A GC-MS-based Metabolomic Strategy to Investigate the Protective Effects of Mulberry Polysaccharide on CCl4-induced Acute Liver Injury in Mice

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Abstract

Background

Mulberry (Morus alba) fruits of the woody mulberry tree (family: Moraceae Morus) is a type of mulberry fruit grown in the southern Xinjiang region,which polysaccharides have antioxidant and liver protective effects.This article further preliminary study on the protective effects of mulberry polysaccharide (MP) on liver.

Methods

A detection kit was used to assess serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), liver malondialdehyde (MDA), superoxide dismutase (SOD) and other indicators. Liver tissue sections were stained with hematoxylin and eosin (H&E) and observed under a microscope. The entire endogenous metabolite profiling was acquired *via* metabolomics strategy using gas chromatography-mass spectrometry (GC-MS) to assess the underlying protective mechanisms of MPs.

Results

Results indicated that MPs exerted a hepatoprotective effect on acute liver injury by decreasing serum ALT and AST levels, hepatic MDA, and restored hepatic SOD glutathione peroxidase (GSH-Px) activities. A total of 33 possible endogenous metabolites associated with lipid, glucose, and energy metabolism including amino acids, sugars, and fatty acids, were found.

Conclusions

The results of the present study provide a reference for elucidating the protective mechanisms of MPs against acute liver injury.

1. Introduction

Acute liver injury is characterized by hepatocyte damage and inflammation. The main mechanism of liver damage is caused by free radical production and lipid peroxidation, which can induce liver cancer and cirrhosis[1]. In many studies, various factors such as ethanol, high-fat[2], CCl_4, and fulminant were shown to damage the liver in various aspects. Overproduction of free radicals may result in structural and functional damage to the membrane and eventually cause serious toxicity to hepatocytes[3]. Nevertheless, the reports of its pathogenetic mechanisms are currently limited.

Natural products, an abundant resource to be utilized in drug development, have received an increased attention due to their specific functions. A number of polysaccharides with hepatoprotective effects have been reported, such as *Crassostrea gigas*, *Mori Fructus*, *Anoectochilus roxburghii*.According to traditional Oriental medicine, mulberry fruits can protect against liver and kidney damage[4] .In Korea, mulberry has been used as a traditional medicine due to its beneficial health effects stemming from its anti-

inflammatory, antioxidant,and hepatoprotective properties[5,6].The medicinal mulberry *Morus L. black* mulberry (*Morus nigra* Linn.) was a type of mulberry fruit grown in the southern Xinjiang region and used as an edible medicinal material by local residents.Hu Junping, a professor in the department of Pharmacognosy, Xinjiang Medical University, identified it as xinjiang medicinal mulberry, and the research group also conducted animal pharmacodynamics research on its anti-inflammatory and hepatoprotective effects. Lee[7] et al. explored the effects of medicinal mulberry on pneumonia caused by bronchi and respiratory tract infections by establishing *in vitro* and *in vivo* models. In previous studies, the polysaccharide content in medicinal mulberries in Xinjiang was shown significantly higher than in common mulberry varieties, indicating the medicinal mulberry has a higher utilization value[8]. Based on our preliminary research regarding the effects of polysaccharides in the medicinal mulberry and literature review[9], investigating the protective mechanistic effects of mulberry polysaccharides (MPs) on acute liver injury is important.

Metabolomics is used to assess pathological states in humans and animals by monitoring small molecular changes[10], which can provide a more in-depth understanding of the biochemical effects of drugs and help further determine the pathological process of diseases[6]. Metabolomics research showed compounds can be biotransformed into hydrophilic molecules[11], which are easily excreted through the kidneys in urine. When liver injury occurs, the metabolic capacity of the liver is weakened and the levels of most metabolites change. Therefore, detecting changes in metabolites to evaluate the protective effects of drugs on liver injury is feasible.

Substantial modern technical advances have enabled robust and comprehensive profiling of molecular markers in the study of human health and disease. Numerous metabolomics technologies, including high-resolution nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-MS (LC-MS)[12-14]have been applied for disease diagnosis, metabolic marker exploration, toxicology research, new drug development, and drug screening by analyzing biological samples such as urine, blood, and tissue. In addition, GC-MS is considered the standard technique for metabolomics research[15].

In the present study, the protective effects and different doses of medicinal MPs on CCl_4-induced acute liver injury in mice were investigated. In addition, biochemical indicators and metabolic markers in liver pathological conditions were analyzed using metabolomics with GC-MS technology.

2. Material And Methods

2.1 Experimental reagents

Commercial kits used for determination of alanine transaminase (ALT), aspartate transaminase (AST), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) malondialdehyde (MDA), and protein levels were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Other chemicals and solvents were purchased from Damao Chemical Reagent Factory (Tianjin, China). N-methyl-N-

trimethylsilyl trifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS), heptadecanoic acid, and methylhydroxylamine hydrochloride were purchased from Sigma(America). Pyridine. CCL_4, and anhydrous ethanol were analytical grade.

2.2 Preparation of mulberries

Mulberries (*Morus nigra* Linn.) were obtained from a local market in Akesu Xinjiang, and only fruits without bruises were kept at room temperature and stored until extraction. Under optimized conditions, the MP was extracted using the ultrasonic-related cellulose method, and the monosaccharide composition of the MP was determined using High Performance Liquid Chromatography. From the reported article that MP was composed of Man, Rah, GalUA, Glc, Gal, Arab in a molar ratio of 2.96:1:1.57:3.92:2.83:1.08.

2.3 Liver injury model construction and treatment

The hepatoprotective effects in *vivo* were evaluated using CCl_4-induced hepatotoxicity model[16,17]. Male Kunming mice with body weight (BW) of 20 ± 2 g were provided by the Experimental Animal Center of Xinjiang Medical University, Urumqi, China. Mice were housed in cages at 21 ± 1°C with 50–60% relative humidity under 12 h light/dark cycle conditions. Food and water were available *ad libitum*. All animal experiments were approved by the Animal Ethics Committee of Xinjiang Medical University (No. A-20100920002).

