H-NMR-based metabolomics study on the effects of raw and processed radix *Wikstroemia indica* on endogenous metabolites in rat plasma

Xue-Li Song, Guo Feng, Lai-Lai Li, Wei Li, Zeng-Guang Wu, Wen-Jing Wang, Hong-Mei Su, Chen-Chen Ren, Guang-Lin Zhu, Zheng-Yan He and Ju Zhang

Department of Chinese Materia Medica, Guizhou University of Traditional Chinese Medicine, Guiyang, Guizhou, P.R. China

**Abstract:** Based on metabolomics, to study the mechanism of Radix *Wikstroemia indica* (RWI) “Sweat soaking method” processing detoxification. The raw drug group and processed products was given raw RWI and processed RWI respectively by gavage. The control group was given the same amount of 1% sodium carboxy methyl cellulose distilled water by gavage. After 7 days of continuous gavage, blood samples were collected. The blood samples of rats in each group were analyzed by 1H-NMR technology to explore the changes of endogenous metabolism and the possible metabolic pathways to rats before and after processing. Compared with the control group, the raw RWI could significantly reduce 16 metabolites and increase 10 metabolites. The processed RWI can increase the levels of most metabolites that decrease to the raw RWI, such as 13 metabolites such as alanine, L-glutamine, L-valine, L-serine, betaine and glutamic acid; At the same time, the metabolites that increased in the level of crude products were down-regulated, such as asparagine, lactate acid, 2-hydroxyisobutyric acid, sucrose, glucose and D-glucose. Compared with raw products, RWI treated with “Sweat soaking method” can reversely regulate or reduce amino acid, choline metabolism, energy and carbohydrate metabolism, thereby reducing hepatotoxicity and nephrotoxicity.

**Keywords:** H-NMR, metabolomics, radix *Wikstroemia indica* (RWI), raw RWI, processed RWI, endogenous metabolism, “sweat soaking method”.

**INTRODUCTION**

The Miao medicine Radix *Wikstroemia indica* is the root and root bark of *Wikstroemia indica* (L.) C. A. Mey. (RWI), which belongs to the Thymelaeaceae family. It is first recorded in the “Lingnan Collection of Medicines” and is one of the commonly used Miao medicines in Miao areas (Su et al., 2023). Modern studies have shown that RWI contains lignin, flavonoids, coumarins, steroids, volatile oils, acids, esters, alcohols, terpenoids and other compounds (Wang et al., 2018; Yin et al., 2018; Wang et al., 2019; Jegal et al., 2020; Li et al., 2021; Tang et al., 2021). It has pharmacological activities such as antibacterial (Chen et al., 2016), anti-inflammatory (Lee et al., 2020; Zheng et al., 2020), antiviral (Zhou, et al., 2022), cytotoxic (Shao et al., 2016), antioxidant (Zhou et al., 2020) and inhibiting the growth of tumor cells (Jiang, et al., 2014). It has been clinically applied to acute tonsillitis, acute pharyngitis, acute tracheobronchitis, pneumonia, mastitis and so on (Zhang et al., 2014). However, its hepatorenal toxicity limits its clinical application to some extent (Feng et al., 2017; Feng et al., 2018). In order to reduce the toxicity of the RWI, our research group carried out in-depth research on its processing and attenuation methods and found that the “Sweat soaking method” can effectively reduce the toxicity of the RWI, but the mechanism of its attenuation is not clear. Therefore, it is necessary to clarify the mechanism of “Sweat soaking method” in processing the detoxification of the RWI. As one of the high-throughput technologies, metabolomics aims to reveal various metabolic characteristics of disturbance on biological systems by analyzing small molecules (<1kDa) in biological samples (Bhatia A et al., 2019; Muthubharathi BC et al., 2021). Metabolome is located in the final “genomics” level of biological systems. Metabolites has clear functions and can provide the most “functional” information on genomics technology. As one of the most commonly used methods of metabolomics research, 1H-NMR-based metabolomics is considered as an attractive tool for its simple sample preparation, high reproducibility and rapid analysis.

