Systematic analysis and identification of the absorption and metabolic components of Zengye decoction in type 2 diabetic rats by HPLC-ESI-Q-TOF–MS/MS

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Abstract

Background: Zengye decoction (ZYD) has been widely used in the treatment of type 2 diabetes mellitus (T2DM). Exploring the fate of various components of ZYD in vivo is of considerable significance for pharmacological research and molecular mechanism elaboration. However, the systematic analysis on the metabolic behavior of chemical components of ZYD in T2DM rats has not been reported.

Methods: To screen and characterize the complex chemical compositions of ZYD, and metabolism fate in plasma, urine, bile, and feces of T2DM rats, the model of T2DM rats was prepared. A rapid procedure using high-performance liquid chromatography coupled with electrospray ionization quadrupole time of flight tandem mass spectrometry (HPLC-ESI-Q-TOF–MS/MS) was established. Data were acquired and analyzed by Agilent MassHunter Workstation Qualitative Analysis software version B.07.00 and PCDL manager B.07.00.

Results: A total of 80 compounds were identified or tentatively characterized in ZYD, 31 more than previously detected. Besides, 36 prototype components and 49 metabolites of ZYD were found and characterized in T2DM rats, and the proposed fragmentation pathways and possible metabolic behaviors of the main types of compounds were described.

Conclusions: This study developed the understanding of the composition of ZYD as well as the cleavage rules and metabolic pathways of the prototype compounds. Besides, this study provided abundant data for further research and for study of the metabolism of traditional Chinese medicine prescriptions.

Keywords: Zengye decoction, Type 2 diabetes mellitus, HPLC-ESI-Q-TOF–MS/MS, Qualitative analysis, Metabolite identification

Background

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes, accounting for 90%–95% of all diabetic patients, which is primarily due to the relative lack of insulin secretion or reduced sensitivity to insulin [1, 2]. According to the latest report, the worldwide prevalence of adult diabetes has reached 9.3%, equivalent to 463 million adults worldwide with diabetes [3]. T2DM has become a serious global public health problem.
Therefore, it is of practical significance to develop new drugs for the treatment of T2DM. Traditional Chinese medicine (TCM) has been widely used in health care in many Asian countries for thousands of years. With the release of the detailed description of TCM by the 11th version of the International Statistical Classification of Diseases and Related Health Problems (ICD), TCM is more widely and gradually accepted around the world. Zengye decoction (ZYD) is a well-known TCM prescription used to treat ‘wasting thirst syndrome’, which would probably be diagnosed as T2DM according to the nationwide unified western medicine diagnostic criteria [4, 5]. ZYD was initially recorded in wen bing tiao bian written by Wu tang in Qing Dynasty of Chinese history (1936 AD-1912 AD) and is composed of Scrophulariae Radix, Rehmanniae Radix and Ophiopogonis Radix. Modern pharmacological studies show that ZYD exhibits hypoglycemic effect [6, 7]. However, the mechanism corresponding to its hypoglycemic effect is still unclear, due to the sophisticated features of multi-components and biological multi-effect of TCM [8]. Therefore, it is necessary to evaluate the therapeutic substances of hypoglycemic effect using modern scientific research methods, not only the herbal phytochemical compositions but also the absorption and metabolism of active ingredients in vivo of T2DM.

There have been some researches on the chemical constituents and herbal ingredients of ZYD in previous studies [9, 10]. A few reports have studied the absorption of a few compounds of ZYD [11]. We have demonstrated that ZYD improves insulin resistance in T2DM rats [12]. However, the metabolism of ZYD in experimental diabetes models has not reported. Actually, in the pathological state of diabetes, the absorption, distribution, metabolism, and excretion of ZYD may be different from those in the natural and healthy state [13, 14]. This ambiguity presents the greatest obstacle to deeper pharmacological mechanism investigation and scientific connotation interpretation. Therefore, a comprehensive, systematic analysis of the absorption and metabolic components of ZYD in vivo under the diabetic state is urgent.

In this paper, a rapid procedure using high-performance liquid chromatography coupled with electrospray ionization quadrupole time of flight tandem mass spectrometry (HPLC-ESI-Q-TOF–MS/MS) was established to characterize complex chemical compounds and metabolic components. In actual, HPLC-ESI-Q-TOF–MS/MS has been substantially applied to qualitative analysis of multiple components and metabolites in the complex mixture especially for the TCM prescriptions owing to its extraordinary performance, high resolution, accurate mass measurement, and rapid scan speed [15]. In the present study, a rat model of T2DM was established, and the absorbed components and metabolic ingredients in plasma, bile, urine, feces were screened after oral administration of ZYD. At the same time, the proposed fragmentation pathways and possible metabolic behaviors of the composition of ZYD in vivo were described in detail.

**Methods**

**Chemicals and reagents**

HPLC-grade acetonitrile was purchased from Tedia (Fairfield, OH, USA). HPLC-grade methanol was purchased from CINC High Purity Solvents Co. Ltd (Shanghai, China). The purified water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Formic acid of HPLC-grade was purchased from Aladdin Bio-Chem Technology Co. Ltd (Shanghai, China). n-Butanol was purchased from Sinopharm Chemical Reagent Co. Ltd (Nanjing, China). Catalpol, leonuride, acteoside, isoacteoside, harpagide, harpagoside, were obtained from Sichuan Weikeqi biological technology Co., Ltd (purity ≥ 98%). Cinnamic acid, p-coumaric acid, ferulic acid, were obtained from Chengdu Biopurify Phytochemicals Ltd (purity ≥ 98%). Streptozotocin (STZ) was purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade.

**Preparation of Zengye decoction extract**

The crude drugs of Scrophulariae Radix, dried Rehmanniae Radix, Ophiopogonis Radix were purchased from Nanjing Traditional Chinese Medicine Clinics (Nanjing, China). The three herbs were authenticated by professor Jin Qi (Jiangsu Key Laboratory of TCM Evaluation and Translational Research). The crude drugs (130 g. Scrophulariae Radix: dried Rehmanniae Radix: Ophiopogonis Radix, 5:4:4, w/w/w) were soaked in double-distilled water and extracted three times by boiling in distilled water (1300 mL, 1040 mL, 780 mL) under reflux for 1 h. And then, the collected filtrates were combined, concentrated, and freeze-dried to obtain a lyophilized powder.