Mice were randomly divided into six groups of 11 animals each. The mice in the control group were treated with saline (10 mL/kg b.w. i.g.) and the mice in the positive control group were treated with silymarin by gavage (100 mg/kg b.w. i.g.). The treatment groups were administered MP at 50, 100, or 200 mg/kg per day by gavage. All groups were treated for 2 weeks. On the 14th day, all mice except those in the normal control group,model group were intraperitoneally injected with 0.3% CCl_4 dissolved in olive oil (10 ml/kg b.w.), and mice in the normal group were injected with equal amount of olive oil instead of CCl_4[18].

2.4 Determination of ALT, AST, SOD, GSH-Px, and MDA levels

All mice were starved for 16 h and then sacrificed. Blood was taken from the eyeball and centrifuged at 3,000 g for 10 min at 4°C to extract serum. The liver was immediately removed, washed, and homogenized in ice-cold physiological saline to prepare a 10% (w/v) homogenate. The obtained homogenate was centrifuged at 4,000 g and 4°C for 10 min to remove cellular debris and the supernatant was collected for analysis. The serum ALT and AST levels and the SOD, GSH-Px, MDA, and protein levels in liver were determined using the reagent test kits.

2.5 Histopathological observation

Part of the liver tissue was fixed in 10% formalin solution and embedded in paraffin. Sections (5 μm in thickness) were cut, stained with hematoxylin and eosin (H&E), and examined under a Nikon Eclipse E600

microscope (Nikon Corp., Tokyo, Japan) at 200× magnification.

2.6 Metabolic profiling analysis

The liver homogenate samples frozen at -80°C were reconstituted. Acetonitrile solution (250 μL) was added into the sample (100 μL), which was then vortexed for 1.5 min, placed into an ultrasonic bath for 10 min, and finally centrifuged at 10,000 r/min and 4°C for 10 min. The supernatant was removed and placed into a new Eppendorf tube and evaporated using N_2. Oximation was performed at 70°C for 1 h after the addition of 50 μL methoxyamine pyridine solution (15 g/L). Trimethylsilylation was performed at 70°C for 1 h after addition of 50 μL derivatization reagent MSTFA (1% TMCS). Then, 150 μL heptanoic acid-containing acetonitrile solution (0.9 mg/mL) was added as a reference and centrifuged (10,000 r/min, 10 min, 4°C). Finally, the supernatant was transferred into the inner tube of the microinjection bottle for GC-MS analysis.

The sample was analyzed with Agilent 7890B/5977A GC-MS equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J & W Scientific, Inc., Folsom, CA, USA). Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. The temperature program was as follows: the initial temperature was 85°C, followed by holding for 5 min, then elevated to 300°C at a rate of 10°C/min, and maintained for 6 min. The temperatures of the injector, transfer line, and ion source were set to 270°C, 270°C, and 230°C, respectively. The mass range (50–600 m/z) in a full-scan mode for electron impact ionization (70 eV) was applied.

2.7 Date processing and statistical analysis

The peaks and representative peaks shared by the GC-MS TIC map were retrieved and each peak area was integrated. The retention time, each peak area, and internal standard peak were imported into the Excel table for use. The ratio of the peak area of the sample to the internal standard peak (relative peak area) indicates the number of metabolites[19], and SPSS 17.0 software was used to analyze the difference between the groups based on *t*-test. Pattern recognition multivariate analysis of normalized data was performed using the SIMCA-P12.0 software, using principal component analysis (PCA), and partial least squares-discrimination analysis (PLS-DA), and maximizing the difference between groups[20]. Finally, data were presented as means ± standard deviation (SD) for test drug groups using SPSS 17.0. A *P*-value < 0.05 was considered statistically significant and highly significant when $P < 0.01$.

3. Results

3.1 Effects of MP on body weight and relative liver weight

MP had no effect on body weight and relative liver weight (Table 1). Body weight was not significantly different among the groups ($P > 0.05$). However, compared with the normal group, the relative liver weight in the CCl_4 model group significantly increased ($P < 0.05$), indicating CCl_4 could induce hypertrophy of

liver tissues in mice. Conversely, the treatment with silymarin (100 mg/kg b.w.) or MP (100 mg/kg) significantly decreased relative liver weight compared with CCl_4 model group ($P < 0.05$).

Table 1. Effects of MP on body weight and relative liver weight in mice（n=6)

Group	Dosage (mg/kg)	Body weight (g)	Relative liver weight (g/100 g body weight)
Normal group		32.34±2.25	4.15± 0.57
Model group		30.93±1.90	4.57±0.39*
L group	50	30.67±3.88	4.81±0.77△
M group	100	32.16±3.67	4.23± 0.31△
H group	200	31.64±1.33	4.46± 0.35
Silymarin group	100	33.12±2.87	3.94±0.89△△

50mg/mL was for the low-dosed group(L), 100mg/mL was for the medium-dosed group(M), and 200mg/mL was for the high-dosed group(H).*$P < 0.05$ and **$P < 0.01$ when compared with control; △$P < 0.05$ and △△$P < 0.01$ when compared with model.

3.2 Effects of MP on ALT and AST levels

Serum ALT, AST levels and other enzyme levels are quantitative markers of liver cell injury. As shown in Table 2, compared with the normal control group, the serum ALT and AST levels in the CCl4 group were significantly increased ($P < 0.05$), which was indicative of hepatic failure. However, the treatment with different dosages of MP (100 and 200 mg/kg) in mice significantly reduced the ALT and AST levels compared with the CCl_4 treatment group ($P < 0.05$); the effects were similar to the silymarin group.

