumaric acid	organic acid	C ₉ H ₈ O ₃	164.16	165.0541	147.0433 91.0538	[M+H] ⁺
14	7.55	Riboflavin	Vitamins	C ₁₇ H ₂₀ N ₄ O ₆	376.40	377.1435	172.0864 198.0663	[M+H] ⁺
15	57.92	Betaine	alkaloid	C ₅ H ₁₁ NO ₂	117.15	118.0858	58.0662 59.0743	[M+H] ⁺
16	15.78	Petunidin 3-galactoside	Other	C ₂₂ H ₂₃ O ₁₂	479.40	479.1168	317.0669 274.0492	[M+H] ⁺
17	15.76	Narcissoside	Flavonoids	C ₂₈ H ₃₂ O ₁₆	624.50	625.1740	317.0582 85.0277	[M+H] ⁺
18	8.84	Baicalin	Flavonoids	C ₂₁ H ₁₈ O ₁₁	446.40	445.0776	269.0459 175.0244	[M-H] ⁻
19	45.02	Cholesterol sulfate	Steroids	C ₂₇ H ₄₆ O ₄ S	466.70	465.3001	97.1336 255.2288	[M-H] ⁻
20	45.70	Ursolic acid	Terpenoids	C ₃₀ H ₄₈ O ₃	456.70	455.3532	191.0428 344.7544	[M-H] ⁻
21	44.72	Betulonic acid	Terpenoids	C ₃₀ H ₄₆ O ₃	454.70	453.3409	436.3500 349.2507	[M-H] ⁻
22	1.89	Citric acid	Organic acids	C ₆ H ₈ O ₇	192.12	191.0194	111.1999 87.1249	[M-H] ⁻

Notes: "[M+H]⁺" and "[M-H]⁻" indicate the quasimolecular ions of the target in ESI (+) and ESI (-) mode, respectively.

In-Vivo Hepatoprotective Effects of CAROL

Effects on Body Weight and LI

The body weight and LI of rats can reflect the health of the liver, as shown in [Supplementary Figure 2](#). After alcohol ingestion, rats in the model group exhibited a depressed mood and significantly lower body weight compared to the normal group ($P < 0.01$) while their hepatic biochemical parameters were elevated ($P < 0.05$). Relative to the model group, the body weight of rats increased in all administration groups, but the highest dose of CAROL group showed the greatest increase ($P < 0.05$). However, no significant difference was found in the LI between the administration groups and the model group, which may be related to the relatively short duration of the experiment.

Effects on Biochemical Indicators

Serum levels of ALT and AST are commonly used indicators of liver injury. As shown in [Figure 1](#), there was liver damage in the model group as indicated by the serum ALT and AST levels being significantly higher than in the normal group ($P < 0.01$). Both levels significantly decreased after CAROL administration ($P < 0.05$). The serum AST levels of rats treated with high-dose CAROL (10.8 mL/kg) showed a more significant decrease than those administered with low-dose CAROL (5.4 mL/kg).

The biochemical parameters SOD, GSH, MDA, and TG in liver tissue homogenates are important indicators reflecting liver injury. As shown in [Figure 2](#), SOD and GSH levels were significantly lower in the liver tissue of the model group compared to the normal group ($P < 0.05$), whereas the MDA content was significantly higher ($P < 0.05$). Moreover, the SOD content in the liver tissues of all administered groups was significantly higher than that in the model group ($P < 0.01$), whereas the MDA content was significantly lower ($P < 0.05$). The GSH content in the high-dose CAROL groups significantly increased ($P < 0.05$). On the contrary, the GSH content in the low-dose group and the positive-control group increased, but there was no significant difference compared to the model group. As shown in [Figure 3](#), the TG levels were significantly higher in the model group ($P < 0.01$) than those of the normal group, all TG levels decreased significantly ($P < 0.01$) after administration of CAROL ($P < 0.01$).

Effects on Cytokines

The levels of IL-6, TNF- α , and NF- κ B in the liver tissues of the model group significantly increased ($P < 0.01$), as depicted in [Figure 3](#). After administration of CAROL, a significant reduction in TNF- α levels was observed ($P < 0.01$). Moreover, the high-dose CAROL groups showed a significant decrease in IL-6 and NF- κ B levels ($P < 0.01$), whereas no such effect was observed in the low-dose CAROL groups.

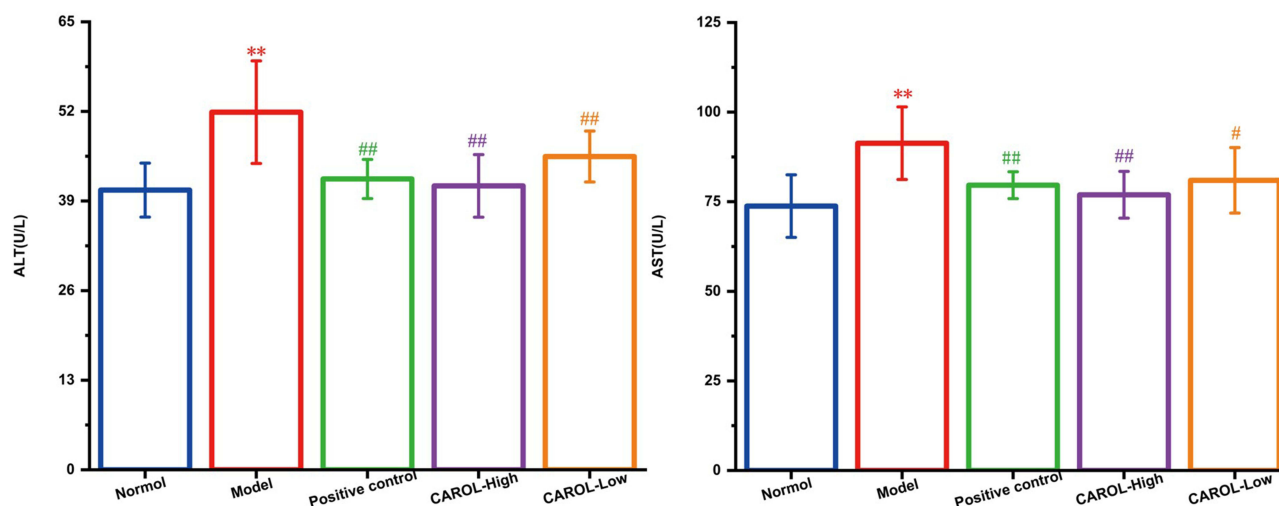


Figure 1 Effect of CAROL on ALT and AST in alcoholic liver rats ($\bar{x} \pm s$, $n = 8$). Compared with the normal group, * $P < 0.05$, ** $P < 0.01$; compared with the alcohol group, # $P < 0.05$, ## $P < 0.01$.

