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Differential Analysis of Components in Different Parts of Viburnum Cylindricum Based on Untargeted Metabolomics

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ABSTRACT

Objective To analyze the main metabolites and their relative contents in leaves, shoots, fruits and barks of Mahogany by non-targeted metabonomics, so as to provide a scientific basis for improving the medicinal value of Mahogany. Methods High performance liquid chromatography-mass spectrometry (HPLC-MS) was used to collect the data of metabolites in different parts of Mahogany, and then the main metabolites were analyzed by non-targeted metabonomics. The results showed that leaves, twigs, fruits and barks all contained flavonoids, benzene and its substituted derivatives, carboxylic acids and their derivatives, fatty acids, phenylpropanoids and other metabolites, with the highest content of flavonoids in leaves and the richest content of carboxylic acids and their derivatives in fruits. The main metabolic pathways of fruit and bark are amino acid metabolism, flavonoids biosynthesis, flavonoids and flavonols biosynthesis. Conclusion The leaves, twigs, fruits and barks of Mahogany contain a variety of metabolites with medicinal functions, and the highest content of metabolites is found in different parts. Therefore, targeted collection of medicinal parts can be considered in the development and utilization to improve the utilization rate of resources.

KEYWORDS

Water mahogany; Non-targeted metabonomics; Different parts; Metabolite

1. CHINESE LIBRARY CLASSIFICATION NUMBER: DOCUMENT IDENTIFICATION CODE: ARTICLE NUMBER:

Viburnum cylindricum Buch. Ham. ex D. Don is a green shrub or small tree [1] of Viburnum. Its leaves turn white when gently rubbed, so it is also commonly known as Rubbing White Leaves [2]. Commonly used medicine by Dai folk doctors, the leaves have significant effects on dysentery, acute gastroenteritis, stomatitis, urinary tract infection [3], lung cancer, stomach cancer, colon cancer, etc. [4]; The root has the effect of expelling wind and activating collaterals, and the flower can moisten the lung and stop coughing [5].

The material basis of the quality of Chinese medicinal materials is mostly the secondary metabolites of medicinal plants [6]. According to the research, the chemical constituents of water mahogany include triterpenes [7], phenols [8], iridoids [9], flavonoids [10], etc. Chen Xiao zhen et al. conducted research on the leaves of water mahogany and found that the triterpenoids in its leaves include lupineol β- Sitosterol β- Carotene, oleanolic acid, ursolic acid, etc. [7]; Yang Jun et al. studied the chemical constituents of the Dai medicine Shuimahogany, and found that the chemical constituents of flavonoids in the leaves of Shuimahogany include quercetin, amantadine, apigenin, luteolin, rutin, etc. [10]. Wang Li - Xia et al. found two new iridoids through chemical composition analysis of the branches and leaves of water mangrove 7 α- Galloyloxysweroside and 7 β- galloyloxysweroside [9]. Zhu Xiangdong et al. used the stems and leaves of water dried mahogany as research materials to

conduct research, which showed that there were two phenolic compounds [8], namely, cyclindrin A and cyclindrin B, in the stems and leaves. These studies show that there are many kinds of compounds in the water mahogany, but most of the studies focus on the stems and leaves of the water mahogany, and there is no report on the chemical composition of the bark and fruit. In order to enable the development of the medicinal plant water mahogany to reasonably collect an organ according to needs, the main metabolic components in different organs of the water mahogany deserve our further study.

2. MATERIALS AND METHODS

2.1. Experimental Materials and Instruments

2.1.1. Experimental materials

In October 2022, mature and complete leaves (Y), twigs (J) connecting leaves, mature fruits (G), and bark (P) of fresh water mahogany on the west side of Lanba, Simao District, Puer City, Yunnan Province were collected as experimental materials, with 3 replicates for each sample. After cleaning, the experimental materials were immediately put into liquid nitrogen for freezing storage and sent to Beijing Qingke Biotechnology Co., Ltd. Kunming Branch for sample preparation and metabonomics monitoring and analysis.