In this study, metabolomics based on 600 MHz high resolution 1H nuclear magnetic resonance (NMR) was used to examine the plasma metabolic profiles of rats after gastric administration of raw and processed RWI. This study aims to clarify the endogenous metabolic changes induced by RWI and explore the possible metabolic pathways, so as to provide important information about understanding the potential mechanism of raw RWI toxicity and reducing the potential mechanism of processed RWI toxicity.

**MATERIALS AND METHODS**

**Instrument**

SK8210HP Ultrasonic instrument (Shanghai Kedao Ultrasound Instrument Co., Ltd.); Unity-Inova 600 Superconducting Nuclear Magnetic Resonance Spectrometer (Varian, Inc. USA); MTN-2800D Nitrogen blowing instrument (Tianjin Automatic Science Instrument...
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Co., Ltd. Tianjin, China); Targin VX-02 Multi-tube Vortex oscillator (Tadjin Technology Co., Ltd. Beijing, China); H2050R Centrifuge (Hunan Xiangyi Laboratory Instrument Development Co., Ltd. Hunan, China); AUW120D Electronic analytical balance (Shimadzu Philippines Factory, Kyoto, Japan); 202-3AB drying oven (TaiSite Instrument Co., Ltd. Tianjin, China); BJ1 stainless steel metabolic cage (Changsha Tianqin Biotechnology Co., Ltd. Hunan, China).

**Drugs and reagents**

The medicinal materials of Radix Wikstroemia Indica (RWI) were purchased from Yulin Yinfeng International Chinese Medicine Harbor (Batch No.: 220160115); Raw ethanol extract RWI and processed ethanol extract RWI (Guizhou University of Traditional Chinese Medicine Pharmaceutical Laboratory, China, Batch No.: YC20160410, PZ20160420); Deuterated deuterium oxide (D2O, 99.9% D) Cambridge Isotope Laboratory, USA); 2, 2, 3, 3, -d4 -3 (trimethylsilyl) propionate sodium salt (TSP) (Merck company, Germany); Pentobarbital sodium (Sigma Company, USA, Batch No. 922LO310). Other reagents are analytically pure.

**Preparation of synthetic perspiration**

The three solutes (table 1) were dissolved in water, whose pH was adjusted to 5.5 by NaOH at a concentration of 0.05M.

**Table 1:** The formula of synthetic perspiration

<table>
<thead>
<tr>
<th>Formula</th>
<th>Concentration(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Histidine HCL</td>
<td>0.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
</tr>
<tr>
<td>NaH2PO4 2H2O</td>
<td>2.2</td>
</tr>
</tbody>
</table>

**Preparation of the processed RWI**

Coarse powder of RWI was sprayed synthetic perspiration (RWI: synthetic perspiration=100:30) and dried in the oven at 37.0±0.5°C for 24h. These operations were repeated for 14 days.

**Preparation of extract of raw and processed RWI**

Preparation of raw RWI and processed RWI were performed according to the method previously described (Feng G et al., 2018).

**Animals and administration**

A total of 18 male Sprague-Dawley rats (200±20) g with specific pathogen freees (SPF) were purchased from the Changsha Tianqin Biotechnology Co., Ltd (Hunan, China, animal certificate number: 43000200002163). All of the animals were to feed freely on a 12h light/dark cycle and were reared in a steady environment (lights on: 07:00 to 19:00, temperature: 25.0±1.0°C and relative humidity: 50.0±10.0%) with free access to food and water. The animal experiments were reviewed and approved by the Ethics Review Committee for Experimental Animals of Guizhou University of Traditional Chinese Medicine and met the 22 relevant requirements for animal welfare. (Ethical review report number: 20210089)

After acclimatization, all rats were randomly divided into three groups (control group, raw RWI group, processed RWI group (n=6)). Two experimental groups treated with RWI (0.3175g/kg/d; raw and processed respectively) and one control group received equivalent volume of 1.0% CMC-Na. Every 1 days for 7 days. During administration, food and water are given normally.

