Abstract. Radix *Wikstroemia indica* (L.) C.A. Mey. (RWI) is a toxic medicinal species primarily present in the Miao area of China. The toxicity of RWI is effectively reduced whilst maintaining the therapeutic effect when processed using the ‘sweat-soaking method’, which is a common method of Traditional Chinese Medicine preparation. However, there is a lack of scientific and medical evidence to explain the potential mechanisms by which the toxicity of RWI is reduced after preparation using this method, and the endogenous systemic metabolic effect of RWI remains uncertain. The aim of the present study was to explore the endogenous metabolic alterations caused by RWI and to examine the possibility of reducing the toxicity of RWI using the sweat-soaking method using proton nuclear magnetic resonance (NMR) metabolomic analysis in rats. Principal Component Analysis, Partial Least Squares-Discriminant Analysis (PLS-DA) and Orthogonal PLS-DA were used to assess individual proton NMR spectra. A total of 34 metabolic products were altered after delivering raw RWI, and 32 endogenous metabolites were induced by processed RWI. The metabolic pathways that lead to a significant impact on energy and carbohydrate, amino acid, organic acids and lipid metabolism following raw and processed RWI use were identified. The mitochondria of hepatic and renal tubules of rats were injured in the raw RWI group, whereas the processed product reduced or interfered with energy substrate, carbohydrate and amino acid metabolism, whilst reducing the levels of metabolic markers of hepatotoxicity and nephrotoxicity, without causing damage to the mitochondria. Our previous study showed that the median lethal dose (*LD*$_{50}$) value of raw RWI was 4.05 g/kg in rats after oral administration; however, the *LD*$_{50}$ value of the processed RWI could not be measured. The maximum tolerated dose and minimum lethal dose were 20 and 30 g/kg for the processed RWI, respectively, corresponding to 109 and 164 times the clinical daily dose (0.029 g/kg). Thus, the sweat-soaking method reduced the toxicity of RWI. Moreover, after processing, the toxic component YH-10 was converted into a YH-10 + OH compound, reducing the content of the toxic YH-10 by 48%, whilst also reducing the contents of the toxic components YH-12 and YH-15 by 44 and 65%, respectively. In conclusion, the present study showed that the sweat-soaking method reduced the toxicity of RWI, as evidenced by the reduction of the levels of metabolic markers and the activity of metabolic pathways, thus providing a basis for processing of RWI for clinical use.

Introduction

Radix *Wikstroemia indica* (L.) C.A. Mey. (RWI) is a toxic Chinese herbal medicine (CHM) that functions by clearing away toxic material (such as anti-respiratory syncytial virus) (1), reducing swelling and exerting an analgesic effect (2). The primary chemical ingredients of RWI are coumarin, lignans, flavonoids, anthraquinone, steroids, saponins, terpenoids, amides, polysaccharides and volatile oils (3-12). Research has shown that RWI has antibacterial, anti-inflammatory, anti-viral and anti-tumorigenic effects (13-17). In Traditional Chinese Medicine (TCM), it is primarily used to treat acute tonsillitis, chronic bronchitis, hepatitis, liver cirrhosis, nephritis, limb pain and cancer (2). However, research on RWI has primarily focused on the separation of the chemical components to address their individual biological activities, with less of a focus on metabolism, the mechanisms of toxicity or reducing the toxicity of RWI. There are no clear definitions for a suitable treatment dose, and what dose would be considered to exert toxic effects if used clinically (18).

RWI was excluded from the China Pharmacopeia due to its toxic side effects (19). Studies have confirmed that RWI
is hepatotoxic and nephrotoxic product (20). The aim of processing is to improve the efficacy of CHMs, or weaken or eliminate their toxicity. RWI is a medicine that is primarily used by people of Miao descent. Therefore, it is not included in the current Chinese Pharmacopoeia (21), but it is included in the quality standard of Traditional Chinese Medicine in the Guizhou Province. ‘Sweat-soaking’ is a special processing method commonly used in ethnic minority areas and believed to reduce the toxicity and improve the safety of TCM products. Analyses of active ingredients (22,23), pharmacodynamics (24) and toxicology (25,26) provide evidence supporting the use of this method to significantly reduce the toxicity of RWI, whilst preserving its pharmacological properties. Indeed, three highly toxic components (YH-10, YH-12 and YH-15) have been identified in RWI, and their levels were significantly reduced after sweat-soaking (27). Nevertheless, there is a lack of studies on the endogenous metabolites of the raw and processed RWI, and the detoxification mechanism of processing RWI using the sweat-soaking method remains uncertain.