2.1.2. Drugs and reagents

Table 1. List of experimental reagents

name	CAS	pureness
methyl alcohol	67-56-1	≥99.0%
acetonitrile	75-05-8	≥99.0%
2- chlorophenylalanine	103616-89-3	98.5%
formic acid	64-18-6	LC-MS grade
ammonium formate	540-69-2	≥99.9%

2.1.3. Main instruments

Table 2. List of experimental instruments

instrument	refrigerated centrifuge	Mixer	Ultrasonic cleaner	Tissue grinder	filter membrane	liquid chromatograph	mass spectrometer/spectrograph/spectroscope
model	H1850-R	BE-2600	KQ-100TDV	MB-96	0.22μm PTFE	Vanquish	QE-HF-X

2.2. Experimental Methods

2.2.1. Extraction of Metabolites

References for extraction methods of metabolites from different parts of Mahogany [11-12]

(1) Accurately weigh an appropriate amount of sample into a 2 mL EP tube, add 0.6 mL of 2-chlorophenylalanine (4 ppm) methanol (- 20 ℃) for preparation, and vortex for 30 s;

(2) Add 100 mg glass beads, put them into a tissue grinder, and grind them at 55 Hz for 60 s;

(3) Room temperature ultrasound for 15 min;

(4) Centrifuge at 12000 rpm at 4 ℃ for 10 min and take 300 supernatant μ L over 0.22 μ M membrane filtration, and the filtrate is added to the detection bottle;

(5) Take 20 from each sample to be tested μ L mixed into QC sample;

(6) Carry out LC-MS test with the remaining samples to be tested according to the method in [13-14].

2.2.2. Computer detection

Chromatographic condition: ACQUITY UPLC ® HSS T3 1.8 μ M (2.1 × 150 mm) chromatographic column, the temperature of the autosampler is set to 8 ℃, the flow rate is 0.25 mL/min, the column temperature is 40 ℃, and sample injection is 2 μ L, the mobile phase is positive and negative ion 0.1% formic acid water (C) - 0.1% formic acid acetonitrile (D). The gradient elution procedure is 0~1 min, 2% D; 1~9 min,2%~50% D; 9~12 min, 50%~98% D; 12~13.5 min, 98% D; 13.5~14 min, 98%~2% D; 14~20min, 2% D.

Mass spectrum conditions: the instrument adopts electrospray ion source (ESI), positive and negative ion ionization simultaneous acquisition mode, spray voltage is ± 3.50 kV, sheath gas is 30 arb, and auxiliary gas is 10 arb. The capillary temperature is 325 ℃, the resolution is 60000, the scanning range is 81~1000, and HCD is used for secondary cracking, the collision voltage is 30 eV, and the dynamic exclusion is used to remove unnecessary MS/MS information.

2.2.3. Data analysis

After the original data is converted into the mzXML format by ProteoWizard software, the R package (XCMS as the core) prepared independently is used for peak recognition, peak extraction, peak alignment and integration. The number of positive ion metabolites identified is 2930 (left in Figure 1), and the number of negative ion metabolites identified is 2415 (right in Figure 1). Then match with the secondary mass spectrometry database for substance annotation, and the Cutoff value scored by the algorithm is set to 0.3. Next, multivariate statistical analysis of metabolites was carried out, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), etc., to reveal the difference of metabolites in different groups. VIP value represents the contribution rate of the difference of metabolites in different groups; The Fold Change (FC) is the ratio of the mean repeated quantitative values of all organisms in the comparison group for each metabolite; In combination with the P value of T-test, we searched for differentially expressed metabolites, set the threshold value as VIP>1.0, difference multiple FC>1.2 or FC<0.833 and P value<0.05, and screened 2560 metabolites with significant differences between fruits and bark. Hierarchical clustering (HCA) and metabolite correlation analysis were used to reveal the relationship between samples and between metabolites and metabolites. Finally, the biological significance of metabolites was explained through functional analysis such as metabolic pathways.