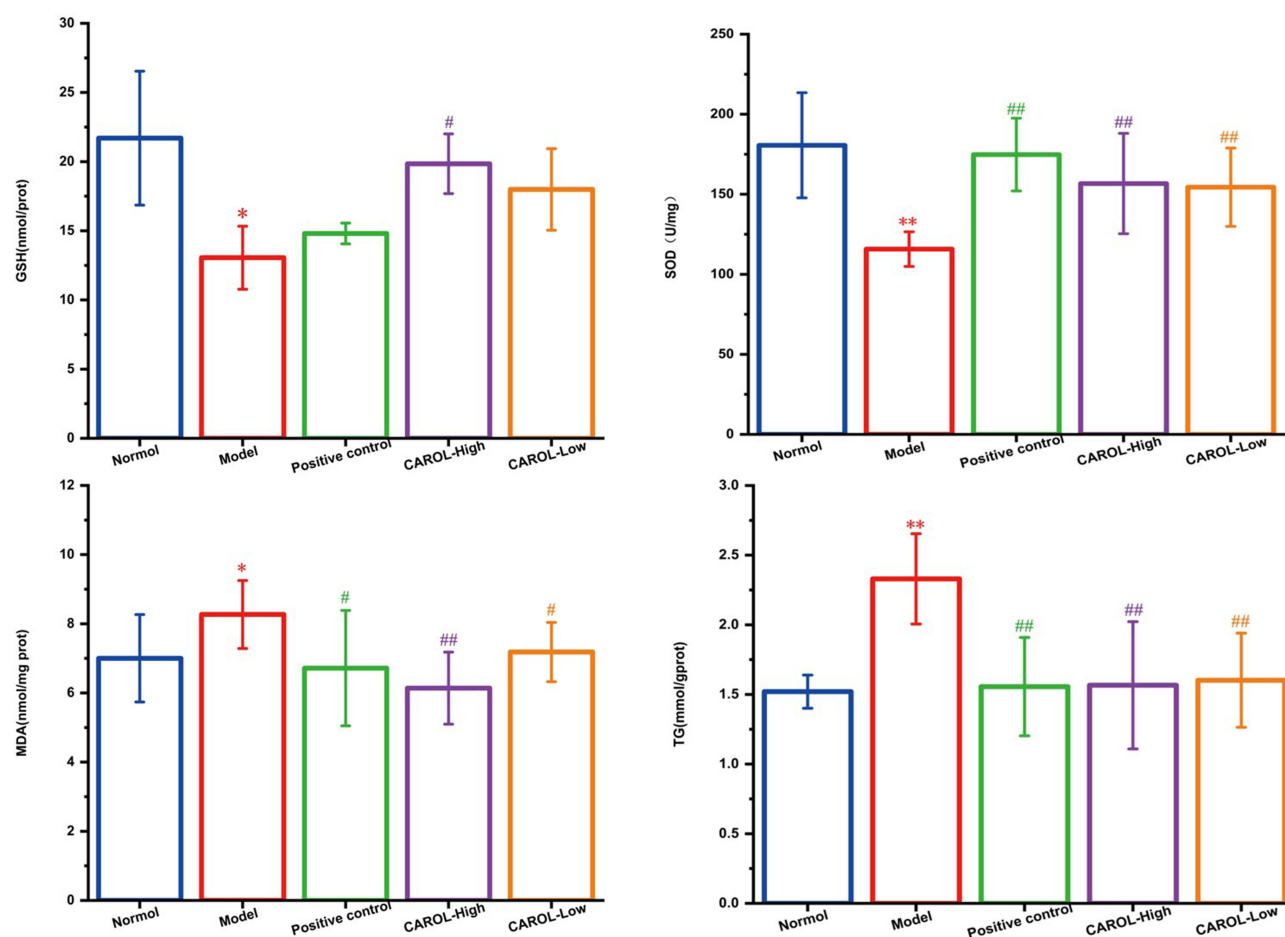


Figure 2 Effect of CAROL on GSH, SOD, MDA, and TG in alcoholic liver rats ($\bar{x} \pm s$, $n = 8$). Compared with the normal group, * $P < 0.05$, ** $P < 0.01$; compared with the alcohol group, # $P < 0.05$, ## $P < 0.01$.

Pathological Findings

The H&E staining results of the liver tissues are presented in Figure 4. In the normal group (Figure 4A), the liver lobules exhibited a well-defined structural organisation, with hepatocytes appearing healthy and arranged radially around the central vein. The cytoplasm was uniformly stained and no signs of degenerative necrosis or inflammatory infiltrates were observed. Conversely, the model group displayed disorganised liver lobules accompanied by inflammatory infiltrates and slight fat vacuoles (highlighted in Figure 4B). Both the positive control group and CAROL groups (Figures 4C-E) showed clearer structures, more consistent staining, and rounder nuclei compared to the model group. Furthermore, the high-dose CAROL groups exhibited better delineation and alignment than the low-dose CAROL groups and the positive control group, suggesting a superior therapeutic effect.

Further, the high-dose CAROL groups were more clearly outlined and more regularly aligned than the low-dose CAROL groups and the positive-control group, indicating superior effect.

Analysis of the Metabonomic Profile

Identification of Serum Metabolites

The biochemical parameters and HE-stained sections of the liver demonstrated the superior therapeutic effects of high-dose CAROL over silymarin and low-dose CAROL. Therefore, samples of high-dose CAROL groups were selected for metabolomic analysis, hereafter collectively referred to as CAROL.

The serum samples were analysed separately by UHPLC–QTOF/MS in both positive and negative ion modes to achieve a broader coverage of metabolites and improved detection sensitivity. Preprocessing of the raw data involved