Table 2. Effects of MP on serum ALT and AST levels （n=6)

Group	Dosage (mg/kg)	ALT (U/L)	AST (U/L)
Normal group		79.91± 9.08	50.81± 7.16
Model group		125.75± 19.27**	80.96± 5.24**
L group	50	124.75 ±13.78	76.48 ±2.61
M group	100	113.72± 13.78	49.63 ±4.77△△
H group	200	85.50± 16.33△△	55.92 ±8.05△△
Positive group	100	77.93± 12.63△△	46.60 ±7.97△△

50mg/mL was for the low-dosed group(L), 100mg/mL was for the medium-dosed group(M), and 200mg/mL was for the high-dosed group(H).*$P < 0.05$ and **$P < 0.01$ when compared with control; $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$ when compared with model

3.3 Effects of MP on the activities of hepatic antioxidant enzymes

SOD, MDA, and GSH-Px are the main antioxidant enzymes in liver tissues and can protect against oxidative damage. As shown in Table 3, compared with the normal control group, the SOD and GSH-Px levels in the CCl_4 model group were significantly decreased ($P < 0.05$). However, MDA levels in the CCl_4 treatment groups were significantly higher than in the normal control group when mice were pretreated with silymarin ($P < 0.05$). MP dosages of 100 mg/mL and 200 mg/mL significantly lowered the MDA levels compared with the model group ($P < 0.05$).

Table 3. Effects of MP on hepatic antioxidant enzymes（n=6)

Group	Dosage (mg/kg)	SOD (U/mg pro)	MDA (nmol/mg pro)	GSH-Px (mg/g prot)
Normal group		251.11±19.18	2.18±0.22	5.09±0.79
Model group		194.09±11.77*	5.89±1.03*	2.69±0.58*
L group	50	200.94±19.39	3.48±0.31	3.85±0.72
M group	100	228.75±3.38△	3.00±0.69△△	4.93±0.48△
L group	200	221.69±5.21△	3.02±0.61△△	4.69±0.16△
Positive group	100	224.83±6.79△	3.48±0.83△△	4.75±0.76△

50mg/mL was for the low-dosed group(L), 100mg/mL was for the medium-dosed group(M), and 200mg/mL was for the high-dosed group(H). *$P < 0.05$ and **$P < 0.01$ when compared with control; $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$ when compared with model

3.4 Histopathological observations

Histological liver slices in the normal control group showed general liver parenchyma, including hepatic lobules, and blood sinuses surrounding normal hepatocytes in hepatic lobules were distributed radially towards the central lobe veins (Fig. 3 A). However, a massive inflammation and infiltration area around the central veins was observed in mice in the CCl_4 model group. Focal central vein congestion, vacuolization, and necrosis with inflammation was observed (Fig. 3B). Compared with mice in the CCl4 group, mice with 100 mg/kg MP had moderate liver injury and the area of inflammation and infiltration was decreased. Liver sections showed mild central vein congestion, vocalization, and necrosis with sinusoidal dilatation (Fig. 3C). In mice that received an increased MP dosage, liver sections showed

absence of vacuolization, inflammatory cells, and regeneration of hepatocytes around the central veins, however, slight congestion was observed in the central veins and the liver sections were almost similar to normal liver architecture and had higher hepatoprotective activities. The effects of high MP dosage was similar to silymarin (Fig. 3D–F).

(A) normal control, (B) CCl_4 model control, (C) silymarin (100 mg/kg) (D) MP (50 mg/kg), (E) MP (100 mg/kg), and (F) MP (200 mg/kg)

3.5 GC-MS analysis

3.5.1 Identification of each metabolite map in the NIST map

A total of 11 liver tissue metabolite peaks were extracted using GC-MS and their peak areas manually integrated. The TIC chromatograms obtained from the control, model, low-dose, middle-dose, high-dose, and positive drug groups are shown in Figure 2. The results indicated the difference between the MP groups (therapy group) and the normal control group was slight but sufficient to elucidate the metabolite changes between the groups. Liver tissue sample data collection was performed using the XCMS Online software and typical TIC maps for each group were obtained; 33 common metabolites were identified based on MS fragment information and HMDB (WWW.hmdb.ca/metabolites) online data. Then, the peak areas extracted from the TIC map in the NIST library were compared.

An obvious separating tendency was observed in the PCA (Fig. 3A) and Validate Model (Fig. 3B) of the two groups and the $R^2Y = 0.927$ and $Q^2Y = 0.678$. The results indicate the models were valid and reliable.

3.5.3 PLS-DA of metabolites

PLS-DA was performed to discriminate differences among groups. Differential metabolites contributing to the group separation were identified using VIP and *P*-values. In addition, the metabolites that matched the condition (VIP > 1.0 and $P < 0.05$) were recognized as candidate compounds. Generally, a threshold of VIP > 1 and $P < 0.05$ in the Mann-Whitney *U* test is considered statistically significant. In summary, 14 discriminating metabolites were filtered and recognized as candidate metabolites which could directly perform antioxidant and liver protective effects (Table 4) or activate the scavenging peroxide mechanism to enhance the antioxidant function of the tissue *in vivo*.

The normalized peak area relative values were imported into SIMCA-P12.0 statistical software for PLS-DA. To determine the accuracy of the data of each group and the difference among all treatments, PCA was performed using SIMCA. As shown in Figure 4, the mice in each group were scattered based on different metabolite patterns. The differences between the three-dimensional map groups were significant and had obvious clustering characteristics, indicating the data of each group were reliable and the CCl_4-induced liver damage may affect the physiological environment and metabolite metabolism in the mouse liver tissue[21].

The metabolites contributing to the group separation are shown in the Figure and were recognized as key metabolites with antioxidant ability.The PLS-DA model (construction of variable importance in projection, VIP) was combined to reflect metabolite differences in groups and to search for possible endogenous metabolites (Fig. 5). Based on the PLS-DA results (Fig. 5 and Table 4), compared with the normal group, a total of eight metabolites were upregulated and seven were downregulated in the model group and each MP group, involving amino acids, amides, sugars and arachidonic acid. These potential endogenous metabolites (VIP > 1) were considered potential biomarkers. Therefore, six potential biomarkers that could provide novel clues for subsequent studies of their protective effects on liver damage were identified. However, these potential biomarkers need further verification and investigation.

