Chemical Profiles and Metabolite Study of Raw and Processed Cistanche Deserticola in rats by UPLC-Q-TOF-MSE

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Research

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Chemical profiles and metabolite study of raw and processed Cistanche deserticola in rats by UPLC-Q-TOF-MS

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ABSTRACT:
Background: Chinese materia medica processing is a distinguished and unique pharmaceutical technique in traditional Chinese Medicine (TCM), which has played an important role in reducing side effects, increasing medical potencies, altering the properties and even changing the curative effects of raw herbs. The efficacy improvement in medicinal plants is mainly caused by changes in the key substances through an optimized processing procedure. The effect of invigorating the kidney-yang for rice wine-steamed Cistancha deserticola was more strengthened than raw C. deserticola (CD).

Methods: To evaluate the effect of processing, a comparative analysis was conducted by utilizing the UPLC-Q-TOF-MSE with the UNIFI informatics platform. In vitro studies were performed for the characterization of constituents as well as metabolites in vivo, and chemical components were determined in CD and its processed products. The multivariate statistical analyses were conducted to evaluate variations between them. OPLS-DA was used for pairwise comparison which revealed their marked differences.

Results: In this study, the obtained results revealed considerable variations in phenylethanoid glycosides (PhGs) and iridoids after processing. The detection of 97 compounds was carried out in the extracts of CD and its processed product. In an in-vivo study, 10 prototype components and 44 metabolites were evaluated in rat plasma, feces, and urine. The obtained results revealed that processing leads to the considerable variation in the chemical constituents of CD and affects the disposition of the compounds in-vivo, and phase II metabolic processes were the key cascades of each compound and most of the metabolites were associated with echinacoside or acteoside.

Conclusions: According to our literature search, the existing study reveals a comparative study of raw and processed CD for the first time. The obtained data help us to understand the impact of CD processing for further studies.

Keywords: Cistanche deserticola; Processing; UPLC-Q-TOF-MS; Chemical profiles; metabolites in vivo
1 Introduction

*Chinese materia medica* (CMM) processing has shown considerable applications in the clinical practice of TCM and for several centuries it has been considered as a potential remedy. This is a unique pharmaceutical technology that has been derived from the theory of Traditional Chinese medicine (TCM). Post-processing, considerable variations have been observed in the appearance, chemical constituents, properties, and medicinal significance of all kinds of TCMs and it has been assumed that processing could promote the efficacy or decrease the toxic effects of the TCM.

For hundreds of years, *Cistanche deserticola* (*Roucongrong* in Chinese, CD) is commonly used in TCM clinical practice, which supplements the functions of the kidney. It also helps in the moisturizing of the intestine that leads to relaxing bowel [1]. The first time, Cistanche was recorded in *ShenNongBencaoJing*. It is commonly occurred in arid and semi-arid habitats across Eurasia and North Africa, including Iran, China, India, and Mongolia [2]. The processing of CD has been carried out by steaming with rice-wine under normal pressure, which is a preparation method documented in the Chinese pharmacopeia (*Jiucongrong* in Chinese, hereinafter called “CD-NP”). And CD steaming with rice-wine under high pressure is a more effective preparation method (hereinafter calling “CD-HP”) [3-4]. Several studies have been revealed that the pharmacological effects of CD are different from its processed products [5]. CD may tonify kidney-yang and relax bowel, while after being steamed by rice-wine, the effect of replenishing the kidney-yang would be strengthened. In our earlier study, it has been found that CD-NP could enhance tonification of the kidney and support yang, and relieve the effect of moistening intestines and defecating [6-8]. In clinical practice, the processed products are the most commonly used form.

Up to date, several studies have analyzed the chemical components of CD, followed by isolation and identification of more than 100 compounds [9-11], such as phenylethanol glycosides (PhGs), iridoids, lignans, and oligosaccharides as its main chemical constituents. It has also been reported that there are many pharmacological activities of PhGs including immunomodulatory, neuroprotective, hepatoprotective, anti-inflammatory, and anti-oxidative, etc. [12-14]. Iridoids possess anti-inflammatory activities [15-16]. It has also been revealed by earlier studies that some chemical components showed variations during the processing [17-20]. Based on underlined reports, it has also been considered that post-processing, the variations in chemical composition led to various pharmacological effects, which needs to be further explored.

In the current study, a sensitive and effective method *i.e.*, ultra-high performance liquid chromatography coupled with TOF-MS*È* (UPLC-Q-TOF-MS*È*) was performed for comparative analysis, and *in-vitro* studies were performed to qualitatively analyze the extracts of CD, CD-NP, and CD-HP which provided clarity in chemical profiles. Generally, the exogenous chemicals with high exposure in target organs were regarded as effective components. Therefore, in rats, CD and its processed products were orally administered respectively, followed by their characterization.
According to our literature search, the existing study reveals a comparative study (both in vitro and in vivo) of raw and processed CD for the first time. The obtained results would explore our knowledge regarding the effect of CD processing, which might be helpful for further studies.

**Materials and Methods**

**Materials**

Ajugol (180120) and standard compounds of 2'-actylacetoside (M0601AS) have been provided by Chendu Pure Chem-Standard Co., Ltd (Chengdu, China). Cistanoside F (MUST-17022620), echinacloside (D1105AS), cistanoside A(M0906AS), and isoacteoside (M0106AS) have provided by Must company (Sichuan China); acteoside (O0618AS), salidroside (J0526AS), catapol (S0728AS), geniposide (A0407AS), and geniposidic acid (MB6001-S) have acquired from Dalian Meilun Bio.Co., Ltd (Dalian, China). 8-Epideoxyloganic acid (B31123) have been obtained from Shanghai Yuanye Biological Technology Co., Ltd, China. Methanol and acetonitrile were of MS-grade and have been obtained from Merck KGaA, Darmstadt, Germany. Methanoic acid (CH$_2$O$_2$) of HPLC grade has been provided by Merck KGaA (Darmstadt, Germany). The water, used in the existing study has further been processed via the Milli-Q system (18.2 MΩ, Millipore, Ma, USA). Rice-wine has been provided by Brand Tower Shaoxing Wine Co., Ltd. (Zhejiang, China).

*Cistanch deserticola* was collected from Neimenggu wangyedi cistanche Co. Ltd. The underlined samples have been recognized via Prof. Yanjun Zhai (school of pharmacy, Liaoning University of TCM). The specimens have been kept at the Liaoning University of Traditional Chinese Medicine.

**Animals**

Sprague-Dawley male rats (SPF grade) with 180-220 gram of total body weight were provided by Liaoning Changsheng biotechnology Co. Ltd. (Laboratory Animal Resource Center of Liaoning Province, license number: SCXK-2015-0001). These rats were housed in a breeding room with well-maintained temperature, and humidity i.e., 20–26 °C, 50-70% accordingly for one week. The rats were fed with usual lab food and water before experimentation. The food and water intake were fasted overnight, however, the water ad libitum was provided before the experimentation. The underlined rats were executed via a 10 percent of chloral hydrate anesthetic. The approval for the existing experimentations and protocols was obtained by the Institutional Animal Ethics Committee of Liaoning Provincial Hospital of Chinese Medicine (2019.3.25, 2019015).

**Preparation of CD, CD-NP, and CD-HP extract**

CD-NP, CD-HP were processed from the same batch of *Cistanch deserticola*. To prepare CD-NP, dry CD sieces (5 mm thick, 100 g) were moisturized by rice-wine (30 mL) and were steamed at 100 °C for 16 hrs., followed by drying at 55 °C via drying oven. While CD-HP was
prepared via infiltration of dry CD pieces (5 mm thick, 100 g) with rice-wine (30 mL), followed by steaming at high-pressure for 4 hrs. (1.25 atmospheric pressure), dried in a drying oven at 55 °C (Liu BN et al).

In a 100 mL measuring flask, one gram of the powder was sieved via sieve#4, followed by adding 50 percent of methanol (50 mL) and then tightly covered and mixed. This mixture was weighed, followed by half hrs. maceration. After maceration, the underlined mixture was ultrasonicate (power 250W, frequency 35kHz) for 40 min, followed by cooling, and weighing again. Replenished the loss of the weight with 50 percent methanol, properly mixed, and allowed to stand, followed by filtering the supernatant and then used the obtained filtrate as the test solution.

**MS analysis of active components**

Preparation of standard substances: precision weighing tubuloside-A, echinacoside, 2'-acetylaceoteoiside, acteoside, isoacteoside, cistanoside-F, salidroside, geniposide, ajugol, catalpol, geniposidic acid, and 8-epideoxyloganic acid of 3.02 mg, 3.00 mg, 2.34 mg, 2.45 mg, 0.61 mg, 2.14 mg, 3.39 mg, 2.84 mg, 1.58 mg, 2.39 mg, 2.56 mg, and 2.34 mg accordingly were added into a 10 mL volumetric flask, added methanol constant volume to scale, configured into a corresponding concentration reference solution. Each of the 100 μL was configured into a mixed reference solution.