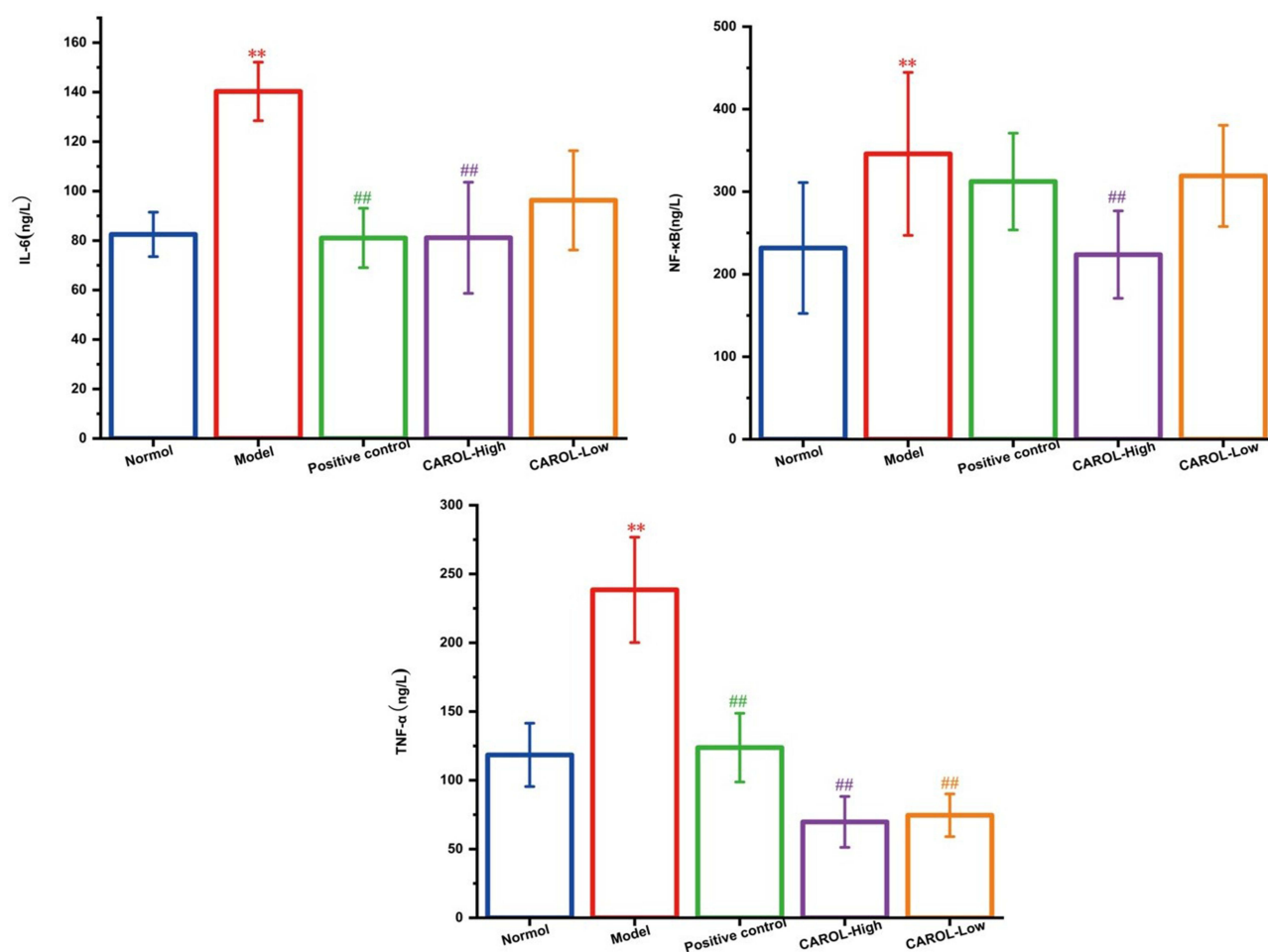


Figure 3 Effect of CAROL on IL-6, TNF- α , and NF- κ B in alcoholic liver rats ($\bar{x} \pm s$, $n = 8$). Compared with the normal group, * $P < 0.05$, ** $P < 0.01$; compared with the alcohol group, # $P < 0.05$, ## $P < 0.01$.

procedures such as noise removal, outlier filtering, simulation and filling in of missing values, and normalization. A total of 3637 peaks were detected in both positive and negative ion mode.

Multivariate Statistical Analysis

PCA, as the most widely used unsupervised pattern recognition method, is used to reduce dimensions and observe the overall distribution between samples and stability of the entire analytical process. The difference of metabolites in the serum of the normal, model, and CAROL groups could be observed through a PCA diagram (Figure 5A). The horizontal and vertical coordinates indicate PC1 and PC2 and their contributions, respectively. The green, red, blue, and purple dots represent samples from the normal, model, CAROL, and QC groups, respectively. Each dot represents one sample. The PCA score plot of the QCS data showed clustering, and all samples fell inside the 95% confidence range, indicating that the data are reliable and stable. This finding showed that the instrument system has good stability. The three sets of samples exhibited intra-group clustering and inter-group separation, indicating that the endogenous metabolites in the rat serum varied across the groups.

The data were further analysed using supervised PLS-DA, which is known for its superior ability to extract information about variation between groups compared to PCA. The results of PLS-DA analysis on serum metabolite data (Figure 5B) demonstrated significant separation among the three groups, substantial metabolic alterations following modeling. Moreover, differential metabolites were identified across the three groups. In serum samples, the R^2Y and Q^2 parameters for normal, model, and CAROL groups were determined as 0.991 and 0.85, respectively. All parameters exceeded 0.5, indicating a high interpretability rate and predictive capability of the established PLS-DA model. The

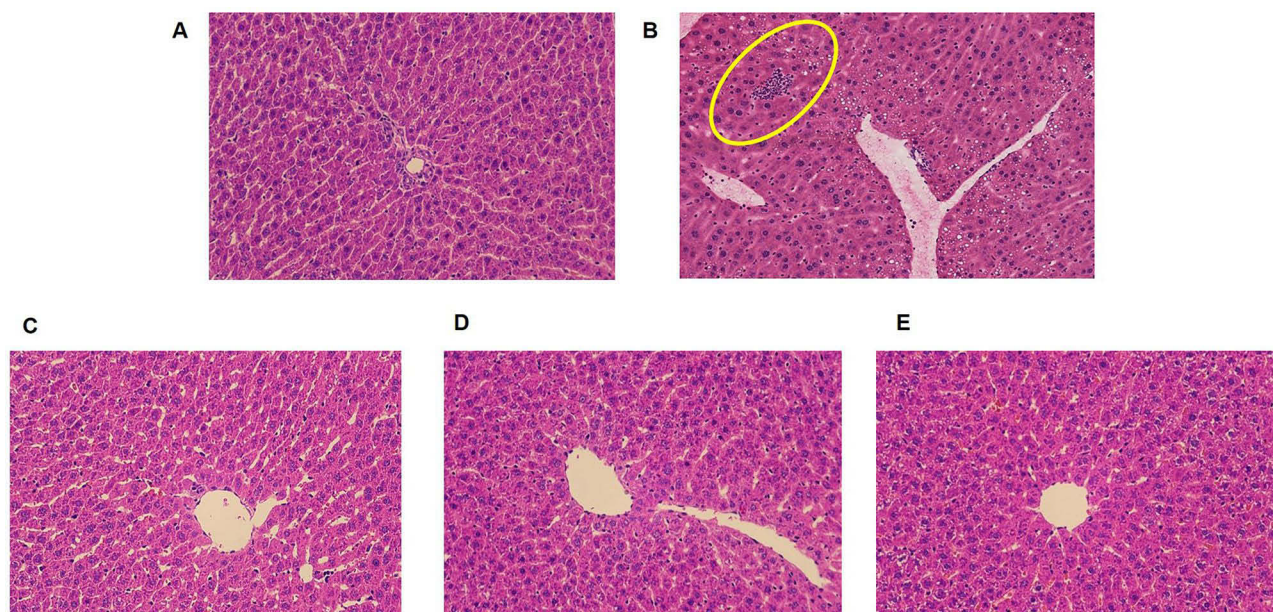


Figure 4 HE staining pathological slice observation of liver (×200). (A) Normal group; (B) model group; (C) high-dose CAROL groups; (D) low-dose CAROL groups; and (E) silymarin-positive control group.

