Compound Sophorae Decoction: treating ulcerative colitis by affecting multiple metabolic pathways

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[ABSTRACT] Ulcerative colitis (UC) is a chronic refractory non-specific intestinal inflammatory disease that is difficult to be cured. The discovery of new ulcerative colitis-related metabolite biomarkers may help further understand UC and facilitate early diagnosis. It may also provide a basis for explaining the mechanism of drug action in the treatment of UC. Compound Sophorae Decoction (CSD) is an empirical formula used in the clinical treatment of UC. Although it is known to be efficacious, its mechanism of action in the treatment of UC is unclear. The purpose of this study was to investigate the changes in endogenous substances in UC rats and the effects of CSD on metabolic pathways using the metabonomics approach. Metabolomics studies in rats with UC and normal rats were performed using LC-MS/MS. Rats with UC induced using TNBS enema were used as the study models. Metabolic profiling and pathway analysis of biomarkers was performed using statistical and pathway enrichment analyses. 36 screened potential biomarkers were found to be significantly different between the UC and the normal groups; it was also found that CSD could modulate the levels of these potential biomarkers. CSD was found to be efficacious in UC by regulating multiple metabolic pathways.

(KEY WORDS) Compound Sophorae Decoction; Ulcerative colitis; LC-MS/MS; Biomarker; Metabolomics


Introduction

UC is a chronic non-specific inflammatory disease of the colon and rectum with an unknown etiology. The initial manifestations of UC are not typical, but bloody diarrhea is the most common early symptom accompanied with abdominal pain, blood in the stool, weight loss, and vomiting \(^{[1,2]}\). The incidence of UC in Europe, North America, and Asia is 24.3/100 000, 19.2/100 000, and 6.3/100 000, respectively. In recent years, the incidence of UC worldwide has increased rapidly and its incidence among women is higher than that in men. Despite the relatively low prevalence in developing countries, the number of cases has increased over the past few years and the ages of most patients are between 20 and 40 years. UC is accompanied with debilitating symptoms and associated with a poor quality of life. It is currently believed that the occurrence of this disorder is the result of a combination of immune, genetic, environmental, and inflammatory factors. UC patients often present autoimmune diseases such as nodular erythema, rheumatoid spondylitis, and sclerosis cholangitis. These may result in serious impacts on the quality of life in patients \(^{[3-8]}\). Therefore, early diagnosis of UC and the provision of safe and effective treatments can help alleviate patient suffering and improve their quality of life.

The efficacy and safety of Traditional Chinese medicine (TCM) has been tested in the five-thousand-year history of China. UC whose course is repeated is difficult to be cured and directly affects the quality of life of patients. TCM has unique advantages in the treatment of UC. Clinical symptoms including abdominal pain, bloating, bloody stools, and...
mucus stools can be quickly relieved by administering Chinese medicines to UC patients, and the effect is remarkable. At the same time, the colonic mucosa of patients who ensured completion of the treatment course and adhered to the medication was almost perfect. This indicates that TCM can relieve clinical symptoms and reduce the relapse rate of the disease. Moreover, dialectical treatment with TCM also has anti-inflammatory and anti-infective effects; it also suppresses immune responses and improves the internal environment. In particular, biological agents or immunosuppressants supplemented with TCM are found to be more effective in patients. The incidence of adverse reactions and side effects of TCM is generally low. Patients with UC treated with TCM show small changes in liver and kidney function indicators; however, no serious liver and kidney function damages have occurred, whereas patients using immunosuppressants and salicylic acid preparations display severe immunosuppression and adverse gastrointestinal reactions. Therefore, in patients with severe UC, it is recommended to use a combination of Chinese and Western medicines to reduce potential side effects.

CSD is a Chinese herbal compound that consists of five herbs: Radix sophorae flavescens (Kushen), Radix sanguisorbae (Diyu), Rhizoma bletilieae (Baiji), Radix glycyrrhizae (Gancao), and Indigo naturalis (Qingdai). As a peroral decoction, CSD has been used to treat thousands of patients with UC and has demonstrated to be efficacious. In our previous study, we found that miR-155 inhibition ameliorates TNBS-induced colitis by regulating the differentiation and function of Th17 cells; Jarid2/notch1 is closely related with the process with the possible co-ordination of STAT3 with AhR to regulate the differentiation of Th17 and Treg cells [11-12]. Overexpression of microRNA-200b reduced tumorgenesis and mortality by inhibiting AOM/DSS-induced inflammation and EMT (epithelial-mesenchymal transition). The anti-inflammatory effect of microRNA-200b was achieved by inhibiting the pro-inflammatory cytokines (including TNF-α, TGF-β, and NF-κB/IL-6/STAT3) [13]. Additionally, it was found that CSD could improve the symptoms and pathological damage in mice with colitis. The administration of CSD reduced the level of inflammatory factors including interleukin IL-1β, tumor necrosis factor TNF-α, and phospho-NF-κBp65, and also decreased the proportion of Th17 cells in the spleen and MLNs (mesenteric lymph nodes) and the expression of ROR-γT, IL-17A, STAT3, IL-6 in colonic tissues. The percentage of Treg cells in the spleen and MLNs and the expression of FOXP3, transforming growth factor TGF-β1, and IL-10 in colonic tissues were up-regulated ere up-regulated ere up-regulated [14]. However, the manner in which CSD exerts its therapeutic role is not clear and we were unable to find any relevant studies when we conducted a systematic search on pathological organisms in our series of studies. The lesions caused by UC are mainly observed in the digestive tract. Further studies are required to elucidate whether these lesions affect the metabolism in the whole body, to determine of metabolic pathways that may be affected, and to ascertain if CSD has a positive effect on the organism of UC. Therefore, exploring the effects of CSD on rats with UC from the perspective of metabolomics may help understand the mechanism of CSD.