The aim of the present study was to explore the endogenous metabolic alterations and potential mechanisms of detoxification of RWI using the sweat-soaking method based on proton nuclear magnetic resonance (1H-NMR) metabolomic analysis. The urine metabolism spectrum was monitored using 600-MHz high-resolution 1H-NMR after intragastric analysis. The urine metabolism spectrum was captured on a Varian Unity-Inova 600 MHz spectrometer. A standard one-dimensional (1H) NMR spectrum using the NOESY presaturation pulse sequence of pre-saturation was used with the following parameters: Spectral width, 8,000 Hz; mixing time, 500 ms; number of scans, 64; acquisition time, 6.24 s; and pulse delay, 1 s.

Materials and methods

**Instruments.** A Unity-Inova 600 Superconducting Nuclear Magnetic Resonance Spectrometer (Varian, Inc.), Ultrasonic instrument (Kunshan Ultrasonic Instrument Co., Ltd.); FA2004 Electronic analytical balance (Hengping Instrument Factory), Eppendorf MiniSpin Plus centrifuge (Eppendorf); nitrogen-blowing instrument (Yi Yao Instrument Technology Development Co., Ltd.); Targxin VX-02 Multi-tube Vortex oscillator (Tadjin Technology Co., Ltd.), BJ 1 stainless steel metabolic cage (Changsha Tianqin Biotechnology Co., Ltd.), and 202-3AB drying oven (Yi Yao Instrument Technology Factory), Eppendorf MiniSpin Plus centrifuge (Eppendorf); nitrogen-blowing instrument (Yi Yao Instrument Technology Development Co., Ltd.); Targxin VX-02 Multi-tube Vortex oscillator (Tadjin Technology Co., Ltd.), BJ 1 stainless steel metabolic cage (Changsha Tianqin Biotechnology Co., Ltd.), and 202-3AB drying oven (Yi Yao Instrument Technology Factory).

**Drugs and reagents.** RWI was collected from Guangxi Yinfeng International (lot no. 20160115). The raw and processed RWI ethanol extract was produced by the Pharmaceutical Preparation Laboratory of Guizhou University of Traditional Chinese Medicine (lot nos. YC20160410 and PZ20160420). 3-Trimethylsilyl-2,2,3,3-d4 acid sodium salt (TSP) (Merck Company, Germany), sodium pentobarbital (Sigma-Aldrich; Merck KGaA; lot no. 922L0310), acetonitrile (chromatographic grade; Fluca Company) and deuterated deuterium oxide (D2O; Cambridge Isotope Laboratories, Inc.) were used in the present study. All other reagents mentioned were of analytical grade.

**Animals and administration.** Specific pathogen-free Sprague-Dawley rats (weight, 200±20; age, 6 weeks), were purchased from Changsha Tianqin Biotechnology Co., Ltd. (animal license no. 43000200002163). Animals were provided ad libitum access to standard food and water under a 12-h light/dark cycle in a maintained environment (temperature, 25±1°C; relative humidity, 50±10%). All animal care and experimental operations were implemented under the Animal Management Rules of the Ministry of Health of the People’s Republic of China.

A total of male SD rats were randomly divided into a control group, a raw RWI group and a processed RWI group. After 1 week of adaptive feeding in a metabolic cage, the two experimental groups were given orally with raw and processed RWI (0.3175 g/kg), respectively, at 9:00 am everyday (7 days in total), whereas the control group was administrated with an equivalent volume of 1% CMC-Na for 7 days. The urine of the rats was collected in the metabolic cage for 24 h at 0, 1, 3, 5 and 7 days. Urine was thawed before use over a 0.22-µm microporous membrane. These samples were centrifuged at 12,100 x g for 10 min at 4°C, and the supernatant was collected and stored at -20°C. During administration of RWI, food and water were provided as normal. The rats were euthanized on the 8th day by intraperitoneal injection of 200 mg/kg sodium pentobarbital, and death was confirmed by cessation of breathing and heartbeat. 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 relevant requirements for animal welfare.

Preparation of ‘artificial sweat’ and ‘sweat-soaking’ of RWI. To prepare artificial sweat, histidine HCL (0.5 g/l), NaH₂PO₄·2H₂O (2.2 g/l) and NaCl (5.0 g/l) were dissolved in ultrapure water. The pH was adjusted to 5.5 using NaOH (0.05 mol/l). For sweat-soaking, the raw RWI root was sliced and sprayed with artificial sweat evenly. A total of 100 kg raw RWI root consumed 30 kg ‘artificial sweat’. Then the mixtures were placed in an oven for 24 h (37±0.5°C in the light for 12 h, and then at room temperature in the dark for 12 h). The procedure was repeated 14 times as described previously (27).