**Preparation model of T2DM rats and drug administration**

Male Wistar rats weighing 140–160 g were purchased from Comparative Medicine Center of Yangzhou University (Yangzhou, China). The rats were raised in an air-conditioned room at 23 ± 1 °C and a 12 h light/dark cycle. The animals were adaptively fed for 1 week prior to use. All the operations were allowed by the Animal Ethics Committee of China Pharmaceutical University, China Pharmaceutical University, Nanjing, Jiangsu, China.

The model of T2DM rats was induced by high-fat diet combined with low-dose STZ (35 mg/kg) [16]. The method was improved according to the previous reports and laboratory studies [10, 17]. Briefly, the animals were
fed a high-fat diet for 3 weeks followed by intraperitoneal injection of STZ (35 mg/kg) which dissolved in cold citrate buffer (pH 4.3, 0.05 M). The fasting blood glucose (FBG) of rats was measured 3 days after injection, the level was higher than 11.1 mmol/L for subsequent experiments.

The T2DM rats were randomly divided into two groups. One group received ZYD (13 g/kg body weight, twice a day) via oral administration for 7 days. The other group received water, as the model control group. In addition, a normal healthy rats group received water, as the normal control group. The rats were fasted but with free access to water for 12 h before experiment.

Samples collection and pretreatment
All samples were obtained after drug administration. The blood samples (n = 4) were collected from T2DM rats in the heparinized centrifugal tube at 0.5 h, 1 h, 2 h, 4 h and 8 h by retro-orbital venipuncture and immediately centrifuged at 1200 × g for 15 min to obtain plasma. The urine and feces (n = 4) were collected at 0–24 h in independent metabolic cages. The feces samples were naturally dried in the fume hood and then crushed into powder. The bile (n = 4) was collected at 0–8 h by bile duct intubation and drainage under general anesthesia induced by 1% pentobarbital sodium, 55 mg/kg. All biological samples of the same type in the same group at each time point were equally combined into one sample, and stored at −80 °C before pretreatment and analysis.

An aliquot of 1 mL plasma sample was added into triple volume of acetonitrile and vigorously vortexed for 1 min. Then the mixture was centrifuged at 1500 xg for 15 min. The supernatant was transferred to another centrifuge tube and evaporated to dryness under a gentle stream of nitrogen at 37 °C. The residual was re-dissolved in 200 μL methanol: water mixture (7:3, v/v) and then centrifuged at 13,700 × g for 15 min. The supernatant was filtered through 0.22 μm nylon microporous filter membrane. The filtrates were analyzed by HPLC-ESI-Q-TOF–MS/MS. An aliquot of 1 mL urine sample was added into 3 mL methanol and vortexed for 1 min. A weight of 0.8 g feces was extracted within 8 mL methanol for 30 min under ultrasonic. Afterwards, the mixture was centrifuged at 13,700 × g for 15 min. The supernatant was transferred to another centrifuge tube and evaporated to dryness under a gentle stream of nitrogen at 37 °C. The residual was re-dissolved in 200 μL reconstituted solvent (methanol: water, 7:3, v/v) and centrifuged at 13,700 × g for 15 min, and the solution was filtered through 0.22 μm nylon microporous filter membrane. The bile was pre-treatment in the same way as urine.

Results
Identification of chemical profile of Zengye Decoction
In order to more accurately characterize the intracorporal process of ZYD in the T2DM rats, the chemical constituents of ZYD were initially identified by HPLC-ESI-Q-TOF–MS/MS. In the present study, 80 compounds from ZYD were tentatively identified by comparing with the reference standards, the retention time, and reviewing literature [9, 10, 19–25]. The total ion chromatogram (TIC) of ZYD in negative mode was showed in Fig. 1a, and the detailed compounds information was summarized in Additional file 1: Table S1. Thirty-one previously undetected compounds were found compared with previous reports [9, 10]. In addition, the structural types of all compounds in ZYD were mainly iridoid glycosides, phenethylalcohol glycosides and phenylpropanoid glycosides, aromatic acid, homoisoflavonoids, steroidal saponins.

Characterization of ZYD prototype components absorbed in T2DM rats
In this study, the biological samples, including plasma, urine, feces, and bile of T2DM rats treated with ZYD, were analyzed by HPLC-ESI-Q-TOF–MS/MS under
Fig. 1  a Total ion chromatogram (TIC) of Zengye decoction (ZYD) in the negative ion mode and b–e extracted ion chromatograms (EICs) of ZYD in biological and blank samples in the negative ion mode. b-1 ZYD plasma sample and b-2 blank plasma sample; c-1 ZYD bile sample and c-2 blank bile sample; d-1 ZYD urine sample and d-2 blank urine sample; and e-1 ZYD feces sample and e-2 blank feces sample.
constant conditions. Peaks which displayed at the same position on the chromatograms of both drug-containing biological samples and ZYD, but not in controlled blank biological samples were considered as absorbed and metabolic components of ZYD. Both TIC and EIC (extracted ion chromatograms) profiles were used to
screen the absorbed prototype components. Finally, 11 compounds were observed in plasma, 17 were in urine, 5 were in bile, 35 were in feces in T2DM rats treated with ZYD. The EICs were showed in Fig. 1b1–e2 and the prototype compounds were listed in Table 1. Furthermore, rehamapicrogenin was chosen as a representative absorbed component to demonstrate EICs further in Fig. 2. In contrast to the controlled blank biological samples, rehamapicrogenin showed remarkable peaks in drug-containing groups.

