Contents lists available at ScienceDirect

Chinese Herbal Medicines

journal homepage: www.elsevier.com/locate/chmed

Original Article

Metabolic regulation mechanism of *Aconiti Radix Cocta* extract in rats based on ^{1}H-NMR metabonomics

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A R T I C L E   I N F O

A B S T R A C T

Objective: To establish a metabonomics research technique based on the combination of ^{1}H-NMR and multivariate statistical analysis, so as to explore the metabolic regulation mechanism of *Aconiti Radix Cocta* extract (ARCE) in rat tissues and serum.
Methods: SD rats were randomly divided into blank group, female group and male group. The ^{1}H-NMR technique was used to collect the information of rat tissues and serum samples in each group. The principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and other methods were used for data pattern recognition, so as to screen out potential differential metabolites and metabolic pathways, and then network analysis and KEGG database were used to analyze the relationship between metabolites, metabolic pathways and diseases.
Results: The external features and ^{1}H-NMR analysis showed that the sex of rats had no obvious effect on the drug action. A total of 15 potential differential metabolites and six metabolic pathways were screened out through data pattern recognition. Through network analysis and KEGG pathway analysis, three target diseases closely related to differential metabolites were found, and the metabolic pathway related to lung cancer was the central carbon metabolism of cancer.
Conclusion: This study shows that *Aconiti Radix Cocta* (ARC) may regulate the energy metabolism of the body by influencing arginine synthesis, so as to play the roles of anti-inflammation, analgesia, anti-tumor and immune regulation.

1. Introduction

Aconiti Radix is the dry mother root of *Aconitum carmichaelii* Debx. of Ranunculaceae plant, which was first recorded in *Shennong's Classic of Materia Medica*, and tastes bitter, pungent, hot and toxic. It has the effects of dispelling wind and removing dampness, warming meridians, dispelling cold and relieving pain (Hui et al., 2020). Modern pharmacological studies showed that *Aconiti Radix* has anti-inflammatory, analgesic, anti-tumor and immunomodulatory effects, and its chemical components are mainly alkaloids, polysaccharides, volatile oils and flavonoids, etc. (Chen, Wang et al., 2021, Chen, Cheng et al., 2021; Ma et al., 2019), among which *Aconitum* alkaloids are not only the main pharmacodynamic components, but also the toxic components. In order to avoid the toxicity of *Aconiti Radix* and exert the maxi-

mum curative effect, raw *Aconiti Radix* needs to be processed into *Aconiti Radix Cocta* (ARC) before use. Modern processing methods of *Aconiti Radix* mostly adopt steaming and boiling, which makes the toxic components hydrolyze (Cao, 2020).

Metabonomics study is a modern biological analysis technology that analyzes the metabolites and their changing rules in living organisms as a whole. Now it has been widely used in drug toxicity research, new drug safety evaluation, disease diagnosis and other fields (Li et al., 2020; Liu et al., 2020; Man et al., 2020). The metabonomics analysis is to use modern instrumental analysis methods such as HPLC, ^{1}H-NMR or MS to qualitatively and quantitatively detect as many endogenous metabolites as possible in organisms and monitor the dynamic changes of metabolites. ^{1}H-NMR technology can be used for rapid efficacy analysis and provide stable and reproducible results (Crook & Powers, 2020; Wishart, 2019). At present, ^{1}H-NMR technology is widely used in metabolomics research to determine natural products and biological samples.

In this study, ^{1}H-NMR metabonomics was used to comprehensively and deeply study the metabolism of *Aconiti Radix Cocta* extract (ARCE) in rat heart, liver, kidney and serum, so as to explore the metabolic differences of ARC in different tissues and serum for the first time. Potential biomarkers combined with multivariate statistical analysis probably find possible metabolic pathways related to pharmacological mechanism of ARC. The purpose of this study is to provide useful reference value for the further development and utilization of *Aconitum* traditional Chinese medicine and the clinical research of its preparation. The specific experimental process was shown in Fig. 1.

2. Materials and methods

2.1. Medicinal materials and experimental animals

Aconiti Radix was purchased from Guangdong Lianfeng Chinese Herbal Pieces Co., Ltd. and identified by Associate Professor Ruolei Xiao from Hubei University of Science and Technology. A total of 18 eight-week-old SD rats (half male and half female) were provided by Hubei Medical Experimental Animal Center (Certificate No. SCXK(E) 2015–0018). The animal protocols were in accordance with the Guide for the Care and Use of Laboratory Animals, and the use of the animals was approved by the Institutional Animal Care and Use Committee at Hubei University of Science and Technology.

2.2. Instruments and reagents

The following equipment was used in this experiment: Nuclear Magnetic Resonance instrument (AVANCE III, Bruker, Switzerland); HR/T20M Desktop High-speed Refrigerated Centrifuge (Hunan Hexi Instrument Equipment Co., Ltd., Changsha, China); rE-5299 Rotary Evaporator (Zhengzhou Yarong Instrument Co., Ltd., Zhengzhou, China); SHZ-D (III) circulating water type multipurpose vacuum pump (Zhengzhou Boko Instrument Equipment Co., Ltd.); FSJ-A03D1 Pulverizer (Guangdong Foshan Shangdian Electric Appliance Co., Ltd., Foshan, China); LGJ-10A Vacuum Freeze Dryer (Shanghai Hefan Instrument Co., Ltd., Shanghai, China); KW-1000DC digital display constant temperature water bath pot (Jiangsu Jintan Yitong Electronics Co., Ltd., Jintan, China); KQ 3200 DB Numerical Control Ultrasonic Cleaner (Kunshan Test Instrument Co., ltd); DZF-6050 Vacuum Drying Oven (Shanghai Bullun Industrial Co., Ltd. Medical Equipment Factory, Shanghai, China); FA2004B Electronic Analytical Balance (Shanghai Yueping Scientific Instrument Co., Ltd., Shanghai, China).

Methanol, acetonitrile (Merck, Germany) and isopropanol (Tianjin Kemeo Chemical Reagent Co., Ltd., Tianjin, China) were chromatographic grade; Ammonia water (Tianjin Hengxing Chemical Reagent Manufacturing Co., Ltd., Tianjin, China) and ethyl acetate (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were analytical reagent grade; D_2O (Sigma-Aldrich, USA).

2.3. Methods

2.3.1. Processing and alkaloid extraction of Aconiti Radix

According to the relevant regulations of the *Pharmacopoeia of the People's Republic of China* (2020 edition) (Chinese Pharmacopoeia Commission, 2020), *Aconiti Radix* was steamed for 6 h, dried in a vacuum drying oven at 40 °C for 5–6 h, crushed and passed through No.3 sieve for later use. *Aconiti Radix* was steamed for 6 h, and put the slices into a vacuum drying oven to dry at 40 °C for 5–6 h, then crushed and passed through the No.3 sieve for later use. The total alkaloids in ARC were extracted by the method in references (Bi et al., 2021; Fan et al., 2021), and the extract was concentrated under reduced pressure and freeze-dried to obtain ARCE.