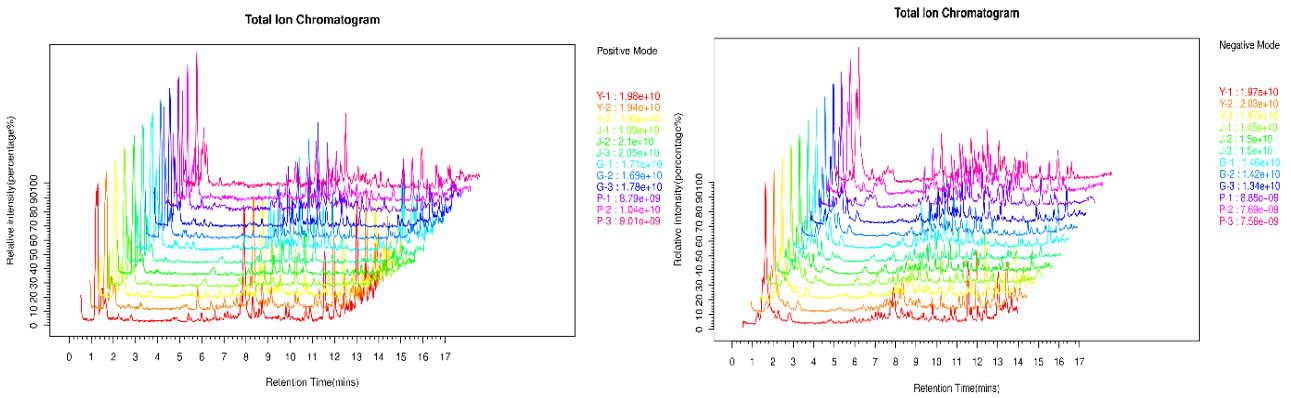

Figure 1. chromatogram of base peaks of samples from different parts of mahogany in positive and negative ion mode (left: positive ion mode, right: negative ion mode)

3. RESULTS

3.1. Evaluation of Mass Spectrum Data of Metabolites in Different Parts of Mahogany

In order to evaluate the stability and validity of the mass spectrometry data of water mahogany metabolites, the correlation analysis between QC samples was carried out according to the peak area values. The results showed that the positive and negative ion mode correlations of QC samples were greater than 0.85 (Figure 2), indicating that the experiment had good repeatability. PCA results showed that the contribution rate of the first principal component was 80.91%, and the contribution rate of the second principal component was 10.53%. The same samples in the group were gathered together, indicating that the variation was small and the data was stable and reliable; The obvious separation of samples from different tissue parts indicates that there are differences in metabolites among leaves (Y), twigs (J), fruits (G), and bark (P) (Figure 3).

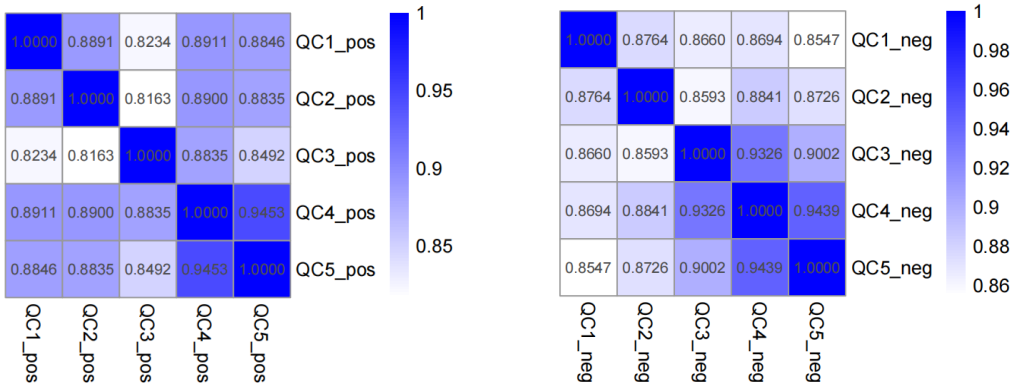

Figure 2. Correlation analysis of QC samples (left: positive ion mode, right: negative ion mode)

Note: The closer R2 is to 1, the higher the correlation of QC samples is, and the better the stability of the whole testing process is.