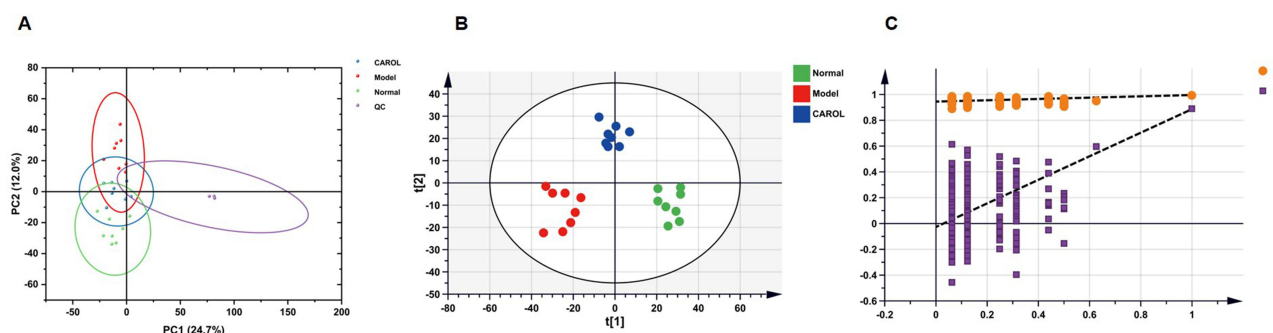


Figure 5 Results of multivariate statistical analysis. PCA score plots of samples and QC samples (A); PLS-DA results based on the serum metabolic profiling (B); The results of 200 permutation tests of serum samples (C).

dispersion of the groups (Figure 5B) obviously indicated that alcohol could induce hepatic metabolic disorder. Compared with the model group, the CAROL group showed a trend of returning to normal, suggesting potential reversal of alcohol-induced metabolic disorder by CAROL treatment. To ensure no overfitting in our models, we performed 200 permutation tests on the PLS-DA model as shown in Figure 5C, where $Q^2 < 0$ indicated absence of overfitting.

ALD Rat Biomarker Analysis After CAROL Intervention

According to the methodology described in “Data processing and statistical analysis”, the statistical analysis method of Student’s *T*-test and the VIP of the multivariate data analysis were used to identify metabolites that exhibited differential expression between the two comparison groups. A total of 52 differentially expressed metabolites by removing duplicates were derived among the three groups, as shown in Table 2.

Differential metabolites, including isocaproic acid, glycochenodeoxycholate, thymine, L-phenylalanine, pyridoxal (vitamin B6), thymidine, Val-Gln, 6-hydroxydopamine, Val-Asn, 5-hydroxytryptophol, Lys-Trp, NG-dimethy-L-arginine, cholic acid, Sn-glycerol-1-phosphate, glycitein, Met-Tyr, pregabalin, Lys-Pro, L-leucine, dihydroxyfumarat, L-carnitine, cholic acid, uracil, deoxycholic acid, (4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10,13,16,19-docosahexaenoic acid dihomogamma-linolenic acid, thromboxane B2, palmitic acid, hippuric acid, helenalin, pentadecanoic acid, heptadecanoic acid, and

Table 2 Differential Metabolites in Serum Samples

NO.	Metabolites	Formula	m/z	TR(min)	KEGG Code	TREND	
						Model vs Normal	CAROL vs Model
1	Trimethylamine N-oxide	C ₃ H ₉ NO	76.076	5.381	C01104	↓*	↑ [#]
2	Larixinic Acid	C ₂₀ H ₂₈ O ₄	127.039	0.665	-	↓*	↑ [#]
3	Thymine	C ₅ H ₆ N ₂ O ₂	127.050	1.655	C00178	↑*	↓ [#]
4	L-Leucine	C ₆ H ₁₃ NO ₂	132.102	4.194	C00123	↑*	↓ [#]
5	Dihydroxyfumarate	C ₄ H ₄ O ₆	149.082	4.132	C00975	↑*	↓ [#]
6	Pyrocatechol	C ₆ H ₆ O ₂	152.070	4.553	C00090	↓*	↑ ^{##}
7	Isocaproic acid	C ₆ H ₁₀ O ₃	158.117	0.727	C03467	↑*	↓ [#]
8	L-Phenylalanine	C ₉ H ₁₁ NO ₂	166.086	4.347	C00079	↑*	↓ [#]
9	Pyridoxal (Vitamin B6)	C ₈ H ₁₁ NO ₃	168.065	0.710	-	↑*	↓ [#]
10	6-Hydroxydopamine	C ₈ H ₁₂ ClNO ₃	170.081	4.185	-	↑*	↓ [#]
11	5-Hydroxytryptophol (5HTOL)	C ₁₀ H ₁₁ NO ₂	177.080	3.006	-	↑*	↓ [#]
12	Pregabalin	C ₈ H ₁₇ NO ₂	182.118	0.722	-	↑*	↓ ^{##}
13	NG,NG-dimethyl-L-arginine(ADMA)	C ₈ H ₁₄ D ₆ Cl ₂ N ₄ O ₂	203.150	8.293	-	↑*	↓ [#]
14	Thymidine	C ₁₀ H ₁₄ N ₂ O ₅	243.098	1.655	C00214	↑*	↓ [#]
15	Lys-Pro	C ₁₁ H ₂₂ ClN ₃ O ₃	261.189	3.622	-	↑*	↓ [#]
16	Alpha-N-Phenylacetyl-L-glutamine	C ₁₃ H ₁₆ N ₂ O ₄	265.118	3.582	C04148	↓*	↑ [#]
17	Glycitein	C ₁₆ H ₁₂ O ₅	285.076	0.776	C14536	↑*	↓ ^{##}
18	Val-Asn	C ₉ H ₁₅ N ₃ O ₃	292.151	5.099	-	↑*	↓ [#]
19	Val-Gln	C ₁₀ H ₁₇ N ₃ O ₃	309.156	4.108	-	↑**	↓ [#]
20	Met-Tyr	C ₁₄ H ₁₈ N ₂ O ₃ S	312.111	5.545	-	↑*	↓ ^{##}
21	sn-glycerol -l-phosphate	C ₃ H ₉ O ₆ P	345.150	3.358	C00623	↑*	↓ [#]
22	Cholic acid	C ₂₄ H ₄₀ O ₅	373.274	3.590	C00695	↑***	↓ ^{##}
23	Lys-Trp	C ₁₇ H ₁₈ N ₃ O ₅	374.225	4.424	-	↑*	↓ [#]
24	Riboflavin	C ₁₇ H ₂₀ N ₄ O ₆	377.145	3.474	C00255	↓**	↑ [#]
25	Glycochenodeoxycholate	C ₂₆ H ₄₃ NO ₅	467.347	3.548	C05466	↑*	↓ [#]
26	Decanoyl-CoA	C ₃₁ H ₅₄ N ₇ O ₁₇ P ₃ S	886.238	3.420	C05274	↓*	↑ ^{##}
27	Salicylic acid	C ₇ H ₆ O ₃	137.026	0.650	C00805	↓*	↑ [#]
28	L-Carnitine	C ₇ H ₁₅ NO ₃	142.088	2.314	C00318	↑*	↓ [#]
29	Raffinose	C ₁₈ H ₃₂ O ₁₆	503.162	7.316	C00492	↓*	↑ ^{##}
30	Palmitic acid	C ₁₆ H ₃₂ O ₂	511.473	0.725	C00249	↑*	↓ [#]
31	3-Guanidinopropanoate	C ₄ H ₉ N ₃ O ₂	152.045	2.534	C03065	↓*	↑ ^{##}

(Continued)

Table 2 (Continued).