2.3.2. Animal experiment and sample collection

Eighteen SD rats (half male and half female) were fed adaptively in SPF laboratory with temperature for (25 ± 0.5) °C and humidity for (50 ± 5)%, light and dark cycle of 12 h. Animals could eat and drink freely during the experiment. Eighteen rats were divided into female and male groups according to sex. Three rats were randomly selected from each group to form a blank group. The ARCE was added into CMC-Na aqueous solution, and made into uniform suspension by ultrasonic and vortex. Then it was gavaged to the female and male groups twice at 8: 00 a.m. and 8: 00 p.m. respectively, in a dose of 20 mg/kg, while the blank group was gavaged the same amount of normal saline for one week.
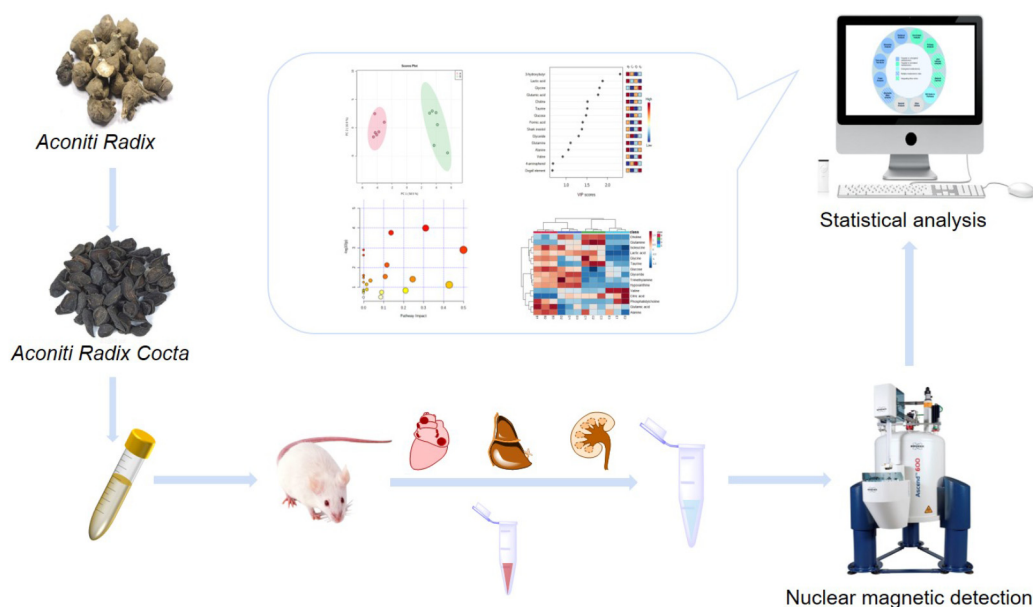

Fig. 1. Flow chart of metabolism experiment of ARC.

Body weight and external features of experimental rats were observed and recorded at any time. After the experiment, all rats were fasted for 12 h, then blood, heart, liver and kidney tissues of rats were taken. The blood was stored at room temperature for 1 h, the supernatant was centrifuged at 4 °C and 3500 r/min for 15 min, and the rat serum was collected and freeze-dried at 4 °C for storage. The tissue was rinsed with cold acetonitrile water (1:1), and then rinsed again with normal saline to remove blood and excess water. After the cleaning solution was sucked dry with filter paper, the tissue sample was put into a beaker. After the tissues were sheared, they were transferred to a tissue homogenizer together with appropriate amount of normal saline for homogenization, then the homogenate was centrifuged at 4 °C and 3500 r/min for 15 min, and finally the supernatant was freeze-dried and stored at 4 °C.

2.3.3. Sample preparation

The freeze-dried serum and tissue samples were vortexed with 500 μL of D_2O for 10 s, and centrifuged at 10 000 r/min for 10 min at 4 °C. Finally, 500 μL of the supernatant was transferred to a 5 mm NMR tube for analysis.

2.3.4. ^1H-NMR detection conditions

Tissue and serum samples of rats in each group were detected by nuclear magnetic resonance spectrometer (Bruker Biospin GmbH). Parameters collected on NMR spectrometer are as follows: frequency is 400 Hz, zg30 pulse sequence, scanning times are 16 times, spectral width is 8012.8 Hz, pulse width is 10.26 Hz, sampling time is 4.0894 s, relaxation delay is 3 s, and temperature is room temperature.

2.3.5. Nuclear magnetic data processing

All NMR spectra were analyzed and processed by MestReNova professional software. Manual phase correction and baseline correction were carried out on the spectrogram, and the proton signal of lactic acid (δ = 1.33) was used for calibration. According to the electronic database HMDB (https://www.hmdb.ca/) and BMRB (https://www.bmrb.wisc.edu/), the metabolites of ^1H-NMR chemical shift were identified. Taking δ 0.01 as the unit, all the δ 0.50–9.50 regions of the map were integrated in segments, in which δ 4.50–5.20 regions were removed to eliminate the influence of residual water peaks. Finally, the total peak area integration data were normalized with the sum as 1, and the obtained data were converted into Excel tables and saved for statistical analysis.

2.3.6. Statistical analysis

MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) database was used for statistical analysis. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were used to optimize the differences among groups. And PLS-DA combined with *t*-test was used to analyze NMR data, the metabolites with VIP > 1 and *P* < 0.05 in PLS-DA model were screened out as differential metabolites. The information of the differential metabolites was imported into the metabolic pathway analysis module of MetaboAnalyst 5.0, which combined the pathway enrichment analysis with the pathway topology analysis to screen out the key metabolic pathways. Finally, the network analysis and KEGG database were used to analyze the correlation between differential metabolites, metabolic pathways and diseases in ARC.

3. Results

3.1. External features

During the experiment, the rats in the female and male groups were in good condition as a whole, and there was no significant difference in behavior, appearance and weight compared with the blank group. In addition, the results of histopathological examination showed that there were no obvious pathological changes in the heart, liver and kidney tissues of rats in the administration group (Fig. 2). All these results indicated that *Aconitine* alkaloids had low toxicity after processing and had no significant difference in the effects of female and male groups.