Identification of phenethyalchol glycosides and phenylpropanoid glycosides
Totally 11 phenethyalchol glycosides and phenylpropanoid glycosides were detected in T2DM rats, and their structures usually included phenylethanol, phenylpropanoid, and glycoside units. Phenylethanol group often involved loss of neutral molecules like H₂O, HCHO, and CH₃OH. Phenylpropionic acid easily loses H₂O due to the presence of hydroxyl and carboxyl groups. In another way, different phenylpropionic acid shows different typical fragments, such as ferulic acid (193, 178, 175, 149, 134), p-coumaric acid (163, 145, 119), cinnamic acid (147, 129, 103). In the case of angoroside C, the fragmentation pathway diagram was shown in Fig. 3. The quasi-molecular ion peak of angoroside C could lose a series of residuals to generate ion peaks including 651.2173 [M-Ara(132 Da)–H]⁻, 633.2078 [M-Ara-H₂O–H]⁻, m/z 607.2299 [M-Feruloyl(176 Da)-H]⁻, m/z 589.2192 [M-Feruloyl-H₂O–H]⁻, m/z 637.2163 [M-Rha(146 Da)–H]⁻, m/z 475.1792 [M-Ara-Feruloyl-H]⁻, m/z 461.1707 [M-Rha-Feruloyl-H]⁻, m/z 443.1553 [M-Rha-Feruloyl-H₂O–H]⁻, m/z 329.1226 [M-Ara-Rha-Feruloyl-H]⁻, m/z 311.1474 [M-Rha-Ara-H₂O–H]⁻, and typical fragments where the residues further to break, such as feruloyl (175,134), 3-methyldihydroxytyrosol (167), etc. Likewise, other compounds could produce similar fragments (CH₂CHO, 44 Da), formaldehyde (HCHO, 30 Da), and H₂O. Typical fragmentation pathway provided reliable information for the identification of iridoid glycosides. For instance, leonuride was the main iridoid glycosides in Rehmanniae Radix. The precursor ions were m/z 393.1503 [M+HCOOH–H]⁺, m/z 383.1210 [M+Cl]⁺, m/z 347.1439 [M–H]⁻, which produced m/z 185.0860 [M-Glc–H]⁻, m/z 167.0741 [M-Glc-H₂O–H]⁻, m/z 149.0606 [M-Glc-2H₂O–H]⁻, m/z 137.0203 [M-Glc-HCHO–H]⁻, m/z 123.0444 [M-Glc-CH₃CHO–H]⁻. At the same time, the glucosyl residue also produced typical fragments such as m/z 161.0462, m/z 113.0227. In order to more intuitively display the fragmentation pathway of this type compounds, the proposed fragmentation pathways of leonuride in negative ion mode was exhibited in Fig. 4a.

Identification of homoisoflavones
Seven homoisoflavones were detected in T2DM rats. Homoisoflavones are a particular class of flavonoids, which are connected by methylene group (CH₂) between B and C rings. Compound P79 was confirmed as methyllophiochogonane A, the MS/MS spectrum and proposed fragmentation diagram were shown in Fig. 4b. P79 gave [M–H]⁻ at m/z 341.1051, in which fragmentation at m/z 206.0580 that originated from the initial loss of B-ring and CH₂ to generate [M-B-ring–CH₂–H]⁻. The ion at m/z 178.0629 was attributed to a loss of CO from m/z 206.0580. Besides, [M–H]⁻ ion just eliminated B-ring to produce the [M-B-ring–H]⁻ at m/z 219.1341. Analogously, the remaining six compounds were identified, respectively.

Identification of other compounds
Based on their exact molecular mass and MS/MS spectra, P14, P15, P20, P23, and P39 were temporarily identified as rhamnopyranosyl vanilloyl, darendoside B, rehamapicrogenin, rehmaionoside A/B, jiocarotenoside A1/A2, respectively.

Tentative characterization of the ZYD metabolites in T2DM rats
Potential metabolic pathways of the ZYD components were determined by comparing data from public databases and relevant publications with the cleavage results of ZYD component mother nuclei. As a result, a total of 49 presumptive metabolites in plasma, feces, urine, and bile were preliminary illuminated. All metabolic components were listed in Table 2.