MS analysis condition: The mass value was corrected before the experiment, and the negative ion mode was used. The range of mass was 50–1200 Da, and the sample was injected through a flow injection pump. The cone velocity was 100 L/hrs, the dissolved flow rate was set at 800 L/h. The capillary and cone voltages were fixed at 2500 and 40 V, accordingly. The temperature of the ion source and dissolved gas were 100 °C and 400 °C, signal acquisition frequency was 0.5 S⁻¹.

**UPLC-Q-TOF-MS analysis of CD extract**

Chromatographic evaluations were carried out in a Waters ACQUITY I-CLASS UPLC system (Waters Corporation, Milford, MA, USA). Including ACQUITY UPLC® BEH C₁₈ column (50 × 2.1 mm, 1.7 μm, Waters). The mobile phase was comprised of water having 0.1 percent formic acid (A) and acetonitrile contains 0.1 percent formic acid (B), and the elution condition was as follows: 97% to 85% A (0~1min), 97% to 85% A (1~5 min), 85% to 75% A (5~15 min), 75% to 65% A (15~16 min), 65% to 55% A (16~18 min). The rate of flow and temp of the auto-sampler room and column was fixed at 0.3 mL·min⁻¹, 30 °C, and 8 °C, accordingly. The injection volume of sample and standard substance solutions was 1 μL.

The mass spectrometric evaluation was carried out via Waters XEVO G2-XS QTOF MS (Waters Corporation, Milford, MA, USA), comprised of an ESI source. The flow rate of nitrogen gas was fixed at 800 L·hrs⁻¹ with a temp of 400 °C, the source temp was fixed at 100 °C, and the cone gas was set at 50 L·hrs⁻¹. The voltage of cone and capillary was adjusted at 40 and 2000 V, accordingly. The collision energy of the ramp was used in the range of 20-30 V. The centroided
Data of all samples were obtained from 50-1200 Da, with a 5-scan time of 0.5 s over an analysis time of 10 min. LockSpray TM was employed for the validation of the mass precision. The [M-H]- ion of leucine enkephalin (200 pg·μL-1 infusion flow rate 10 μL·min-1) at m/z 554.2615 was used as the lock mass. The MassLynx V4.1 software (Waters Co., Milford, USA) was employed for the accurate mass, the composition of the precursor ions, and the fragment ions calculation.

**Data analysis in Masslynx platform**

Furthermore, we set up an in-house library comprising the name of the compound, its structure, and molecular formula (in mol.) which is based on literature. All the compounds were noted in a special template, made in Excel. In addition, the mol files (Chemdraw Ultra 8.0, Cambridge soft, USA) and the Excel files of all the individual compound structures were also saved on the local PC. The established Excel-sheet having important data was directly imported into the scientific library in UNIFI.

UNIFI 1.8.2, Waters, Manchester, UK was employed for the evaluation of structural characteristics, particularly for the characteristic fragments and MS fragmentation. The key parameters were adjusted as follows: a minimum peak area of 500 was set for the 2D peak detection. During revealing 3D peaks, a low energy peak intensity of more than 300 counts and elevated energy peak intensity of more than 80 counts were chosen. The error of mass was found to be up to ±10 ppm for known compounds, and the retention time tolerance was set in the range of ±0.1 min. We selected the negative adducts containing -H, +HCOOH. The processing of the raw data obtained from MS was carried out via streamlined UNIFI software to rapidly pinpoint the chemical components that met the standards with the self-built database and the in-house Traditional Medicine Library.

Next, to verify the chemical structure of each target compound, the isomers were distinguished by their characteristic MS fragmentation patterns which were revealed in the reported studies, and by comparing the retention times of reference standards.

**Metabolomics Analysis Based on Multivariate Statistical Analysis**

Before processing the raw data, the parameters were set, such as mass ranging from 150 to 1200 Da, range of retention time (0 to 20 min), threshold intensity (2000 counts), mass tolerance i.e., 5 mDa, while mass and retention time window was 0.20 min and 0.05 Da, accordingly. In the subsequent list of the database, the identifier of ions was the RT-m/z pairs with respect to their elution times. The same values for RT and m/z in various batches of samples were considered as the same compound.

Multivariate statistical analysis was conducted to evaluate effective biomarkers that considerably contributed to variations among different groups. During the analysis, principal component analysis (PCA) was employed to indicate the maximum differences and pattern recognition for obtaining an overview and classification. The OPLS-DA is a modeling tool that provides visualization of the OPLS-DA predictive component loading to assist model evaluation.
Variable importance for the projection (VIP) was used for assessing the evaluation of various components, and the metabolites with VIP values > 1.0 and \( P\)-value < 0.05 were regarded as effective markers. Furthermore, a permutation test was conducted for providing reference distributions for the \( R^2/Q^2 \) values that could show the statistical significance.

**Animal experiments**

The rats were randomly categorized into four groups (\( n=6 \) for each group), followed by the oral administration of various extracts: (1) Blank control group: the rats were given normal saline (2 mL/100 g); (2) CD group: the rats were given CD extract (2 mL/100 g); (3) CD-NP group: the rats were given CD-NP extract (2 mL/100 g); (4) CD-HP group: the rats were given CD-HP extract (2 mL/100 g). The further categorization of all groups was carried out into three sub-groups for plasma, urine, and feces, accordingly. Two hours later, each rat was orally administered with the same and equal amount of extracts.

Post administration, the collection of blood samples was carried out at 1.0 h, 2.0 h, and 4.0 h in heparinized 1.5 mL polythene tubes (from orbital veins), followed by centrifugation (at 4,500 rpm) of all samples for 15 min. Next, supernatant (200 \( \mu \)L) was taken out and the plasma samples from the same rat were mixed.

For urine and feces samples, the rats were held in metabolism cages, and then the collection of urine and feces samples was carried out for 24 h after administration. The centrifugation of urine samples was carried out at 4,500 rpm for 15 min, while feces samples were dried in the shade, ground into powder, then 0.2 g was taken, and added into 0.5 mL saline solution, then ultrasound for 5 min, and centrifuged at 12000 rpm for 15 min. All the bio-samples were kept at -80 \(^\circ\)C until analysis.

**Preparation of biological samples**

The addition of plasma, urine, and feces samples was carried out with 3 volumes of methanol, followed by vortexing for 3 min. Next, the centrifugation (at 12000 rpm) of the mixtures was carried out for 10 min, followed by transferring of supernatant into the EP tube, and then dried by nitrogen at 37 \(^\circ\)C. Furthermore, the addition of 200 \( \mu \)L of HCN-H\(_2\)O (50\%) solution was carried out. Then, the vortex was used for mixing (1 min), followed by centrifugation (at 12000 rpm) for 5 min. The supernatant (5 \( \mu \)L) of the treated samples was injected into the UPLC-Q-TOF-MS\(^E\) system.

**Liquid chromatographic and mass spectrometric condition**

The analysis for metabolites was also performed by Waters UPLC instrument through an ESI interface. Separations were carried out using an Acquity UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 \( \mu \)m), the mobile phase was 0.1 % formic acid (A): Acetonitrile (B), the gradient elution condition was 0–3 min (99.8%\( \to\)98%A), 3–5 min (98%\( \to\)95%A), 5–8 min (95%\( \to\)90%A), 8–12 min (90% \( \to\)85%A), 12–17 min (85%\( \to\)70%A), 17–22 min (70%\( \to\)60%A), 22–23 min
(60%→58%A), 23–25 min (58%A), 25–32 min (58%→45%A), and 32–37 min (45%→35%A), 0.4 mL·min⁻¹ was the flow rate. Temperature for column and sample room was set at 40 °C and 8 °C. The above conditions of mass spectrometry were used.

**Strategy for systematic analysis of metabolites in bio-samples**

UNIFI (1.8.2) software was employed for data processing. The Binary Compare function was used for the identification of effective metabolites. Evaluated metabolites were not existing in the equivalent control sample or exist at low ion intensities. The relative intensity threshold was set at 3 or 5, and metabolites that fulfilled the underlined criteria could be evaluated. Common and predictable metabolites were then determined by EIC. For searching of two-phase metabolites, the NLF function was applied. For example, in the UNIFI software, the parameters could be set at 176.0321 for searching for possible glucuronic acid conjugates. Post-processing, a neutral loss can be set in the method or identified. MassFragment was used for determining or characterization of detected metabolites’ structures, a UNIFI’s spectral interpretation function is the main function used to analyze secondary fragmentation of parent components. And the underlined function can be used for rapid verification of the fragmentation path whether reasonable.