NO.	Metabolites	Formula	m/z	TR(min)	KEGG Code	TREND	
						Model vs Normal	CAROL vs Model
32	Pelargonic acid	C ₉ H ₁₈ O ₂	157.125	2.618	C01601	↓*	↑ [#]
33	L-Alanine	C ₃ H ₇ NO ₂	88.042	5.656	C00041	↓*	↑ [#]
34	Hippuric acid	C ₉ H ₉ NO ₃	178.052	3.097	C01586	↑**	↓ ^{##}
35	Ferulic acid	C ₁₀ H ₁₀ O ₄	193.052	3.261	C01494	↓*	↑ [#]
36	2-hydroxy-butanoic acid	C ₄ H ₈ O ₃	103.041	3.376	-	↓*	↑ ^{##}
37	Stavudine	C ₁₀ H ₁₂ N ₂ O ₄	224.080	6.278	C07312	↓*	↑ [#]
38	Uracil	C ₄ H ₄ N ₂ O ₂	111.022	1.432	C00106	↑*	↓ [#]
39	Pentadecanoic Acid	C ₁₅ H ₃₀ O ₂	241.218	0.755	C16537	↑*	↓ [#]
40	Helenalin	C ₁₅ H ₁₈ O ₄	243.104	1.928	C09473	↑*	↓ [#]
41	Pyridoxal 5'-phosphate	C ₈ H ₁₀ NO ₆ P	247.030	0.650	C00018	↓**	↑ [#]
42	L-Proline	C ₅ H ₉ NO ₂	114.058	5.102	C00148	↓*	↑ [#]
43	3-methylcytidine	C ₁₀ H ₁₅ N ₃ O ₅	256.095	3.704	-	↓*	↑ [#]
44	12-Oxo-2,3-dinor-10,15-phytodienoic acid	C ₁₆ H ₂₄ O ₃	263.166	0.810	-	↓**	↑ [#]
45	Heptadecanoic acid	C ₁₇ H ₃₄ O ₂ Pb	269.250	0.725	-	↑*	↓ [#]
46	Deoxycytidine	C ₉ H ₁₃ N ₃ O ₄	286.105	3.366	C00881	↓*	↑ [#]
47	Dihomo-γ-Linolenic Acid	C ₂₀ H ₃₄ O ₂	305.249	2.141	C03242	↑*	↓ [#]
48	(4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10,13,16,19-Docosahexaenoic acid	C ₂₂ H ₃₂ O ₂	327.235	0.706	-	↑*	↓ [#]
49	Ginkgolic Acid	C ₂₂ H ₃₄ O ₃	345.244	0.798	C10794	↑*	↓ [#]
50	L-Asparagine	C ₄ H ₈ N ₂ O ₃	131.048	6.392	C00152	↓*	↑ ^{##}
51	Thromboxane B2	C ₂₀ H ₃₄ O ₆	369.229	2.750	C05963	↑*	↓ [#]
52	Deoxycholic acid	C ₂₄ H ₄₀ O ₄	391.286	2.495	C04483	↑*	↓ [#]

Notes: “-” indicates that the corresponding kegg code was not found. “↓” or “↑” indicates increased or decreased levels of the metabolites, respectively. Compared with the normal group, P* <0.05 , P** <0.01 , P*** <0.001 ; Compared with the model group, P# <0.05 , P## <0.01 .

ginkgolic acid were upregulated in the model group under the influence of alcohol. After the CAROL intervention was applied, 32 of these metabolites had a tendency to reverse modulation (downregulation), similar to the normal group. Meanwhile, the expression levels of riboflavin, alpha-N-phenylacetyl-L-glutamine, decanoyl-CoA, trimethylamine N-oxide, larixinic acid, pyrocatechol, stavudine, 12-oxo-2,3-dinor-10,15-phytodienoic acid, salicylic acid, deoxycytidine, raffinose, pyridoxal 5'-phosphate (PLP), 3-methylcytidine, pelargonic acid, 2-hydroxy-butanoic acid, L-asparagine, ferulic acid, L-proline, 3-guanidinopropanoate, and L-alanine were upregulated after CAROL invention compared to those in the model groups. These findings indicate that alcohol-induced alterations in metabolite profiles tend to regress with CAROL treatment.

In order to better illustrate the patterns in metabolite alterations and the counter-regulatory effects of CAROL, heatmaps were drawn based on the relative intensities of 52 metabolites in the normal, model, and CAROL groups (shown in Figure 6). Each row in Figure 6 represents the relative expression levels of a specific metabolite displayed on

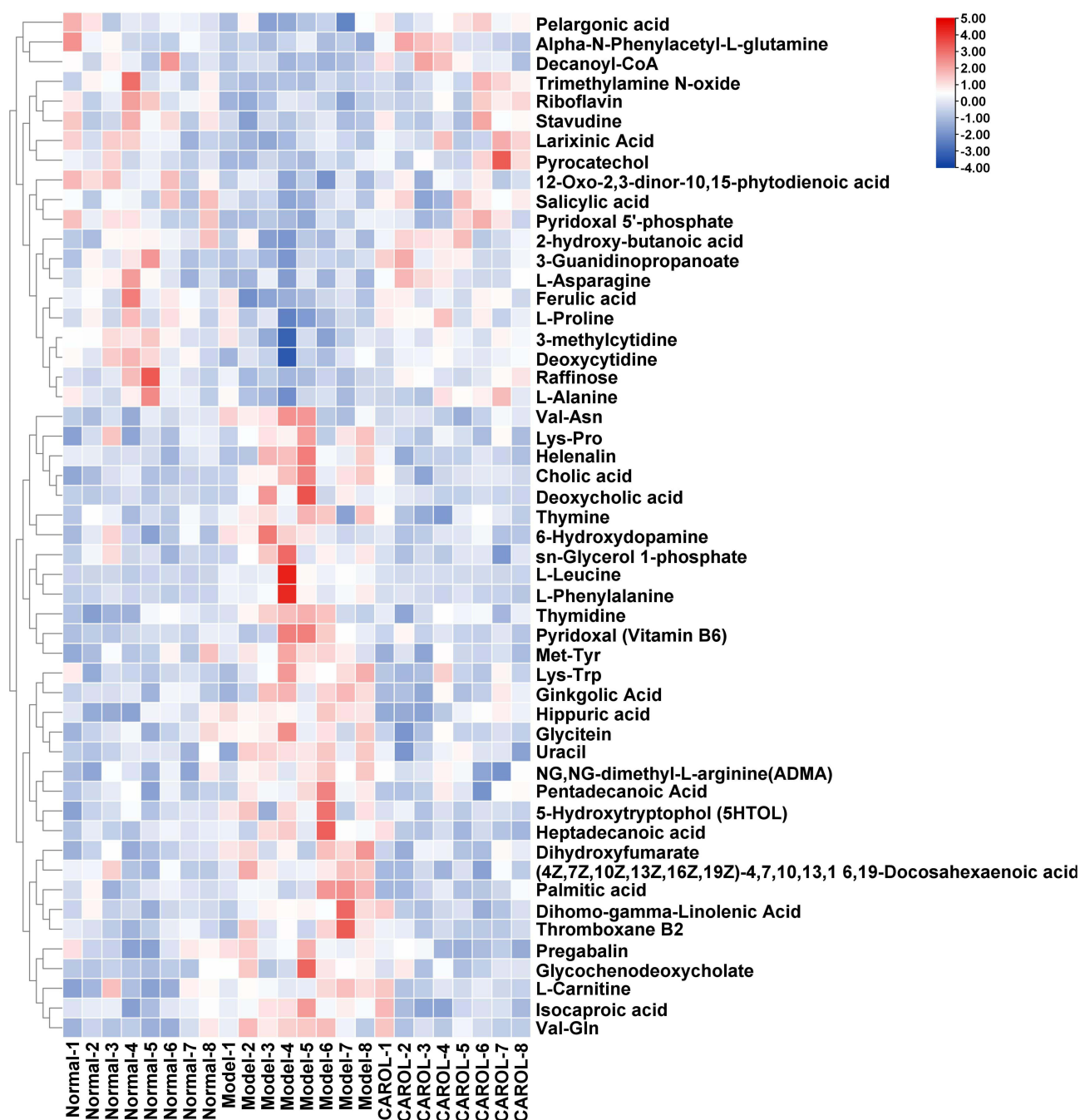


Figure 6 Heatmap of serum metabolites differentially expressed. Rows correspond to samples, and columns correspond to metabolites.

the right-hand side, while each column corresponds to the normal, model, and CAROL groups. The intensity level of the metabolite expression is shown by different colors, with red denoting high expression and blue denoting low expression.