Table 4. Possible endogenous substance analysis

No	Time (min)	Possible endogenous substances	MS (molecular weight)	VIP	Trend
1	4.11	Glucuronamide	193.15	**1.29**	↓
2	7.00	Octadecanoic acid	284.48	**2.52**	↑
3	9.38	D-Tyrosine	181.19	**1.75**	↓
4	10.75	Arachidonic acid	184.20	0.17	↓
5	11.14	D-leucine	131.17	0.83	↓
6	14.34	Hexadecanoic acid	256.42	0.39	↓
7	15.01	Adenine	135.13	**1.87**	↑
8	15.54	Glycine	75.07	0.41	↑
9	15.81	D-glucose	198.17	**1.66**	↑
10	16.33	Linoleic acid	280.44	0.34	↑
11	18.55	Benzoic acid	122.12	0.89	↓
12	19.62	D-galactose	180.16	0.51	↓
13	26.09	Ergosterol	396.65	**1.46**	↑
14	26.46	Proline	115.13	0.52	↓

Variable importance in projection (VIP) > 1 was used to filter the biomarker ↑ or ↓ compared with normal

3.6 Pathway analysis and biological interpretation

The integration of metabolic pathway metabolites were further performed. The significant relevant pathways affected by *Dioscorea bulbifera* rhizome (DBR) administration were analyzed using MetaboAnalyst 3.0 (www.metaboanalyst.ca), a web-based server that supports pathway analysis, integrated analysis, and pathway enrichment topology. As shown in Figure 6, elevated purine, amino

sugar, and nucleotide sugar metabolism were the most relevant pathways affected by MPs with the most significant impact value. Furthermore, MP-induced pathways were associated with phenylalanine, thiamine, and tyrosine metabolism and glycolysis. Among the pathways induced by MPs, tyrosine metabolism was the most significant. In addition, a metabolic network was built using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp) database (Fig. 6). The data indicate that changes in the comprehensive metabolic profile of mice with MP-induced liver protection were mainly associated with phenylalanine and tyrosine metabolism. Consequently, amino acid metabolism was identified as the key metabolic pathway stimulated by MPs indicating MPs mainly influence the biosynthesis of amino acids in liver tissue with specific biochemical metabolites. In addition, MPs promoted the biosynthesis of starch and sucrose which also reflects the influence of MPs on the liver tissue to some extent.

Pathway analysis of the candidate metabolites identified in this study. All the pathways enriched when filtered based on *P*-value are shown in the Figure. The color of the circle represents the significance and the size of circle represents the impact factor of each pathway. Red represents significant and blue non-significant ($P < 0.05$).

4. Discussion

MPs are a natural resource combining medicinal and nutritional properties and exert antioxidant effects due to specific biochemical metabolites. Reportedly, the hypoglycemic and lipid-lowering effects are mainly due to the MP antioxidant activity[22]. Water-soluble polysaccharides can play an important role in free radical scavengers and prevent many free radical-mediated chronic diseases such as acute liver damage[23]. In the present study, the *in vitro* antioxidant activity of MPs was determined using four free radical assays. The results indicated that MPs have a protective effect on CCl_4-induced liver toxicity in mice which could potentially be used to treat liver disease in humans.

CCl_4, a commonly used chemical for inducing liver damage, can significantly increase the serum ALT and AST levels. The serum ALT and AST levels were elevated in the CCl_4 group (Table 2) compared with the normal control group ($P < 0.05$). However, ALT and AST levels were significantly lower in mice pretreated with silymarin and two doses of MP (100 and 200 mg/kg), indicating that MP has a hepatoprotective effect on CCl_4-induced liver injury in mice.

In addition, the liver SOD, MDA, and GSH-Px levels were evaluated in each group. The SOD and GSH-px levels in the silymarin and MP treatment groups were significantly increased (Table 3). This result could also be improved by the same treatment (Journal of China Institute of Pharmacy). The liver enzyme antioxidant levels in the 200 mg/kg MP group were the same as in the silymarin treatment group, indicating that MPs can effectively enhance the antioxidant ability of liver tissue.

Metabolomics is a popular metabolite analysis technology and has been rapidly developed and applied. Metabolomics is a new technology developed after genomics and proteomics and used in many fields,

including NMR, MS, and HPLC/GC. GC-MS is commonly used to detect metabolites of steroid-containing biological samples[24] (urine, serum, and tissue). Shiman[25] et al. used capillary electrophoresis (CE)-MS/MS combined with GC time-of-flight (TOF)-MS to identify potential biomarkers of hepatotoxicity. In the present study, the acute liver injury caused by CCl_4 was explored using GC-MS metabolomic technology. The results indicated significant differences among various MP doses. Furthermore, a total of 14 possible endogenous substances were found that involved amino acid, glucose, and lipid metabolism. The use of metabolomics shows that combining physiological and metabolite information with drug action can provide a new methodology for exploring clinical and basic research which has been applied in some studies[26].

The MPs significantly affected amino acid metabolism. In particular, the most notable changes were observed in phenylalanine and thiamine metabolism. Phenylalanine was predominant in 13 altered metabolites and the levels were increased in the treated groups compared with the control group. In addition, the most relevant pathways affected by MPs were amino acid metabolism pathways such as phenylalanine and tyrosine. Furthermore, phenylalanine, tyrosine, and tryptophan biosynthesis showed MPs, to a certain extent, exert protective effects by mainly influencing amino acid metabolism.

