Volatile components in Yinchenzhufu decoction and their pharmacokinetics after oral administration in rats†

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In China, Yinchenzhufu decoction (YCZFD) has been used to treat cholestatic liver disease in clinical practice for hundreds of years. Nonvolatile components in YCZFD, their composition, components absorbed in blood, and pharmacokinetic characteristics have been clarified. However, information about its volatile components is limited. The aim of the present study was to identify the components of the volatile oil (VO) of YCZFD, quantify the major volatile components in YCZFD, and reveal their pharmacokinetic characteristics. In YCZFD, 85 components representing 95.36% of the total oil composition were identified by gas chromatography-mass spectrometry. Next, 11 highly abundant components were quantified in YCZFD and YCZFD VO. Finally, a sensitive headspace solid-phase dynamic extraction-chromatography-quadruple mass spectrometry method for determining 8 volatile components in rat plasma was established and applied to compare the pharmacokinetics of YCZFD and YCZFD VO after oral administration in rats. These volatile components were rapidly absorbed and eliminated, and they presented highly different exposure levels. The area under the concentration–time curves of some volatile components in YCZFD was higher than that in YCZFD VO. The results showed that the water extract of YCZFD increased the exposure of volatile components. Our study provides valuable information for understanding the potential effective components of YCZFD.

1. Introduction

Yinchenzhufu decoction (YCZFD) consists of Artemisia capillaris Thunb. (ACT), Rhizoma Atractyloides Macrocephala (RAM), Radix Glycyrrhiza (RG), Radix Aconiti Lateralis Preparata (RALP), Rhizoma Zingiberis (RZ), and Cinnamomum cassia Presl (CCP). It is a classic Chinese herbal prescription that has been used to treat cholestatic liver disease since the Qing Dynasty (18th century CE). In China, YCZFD is still widely used to treat chronic liver failure with jaundice in modern clinical practice. Experimental studies have shown that YCZFD decreases jaundice, improves liver function, and alleviates liver damage in animal models. However, to date, the effective components associated with the effect of YCZFD against liver injury have not been very clear in vivo.

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2 Material and methods

2.1 Chemicals, reagents, and materials

α-Pinene, β-phellandrene, copaene, zingiberene, and curcumene were supplied by Shanghai ZZBio Co., Ltd (Shanghai, China). Camphene, eucalyptol, borneol, and naphthalene (internal standard, IS) were provided by Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). Caryophyllene and trans-cinnamaldehyde were purchased from the National Institutes for Food and Drug Control (Beijing, China). Atractylon was supplied by Nanjing Spring & Autumn Biological Engineering Co., Ltd (Nanjing, China). The chemical structures of these analytes are shown in Fig. 1. C7–C30 saturated alkanes and dichloromethane of GC grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Methyl tert-butyl ether was obtained from TCI (Shanghai) Development Co., Ltd (Shanghai, China). The crude herbs ACT, RAM, RG, and CCP were obtained from Shanghai Dehua Pharmaceutical Co., Ltd (Shanghai, China). RALP and RZ were purchased from Shanghai Kangqiao Chinese Medicine Tablet Co., Ltd (Shanghai, China). Sodium sulfate anhydrous was supplied by Sinopharm Chemical Regent Co., Ltd.

2.2 Instrument

The gas chromatography-tandem mass spectrometry (GC-MS/MS) system consisted of an Agilent 7890A gas chromatograph combined with a 7000B triple-quadrupole mass spectrometry detector (Agilent, Palo Alto, USA).

2.3 Preparation of extracts

2.3.1 VO of YCZFD. Crude materials including ACT (900 g), RAM (1800 g), RALP (450 g), RZ (450 g), RG (900 g), and CCP (300 g), were immersed in a 10-fold amount of water for 2 h, and then extracted via hydro-distillation process using a Clevenger-type apparatus for 6 h. VO was collected and dried with anhydrous sodium sulfate. VO at 0.12% (v/w) yield rate was stored in sealed dark vials at −80°C.

