Therapeutic Effects of Selaginella tamariscina on the Model of Acute Gout with Hyperuricemia in Rats Based on Metabolomics Analysis

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Gout is a disease of purine metabolic disorders which results from long-term hyperuricemia and the sodium urate deposition in and around the joints. Selaginella tamariscina (ST) is an important traditional Chinese herbal medicine and is used for the treatment of gout and hyperuricemia. In this study, the rat model of acute gout with hyperuricemia was established by intraperitoneal injection of xanthine and oxonic acid potassium salt and articular injection monosodium urate (MSU). The effect of ST in the treatment of gout was investigated by measuring joint swelling, the expression of IL-1β in serum and histological changes of joint by haematoxylin eosin (H&E) staining. Subsequently, urine metabolomics analysis for biomarkers discovery in acute gout with hyperuricemia rats was performed by the ultra-performance liquid chromatography-electrospray ionization quadruple time-of-flight mass spectrometry (UPLC-ESI-QTOF/MS) combined with chemometric approach. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were used to detect potential biomarkers. A total of 18 potential biomarkers were identified mainly including tryptophan metabolism; tyrosine metabolism; lysine methylation; pyrimidine metabolism; purine metabolism; TCA cycle and fatty acid metabolisms. This study indicates that ST could efficiently ameliorate the disease of acute gout with hyperuricemia in rats. The related metabolic biomarkers could provide useful information and the metabolic mechanism could be used for further study about the model of acute gout with hyperuricemia in rats.

Keywords Gout, Selaginella tamariscina, metabolomic, UPLC-ESI-QTOF/MS

Introduction

Gout, a disorder of purine metabolism and a type of inflammatory arthritis, is induced by uric acid accumulation in joint. Hyperuricaemia is not only strongly associated with causing gout, but main factor for the development of gout. The serum level is higher or equal to 360 μmol/L that could regard as the basis of gouty initiation. Gout attack caused a number of important complications which associated with metabolic, cardiovascular, hypertension and renal morbidity. The treatment of gout is primarily on two aspects: inhibiting pain and lowering uric acid level in serum. Therapies for acute gout include nonsteroidal anti-inflammatory drugs (NSAIDs), colchicine, allopurinol, febuxostat and so on. However, all acute and prophylactic therapies are associated with adverse events, for example, renal injury and allergy. There is a growing need to find more effective and safe alternate therapies to reduce the side effects of synthesized medicine from traditional Chinese medicine (TCM).

Selaginella tamariscina (ST), belonging to the genus Selaginellaceae, is an important traditional Chinese herbal medicine. It is used for the treatments of cancer, hepatitis, diabetes, and skin diseases as well as for the treatments of gout and hyperuricemia. In addition, ST could effectively inhibit productions of pro-inflammatory cytokines VCAM-1, E-selectin, IL-6 and IL-8 and inhibit xanthine oxidase (XOD) activity. They are major mediators of gouty inflammation and lower uric acid. In this study, the exact mechanism on the treatment of acute gout with hyperuricemia was discussed by metabolite analysis method. Modern analytical techniques are often used in metabolomics research, especially in the analysis and identification of biomarkers. Nowadays the most popular methods used for metabolomics research include...
nuclear magnetic resonance spectroscopy (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and ultra performance liquid chromatography-mass spectrometry (UPLC-MS). Among them, LC-MS is the most widely used chemical analytical technology and an ideal means to identify the metabolites and metabolic end products of urine. UPLC-MS possessing high sensitivity and accuracy has been used for identification of the chemical constituents in TCM[11] or biological samples. The method of metabolomics based on chemical analysis is a convenient, rapid and accurate tool for the detection of drug metabolism, in which, the metabolic profiling can be obtained and the biomarkers can be found by the measurements of masses, retention times and the chemical components.[12] For identifying potential biomarkers, biochemistry databases such as HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/) and KEGG (http://www.kegg.com/) are always used.

In this study, an UPLC-ESI-QTOF-MS method was established to gain the global profiling of the endogenous metabolites in urine which was combined with chemometric approaches PCA and OPLS-DA. The endogenous metabolites were investigated for the metabolite pathway changes after treatment of the acute gout model with hyperuricemia by using ST.