3.2. ^1H-NMR analysis of tissue and serum metabonomics

There was no significant difference in ^1H-NMR spectra of tissues and serum between female and male rats, indicating that the sex of rats had no obvious influence on the administration effect of ARC. The representative ^1H-NMR spectrum was shown in Fig. 3. Metabolites in heart, liver, kidney and serum of rats were identified by analyzing chemical shift, coupling constant and peak splitting, combining with HMDB and BMRB databases and metabolite data reported in references (Lü et al., 2020; Nie et al., 2019). A total of 40 metabolites were identified, including amino acids, short chain fatty acids, sugars, lipids, tricarboxylic acid cycle intermediates, choline metabolites, purine metabolites, etc. (Table 1). Among them, 34, 24, 32 and 18 kinds of metabolites were identified in heart, liver, kidney and serum respectively, and the metabolites were mainly distributed in heart and kidney tissues, indicating that the metabolism of ARC may be carried out in kidney, and the metabolic activity of rats was the most abundant in heart after administration. *Aconiti Radix* belongs to heart, liver, kidney and spleen channels, and the drug action has certain selectivity to heart, liver and kidney, which can provide basis for clinical medication based on syndrome differentiation.

3.3. Multivariate statistical analysis

Multivariate statistical analysis was carried out on metabonomics data of blank group and ARC administration group. Firstly, the ^1H-NMR spectra of hearts in blank group and administration group were statistically analyzed by PCA to observe the inherent metabolic changes in rats, and then PLS-DA and OPLS-DA were used to analyze the differences between groups.

As shown in Fig. 4A, unsupervised discriminant model PCA can deliver the natural clustering trend between samples and other groups, and reflect the overall changes of the body affected by external intervention. Blank group and administration group had a certain tendency to gather in groups, and the data was mainly distributed on the left and right sides of the plane, with an obvious separation trend between the two groups of samples, which showed the endogenous metabolites of rats had changed after administration of ARCE. As shown in Fig. 4B and C, PLS-DA and OPLS-DA technologies with supervised discrimination mode can maximize the difference between groups according to the predefined classification, and obtain better separation effect than PCA. Under the supervision, the blank group and the administration group had stronger aggregation, and the samples can be separated more effectively. And the model validation parameters R2X = 0.473, R2Y = 0.972, Q2 = 0.859, which showed that the model had good fitting effect and high reliability.

PLS-DA analysis was carried out on the metabonomics data of the tissues and serum of rats in the administration group of ARC. Predictive importance method (VIP) was used to study the changes
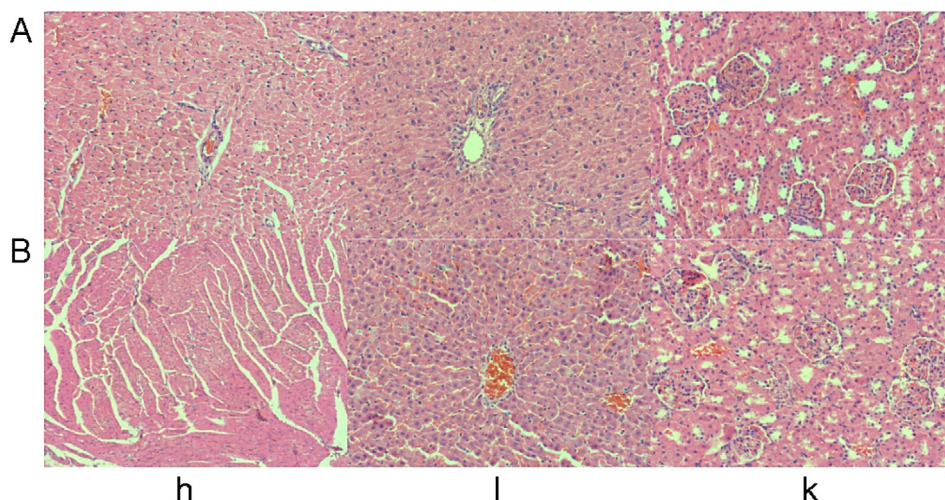
Fig. 2. Histopathological charts of heart (h), liver (l) and kidney (k) tissue in female (A) and male (B) groups.
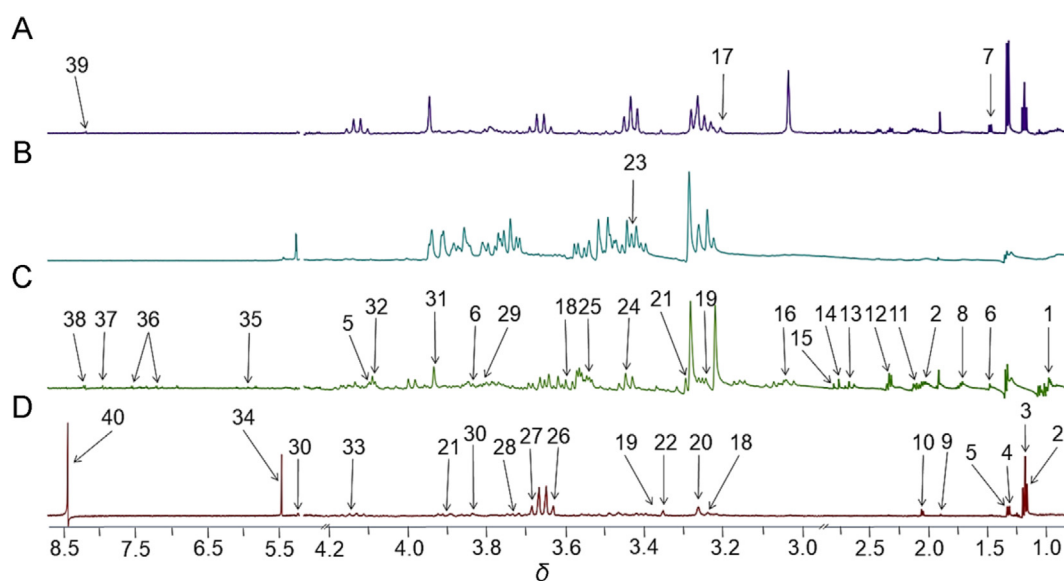

Fig. 3. ^{1}H-NMR spectra of metabolites in rat heart (A), liver (B), kidney (C) and serum (D).

of metabolites in tissues and serum and screen for potential differential metabolites.

The four groups of heart, liver, kidney and serum were pattern recognized by PLS-DA, as shown in Fig. 5A and B. The samples of each group were separated effectively, indicating that the metabolites in tissues and serum changed differently after administration of ARCE. And the model validation parameters R2 = 0.958, Q2 = 0.838, which shows that the model fits well and the prediction is reliable. According to VIP value (>1), the important metabolites were classified and identified to obtain 12 common differential metabolites, namely 3-hydroxybutyric acid, lactic acid, glycine, glutamic acid, choline, taurine, glucose, formic acid, shark inositol, glyceride, glutamine and alanine.

3.4. Screening of differential metabolites

In order to screen out further potential differential metabolites, the ^{1}H-NMR data of the heart of the blank group and the ARC administration group was analyzed by PLS-DA combined with t-test. Metabolites with VIP > 1 in PLS-DA model and $P < 0.05$ in t-test were

selected as differential metabolites, and the concentration change of differential metabolites in the heart metabolism of the two groups was further analyzed by biomarker analysis function.