2.3.2 Water extract (WE) of YCZFD. The crude materials (mentioned in Section 2.3.1) were prepared in accordance with the traditional preparation method of YCZFD. Briefly, crude materials were immersed in water and boiled twice. After filtration, the two decoctions were mixed, concentrated with a rotary evaporator, and then freeze-dried to obtain the WE powder of YCZFD.

2.3.3 YCZFD extract (VO + WE). Before use, the WE powder was dissolved in water and VO was proportionally added, then both were mixed with ultrasound to obtain YCZFD extract suspension.

2.4 Identification of components in the VO of YCZFD

The GC-MS/MS system with an HP-5 MS 5% phenyl methyl siloxane column (30 m × 0.25 mm, 0.25 μm) and NIST11 library was used to identify volatile components in the VO of YCZFD. The carrier gas was helium applied at a velocity of 1.0 mL min⁻¹, the oven temperature program was initial at 40°C, then raised to 250°C at 3°C min⁻¹, and finally held at 250°C for 5 min. The injection was in split mode at the split ratio of 1:20 with the injection volume of 1 μL and the injector temperature of 250°C. A mass spectrometer was used in the electron impact (EI) mode, in which a filament attached to the source body emits electrons into the ionization chamber through the guidance of a magnetic field, with ionization potential set at 70 eV, ionization current at 150 μA, and mass range at 50–500. The YCZFD VO/C7–C30 saturated alkanes were serially diluted with n-hexane and the supernatant was analyzed after centrifuging at 4832 × g for 10 min. According to Kovats method, the linear retention index (RI) was

![Fig. 1](image-url) Chemical structures of 11 volatile components and internal standards. (A) α-Pinene, (B) camphene, (C) β-phellandrene, (D) eucalyptol, (E) copaene, (F) caryophyllene, (G) borneol, (H) zingiberene, (I) curcumene, (J) trans-cinnamaldehyde, (K) atractylon, (L) naphthalene.
calculated for all components in the YCZFD VO sample using the retention time (RT) of a homologous series of n-alkanes (C7–C30) injected in the same conditions as the reference. The components in the YCZFD VO were identified based on RI relative to that of n-alkanes, computer matching with those in the NIST11 library, and comparisons of the fragmentation pattern of the mass spectra with the data in the database. In addition, 11 standard compounds were used for comparison and final confirmation.

2.5 Quantification of 11 volatile components in YCZFD

2.5.1 Experimental conditions. The GC-MS/MS system was used for the quantitative analysis. The capillary column was CP2025 VF-WAXms (30 m × 0.25 mm, 0.25 μm). GC temperature program was initiated at 45 °C for 3 min, then raised to 130 °C at 2 °C min⁻¹ and to 250 °C at 13 °C min⁻¹, and finally held at 250 °C for 5 min. The solvent delay was set at 4 min and the sample was injected in the split mode at the split ratio of 1 : 15 with the injection volume of 1 μL. Mass spectrometer was used in the EI source multiple reaction monitoring (MRM) mode with ionization potential set at 70 eV. The MRM parameters for the 11 volatile components and ISs are listed in Table 1. The temperature of the injector and ion source was 250 °C and 230 °C, respectively. The carrier gas was helium applied at a velocity of 1.0 mL min⁻¹, and collision cell gases were helium and nitrogen at a velocity of 2.25 and 1.5 mL min⁻¹, respectively.

2.5.2 Method validation. The developed GC-MS/MS method was validated for specificity, linearity, precision and accuracy, recovery, repeatability, and stability according to the US FDA guidelines (Draft Guidance for Industry on Analytical Procedures and Methods Validation, 2000) (details are presented in the ESI†).

2.5.3 Sample determination. YCZFD/YCZFD VO was serially diluted with methyl tert-buty1 ether:dichloromethane (50 : 50, v/v) and centrifuged at 4832 for 10 min. Next, 90 μL of the supernatant was mixed with 10 μL of IS solution. One microliter of sample was injected into the GC-MS/MS system to determine the content of 11 volatile components.