An important metabolic pathway *in vivo* is the phenylalanine to tyrosine conversion process which mainly occurs in the liver. The reduced phenylalanine-to-tyrosine conversion ratio in blood could promote secondary effects. In addition, liver damage changes the activity of many enzymes. For example, MDA changes can lead to reduced hepatic lipid and glucose metabolism. Changes in phenylalanine and tyrosine metabolism were observed in the present study, indicating MPs protect the liver with their hepatoprotective activities. The tricarboxylic acid (TCA) cycle is involved in aerobic oxidation of glucose and is the main process for lipid and amino acid metabolism, thus, inhibition of the TCA cycle may lead to organ degeneration. Some altered saccharide metabolites, including fructose, glucose, and galactose, which are involved in galactic metabolism as well as the TCA cycle pathway, showed a strong association with energy metabolism in DBR-treated groups. In epidemiological and clinical studies, stearic acid was associated with lower LDL cholesterol more than other saturated fatty acids. In addition, when a significant increase in stearic acid was observed in the plasma, stearic acid excretion was significantly reduced in the feces, which may be due to elevated liver damage caused by stearic acid. Furthermore, the above-mentioned results could provide more accurate targets for clinical treatment[27,28]. In conclusion, the results of the present work showed MPs had a hepatoprotective effect on CCl_4-induced liver injury in mice. In addition, 13 metabolites were identified as biomarkers and need further verification. Using a multiple integrated metabolomics method, the biomarkers associated with liver damage were identified and further investigated using PLS-DA with a panel of the integrated characteristic metabolites. These newly identified pathways could help further analyze the mechanisms associated with liver damage and explore novel potential therapeutic targets, which should be investigated in future studies.

Declarations

The data availability statement

All the authors in the manuscript have published the usability of the data and a declaration of consent for publication in this journal.

Novelty statement

In this study, we applied GC-MS metabolomics technology to detect the metabolic differences between CCl4-induced acute liver injury in mice models and investigating the protective mechanistic effects of mulberry polysaccharides (MPs) on acute liver injury.Combined with multivariate statistical analysis to study the metabolic markers on CCl4-induced acute liver injury in mice models with GC-MS technology.

Conflict of interest

The authors declare that they have no conflict of interest with the publication of this article and agree that the data in the manuscript will be published in this journal.

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Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and PMN, polymorphonuclear leukocyte. *Biochemical Pharmacology, 62*(9).

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Figures

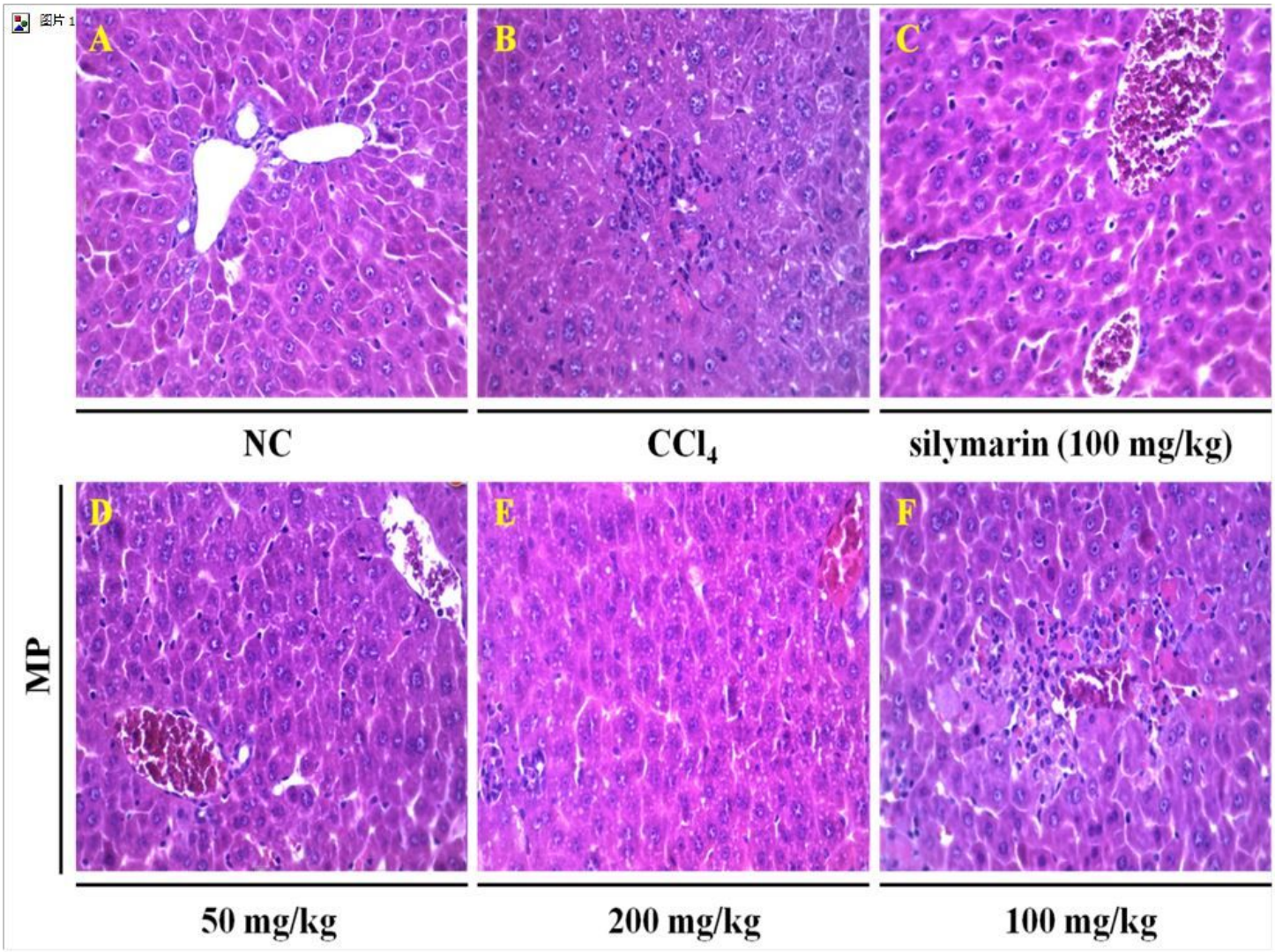

Figure 1

Histopathological observation (using light microscope) of the liver tissues of mice suffering from CCl4-induced acute liver injury after treatments at different doses (HE×40) (A) normal control, (B) CCl4 model control, (C) silymarin (100 mg/kg) (D) MP (50 mg/kg), (E) MP (100 mg/kg) (F) MP (200 mg/kg)