Seventeen possible metabolites were screened according to VIP value (>1), as shown in Fig. 6. T-test was carried out according to the relative peak area of metabolites, and potential differential metabolites were screened according to $P < 0.05$. Fifteen different metabolites were identified, including choline, glutamine, phosphorylcholine, lactic acid, glutamic acid, isoleucine, valine, glycine, alanine, glucose, taurine, glyceride, citric acid, trimethylamine and hypoxanthine. According to the analysis of biomarkers, the contents of choline, glutamine, valine and citric acid in the treatment group were significantly reduced compared with the blank group, while the contents of other metabolites were significantly increased. The receiver operating characteristics (ROC) curves of biomarkers were further analyzed to evaluate their predictive ability and diagnostic accuracy. The results showed the highest AUC was 1, indicating that these metabolites may be potential biomarkers for diagnosis and early detection of diseases. Analysis results were shown in Table 2.

Table 1
Metabolites analysis in heart, liver, kidney and serum of rats.

No.	δ_H	Metabolites	Heart	Liver	Kidney	Serum
1	1.01 (d)	Isoleucine	+	+	+	−
2	1.16 (s), 2.10 (m)	Proline	+	+	+	+
3	1.19 (d)	3-hydroxybutyric acid	+	+	−	+
4	1.32 (d)	Threonine	+	−	+	+
5	1.33 (d), 4.10 (q)	Lactic acid	+	+	+	+
6	1.47 (d), 3.82 (d)	Alanine	+	+	+	−
7	1.49 (m)	Lysine	+	−	−	−
8	1.74 (s)	Ornithine	−	−	+	−
9	1.92 (s)	Acetic acid	+	+	+	+
10	2.07 (m)	Glutamate	+	−	+	+
11	2.14 (s)	O-acetyl glycoprotein	+	−	+	−
12	2.35 (s)	Pyruvate	+	−	+	−
13	2.69 (d)	Citric acid	+	−	+	−
14	2.71 (s)	Dimethylamine	−	−	+	−
15	2.79 (m)	Fatty acid	+	+	+	−
16	3.04 (s)	Creatinine	+	−	+	−
17	3.20 (s)	Phosphatidylcholine	+	−	−	−
18	3.23 (t), 3.59	Arginine	+	+	+	+
19	3.24 (t), 3.40 (t)	Taurine	+	+	+	+
20	3.26 (s)	Trimethylamine	+	+	+	+
21	3.28 (s), 3.90 (s)	Betaine	+	+	+	+
22	3.35 (s)	Shark inositol	+	−	−	+
23	3.43 (t)	Oxgall element	+	+	−	−
24	3.44 (m)	β-glucose	+	+	+	−
25	3.56 (s)	Glycine	+	+	+	−
26	3.63 (d)	Valine	+	−	+	+
27	3.66 (s)	Glyceride	+	+	+	+
28	3.72 (s)	Dimethyl glycine	+	+	−	+
29	3.78 (t)	Glutamine	+	+	+	−
30	3.83 (m), 5.25 (d)	α-glucose	+	+	+	+
31	3.93 (s)	Creatine	+	+	+	
32	4.08 (s)	Choline	+	+	+	−
33	4.14 (d)	2-hydroxyisobutyrate	+	+	+	+
34	5.41 (m)	Hepatin	−	+	−	+
35	5.81 (d)	Uracil	−	−	+	−
36	7.21 (t), 7.55 (d)	Tryptophan	−	+	+	−
37	8.19 (s)	Adenosine	−	−	+	−
38	8.21 (s)	Hypoxanthine	+	−	+	−
39	8.35 (s)	4-aminophenol	+	−	−	−
40	8.45 (s)	Formic acid	+	+	+	+

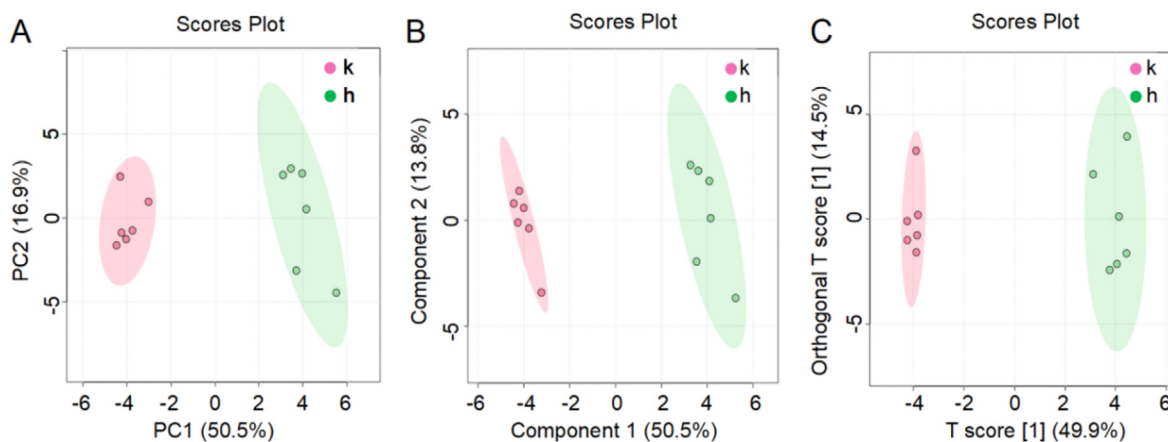Note: +: detection; −: not detected.

Fig. 4. Multivariable statistical model of ^{1}H-NMR cardiac metabolomics data. It showed the group separation achieved by PCA (A), PLS-DA (B) and OPLS-DA (C); k represents the blank cardiac metabolism group, and h represents the administration cardiac metabolism group.