2.6 Quantitation of the 8 volatile components of YCZFD in rat plasma

2.6.1 Experimental conditions. The solid-phase dynamic extraction (SPDE) device comprised a syringe, heater, gas station for aspiration of the desorption gas, and heatable flush station for preventing analyte carryover through flushing with argon, and they were assembled on a CTC Analytics GC PAL-autosampler. The replaceable SPDE cannulas were coated on the inner surface with a polydimethylsiloxane (PDMS) phase. The GC-MS/MS conditions, including capillary column, column temperature program, injector temperature, ion source temperature, carrier gas, collision cell gases, and MRM parameters, were the same as those in Section 2.5.1. The sample was injected in the splitless mode. The multiplier voltage was 1375 V.

2.6.2 Headspace solid-phase dynamic extraction. For extraction, 100 μL of plasma sample was spiked with 5 μL of IS solution and 10 μL of HCl (0.1 M), and then placed in a PTFE/silicone screw-cap glass vial. Next, 40 mg of sodium chloride was added, and the vial was sealed airtight and vortex-mixed for approximately 20 s. The vial was kept in a single point heater set at 50 °C with an autosampler during extraction.

The SPDE method has been previously reported; here, it was partially modified as follows: the SPDE casing was inserted through the diaphragm to a depth of 20 mm, 40 times each time, and the volume of sampling headspace volume was 500 μL, and the extraction speed was maintained at 100 μL s⁻¹. The sample was injected at 20 μL s⁻¹. A blank run was appended to show the absence of carryover effects. To achieve the highest extraction efficiency, some parameters affecting the extraction rate were optimized, such as the type of SPDE sorbent coating, salting-out effect, concentration and volume of HCl added to the sample, number of extraction cycles, extraction temperature, preincubation time, desorption volume, and desorption flow speed.

2.6.3 Preparation of the calibration standards and quality control (QC) samples. The stock solutions of the 8 volatile analytes and IS (naphthalene) were prepared in methyl tert-butyl ether dichloromethane (50 : 50, v/v) and stored at −20 °C.

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Table 1  Multiple reaction monitoring parameters for 11 volatile components and Internal Standard (IS)

| Components                | Precursor ion | Product ion | Dwell time (ms) | CE (V) | RT (min) |
|----------------------------|---------------|-------------|-----------------|--------|----------|
| α-Pinene                   | 136           | 93          | 50              | 8      | 7.2      |
| Camphene                   | 136           | 93          | 50              | 8      | 8.6      |
| β-Phellandrene             | 136           | 93          | 50              | 8      | 15.1     |
| Eucalyptol                 | 154           | 139         | 50              | 2      | 14.9     |
| Copaene                    | 204           | 161         | 50              | 10     | 31.3     |
| Caryophyllene              | 189           | 105         | 50              | 22     | 37.2     |
| Bornol                     | 95            | 67          | 50              | 15     | 43.2     |
| Zingiberene                | 204           | 119         | 50              | 8      | 44.5     |
| Curcumene                  | 202           | 132         | 50              | 10     | 46.8     |
| trans-Cinnamaldehyde       | 131           | 77          | 50              | 30     | 51.1     |
| Atractylon (IS)            | 216           | 108         | 50              | 20     | 52.0     |
| Naphthalene (IS)           | 128           | 102         | 50              | 30     | 44.7     |
Next, the 8 volatile analyte stock solutions were mixed and diluted with tert-butyl ether:dichloromethane (50 : 50, v/v) to prepare a mixed working solution. The mixed working solution was serially diluted with blank rat plasma to prepare standard and QC samples.

2.6.4 Method validation. The GC-MS/MS method of the 8 volatile components of YCZFD in rat plasma was validated for specificity, linearity, precision and accuracy, recovery, and stability according to the FDA guidelines for biological sample determination methods (Bioanalytical Method Validation Guidance for Industry, 2018) [details are presented in the ESI†].