**Results**

**Mass Fragmentation Rule of Phenylethanoid glycosides and iridoids**

Phenylethanoid glycosides are the main chemical constituents of CD. The standard solutions of isoacteoside, cistanoside F, tubuloside A, echinacoside, acteoside, and 2′-actylacteoside were taken, followed by providing a different level of collision energies, and then corresponding MS² maps were obtained (Figure 1).

| Components | Molecular Formula | Theoretical Mass (Da) | Detected Mass (Da) | Fragment | Energy (v) |
|------------|------------------|-----------------------|--------------------|----------|------------|
| isoacteoside | C_{23}H_{31}O_{15} | 623.1976[M-H]⁻ | 623.1956[M-H]⁻ | 461.1636, 315.1636, 179.0354, 161.0232 | 35 |
| cistanoside F | C_{21}H_{28}O_{13} | 487.1452[M-H]⁻ | 487.1423[M-H]⁻ | 179.0326, 135.0440 | 20 |
| tubuloside A | C_{27}H_{40}O_{17} | 827.2610[M-H]⁻ | 827.2852[M-H]⁻ | 665.2477, 623.2388, 477.1722, 161.0202 | 40 |
| echinacoside | C_{35}H_{46}O_{20} | 785.2505[M-H]⁻ | 785.2651[M-H]⁻ | 623.2286, 477.1677, 179.0374, 161.0250 | 45 |
| acteoside | C_{25}H_{36}O_{15} | 623.1976[M-H]⁻ | 623.2050[M-H]⁻ | 461.1691, 179.0374, 161.0250 | 25 |
| 2′-actylacteoside | C_{23}H_{30}O_{14} | 665.2082[M-H]⁻ | 665.2108[M-H]⁻ | 623.1979, 503.1784, 461.1647, 179.0374, 161.0250 | 30 |
| ajugol | C_{15}H_{24}O_{9} | 347.1342[M-H]⁻ | 347.1410[M-H]⁻ | 185.0845, 167.0721, 149.0624, 127.0413 | 25 |
| catalpol | C_{36}H_{26}O_{11} | 361.1135[M-H]⁻ | 361.1131[M-H]⁻ | 199.0586, 169.0486, 151.0380, 125.0332 | 50 |
| geniposidic acid | C_{26}H_{32}O_{10} | 373.1135[M-H]⁻ | 373.1143[M-H]⁻ | 211.0602, 193.0500, 149.0608, 167.0703, 123.0453 | 30 |
| geniposide | C_{25}H_{30}O_{12} | 387.1291[M-H]⁻ | 387.1313[M-H]⁻ | 225.0787, 207.0678, 123.0453 | 10 |
| 8-epideoxyloganic acid | C_{21}H_{24}O_{9} | 359.1342[M-H]⁻ | 359.1345[M-H]⁻ | 197.0810, 153.0916, 135.0823 | 40 |

The mass spectrometric analysis revealed that phenylethanoid glycosides have similar mass spectrum fragmentation patterns, the cleavage pathways in the negative-ion mode mainly include:

1. **Ester bond cleavage:** loss of neutral caffeoyl group (C₉H₇O₆, 162.03) and neutral acetyl group
(C\textsubscript{2}H\textsubscript{2}O, 42.00); (2) Glycosidic cleavage: loss of neutral rhamnose residues (C\textsubscript{6}H\textsubscript{10}O\textsubscript{4}, 146.05) and neutral glucose residue (C\textsubscript{6}H\textsubscript{10}O\textsubscript{5}, 162.05). From high-resolution mass spectrometry, caffeoyl (162.03) and glucose residue (162.05) could be distinguished.
Iridoids ajugol, catalpol, geniposidic acid, geniposide, 8-epideoxyloganic acid standard solutions were taken, followed by providing different collision energies, and then corresponding MS² maps were obtained (Figure 2).
A. ajugol  B. catalpol  C. geniposidic acid  D. geniposide  E. 8-epideoxyloganic acid

Figure 2  Mass Spectrogram and cleavage pathway of iridoid glycosides

Iridoid glycosides have similar mass spectrum fragmentation patterns, the cleavage pathways in the negative-ion mode mainly include (1) Glycosidic cleavage: Loss of neutral glucose residue (C₆H₁₀O₅, 162.05); (2) Loss of neutral CO₂ (43.99) and H₂O (18.01).