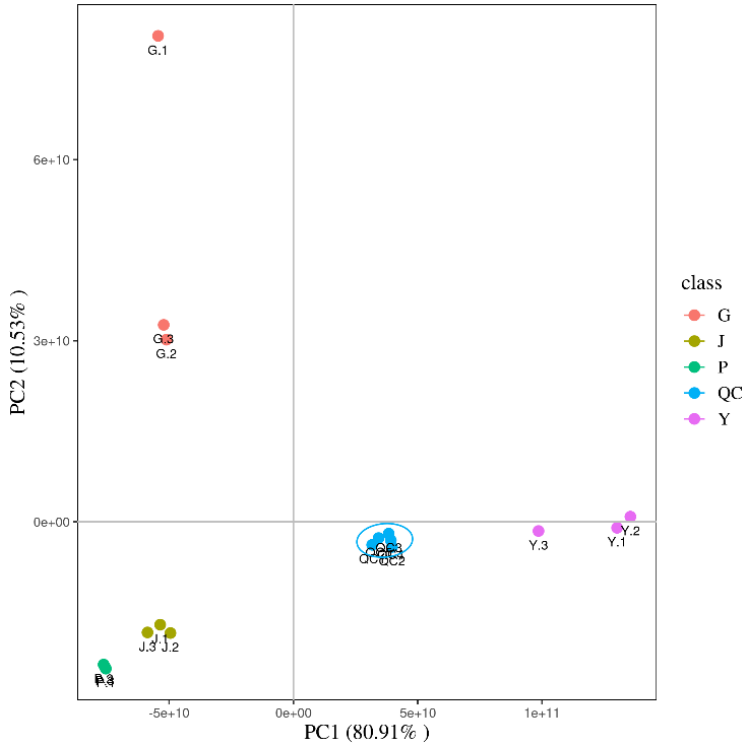

Figure 3. PCA Analysis of Total Samples (Positive Ion Mode)

3.2. Metabonomics Analysis Results of Metabolites in Different Parts of Mahogany

By matching the fragment ion, collision energy and other information of each compound in the mzCloud database, the metabolites in the biological system were identified. Under the positive and negative ion mode, a total of 2560 differential metabolites were obtained in water mahogany, with 1134 significantly up-regulated and 1426 significantly down-regulated. It can be seen that there are very rich differential metabolites in water mahogany, it is difficult to conduct complete analysis with traditional analysis methods. Further annotation of KEGG function was carried out on the differential metabolites of different tissue structure species of water mahogany. It was found that 642 metabolites were involved in the metabolism of Global and overview maps under the metabolism mode under the positive ion mode. This pathway was the most involved in all metabolic pathways, so these metabolites were analyzed in the next step (Figure 4).