Identification of iridoid glycoside-related metabolites
Totally 12 metabolites of the iridoids were initially identified, most of which were derived from the
| Peak No. | t<sub>0</sub>(min) | Precursor ions (m/z) | Formula | Error (mDa) | Fragment ions | Identification | P | U | F | B | Refs. |
|---------|----------------|-------------------|---------|-------------|--------------|----------------|---|---|---|---|-------|
| P1      | 10.332         | 407.1282*         | C₁₇H₂₂O₁₀ | 8.70        | 361.1038, 199.0607, 181.0507, 169.0505 | Catalpol | + | + | + | − | [10] |
| P2      | 20.658         | 391.1284*         | C₁₅H₂₂O₁₉ | 3.81        | 211.1011, 183.0662, 165.0543, 139.0410 | Aucubin | + | + | + | − | [10] |
| P3      | 23.285         | 731.2325*         | C₁₇H₂₂O₁₀ | 7.35        | 685.2276, 505.1599, 341.1118, 179.0561 | Rehmannioside D | − | − | − | − | [10] |
| P4      | 25.065         | 409.1384*         | C₁₉H₂₂O₁₉ | 3.05        | 201.0771, 183.0662, 165.0556, 157.0504 | Harpagide | + | + | + | − | [10] |
| P5      | 26.149         | 393.1443*         | C₁₉H₂₀O₉  | 4.06        | 185.0560, 179.0526, 167.0716, 113.0238 | Leonuride | + | + | + | + | [10] |
| P6      | 29.733         | 373.1216          | C₁₇H₂₂O₁₀ | 7.58        | 331.0899, 221.0893, 167.0426, 149.0622 | Geniposidic acid | − | − | − | − | [18] |
| P7      | 30.113         | 421.1332*         | C₁₉H₂₂O₁₀ | 1.95        | 213.0732, 195.0662, 183.0657, 169.0437 | 6-O-methylcatalpol | + | + | + | + | [19] |
| P8      | 30.783         | 461.1698          | C₁₇H₂₀O₁₂ | 3.35        | 461.1698 | Decaffeoylacteoside | − | − | − | − | [10] |
| P9      | 31.790         | 375.1317          | C₁₇H₂₂O₁₀ | 2.03        | 375.1317 | 8-epilogenic acid | − | − | − | − | [9] |
| P10     | 36.172         | 487.1527          | C₁₉H₂₂O₁₉ | 6.99        | 487.1527 | Cistanoside F | − | − | − | − | [10] |
| P11     | 41.765         | 313.0938          | C₁₃H₁₈O₈   | 0.91        | 313.0938, 229.1749, 137.0605, 123.0439 | Rhamnopyranosyl vanilloyl | − | − | − | − | [20] |
| P12     | 42.066         | 475.1845          | C₁₉H₂₂O₁₀ | 2.4         | 475.1845 | Darendoside B | − | − | − | − | [10] |
| P13     | 43.203         | 607.2290          | C₂₀H₂₄O₁₆ | 4.64        | 607.2298, 473.1859, 463.1697, 443.1586, 149.0457, 131.0351 | ⍺-(3-hydroxy-4-methoxyphenyl) ethyl-O-β-L-arabinopyranosyl(1→6)-O-[6-α-L-rhamnopyranosyl(1→3)-β-D-glucopyranoside] | − | − | − | − | [10] |
| P14     | 49.444         | 183.1028          | C₁₀H₁₄O₃  | 0.13        | 183.1036, 139.1123, 123.0792 | Rehmaphigenin | + | + | + | + | [10] |
| P15     | 54.309         | 435.2269*         | C₁₉H₂₂O₁₀ | 3.33        | 435.2236, 279.1593, 161.0451, 119.0348 | Rehmabioside A/B | − | − | − | − | [10] |
| P16     | 56.018         | 799.2778          | C₂₀H₂₄O₁₆ | 11.18       | 799.2778 | Jionioside A1/A2 | − | − | − | − | [21] |
| P17     | 56.295         | 163.0399          | C₁₂H₂₂O₈  | 0.17        | 145.0270, 119.0499 | p-coumaric acid | + | + | + | − | [10] |
| P18     | 60.152         | 193.0511          | C₁₇H₂₂O₈  | 0.47        | 178.0269, 149.0598, 134.0369, 121.0282 | Ferulic acid | − | − | − | − | [10] |
| P19     | 61.894         | 623.2049          | C₁₇H₂₂O₁₅ | 6.76        | 461.1676, 315.1084, 161.0241, 135.0445 | Acteoside | − | − | − | − | [10] |
| P20     | 63.221         | 769.2634          | C₁₉H₂₂O₁₉ | 7.35        | 769.2634 | Scrophuloside B1/B2 | − | − | − | − | [22] |
| P21     | 63.473         | 623.2021          | C₁₇H₂₂O₁₅ | 3.96        | 623.2021 | Isoacteoside or Forsythoside A | − | − | − | − | [10] |
| P22     | 65.538         | 525.1665          | C₁₇H₂₂O₁₃ | 5.14        | 525.1665 | 8-O-caffeoyl harpagide | − | − | − | − | [19] |
| P23     | 67.938         | 429.2171          | C₁₇H₂₂O₈  | 4.09        | 429.2130, 249.1512, 231.1370, 187.1472 | Jiocarotenoside A1/A2 | − | − | − | − | [23] |
| P24     | 69.475         | 783.2798          | C₂₀H₂₂O₁₉ | 8.10        | 607.2294, 589.2154, 461.1663, 193.0500 | Angioside C | + | + | + | − | [10] |
| P25     | 70.273         | 783.2840          | C₂₀H₂₂O₁₉ | 12.30       | 783.2840, 829.4135 | Isoangioside C | + | + | + | − | [10] |
| P26     | 70.428         | 631.2199          | C₁₇H₂₂O₁₅ | 6.11        | 461.1706, 193.0512, 149.0577, 134.0372 | Leucosceptoside A | − | − | − | − | [21] |
| P27     | 73.561         | 651.2397          | C₁₇H₂₂O₁₅ | 10.26       | 651.2397 | Cistanoside D | − | − | − | − | [24] |
| P28     | 79.343         | 539.1823*         | C₁₇H₂₂O₁₁ | 5.29        | 493.1715, 345.1210, 183.0662, 165.0556 | Harpagoside | + | + | + | + | [10] |
| P29     | 81.455         | 147.0452          | C₁₂H₂₂O₄  | 0.15        | 147.0450 | Cymaric acid | + | + | + | − | [10] |
| P30     | 98.429         | 345.1014          | C₁₇H₂₂O₇  | 3.42        | 345.1014 | 5,7,2'-tetradihydroxy-8-methoxyl-6-methyl-homoisoflavone | − | − | − | − | [10] |
Table 1 (continued)

| Peak No. | t_R(min) | Precursor ions (m/z) | Formula | Error (mDa) | Fragment ions | Identification | P | U | F | B | Refs. |
|---------|----------|----------------------|---------|-------------|---------------|----------------|---|---|---|---|-------|
| P74     | 104.020  | 359.1161             | C_19H_20O_7^- | -2.47       | 359.1161      | Ophiopogonanone E |   |   | + | + | [10]  |
| P75     | 105.884  | 343.1215             | C_19H_20O_6^- | -2.79       | 343.1215      | 5-7-4'-trihydroxy-3'-methoxy-6,8-dimethyl hamoisoflavone |   |   |   | + | [25]  |
| P77     | 107.848  | 327.0905             | C_18H_16O_6^- | -3.09       | 327.0905      | Ophiopogonone A |   |   |   | + | [10]  |
| P78     | 110.065  | 339.0898             | C_19H_18O_6^- | -2.39       | 339.0898      | Methylphiopogonone A |   |   | + | + | [10]  |
| P79     | 110.853  | 341.1059             | C_19H_18O_6^- | -2.84       | 206.0587, 178.0258 | Methylphiopogonanone A |   |   |   | + | [10]  |
| P80     | 111.626  | 327.1260             | C_19H_20O_5^- | -2.20       | 327.1260      | Methylphiopogonone B |   |   |   | + | [10]  |

* t_R, retention time; *, [M+HCOOH−H]^-; *, [M−H]^-; P, plasma; U, urine; F, feces; B, bile. +, containing; −, not
metabolism of harpagide and its derivatives. Harpagide and its derivatives easily lose a glycosyl group and are converted to harpigenin by hydrolases. M1 displayed [M−H]− at m/z 201.1129, which typical product ions were basically consistent with the above description of harpagide. M42 showed [M−H]− at m/z 377.1471, and the fragment ion at m/z 201.1129 was yielded obviously by the loss of a GluA group (176 Da). Simultaneously, the deglucose products of some other iridoid glycosides were also found. M6 displayed [M−H]− at m/z 185.0819, a neutral loss of 162 Da (Glc) comparing with leonuride, indicating it was deglucosylated leonuride. M32 exhibited [M−H]− at m/z 331.1208. Comparing to harpagoside, the metabolite underwent a loss of glucosyl group. Possible metabolic pathways of harpagide and leonuride were shown in Fig. 5a, b.