Identification of the compounds in CD, CD-NP, and CD-HP extracts

UPLC-QTOF-MS² analysis

The optimization of chromatographic conditions was carried out. Next, the compounds of Cistanche Herba were evaluated in both negative and positive ion modes with high as well as low CEs. The obtained results revealed that the compatibility of the negative mode was higher relative to the positive mode for the underlined compounds. Figure 1 depicts MS basic peak ion (BPI) chromatogram traced with numbered peaks. The intensity of each detected ion in UPLC-Q-TOF-MS² analysis was normalized with respect to the whole ion count for the generation of a data matrix which comprised of m/z value, the normalized peak area, and retention time.
A total of 97 compounds were identified with -SEM (n=6) mode from CD and its processed products (Table 2), including phenylethanoid glycosides (PhGs), iridoids, lignans, and oligosaccharides. The 95, 91, and 94 components were detected in CD, CD-NP, and CD-HP, accordingly. Among them, 64 were phenylethanoids, 13 were iridoids, and 20 other kinds of compounds were determined. There was a similarity in the chemical composition of CD and their processed product, however, the quantity of the components was found to be different among CD and its processed product.
| NO | RT | Identification          | Molecular formula | Adducts | Experimental (ppm) | Theoretical (ppm) | Error (ppm) | MS/MS fragmentation | source |
|----|----|-------------------------|-------------------|---------|-------------------|------------------|------------|---------------------|--------|
| 1  | 1.10 | Kankanoside B | C_{34}H_{52}O_{24} | -HCOO | 409.1348 | 364.1369 | 0.2 | 363.1280, 183.06693, 153.05619 | CD, CD-HP |
| 2  | 1.22 | 6-Deoxycatalpol | C_{34}H_{52}O_{24} | -HCOO | 391.1245 | 346.1260 | 0.5 | 391.1244, 341.10886, 183.06693 | CD, CD-HP |
| 3  | 1.44 | 6-Deoxycatalpol | C_{34}H_{52}O_{24} | -HCOO | 391.1245 | 346.1260 | 0.5 | 391.1244, 183.06662 | CD, CD-HP |
| 4  | 2.04 | Androsin | C_{34}H_{52}O_{24} | -HCOO | 373.1143 | 328.1163 | 0.8 | 373.1143, 211.06188, 193.05342 | CD, CD-HP |
| 5  | 2.25 | 6-Deoxycatalpol | C_{34}H_{52}O_{24} | -HCOO | 391.1245 | 346.1260 | 0.5 | 391.1244, 229.07232, 167.03571 | CD, CD-HP |
| 6  | 2.58 | Androsin | C_{34}H_{52}O_{24} | -HCOO | 373.1147 | 328.1166 | 1.2 | 373.1147, 299.11294, 211.06206, 149.06137 | CD, CD-HP |
| 7  | 2.64 | Kankanoside L | C_{34}H_{52}O_{24} | -HCOO | 393.1404 | 348.1421 | 0.7 | 393.1404, 315.10749, 206.00863, 134.04736 | CD, CD-HP |
| 8  | 2.93 | kankanoside M | C_{34}H_{52}O_{24} | -HCOO | 375.1297 | 330.1318 | 0.6 | 375.12966, 213.07775, 125.06127 | CD, CD-HP |
| 9  | 3.14 | 3,4-dimethoxybenzyl-β-D-glucoside | C_{34}H_{52}O_{24} | -H | 375.1299 | 376.1373 | 0.8 | 375.12994, 255.08683, 213.07767, 151.07707 | CD, CD-HP |
| 10 | 3.22 | Decaffeoylacteoside | C_{34}H_{52}O_{24} | -H | 461.1659 | 462.1734 | 0 | 461.16591, 315.10891, 135.04591 | CD, CD-HP |
| 11 | 3.24 | Kankanoside F | C_{34}H_{52}O_{24} | -H | 623.2192 | 624.2273 | 0.5 | 623.21920, 461.16678, 315.10994, 135.04591 | CD, CD-HP |
| 12 | 3.25 | Gluicoside | C_{34}H_{52}O_{24} | -H | 377.1449 | 332.1463 | 0.1 | 377.14491, 461.16609, 315.10891, 135.04591 | CD, CD-HP |
| 13 | 3.31 | Cistanthulose A1 | C_{34}H_{52}O_{24} | -H | 649.1987 | 650.2068 | 0.7 | 649.19871, 537.18251, 335.09146, 179.03598 | CD, CD-HP |
| 14 | 3.35 | 6-deoxycatalpol | C_{34}H_{52}O_{24} | -H | 345.1193 | 346.1271 | 0.7 | 345.11929, 299.11424, 179.03598 | CD, CD-HP |
| 15 | 3.40 | Adoxoside acid | C_{34}H_{52}O_{24} | -H | 375.1302 | 376.1373 | -1.1 | 213.07683 | CD, CD-HP |
| 16 | 3.54 | Cistanoside F | C_{34}H_{52}O_{24} | -H | 487.1451 | 488.1532 | -0.1 | 487.14512, 325.09503, 251.05822, 179.03637 | CD, CD-HP |
| 17 | 3.65 | Sinapic aldehyde 4-O-β-D-glucopyranoside | C_{34}H_{52}O_{24} | -HCOO | 401.1087 | 356.1109 | 0.3 | 401.10872, 301.09397, 283.08371, 193.05148 | CD, CD-HP |
| 18 | 3.77 | Kankanose | C_{34}H_{52}O_{24} | -H | 649.1985 | 650.2063 | 0.5 | 649.19853, 461.16139, 293.12495, 179.03612 | CD, CD-HP |
| 19 | 3.79 | 3-Methyl-but-2-en-1-yl-β-D-glucopyranoside | C_{34}H_{52}O_{24} | -HCOO | 293.1249 | 248.1264 | 1.3 | 293.12494, 195.06673, 179.03612 | CD, CD-HP |
| 20 | 3.81 | Demethylsyringic acid | C_{34}H_{52}O_{24} | -H | 357.1193 | 358.1266 | 0.7 | 357.11931, 251.05778, 195.06678, 179.03653 | CD, CD-HP |
| 21 | 3.82 | Cistanoside G | C_{34}H_{52}O_{24} | -H | 445.1709 | 446.1756 | -0.1 | 445.17092, 375.13013, 293.12548, 195.06823, 179.03678 | CD, CD-HP |
| 22  | 3.84 | Cistanoside F | C_{21}H_{20}O_{13} | -H | 487.1458 | 488.1523 | -0.6 | 487.14577, 445.17102, 323.08273, 179.03678. | CD - CD-NP, CD-HP |
| 23  | 3.96 | 3-Methylbut-2-en-1-yl-β-D-glucopyranoside | C_{21}H_{20}O_{13} | -HCOO | 293.1251 | 248.1268 | 1.5 | 0.9 | 293.12505, 161.04759. | CD - CD-NP, CD-HP |
| 24  | 3.98 | Glucoside | C_{13}H_{12}O_{10} | -HCOO | 377.1455 | 332.1476 | 0.7 | 377.14547, 293.12505, 179.03614. | CD - CD-HP |
| 25  | 4.03 | Cistanoside F | C_{21}H_{20}O_{13} | -H | 487.1457 | 488.1538 | 0.5 | 487.14668, 223.06196, 179.03779. | CD - CD-NP, CD-HP |
| 26  | 4.16 | Kankanoside D | C_{13}H_{12}O_{10} | -HCOO | 363.1663 | 318.1679 | 0.8 | 363.1663, 315.10883, 179.03855, 161.04465. | CD - CD-NP, CD-HP |
| 27  | 4.19 | Cistanoside E | C_{13}H_{12}O_{10} | -H | 475.1869 | 476.1888 | 5.3 | 475.18694, 363.16559, 179.0385. | CD - CD-NP, CD-HP |
| 28  | 4.25 | Cistanoside I | C_{21}H_{20}O_{13} | -H | 471.1505 | 472.1577 | 0.2 | 471.15048, 369.11987, 471.15071, 179.03589. | CD - CD-NP, CD-HP |
| 29  | 4.32 | Cistanoside F | C_{13}H_{12}O_{10} | -H | 487.1466 | 488.153 | 1.4 | 487.14656, 323.07922, 251.05793, 179.03699. | CD - CD-NP, CD-HP |
| 30  | 4.53 | Cistanoside F | C_{13}H_{12}O_{10} | -H | 487.1464 | 488.1537 | 1.2 | 487.14637, 323.08028, 251.05805, 179.03769. | CD - CD-NP, CD-HP |
| 31  | 4.55 | Androsin | C_{13}H_{12}O_{10} | -H | 327.1092 | 328.1158 | 1.2 | 327.10918, 251.05805, 179.03769, 131.07201. | CD - CD-NP, CD-HP |
| 32  | 4.86 | Cistanoside H | C_{13}H_{12}O_{10} | -H | 503.1761 | 504.1835 | -0.4 | 503.17610, 461.16590, 375.13036, 315.10972. | CD - CD-NP, CD-HP |
| 33  | 4.88 | Kankanoside E | C_{13}H_{12}O_{10} | -HCOO | 393.1760 | 348.1784 | -0.1 | 393.17603, 241.11923, 375.13036. | CD - CD-HP |
| 34  | 4.92 | Cistamabuloside C1/C2 | C_{13}H_{12}O_{10} | -H | 801.2487 | 802.2556 | 3.4 | 801.24867, 623.20258, 110.03824. | CD - CD-NP, CD-HP |
| 35  | 5.29 | (2E,6Z)-2,6-Dimethyl-2,6-octadienoic acid | C_{13}H_{13}O_{5} | -H | 391.1608 | 346.1628 | 0.4 | 391.16080, 345.15509, 163.03730. | CD - CD-NP, CD-HP |
| 36  | 5.47 | Kankanoside E | C_{13}H_{12}O_{10} | -HCOO | 393.1766 | 348.1783 | 0.5 | 393.17656, 283.07834, 179.03768. | CD - CD-NP, CD-HP |
| 37  | 5.56 | Campneoside II | C_{13}H_{12}O_{10} | -H | 639.1926 | 640.2003 | 0.1 | 621.19264, 361.15007, 161.02704. | CD - CD-NP, CD-HP |
| 38  | 5.73 | Echinacoside | C_{13}H_{12}O_{10} | -H | 785.2549 | 786.2618 | 4.5 | 785.25485, 623.21903, 392.11650, 179.03596. | CD - CD-NP, CD-HP |
| 39  | 5.81 | 8-Hydroxygeraniol-1-β-D-glucopyranoside | C_{13}H_{12}O_{10} | -HCOO | 377.1813 | 332.1835 | 0.1 | 377.18129, 331.14023, 164.07382. | CD - CD-NP, CD-HP |
| 40  | 5.86 | Cistanoside E | C_{13}H_{12}O_{10} | -H | 475.1813 | 476.1888 | -0.3 | 347.17188, 251.05915. | CD - CD-NP, CD-HP |
| 41  | 5.93 | Liriodendrin | C_{13}H_{12}O_{10} | -H | 787.2670 | 742.2689 | 0.9 | 787.26703, 579.20978, 475.18047, 417.15414. | CD - CD-NP |