2.6.5 Pharmacokinetic study. The animal experiments were approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (the approval number PZSHUTCM190628025). Sprague-Dawley rats (6 males and 6 females) weighing 210–260 g were randomly divided into the YCZFD and YCZFD VO groups. After fasting for 12 h, both group rats were separately intragastrically administered YCZFD extract of 4.80 g kg⁻¹ (equalling 24.0 g crude herbs per kg and containing camphene 1.17, β-phellandrene 2.81, eucalyptol 0.552, copaene 0.286, borneol 0.518, zingiberene 2.16, curcumene 0.593, and atractylon 11.0 mg kg⁻¹) and YCZFD VO 29.2 mg kg⁻¹ (equalling 24.0 g crude herbs per kg and containing camphene 1.14, β-phellandrene 2.90, eucalyptol 0.542, copaene 0.281, borneol, 0.502, zingiberene 2.14, curcumene 0.569, and atractylon 11.1 mg kg⁻¹). Thereafter, 220 μL of blood was collected at 0, 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h post administration. The blood samples were immediately centrifuged to obtain plasma, and then stored at −80 °C until analysis.

The pharmacokinetic parameters of the volatile components were calculated using non-compartmental methods with Phoenix WinNonlin 6.1 (Pharsight, Mountain View, CA, USA) software. The observed value of C_max was obtained from the observed data and the observed value of AUC_0-1 was calculated using the trapezoidal rule. C_max/dose and AUC_0-1/dose (the dose-normalized values) were calculated by dividing the observed values of C_max and AUC_0-1 by the dose in each extract. Data are expressed as mean ± standard deviation (SD). The statistical differences between groups were determined using t-test, and p < 0.05 were considered statistically significant.

3. Results and discussion

3.1 Identification of multiple volatile components in the VO of YCZFD

In order to improve the reliability of the qualitative analysis of volatile components, we used the automated mass spectral deconvolution and identification system for purification of the chromatographic peak; the relatively “pure” mass spectrum was compared and matched with that in Wiley libraries (NIST11). Furthermore, values were confirmed by calculating linear retention indices (RI) of the test compounds to n-alkanes (C7–C30) under the same conditions as those of the reference. Furthermore, the RI was compared with values in the literature to accurately identify similar compounds that matched NIST11 well. In addition, we also used as many commercially available standard compounds as possible for comparison and final confirmation. Based on these three approaches, the components in YCZFD VO were identified.

A typical chromatographic profile of the VO of YCZFD is presented in Fig. 2. The volatile components in the VO of YCZFD are listed in the order of their elution time in Table 2. Eighty-five volatile components were identified in the VO, which accounted for 95.4% of the total oil composition by normalization of whole chromatographic peak area. Sesquiterpene hydrocarbons (39.5%) and oxygenated sesquiterpene (30.5%) were the most abundant components. In this study, the composition of volatile components in VO of YCZFD was clarified for the first time.

3.2 Quantification of the 11 major components in YCZFD

3.2.1 Method development. As shown in Table 2, YCZFD is a complex mixture of volatile components, and we selected 11 major components (α-pinene, camphene, β-phellandrene, eucalyptol, copaene, caryophyllene, borneol, zingiberene, curcumene, trans-cinnamaldehyde, and atractylon) for quantification. These components are relatively more abundant, which the peak area is relatively large by normalization method of chromatographic peak area, and the standard reference substances are available.

The calibration curves for all analytes showed good linearity (r² > 0.9962) and the variation in intra- and inter-batch precisions for all analytes was less than 6.92%. The recovery rate varied from 92.1% to 105%. The corresponding RSDs did not exceed 11.1%. The repeatability (RSD < 3.87%) and stability (RSD < 9.65%) were also within the acceptable limits. The results indicated that the analytical method was sensitive, and reliable for the quantification of the 11 volatile constituents in YCZFD (details are presented in the ESI†).