Preparation of raw and processed RWI ethanol extracts. The preparation of RWI ethanol extracts was performed as previously described (27). Briefly, the raw and processed RWI samples were percolated with 70% ethanol solvent (volume of 70% ethanol is 14 times that of medicinal materials) at a seepage rate of 5 ml/min/kg. The infiltrate was concentrated on a rotary evaporator under negative pressure, and finally dried using a lyophilizer to obtain the freeze-dried powder. The powder was stored at -20°C until required.

Urine sample preparation and 1H-NMR spectra acquisition. A total of 350 μl urine was mixed with 350 μl PBS (0.2 M; pH 7.4), then centrifuged at 12,100 x g for 10 min at 4°C. The supernatant (600 μl) was transferred to NMR tubes (5 mm) containing 30 μl TSP/D2O solutions (1 mg/ml), and the mixtures were immediately stored at -4°C until required.

The urine spectra were captured on a Varian Unity INOVA 600 MHz spectrometer. A standard one-dimensional NOESY presaturation pulse sequence of pre-saturation was used with the following parameters: Spectral width, 8,000 Hz;
Data processing and statistical analysis. The data were corrected by exporting the detection data table. The data filling principle was used to retain all detected ppm chemical shifts, in which the null value was filled with the minimum value. The smoothing data filtering conditions were: A robust estimate, such as the interquartile range; <250 variables, 5% was filtered; 250-500 variables, 10% was filtered; 500-1,000 variables, 25% was filtered; >1,000 variables, 40% was filtered; sample standardization was based on normalization using the total integral strength, and data normalization was based on the mean, centered and divided by the standard deviation of each variable.

Each 1H-NMR spectrum was phase-adjusted and baseline-corrected manually. For cpmgpppr2d (CPMG) data, segmentation of 0.4-4.4 spectra was performed using 0.04 ppm per segment. For ledhpgppr2d (LED) data, the 0.1-6.0 ppm spectra were segmented to 0.04 ppm per segment. Residual water (δ 4.6-δ 5.0) was excluded from the analysis. The remaining integral was then normalized to the sum of the spectral integrals using Microsoft Excel 2020 (Microsoft Corporation).

Multivariate statistical analysis was implemented using Metabo Analyst 3.0 software (Umetrics Corporation). After mean-centering and scaling the 1H-NMR datasets to default unit variance, principal component analysis (PCA) was performed to identify intrinsic clusters and determine obvious outlier values. Partial Least Squares Discriminant Analysis (PLS-DA) was further performed as a supervised pattern recognition analysis, to strengthen the difference between groups and identification variables responsible for separation. Orthogonal (O)PLS-DA was also used for predictive classification. The results of the analysis are expressed in the form of scores plot. The variable importance in projection weight variable importance in projection (VIP) was obtained, which is a value denoting the variable importance factor. Generally, a VIP value >1 can be considered as different, and the greater the VIP value, the more obvious the difference is (28). The reliability of the PCA, PLS-DA and OPLS-DA model was verified using a paired Student's t-test, and the differences between the variables were screened. Metabolic pathway mapping was established based on all identified endogenous metabolites, and the biological relevance was examined using Kyoto Encyclopedia of Genes and Genomes (KEGG) (29) annotation. Finally, metabolites were searched for and identified using Q1 data (the common databases were KEGG compound database, Massbank database, Lipidmaps database, Human Metabonomics database, Metlin database and Pubchem compound database).

Results

1H-NMR spectroscopic analysis of urine samples. As shown in Fig. 1, compared with the control group (Fig. 1A), the peak intensities of the 1H-NMR spectrum of urine on day 1 (Fig. 1B), day 3 (Fig. 1C), day 5 (Fig. 1D) and day 7 (Fig. 1E) day after administration in the raw RWI group were reduced, especially with the extension of administration time, the decrease of peak intensity became more obvious. However, some peaks also increased in intensity. Fig. 2 shows the 1H-NMR spectra of urine from the processed group on day 1 (Fig. 2A), day 3 (Fig. 2B), day 5 (Fig. 2C) and day 7 (Fig. 2D) after administration. Compared with the control group, the 1H-NMR spectra of processed urine group were altered. The majority of peaks increased in intensity, although the changes intensity were not as marked as those of the raw product group.