Experimental

Reagents and materials

Xanthine and sodium urate were purchased from Sigma (St. Louis, MO, USA). Oxonic acid potassium salt was purchased from Xiya Reagent (Chengdu, China). Acetonitrile of HPLC grade was obtained from Fisher Scientific (Loughborough, UK). Formic acid of HPLC grade was bought from ROE Scientific Inc. (Newark, USA). Rat IL-1β ELLSA kits were taken from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Selaginella tamariscina was taken from Jilin Pharmacy (Changchun, China) which belongs to Selaginella tamariscina (Beauv.) Spring. Dried and pulverized Selaginella tamariscina of 500 g was refluxed with 4 L of 70% ethanol for 40 min, two times. The filtrates were combined and concentrated. After cooling, the extracted solutions were freeze-dried into powder, which was dissolved in water for using.

Animal model construction and treatment

Adult male rats of Sprague-Dawley rats (weighing 200 to 220 g) obtained from Liaoning Changsheng Technology and Biology Institute were used for the experiments. The animals were acclimatized for one week before the experiment to begin freely accessing food and water. After that, the rats were randomly divided into three groups (each group consists of 10 rats): control group (CG), model group (MG) and Selaginella tamariscina-treated group (StG). The rats of CG were intraperitoneally injected identical volume of normal saline. The rats of MG and StG were injected with the model drug (60 mg/mL of xanthine and oxonic acid potassium salt) at the dose of 0.5 mL/100g twice daily for 7 d, in which, the StG group received Selaginella tamariscina extract at the dose of 210 mg/kg for 7 d[13]. On the 6th day, 100 μL sodium urate solution (30 mg/mL) was injected into the rat of right articular cavities for MG and StG groups. Joint swelling was measured by a soft rule for the right ankle of each rat for circumferences 3, 6, 12, 24, and 48 h. Joint swelling ratio was calculated using the following formula: Joint swelling ratio = (A − B)/B×100%, where A is the circumference of each group at different time; B is the circumference of each group at 0 h.

All animal experiments were performed in accordance with the operation rules for animal testing which were approved by the Ethics Committee for the Use of Experimental Animals of Jilin University.

Sample collection and preparation

After 7 d, the blood of rats was centrifuged at 3000 r/min for 10 min and was stored frozen at −80 °C for ELISA. The right joint of rats were fixed with 10% formalin and embedded in paraffin. Then, the tissues were stained with H&E staining for general evaluation. The collected twelve-hour urine samples were centrifuged at 4000 r/min for 10 min and were stored frozen at −80 °C until analyzing. All the stored frozen urine samples were thawed and centrifuged at 10000 r/min for 10 min at 4 °C, then filtered through a 0.22 mm membrane. Subsequently, the samples were diluted at a ratio of 1 : 8 with water before UPLC-ESI-QTOF/MS analyzing.

Chromatography conditions

Urine samples analyses were performed on a Waters ACQUITY UPLC system. Chromatographic separation was executed on an UPLC BEH C18 column (1.7 μm, 2.1×50 mm, Acquity) with a temperature of the column set at 35 °C at a flow rate of 0.3 mL/min. The samples were maintained at 4 °C during the analysis. The mobile phases were solvent A (acetonitrile) and solvent B (0.1% formic acid in water). The gradient elution of A was performed as follows: 0−2 min, 5%−10% A; 2−5 min, 10%−20% A; 5−6 min, 20−50 A; 6−9 min, 50%−70% A; 9−10 min, 70%−100% A. The injection volume was 10 μL.

Mass spectrometry conditions

The ESI source in both positive and negative ion modes was used in MS analysis. The source parameters were set as follows: the source temperature was 120 °C, desolvation gas temperature was 350 °C, cone gas flow was 50 L/h, desolvation gas flow was 700 L/h. In positive ion mode, capillary, cone and extraction cone voltages were 3.0 kV, 40 V and 5.0 V, respectively. In negative ion mode, capillary, cone and extraction cone volt-
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Figure 1 (A) Joint swelling of CG, MG and StG in 48 h. (B) The expression of IL-1β of CG, MG and StG in serum. *p<0.05, **p<0.01, compared with the MG.

Statistical analysis

The MS data were processed using MassLynx V4.1 (Waters Corporation, Milford, USA). EZinfo 2.0 software was applied for principal component analysis (PCA) and orthogonal projection to latent structures squares-discriminant analysis (OPLS-DA). The variable importance in the projection (VIP) values could be generated after OPLS-DA processing. The statistical analysis was performed using one-way analysis of SPSS18.0 and significance t test (p<0.01 or p<0.05) was carried out. Biochemical databases were used to identify potential markers, such as HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/) and KEGG (http://www.kegg.com/).