Pathological disorders of the body and changes in the types and concentrations of endogenous substances can be reflected using metabolomics. A comprehensive metabolomics analysis of organisms not only helps people better understand the process of disease development and the metabolic pathways of substances in the body, but also helps to screen out the early biomarkers of a disease to achieve the goal of early clinical diagnosis. Metabolomics is a newly developed field after genomics and proteomics that is contributing to early diagnosis of disease [15-16]. Metabolomics is an important part of systems biology and has been routine and popular in the search for disease biomarkers and research mechanisms of drug action [17-19]. At present, metabolomics has been widely used to study the disease states of diabetes, prostate cancer, and liver damage among others [20-21]. Metabolomics relies heavily on advanced instrumental analyses, with LC-MS/MS, NMR, and GC-MS being more common; LC-MS/MS is more advantageous owing to its high throughput screening- and high-resolution abilities [22-26]. In this study, we used an LC-MS/MS-based metabolonomic approach to identify metabolite markers associated with UC, evaluate the effects of UC on metabolic pathways, and explain the mechanism of CSD in the treatment of UC.

Methods

Preparation of CSD aqueous extract

CSD consists of five Chinese herbs, namely, Kushen, Diyu, Baiji, Gancao, and Qingdai that were purchased from Hubei Provincial Hospital of TCM (Hubei, China). Samples of all plants were deposited in the herbarium of the Hubei Province Academy of Traditional Chinese Medicine and authenticated by Professor WU He-Zhen. The proportion of daily raw herbs dose was 15 : 15 : 3 : 10 : 10 (Table 1). The decoction was prepared according to a previous preparation method [16]. Briefly, the herbs were mixed and soaked in cold distilled water for half an hour. The contents were boiled over high heat, then cooked over mild fire for half an hour and the decoction was filtered. Boiling water was added to the dregs of the decoction and the above decocting methods were performed twice. Subsequently, three filtrates were collected in

Table 1  The composition of CSD

<table>
<thead>
<tr>
<th>Chinese Name</th>
<th>Latin Name</th>
<th>Daily dose/g</th>
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<tbody>
<tr>
<td>Kushen</td>
<td><em>Sophora flavescens</em> Ait.</td>
<td>15</td>
</tr>
<tr>
<td>Diyu</td>
<td><em>Sanguisorba officinalis</em> L.</td>
<td>15</td>
</tr>
<tr>
<td>Qingdai</td>
<td><em>Polygonum tinctorium</em> Ait.</td>
<td>3</td>
</tr>
<tr>
<td>Baiji</td>
<td><em>Bletilla striata</em> (Thunb. Reichb. f.) Diels.</td>
<td>10</td>
</tr>
<tr>
<td>Gancao</td>
<td><em>Glycyrrhiza uralensis</em> Fisch.</td>
<td>10</td>
</tr>
</tbody>
</table>
the presence of loose stools, mucus, and bloody stools, mod-
days, model rats with a score of 1 or less were excluded. In
itored. The parameters of the disease activity index (DAI)
stool consistency, and stool occult blood were daily mon-
movement. The food and water intake, body weights,
modeling, and were given access to free diet after unrestric-

mL·kg\(^{-1}\)) solution in a proportion of 12 : 5 and the mixture was injec-
to a UC model using TNBS (mix 5\% TNBS and 50\% pento-

Laboratory Animals (NIH Publications No. 8023, revised

Saline was provided by Sichuan Cologne Pharmaceutical-
al Co., Ltd. (Sichuan, China); ultrapure water was prepared
using a UPT-11-1OT water-purification system (Ulpure, China); acetonitrile and methanol (MS grade) were pur-
chased from Merck (Germany); formic acid was bought from
Aladdin Industrial Corporation (USA); 5\% TNBS and pento-
barbital sodium were purchased from Sigma Aldrich (USA);
absolute ethanol was bought from Sinopharm Chemical Re-
agent Co., Ltd. (Shanghai, China); 8,11,14-icosaatrienoic acid,
pyroglutamatic acid, taurocholic acid, adenosine 5’-monophos-
phate monohydrate, thiamin hydrochloride, carnosin, 2,6-
diaminopimelic acid, xanthurenic acid, orotic acid, nicotin-
amide, 5-methoxytryptamine, oleic Acid, glutathione, pyro-
glutamatic acid, and cysteine were purchased from Sigma Ald-
rich (USA). Tyrosine, aspartic acid, phenylalanine, lysine,
citric acid, and L-histidine were purchased from the National
Institutes for Food and Drug Control (Beijing, China).
Arachidonic acid, cytidine, and lactic acid were purchased from
Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai,
China).

Animal and treatment
All experiments complied with the ARRIVE guidelines and
carried out in accordance with the U.K. Animals (Sci-
entific Procedures) Act, 1986 and associated guidelines, EU
Directive 2010/63/EU for Animal Experiments, or the Na-
tional Institutes of Health Guide for the Care and Use of
Laboratory Animals (NIH Publications No. 8023, revised
1978).