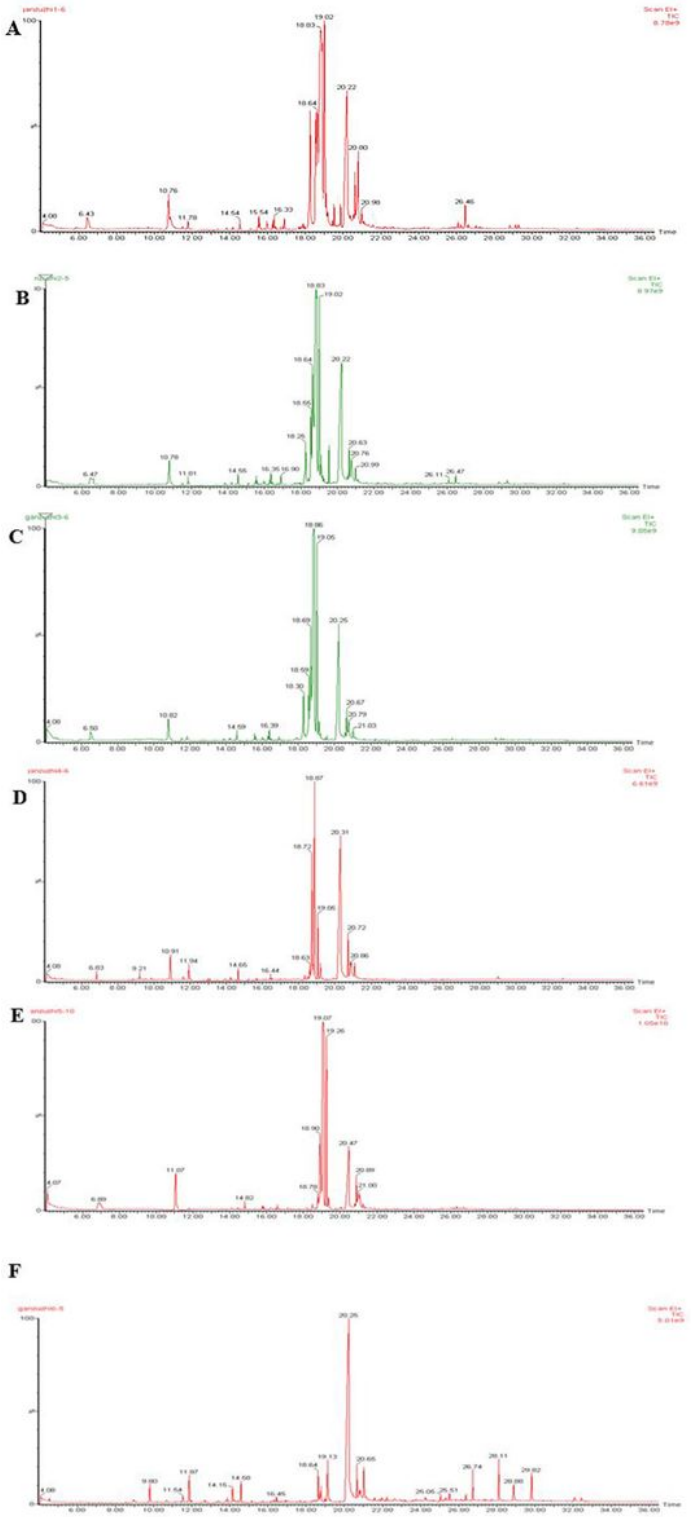

Figure 2

Total ion chromatography of normal group (A), model group (B), high dose group (C), middle dose group (D), low dose group (E) and silymarin group (F).

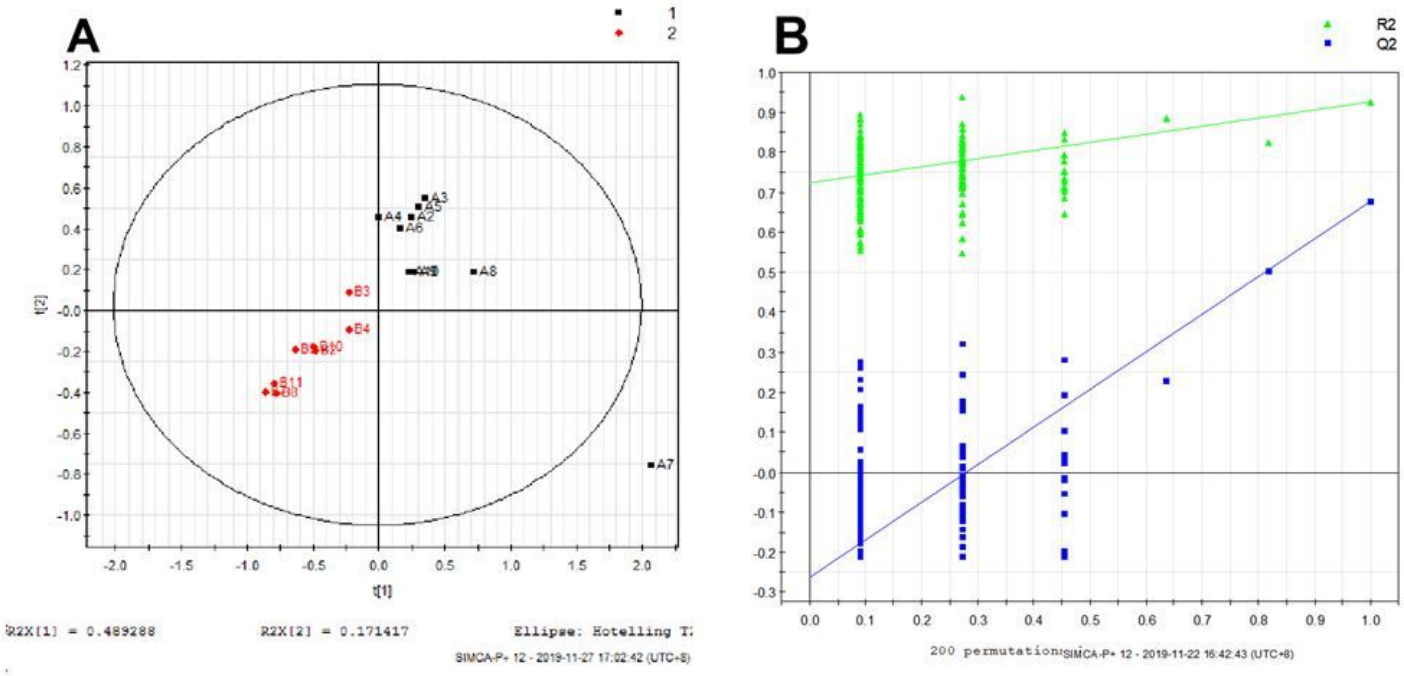

Figure 3

The PCA (Fig.3A) and Validate Model(Fig.3B) of the normal groups(■) and model control(■)