Identification of phenylpropanoid-related metabolites
A total of 23 constituents were initially identified as generating from the metabolism of phenylpropanoid-related compounds. The metabolic pathways were shown in Fig. 6a–c. [M−H]− ions, M19 at m/z 341.0890 and M34 at m/z 245.0133, had formed by the addition of 176 Da (GluA, C6H8O7) and 80 Da (SO3), respectively, to m/z 165.0554. Both had neutral losses of H2O and CO2 (44 Da). The metabolic profile was consistent with p-coumaric acid, indicating that the two metabolites were the glucuronidation and sulfonation products of dihydro p-coumaric acid. At the same time, the demethylation and hydrogenation products of ferulic acid were also observed. The demethylated product M22, called caffeic acid, neutral losing CO2 to produce abundant fragments of m/z 135.0450. The neutral loss of hydrogenated product M29 was the same as that of ferulic acid, including.
Fig. 3 Proposed fragmentation pathway diagram of angoroside C in the negative ion mode. Ara, arabinosyl; Rha, rhamnosyl; Glc, glucosyl; Feruloyl, Ferulic acid dehydration.
the loss of H₂O to produce m/z 177.0570, and the loss of CO₂ and methyl group (CH₃, 15 Da) to produce typical debris m/z 136.0524, etc. The other 19 metabolites were identified in a similar manner.

**Identification of hydroxytyrosol-related metabolites**

Hydroxytyrosol (HT) was mainly derived from phenylethanoid glycosides, which was produced by hydrolysis and formed different metabolites through various metabolic pathways in vivo. In this study, ten HT-related metabolites were identified in vivo, and the specific metabolic pathways were shown in Fig. 6d. M15 showed [M−H]⁻ at m/z 151.0405, the loss of 2 Da (2H) from HT, suggesting that the metabolite was generated from the dehydrogenation of HT. Moreover, M18 and M31 both yielded [M−H]⁻ at m/z 233.0139, linkage of an SO₃ group separately, suggesting it to be hydroxytyrosol sulfated. Furthermore, both M25 and M41 showed [M−H]⁻ at m/z 247.0288, the addition of methyl to M18 and M31, respectively.

**Identification of metabolic components of other compounds**

Analysis of the fragmentation pattern of rehmaionoside A found that M9 showed [M+HCOOH-H]⁻ at m/z 451.2234, an addition of 16 Da to [rehmaionoside A-H]⁻, suggesting that M9 was tentatively identified as hydroxyrehmaionoside A. The product ion m/z 243.1105, and 225.0782 were losses of glucosyl group and water from M9. The product ions of M23 included m/z 183.1025, m/z 139.1122, m/z 175.0294, and m/z 113.0232. m/z 183.1025, and m/z 139.1122 were product ions of P20 and m/z 175.0294, m/z 113.0232 were the characteristic fragments of glucuronic acid. M23
Table 2 Identification of ZYD metabolic components in T2DM rats