| 42  | 6.00 | Isolariciresinol-9'-O-β-D-glucopyranoside | C_{13}H_{12}O_{10} | -HCOO | 567.2076 | 522.2095 | -0.2 | 567.20755, 359.14970, 329.13966, 178.06231. | CD - CD-NP, CD-HP |
| 43  | 6.06 | Campneoside II | C_{13}H_{12}O_{10} | -H | 639.1936 | 640.2003 | 1.1 | 639.19362, 487.14472, 251.05630. | CD - CD-NP, CD-HP |
| 44  | 6.26 | Kankanosides K1/K2 | C_{13}H_{12}O_{10} | -H | 815.2636 | 816.2701 | 2.6 | 815.26358, 783.23518, 637.1979, 381.15558, 179.03631. | CD - CD-NP, CD-HP |
| 45  | 6.34 | Cistamabuloside B1 | C_{13}H_{12}O_{10} | -H | 769.2573 | 770.2655 | 1.8 | 769.25732, 623.21303, 420.06489, 163.03926. | CD - CD-NP, CD-HP |
| 46  | 6.36 | 8-Hydroxygeraniol-1-β-D-glucopyranoside | C_{13}H_{12}O_{10} | -HCOO | 377.1820 | 332.1833 | 0.8 | 377.18204, 367.15243, 163.04196. | CD - CD-NP, CD-HP |
47 6.42 Kankanoside N  C_{21}H_{29}O_{11}  -H  345.1563  346.1637  1.4  345.1560, 197.80891, 113.02490  CD, CD-NP, CD-HP
48 6.50 Cistanoside A  C_{23}H_{29}O_{12}  -HCOO  845.2769  800.2781  5.4  845.2769, 799.27001,681.20502  CD, CD-NP, CD-HP
49 6.47 Kankanoside I  C_{29}H_{30}O_{11}  -HCOO  799.2703  754.2718  4.2  799.27031, 365.08428, 161.02522  CD, CD-NP, CD-HP
50 6.79 (2E,6E)-2-β-D-glucopyranosyloxy-2,6-dimethyl-2,6-octadienoic acid  C_{21}H_{29}O_{11}  -H  345.1565  346.1632  1.6  345.15649, 165.99327  CD, CD-NP, CD-HP
51 6.96 Kankanoside A  C_{21}H_{29}O_{11}  -H  345.1565  346.1633  1.6  345.15647, 195.86666, 179.03628  CD, CD-NP, CD-HP
52 7.03 Cistanoside C  C_{23}H_{29}O_{12}  -HCOO  683.2198  638.2208  1.1  683.21978, 489.14915, 417.15349, 335.20636, 197.80796  CD, CD-NP, CD-HP
53 7.09 kankanoside E  C_{21}H_{29}O_{11}  -H  347.1716  348.1781  1.0  347.17157, 195.81307, 167.10929  CD, CD-NP, CD-HP
54 7.19 Acteoside  C_{21}H_{29}O_{11}  -H  623.1992  622.1892  1.6  623.19917, 461.16657, 315.10988, 161.02530  CD, CD-NP, CD-HP
55 7.25 Tubuloside A  C_{21}H_{29}O_{11}  -H  827.2655  828.2721  4.5  827.26548, 621.18343, 469.13652, 379.19635  CD, CD-NP, CD-HP
56 7.51 Cistanoside B  C_{21}H_{29}O_{11}  -HCOO  859.2913  814.2931  4.1  859.2913, 679.18910, 565.19246  CD, CD-NP, CD-HP
57 7.58 Cistanoside J  C_{21}H_{29}O_{11}  -HCOO  739.2409  694.2482  4.0  739.24093, 345.15468, 161.02597  CD, CD-NP, CD-HP
58 7.60 Tubuloside A  C_{21}H_{29}O_{11}  -H  827.2649  828.2727  3.9  827.26486, 739.24745, 579.22756, 345.15468, 161.02597  CD, CD-NP, CD-HP
59 7.7 Kankanoside E  C_{21}H_{29}O_{11}  -H  347.1719  348.1791  1.3  347.17191, 303.18323, 211.13616, 185.11917  CD, CD-NP, CD-HP
60 7.86 Acteoside  C_{21}H_{29}O_{11}  -H  623.1995  624.2067  1.9  623.19954, 461.16624, 161.02546  CD, CD-NP, CD-HP
61 7.94 Crentoside a  C_{21}H_{29}O_{11}  -H  621.1833  622.1907  1.4  621.18331, 387.14418, 179.03640  CD, CD-NP, CD-HP
62 8.06 Kankanosides K1/K2  C_{21}H_{29}O_{11}  -H  815.2631  816.2688  2.1  499.1811, 197.80800, 160.8423  CD-HP
63 8.33 Kankanoside H1  C_{21}H_{29}O_{11}  -H  812.2731  812.2739  -0.8  607.20431, 445.17033, 161.02556  CD, CD-HP
64 8.36 Isoysingalide-3′-α-L-rhamnopyranoside  C_{21}H_{29}O_{11}  -H  607.2034  608.2119  0.7  607.20341, 461.16447, 315.10906, 145.03063  CD, CD-NP, CD-HP
65 8.53 Campneoside I  C_{21}H_{29}O_{11}  -H  653.2084  654.2161  1.6  607.20440, 461.16367, 443.15204, 145.03081  CD, CD-NP, CD-HP
66 8.78 Cis-isocistanoside C  C_{21}H_{29}O_{11}  -H  637.2147  638.2222  1.5  637.21474, 475.18074, 329.12012, 161.02576  CD, CD-NP, CD-HP
67 8.84 Citrusin A  C_{21}H_{29}O_{11}  -HCOO  581.2235  536.2280  0.1  581.22351, 433.15241,371.13360,343.1457  CD, CD-NP, CD-HP
68 9.17 Isoysingalise-3′-α-L-rhamnopyranoside  C_{21}H_{29}O_{11}  -H  607.2033  608.2122  0.6  607.20334, 461.15822, 161.02611  CD, CD-NP, CD-HP
69 9.50 Isocampneoside I  C_{21}H_{29}O_{11}  -H  653.2094  654.2161  1.2  607.2094, 461.16616, 307.08417, 145.03058  CD, CD-NP, CD-HP
70 9.50 Syringalide  C_{21}H_{29}O_{11}  -H  607.2037  608.2127  1.0  607.20372, 461.16616, 307.08417, 145.03089  CD, CD-NP, CD-HP
71 9.57 isocistanoside C  C_{21}H_{29}O_{11}  -H  637.2150  638.2221  1.8  637.21503, 445.15153, 323.07862, 251.05653  CD, CD-NP, CD-HP
72 9.62 Cis-Tubuloside B  C_{21}H_{29}O_{11}  -H  665.2103  666.2169  2.1  665.21032, 503.17680, 305.06585, 161.02529  CD, CD-NP, CD-HP
73 9.76 Crentoside a  C_{21}H_{29}O_{11}  -H  621.1826  622.1891  0.7  621.18264, 487.14611, 323.07878, 179.03579  CD, CD-NP, CD-HP
| No. | Name                  | Formula    | CD   | CD-NP | CD-HP |
|-----|-----------------------|------------|------|-------|-------|
| 74  | cistanoside C         | C_{10}H_{10}O_{6} | -H   | 637.2139 | 638.2209 | 0.7 | 637.21389, 591.20888, 445.16991, 163.04078, 145.03032 |
| 75  | Osmanthuside B        | C_{10}H_{10}O_{6} | -H   | 591.2080 | 592.2158 | 0.2 | 591.20804, 445.16991, 160.84291, 145.03032 |
| 76  | Eutigoside A          | C_{10}H_{10}O_{6} | -H   | 445.1501 | 446.1571 | 0.2 | 445.15008, 163.03943, 145.03004 |
| 77  | Cistanoside M         | C_{10}H_{10}O_{6} | -HCOO | 667.2245 | 622.2248 | 0.7 | 667.22446, 621.21761, 555.20753, 161.02534 |
| 78  | Isomartynoside        | C_{10}H_{10}O_{6} | -HCOO | 697.2356 | 652.2372 | 1.2 | 697.23563, 651.22859, 475.17960, 175.04062 |
| 79  | Salsaside B           | C_{10}H_{10}O_{6} | -H   | 577.1929 | 578.1999 | 0.8 | 503.17758, 323.07755, 161.02527 |
| 80  | 2'-acetyllactoside    | C_{10}H_{10}O_{6} | -H   | 665.2108 | 666.2173 | 2.6 | 665.21076, 503.17758, 305.60712, 161.02527 |
| 81  | Osmanthuside B        | C_{10}H_{10}O_{6} | -H   | 591.2084 | 591.2093 | 0.6 | 445.1579, 163.04080, 145.0301 |
| 82  | Plantainoside C       | C_{10}H_{10}O_{6} | -H   | 637.2141 | 638.2238 | 0.9 | 591.20986, 445.15921, 145.03022 |
| 83  | Kankanosides J1/J2    | C_{10}H_{10}O_{6} | -H   | 695.2190 | 696.2267 | 0.3 | 695.21902, 649.21477, 503.17505, 145.03017 |
| 84  | Salsaside F           | C_{10}H_{10}O_{6} | -H   | 649.2140 | 650.2199 | 0.8 | 649.21399, 503.17505, 347.16994, 145.03017 |
| 85  | Cistanosinomide A     | C_{10}H_{10}O_{6} | -H   | 679.2246 | 680.2324 | 0.8 | 679.22464, 623.19749, 161.02503 |
| 86  | Isomartynoside        | C_{10}H_{10}O_{6} | -HCOO | 697.2360 | 652.2364 | 1.6 | 697.23604, 651.22862, 505.15921, 175.04095 |
| 87  | Salsaside A           | C_{10}H_{10}O_{6} | -H   | 577.1932 | 578.1982 | 1.1 | 577.19316, 501.16521, 469.13425, 179.03548, 161.02496 |
| 88  | Salsaside Ca/Cb        | C_{10}H_{10}O_{6} | -H   | 561.1978 | 562.2044 | 0.6 | 561.19776, 415.16021, 163.04118, 145.03011 |
| 89  | Salsaside F           | C_{10}H_{10}O_{6} | -H   | 649.2144 | 650.2212 | 1.2 | 649.21443, 503.17434, 461.16538 |
| 90  | Kankanosides J1/J2    | C_{10}H_{10}O_{6} | -H   | 695.2195 | 696.2274 | 0.8 | 695.21948, 649.21421, 607.20459, 503.17401, 149.02394 |
| 91  | Osmanthuside B        | C_{10}H_{10}O_{6} | -H   | 591.2085 | 592.2162 | 0.7 | 591.20845, 429.17830, 161.02556 |
| 92  | Jionoside D           | C_{10}H_{10}O_{6} | -H   | 637.2131 | 638.2210 | 0.1 | 591.20842, 161.02556 |
| 93  | Salsaside D           | C_{10}H_{10}O_{6} | -H   | 649.2140 | 650.2199 | 0.8 | 649.21401, 607.19810, 329.16152 |
| 94  | cistanosinomide A     | C_{10}H_{10}O_{6} | -H   | 679.2251 | 680.2314 | 1.3 | 679.22512, 637.21464, 461.16791, 161.02601 |
| 95  | Osmanthuside B6(Z)    | C_{10}H_{10}O_{6} | -H   | 591.2085 | 592.2152 | 0.7 | 591.20854, 489.26955, 445.15395, 161.02652 |
| 96  | sinenside A           | C_{10}H_{10}O_{6} | -H   | 679.2255 | 680.2328 | 1.7 | 679.22547, 633.22252, 591.20916, 145.03024 |
| 97  | Cistanoside M         | C_{10}H_{10}O_{6} | -H   | 621.2184 | 622.2258 | 0.1 | 591.20868, 489.27111, 161.02535 |
Variations in chemical components of processed products