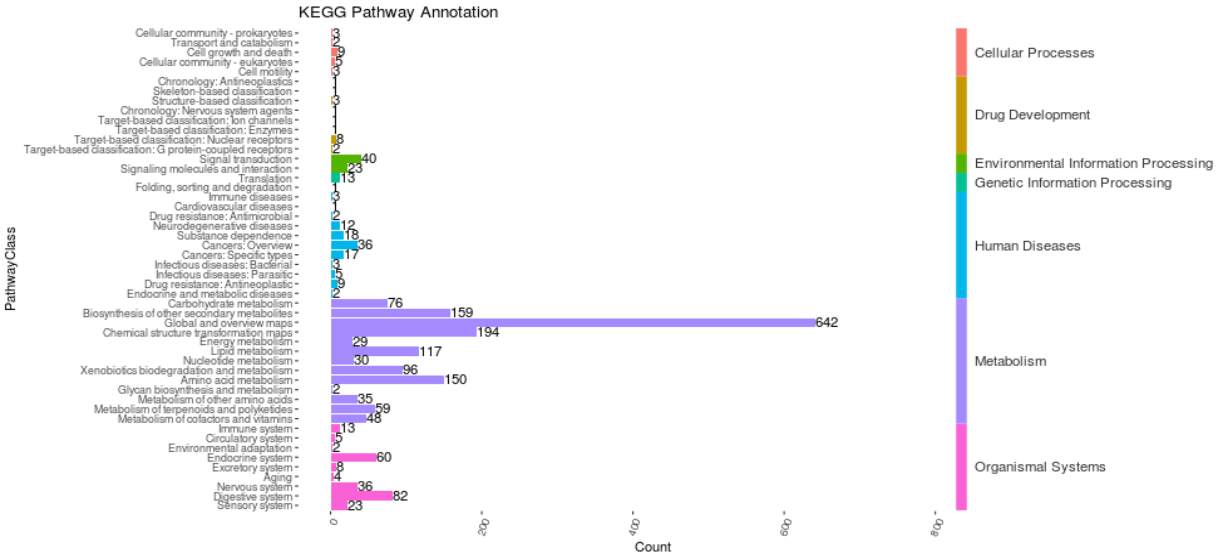

Figure 4. KEGG Function Notes

Note: The abscissa represents the number of metabolites, and the ordinate represents the KEGG entry noted.

3.3. Contents of Main Plant Metabolites in Different Parts of Mahogany

The metabolites in 642 leaves, fruits, twigs and barks were classified and found that there were 68 benzene and its substituted derivatives, 60 carboxylic acids and their derivatives, 53 fatty acids, 39 flavonoids, 32 organic oxygen compounds, 28 pregnenolone esters, 27 phenols, 25 sterols and steroid derivatives, 19 terpenoids, 18 phenylpropanoids and 14 endogenous metabolites. Then, the content analysis of substances with more than 14 metabolites shows that benzene and its substituted derivatives are abundant, but the content is not the highest. Among these metabolites, the content of flavonoids is the highest, followed by carboxylic acid and its derivatives, benzene and its substituted derivatives, fatty acids, etc.

In four different tissue parts, the content of flavonoids metabolites in leaves is higher than that in other structures, followed by the content in fruits. The content of carboxylic acid and its derivatives in fruits is the highest, followed by shoots and leaves. The content of benzene and its substituted derivatives is relatively high in bark, followed by fruit; The content of fatty acids in bark is higher than that in other three parts. The contents of steroids and sterol derivatives in fruits are high. This shows that there are differences in the contents of metabolites in different tissue structures.

The full name of HMDB is Human Metabolome Database, which contains detailed information about small molecule metabolites found in human body and their biological effects, physiological concentrations, disease associations, chemical reactions, metabolic pathways, etc. The annotation

results of HMDB showed that 268 metabolites were involved in the synthesis of organic heterocyclic compounds, 259 metabolites were involved in the synthesis of lipids and lipid molecules, 229 metabolites were involved in the synthesis of benzene ring compounds, 215 metabolites were involved in the synthesis of organic acids and their derivatives, and 170 metabolites were involved in the synthesis of phenylpropane and polyketones (Figure 5).

In order to understand the metabolic activities of water mangrove tissues, the differential metabolites in fruits and bark were mapped to the bubble diagram of the KEGG pathway enriched. As shown in the figure (Figure 6), more than 65 differential metabolites are involved in amino acid metabolism; More than 25 differential metabolites are involved in the biosynthesis of flavonoids, and 20 differential metabolites are involved in the biosynthesis of flavonoids and flavonols, which indicates that; Amino acid metabolism, flavonoid biosynthesis, flavonoid and flavonol biosynthesis are significantly enriched compared with other pathways. These three metabolic pathways are important metabolic pathways in the fruits and bark of water mahogany, and participate in the synthesis of a variety of metabolic substances. In addition, differential metabolites are also involved in steroid hormone biosynthesis, pyrimidine and purine metabolism, protein digestion and absorption, phenylpropane biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, arachidonic acid metabolism, etc. (Figure 6).