| Peak No. | \(t_R\) (min) | Precursor ions (m/z) | Formula | Fragment ions (m/z) | Identification | Metabolic type | P | U | F | B |
|----------|----------------|---------------------|---------|---------------------|----------------|---------------|---|---|---|---|
| M1       | 12.985         | 201.076             | C_{21}H_{36}O_{14} | 201.0762, 157.0529, 139.0751 | Harpagnin | Hydrolyzation | + | − | + | − |
| M2       | 24.773         | 377.1460            | C_{16}H_{26}O_{10} | 377.1460, 217.0938, 169.0841, 161.0465 | Dihydrogen methylcatalpol | Methylation, hydrogenation | + | + | − | − |
| M3       | 26.057         | 263.0240            | C_{9}H_{12}O_{7}S | 263.0240, 183.0455, 165.0588, 121.0291 | Dehydrated harpagenin sulfure | Dehydration, sulfation | − | − | − | − |
| M4       | 26.304         | 153.0555            | C_{9}H_{12}O_{7}S | 153.0576, 123.0448, 135.0412, 121.0281 | Hydroxytyrosol | Hydrolyzation | + | − | + | − |
| M5       | 26.862         | 583.1938            | C_{27}H_{36}O_{14} | 583.2279, 195.0671, 151.0761, 179.0566, 161.0460, 149.0501 | Acetyl 6-O- dihydro-feruloyl harpagide | Hydrogenation, acetylation | − | − | − | − |
| M6       | 29.227         | 185.0819            | C_{9}H_{12}O_{4} | 185.0819, 141.0891 | Deglucosylated leonuride | Deglucosylation | + | + | − | − |
| M7       | 36.029         | 225.0774            | C_{9}H_{12}O_{4} | 225.0801, 210.0793, 165.0556 | Methyl hydrated ferulic acid | Methylation, hydration | − | − | − | − |
| M8       | 31.271         | 181.0506            | C_{9}H_{12}O_{4} | 181.0513, 163.0398, 137.0514, 119.0498 | Hydrated p-coumaric acid | Hydrolyzation | + | + | + | + |
| M9       | 35.171         | 451.2234*           | C_{19}H_{34}O_{9} | 451.2234, 225.0505, 179.0553, 161.0482 | Hydroxy rehmanioside A | Hydroxylation | − | − | − | − |
| M10      | 36.81          | 363.1109            | C_{16}H_{20}O_{8} | 363.1316, 345.1053, 183.0626, 179.0445, 165.0555, 139.0667 | Deglucosylated 8-O-cafeoil-harpagide | Deglucosylation | − | − | − | − |
| M11      | 37.114         | 137.0602            | C_{9}H_{12}O_{4} | 137.0602, 122.0397, 111.0424, 107.0455 | 8-O-deoxyhydroxytyrosol | Deoxygenation | − | − | − | − |
| M12      | 37.366         | 489.1641            | C_{15}H_{16}O_{9} | 489.1641, 179.0363, 165.0542, 113.0249 | 8-O-dehydrated caffeoyl harpagide | Dehydration | − | − | − | − |
| M13      | 37.895         | 339.0709            | C_{9}H_{10}O_{3} | 339.0709 | p-coumaric acid glucuronide | Glucuronidation | − | − | − | − |
| M14      | 37.895         | 181.0506            | C_{9}H_{12}O_{4} | 181.0506, 163.0773, 137.0601, 121.0653 | Dihydroxy caffeic acid | Hydrogenation | − | − | − | − |
| M15      | 39.276         | 151.0405            | C_{9}H_{12}O_{4} | 151.0405, 123.0439, 107.0501 | Dehydrogen hydroxytyrosol | Dehydrogenation | − | − | − | − |
| M16      | 39.401         | 371.0984            | C_{16}H_{20}O_{10} | 371.0993, 195.0657, 177.0549, 193.0334, 175.0255, 113.0242 | Didehydrated 8-O-cafeoil harpagide | Dehydration | − | − | − | − |
| M17      | 41.295         | 369.0842            | C_{16}H_{20}O_{10} | 369.0842, 195.0504, 178.0272, 149.0599, 134.0369, 113.0232 | Dihydro-ferulic acid glucuronide | Hydrogenation, glucuronidation | − | − | − | − |
| M18      | 41.618         | 233.0131            | C_{21}H_{36}O_{14} | 233.0131, 153.0554, 135.0444, 123.0449, 121.0299, 109.0274 | Ferulic acid glucuronide | Glucuronidation | + | + | − | − |
| M19      | 42.554         | 341.0800            | C_{18}H_{20}O_{8} | 341.0890, 165.0556, 121.0657, 175.0233, 149.0598, 113.0236 | Dihydro-p-coumaric acid glucuronide | Hydrogenation, glucuronidation | − | − | − | − |
| M20      | 42.703         | 357.0818            | C_{14}H_{16}O_{10} | 357.0822, 339.0646, 175.0585, 131.0406 | Hydroxyp-coumaric acid glucuronide | Glucuronidation, hydration | − | − | − | − |
| M21      | 45.675         | 181.0870            | C_{9}H_{12}O_{4} | 181.0376, 137.0971, 122.0663, 121.0653 | Dehydro-rehmaprogenin | Dehydrogenation | + | − | − | − |
| Peak No. | t<sub>R</sub> (min) | Precursor ions (m/z) | Error (mDa) | Formula | Fragment ions (m/z) | Identification | Metabolic type | P | U | F | B |
|---------|------------------|---------------------|-------------|---------|-------------------|---------------|---------------|---|---|---|---|
| M22     | 45.826           | 179.0350            | 0.02        | C<sub>9</sub>H<sub>8</sub>O<sub>4</sub> | 179.0613, 135.0450, 161.4731, 121.0657 | Caffeic acid | Hydrolyzation | – | + | + | – |
| M23     | 47.591           | 359.1361            | 1.34        | C<sub>16</sub>H<sub>24</sub>O<sub>9</sub> | 359.1361, 183.1025, 139.1122, 113.0232 | Rehmapicrogenin glucuronide | Glucuronidation | – | – | – | + |
| M24     | 47.813           | 151.0398            | 0.27        | C<sub>8</sub>H<sub>8</sub>O<sub>3</sub> | 151.0398, 107.0500 | Dehydrogen hydroxytyrosol | Dehydrogenation | – | – | – | – |
| M25     | 48.213           | 247.0288            | 0.62        | C<sub>9</sub>H<sub>12</sub>O<sub>6</sub>S | 247.0288, 167.0711, 152.0474, 149.0285 | Methyl hydroxytyrosol sulfate | Methylation, sulfation | – | + | – | – |
| M26     | 49.757           | 385.1154            | 1.38        | C<sub>17</sub>H<sub>22</sub>O<sub>10</sub> | 385.1154, 209.0821, 191.0689, 175.0120, 113.0244 | Dihydro-methyl ferulic acid glucuronide | Hydrogenation, methylation, glucuronidation | – | + | + | – |