Wistar rats (200−220 g) were supplied by the Beijing Vi-
tal River Laboratory Animal Technology Co., Ltd. (Beijing,
China). The animals were housed under standard laboratory
conditions at a temperature of 25 ± 2 °C, relative humidity
of 50%−55%, a 12 h light/dark cycle, and acclimatized to the
environment at least one week before animal experiments.
After 24 h of fasting, all rats were anesthetized with 1% pentob-
arbital sodium (40 mg·kg\(^{-1}\) body weight), and 14 rats
(7 male and 7 female) were randomly selected, which constit-
tuted the normal group. The remaining rats were induced in-
to a UC model using TNBS (mix 5\% TNBS and 50\% ethanol
solution in a proportion of 12 : 5 and the mixture was injec-
ted into the rat colon (4.25 mL·kg\(^{-1}\) body weight) through the
anus with a rubber hose, 2 mm in diameter). The rats in the
normal group were injected saline into the colon (4.25 mL·kg\(^{-1}\) body weight). The rats were laid flat after the end of
modeling, and were given access to free diet after unrestric-
ted movement. The food and water intake, body weights,
stool consistency, and stool occult blood were daily mon-
tored. The parameters of the disease activity index (DAI)
used to evaluate mouse colitis are listed in Table 2. After 7
days, model rats with a score of 1 or less were excluded. In
the presence of loose stools, mucus, and bloody stools, mod-
el rats that exhibited a loss of appetite, laziness, weight loss,
arched back, dullness, etc. were used for later studies \cite{27, 28}.
The TNBS-induced UC model was randomly divided into four
groups (n = 12): low (0.25 g·mL\(^{-1}\)), medium (0.50 g·mL\(^{-1}\)), and high (1.0 g·mL\(^{-1}\)) CSD gavage groups, and a
model group. Normal and model groups received gavages
with the same volume of pure water (All groups received
gavages at 5.0 mL·kg\(^{-1}\) body weight). Each group was admin-
istered either the drug or water once a day between 9: 30 and
11: 00 a.m. for 10 days.

Sample collection and pretreatment
After ten days of CSD intervention, rat urine was collect-
ted. Blood was collected from the abdominal aorta under an-
esthesia [1% sodium pentobarbital (40 mg·kg\(^{-1}\) body
weight)], then the colon, liver, and kidneys were collected.
Body fluid samples were centrifuged at 3000 r·min\(^{-1}\) for 10
minutes at 4 °C and the serum and urine supernatants were
separated. All samples were stored at −80 °C until further use.

LC-MS/MS sample preparation
After the serum samples thawed at 4 °C, 10 μL of each
sample was accurately taken and mixed to obtain a serum
combined quality control (QC) sample. To the accurately as-
pirated 100 μL of serum sample, 400 μL of methanol in a
volume ratio of 1:4 was added, vortexed for 30 seconds, and
centrifuged at 3000 r·min\(^{-1}\) for 10 minutes at 4 °C to deposit
protein. Then, 300 μL of the supernatant was accurately col-
lected and blow-dried with nitrogen at 40 °C. The residue
was dissolved in 100 μL of 50% methanol solution and cen-
trifuged at 12 000 r·min\(^{-1}\) for 5 min at 4 °C. The supernatant
was transferred to auto-sampler vials for LC-MS/MS analy-
sis.

After thawing at 4 °C, 15 μL of each urine sample was
mixed to get a QC sample. 400 μL urine samples were added
to 800 μL methanol to a volume ratio of 1 : 2. The mixture
was vortexed for 1 min and centrifuged at 12 000 r·min\(^{-1}\) for
10 min at 4 °C. The supernatant was transferred to the auto-
sampler vials for analyzing by LC-MS/MS.

All tissue samples were thawed at 4 °C. Tissue samples
(150 mg) from the same part of each organ were weighed
accurately and 750 μL of methanol aqueous solution (V/V = 2 : 1)
stored at −20 °C for 12h was added at a ratio of 1 mg: 5
μL. It was then homogenized using a high-throughput tissue
grinder (Model SCIENTZ-48, Ningbo Xinzhi Biotechnology
Co., Ltd. China). Then, 50 μL from each homogenate was
centrifuged at 12 000 r·min\(^{-1}\) for 10 min at

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Details of DAI score</th>
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<td>Score</td>
<td>Weight loss</td>
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<td>0</td>
<td>——</td>
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<tr>
<td>1</td>
<td>1%−5%</td>
</tr>
<tr>
<td>2</td>
<td>5%−10%</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 10%</td>
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</table>

4 °C and 450 μL of the supernatant was transferred to the EP tube. To the remaining tissue precipitate, 500 μL of aqueous methanol solution (V/V = 2 : 1) was added and homogenized, and this method was conducted twice as described above. All supernatants (total 1350 μL) were mixed and blow-dried using nitrogen gas at 40 °C. The residue was dissolved with 100 μL of 50% methanol solution and centrifuged at 12 000 r·min⁻¹ for 5 min at 4 °C. The supernatant was transferred to the auto-sampler vials for LC-MS/MS analysis.

The pooled QC sample was run at the beginning of the whole sample list for method validation and to condition and equilibrate the system, following which, six samples were analyzed at a time during the analytical run to further monitor or system stability. It was critical to acquire QC data for assessing the changes in the analytical results and to monitor the reliability of the metabolites.