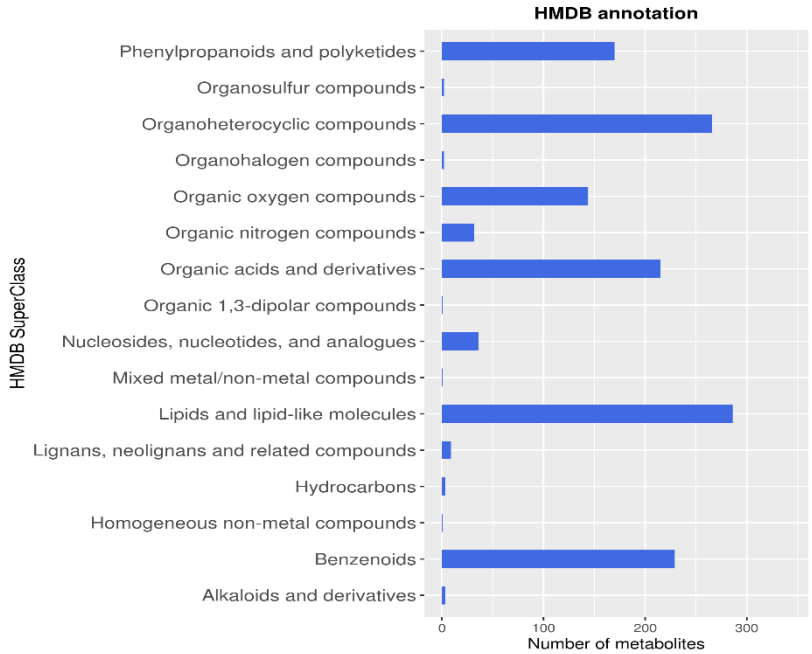

Figure 5. HMDB classification notes (including all positive and negative ion metabolites)

Note: The abscissa represents the number of metabolites, and the ordinate represents the annotated HMDB entry.

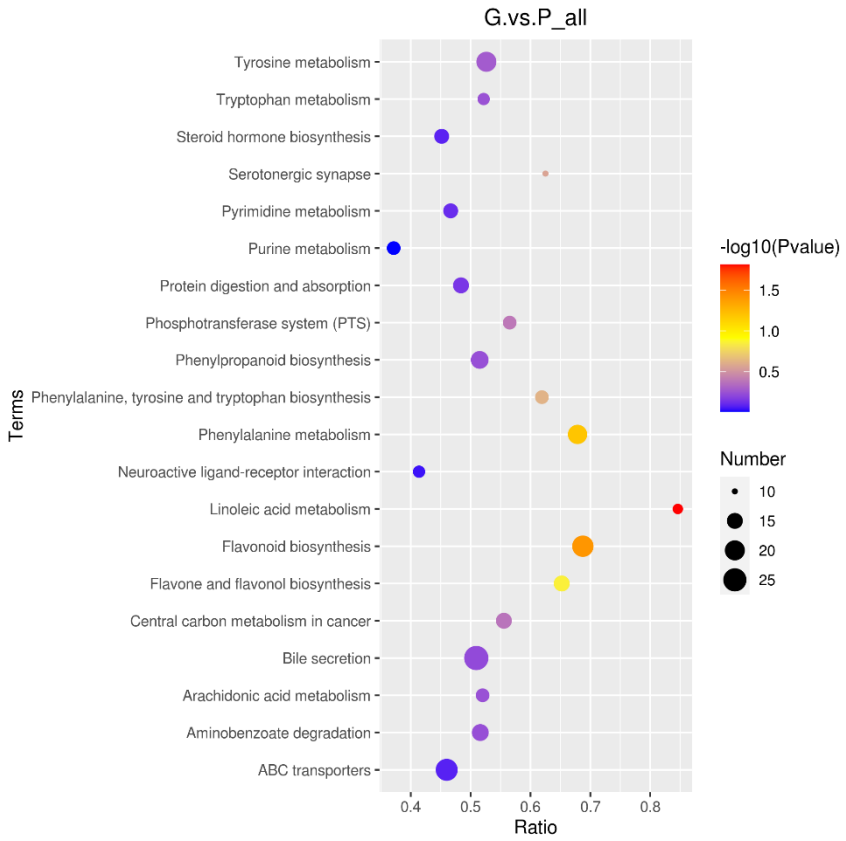

Figure 6. KEGG Enrichment Bubble Diagram (Positive and Negative Ion Metabolites)