3.5. Thermogram analysis of differential metabolites

In order to explore the significant changes of different metabolites in tissues and serum metabolisms of rats administered with ARC, the metabolites were analyzed by cluster analysis thermogram, as shown in Fig. 7. Thermogram analysis visually showed the relative increase or decrease of differential metabolites in rats of each group. Compared with liver and serum groups, the contents of many metabolites in the heart and kidney groups were increased significantly, especially the contents of phosphoacylcholine, lactic acid, glutamic acid, isoleucine and alanine in the more influential heart metabolism groups were increased
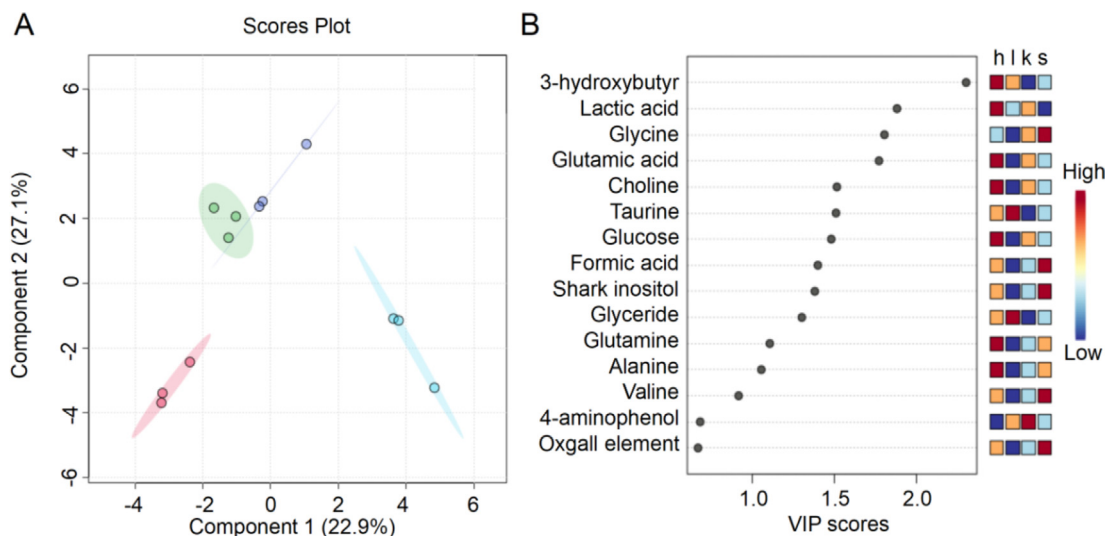
Fig. 5. Multivariate statistical model of ^{1}H-NMR tissue and serum metabolomics data. PLS-DA score map (A) and VIP map (B) of tissue and serum obtained by data analysis; h, l, k and s represent the heart, liver, kidney and serum metabolism groups respectively.
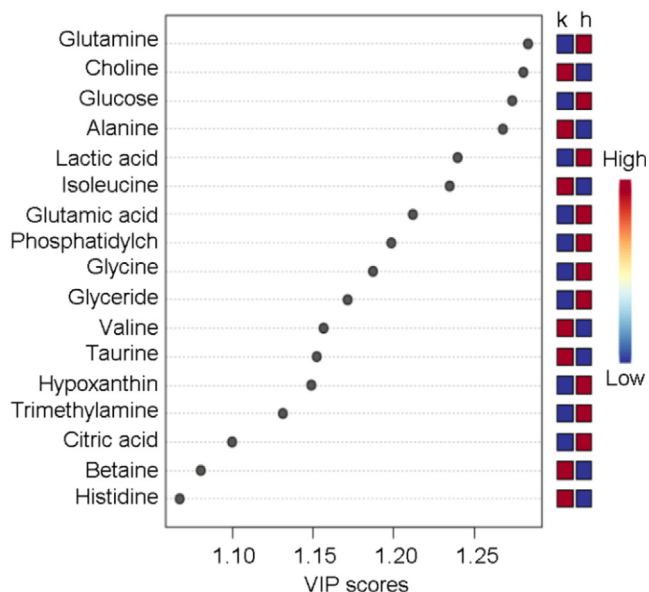

Fig. 6. VIP map of cardiac metabolism in rats. k represents the blank cardiac metabolism group, and h represents the administration cardiac metabolism group.

significantly. This indicated that many metabolites jointly regulate cardiometabolic activity, and the regulatory effect of these five metabolites was particularly significant. The changes of metabolites can directly reflect the physiological changes of rats after administration, and the effects and risks of drug treatment can be visualized by monitoring metabolites.

3.6. Metabolic pathway analysis

In order to find out the relationship between differential metabolites and metabolic pathways in rats, 15 differential metabolites were imported into MetaboAnalyst 5.0 database for high-throughput metabolic pathway analysis, which were shown in Fig. 8A. The size and color of bubbles are related to the P value and Impact value of each channel, which is calculated by topological analysis. Metabolic pathways with $P < 0.05$ and Impact > 0.1 indicated that this pathway may be closely related to potential target pathways. According to the analysis results, six related pathways were found as follow: alanine, aspartate and glutamate metabolism; glyoxylate and dicarboxylate metabolism; glutamine and glutamate metabolism; arginine biosynthesis; glutathione metabolism; glycine, serine and threonine metabolism.

A total of 15 differential metabolites were imported into the MetaboAnalyst 5.0 database for enrichment analysis, as shown in Fig. 8B. The longer column means higher enrichment value.
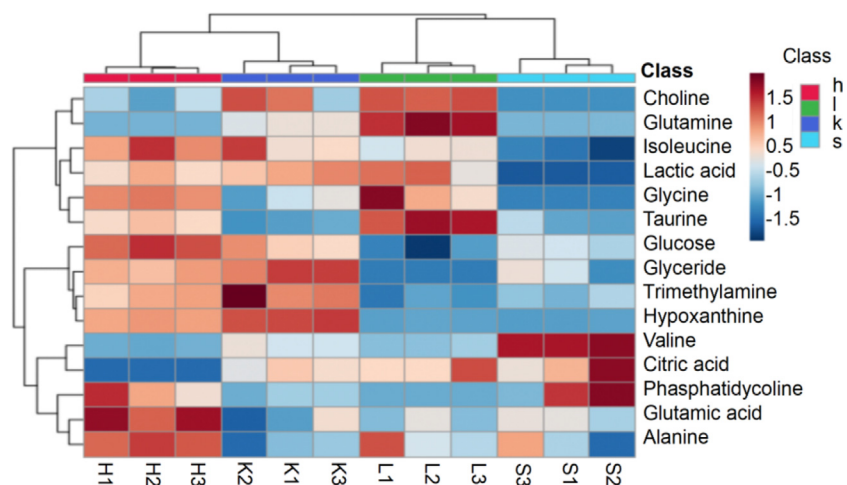
Table 2
Analysis results of differential metabolites.