**Sample collection**

At the end of the experiment, all rats were anesthetized (pentobarbital sodium, 0.2mL/100g) after 12h of fasting. Before the rats were sacrificed, blood samples were drawn in blood collection tube (Anticoagulant) from the abdominal aorta and placed at room temperature for 30 minutes. After centrifugation at 4500rpm for 15 min, plasma was collected and was kept at -20°C until further analysis.

**Sample Preparation and 1H-NMR Spectra Acquisition**

For NMR analysis, plasma samples were removed from -20°C storages and thawed at room temperature, 350μL of plasma samples followed by centrifugation (13,000 rpm, 10 min, 4°C). The supernatants (300μL) were mixed with 100μL 2, 2, 3, 3, -d4 -3 (trimethylsilyl) propionate sodium salt (TSP, 1mg/mL) and 200μL D2O, A total of 600μL of the supernatant, was then transferred into a 5 mm high quality NMR tube.

Spectra were acquired at 27°C on a Varian Unity INOVA 600 MHz spectrometer (Varian Inc., USA). The 1H NMR spectra of plasma samples were recorded with the relaxation edited Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence and the LED diffusion edit pulse sequence, using a spectral width of 8000Hz, 64k data points, 64 scans. Water peak inhibition by pre-saturation. The spectra were automatically Fourier transformed. CPMG was calibrated with the left peak of bimodal lactic acid with a chemical displacement of δ1.33 and LED was calibrated with spectral center.

**STATISTICAL ANALYSIS**

Non zero filled spectra were manually phased, the baseline was corrected, calibrated to TSP at 0.00ppm and using the profiler module. The spectral regions of δ 0.4-4.4ppm for CPMG and δ 0.1-6.0ppm for LED were segmented (0.04ppm). Residual water (δ 4.6-5.0) was excluded from the analysis. Remaining bins were then normalized to sum of the spectral integrals, extracted with Microsoft Excel. The normalized data set was imported into Metabo Analyst 3.0 for multivariate statistical analysis. To generate a group clustering overview and to search for potential outliers, PCA was carried out on the 1H-NMR datasets. The metabolites that altered significantly on 1H-NMR datasets.
driven group clustering were explored by the PLS-DA. The reliability of the PCA and PLS-DA model was verified by Student’s t-test and the difference variables were screened.

Pathway analysis
For the metabolites that significantly changed into experimental groups, pathway analysis was performed using KEGG database. Here, those pathways with the impact value >1.0 and \( p<0.05 \) were considered as the most relevant pathways involved in the raw and processed RWI groups.

RESULTS

1H NMR spectra of plasma sample
Representative \(^1\)H NMR spectra from plasma of control, raw and processed RWI were shown in fig. 1, with metabolites indicated based on their chemical shifts. Assignment to metabolites was achieved using Q1 Data (Massbank database, Human metabolome database, Metlin database, Kegg compound database, Pubchem compound database, Lipidmaps database). The 1D-CPMG \(^1\)H NMR spectra of plasma showed signals mainly from amino acid, organic acid, choline, betaine and guanosine energy metabolites. Major metabolites were labeled with the spectra. A number of perturbations in endogenous metabolites were observed in the \(^1\)H NMR spectra of plasma from raw and processed RWI. Totally, 26 metabolites of plasma in raw RWI (table 2) and 23 metabolites in processed RWI (table 3) were identified as listed along with their chemical shift and trend. Most visual inspection of the spectra suggested that prominent changes in the raw RWI group compared with the control group were the increases in asparagine, histidine, 2-hydroxyisobutyric acid, ornithine, leucine, lactic acid, glucose, D-glucose, sucrose, Choline and the decreases in 4-aminobutyric acid, succinate, alanine, glutamate, L-proline, beta-alanine, L-serine, L-valine, phenylalanine, L-glutamidc, citric acid, betaine, etc. Meanwhile, compared with the control group, the type, concentration and relative proportion of endogenous metabolites in plasma of processed RWI group changed significantly. Contrarily, most of the rising metabolites in raw RWI were down-regulated in processed RWI, such as D-glucose, lactic acid, glucose and metabolites, with reduced in raw RWI up regulated in processed RWI. Such as citric acid, L-valine, L-serine, L-proline, phenylalanine, beta-alanine, asparagine, etc. This phenomenon indicated that raw RWI was processed with auxiliary materials or heating process could bring about significantly quantitative and/or qualitative changes in the chemical ingredients, which might result in alleviating the toxicity of the RWI and enhancing pharmacological actions. To establish a global overview of the discrimination of metabolic patterns of control and RWI groups, multivariate data analysis of all NMR spectra was subsequently performed.