Analysis of Metabolic Pathways in CAROL Intervention of ALD Rats

By analyzing the multiple metabolic and regulatory pathways involved, the biological alterations in ALD rats under the effect of CAROL could be systematically understand, thereby elucidating the mechanism of CAROL's hepatoprotective actions in ALD rats. The metabolic pathway was obtained by mapping differentially expressed metabolites into the KEGG pathway database. Enrichment and topological analyses were performed using the MetPA pathway database to screen the top 10 pathways most closely related to the effects of CAROL (Table 3). The degree of influence is

Table 3 Metabolite Pathway Changes with MetPA

NO.	Pathway Name	Total	-ln(P)	Impact
1	Phenylalanine, tyrosine and tryptophan biosynthesis	4	3.2388	0.50
2	Riboflavin metabolism	4	3.2388	0.50
3	Phenylalanine metabolism	12	2.1771	0.36
4	Vitamin B6 metabolism	9	2.0864	0.25
5	Pyrimidine metabolism	39	2.8951	0.10
6	Arginine and proline metabolism	38	0.8400	0.08
7	Galactose metabolism	27	1.1096	0.03
8	Fatty acid degradation	39	1.1206	0.02
9	Primary bile acid biosynthesis	46	2.6009	0.02
10	Aminoacyl-tRNA biosynthesis	48	1.0998	0.01

proportional to the size of each bubble. Bubble color and ordinate ($-\ln P$) represent the P value obtained from enrichment analysis, with deeper red hues indicating higher degrees of enrichment. Major pathways that affect metabolism were identified, including phenylalanine, tyrosine and tryptophan biosynthesis, riboflavin metabolism, phenylalanine metabolism, pyrimidine metabolism, vitamin B6 metabolism, and arginine and proline metabolism. To sum up, six main metabolic pathways were identified two related to vitamin B group metabolism, three related to amino acid metabolism, and one is nucleotide metabolism (Figure 7).

Discussion

In this study, 22 active ingredients in CAROL were identified by UHPLC -QTOF-MS. It has been reported in the literature that these 22 active ingredients have 15 compounds were identified as having ameliorative effects on liver injury, including vanillin,¹⁸ rutin,¹⁹ baicalein,²⁰ hesperidin,²¹ uridine,²² guanosine,²³ adenine,²⁴ scopoletin,²⁵ riboflavin,²⁶ betaine,²⁷ narcissoside,²⁸ baicalin,²⁹ ursolic acid,³⁰ betulonic acid,³¹ and citric acid.³² The hepatoprotective effects of CAROL are attributed to these 15 active ingredients.

The pathological mechanism of alcoholic liver injury is complex, the therapeutic effect of CAROL on alcoholic liver injury has not been reported in the literature. To investigate this, a sprague-dawley (SD) rats model of alcoholic liver injury was established and treated with CAROL. The hepatoprotective effect of CAROL and its mechanism were investigated by observing liver histological lesions, examining the changes of biochemical index activities and cytokines in serum and liver homogenates, as well as conducting metabolomic analysis. Indicators of liver weight and LI are macroscopic indicators of response to liver injury. Alcohol intake can cause macroscopic changes in the liver, while an increase in LI indicates successful modeling.³³ The rats in the high dose administration group showed a significant increase in body weight. Although there was no significant change in LI between each dosing group and the model group, which may be related to the relatively short duration of the experiment. The ALT and AST in serum are commonly used indicators of liver injury, and they often manifest as elevated biochemical levels.^{34,35} Serum ALT and AST levels were significantly elevated in rats after gavage alcohol, and then decreased significantly after CAROL administration. It showed that the rat model of liver injury was successfully established and CAROL had a therapeutic effect on liver injury. And the effect of high-dose CAROL was better than that of low-dose CAROL group, indicating a certain dose-effect dependence. Reactive oxygen species (ROS) are highly produced when alcohol is consumed, and these ROS can lead to oxidative stress in the body. Oxidative stress can induce DNA damage and abnormal protein expression in the body, leading to irreversible cellular damage and ultimately resulting in the occurrence of diseases.³⁶ MDA is the end product of lipid peroxidation and widely used to reflect the extent of cellular damage. GSH is a scavenger of toxic

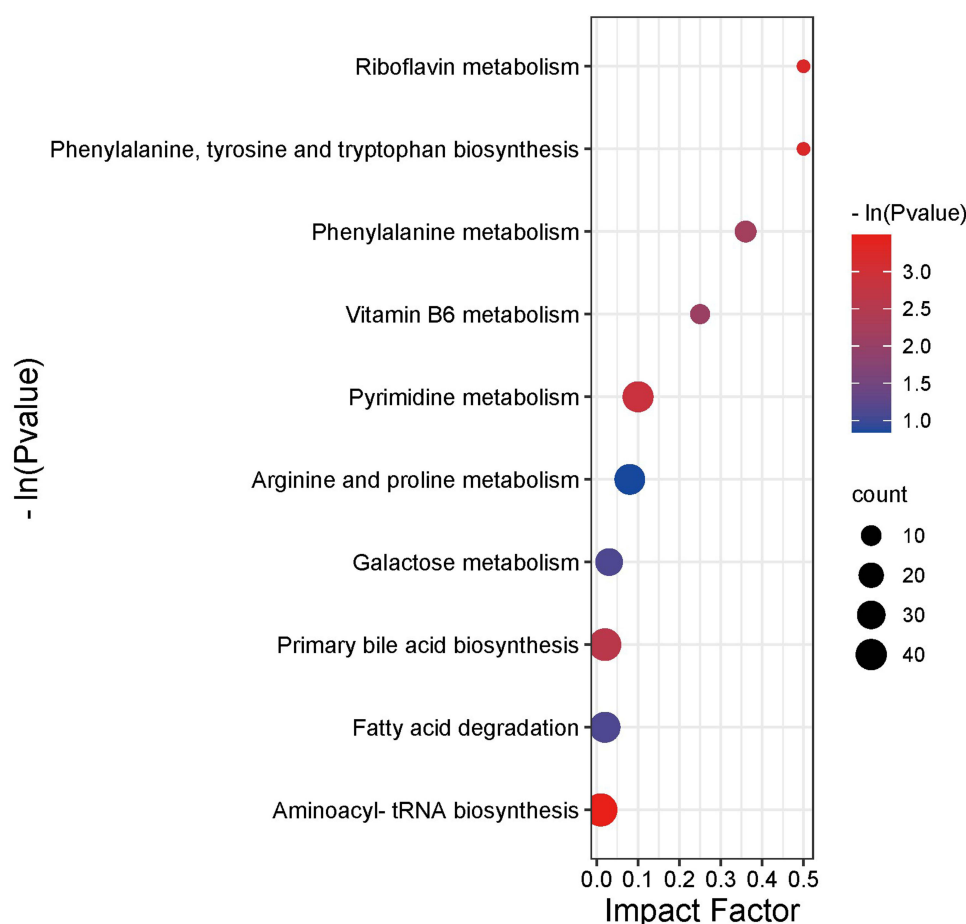


Figure 7 Path analysis bubble chart.