**LC-MS/MS conditions**

Metabolic profiling of serum and urine was conducted on an Agilent-1260 LC system coupled with an AB SCIEX 4000 QTRA mass spectrometer, which was equipped with an electrospray ionization (ESI) ion source with a positive and negative ion mode. Chromatographic separation was performed on an Agilent Zorbax SB-C₁₈ column (2.1 mm × 150 mm, 5 μm) and protected with an Agilent Zorbax SB-C₁₈ column (2.1 mm × 12.5 mm, 5 μm) with the column temperature set at 30 °C. The flow rate was 0.3 mL·min⁻¹ and the mobile phase was ultrapure water with 0.015% formic acid (A) and acetonitrile (B). The gradient elution programs for serum of A was performed as follows: at 0–2 min 93% A, at 2–4 min 93%–50% A, at 4–10 min 50%–47% A, at 10–15 min 47%–20% A, at 15–20 min 20%–6% A, at 25–35 min 6% A. The gradient elution for urine samples were as follows: at 0–2 min 96% A, at 2–3 min 96%–78% A, at 3–7 min 78% A, at 7–12 min 78%–70% A, at 12–15 min 70%–52% A, at 15–18 min 52%–15% A, at 18–22 min 18%–5% A, and at 22–35 min 5% A. Gradient elution programs for each tissue extract were performed as showed in Table 3. The sample injection volume was 3 μL.

The parameters of mass detection were set as followed: In positive ion mode, the setting was Curtain Gas (CUR): 35 psi, IonSpray Voltage (IS): 5500 V, Temperature (TEM): 350 °C, Ion Source Gas1 (GS1): 50 psi, Ion Source Gas2 (GS2): 55 psi; In negative mode, CUR: 35 psi, IS: –4500 V, TEM: 350 °C, GS1: 50 psi, GS2: 55 psi; scan range was from m/z 70 to 1000 Da.

**Multivariate data analysis and data processing**

All raw data collected by the LC-MS/MS system was converted to document format using Analysis Base File Converter and then processed using the MS-Dial with default settings to carry out baseline correction, retention time correction, peak discrimination, and alignment based on the QC sample. Then, a data matrix containing information such as m/z, retention time, and corresponding peak area was obtained. The data matrix was imported into SIMCA-P (version 14.1, Umetrics) software in the “.csv” format for multivariate statistical analysis; PCA and PLS-DA were performed for pattern recognition. Variable importance projection (VIP) produced by PLS-DA was applied to discover the contributable-variable for classification. GraphPad Prism7 software was used for student’s t-test and a value of P < 0.05 was considered to be statistically significant. The variables with VIP values > 1.5 and P < 0.05 were considered as potential biomarkers. Deep fragmentation of these potential biomarkers using the MS2 model was performed and searched in databases including HMDB (http://www.hmdb.ca/), KEGG (https://www.genome.jp/kegg/), and Metlin (https://metlin.scripps.edu/) for the identification of biomarkers. Lastly, some of the identified compounds were verified using commercial reference standards. The data obtained from the above analysis were uploaded to MetaboAnalyst (https://www.metaboanalyst.ca/) data processing platform for statistical verification and metabolic-pathway analysis.

**Results**

**Data acquisition based on LC-MS/MS**

The typical total ion current (TIC) chromatograms of serum and urine, and the colon, liver, and kidney tissues of the study groups were obtained using LC-MS/MS. The typical base peaks chromatogram of all samples are shown in Fig. 1. QC samples were used to evaluate the repeatability and ac-

<table>
<thead>
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<th>Table 3  Gradient elution conditions of the test materials for each tissue extract</th>
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<td>t/min</td>
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<td>0–2</td>
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<tr>
<td>25–35</td>
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accuracy of the LC-MS/MS system. Before data collection, 8 QCs were started to condition the analysis prior to testing the samples. A QC sample was analyzed once for every 6 samples analyzed. Based on the retention time (Rt) and peak

Fig. 1    The typical TIC chromatograms of serum (A), urine (C), colon extract (E), liver extract (G), kidney extract (I) in positive mode. The typical TIC chromatograms of serum (B), urine (D), colon extract (H), liver extract (F), kidney extract (J) in negative mode.
area changes, six ions were selected to evaluate the accuracy, stability, and repeatability of the established method (serum: Rt-m/z 3.81–205.1, 13.07–432.1, 18.12–303.4 in positive ion mode and 3.79–203.5, 9.42–228.2, 22.49–303.3 in negative ion mode; urine: Rt-m/z 2.94–199.2, 12.23–279.3, 19.92–426.5 in positive ion mode and 3.81–254.3, 18.10–269.0, 25.14–339.3 in negative ion mode; colon extract: Rt-m/z 4.42–187.9, 15.91–426.7, 25.92–88.2 in positive ion mode and 4.40–203.5, 14.57–225.3, 28.56–62.5 in negative ion mode; kidney extract: Rt-m/z 4.24–187.7, 14.00–118.5, 23.76–86.4 in positive ion mode and 2.97–97.5, 12.41–349.6, 26.97–255.6 in negative ion mode; liver extract: Rt-m/z 3.37–77.1, 14.10–932.1, 22.12–184.3 in positive ion mode and 2.54–135.5, 15.02–225.5, 28.74–279.3 in negative ion mode). The RSD of retention time was less than 15%, negative ion mode (cum) was used to estimate the degree of fit of the model, and compared to PCA, PLS-DA was a supervised analytical approach that focused on discovering differences in the dataset. Compared to PCA, PLS-DA was a supervised analytical approach that focused on discovering differences in the dataset. The PCA and PLS-DA models were used to distinguish different groups. Although the unsupervised PCA could distinguish between different groups, the PLS-DA model was more distinct than the PCA model. The main role of PCA was to find intrinsic changes in the dataset. Compared to PCA, PLS-DA was a supervised analytical approach that focused on discovering changes in data. The $Q^2$ and $R^2$ values were calculated based on the results from the SIMCA-P package. The value of $R^2$ (cum) was used to estimate the degree of fit of the model, which represented the fraction of the interpreted y-variation.