Data analysis of NMR data
To visualize the general clustering trends between control and RWI groups, PCA and PLS-DA were applied to the metabolic profiling of plasma obtained from the three groups. In PCA scores plots, a clustering trend was observed between control and processed RWI (fig. 3A) but it was not obvious between control and raw RWI (fig. 2A). This indicates a substantial perturbation of the rats metabolome after seven days’ treated with raw and processed RWI. Because the clustering trend of raw products and processed products is different, it shows that the chemical composition of RWI have changed after the processing of “sweat soaking method”. As shown by the PLS-DA scores plot, not only the control group and processed group could be separated from distinct clusters (fig. 3B) but also control group and raw group could be (fig. 2B). The normal rats were located in the negative PCI region, while treated with RWI (raw and processed respectively) rats were clustered distinctly in the positive PCI region. Moreover, the plots of the raw RWI and processed RWI groups were also different, which once again indicated that the chemical composition of the RWI were changed after “sweat soaking method” processing.

Each independent variable has a key parameter derived from the PLS-DA mode, called the variable importance in the project (VIP) value, which, when high, holds greater relevance is in classification. Thus, each peak’s VIP value was determined to discern its role in the classification. The results are shown in figure (fig. 2C, fig. 3C). The identified metabolites were further quantified in all the three groups using univariate statistical method. Finally, 26 metabolites of plasma in raw RWI and 23 metabolites in processed RWI were identified on the basis of Student’s t-test (\( P<0.05 \)) and VIP threshold (VIP >1).

Pathway analysis
A metabolic pathway map was established according to all the identified plasma metabolites and the correlations were computed by referring to the Kyoto Encyclopedia of Genes and Genomes (KEGG), a web-based free database resource (Kanehisa M et al., 2023). The result showed 31 metabolic pathways were disturbed in raw RWI rats (table 4) and 21 metabolic pathways were disturbed in processed RWI rats (table 5). From the metabolic pathway enrichment analysis diagram, for raw RWI rats, In totally, 20 metabolic pathways (Impact value >1.0 and \( P<0.05 \)) that are closely related to the identified plasma metabolites were tabulated in the schematic diagram with the aid of the KEGG online database (fig 4). Out of the 20 metabolic pathways, the main metabolic pathways with great influence including amino acids, choline metabolism, energy and carbohydrate metabolism pathways. Besides, 15 metabolic pathways were the most influenced metabolic pathways (Impact value >1.0 and \( P<0.05 \)) associated with processed RWI (fig 5). The general map of metabolic pathway and the main metabolic pathway are similar to the raw RWI group, but the effect on the metabolic pathway is not the same.
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**Table 2:** Summary of significantly changed metabolites in rats related to raw RWI (raw RWI group vs. control group).