The toxicity of raw products has a great impact on their metabolism. Combined with map analysis, the metabolites corresponding to each peak, as well as the differences between the metabolites of the processed and raw RWI group were identified. The 1H-NMR spectrum of urine had strong signals primarily from amino acids, energy substrates and carbohydrates; additionally, organic acids, choline, creatinine, betaine and guanosine metabolites, as evidenced by the Q1 Data. The major metabolites were marked in the spectrum. A number of alterations in endogenous metabolites in the 1H-NMR spectra of urine from the raw and processed RWI groups were observed. In total, 34 metabolites in the urine of rats treated with raw RWI (Table I) and 32 metabolites in the rats treated with the processed RWI (Table II) were identified. Compared with the control group, visual analysis of the spectra indicated the raw RWI group displayed an increase in lysine, proline, L-Alanine, tyrosine, phenylalanine, tryptophan, lactic acid, citric acid, succinic acid, fructose, sucrose, glucose, creatinine and choline, and the decreases in betaine, alanine, β-alanine, asparagine, glycine, ornithine, L-proline, valine and L-serine. Compared with the raw RWI group, the type, concentration and relative proportion of endogenous metabolites in the urine of the processed RWI group markedly changed. Relative to the control group, the majority of metabolites that increased in the raw RWI group, such as creatinine, D-glucose, fructose, phenylalanine, L-alanine and asparagine; however, acetic acid, lactic acid, citric acid, L-lactic acid and pyruvic acid, were increased in the processed RWI group. In addition, unique endogenous metabolites were also produced in the processed RWI group, such as glutamic acid, L-proline, glutamic acid, threonine, leucine, methotrexate, isomylene and cyclopentane. This indicated that the chemical ingredients of RWI were altered quantitatively and/or qualitatively after processing with artificial sweat and/or by the heating process, which may result in reduced toxicity of RWI and enhanced the pharmacological actions.

Data analysis of metabolic biomarkers. PCA, PLS-DA and OPLS-DA were used to analyze the general clustering trends and the metabolic profiles of the urine of the control and experimental groups. Unsupervised PCA was initially used on the normalized 1H-NMR spectral data, and score plots of PCA on the data did not show obvious clustering between the control and raw RWI groups when compared by day. Similarly, the PCA score plots showed there was no obvious trend for clustering between the processed RWI group (when compared by day) and the control group, but the patterns of these samples on the 7th day did differ compared with the control group. As
the clustering trend of raw products and processed products were different, this showed that the chemical composition of RWI had changed after processing using the sweat soaking method; therefore, the resulting difference in interference with the endogenous metabolites also differed. In order to further separate the groups and identify discriminatory metabolites, PLS-DA, a supervised projection model, was used to identify the changes in the metabolite biomarkers. The control group and rats treated with the raw RWI at different time points could be separated; however, some of the 95% confidence intervals still overlapped, which was also true of the processed group. A high percentage of similarity in the treated groups on days 1, 3, 5 and 7 (in both the raw and processed RWI groups, respectively) was observed, based on the close proximity

Figure 1. Representative 1H-nuclear magnetic resonance spectra of the urine from the control and raw RWI groups. (A) Spectra of the urine from the control group. (B-E) Spectra of the urine from the raw RWI at (B) day 1, (C) day 3, (D) day 5 and (E) day 7. RWI, Radix Wikstroemia indica (L.) C.A. Mey.
observed in the PLS-DA score plots. In order to minimize the possible contributions of intergroup variability and to further improve the separation, OPLS-DA was used. Using OPLS-DA, the control and raw groups could be separated into distinct clusters (Fig. 3). Likewise, the control group and processed group could also be separated (Fig. 4). As the duration of administration increased, the spectrum of metabolites in rats changed more notably (Figs. 5 and 6). Moreover, the plots of the raw RWI groups and processed RWI groups also differed, which once again suggested that the chemical composition of RWI was altered following sweat-soaking.

The VIP value of each peak was used to identify its role in classification. Univariate statistical methods were used to further analyze the identified metabolites. Finally, 34 urinary metabolites in the original RWI and 32 metabolites in the processed RWI groups were identified, based on a VIP value >1, and a Student’s t-test significant difference threshold of P<0.05. The results of the analysis are shown in Figs. 7 and 8.