The Simca-P 13.0 software was employed for analyzing the multivariate data matrix. Before PCA, all variables were mean-centered and pareto-scaled, followed by identification of potential discriminant variables. In a PCA score plot, every point showed an individual sample. Samples that showed similarity in their chemical components were scattered adjacent to each other, while those which showed variations in their components were divided. As seen in PCA (Figure 4), the group of CD-HP was separated from the groups of CD and CD-NP.

To distinguish CD from CD-HP and CD-NP, OPLS-DA, permutation test, S-plot, and VIP value were developed. (Figures 5, 6, 7) The obtained results revealed that many components were key characteristic components of each product. The screening condition was the VIP >1 and $P < 0.05$. From the date of the S-plot, the characteristic components were evaluated, which were commonly existing in the three groups.
Figure 5 The OPLS-DA/permutation test/S-plot/heat map indicating the intensities of potential biomarkers between CD-NP and CD-HP.

Compounds 9, 10, 14, 32, 59, 60, 68, 70, 74, 75, 80, 81, 82, and 84 are the differential components of CD-NP, while compounds 11, 15, 16, 45, 48, 66, and 72 are the differential components of CD-HP.

Figure 6 The OPLS-DA/permutation test/S-plot/heatmaps indicating the intensities of effective biomarkers between CD and CD-NP.
Compounds 13, 15, 16, 37, 49, 63, 66, 72, 74, 75, and 85 are the differential components of CD, while compounds 10, 11, 32, 59, 60, 68, 70, 71, 80, 81, and 82 are the differential components of CD-NP.

Figure 7 The OPLS-DA/ permutation test/ S-plot/ heatmaps revealing the intensities of effective biomarkers between CD and CD-HP.

Compounds 9, 14, 16, 59, 63, 66, 72, 74, 75, 80, 82, 84, 85, and 94 are the differential components of CD, and 11, 15, 45, 49, 50, 60, and 71 are the differential components of CD-HP.

Identification of the metabolites in rats

From high-resolution mass spectrometry data, the accurate molecular weight and elemental composition for metabolites and protomolecule compounds were analyzed and compared. As the same kinds of compounds in TCM showed similarity in metabolic modifications, the correlations of phytochemical constituents \textit{in-vitro} can extend to their metabolites \textit{in-vivo}. Meanwhile, based on conventional biotransformation pathways, a reasonable change of molecular weight was inferred. Finally, the metabolites were identified by analyzing the MS$^5$ mass spectra of the metabolites and proto-compounds fragmentation pathway in the mass spectrum [21-22].
Compared with the blank sample, its components were identified *in vivo* based on the information provided by chromatogram-mass spectrum, the possibility of a metabolic reaction, the characteristics of the compound structure, and the fragmentation rule of its mass spectrum. See Table 3.
| NO | rtmed | Measured Mass | Error (mDa) | Formula | distribution | Identification | status |
|----|-------|---------------|-------------|---------|--------------|----------------|--------|
| 1  | 0.77  | 179.0389      | 4.4         | C_{6}H_{5}O_{4} | U_{CD,NP,HP}, F_{NP,HP} | Caffeic acid metabolites |
| 2  | 0.81  | 149.0653      | 5.0         | C_{4}H_{3}O_{2} | U_{CD,NP,HP} | 3-phenylpropionic acid metabolites |
| 3  | 0.93  | 195.0623      | -3.5        | C_{10}H_{12}O_{4} | U_{CD}, S_{NP}, F_{HP} | methylated 3,4-dihydroxybenzenepropionic acid metabolites |
| 4  | 1.02  | 193.0524      | 2.3         | C_{10}H_{10}O_{4} | U_{CD,NP,HP} | methylated caffeic acid metabolites |
| 5  | 1.18  | 167.0762      | 5.4         | C_{4}H_{12}O_{3} | U_{NP} | methylated HT metabolites |
| 6  | 3.31  | 185.1117      | -0.8        | C_{10}H_{18}O_{3} | U_{CD,HP}, F_{HP} | Ajugol deglycosylation product metabolites |
| 7  | 3.52  | 167.0536      | -0.9        | C_{4}H_{8}O_{4} | U_{NP} | HT oxidation metabolites |
| 8  | 4.48  | 361.1491      | -0.8        | C_{14}H_{26}O_{9} | U_{CD,NP,HP}, F_{NP,HP} | hydroxylated kankanoside A or isomer metabolites |
| 9  | 4.70  | 541.1144      | -8.3        | C_{10}H_{15}O_{15}S | S_{NP,HP}, F_{HP} | decaffeoylacteoside sulfate conjugation metabolites |
| 10 | 4.73  | 153.0504      | -4.8        | C_{10}H_{10}O_{3} | U_{CD,NP,HP}, F_{NP,HP} | HT metabolites |
| 11 | 4.85  | 123.0821      | 1.1         | C_{4}H_{12}O_{4} | U_{CD,NP,HP}, F_{CD,HP} | Geniposide hydrolysed product metabolites |
| 12 | 5.14  | 246.9911      | -1.4        | C_{4}H_{10}O_{3}S | U_{CD,NP,HP}, F_{NP} | 3,4-dihydroxyphenylacetic acid sulfate conjugation metabolites |
| 13 | 5.23  | 361.1471      | -2.8        | C_{10}H_{20}O_{9} | U_{CD,NP,HP}, F_{NP,HP} | hydroxylated kankanoside A or isomer metabolites |
| 14 | 5.35  | 313.0962      | 3.9         | C_{14}H_{13}O_{8} | U_{CD,NP,HP}, F_{NP,HP} | tyrosol glucuronide conjugation metabolites |
| 15 | 5.63  | 217.0138      | -3.3        | C_{10}H_{10}O_{3}S | U_{CD,NP,HP}, F_{NP,HP} | tyrosol sulfate conjugation metabolites |
| 16 | 5.73  | 329.0851      | -2.2        | C_{14}H_{13}O_{8} | U_{CD,NP,HP} | HT-glucuronide conjugation metabolites |
| 17 | 5.98  | 233.0170      | -5.0        | C_{10}H_{10}O_{3}S | U_{CD,NP,HP}, F_{CD,NP,HP} | HT sulfate conjugation metabolites |
| 18 | 6.54  | 185.1114      | -6.4        | C_{10}H_{10}O_{3} | U_{CD,NP,HP}, F_{HP} | deglycosylated kankanoside N metabolites |
| # | TIC | Retention Time | Purity % | MRM Information | Parent Molecule | Metabolite Information |
|---|-----|---------------|----------|----------------|-----------------|------------------------|
| 19 | 6.76 | 261.0084 | 1.5 | C4H10O4S | UCD,NP,HP, FNP | 3,4-dihydroxybenzene propionic acid sulfate conjugation metabolites |
| 20 | 7.01 | 183.1085 | 6.4 | C10H16O5 | UCD,NP,HP | deglycosylated kankanoside A or isomer metabolites |
| 21 | 7.16 | 461.1605 | -5.4 | C20H30O12 | FNP | Decaffeoylacteoside proto |
| 22 | 7.19 | 247.0278 | 0.1 | C16H25O8 | UCD,NP,HP, SCD,HP | methylated HT sulfate conjugation metabolites |
| 23 | 7.28 | 345.1476 | -7.3 | C16H25O8 | UCD,NP,HP, SCD,HP | kankanoside A or isomer proto |
| 24 | 7.57 | 247.0278 | 0.1 | C16H25O8 | UCD,NP,HP, SCD,HP | methylated HT sulfate conjugation metabolites |
| 25 | 7.69 | 355.0704 | 3.9 | C20H30O12 | UCD,NP,HP, SCD,HP | CA sulfate conjugation metabolites |
| 26 | 7.78 | 343.1037 | 0.8 | C15H23O8 | UCD,NP,HP, SCD,HP | methylated HT sulfate conjugation metabolites |
| 27 | 7.81 | 258.994 | 0.2 | C16H25O8 | UCD,NP,HP, SCD,HP | CA sulfate conjugation metabolites |
| 28 | 8.19 | 375.1284 | -0.7 | C16H25O8 | UCD,NP,HP, SCD,HP | 8-epiloganic acid proto |
| 29 | 8.52 | 245.0125 | 0.5 | C16H25O8 | UCD,NP,HP, FNP | 3-HPP sulfate conjugation metabolites |
| 30 | 8.53 | 193.0531 | 0.8 | C10H16O4 | UCD,NP,HP | Geniposidic acid deglycosylation dehydration product metabolites |
| 31 | 8.90 | 341.0942 | 6.9 | C15H23O8 | UCD,NP,HP, SCD,HP | 3-HPP glucuronide conjugation metabolites |
| 32 | 9.02 | 242.9951 | -2.1 | C15H23O8 | UCD,NP,HP, SCD,HP | dehydroxylated CA sulfate conjugation metabolites |
| 33 | 9.06 | 181.0491 | -1.0 | C15H23O8 | UCD,NP,HP, FCD,HP | 3,4-dihydroxybenzene propionic acid metabolites |
| 34 | 9.08 | 151.0352 | -4.3 | C15H23O8 | UCD,NP,HP, FCD,HP | catalpol deglycosylated dehydration product metabolites |
| 35 | 9.58 | 273.0064 | -0.5 | C10H16O4 | UCD,NP,HP, FNP | methylated CA sulfate conjugation metabolites |
| 36 | 10.02 | 275.0209 | -1.6 | C10H16O4 | UCD,NP,HP, FNP | methoxylated 3-HPP sulfate conjugation metabolites |
| 37 | 10.13 | 583.1320 | -1.3 | C22H32O12 | UCD,NP,HP, SCD,HP | Cistanoside H sulfate conjugation metabolites |
| 38 | 10.28 | 299.1108 | -2.3 | C14H18O7 | UCD,NP,HP, SCD,HP | salidroside proto |
| 39 | 10.4 | 163.04 | 0.5 | C4H4O3 | UCD,NP,HP, FCD,HP | dehydroxylated CA metabolites |
| 40 | 10.91 | 199.0641 | 3.5 | C4H4O3 | UCD,NP,HP, FNP | catalpol hydrolysated product metabolites |
| 41 | 11.17 | 521.1816 | -5.4 | C22H32O12 | UCD,NP,HP, SCD,HP | 6-deoxycatalpol glucuronide conjugation metabolites |
| 42 | 11.29 | 165.0558 | 0.6 | C4H4O3 | UCD,NP,HP, FCD,HP | 3-HPP metabolites |
|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 43 | 11.31 | 332.1479 | 0.8 | $\text{C}_8\text{H}_{11}\text{O}_8$ | $U_{\text{CD},\text{NP},\text{HP}}$ | Gluicoside metabolettes |
| 44 | 11.31 | 211.0665 | 5.8 | $\text{C}_{10}\text{H}_{12}\text{O}_5$ | $U_{\text{CD},\text{NP},\text{HP}}, \ F_{\text{CD},\text{NP},\text{HP}}$ | deglycosylated geniposidic acid metabolettes |
| 45 | 12.15 | 169.0487 | -1.4 | $\text{C}_4\text{H}_6\text{O}_4$ | $U_{\text{CD},\text{NP},\text{HP}}$ | catalpol deglycosylated product metabolettes |
| 46 | 12.15 | 785.2552 | 4.8 | $\text{C}_{35}\text{H}_{45}\text{O}_{20}$ | $F_{\text{CD},\text{NP},\text{HP}}$ | echinacoside metabolettes |
| 47 | 13.66 | 345.1571 | 2.2 | $\text{C}_{16}\text{H}_{25}\text{O}_8$ | $U_{\text{CD},\text{NP},\text{HP}}, \ S_{\text{NP}}$ | 6-deoxycatapol metabolettes |
| 48 | 13.95 | 489.1514 | -9.4 | $\text{C}_{21}\text{H}_{31}\text{O}_{13}$ | $U_{\text{HP}}$ | cistanoside F reduction metabolettes |
| 49 | 14.40 | 487.1480 | 2.8 | $\text{C}_{21}\text{H}_{25}\text{O}_{13}$ | $F_{\text{CD},\text{NP}}$ | cistanoside F metabolettes |
| 50 | 14.53 | 347.1747 | -4.1 | $\text{C}_{16}\text{H}_{27}\text{O}_8$ | $U_{\text{CD},\text{HP}}$ | kankanasonide N metabolettes |
| 51 | 14.55 | 477.1193 | -0.4 | $\text{C}_{21}\text{H}_{25}\text{O}_{11}$ | $U_{\text{HP}}$ | calceolarisolide A metabolettes |
| 52 | 14.84 | 315.1174 | 9.4 | $\text{C}_{14}\text{H}_{30}\text{O}_8$ | $F_{\text{NP}}$ | 3,4-dihydroxyphenethyl glycoside metabolettes |
| 53 | 15.03 | 197.0833 | 1.9 | $\text{C}_{10}\text{H}_{11}\text{O}_4$ | $U_{\text{CD},\text{NP},\text{HP}}$ | deglycosylation products of 8-epideoxyloganic acid metabolettes |
| 54 | 16.43 | 230.9984 | 1.0 | $\text{C}_4\text{H}_6\text{O}_5$ | $U_{\text{CD},\text{HP}}, \ F_{\text{NP}}$ | 4-phenylacetoyl sulfate conjugate metabolettes |
Identification of phenylethanol glycosides related metabolites