Results and Discussion

Joint swelling analysis

Joint swelling is the typically diagnostic basis of acute gout on the rapid development of monoarticular arthritis. In this study, the rat articular was injected MSU on the 6th day to establish the model of acute gout. The result in Figure 1(A) shows that the joint swelling increased as increasing of the time and reached a peak at ca. 12 h, and then decreased after that time in MG. Compared with MG, the joint swelling in StG decreased during the observed period. It revealed that ST could decrease joint swelling and restrain MSU-induced inflammatory respond and development. Meanwhile, the expression of IL-1β was detected by ELISA to explore the ability of ST on restraining inflammatory factors. IL-1β is a crucial mediator for gout and MSU crystal-induced inflammation. From Figure 1 (B) we can see that ST could decrease the level of IL-1β in serum compared with MG and showed a better effect in acute gout with hyperuricemia rats.

Histological changes

The uric acid accumulation in or around joint could cause relevant morphological changes. The joint samples were collected after 7 d and H&E staining was used to evaluate the histopathological changes and reflect the real disease state. As shown in Figure 2, we can observe that there is no histopathological change in the morphology of the normal joint. On the contrary, the characteristic of MG is inflammatory cell infiltration, necrosis and edema of tissue by injection with MSU crystals compared with CG. After ST treatment, the above mentioned pathological abnormalities were ameliorated obviously. From the results, it can be seen that ST exhibits significant therapeutic effects on the model of acute gout with hyperuricemia in rats.

Metabolic profiling of UPLC-ESI-QTOF-MS

The CG, MG and StG urine samples were analyzed by UPLC-ESI-QTOF-MS in both positive and negative ionization modes. The typical base peak intensity (BPI)
chromatograms of urine samples in positive mode and negative mode for CG, MG and StG samples are shown in Figure 3. It can be seen that the low molecular mass metabolites could achieve a good separation in 10 min. In this study, the data were analyzed by metabolic profiling combined with PCA and OPLS-DA to understand the metabolic changes. OPLS-DA score plots between MG and StG in positive and negative ion modes are respectively showed in Figure 4(A) and 4(B), from which, we can see that in any model, i.e., in positive model or in negative model, MG and StG could be scattered clearly in different areas, that is to say, could be well separated. The S-plot of each spot represents an endogenous substance which was usually used to identify the features contributing to the group discrimination in Figure 4(C,D). The VIP values could be generated after OPLS-DA processing, which reflect the relative contribution of each x variable to the model. A compound whose VIP was above 1.0 and \( P < 0.05 \) for significance t test between two groups was considered as a potential biomarker. The structure informations of potential biomarkers were confirmed by searching freely accessible databases of HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/) and KEGG (http://www.kegg.com/). Accordingly, 18 potential biomarkers in urine (11 from the positive mode and 7 from the negative mode) were identified between MG and StG. They are summarized in Table 1, which provides information of compounds including retention time, mass \( (m/z) \), metabolite identification, formula, VIP, error (ppm) and the pathway. And the primarily metabolism pathways involved tryptophan metabolism, lysine degradation, tyrosine metabolism, pyrimidine metabolism, purine metabolism, TCA cycle and fatty acid metabolism.

The relative intensities of 18 potential biomarkers are shown in Figure 5. Comparing CG and StG with MG, the relative intensities had significant differences in all potential biomarkers. Deoxycytidine, 1-methyladenosine and homocysteinesulfinic acid were down-regulation in MG, and then up-regulation after ST treatment. The other 15 potential biomarkers were increased in MG and decreased after treatment.

![Figure 3](image_url)

**Figure 3** The typical urinary base peak intensity (BPI) chromatograms of CG, MG and StG obtained in positive mode and in negative mode based on UPLC-ESI-QTOF/MS. The left BPI chromatograms were the groups in the positive ion mode and the right BPI chromatograms were the groups in the negative ion mode.
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### Figure 4
OPLS-DA score plots of urine metabolic profiling of MG and StG in positive mode (A) and negative mode (B) for MG and StG. MG and StG could be scattered clearly in different areas; OPLS-DA S-plot in positive mode (C) and negative mode (D) for MG and StG. The S-plot of each spot represents an endogenous substance which is usually used to identify the features contributing to the group discrimination.