Pathway analysis. In total, 36 metabolic pathways in the raw RWI group and 22 metabolic pathways in the processed RWI group were altered. Using KEGG metabolic pathway enrichment analysis, 20 metabolic pathways (VIP>1.0, P<0.05) were found to have the greatest impact on the identified urine metabolites in the raw RWI group (Fig. 9). Out of the 20 metabolic pathways, the primary metabolic pathways with the greatest effect were amino acid metabolism (including ‘Glycine, serine and threonine metabolism’, ‘Phenylalanine, tyrosine and tryptophan biosynthesis’, ‘Alanine, aspartate and glutamate metabolism’, ‘Valine, leucine and isoleucine biosynthesis’ and ‘Phenylalanine metabolism’), carbohydrate metabolism (including ‘Citrate cycle (TCA cycle)’, ‘Pyruvate metabolism’ and ‘Starch and sucrose metabolism’) and energy metabolism (mainly ‘Methane metabolism’). Additionally, 13 metabolic pathways in the processed RWI group with the most influence (VIP>1.0, P<0.05; Fig. 10), exhibited a general map of primary metabolic pathways that were similar to the raw RWI group, although the significance of the influences on the metabolic pathways differed. For example, compared with the raw RWI group, the ‘Citrate cycle (TCA cycle)’ was not involved. In addition, by comparing the metabolic pathway diagrams of raw materials and processed products, it could be seen that where the metabolic pathways of ‘alanine, aspartate and glutamate metabolism’ and ‘valine, leucine and isoleucine biosynthesis’ corresponded with the abscissa and ordinate values in the processed group had increased, which showed
that there was an increased contribution of the two metabolic pathways to the processed group.

Discussion

Our previous study demonstrated that the seat-soaking method could reduce the toxicity of RWI. After processing, the toxic component YH-10 was converted into YH-10 + OH, the content of YH-10 decreased by 48%, and the contents of the other toxic components YH-12 and YH-15 also decreased by 44 and 65% respectively (25). The present study primarily focused on the effect of sweat-soaking on the effect of RWI in terms of metabolic markers and metabolic pathways.

In the present study, it was noted that several amino acids were perturbed in the urine from rats treated with raw RWI. The levels of lysine, proline, L-alanine, tyrosine, phenylalanine and tryptophan were increased, whereas those of alanine, β-alanine, asparagine, glycine, ornithine, valine and metabolites were reduced. Disruption of amino acid metabolism in raw products may be caused by hepatic injury, as the liver is the primary site of amino acid catabolism (30). In addition, alanine is a non-essential amino acid, and as a major energy

Table I. Identification of endogenous compounds in the urine of rats treated with raw RWI.

| Chemical shift (ppm) | f.value | P-value | -Log_{10}(P-value) | FDR | Identified compounds          |
|----------------------|---------|---------|---------------------|-----|-------------------------------|
| 1.33                 | 3.7897  | 0.0398  | 1.4000              | 0.87312 | Lactate                      |
| 1.34                 | 4.5927  | 0.0231  | 1.6372              | 0.87312 | 2-Hydroxyisobutyrate          |
| 1.73                 | 3.8113  | 0.0392  | 1.4067              | 0.87312 | Lysine                        |
| 1.91                 | 3.5528  | 0.0473  | 1.3251              | 0.87312 | Acetate                       |
| 1.98                 | 4.5496  | 0.0237  | 1.6251              | 0.87312 | Ornithine                     |
| 2.02                 | 6.2679  | 0.0086  | 2.0644              | 0.81534 | L-proline                     |
| 2.33                 | 3.8748  | 0.0375  | 1.4263              | 0.87312 | Proline                       |
| 2.41                 | 4.1354  | 0.0313  | 1.5051              | 0.87312 | Succinic acid                 |
| 2.44                 | 5.2052  | 0.0157  | 1.8030              | 0.81534 | L-glutamine                   |
| 2.46                 | 4.6101  | 0.0228  | 1.6421              | 0.87312 | Pyruvic acid                  |
| 2.53                 | 4.3524  | 0.0270  | 1.5688              | 0.87312 | Citric acid                   |
| 2.54                 | 4.2634  | 0.0287  | 1.5429              | 0.87312 | β-alanine                     |
| 2.76                 | 4.2561  | 0.0288  | 1.5408              | 0.87312 | Citrate                       |
| 2.84                 | 3.8594  | 0.0379  | 1.4216              | 0.87312 | Asparagine                    |
| 2.94                 | 4.7835  | 0.0204  | 1.6902              | 0.87312 | Asparagine                    |
| 3.02                 | 6.1216  | 0.0093  | 2.0302              | 0.87312 | Glucose                       |
| 3.10                 | 8.9168  | 0.0025  | 2.6074              | 0.87312 | Creatinine                    |
| 3.19                 | 3.5533  | 0.0479  | 1.3195              | 0.81534 | Choline                       |
| 3.20                 | 4.4520  | 0.0253  | 1.5974              | 0.64726 | Glucose                       |
| 3.25                 | 3.5767  | 0.0465  | 1.3328              | 0.87312 | Betaine                       |
| 3.56                 | 3.6898  | 0.0428  | 1.3688              | 0.87312 | Glycine                       |
| 3.60                 | 3.5101  | 0.0488  | 1.3114              | 0.87312 | L-valine                      |
| 3.68                 | 5.9241  | 0.0104  | 1.9831              | 0.87312 | Fructose                      |
| 3.76                 | 3.7921  | 0.0397  | 1.4008              | 0.87312 | L-alanine                     |
| 3.84                 | 3.9119  | 0.0365  | 1.4377              | 0.87312 | L-serine                      |
| 3.91                 | 5.5599  | 0.0128  | 1.8937              | 0.84073 | D-glucose                     |
| 4.01                 | 4.3134  | 0.0277  | 1.5575              | 0.87312 | Fructose                      |
| 4.05                 | 3.6775  | 0.0432  | 1.3649              | 0.87312 | Sucrose                       |
| 4.29                 | 6.9312  | 0.0061  | 2.2134              | 0.87312 | L-lactic acid                 |
| 5.25                 | 5.1374  | 0.0164  | 1.7852              | 0.87312 | Maltose                       |
| 7.20                 | 6.2130  | 0.0089  | 2.0517              | 0.87312 | Tyrosine                      |
| 7.34                 | 3.5071  | 0.0489  | 1.3104              | 0.81534 | Phenylalanine                 |
| 7.55                 | 3.8559  | 0.0380  | 1.4205              | 0.87312 | Tryptophan                    |
| 8.02                 | 3.6479  | 0.0441  | 1.3555              | 0.81534 | Guanosine                     |