No.	Metabolites	P	VIP	FDR	AUC	FC
1	Choline	3.57E−5	1.281	3.86E−4	1	−1.45
2	Glutamine	3.92E−5	1.283	4.87E−4	1	−0.67
3	Phosphatidylcholine	1.26E−4	1.199	2.84E−4	1	1.83
4	Lactic acid	2.65E−4	1.240	6.63E−4	1	2.21
5	Glutamic acid	3.14E−4	1.212	1.63E−4	1	2.09
6	Isoleucine	3.28E−4	1.235	6.07E−4	1	1.72
7	Valine	4.25E−4	1.158	5.39E−4	0.94	−0.59
8	Glycine	5.69E−4	1.188	1.03E−3	1	1.34
9	Alanine	7.45E−4	1.269	5.17E−3	1	1.52
10	Glucose	1.11E−3	1.273	3.07E−3	1	0.95
11	Taurine	2.32E−3	1.152	5.81E−3	1	2.13
12	Glyceride	3.76E−3	1.171	8.23E−3	1	1.07
13	Citric acid	8.59E−3	1.099	1.58E−2	1	−1.73
14	Trimethylamine	1.54E−2	1.131	2.71E−2	0.89	0.94
15	Hypoxanthine	3.36E−2	1.149	2.80E−2	0.97	1.20

Fig. 7. Thermogram of differential metabolites in tissues and serum of rat. h, l, k and s represent the heart, liver, kidney and serum metabolism groups respectively.
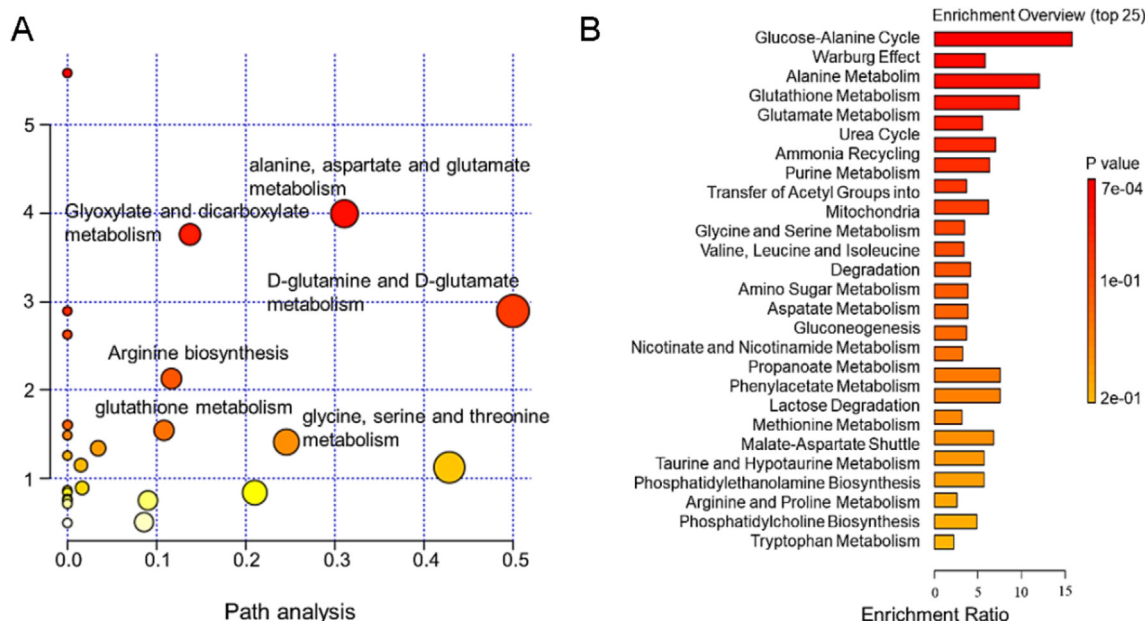

Fig. 8. Pathway analysis (A) and enrichment analysis (B) diagram of differential metabolites.

Meanwhile, the higher column means smaller *P* value and greater significance level of metabolic pathway. The metabolic pathways found by enrichment analysis, such as alanine metabolism, glutathione metabolism, glutamate metabolism, glycine and serine metabolism are consistent with those found by our organization and serum metabonomics, and these metabolic pathways may play an important role in targeted metabolism of diseases.

3.7. Network analysis

The interaction network of metabolites and diseases was studied by network analysis to show the potential functional relationship between differential metabolites involved in the regulation of ARC and target diseases. And the correlation analysis between metabolic pathways involved in the regulation of ARC and target diseases was established by using KEGG database to explore the potential pharmacological activities of *Aconiti Radix*. According to

the lowest network, the three diseases with the most closely related differential metabolites are schizophrenia, lung cancer and Alzheimer's disease. Each degree center values of glycine and valine are three, while the degree center of differential metabolites was the highest, indicating that these two metabolites were most related to three diseases (Table 3).

According to KEGG pathway analysis, the target disease most closely related to the metabolism in ARC is cancer, and the related metabolic pathway is the central carbon metabolism of cancer (Fig. 9). Six different metabolites such as glycine, citric acid, lactic acid, glutamine, glutamic acid and alanine and three metabolic pathways such as alanine, aspartic acid and glutamic acid metabolism, glutamine and glutamic acid metabolism and glycine, serine and threonine metabolism mainly mediate the tricarboxylic acid circulation pathway. The results of network analysis and KEGG path analysis showed that ARC had certain preventive and therapeutic effects on lung cancer.

Table 3
Analysis results of metabolite-disease interaction network.

Id	Label	Degree	Betweenness
181500	Schizophrenia	9	35.96
211980	Lung cancer	7	22.96
104300	Alzheimer disease	6	12.08
C00037	Glycine	3	4.52
C00183	L-Valine	3	4.52
C00114	Choline	2	1.79
C00245	Taurine	2	1.75
C00158	Citric acid	2	1.75
C00041	L-Alanine	2	1.75
C00025	L-Glutamic acid	2	0.98
C00407	L-Isoleucine	2	0.98
C00064	L-Glutamine	2	0.98
C00186	L-Lactic acid	1	0
C00262	Hypoxanthine	1	0

4. Discussion

In this study, ^{1}H-NMR combined with multivariate statistical analysis was used to analyze the metabonomics of rats after oral ARCE. In the early stage of the experiment, we used steaming method to reduce the toxicity of *Aconiti Radix*. *Aconitum* alkaloids are the toxic components of *Aconiti Radix*, among which diester aconitine is the most toxic. Steaming method can promote the hydrolysis of diester alkaloids, so that the acetyl group at C8 position loses a molecule of acetic acid in the hydrolysis process, and the corresponding benzoyl monoester aconitine is obtained. The toxicity of benzoyl monoester alkaloid and aconitine at C14 position will be reduced by continued hydrolysis (Chen, Wang et al., 2021, Chen, Cheng et al., 2021; Ye et al., 2020), so as to achieve the purpose of detoxification and ensure its safe and effective use. Next, PCA, PLS-DA and OPLS-DA were then used to recognize the pattern of rat heart metabolism data, which showed that the

administration group was completely separated from the blank group. Meanwhile, PLS-DA was used to analyze the pattern of rat tissues and serum, and it found that each group could be separated effectively and indicated that the content of endogenous metabolites in rats was changed significantly after administration of ARCE. Most metabolites were detected, among which 15 metabolites can be used as differential metabolites after oral administration in rats, such as choline, glutamine, phosphorylcholine, lactic acid, glutamic acid, isoleucine, valine, glycine, alanine, glucose, taurine, glyceride, citric acid, trimethylamine and hypoxanthine. Except for choline, glutamine, valine and citric acid, the content of other metabolites was increased obviously, which indicated that metabolic detection based on ^{1}H-NMR can characterize the metabolic changes of ARC. At the same time, these markers play an important role in the processes of glucose metabolism, protein metabolism and lipid metabolism, which indicates that ARC can cause the changes of energy metabolism by regulating the changes of metabolites such as glucose, amino acids and fatty acids.