### Table 1
Potential biomarkers and their metabolic pathways

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time/min</th>
<th>Measured ((m/z))</th>
<th>Compound</th>
<th>Formula</th>
<th>VIP</th>
<th>Error/ppm</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.36</td>
<td>118.066</td>
<td>Indole</td>
<td>C₉H₇N</td>
<td>1.3</td>
<td>3.39</td>
<td>Tryptophan metabolism</td>
</tr>
<tr>
<td>2</td>
<td>6.69</td>
<td>161.1289</td>
<td>N(6)-Methyllysine</td>
<td>C₇H₁₆N₂O₂</td>
<td>1.03</td>
<td>3.01</td>
<td>Lysine degradation</td>
</tr>
<tr>
<td>3</td>
<td>4.32</td>
<td>164.071</td>
<td>3-Methyldioxyindole</td>
<td>C₉H₁₀N₂O₂</td>
<td>2.18</td>
<td>2.44</td>
<td>Tryptophan metabolism</td>
</tr>
<tr>
<td>4</td>
<td>6.39</td>
<td>179.071</td>
<td>4-Methoxycinnamic acid</td>
<td>C₁₀H₁₀O₃</td>
<td>1.19</td>
<td>4.47</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
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<td>Glucosamine</td>
<td>C₆H₁₃NO₅</td>
<td>1.34</td>
<td>3.89</td>
<td>Amino sugar and nucleotide sugar metabolism</td>
</tr>
<tr>
<td>6</td>
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<td>L-Tyrosine</td>
<td>C₉H₁₀NO₃</td>
<td>1.10</td>
<td>0.55</td>
<td>Tyrosine metabolism</td>
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<td>7</td>
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<td>189.1594</td>
<td>N6,N6,N6-Trimethyl-L-lysine</td>
<td>C₉H₂₀N₂O₂</td>
<td>1.12</td>
<td>−1.59</td>
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<tr>
<td>8</td>
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<td>208.0607</td>
<td>4-(2-Aminophenyl)-2,4-dioxobutanoic acid</td>
<td>C₁₀H₁₀NO₄</td>
<td>1.38</td>
<td>1.44</td>
<td>Tryptophan metabolism</td>
</tr>
<tr>
<td>9</td>
<td>0.67</td>
<td>228.0986</td>
<td>Deoxycytidine</td>
<td>C₉H₁₉N₅O₄</td>
<td>1.28</td>
<td>3.51</td>
<td>Pyrimidine metabolism</td>
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<tr>
<td>10</td>
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<td>1-Methyladenosine</td>
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<td>Purine metabolism</td>
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<td>11</td>
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<td>Tetracosahexaenoic acid</td>
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<td>Alpha linolenic acid and acid metabolism</td>
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<tr>
<td>13</td>
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<td>Homocysteinesulfonic acid</td>
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<td>cis-Aconitic acid</td>
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<td>2.89</td>
<td>TCA cycle</td>
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<td>3.89</td>
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<td>Suberic acid</td>
<td>C₁₀H₁₈O₄</td>
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<td>0.58</td>
<td>Fatty acid metabolism</td>
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<td>191.0201</td>
<td>Citric acid</td>
<td>C₈H₁₈O₇</td>
<td>3.35</td>
<td>2.09</td>
<td>TCA cycle</td>
</tr>
<tr>
<td>16</td>
<td>2.91</td>
<td>212.0019</td>
<td>Indoxyl sulfate</td>
<td>C₁₀H₁₆NO₅S</td>
<td>3.17</td>
<td>−1.89</td>
<td>Tryptophan metabolism</td>
</tr>
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<td>17</td>
<td>4.45</td>
<td>229.1446</td>
<td>Dodecanedioic acid</td>
<td>C₁₀H₂₀O₄</td>
<td>1.30</td>
<td>0.44</td>
<td>Fatty acid metabolism</td>
</tr>
<tr>
<td>18</td>
<td>7.32</td>
<td>313.2377</td>
<td>Octadecanedioic acid</td>
<td>C₁₈H₃₄O₄</td>
<td>1.53</td>
<td>−2.23</td>
<td>Fatty acid metabolism</td>
</tr>
</tbody>
</table>

**Metabolic changes under the treatment of StG**

The 18 potential biomarkers were important for the development of disease on acute inflammation. The five important metabolic pathways included tryptophan metabolism, pyrimidine metabolism, TCA cycle, lysine degradation and fatty acid metabolism. Most of them were directly or indirectly connected with each other, for which the metabolic networks are structured in...
Figure 5. Relative intensities of potential biomarkers in CG, MG, and StG (*p<0.05, **p<0.01, compared to the MG).

Figure 6.