FDR, false discovery rate.
Table II. Identification of endogenous compounds in the urine of rats treated with processed RWI.

| Chemical shift (ppm) | f.value  | P-value  | Log₁₀(P-value) | FDR     | Identified compounds                  |
|----------------------|----------|----------|----------------|---------|---------------------------------------|
| 0.94                 | 14.790   | 0.0013   | 2.9012         | 0.0587  | Leucine                               |
| 1.16                 | 4.1944   | 0.0466   | 1.3319         | 0.2122  | Proline                               |
| 1.21                 | 6.0518   | 0.0187   | 1.7279         | 0.1581  | Pyruvic acid                          |
| 1.32                 | 5.2584   | 0.0270   | 1.5694         | 0.1759  | Threonine                             |
| 1.40                 | 7.4677   | 0.0105   | 1.9797         | 0.1302  | D-lactic acid                         |
| 1.48                 | 11.867   | 0.0026   | 2.589          | 0.0842  | 2-Aminoisobutyric acid                |
| 1.51                 | 7.9331   | 0.0088   | 2.0552         | 0.1302  | Cyclopentane                          |
| 1.52                 | 4.2532   | 0.0451   | 1.3459         | 0.2086  | β-alanine                             |
| 1.7                  | 4.6149   | 0.0372   | 1.4296         | 0.1963  | Citrate                               |
| 1.77                 | 14.718   | 0.0013   | 2.8942         | 0.0587  | Senecioic acid                        |
| 1.86                 | 8.4776   | 0.0073   | 2.1395         | 0.1240  | Asparagine                            |
| 1.90                 | 6.0531   | 0.0187   | 1.7282         | 0.1581  | Creatinine                            |
| 2.07                 | 5.9621   | 0.0195   | 1.7107         | 0.1589  | Choline                               |
| 2.08                 | 8.4082   | 0.0074   | 2.1290         | 0.1240  | L-proline                             |
| 2.22                 | 11.663   | 0.0027   | 2.5651         | 0.0864  | Acetate                               |
| 2.28                 | 9.4745   | 0.0052   | 2.2841         | 0.1147  | 4-Aminobutyrate                       |
| 2.52                 | 5.1081   | 0.0290   | 1.5377         | 0.1792  | L-Valine                              |
| 2.54                 | 6.7094   | 0.0141   | 1.8494         | 0.1457  | Beta-alanine                          |
| 2.61                 | 5.2581   | 0.0270   | 1.5694         | 0.1759  | Fructose                              |
| 2.69                 | 10.686   | 0.0036   | 2.4452         | 0.1008  | L-alanine                             |
| 2.79                 | 4.5526   | 0.0384   | 1.4154         | 0.1990  | Aspartate                             |
| 2.89                 | 4.9909   | 0.0307   | 1.5126         | 0.1818  | D-glucose                             |
| 3.05                 | 5.4644   | 0.0244   | 1.6120         | 0.1714  | Ornithine                             |
| 3.20                 | 6.4374   | 0.0158   | 1.8001         | 0.1500  | Sucrose                               |
| 3.76                 | 17.902   | 0.0007   | 3.1817         | 0.0528  | Glutamic acid                         |
| 3.98                 | 9.8598   | 0.0046   | 2.3370         | 0.1077  | L-serine                              |
| 4.27                 | 7.0147   | 0.0125   | 1.9030         | 0.1405  | L-lactic acid                         |
| 6.07                 | 13.313   | 0.0018   | 2.7504         | 0.0667  | Tyrosine                              |
| 6.67                 | 4.9699   | 0.0310   | 1.5081         | 0.1822  | Phenylalanine                         |
| 7.28                 | 9.0751   | 0.0059   | 2.2276         | 0.1192  | Tryptophan                            |
| 7.81                 | 8.3600   | 0.0076   | 2.1216         | 0.1246  | Lactate                               |
| 8.86                 | 11.241   | 0.0031   | 2.5143         | 0.0888  | Purine                                |