metabolites and free radicals, and oxidative stress leads to depletion of GSH. In addition, SOD is associated with inflammatory diseases and oxidative stress.^{37,38} The increase in SOD and GSH levels and the decrease in MDA content suggest that high doses of CAROL may protect the liver by inhibiting lipid peroxidation and enhancing the antioxidant defense system. In rats intervened with CAROL, the hepatoprotective effects of CAROL were, at least in part, caused by their radical scavenging and antioxidant activities. Fatty liver, which is caused by lipid accumulation, is the most common pathological change caused by alcohol and one of the first pathologies revealed in ALD.³⁹ CAROL can regulate TG levels and thus alleviate liver injury.

In addition to oxidative stress, inflammatory factors may be involved in the development of ALD and predispose individuals to hepatitis. The elevated levels of IL-6, TNF- α and NF- κ B in liver tissues of the model group may result from the activation of the NF- κ B pathway, which subsequently accelerates the synthesis of inflammatory mediators, including IL-6 and TNF- α . The upregulation of the synthesis of these inflammatory factors leads to cytokine imbalance and immune dysfunction, ultimately resulting in liver hypofunction.³⁸ However, high-dose CAROL intake can exert hepatoprotective effects by downregulating inflammatory factors and ultimately alleviate alcohol-induced hepatotoxicity.

Alcoholic liver injury is usually associated with metabolic disturbances caused by changes in the metabolite profile.⁴⁰ Serum liver indices, as well as HE-stained sections, confirmed that CAROL can effectively reduce oxidative stress and hepatocyte injury with hepatoprotective effects. On this basis, UPLC-QTOF-MS non-targeted metabolomics technique was used to study the changes of metabolites in liver-injured rats after drug administration. It is hypothesised that CAROL may exert hepatoprotective effects by regulating metabolic pathways such as riboflavin metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis metabolism; phenylalanine metabolism; pyrimidine metabolism; arginine and proline metabolism; and vitamin B6 metabolism.

Vitamin B metabolism is an important metabolic pathway affecting alcoholic liver injury, and riboflavin is a compound that regulates the metabolism of vitamin B2. In this experiment, the metabolism of riboflavin was downregulated in the model group compared to that in the normal group, which may indicate that alcohol interferes with the digestion and absorption of riboflavin from food in rats.⁴¹ Riboflavin is a necessary for GSH production, which is crucial for antioxidant defense, especially when active oxygen is produced due to alcohol induction.⁴² Therefore, a deficiency in riboflavin could lead to the destruction of mitochondrial respiratory chain, thus overwhelming the cellular antioxidant, and oxidative stress is conducive to the occurrence of liver disease.⁴³ Compared to the alcohol group, CAROL upregulated this metabolism. This finding is consistent with the results of the biochemical index tests in section “Effects on biochemical indicators”. Research showed that vitamin B6 could significantly improve inflammatory cell infiltration and liver cell edema and degeneration.^{44,45} PLP is a compound that regulates the metabolism of vitamin B6. The absorption of ethanol could change the liver metabolism of PLP in an acute and chronic manner. Acetaldehyde produced by ethanol has a harmful effect on the metabolism of PLP, and it could enhance the enzymatic hydrolysis of PLP by phosphatase.⁴⁶ Compared to the alcohol group, the CAROL group affected the metabolism of Vitamin B6 by upregulating PLP. In summary, CAROL could improve alcoholic liver injury by regulating the vitamin B metabolic pathway.

The disorder of amino acid metabolism often occurs in patients with liver disease or mild liver injury, indicating that amino acid metabolism could be significantly interfered by liver injury. Some studies have shown that liver injury has a specific and repeatable plasma amino acid pattern, such as an increase in the concentration of aromatic amino acids (AAAs) and a reduction in the concentration of branched chain amino acids (BCAAs). This phenomenon is known as Fisher’s ratio (BCAAs/AAAs), which has been reported as an important indicator for determining the liver’s metabolism, reserve of liver function, and degree of liver disease.⁴⁷ The biosynthesis and biological metabolism of tyrosine, tryptophan, and phenylalanine are all influenced by oxidative stress, inflammation, and lipid metabolism.⁴⁸ The increased content of phenylalanine, tyrosine, and tryptophan could interfere with the mitochondrial TCA cycle and thus affect liver injury.⁴⁹ In the present study, the metabolism of phenylalanine, tyrosine, and tryptophan was one of the metabolic pathways of ALD regulation by CAROL. Phenylalanine was significantly upregulated in rat serum after alcohol intake, in agreement with the literature.⁵⁰ Compared to the alcohol group, the concentration of phenylalanine in the serum of ALD rats after CAROL intervention was reduced. CAROL may improve in-vivo lipid metabolism, inflammation, and oxidative stress in ALD rats by regulating the biosynthesis of phenylalanine, tyrosine, and tryptophan.

Proline is closely related to arginine and proline metabolism, playing a significant role in protecting the liver from damage. It possesses antioxidant and ROS scavenging properties, helping to maintain stable levels of glutathione. Furthermore, proline metabolism in the mitochondria can prevent liver damage associated with cholestasis, contributing significantly to the liver’s defense. In addition, proline has an inhibitory effect on liver inflammation.⁵¹ In this study, the model group exhibited downregulated level of proline compared to the normal group. Alcohol enhanced oxidative stress by downregulating proline levels, leading to liver injury. Therefore, CAROL may ameliorate liver injury by regulating arginine and proline metabolism and upregulating proline. This is consistent with literature reporting that proline is upregulated to protect against liver injury.⁵¹

Pyrimidine nucleotides are essential for base pairing in DNA synthesis. The derivatives of pyrimidine nucleotides may regulate cell signaling and energy metabolism to maintain intracellular homeostasis.⁵² Different metabolites of thymidine, thymine, uracil, and deoxycytidine are closely associated with pyrimidine metabolism. Alcohol upregulated uracil, thymidine, and thymine and downregulated deoxycytidine compared to the normal group; however, CAROL intervention reversed this trend. A report has shown that liver damage could be caused by disrupted metabolism of pyrimidines.⁵³ The interference of pyrimidine metabolism may occur simultaneously with major liver cell dysfunction, such as oxidative stress, inhibition of bile acid production, and GSH biosynthesis pathway.⁵⁴ CAROL could alleviate ALD by regulating pyrimidine metabolism. The literature also reports that ethanol extract of Baizhi Gentiana can treat alcoholic liver injury by regulating the pyrimidine metabolic pathway.⁵⁵

In conclusion, riboflavin metabolism; biosynthetic metabolism of phenylalanine, tyrosine, and tryptophan; phenylalanine metabolism; pyrimidine metabolism; arginine and proline metabolism; and vitamin B6 metabolism may be the important metabolic pathways for the protective effect of CAROL on alcoholic liver injury in rats. The protection from

CAROL against alcoholic liver injury in rats may act by affecting multiple important metabolic pathways as shown in Figure 8. This work did not quantify the changes in serum levels of several core metabolites in rats after CAROL intervention, and the levels of several core metabolites in serum will be further verified quantitatively by LC-QqQ/MS technique in the future.