UNIFI platform was used for processing. Compared with blank samples, a total of 54 metabolites were identified in rats, including 10 prototype components and 44 metabolites, in which 24, 49, and 6 were in feces, urine, and plasma, accordingly.

A. Urine sample in BC group; B. Urine sample in CD group; C. Urine sample in CD-NP group; D. Urine sample in CD-HP group; E. Feces sample in BC group; F. Feces sample in CD group; G. Feces sample in CD-NP group; H. Feces sample in CD-HP group; I. Plasma sample in BC group; J. Plasma sample in CD group; K. Plasma sample in CD-NP group; M. Plasma sample in CD-HP group

Figure 8 chromatograph of TIC

Based on accurate mass, fragmentation cascade, and predictable neutral losses by biotransformation, a total of 35 phenylethanoid glycosides-associated metabolites were tentatively evaluated. The related metabolites of phenylethanoid glycosides have similar mass spectrum fragmentation patterns, like the typical decaffeoyl fragment \( m/z \) 461.1605, then further hydrolyzed by glycosidic and ester bonds \textit{in vivo}, and metabolized into hydroxytyrosol (HT) \( (m/z \) 153.0504, C\textsubscript{8}H\textsubscript{10}O\textsubscript{3}, 4.73 min) and caffeic acid (CA)\( (m/z \) 179.0389, C\textsubscript{9}H\textsubscript{7}O\textsubscript{4}, 0.77 min), see Figure 9A.
Figure 9  Mass Spectrum of some Metabolites in CDs

M11 indicated [M-H] at m/z 153.0504 with formula i.e., C₈H₁₀O₃, and identified as HT. M16 presented [M-H] at m/z 329.0851, which was 176 Da elevated than that of HT, revealing that it
might be a glucuronidated metabolite of HT. The \([\text{M-H}]^-\) of M26 was at \(m/z\) 343.1037, 14 Da higher than that of HT-glucuronide. Therefore, M26 was identified as HT-methylated glucuronide. M17 was identified as HT-sulfate based on its \([\text{M-H}]^-\) at \(m/z\) 233.0112, 80 Da over the HT, which could be further methylated, then produced M22, which showed the \(m/z\) 247.0278, indicating that it was HT-methylated sulfated metabolite. M7 (\(m/z\) 167.0335) and M5 (\(m/z\) 167.0762) were considered as oxidation products and methylated HT, respectively. \(\text{Figure 9B}\).

M1 indicated \([\text{M-H}]^-\) at \(m/z\) 179.0389, elucidated molecular formula was \(\text{C}_9\text{H}_7\text{O}_4\) and identified as caffeic acid (CA). M25 revealed \([\text{M-H}]^-\) at \(m/z\) 355.0704, which were 176 Da elevated than that of CA, shows that it might be a glucuronidated metabolite of CA. M27 had \(m/z\) 258.994, which was 80 Da higher than that of CA, so we elucidated it as CA sulfate, and it could produce M35 (\(m/z\) 273.0064). As M4 gives the \([\text{M-H}]^-\) at \(m/z\) 193.0524, 14 Da higher than CA, it was identified as CA methylated metabolite. M39 was CA dehydroxylation metabolite, whose \(m/z\) 163.04, it could be sulfated into M32 (\(m/z\) 242.9951).

M33 (\(m/z\) 181.0491, \(\text{C}_9\text{H}_{10}\text{O}_4\), 9.06 min) was the reduction product of CA, that is 3,4-dihydroxybenzenepropionic acid, which could be methylated into M19 (\(m/z\) 195.0623, \(\text{C}_{10}\text{H}_{12}\text{O}_4\), 0.93 min). M33 could be dehydroxeyed into M43, that is 3-HPP (\(m/z\) 165.0558, \(\text{C}_9\text{H}_{10}\text{O}_3\), 11.29 min), and M31 (\(m/z\) 341.0942, \(\text{C}_{15}\text{H}_{17}\text{O}_9\), 8.90 min) and M29 (\(m/z\) 245.0125, \(\text{C}_9\text{H}_{10}\text{O}_8\text{S}\), 8.52 min) were the glucuronidated and sulfated products. \(\text{Figure 9C}\).

For the phenylethanoid glycosides-associated metabolites, the key metabolic cascades were phase II metabolic reactions, \(i.e.,\) glucuronidation, methylation, and sulfation. The proposed metabolic cascades of phenylethanoids are depicted in \(\text{Figure 10}\).
Identification of iridoids related metabolites

By analyzing the elemental composition of the metabolites, MS<sup>E</sup> fragmentation, and associated literature, a total of 19 iridoid-associated metabolites were tentatively evaluated. Iridoid glycosides are hydrolyzed by glycosidic bonds to form their corresponding aglycones. The m/z 185.117 was for M8, 162 Da less than ajugol, was yielded by the loss of glucose residue.

M40 (m/z 199.0641, Rt 10.91 min) was the deglycosylated product of caalpol. M45 (m/z 169.0487, Rt 12.15 min) was less than 30 Da that of catalpol deglycosylated metabolite, and was identified as remove a molecule of CH<sub>2</sub>O metabolite. M34 (m/z 151.0352, Rt 9.08 min), was further loss of H<sub>2</sub>O metabolite.

M44 (m/z 211.0665, Rt 11.31 min) was a deglycosylated metabolite of geniposide, and M37 (m/z 197.0833, Rt 15.03 min) was deglycosylation of 8-epideoxyloganic acid. Metabolic reactions for iridoids could be revealed as phase I metabolism of deglycosylation. Figure 9D.

Comparison of metabolic profiling in plasma, urine, and feces between CD and its processed products

2 prototypes in plasma, 7 in urine, and 3 in feces were compared. There are 7 prototypes absorbed in CD, 7 in CD-NP, and 8 in CD-HP. M21 was only detected in the feces group of CD-NP, and M38 and M51 were detected just in urine groups of CD-HP. Compared with
metabolites, identical metabolites in plasma, urine, and feces were 4, 42, and 21, respectively. There are 34 metabolites absorbed in the CD group, 39 in CD-NP, and 40 in the CD-HP group. M5, M7, M40, and M52 were only detected in CD-NP groups, while M24, M41, and M48 were just detected in CD-HP groups.