**Multivariate data analysis**

LC-MS/MS-based analysis was used to detect abnormal metabolism in the organism, which could more accurately monitor the changes of endogenous metabolites and reflect the state of the biological system. Biomarkers can be used to determine exposure to anomalous substances, thereby providing a platform for monitoring anomalous substances and assessing the harmful effects on the organism. The PCA and PLS-DA models were used to distinguish different groups. Although the unsupervised PCA could distinguish between different groups, the PLS-DA model was more distinct than the PCA model. The main role of PCA was to find intrinsic changes in the dataset. Compared to PCA, PLS-DA was a supervised analytical approach that focused on discovering changes in data. The $Q^2$ and $R^2$ values were calculated based on the results from the SIMCA-P package. The value of $R^2$ (cum) was used to estimate the degree of fit of the model, which represented the fraction of the interpreted y-variation.

**Table 4 Method validation of the analysis system (RSDs)**

<table>
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<td>0.26</td>
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<td>0.88</td>
<td>0.79</td>
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<td>3.33</td>
<td>0.05</td>
<td>9.51</td>
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<td>0.12</td>
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<td>7.24</td>
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and $Q^2$ (cum) estimated the ability to predict. A good model was obtained when the values of $R^2Y$ and $Q^2$ were both higher than 0.5 and the difference between the two was not more than 0.3. Except for the models obtained from the colon and liver tissue extracts in positive ion mode, the $R^2Y$ and $Q^2$ values of PLS-DA analysis of other models were greater than 0.78, and the difference between the two was also less than 0.3, indicating that the model had good interpretation and prediction capabilities.

Consequently, the PLS-DA method was employed to find out the specific variation between the model, normal, and treatment groups. The PCA model obtained by collecting data from the colon and liver tissue extracts in the positive ion mode [Fig. 2 C(1) and D(1)] showed that the biological samples in each group were mixed and the separation was not obvious. It indicated that in these two models, the data change between groups was not obvious. In other words, the difference between the components of the extracts of the two tissues detected in the positive ion mode was not obvious. The PLS-DA scores plots for serum [Fig. 2 A(3) and A(4)], urine [Fig. 2 B(3) and B(4)], colon tissue extract [Fig. 2 C(4)], liver tissue extract [Fig. 2 D(4)], and kidney tissue extract [Fig. 2 E(3) and E(4)] showed that the separation between the normal, model, and treatment groups was obvious, which suggested that TNBS-induced UC disrupted the metabolism of endogenous substances and significantly altered metabolites in rats. The PCA scores plot for serum [Fig. 2 A(1) and A(2)], urine [Fig. 2 B(1) and B(2)], colon tissue extract [Fig. 2 C(2)], liver tissue extract [Fig. 2 D(2)] and kidney tissue extract [Fig. 2 E(1) and E(2)] also showed that there was a satisfactory classification among the clustering of the normal, model, and treatment groups. Compared to the model

![PCA and PLS-DA score plots](image)

**Fig. 2** PCA and PLS-DA score plots. (1) PCA score plots between the five groups in positive mode; (2) PCA score plots between the five groups in negative mode; (3) PLS-DA score plots between the five groups in positive mode. (4) PLS-DA score plots between the five groups in negative mode. A: serum sample; B: urine sample; C: colon tissue extract sample; D: liver tissue extract sample; E: kidney tissue extract sample
group, the CSD medium group was very similar to the normal group, which showed the performance of UC interference metabolic state recovery. 

Potential biomarkers of UC

Variable importance on projection (VIP) values was used to identify the features contributing to group separation.

Based on the PLS-DA model, ions with VIP value above 1.5 were selected (Fig. 3) and an independent sample t-test was conducted. The ions whose $P$-values were less than 0.05 were considered as potential biomarkers. Fragmentation information of potentially differential metabolites were obtained using mass spectrometry in the MS2 mode. Possible molecular

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**Fig. 3** VIP score (serum: A, B are positive ion mode and negative ion mode, respectively. Urine: C, D are positive ion mode and negative ion mode, respectively. Colon tissue extract: E, F are positive ion mode and negative ion mode, respectively. Liver tissue extract: G, H are positive ion mode and negative ion mode, respectively. Kidney tissue extract: I, J are positive ion mode and negative ion mode, respectively)
formulas for potential biomarker information were obtained by searching a freely accessible database of KEGG, Metlin, and HMDB. A total of 36 endogenous metabolites were matched and some of them were confirmed using commercial reference standards. Information of potential biomarkers are summarized in Table 5. Compared to the normal group,