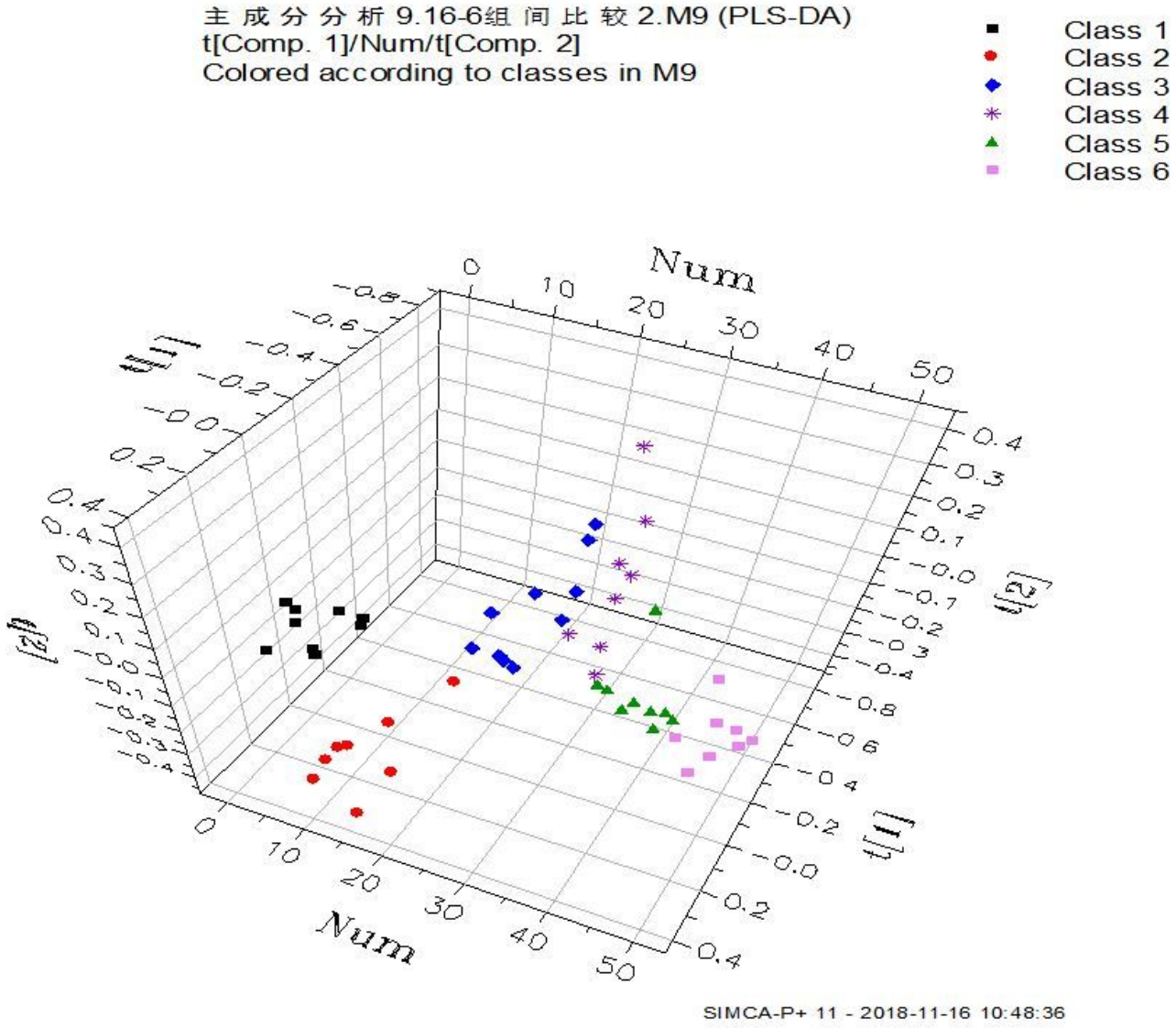

Figure 4

PCA-X map of metabolites in the group, one for the normal group, two for the model group, three for the low dose group, four for the middle dose group, five for the high dose group, six for the biphenyl dilipid group