FDR, false discovery rate.

source, can be metabolized by pyruvate and released from muscles (31).

Fat, sugar and protein, as the major energy sources in our body, are mainly metabolized in liver mitochondria (32). When liver mitochondria are damaged, energy metabolism decreases, and the usage of materials for energy generation increases (33). Increasing the supply of small molecular compounds as a source of energy, such as glucose, fructose and lactic acid, may be due to a decrease in energy metabolism in the body (34). Increased excretion of glucose and amino acids in the urine is a typical biomarker of renal proximal tubule injury and renal toxicity (35). In the present study, the raw RWI had a significant effect on energy metabolism and the levels of metabolites, such as fructose, sucrose and D-glucose, as their levels were increased in the urine, which may be a result of liver and kidney injury and obstruction of starch glucose metabolism. Moreover, when liver mitochondria are injured, the TCA cycle is blocked, which induces citric acid, 2-ketoglutaric acid and succinic acid, and the production of ATP is decreased (36). Glycolysis is increased to compensate for the reduction in ATP production, leading to an increase in lactic acid levels (37). After administration of raw RWI, the levels of metabolites, such as citric acid and succinic acid decreased, but those of lactic acid increased. This may be caused by TCA cycle inhibition and liver injury. Furthermore, liver injury can lead to impaired energy metabolism and thus promote creatinine production (38). Elevated levels of creatinine and acetic acid can be used as a marker for nephrotoxicity (39).
Glucose, lactic acid, hydroxybutyrate, equine, creatinine and ketoglutaric acid in urine can be used as biomarkers of renal tubular injury (40). Increased creatinine levels in the urine in animals treated with raw RWI was indicative of kidney damage in these rats.

Hepatic and nephritic injury can lead to an increase in the levels of choline and a decrease in the levels of metabolites. Choline can be used to synthesize phosphatidylcholine, acetylcholine and oxidized to betaine in mammals. Choline is oxidized to betaine primarily in the mitochondria of the liver (41). When the liver is injured, choline metabolism is blocked, which results in increased choline levels and reduced levels of its metabolite, betaine (42). In the present study, the levels of choline were elevated, and those of betaine were reduced in the urine after treatment with raw RWI. While in the rats treated with the processed RWI, choline levels in the urine were increased only slightly, whereas betaine levels did not exhibit a difference. This demonstrated that the toxicity of the processed RWI was lower compared with that of the raw RWI.

Compared with the raw RWI, the processed form resulted in the opposite effects, reducing interference in amino acid levels,
Figure 4. Orthogonal Partial Least Squares-Discriminant Analysis. score plot from $^1$H-nuclear magnetic resonance metabolic profiling in the rats treated with the processed RWI. (A) Principal component analysis, the contribution of PC1 was 36.5% and that of PC2 was 14.2%. (B) Partial least squares discrimination analysis, the contribution of PC1 was 35.8% and that of PC2 was 9.6%. (C) Orthogonal partial least squares discriminant analysis, the contribution of PC1 was 27% and that of PC2 was 18.3%. Each symbol represents a sample. Red, control; green, day 1; blue, day 3; cyan, day 5; pink, day 7. RWI, Radix Wikstroemia indica (L.) C.A. Mey.

as well as the emergence of new metabolites. For example, the increased lysine, proline, L-Alanine and tyrosine levels observed in the rats treated with the RWI product was decreased in the rats treated with the processed RWI. Similarly, the decreased $\beta$-alanine, asparagine, ornithine, L-proline, L-valine and L-serine levels were upregulated in rats treated with processed RWI. Thus, processing of RWI may reduce liver injury to RWI.