Table 5  Summary of potential biomarker information

<table>
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<tr>
<th>Matrix</th>
<th>Compound</th>
<th>Formula</th>
<th>Model</th>
<th>Medium</th>
<th>Confirm</th>
<th>LOD (ng·mL⁻¹)</th>
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<tr>
<td></td>
<td>8,11,14-Icosatrienoic acid</td>
<td>C₂₀H₃₂O₂</td>
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<td>↓</td>
<td>Y</td>
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<tr>
<td></td>
<td>Citric acid</td>
<td>C₆H₈O₇</td>
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<td>↓</td>
<td>Y</td>
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</tr>
<tr>
<td></td>
<td>3b-Hydroxy-5-cholenoic acid</td>
<td>C₁₂H₂₄O₃</td>
<td>↓</td>
<td>↑</td>
<td>N</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Pyroglutamic acid</td>
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<td>↓</td>
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<td>0.059</td>
</tr>
<tr>
<td></td>
<td>Docosahexaenoic acid</td>
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<td>↑</td>
<td>N</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Arachidonic acid</td>
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<td>↓</td>
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</tr>
<tr>
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<td></td>
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<td>↓</td>
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<td>↓</td>
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<td>↓</td>
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<td></td>
<td>Mono-benzyl phthalate</td>
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<td>↑</td>
<td>N</td>
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<td>↑</td>
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<td>↓</td>
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<td>↓</td>
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<td>Dimethylglycine</td>
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The up- (or down-) arrows represent the relatively increased (or decreased) levels of the potential metabolite markers in model group or in CSD medium dose group. a Compared with normal group. b Compared with model group.
these potential biomarkers had different degrees of up- or down-regulation in the UC group; however, there was a tendency to return to normal after intervention with CSD (Fig. 4). The results effectively indicated that CSD could correct abnormal levels of potential biomarkers and prove to be efficacious in the management of UC.

**Statistical verification and metabolism pathway analysis**

Based on the metabolites that significantly reversed by CSD, statistical verification and detailed analysis of the impact of pathways were performed using MetaboAnalyst. The PCA and PLS-DA scores (Figs. 5A, 5B) between the model and the normal groups showed that the 36 potential biomarkers were different between them; PCA and PLS-DA scores among the five groups (Figs. 5C, 5D) also showed statistical results to be consistent with SIMCA-P analysis. It could also be seen from the results of the heat map that the levels of these 36 potential biomarkers in the model group were either up- or down-regulated to varying degrees relative to the normal group (Fig. 5E). According to pathway impact (Fig. 5F), each point showed a metabolic pathway and the impact of this metabolic pathway positively correlated with the size of the dot and the shade intensity of the color. The analysis showed that the potential biomarkers that were screened had a significant impact on phenylalanine, tyrosine, and tryptophan biosynthesis, and on metabolic pathways including that of phenylalanine, thiamine, glutathione, arachidonic acid, glyoxylate and dicarboxylate, nicotinate and nicotinamide, alanine, aspartate and glutamate metabolism, glycolysis or gluconeogenesis, pyrimidine, purine, and the citrate cycle (TCA cycle).

**Evaluation of biomarkers**

Receiver operating characteristic curve (ROC) is a comprehensive indicator reflecting the continuous variables of sensitivity and specificity. It is used in diagnostic tests to evaluate disease recognition ability. The ROC analysis between the normal and model groups was conducted to assess the diagnostic performance of the 36 biomarkers of UC (Fig. 6). The area under the ROC curve was greater than 0.75 and even most was greater than 0.9, indicating that the diagnostic capabilities of these different metabolites were very good.

**Discussion**

As formulated under the strict theory of TCM, CSD has been used in clinical treatment in more than 3 000 patients with UC, with an effective rate of more than 96%, and without any serious adverse events. CSD is composed of five traditional Chinese medicines. Kushen is the sovereign drug and alkaloid compounds are the main components in Kushen. In our previous research, we characterized the crude extract of CSD and quantitatively analyzed the main components of the sovereign drug. Matrine and oxymatrine in Kushen are clearly detected in CSD. In addition, glycyrrhizin and glycyrrhetic acid in Gancao, and indigo and indirubin in Qingdai were successfully characterization. The main component of Baiji is polysaccharides. In our previous study, we used thin-layer chromatography to detect the presence of Baiji in CSD. Furthermore, we detected the content of matrine and oxymatrine in CSD using HPLC. After analyzing more than ten batches of the test results, we determined the content of matrine to be in the range of 0.3951–0.4729 mg·mL⁻¹ and that of oxymatrine in the range of 0.1001–0.1631 mg·mL⁻¹, and thus preliminarily formulated a detection method to control the quality of CSD. Besides, we also attempted to identify the active constituents in CSD using high-resolution mass spectrometry (HRMS) and part of a summary of the various constituents detected and identified using the channels of methanol and pure water extraction were given. HRMS confirmed oxymatrine as the most abundant ingredient in CSD. The active compounds obtained after the methanol extraction of CSD were as follows: oxymatrine, isoliquiritigenin, (−)-maackiain, DL-stachydrine, cytisine, indirubin, 18-β-glycyrrhetinic acid, licochalcone A, xanthohumol, 7,8-dihydroxy-4-methylcoumarin, and naringenin. Simultaneously, the active ingredients obtained using pure water extraction were as follows: oxymatrine, isoliquiritigenin, DL-stachydrine, cytisine, (−)-maackiain, 18-β-glycyrrhetinic acid, 7, 8-dihydroxy-4-methylcoumarin, and naringenin. We also conducted a chemical analysis and detected matrine, oxymatrine, and glycyrrhizin in the serum after administration of CSD. However, it is not clear how these components in CSD are metabolized in the body and the metabolic changes these components undergo; hence, additional studies to elucidate these processes are required. ZHOU Qi-Hang et al. reviewed the interactions between human drug-metabolizing enzymes and the constituents of Psoraleae Fructus; the major constituents in Psoraleae Fructus along with their chemical structures and metabolic pathways are summarized. The inhibitory and inductive effects of the constituents in Psoraleae Fructus on human drug-metabolizing enzymes (DMEs) will be helpful to pharmacologists to gain a deeper understanding of the metabolic fate of the components of the Chinese herb Psoraleae Fructus in both human and animals. This has guiding significance for the in-depth study of CSD. The use of herbal products as dietary supplements and herbal therapeutics is increasing worldwide. As did in the past and the current issue, the information on the metabolic fates of herbal constituents in the human body and the interactions of herbal constituents with the human metabolic system are useful in avoiding the occurrence of adverse drug events. Therefore, a comprehensive study of the ingredients of Chinese herbal medicine and the study of its overall mechanisms of action are important, which would have a positive impact on promoting the modernization of traditional Chinese medicine.