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>( \log_{10}(FC) )</th>
<th>( p )</th>
<th>-LOG_{10}(p)</th>
<th>Metabolites</th>
<th>Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.89</td>
<td>13.37</td>
<td>0.0352</td>
<td>1.45</td>
<td>4-Aminobutyrate</td>
<td>↓</td>
</tr>
<tr>
<td>7.97</td>
<td>11.24</td>
<td>0.0076</td>
<td>2.12</td>
<td>Guanosine</td>
<td>↓</td>
</tr>
<tr>
<td>2.38</td>
<td>10.60</td>
<td>0.0879</td>
<td>1.06</td>
<td>Alanine</td>
<td>↓</td>
</tr>
<tr>
<td>8.46</td>
<td>3.64</td>
<td>0.0280</td>
<td>1.55</td>
<td>Formate</td>
<td>↓</td>
</tr>
<tr>
<td>1.90</td>
<td>2.22</td>
<td>0.0916</td>
<td>1.04</td>
<td>Acetate</td>
<td>↓</td>
</tr>
<tr>
<td>2.12</td>
<td>2.12</td>
<td>0.0503</td>
<td>1.30</td>
<td>Glutamic acid</td>
<td>↓</td>
</tr>
<tr>
<td>0.97</td>
<td>1.15</td>
<td>0.0836</td>
<td>1.08</td>
<td>Phenylalanine</td>
<td>↓</td>
</tr>
<tr>
<td>2.65</td>
<td>1.03</td>
<td>0.0366</td>
<td>1.44</td>
<td>L-glutamine</td>
<td>↓</td>
</tr>
<tr>
<td>3.89</td>
<td>-1.33</td>
<td>0.0837</td>
<td>1.08</td>
<td>Citric acid</td>
<td>↓</td>
</tr>
<tr>
<td>3.19</td>
<td>-1.12</td>
<td>0.0089</td>
<td>2.05</td>
<td>Choline</td>
<td>↑</td>
</tr>
<tr>
<td>7.08</td>
<td>-1.15</td>
<td>0.0665</td>
<td>1.18</td>
<td>Histidine</td>
<td>↑</td>
</tr>
<tr>
<td>1.34</td>
<td>-1.22</td>
<td>0.0293</td>
<td>1.53</td>
<td>2-Hydroxyisobutyrate</td>
<td>↑</td>
</tr>
<tr>
<td>4.14</td>
<td>12.19</td>
<td>0.0045</td>
<td>2.34</td>
<td>Lactate</td>
<td>↑</td>
</tr>
<tr>
<td>4.18</td>
<td>5.57</td>
<td>0.0947</td>
<td>1.02</td>
<td>Sucrose</td>
<td>↑</td>
</tr>
<tr>
<td>1.83</td>
<td>-1.98</td>
<td>0.0927</td>
<td>1.03</td>
<td>Ornithine</td>
<td>↑</td>
</tr>
<tr>
<td>3.91</td>
<td>-2.32</td>
<td>0.0049</td>
<td>2.31</td>
<td>D-glucose</td>
<td>↑</td>
</tr>
<tr>
<td>0.95</td>
<td>-2.77</td>
<td>0.0658</td>
<td>1.18</td>
<td>Leucine</td>
<td>↑</td>
</tr>
<tr>
<td>3.20</td>
<td>-3.08</td>
<td>0.0773</td>
<td>1.11</td>
<td>Glucose</td>
<td>↑</td>
</tr>
</tbody>
</table>

**Table 3:** Summary of significantly changed metabolites in rats related to processed RWI (processed RWI group vs. control group).