| M27     | 50.395           | 369.0827            | 0.02        | C<sub>10</sub>H<sub>12</sub>O<sub>7</sub>S | 369.0842, 193.0500, 178.0272, 149.0599, 134.0369, 113.0232 | Ferulic acid glucuronide | Glucuronidation | – | – | – | + |
| M28     | 54.181           | 275.0239            | 0.8         | C<sub>10</sub>H<sub>12</sub>O<sub>7</sub>S | 275.0239, 195.0662, 177.0558, 151.0761, 136.0524, 121.0293 | Dihydro-ferulic acid sulfate | Hydrogenation, sulfation | – | – | – | – |
| M29     | 54.078           | 195.0667            | 0.42        | C<sub>8</sub>H<sub>10</sub>O<sub>4</sub> | 195.0676, 177.0570, 136.0524 | Dihydro-ferulic acid | Hydrogenation | + | + | – | – |
| M30     | 55.035           | 165.0556            | 0.12        | C<sub>7</sub>H<sub>8</sub>O<sub>3</sub> | 165.0559, 147.0427, 129.0326, 121.0655 | Hydrated cinnamic acid | Hydration | – | – | + | + |
| M31     | 55.744           | 233.0139            | 1.37        | C<sub>9</sub>H<sub>12</sub>O<sub>6</sub>S | 233.0131, 153.0554, 135.0444, 123.0449 | Hydroxytyrosol sulfate | Sulfation | – | – | – | – |
| M32     | 55.195           | 331.1208            | 2.09        | C<sub>10</sub>H<sub>18</sub>O<sub>5</sub> | 331.1222, 313.1108, 287.0830, 165.0553, 147.0440, 103.0543 | Deglucosylated harpagoside | Deglucosylation | – | – | – | – |
| M33     | 56.101           | 521.1820            | 12.05       | C<sub>25</sub>H<sub>30</sub>O<sub>12</sub> | 521.1785, 503.1661, 183.0665, 157.0509, 139.0404, 113.0247 | Dehydrated harpagoside glucuronide | Dehydration, glucuronidation | – | – | – | – |
| M34     | 56.427           | 245.0133            | 0.77        | C<sub>9</sub>H<sub>12</sub>O<sub>6</sub>S | 245.0133, 165.0554, 147.0432, 121.0657 | Dihydro-p-coumaric acid sulfate | Hydrogenation, sulfation | – | – | – | – |
| M35     | 58.309           | 209.0822            | 0.27        | C<sub>10</sub>H<sub>18</sub>O<sub>5</sub> | 209.0813, 191.0713, 165.0926, 149.0616 | Dihydro-methyl ferulic acid | Hydrogenation, methylation | – | – | – | – |
| M36     | 58.362           | 273.0083            | 0.85        | C<sub>10</sub>H<sub>18</sub>O<sub>5</sub> | 273.0083, 193.0503, 178.0268, 134.0369 | Ferulic acid sulfate | Sulfation | – | – | – | – |
| M37     | 59.756           | 625.2194            | 5.61        | C<sub>23</sub>H<sub>28</sub>O<sub>13</sub> | 625.2194, 461.1695, 315.1104, 181.0510, 163.0407, 153.0538 | Dihydro-acetoside | Hydrogenation | – | – | – | – |
| M38     | 60.258           | 217.1088            | 0.65        | C<sub>10</sub>H<sub>18</sub>O<sub>5</sub> | 217.1087, 199.0936, 186.2198, 171.1025, 155.1062, 153.0895 | Dihydro-methyl harpagoside | Hydrogenation, methylation | – | – | – | – |
| M39     | 61.965           | 135.0449            | 0.25        | C<sub>9</sub>H<sub>8</sub>O<sub>3</sub> | 135.0449, 123.0065, 107.0468, 100.9257 | Dehydrated hydroxytyrosol | Dehydration | + | – | – | – |
| M40     | 63.176           | 361.1518            | 1.39        | C<sub>10</sub>H<sub>18</sub>O<sub>5</sub> | 361.2317, 185.1180, 141.1279, 113.0242 | Dihydrogen rehmapicrogenin glucuronide | Hydrogenation, glucuronidation | – | – | – | – |
| M41     | 64.035           | 247.0294            | 1.22        | C<sub>9</sub>H<sub>12</sub>O<sub>6</sub>S | 247.0537, 167.0706, 152.0476 | Methyl hydroxytyrosol sulfate | Methylation, sulfation | – | – | + | – |
| Peak No. | t<sub>R</sub> (min) | Precursor ions (m/z) | Error (mDa) | Formula | Fragment ions (m/z) | Identification | Metabolic type | P | U | F | B |
|---------|-----------------|---------------------|------------|---------|------------------|---------------|---------------|---|---|---|---|
| M42     | 66.026          | 377.1471            | 377.1453   | −1.78   | C<sub>16</sub>H<sub>26</sub>O<sub>10</sub> | 377.1471, 201.1129, 183.1002, 165.0567, 175.0242, 113.0242 | Harpagenin glucuronide | Glucuronidation | − | + | − | + |
| M43     | 70.428          | 637.2199            | 637.2138   | −6.11   | C<sub>30</sub>H<sub>38</sub>O<sub>15</sub> | 637.2155, 461.1706, 193.0512, 135.0407 | Methyl acteoside | Methylation | − | − | + | − |
| M44     | 72.673          | 275.0246            | 275.0231   | −1.50   | C<sub>10</sub>H<sub>12</sub>O<sub>7</sub>S | 275.0240, 195.0665, 177.0575, 151.0772 | Hydrated ferulic acid sulfate | Hydration, sulfation | + | − | + | − |
| M45     | 72.935          | 245.0135            | 245.0125   | −0.97   | C<sub>9</sub>H<sub>10</sub>O<sub>6</sub>S | 245.0135, 165.0666, 147.0549, 121.0355 | Dihydro-<i>p</i>-coumaric acid sulfate | Hydrogenation, sulfation | − | + | − | − |
| M46     | 78.281          | 273.0081            | 273.0074   | −0.65   | C<sub>11</sub>H<sub>10</sub>O<sub>5</sub>S | 273.0090, 193.0506, 178.0271, 134.0372 | Ferulic acid sulfate | Sulfation | + | − | − | + |
| M47     | 78.166          | 149.0609            | 149.0608   | −0.10   | C<sub>9</sub>H<sub>8</sub>O<sub>2</sub> | 149.0609, 107.0480, 105.0703 | Dihydro-cinnamic acid | Hydrogenation | − | + | − | − |
| M48     | 79.350          | 583.2092            | 583.2032   | −5.97   | C<sub>23</sub>H<sub>38</sub>O<sub>14</sub> | 583.2100, 193.0525, 149.0439, 201.1145, 183.0656, 165.0568 | Acetyl-<i>β</i>-dihydro-feruloyl harpagide | Hydrogenation, acetylation | − | + | − | − |
| M49     | 74.738          | 315.1270            | 315.1297   | 2.67    | C<sub>14</sub>H<sub>20</sub>O<sub>8</sub> | 315.1278, 297.1136, 161.0582, 135.0442 | Hydroxytyrosol glucosylate | Hydrolyzation | − | + | − | − |