In brief, variations were observed in the absorption as well as the metabolism of active compounds in diverse processed products of CD. Furthermore, plasma showed more susceptibility to phase I and then phase II metabolism. Their precursor compounds, such as hydroxytyrosol have anti-tumor, anti-inflammatory, antibacterial, antiviral, and antifungal properties [23]. Caffeic acid possesses anti-inflammatory, anti-cancer, and antiviral activities [24]. It was consistent with the clinical use of CD and its processed products.

**Discussion**

In TCM clinical practice, the processed products of CD have been widely used relative to raw ones. Up to date, many studies have been carried out for chemical and bio-active evaluation of the raw CD. In the current study, the qualitative analysis of CD, CD-NP and CD-HP was carried out and the obtained results showed variations in the contents of phenylethanoids as well as iridoids during the processing. Under high pressure or steaming (for a long time), glucose-combined phenylethanoids were hydrolyzed, the obtained results showed consistency with the reported studies. In-vitro studies revealed variations in chemical composition post-processing, for example, the degradation of few phenylethanoids was carried out, however, elevation was observed in its secondary glycosides or aglycones. While the components showed similarity in CD-NP and CD-HP.

Fragmentation patterns of phenylethanoid glycosides were evaluated by the UPLC-Q-TOF-MSE technique in negative ion mode. UPLC-Q-TOF-MS with UNIFI informatics platform is an analytical method for quick evaluation of chemical ingredients in TCMs sample. In this study, a total of 97 compounds, including phenylethanoid glycosides (PhGs), iridoids, etc. PCA could successfully illustrate the differences for different processing methods samples. CD group was also obviously separated from CD-NP and CD-HP, indicating that these groups could be differentiated. OPLS-DA determined two unique maker ions that could differentiate between CD and processed products. The underlined results indicated that many components were the key characteristic components of each product.

CD is a TCMs and many studies have been reported its key bioactive components, including echinacoside, acteoside, and cistanoside. However, there is a lack of clarity regarding the chemical components absorbed in vivo and metabolites post oral administration of CD and its processed products. In the current study, the evaluation of 10 prototype components and 44 metabolites was carried out in urine, plasma, and feces of rats, in which the metabolic processes of phase II were the key cascades and most of the metabolites were sulfate, glucuronide, and methylated conjugates. Phenylethanol glycosides have low oral absorption and utilization. It is difficult to absorb into the
blood, but it can be metabolized by gut flora. Phenylethanol glycosides, the main components in CD, were used as progenitors to play their roles after metabolic activation in vivo. Iridoid glycosides, the minor components, are more easily absorbed into the blood after being metabolized into the corresponding aglycone. The metabolism and material basis of CD were preliminarily elucidated. It provides a basis for screening its pharmacodynamic substances and clinical mechanism.

In the current study, most of the metabolites were at lower concentrations or not detected in rat plasma, however, higher concentration was observed in feces or the urine, which indicated that there would be no difficulty in the elimination of underlined metabolites via urine or feces. It indicates that phenylethanol glycosides can degrade rapidly in the gastrointestinal tract after oral administration. Post oral administration of CD and its processed products decoction, phenylethanoids produced into phenylethanolaglycone, like hydroxytyrosine (HT) and caffeic acid (CA) and its derivative 3-hydroxyphenylpropionic acid (3-HPP) through the metabolism of the gastrointestinal tract, these metabolites have the same activity as the prototype. Iridoid glycosides are metabolized as their aglycones. These metabolites may be more easily absorbed into the plasma to have a better medicinal effect. As depicted in Table 3, the same compounds were determined in various groups, while considerable variations were found in the concentrations of the metabolites which might be associated with the unequal efficacy of CD and its processed products.

There were differences of metabolites in urine for CD and its processed products, like methylated HT (M7) and HT oxidation (M5), both were detected only in samples of CD-NP. Decaffeoyl acetoside (M21) and 3,4-dihydroxyphenethyl glycoside (M52) were only detected in feces of CD-NP. Cistanoside F (M49) was detected in feces of CD and CD-NP, whereas cistanoside F reduction (M48) existed in CD-HP. HT sulfate conjugation dehydration product (M24), 6-deoxycatalpol glucuronide conjugation (M41), salidroside (M38) and calceolarisolide A (M51) were only detected in CD-HP.

Generally, the components having high exposure in target organs could be effective. As acteoside and echinacoside are the characteristic compounds in CD, their metabolites may be effective components. A sufficient amount of phenylethanoids and their derivatives have been evaluated and determined in vitro, only their degradation products CA and HT derivatives could be evaluated in the bio-samples. The underlined facts reveal that processing can enhance the content of phenylethanoids in CD in vitro instead of in-vivo. So, it may have preferred to explore a class of compounds relative to a single compound in TCM research.

**Conclusion**

In the existing study, the detection of 97 compounds was carried out in the extracts of CD and its processed product. Under a high temperature, the degradation of few glycosides was carried out
under an elevated temperature and as a result, some new isomers and complexes were synthesized. In an *in-vivo* study, prototype components (10) and metabolites (44) were determined or tentatively evaluated in rat plasma, feces, and urine. The underlined results revealed that the phase II metabolic processes were the key cascades of each compound and most of the metabolites were associated with echinacoside or acteoside. Numerous metabolites were only determined in rats to post oral administration of CD or CD-NP, and considerable variations were observed in the concentrations of few metabolites. The obtained results showed that the chemical composition of CD was different post-processing and affected the disposition of the compound in *vivo*.

**Abbreviations**

PhGs: phenylethanoid glycosides; CD: *Cistanche deserticola*; CMM: Chinese Materia Medica; TCM: Traditional Chinese Medicine; CD-NP: *Cistanche deserticola* processed by steaming with rice-wine under normal pressure; CD-HP: *Cistanche deserticola* processed by steaming with rice-wine under high pressure; UPLC-Q-TOF-MS²: Ultra-high performance liquid chromatography coupled with TOF-MS²; PCA: principal component analysis; VIP: Variable importance for the projection; CA: Caffeic acid; HA: hydroxytyrosol

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**Author’s contributions**

LZ, LBN, SJ participated in drafting, writing the manuscript. RJ, LPP assisted with the animal experiments and drafted and finalized all figures and tables. ZC, HY, JTZ assisted with the design and performance of this study and reviewed the manuscript. All authors approved the final version of the manuscript.

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**Availability of data and materials**

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

**Ethics approval and consent to participate**

Ethical approval for using experimental animals for this study had been obtained from the Medical Ethics Committee of Liaoning University of Traditional Chinese Medicine (Approval number:2018YS(DW)-044-01). All experimental procedures in this study were under ethical standards of the medical Ethics Committee of Liaoning University of Traditional Chinese Medicine.

**Consent for publication**

Not applicable.

**Competing interests**

The Authors declare that they have no conflicts of interest to disclose.
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Figures

Figure 1

A. isoacteoside; B. cistanoside F; C. tubuloside A; D. echinacteoside; E. acteoside; F. 2'-actylacteoside

Mass Spectrogram and cleavage pathway of phenylethanoid glycosides
Figure 2

A. ajugol  B. catalpol  C. geniposidic acid  D. geniposide  E. 8-epideoxyloganic acid
Mass Spectrogram and cleavage pathway of iridoid
1. CD, 2. CD-NP, 3. CD-HP The base peak intensity (BPI) of the samples
**Figure 4**

The PCA of CD and its different processed pro

**Roucongrong-tiwait-1.M3 (OPLS-DA)**
Scaled proportionally to R2X
Colored according to classes in M3

**Roucongrong-tiwait-1.M2 (PLS-DA): Validate Model**

| A | Roucongrong-tiwait-1.M2 (PLS-DA): Intercept | R2=(0.0, 0.522), Q2=(0.0, -0.028) |
|---|------------------------------------------|-----------------------------------|

**Roucongrong-tiwait-1.M3 (OPLS-DA)**

R2X[XSide Comp. 1] = 0.0276
Ellipse: Hotelling's T2PS

**Roucongrong-tiwait-1.M1 (PCA-X), PS-Roucongrong-tiwait-1.F**

R2X[1] = 0.645
Ellipse: Hotelling's T2PS (95%)

R2X[2] = 0.273
Figure 5

The OPLS-DA/permutation test/S-plot/heat map indicating the intensities of potential biomarkers between CD-NP and CD-HP

Figure 6

The OPLS-DA/permutation test/S-plot/heatmaps indicating the intensities of effective biomarkers between CD and CD-N
Figure 7

The OPLS-DA/permutation test/S-plot/ heatmaps revealing the intensities of effective biomarkers between CD and CD-HP
Figure 8

A. Urine sample in BC group; B. Urine sample in CD group; C. Urine sample in CD-NP group; D. Urine sample in CD-HP group; E. Feces sample in BC group; F. Feces sample in CD group; G. Feces sample in CD-NP group; H. Feces sample in CD-HP group; I. Plasma sample in BC group; J. Plasma sample in CD group; K. Plasma sample in CD-NP group; M. Plasma sample in CD-HP group chromatograph of TIC
Figure 9

Mass Spectrum of some Metabolites in CDs
Figure 10

Possible Metabolic pathway of phenylethanoids