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>( p ).value</th>
<th>FDR</th>
<th>Metabolites</th>
<th>Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.47</td>
<td>0.0000</td>
<td>0.0000</td>
<td>Alanine</td>
<td>↑</td>
</tr>
<tr>
<td>7.07</td>
<td>0.0000</td>
<td>0.0000</td>
<td>Histidine</td>
<td>↑</td>
</tr>
<tr>
<td>2.16</td>
<td>0.0000</td>
<td>0.0000</td>
<td>L-glutamine</td>
<td>↑</td>
</tr>
<tr>
<td>0.97</td>
<td>0.0000</td>
<td>0.0000</td>
<td>L-Valine</td>
<td>↑</td>
</tr>
<tr>
<td>3.86</td>
<td>0.0000</td>
<td>0.0000</td>
<td>L-Serine</td>
<td>↑</td>
</tr>
<tr>
<td>3.88</td>
<td>0.0000</td>
<td>0.0000</td>
<td>Betaine</td>
<td>↑</td>
</tr>
<tr>
<td>2.13</td>
<td>0.0000</td>
<td>0.0000</td>
<td>Glutamic acid</td>
<td>↑</td>
</tr>
<tr>
<td>1.89</td>
<td>0.0000</td>
<td>0.0001</td>
<td>4-Aminobutyrate</td>
<td>↑</td>
</tr>
<tr>
<td>2.00</td>
<td>0.0000</td>
<td>0.0001</td>
<td>L-proline</td>
<td>↑</td>
</tr>
<tr>
<td>0.95</td>
<td>0.0001</td>
<td>0.0002</td>
<td>Leucine</td>
<td>↑</td>
</tr>
<tr>
<td>7.35</td>
<td>0.0002</td>
<td>0.0006</td>
<td>Phenyllalanine</td>
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</tr>
<tr>
<td>8.48</td>
<td>0.0013</td>
<td>0.0029</td>
<td>Formate</td>
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<tr>
<td>2.22</td>
<td>0.0046</td>
<td>0.0082</td>
<td>Acetate</td>
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</tr>
<tr>
<td>2.37</td>
<td>0.0060</td>
<td>0.0104</td>
<td>Succinic acid</td>
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</tr>
<tr>
<td>2.64</td>
<td>0.0109</td>
<td>0.0175</td>
<td>Citric acid</td>
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</tr>
<tr>
<td>7.95</td>
<td>0.0425</td>
<td>0.0583</td>
<td>Guanosine</td>
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</tr>
<tr>
<td>2.55</td>
<td>0.0018</td>
<td>0.0037</td>
<td>Beta-Alanine</td>
<td>↓</td>
</tr>
<tr>
<td>2.94</td>
<td>0.0035</td>
<td>0.0064</td>
<td>Asparagine</td>
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</tr>
<tr>
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<td>0.0000</td>
<td>0.0000</td>
<td>Lactate</td>
<td>↓</td>
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<tr>
<td>1.33</td>
<td>0.0000</td>
<td>0.0000</td>
<td>2-Hydroxyisobutyrate</td>
<td>↓</td>
</tr>
<tr>
<td>4.19</td>
<td>0.0089</td>
<td>0.0149</td>
<td>Sucrose</td>
<td>↓</td>
</tr>
<tr>
<td>3.21</td>
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<td>0.0000</td>
<td>Glucose</td>
<td>↓</td>
</tr>
<tr>
<td>3.93</td>
<td>0.0000</td>
<td>0.0000</td>
<td>D-glucose</td>
<td>↓</td>
</tr>
</tbody>
</table>
### Table 4: Possible metabolic pathways of differential metabolites in raw RWI