According to differential metabolite pathway analysis and enrichment analysis, six related pathways were found, such as alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, glutamine and glutamate metabolism, arginine biosynthesis, glutathione metabolism, glycine, serine and threonine metabolism, which can be used as the main metabolic pathway of ARCE acting on the body. Amino acids are the basic substances that constitute the protein and needed by human nutrition, which play a very important role in regulating physiological functions and maintaining life activities. Aspartate is a nonessential amino acid, which is obtained by transamination of glutamic acid to oxaloacetate *in vivo*, and has a protective effect on myocardium (Leng et al., 2014). The increase of glutamic acid and alanine *in vivo* can improve the energy metabolism in body. Therefore, the metabolic pathway of alanine, aspartic acid and glutamic acid may be related to the cardiovascular therapeutic effect of ARC. Arginine can enhance humoral immunity and promote the
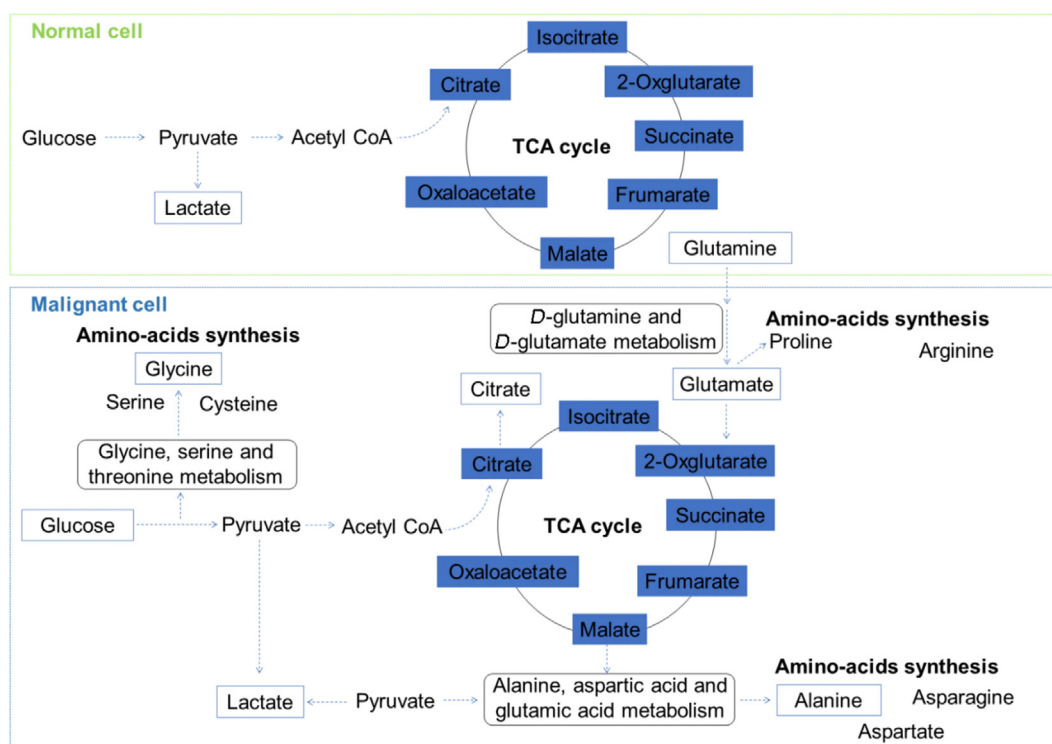

Fig. 9. Correlation analysis chart of metabolites-metabolic pathway-disease.

production of immunoglobulin effectively. At the same time, it can also play an immunomodulatory role by improving the direct or indirect reaction mediated by T lymphocytes (Chen, Wang et al., 2021, Chen, Cheng et al., 2021), while glutamic acid also has an important immunomodulatory role, which is necessary for lymphocyte secretion, proliferation and maintenance of its function. Therefore, arginine biosynthesis, glutamine and glutamic acid metabolism pathway may help ARC to exert its immunomodulatory effect. Glycine, serine and threonine can be transformed mutually. Threonine is mainly metabolized into glycine and acetaldehyde through aldolase, while proper amount of threonine can enhance the immunity of the body. Glycine plays an important role as the key substance of many reactions in the regulation of pain metabolism, mainly through its receptor, chloride ion influx causes postsynaptic potential, reduces the excitability of neurons, inhibits the transmission of pain information, and thus produces analgesic effect (Gundersen et al., 2005). ARC may achieve certain analgesic effect by regulating glycine, serine and threonine metabolism. Glutathione composed of three amino acids can scavenge free radicals and peroxides (Tsutsui et al., 2021; Zhang & Ye, 2020). It can also prevent membrane lipid peroxidation, increase PEG2 and mucosal epithelial defense, and inhibit inflammatory cytokines. Therefore, ARC can play an anti-inflammatory role by regulating glutathione metabolism.

Through network analysis and KEGG pathway analysis, 15 differential metabolites and six metabolic pathways were combined with the target diseases for correlation analysis effectively. The results showed that ARC could realize the prevention and treatment of lung cancer by mediating the central carbon metabolism of cancer. Lung cancer is a primary malignant tumor in the lung, and the malignant transformation of tumor cells needs the specific adaptation of cell metabolism to support its growth and survival. Central carbon metabolism (CCM) is the main source of energy needed by organisms and provides precursors for other metabolism in the body, mainly including glycolytic pathway (EMP), pentose phosphate pathway (PPP) and tricarboxylic acid cycle (TCA) (Wang et al., 2018). Cancer cells can consume a large amount of glucose through central carbon metabolism, keep high glycolysis rate and improve glutamine utilization rate, and synthesize most macromolecules needed to replicate their biomass and genome from the intermediate molecules provided by increasing glycolysis and glutamic acid dissolution (Liu et al., 2021). TCA is a cyclic reaction system composed of a series of enzymatic reactions, which is the most effective way for the body to oxidize sugar or other substances to obtain energy. Glutamine can produce glutamate and citrate as a metabolite of ARC to participate in TCA through glutamine and glutamate metabolism, which affects energy metabolism and substance metabolism of tumor cells. Meanwhile, ARC can affect amino acid synthesis of tumor cells through alanine, aspartic acid and glutamic acid metabolism, and glycine, serine and threonine metabolism. Therefore, it concluded that ARC has certain anti-tumor activity, which can influence the regulation of central carbon metabolism by regulating TCA and amino acid synthesis of malignant tumor cells. The multiple metabolic pathways of ARC *in vivo* enable it to realize different pharmacological activities, so it also has unique advantages of multi-orientation and multi-target in cancer treatment (Yang et al., 2019), which can not only stimulate the immune system of the body, restore the immune function inhibited by anti-tumor drugs, but also relieve the cancer pain of patients.