In this study, we investigated the changes in metabolic profiles by using LC-MS/MS and pattern recognition analysis. The results of LC-MS-based metabonomics analysis of the five samples constituting CSD indicated that the decoction had a clear impact on TNBS-induced UC metabolites dis-
Fig. 4  Comparison of different potential biomarkers of normal group, model group, and CSD-treated group. P < 0.05 vs the model group; a. Normal, b. Model, c. CSD (medium)
Fig. 6  ROC curves for different potential biomarkers of normal group, model group
order and could redress the perturbation of metabolites. The metabolites in the arachidonic acid metabolic pathway mainly include 8,11,14-icosatrienoic acid, docosahexaenoic acid, and arachidonic acid. As an important precursor for prostaglandins and leukotrienes, the level of arachidonic acid is elevated when inflammatory factors are introduced \cite{33-34}. Individuals with relatively high concentrations of arachidonic acid in adipose tissue have a significantly increased risk of UC. Eicosapentaenoic and docosahexaenoic acids are known to protect against UC, and the antagonistic arachidonic acid and its derivative, eicosanoic acid, effectively reduce the severity of UC in individuals \cite{35-36}. There was a significant positive correlation between arachidonic acid and inflammatory response. Our previous study indicated that inflammatory factors IL-1β, TNF-α, and NF-κBp65 are significantly elevated in animals with ulcerative colitis. Arachidonic acid is the key substances in the metabolic pathway of arachidonic acid, and regulating its levels can help reverse inflammatory diseases \cite{37}. The levels of 8,11,14-icosatrienoic and arachidonic acids in the model group were up-regulated compared to the normal and CSD treatment groups; docosahexaenoic acid in the model group was down-regulated compared to the normal and CSD treatment groups, which indicated that CSD played a role in the imbalanced metabolism. Thus, our results suggested that CSD might exert its anti-inflammatory effects by affecting the arachidonic acid metabolism pathway.

Thiamine (vitamin B1) is a water-soluble vitamin and a metabolite of the thiamine metabolic pathway. It is an enzyme cofactor that plays an important role in energy metabolism and is associated with oxidative stress. Thiamine is also an essential vitamin necessary to maintain the functional integrity of brain cells. In the human colon, the microbiota synthesizes large amounts of thiamine in the form of thiamine pyrophosphate (TPP). The expression of human TPP transporter in the colon is high and the TPP produced by the microbiota is absorbable, which may help regulate steady state thiamine levels in the host \cite{38-40}. In the colitis model group, thiamine levels were up-regulated, which may be the result of the improved absorption of thiamine in the colonic mucosa of rats in the pathological state, or the imbalance of microbiota in rats in the pathological state, which resulted in the increased synthesis and abnormal metabolism of thiamine.

Glyoxylate and dicarboxylate metabolism, glycolysis or gluconeogenesis, and TCA cycle are several forms of energy metabolism. Glycolysis supplies energy in an anaerobic state and its metabolite, lactic acid, is one of the main sources of carbon in the TCA cycle. Citric acid in the experiment is closely related to the metabolic glyoxylate pathway, dicarboxylate metabolism, and the TCA cycle, which is the hub of metabolism of sugars, lipids, and amino acids and closely related to a variety of diseases. Citric acid is an important compound which plays a role in the conversion of fats, proteins, and sugars into carbon dioxide. These chemical reactions constitute the core reaction of all metabolic pathways.

Niacinamide, a metabolite of nicotinate and nicotinamide metabolism, effectively reduces serum phosphorus and leads to the potentially beneficial increase of high-density lipoprotein cholesterol. Niacinamide plays an active role in the treatment of various immune diseases and bacterial skin diseases, is involved in the metabolism of proteins and sugars, and is closely related to inflammatory reactions. The deficiency of niacinamide affects the normal respiration and metabolism of cells and causes pellucid. Niacinamide is easily absorbed by the gastrointestinal tract and distributed to the entire body. It is metabolized by the liver and only a small amount is excreted unchanged in the urine \cite{41-43}.

Purine and pyrimidine have long been used as the construction units of nucleic acid synthesis and the intermediate of metabolic energy transfer. Pyrimidine and purine metabolism are metabolically balanced during normal states; a disturbance in their metabolism can cause conditions such as metabolic acidosis and hyperuricemia. Moreover, oxidative stress leads to a general increase in purine catabolism. The abnormal metabolism of purine and purine may lead to diseases related to the nervous, circulatory, and immune systems, and the inhibition of pyrimidine biosynthesis can be effective in treating immune inflammatory diseases \cite{44-46}.

Phenylalanine and tyrosine are closely related to phenylalanine, tyrosine, and tryptophan biosynthesis, and phenylalanine metabolism. Both phenylalanine and tyrosine are essential amino acids, which play an important role in the metabolism and growth of humans and animals. Most of the phenylalanine is converted to tyrosine by the catalytic conversion of phenylalanine hydroxylase and tetrahydrobiopterin. Abnormal metabolism of phenylalanine and tyrosine can cause phenylketonuria and the level of phenylpyruvate has a negative correlation with the oxidative stress state of the body. Studies have also found that chronic immune stimulation is closely related to phenylalanine conversion, and chronic low-grade inflammation is associated with changes in tyrosine metabolism \cite{47-49}.