a): P, plasma; U, urine; F, feces; B, bile. +, containing; −, not
was thus initially identified as a glucuronic acid-binding product of rehmapicrogenin. Also, the dehydrogenation product M21 (m/z 181.087) of rehmapicrogenin, and the hydrogenation product M40 (m/z 361.1518) of M23 were observed. The above fragmentation regularity was consistent with rehmapicrogenin.

Distribution of ZYD metabolites in T2DM rats

According to the distribution of ZYD metabolites in T2DM rats, Fourteen metabolites were found in plasma samples, 23 in urine, 33 in feces, and 11 in bile, which all have been identified and the details were listed in Table 2. Harpagoside, an iridoid glycoside, was the primary bioactive constituent of ZYD, which was detected in plasma, bile and feces samples of T2DM rats and its possible metabolites could be found in all bio-samples. For example, deglucosylation of harpagoside was observed in feces due to the transformation of harpagoside by glycoside hydrolase in the gut. Besides, glucuronic conjugates of p-coumaric acid, harpigenin, ferulic acid, and rehmapicrogenin were detected in bile, which was consistent with the glucuronic conjugates as the primarily metabolites in bile excretion. Meanwhile, p-coumaric acid was found in plasma, urine, and feces, but not in bile. It was likely that p-coumaric acid was metabolized to a glucuronic conjugate in the liver and then excreted into the duodenum via the bile duct and regenerated the prototype by a glucuronidase, and some of them were reabsorbed into the liver through the enterohepatic circulation.

Discussion

According to the results of the pre-experiment, there was no significant difference between the previously established method and the current method in the HPLC optimization process [10]. Hence, the previous performance was used for the next experiment. Besides, the negative ion mode was chosen for further analysis because most of the compounds in ZYD contain functional groups such as hydroxyl, carboxyl, etc., and most components can be detected in the negative ion mode.

Sufficient detection level was the premise of instrument analysis [26]. The low concentrations of TCM components in vivo and possibly substantial interference by the matrix effect posed significant challenges for the analysis in vivo [27]. Samples handling has become the essential part of biological sample analysis, so it was of considerable significance to choose the appropriate pretreatment method. In the current research, dialysis, protein precipitation (PPT), solid phase extraction (SPE), immunoaffinity extraction, and other methods were widely used for the biological samples pretreatment. Among them, the PPT shows the advantages of simplicity, rapidity, and convenience, and it has been widely used in the qualitative analysis of TCM in vivo [28]. So PPT was the preferred method for this experiment.

The effectiveness of TCM for disease prevention and treatment depends on the active ingredients contained in TCM [29]. The absorption of a drug is a prerequisite for its pharmacological activity within the body. Therefore, it was assumed that the absorbable component might be an active ingredient, and that the disease condition might
Fig. 6 Possible metabolic pathways for major ZYD compounds in T2DM rats. a ferulic acid; b cinnamic acid; c $\mu$-coumaric acid; and d Hydroxytyrosol
have some impacts on the absorption process [30]. Diabetes may reduce the expression and function of P-glycoprotein (P-gp) in the intestine [31]. When the intestinal P-gp activity is inhibited, the absorption of some drugs will be enhanced in the intestine [32]. A previous study found that the plasma concentrations of catalpol and harpagide in diabetes-model rats were increased compared with normal rats, and that the clearance rate was slower [11]. Diabetes also changes the expression of cytochrome P450 enzymes, including CYP3A4, CYP2E1, CYP2C9, and CYP2D4 participate in phase I drug metabolism [33–35]. Catalpol has been shown to effect the activity of CYP3A4, CYP2E1 and CYP2C9 that resulted in pharmacokinetic interactions of coadministered drugs [36]. It is possible that differences in the pharmacokinetics of ZYD compounds in diabetic and normal rats may be caused by disease-associated changes in some functional enzymes.

Some of the prototype and metabolic components identified in ZYD have had pharmacological effects in the treatment of T2DM in animal models and in cell lines. Harpagoside in ZYD could activate the PPAR-γ pathway in 3T3-L1 adipocytes to regulate lipid and glucose metabolism similar to the hypoglycemic effects of thiazolidinedione [37, 38]. p-Coumaric acid was shown to promote glucose uptake and utilization by activating the AMPK pathway and upregulating GLUT2 expression [39, 40]. Ferulic acid was reported to promote glucose uptake by activating PI3K-Akt pathway and upregulating the expression of GLUT4. It has also been found to promote glycogen synthesis and inhibit gluconeogenesis by downregulating the expression of PEPCK, G6PC and upregulating glucokinase expression [41–44]. In addition, Ferulic acid was also found to increase intracellular Ca2+ to promote insulin secretion [44, 45]. Among metabolites, caffeic acid was shown to increase insulin sensitivity in HepG2 cells, reduce hepatic glucose output, enhance glucose uptake, promote insulin secretion, and increase antioxidant activity in adipocytes [45–48]. Our previous study showed that ZYD had hypoglycemic activity, improved dyslipidemia, and promoted pancreatic islet-cell function in T2DM model rats [12]. Knowing which of the chemical constituents of ZYD presented in vivo is essential for further investigation of the material basis and mechanism of ZYD in the treatment of T2DM.

Conclusions
ZYD is increasingly used for the treatment of T2DM. However there have been few reports on the component analysis of ZYD in vivo and even fewer in the pathological state of T2DM. This study identified and tentatively characterized previously undetected ZYD ingredients and was the first to systematically analyze the metabolism of ZYD ingredients in T2DM rats. As a consequence, thirty-six prototype components and 49 metabolites were presumed and characterized in vivo, and the proposed fragmentation pathways and possible metabolic behaviors of the main types of compounds were analyzed. In summary, this study added to the understanding of the chemical profile of ZYD and its metabolism information in T2DM rats. It provided essential data for the detailed pharmacokinetic study and pharmacodynamic material basis of ZYD in T2DM. Meanwhile, the study methods are applicable to the study of the metabolism of other TCM prescriptions.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13020-020-00531-z.

Additional file 1: Table S1. Compounds information of ZYD by HPLC-ESI-Q-TOF MS/MS.

Abbreviations
Ara: Arabinosyl group; FBG: Fasting blood glucose; Glc: Glucosyl group; GluA: Glucuronic acid group; HT: Hydroxytyrosol; PPT: Protein precipitation; Rha: Rhamnosyl group; T2DM: Type 2 diabetes mellitus; TCM: Traditional Chinese medicine; STZ: Streptozotocin; ZYD: Zengye decoction.

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Authors’ contributions
JQ and ZQ conceived and designed all the experiments; SC, MW and YT carried out the experiments; SC performed the data analyses and wrote the manuscript, JQ and ZQ revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The dataset supporting the conclusions of this article is included within the article.

Ethics approval and consent to participate
The study protocol and experiments were approved by the Animal Ethics Committee of China Pharmaceutical University, China Pharmaceutical University, Nanjing, Jiangsu, China.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
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