<table>
<thead>
<tr>
<th>serial number</th>
<th>Pathway</th>
<th>Total</th>
<th>Hits</th>
<th>Raw p</th>
<th>FDR</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aminoacyl-tRNA biosynthesis</td>
<td>67</td>
<td>10</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.1379</td>
</tr>
<tr>
<td>2</td>
<td>Alanine, aspartate and glutamate metabolism</td>
<td>24</td>
<td>6</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.5232</td>
</tr>
<tr>
<td>3</td>
<td>Arginine and proline metabolism</td>
<td>44</td>
<td>5</td>
<td>0.0005</td>
<td>0.0108</td>
<td>0.2968</td>
</tr>
<tr>
<td>4</td>
<td>Glycine, serine and threonine metabolism</td>
<td>32</td>
<td>3</td>
<td>0.0140</td>
<td>0.1263</td>
<td>0.2428</td>
</tr>
<tr>
<td>5</td>
<td>Butanoate metabolism</td>
<td>20</td>
<td>3</td>
<td>0.0037</td>
<td>0.0495</td>
<td>0.0290</td>
</tr>
<tr>
<td>6</td>
<td>Nitrogen metabolism</td>
<td>9</td>
<td>3</td>
<td>0.0003</td>
<td>0.0082</td>
<td>0.0000</td>
</tr>
<tr>
<td>7</td>
<td>D-Glutamine and D-glutamate metabolism</td>
<td>5</td>
<td>2</td>
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<td>0.0405</td>
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</tr>
<tr>
<td>8</td>
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<td>beta-Alanine metabolism</td>
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<td>0.2208</td>
<td>0.4444</td>
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<tr>
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<td>Glyoxy late and dicarboxylate metabolism</td>
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<td>0.1814</td>
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<tr>
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<td>Methane metabolism</td>
<td>9</td>
<td>2</td>
<td>0.0086</td>
<td>0.1000</td>
<td>0.4000</td>
</tr>
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### Table 5: Possible metabolic pathways of differential metabolites in processed RWI

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H-NMR-based metabolomics study on the effects of raw and processed radix Wikstroemia indica

Fig. 1: Representative 600 MHz one-dimensional Carr-Purcell-Meiboom-Gill (1D-CPMG) \(^1\)H NMR spectra of plasma samples from a control (A), raw RWI (B) and processed RWI (C) indicating key metabolites.

Fig 2: (A) Plots for the principal component analysis (PCA) score, (B) Plots for the partial least squares-discriminant analysis (PLS-DA) score, VIP score (C) and heat map (D) of rats plasma metabolites after treated with raw RWI: control group, +: raw RWI group.

Fig 3: (A) Plots for the principal component analysis (PCA) score, (B) Plots for the partial least squares-discriminant analysis (PLS-DA) score, VIP score (C) and heat map (D) of rats plasma metabolites after treated with raw RWI: control group, +: processed RWI group.

Fig. 4: Pathway analysis of differential metabolites between control and raw RWI group. Global metabolic alterations of the most relevant pathways induced by raw RWI were revealed using the Metabo Analyst 3.0.
Compared with the raw RWI products, processed RWI have the effect of reverse regulation or reducing interference, as well as the emergence of new metabolites. For example, the increased D-glucose, lactic acid and glucose metabolites in raw RWI products are now decreased; the decreased citric acid, L-valine, L-serine, L-proline, phenylalanine, beta-alanine, asparagine, etc. are now up-regulated.