Glutathione is very important for its resistance to oxidation and for detoxification. It is found in almost every cell in the body and is one of the most abundant antioxidants that helps maintain normal function of the immune system \cite{50-51}.

One of the main purposes of metabolomics research is to investigate the mechanisms of disease pathology and help in disease diagnosis. In this study, 36 potential biomarkers related to UC were screened from rats with UC, involving multiple metabolic pathways in the body. These differences could help understand the development process of UC and provide a new reference in its early screening. We thus identified the metabolic pathways that were mainly affected by TNBS-induced ulcerative colitis (Fig. 7). When the intestinal tract is strongly stimulated, there is an imbalance created in the oxidative and antioxidant systems of rats, which evokes an oxidative stress response. After being stimulated, the body will engage in antioxidant behaviors to counter the occurrence of the oxidative stress response to maintain the balance between the oxidative and antioxidant systems. We detected signific-
ally up-regulated metabolites from the adenosine monophosphate in the purine metabolism pathway associated with oxidative stress and significantly up-regulated thiamine levels involved in oxidative stress. The levels of glutathione and pyroglutamic acid that are closely related to antioxidant and detoxification reactions were significantly upregulated and the phenylalanine metabolite in the phenylalanine metabolic pathway, which was inversely related to oxidative stress, was significantly down-regulated. Intestinal tissue damage along with pyrimidine metabolism and arachidonic acid metabolism pathway disorder could lead to inflammation, which elicits a series of immune responses to inflammation by the immune system. We detected significantly up-regulated levels of orotic acid and carnosine that characterized the level of pyrimidine metabolism; we also found significantly up-regulated levels of arachidonic acid, which are positively correlated with inflammatory factors. Significantly down-regulated levels of docosahexaenoic acid that antagonizes arachidonic acid, and taurocholic acid that inhibits the production of inflammatory mediators were also detected. Inflammation stimulates the nicotinate and nicotinamide metabolism pathways and leads to a reduction in phenylalanine conversion. We detected down-regulated tyrosine levels and an increased level of nicotinamide. Intestinal damage weakens the absorption of nutrients and leads to a significant reduction in energy utilization, which in turn leads to insufficient energy supply and stimulates the process of glycolysis and protein decomposition. Stimulation of glycolysis accelerates the TCA cycle to meet the energy supply of the body. We observed the wasting state of rats with UC and detected significant up-regulation of related metabolites such as lactic acid, citric acid, cysteine, and aspartic acid.

As part of systems biology, metabolomics can explain the mechanism of action of CSD from a holistic perspective. By comparing the CSD, normal, and model groups, it was easy to indicate the efficacy of CSD in UC. However, the absence of a positive drug group was a drawback of this study. In the absence of a positive control, we could not clearly indicate the superiority of CSD to existing marketed drugs (such as Mesalazine). Traditional Chinese medicine and its prescription are used as multi-component, multi-target, and multi-channel therapies in the overall regulation of ailments. Although the use of a single-component listed drug as a positive control cannot be accurately compared to multi-component traditional Chinese medicines, the existence of a positive control is still meaningful. Therefore, in our in-depth re-
search in the next step, adding a positive control group is an important consideration to improve experimental research.

Our study results revealed that CSD could recall the level of differential metabolites caused by UC. The specific performance of CSD may be targeted at the regulation of glutathione and purine metabolism and maintaining the balance of the oxidation and antioxidant systems. CSD improved immunity and recovery of docosahexaenoic and docosapentaenoic acids that are conducive to antagonize arachidonic acid, and regulated the level of inflammatory factors in the body, restored the level of taurocholic acid to inhibit the production of inflammatory mediators, and exerted an anti-inflammatory effect. Additionally, it improved the status of intestinal ulcer, restored the TCA cycle and glycolytic metabolism, and lastly, restored the energy supply state of the body.

**Conclusion**

An LC-MS/MS method was used to profile the metabolites of rats with UC. Using a library search and standard validation, a total of 36 differential metabolites were identified which were involved in multiple metabolic pathways. CSD could affect multiple metabolic pathways in TNBS-induced-UC rats to weaken their pathophysiological symptoms and obtain positive therapeutic effects.

**Abbreviations**

UC: Ulcerative colitis  
CSD: Compound Sophorae Decoction  
TNBS: 2,4,6-trinitrobenzenesulfonic acid solution  
STAT: Signal transducer and activator of transcription  
AhR: Aryl Hydrocarbon Receptor  
Treg cells: Regulatory cells  
AOM/DSS: Azoxymethane/dextran sodium sulfate  
EMT: Epithelial-Mesenchymal Transition  
TNF: Tumor necrosis factor  
TGF: Transforming growth factor  
NF-κB: Nuclear factor kappa-B  
IL-6: Interleukin 6  
MLNs: Mesenteric lymph node cells  
ROR: Retinoic Acid-related Orphan Receptor  
IL-17A: Interleukin 17A  
FOXp3: Forkhead/winged helix transcription factor p3  
QC: Quality control  
VIP: Variable importance projection  
TIC: The typical total ion current  
RSD: Relative Sandard Deviation  
PCA: Principal component analysis  
PLS-DA: Partial least squares discrimination analysis  
ROC: Receiver operating characteristic curve

**References**