The changes in the products of the TCA cycle revealed a disturbance of anaerobic glycolysis in raw RWI treated rats. Increasing compensatory anaerobic glycolysis leads to higher levels of lactate (37). An increase in the lactate levels was observed in the urine of rats treated with raw RWI (Table I), suggesting increased glycolysis. In the processed RWI group, the levels of citrate increased and lactate levels decreased, whereas succinate levels did not differ (Table II). This indicated that the sweat soaking method reduced the toxicity of RWI, thereby reducing liver mitochondrial injury. In addition, liver mitochondria injury can result in decreases in energy metabolism and an increase in carbohydrate levels, such as saccharides (43). The levels of energy-supply materials, including D-glucose and fructose, decreased in the rats treated with the processed RWI product, showing a reversal in the effects observed with the raw RWI.

When the mitochondria of the liver are damaged, the TCA cycle is blocked, and thus, citric acid, 2-keto-glutaric acid and succinic acid levels, as well as ATP production are reduced, and a compensatory increase in glycolysis is observed, thus
increasing lactic acid levels (36). In the rats treated with raw RWI, the mitochondria of the liver were damaged, the TCA cycle was blocked, and the levels of products, such as citric acid and succinic acid were significantly decreased, whereas that of lactic acid was increased. In the rats treated with the processed RWI, the levels of citric acid were increased, lactic...
Acid levels were reduced and succinic acid levels did not differ, suggesting that the sweat-soaking method could reduce injury to liver mitochondria caused by raw RWI. In addition, the damage to liver mitochondria can lead to a decrease from energy metabolism and an increase in the use of carbohydrate, such as saccharides instead. D-glucose and fructose levels...
Figure 7. Univariate, VIP value and thermogram analysis results of urine differential metabolites of raw RWI. (A) Raw RWI univariate analysis. (B) VIP score. The transverse coordinate represents the VIP value, and the longitudinal coordinate represents the ppm of the metabolite. RWI, Radix Wikstroemia indica (L.) C.A. Mey.; VIP, variable importance in projection weight variation importance ranking.

Figure 8. Univariate, VIP value and thermogram analysis results of urine differential metabolites of processed RWI. (A) Processed RWI univariate analysis. (B) VIP score. The transverse coordinate represents the VIP value, and the longitudinal coordinate represents the ppm of the metabolite. (C) Heat map of correlation analysis of differential compounds and groups. RWI, Radix Wikstroemia indica (L.) C.A. Mey.; VIP, variable importance in projection weight variation importance ranking; C, control; D1, day 1; D3, day 3; D5, day 5; D7, day 7.
decreased in the urine in the rats treated with the processed RWI, compared with the rats treated with raw RWI. The creatinine levels were increased in the rats treated with the raw RWI and decreased in those treated with the processed form, which indicated that the administration of the processed product resulted in less injury to the liver and kidney than raw RWI.

In conclusion, intragastric administration of raw RWI induced apparent systemic metabolic changes based on analysis of the urine samples of rats using 1H-NMR-based metabolomics. The metabolomics analysis demonstrated that raw RWI perturbed the metabolism of amino acids, choline metabolism, energy substrates and carbohydrates. However, compared with the raw product, the processed RWI had the opposite effect, or reduced interference to amino acid metabolism, choline metabolism, energy substrates and carbohydrate metabolism. This study revealed the toxic effects of RWI, and confirmed that the sweat-soaking method could reduce the toxicity of RWI, thus highlighting its potential for detoxification of TCM products to improve their potential clinical value.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZRZ and GF performed the experiments, including collection of the plant material, preparation of the raw and processed RWI ethanol extracts, interpretation of the experimental data, and writing of the manuscript. WL and LLL contributed to analysis of the metabonomic data. GF conceived and designed the study. ZGW, CQZ, QX, CCR and LZP all participated in the experiments. All authors have read and approved the final manuscript. ZRZ and GF confirm the authenticity of all the raw data.

Ethics approval and consent to participate

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 relevant requirements for animal welfare.

Patient consent for publication

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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