Indole, 3-methylindoyl, indoxyl sulfate, and 4-(2-aminophenyl)-2,4-dioxobutanoic acid belong to tryptophan metabolism which plays a critical role in inflammation respond. They were up-regulated in MG and down-regulated in StG (see Figure 5). The disorder of tryptophan metabolism is discovered in the development of gout. Tryptophan undergoes degradation via two distinct pathways: the serotonin and kynurenine pathways. The kynurenine pathway as its key step is induced by pro-inflammatory mechanisms via the immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO). Tryptophan converts to kynurenine after IDO is activated, and 4-(2-aminophenyl)-2,4-dioxobutanoic acid increased which is the kynurenine metabolic product. Indoxyl sulfate is a valuable biomarker in chronic kidney disease (CKD) and it increases IL-1β-induced E-selectin and transcription factors expression (for example, NF-κB). The indoxyl sulfate is an important factor that could lead to relative inflammatory system activated. So, tryptophan metabolism is an important pathway for acute gout. In this study, the treatment...
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Figure 6  Correlation networks of main potential biomarkers in response to the therapeutic effects of ST on the model of acute gout with hyperuricemia in rats. The metabolites in the box denote the identified metabolites.

group had a reverse effect on regulation to normal level by inhibiting inflammatory factor release.

Citric acid and cis-aconitic acid are important intermediates of the tricarboxylic acid (TCA) cycle taking place in mitochondria. TCA cycle is the important energy metabolism, because it is the main metabolic pathway for the production of NADH for conversion to ATP. The NADH and ATP to be synthesized in mitochondria are crucial for biomass production and cellular maintenance. cis-Aconitic acid is an intermediate in the TCA cycle produced by the dehydrogenation of citric acid. The TCA cycle is used for the oxidation of fuel molecules eventually, including proteins, fatty acids and carbohydrates. Therefore, ST could reverse the levels of citric acid and cis-aconitic acid (see Figure 5) and maintain the balance of energy metabolism in disease states.

Tyrosine is an essential amino acid in the body of human. The model rats were injected MSU in right articular. MSU crystals could induce the tyrosine phosphorylation and activation of tyrosine kinase Tec in a time and concentration-dependent mode. The activation of tyrosine kinase Tec is necessary for the MSU crystal–induced secretion of IL-1 and IL-8 and for the generation of the chemotactic activity from MSU crystal–stimulated neutrophils. The responses of neutrophils to MSU crystals are considered to represent critical effector cells responsible for gouty inflammation. Therefore, tyrosine is a characteristic marker of MSU-induced gout. In this research, ST decreased the level of tyrosine so that the releasing of neutrophils was reduced and the expressions of IL-1 and IL-8 were inhibited.

Among fatty acid metabolisms, suberic acid, dodecanedioic acid and octadecanedioic acid were confirmed as potential metabolites, because they are related with fatty acid oxidation (FAO). The process of FAO could promote ROS production and initiate pro-inflammatory signal. Thus after ST treatment, the levels of suberic acid, dodecanedioic acid and octadecanedioic acid were decreased and the inflammatory signal was inhibited to reveal a potential effect on the model of acute gout with hyperuricemia in rats.

N(6)-Methyllysine and N6,N6,N6-trimethyl-L-lysine are methylated derivatives of the amino acid lysine. Histone lysine methylation is associated with the synthesis of DNA. Deoxycytidine triphosphate (dCTP) is the source of the deoxycytidine in DNA. In our work those compounds were up-regulated in MG and then ST had a reverse effect on regulation to normal level (see Figure 5).

Metabolomic network has been widely used in the prevention and diagnosis of the diseases. The perturbation biological markers and multiple pathways were used to elucidate mechanisms of disease progression. By metabonomics analysis method, we found that ST exerted therapeutic effects for acute gout with hyperuricemia rats mainly through the regulations of tryptophan metabolism, TCA cycle, lysine degradation, fatty acid metabolism and pyrimidine metabolism.

Conclusions

In this study, the rat model of acute gout with hyperuricemia was established by intraperitoneal injection of xanthine and oxonic acid potassium salt and articular injection monosodium urate (MSU). After ST treatment, the joint swelling was decreased and lesions of joint were relieved. Additionally, a total of 18 potential biomarkers were identified by UPLC-ESI-QTOF-MS method combined with chemometric approaches, PCA and OPLS-DA. The primary metabolic pathways included tryptophan metabolism, tyrosine metabolism, lysine methylation metabolism, pyrimidine metabolism, purine metabolism, TCA cycle and fatty acid metabolism. This study indicates that ST could efficiently ameliorate the disease of acute gout with hyperuricemia in rats. These results are valuable and important for the understanding of the metabolic process and therapeutic mechanism of ST. Meanwhile, the potential biomarkers
could provide medical diagnosis and treatment on the acute gout with hyperuricemia and be the bases for further development of new drugs.

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