5. Conclusion

In conclusion, we studied the toxicity of *Aconiti Radix* in this study firstly. The method of steaming can reduce the toxicity of

Aconiti Radix effectively, so as to maximize the efficacy of *Aconiti Radix* on rats. Secondly, we investigated the overall metabolism of ARCE in rats. The metabolic changes induced by ARCE in tissues and serum of rats could be comprehensively and dynamically monitored by using the metabolomics research method combined with ¹H-NMR and multivariate statistics. Fifteen different metabolites such as choline, lactic acid and glutamic acid and six metabolic pathways such as arginine synthesis were screened out. The results showed that ARC may play an anti-inflammatory, analgesic, anti-tumor and immunomodulatory role by influencing metabolic pathways such as arginine synthesis. Therefore, the clinical application of *Aconiti Radix* as a potential anti-tumor drug will provide a new direction for the research and development of new anti-cancer drugs and further accelerate the clinical popularization of traditional Chinese medicine preparations.

Authors' contributions

Wen fang Jin and Jian li Bi designed this study; Wen fang Jin, Sheng Xu and Meng fan Rao participated in the experiment; Jian li Bi and Qi Wang analyzed the data; Yan Yuan and Bao lei Fan revised the manuscript.

Declaration of Competing Interest

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.

Acknowledgements

References

Bi, J. L., Chen, T., Jing, W. F., Qu, Z. Y., Rao, M. F., Cai, X., ... Fan, B. L. (2021). SPE-HPLC determination of the processing process of *Aconiti Radix* dynamic changes and toxicity analysis of 6 aconitine alkaloids. *Chinese Journal of Pharmaceutical Analysis, 41*(8), 1394.

Cao, R. G. (2020). Effect of processing on alkaloids in *Radix Aconiti. Journal of Practical Traditional Chinese Medicine, 36*(12), 1661–1663.

Chen, L. N., Cheng, X. M., Chen, Y., & Wang, C. H. (2021). Research progress on pharmacological action, toxicity and quality control methods of *Radix Aconiti. Chinese Patent Medicine, 43*(03), 722–729.

Chen, X. Y., Wang, X., Zhou, T., & Yang, Y. (2021). Research progress on amino acid metabolism regulation and tumor immunity. *Pharmaceutical Research, 40*(01), 35–39.

Chinese Pharmacopoeia Commission (2020). *Pharmacopoeia of the People's Republic of China (40)*. Beijing: China Medical Science Press.

Crook, A. A., & Powers, R. (2020). Quantitative NMR-based biomedical metabolomics: Current status and applications. *Molecules, 25*(21), 5128.

Gundersen, R. Y., Vaagenes, P., Breivik, T., Fonnum, F., & Opstad, P. K. (2005). Glycine–an important neurotransmitter and cytoprotective agent. *Acta Anaesthesiologica Scandinavica, 49*(8), 1108–1116.

Leng, W. B., Liu, Y. L., Shi, H. F., Li, S., Zhu, H. L., Pi, D. G., ... Gong, J. (2014). Aspartate alleviates liver injury and regulates mRNA expressions of TLR4 and NOD signaling-related genes in weaned pigs after lipopolysaccharide challenge. *The Journal of Nutritional Biochemistry, 25*(6), 592–599.

Li, T. T., Xu, S., Bi, J. L., Huang, S. T., Fan, B. L., & Qian, C. Q. (2020). Metabolomics study of polysaccharide extracts from polygonatum sibiricum in mice based on ¹H-NMR technology. *Journal of the Science of Food and Agriculture, 100*(12), 4627–4635.

Liu, C. X., Li, H., Chu, F. L., Zhou, X., Xie, R., Wei, Q. Q., ... Lü, M. (2021). Long non-coding RNAs: Key regulators involved in metabolic reprogramming in cancer (review). *Oncology Reports, 45*(5), 1–12.

Liu, J. H., Sun, B. G., Chen, Y., Chen, Z. X., & Zhou, H. M. (2020). Metabolic regulation mechanism of Jianpi Jiedu recipe in treating liver cancer model rats. *Journal of Chinese Medicinal Materials, 43*(07), 1663–1668.

Lü, M., Wang, Y. Z., Zhao, D., Zhao, S. J., Li, S. Y., Qin, X. M., & Liu, X. (2020). Anti-depression mechanisms of Xiaoyao Powder based on fecal metabolomics. *Chinese Traditional and Herbal Drugs, 51*(13), 3482–3492.

Ma, F. G., Zhang, Y. P., Cao, G. Q., Xu, J., & Hu, Z. P. (2019). Determination of six alkaloids in 12 batches of *Radix Aconiti* by HPLC. *Journal of Chinese Medicinal Materials, 42*(06), 1339–1342.

Man, Q., Ma, J., Deng, Y., Li, P. J., Yang, Z. J., & Yang, X. J. (2020). Application of multi-omics in toxicity and detoxification of Chinese materia medicine. *Chinese Traditional and Herbal Drugs, 51*(12), 3117–3125.

Nie, C. X., He, P., Hao, Y. Y., Liu, C., Hao, Y., & Hao, X. L. (2019). Effects of different processed products of *Crataegi Fructus* on hyperlipidemia rat model by ^{1}H-NMR metabolomics. *Chinese Traditional and Herbal Drugs, 50*(10), 2362–2370.

Tsutsui, A., Morishita, Y., Furumachi, H., Fujimoto, T., Hirai, R., Fujita, T., & Machinami, T. (2021). Generation of cyclic glutathione via the thiolactonization of glutathione and identification of a new radical scavenging mechanism. *Tetrahedron Letters, 68* 152836.

Wang, X. Y., Li, W. D., & Hua, B. J. (2018). Lung cancer-related genes and central carbon metabolism. *Chinese Archives of Traditional Chinese Medicine, 36*(01), 90–93.

Wishart, D. S. (2019). NMR metabolomics: A look ahead. *Journal of Magnetic Resonance, 306*, 155–161.

Yang, L., Zhu, H. R., Feng, Y., & Li, Q. (2019). Experimental research progress of Chinese medicine in inducing tumor cell autophagy through regulating related signaling pathways. *Academic Journal of Shanghai University of Traditional Chinese Medicine, 33*(02), 92–96.

Ye, X. T., Zhong, L. Y., Zhang, D. Y., Yang, M., & Lan, Z. L. (2020). Research progress on multi-material and multi-process processing of *Aconiti Radix. Chinese Journal of Experimental Traditional Medical Formulae, 26*(18), 202–209.

Zhang, Y., & Ye, S. (2020). Physiological functions and clinical applications of glutathione. *Chemistry of Life, 40*(12), 2226–2235.