4. DISCUSSION

Metabonomics, as a scientific research method widely used in biomedical field, can analyze many metabolites qualitatively and quantitatively at the same time [15-16]. Non-targeted metabonomics is a highly sensitive and wide-ranging Qualcomm detection technology, which is helpful to the development and utilization of medicinal plant resources to some extent [17]. In recent years, non-targeted metabonomics has made good progress in the analysis of metabolites in different tissue structures, such as the difference analysis of metabolites in different organs of Meconopsis spinosa [18], the characteristics analysis of metabolites in different medicinal parts of Xanthium sibiricum [19] and the difference analysis of metabolites in different parts of Solanum nigrum [20]. As a commonly used medicinal plant in Dai medicine, the metabolites and contents of different medicinal parts are closely related to their medicinal value.

Mahogany has a long history of folk medicine, but the medicinal parts of Mahogany have been concentrated in branches and leaves, while the medicinal value of other parts is recorded less. In this study, leaves, shoots, fruits and barks of Mahogany were taken as the research objects, and the differences of metabolites in different parts were analyzed by non-targeted metabonomics. The results showed that leaves, twigs, fruits and barks all contained benzene and its substituted derivatives, carboxylic acids and their derivatives, fatty acids, flavonoids, organic oxygen compounds, pregnenolone esters, phenols, sterols and steroid derivatives, terpenoids, phenylpropanoids and endogenous metabolites. The detection results of flavonoids, phenols and terpenoids are similar to those of previous studies.

Plant flavonoids have a variety of pharmacological activities, such as anti-inflammatory, antioxidant and anti-tumor, and have good preventive and therapeutic effects on cardiovascular diseases, neurological diseases, liver diseases, kidney diseases and other diseases [21]. Water mahogany often takes its branches and leaves as its medicinal parts, which may be related to its high content of

flavonoids. In addition, the content of flavonoids in fruits is relatively high, so it can be considered to collect fruits as medicinal parts. KEGG enrichment analysis and HMDB classification annotation showed that several differential metabolites were involved in flavonoid biosynthesis.

Studies have shown that plant fatty acids have a variety of pharmacological activities, such as palmitic acid and oleic acid, which can reduce blood lipids, anti atherosclerosis, anti platelet aggregation and anti thrombosis [22]. The content of fatty acids in the bark of water mahogany is relatively high, including jasmonic acid, erucic acid A- Linolenic acid, docosahexaenoic acid and other substances, water mahogany bark is the main part to obtain fatty acids.

Phenylpropanoids include simple phenylpropanoids, lignans, coumarins and other subtypes [23]. They have various structural types and wide biological activities, mainly including anti-tumor [24], antibacterial and antioxidant [25]. The content of phenylpropanoids metabolites in Mahogany is relatively high, especially in bark and fruit, which can be used as the main structure for the development of antibacterial and antioxidant drugs.

The content of carboxylic acid and its derivatives is the highest in fruits, and there are also a lot of this substance in shoots and leaves. Most of these carboxylic acids and their derivatives are amino acids, indicating that the fruit contains a large number of amino acids. Li Yun also confirmed that there are a large number of amino acids [27] in the research on the nutritional components of water mahogany.

5. CONCLUSION

The leaves, twigs, fruits and barks of Mahogany contain a variety of metabolites with medicinal functions, and the highest content of metabolites is found in different parts. When extracting and utilizing the active components in them, we can consider collecting the medicinal parts targeted to improve the utilization rate of resources.

ACKNOWLEDGMENTS

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