**DISCUSSION**

**Effect on amino acid metabolism under raw and processed RWI**

In our current study, we noted a series of amino acids were perturbed in plasma treated with raw RWI. The levels of asparagine, histidine, 2-hydroxyisobutyric acid, ornithine, leucine increasing and the 4-aminobutyric acid, alanine, glutamate, L-proline, beta-alanine, L-serine, L-valine, phenylalanine and L-glutamate decreasing. Because the liver is the main site of amino acid catabolism, disorder of amino acid metabolism under raw products, which may be caused by hepatic injury. Compared with the raw RWI, the processed RWI has the effect of reverse regulation or reducing the regulation degree. For example, 4-aminobutyric acid, alanine, glutamic acid, L-proline, beta-alanine, L-serine, L-valine, phenylalanine and L-glutamate decreased. Raw RWI decreased, processed RWI increased, asparagine, The elevated 2-hydroxyisobutyric acid in raw RWI was down-regulated in processed RWI. Among them, a variety of amino acids up-regulated in the processed RWI have been confirmed to have liver protective effects. For example, 4-aminobutyric acid, which is produced by glutamate decarboxylation through the catalytic activity of glutamate decarboxylase, can reduce hepatocyte necrosis and apoptosis by mediating STAT3 signaling pathway and enhance antioxidant capacity, alleviate liver injury and prolong survival in mice with lethal acute liver failure induced by experiments (Hata et al., 2019). Alanine can reduce the leakage of lactate dehydrogenase (LDH) in primary cultured rat hepatocytes treated with D-galactosamine (D-gal). Rapid administration of alanine can also significantly prevent elevated plasma transaminase levels and histological liver injury in rats treated with CCl4 (Maezono et al., 1996). L-serine can reduce fibrosis in a mouse model of chronic liver injury (Yun HH et al., 2021). Glutamine protects the liver through its antioxidant, anti-inflammatory and chelating properties. (Mahdavifard et al., 2022). Proline can reduce the changes of serum biomarkers of liver injury induced by bile duct ligation, alleviate liver histopathological changes and reduce oxidative stress markers (Heidari et al., 2018). It also protects the liver from D-galactosamine hepatitis by activating IL-6/STAT3 survival signaling pathway (Obayashi et al., 2012). Therefore, the retuning of the above amino acid metabolism level may be one of the mechanisms of “Sweat soaking method” processing to reduce the toxicity of RWI.

**Effect on energy metabolism and carbohydrate metabolism under raw and processed RWI**

TCA cycle is used to generate energy. It is the final metabolic pathway of glucose, fat and amino acids and an important hub of the three nutrients. TCA cycle-related enzymes are essential for maintaining normal cell function. The dysfunction of TCA cycle-related enzymes can lead to human diseases (Kang et al., 2021). Citrate and succinate are important intermediates in tricarboxylic acid cycle (Monchi, 2017; Grimolizzi and Arranz, 2018). Citrate can reduce plasma creatinine level and lactate dehydrogenase activity, partially restore ATP content in tissues and improve renal function (Bienholz et al., 2017). In this study, in processed RWI group, the level of citric acid increased and lactate level decreased and the succinic acid did not
Effect on choline metabolism under raw and processed RWI

Liver and kidney damage can lead to elevated choline levels and reduced metabolites. Choline is mainly oxidized to betaine in liver mitochondria. When liver injury occurs, choline metabolism is blocked, resulting in increased choline and reduced betaine. Betaine is much richer in the kidney and liver than in other mammalian organs. The main function of betaine in kidney is the osmotic protection of medullary cells. In liver, the main function of betaine is the methyl donor in methionine cycle (Kempson et al., 2013). In addition, betaine can prevent cadmium nephrotoxicity by inhibiting lipid peroxidation, increasing total antioxidant status and reducing caspase signaling cascade reactions in renal tissues (Hagar and Al Malki 2014). It also inhibits the activation of NF-xB and caspase-3, improves the histological changes induced by cisplatin and exerts renal protection (Hagar et al., 2015). In addition, betaine can improve liver injury caused by various factors (Wang et al., 2021). In the current study, the plasma choline level increased and the metabolite betaine decreased after the raw RWI was given. However, the level of choline metabolites in the processed group was not significantly affected, which also showed that the damage of liver and kidney after RWI was less than that of raw RWI.

CONCLUSION

In summary, we found that intragastric administration of raw RWI induced apparent systemic metabolic changes in plasma samples of rats by using NMR-based metabolomics approach. The metabolomics analysis demonstrated that raw RWI perturbed amino acid, choline metabolism, energy and carbohydrate metabolism. However, compared to raw product, the processed product have the effect of opposite regulation or reducing interference on amino acids, choline metabolism, energy and carbohydrate metabolism. Our study revealed the toxic mechanism of RWI and also confirmed that the "sweat soaking method" processing can reduce the toxicity of RWI, thus providing a reference frame for the processing and detoxification of the traditional Chinese medicine in clinical application.

REFERENCES