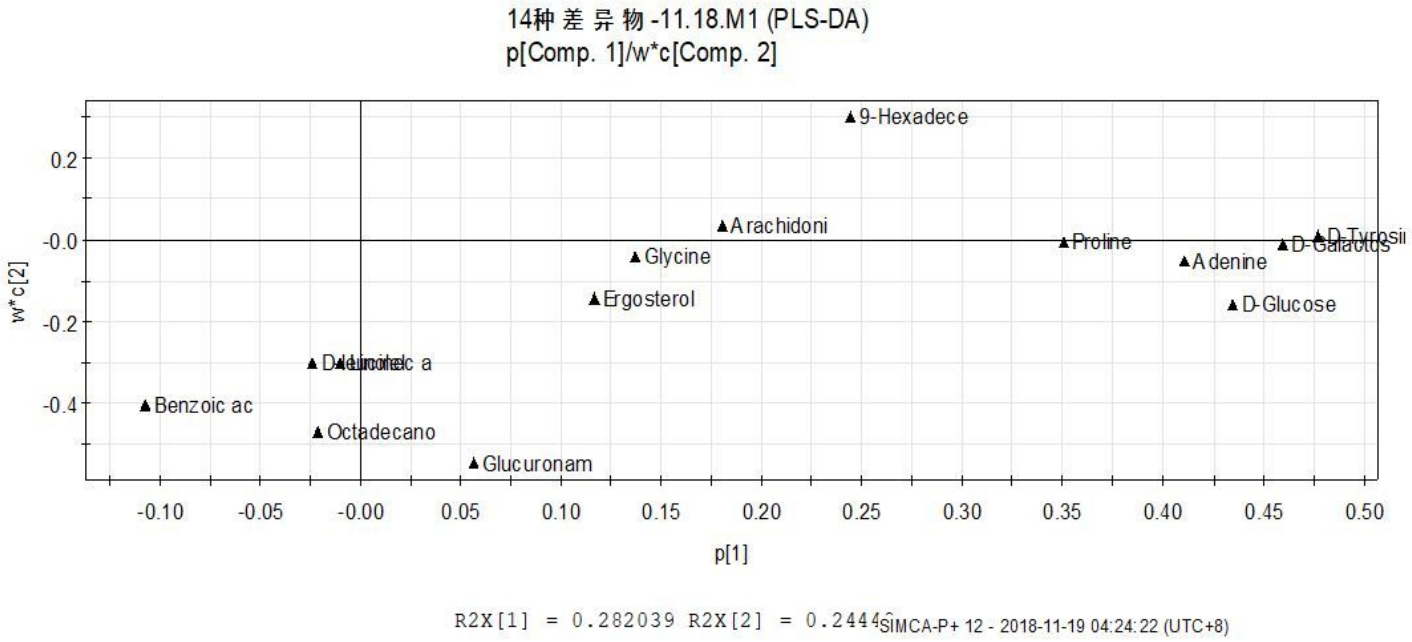

Figure 5

PLS-DA analysis of metabolite results The metabolites contributes to the group separation were shown in the figure which were recognized as key metabolites and have antioxidant ability

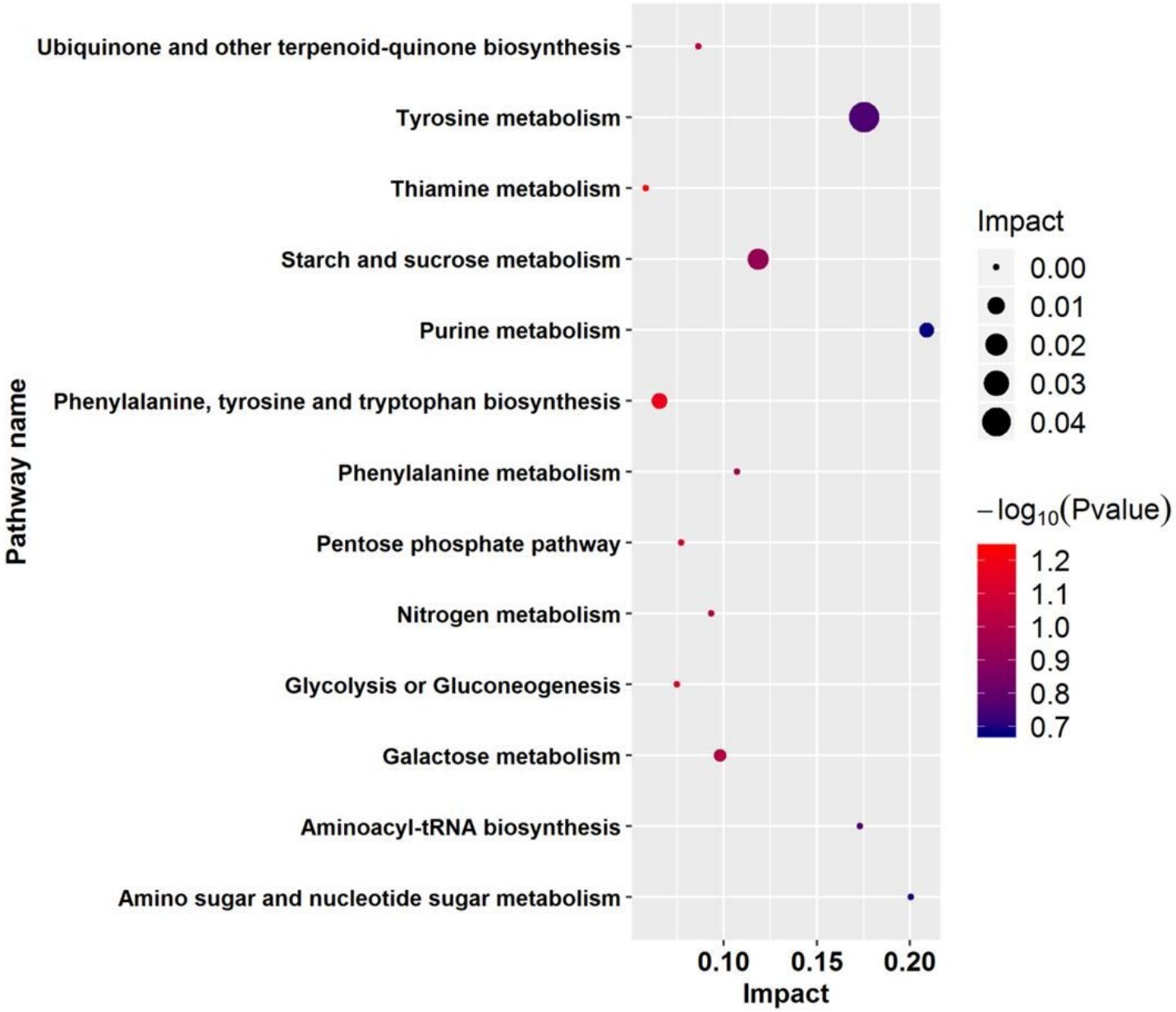

Figure 6

summary of pathway analysis Pathway analysis of the candidate metabolites involved in this study. All the pathways enriched filtered by P-value were shown in the Figure. The color of the circle represents the significance, and the size of circle represents the impact factor of each pathway. Red means significant, and blue means nonsignificant ($P < 0.05$)

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