Conclusion

In this study, 22 active substances in CAROL were identified, 15 of which have hepatoprotective activity. The outcomes of the trial demonstrated that CAROL protects rats' livers from damage brought on by alcohol. By using multivariate statistical analysis and UPLC-QTOF/MS, the potential differential metabolites were identified. A total of 52 differential metabolites that could be reversed by CAROL were identified in positive and negative ion modes, respectively. The analysis of differential metabolites revealed that riboflavin metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis metabolism; phenylalanine metabolism; pyrimidine metabolism; arginine and proline metabolism; and vitamin B6 metabolism are important metabolic pathways related with CAROL therapy in rats with alcoholic liver injury. Comprehensive analysis of the mechanism inferred that CAROL could inhibit inflammatory factors, enhance the activity of antioxidant enzymes, regulate the TCA cycle, and reduce lipid peroxidation to alter related metabolites and their metabolic pathways, thus achieving hepatoprotective effects. This study revealed the hepatoprotective mechanism of CAROL based on metabolomics research findings, thus providing a theoretical basis for studying its clinical application as well as a strategy for exploring the mechanism behind the hepatoprotective effect of Chinese compound medicinal preparations. The target proteins in the key pathways will be further validated by Western blotting (WB) to explore the mechanism of CAROL attenuating liver injury in a more comprehensive and in-depth manner.

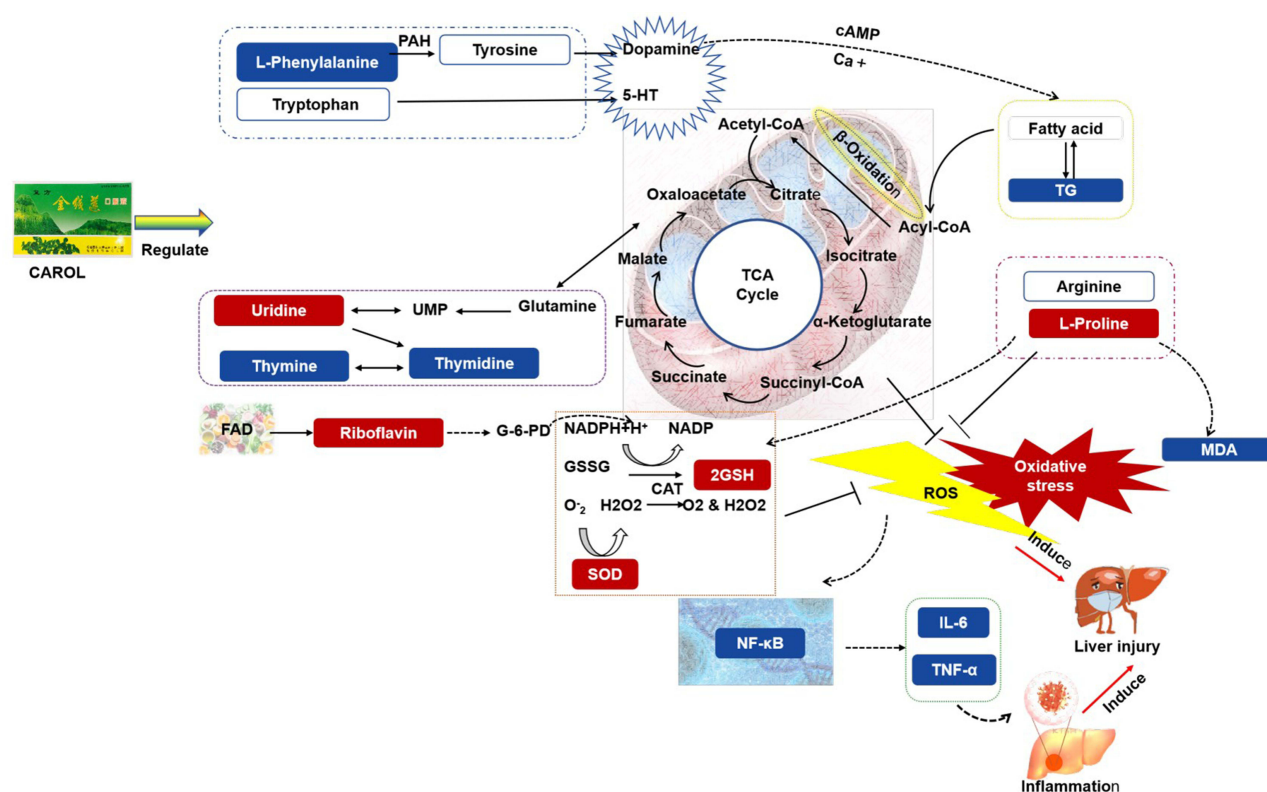


Figure 8 Mechanism of ALD regulation by CAROL. Differential metabolites, biochemical indicators, and inflammatory factors are indicated by the blue and red boxes, respectively. Solid line arrows indicate generative relationships, and dashed arrows indicate regulated relationships.

Abbreviations

CAROL, Compound *Anoectochilus roxburghii* (Wall.) Lindl oral liquid; ALD, Alcoholic liver injury; UHPLC-QTOF/MS Ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry; SD, Sprague-Dawley; KEGG, Kyoto Encyclopedia of Genes and Genomes; TCM, Traditional Chinese medicine; *A. roxburghii*, *Anoectochilus Roxburghii* (Wall.) Lindl; *G. lucidum*, *Ganoderma lucidum*; NMR, Nuclear magnetic resonance; GC/MS, Gas chromatography/mass spectrometry; LC/MS, Liquid chromatography/mass spectrometry; ELISA, Enzyme-linked immunosorbent assay; ESI, Electrospray ionization source in positive mode; IDA, Information dependent acquisition; LI, Liver index; ALT, Alanine transaminase; AST, Aspartate aminotransferase; SOD Superoxide dismutase; GSH, Glutathione; MDA, Malondialdehyde; TG, Triglyceride; TNF- α , Tumor necrosis factor- α ; IL-6, Interleukin-6; NF- κ B, Nuclear regulatory factor- κ B; QC, Quality control; ANOVA, One-way analysis of variance; PCA, Principal component analysis; PLS-DA, Partial least squares discriminant analysis; ROS, Reactive oxygen species; VIP, Variable importance in the projection; MetPA, Metabolic pathway analysis; PLP, Pyridoxal 5'-phosphate; AAAs, Aromatic amino acids; BCAAs, Branched chain amino acids; TCA, Tricarboxylic acid cycle; WB, Western blotting.

Ethics Approval and Consent to Participate

The ethical approval of this study was provided by the Institutional Ethical Committee of Fujian Medical University, Fujian, China (No. FJMU IACUC 2021-NSFC-0010).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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.

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