3.2.2 Sample determination. The level of the 11 volatile components in five batches of YCZFD and YCZFD VO samples is summarized in Tables 3 and 4. The results showed that the levels of the components in the five sample batches were relatively stable. Among the 11 components, atractylon was present at the highest concentration, followed by β-phellandrene, zingiberene, and trans-cinnamaldehyde. There was no significant difference in the level of the 11 volatile components between YCZFD and YCZFD VO. The results also indicated that the established method can provide a basis for the comprehensive quality assessment of volatile components of YCZFD.

3.3 Quantification of the 8 volatile components in YCZFD in rat plasma

3.3.1 Method development. 3.3.1.1 Stability of zingiberene and curcumene. Zingiberene and curcumene are susceptible to oxidation in rat plasma. To prevent oxidation, ascorbic acid at different concentrations were added as an antioxidant agent under dark conditions. The
Fig. 2 Total ion compound (TIC) chromatogram of (A) the volatile oil of Yinchenzhufu decoction and (B) N-alkanes containing 9 to 17 carbons.

Table 2 Chemical composition of the essential oil of Yinchenzhufu decoction

| No. | Components                      | %    | RI  | RI lit. | Identif. |
|-----|---------------------------------|------|-----|--------|----------|
| 1   | 2-Heptanol                      | 0.03 | 901 | 886    | RI, MS   |
| 2   | Cyclene                         | 0.04 | 919 | 918    | RI, MS   |
| 3   | α-piene                         | 1.24 | 930 | 931    | RI, MS, S|
| 4   | Camphene                        | 2.97 | 946 | 943    | RI, MS, S|
| 5   | Benzaldehyde                    | 0.29 | 956 | 929    | RI, MS   |
| 6   | β-Pinene                        | 0.17 | 973 | 970    | RI, MS   |
| 7   | Sulcatone                       | 0.10 | 982 | 960    | RI, MS   |
| 8   | β-Myrcene                       | 0.34 | 988 | 981    | RI, MS   |
| 9   | Pseudolimonen                   | 0.01 | 1001| 993    | RI, MS   |
| 10  | α-Phellandrene                  | 0.30 | 1003| 1007   | RI, MS   |
| 11  | α-Terpinene                     | 0.06 | 1014| 1017   | RI,MS    |
| 12  | β-Cymene                        | 0.10 | 1022| 1013   | RI, MS   |
| 13  | β-Phellandrene                  | 7.19 | 1028| 1030   | RI, MS, S|
| 14  | Eucalyptol                      | 2.56 | 1029| 1023   | RI,MS,S  |
| 15  | γ-Terpinene                     | 0.08 | 1055| 1053   | RI, MS   |
| 16  | α-Terpinolene                   | 0.15 | 1082| 1080   | RI, MS   |
| 17  | 2-Nonanone                      | 0.08 | 1089| 1074   | RI, MS   |
| 18  | 2,3-Epoxypropane                | 0.06 | 1094| 1095   | RI, MS   |
| 19  | β-Linalool                      | 0.42 | 1098| 1082   | RI, MS   |
| 20  | β-Fenchol                       | 0.02 | 1115| 1112   | RI, MS   |
| 21  | cis-p-Menth-2-en-1-ol           | 0.05 | 1121| 1118   | RI, MS   |
| 22  | trans-p-Ment-2-en-1-ol          | 0.04 | 1139| 1138   | RI, MS   |
| 23  | (−)-Camphor                     | 0.07 | 1142| 1139   | RI, MS   |
| 24  | 2-Norbornanol                   | 0.04 | 1150| 1142   | RI, MS   |
| 25  | Benzenepropanal                 | 0.19 | 1157| 1123   | RI, MS   |
| 26  | endo-2-Borneol                  | 1.78 | 1168| 1148   | RI, MS, S|
| 27  | Isomenthol                      | 0.02 | 1174| 1174   | RI, MS   |
| 28  | (−)-4-Terpinol                  | 0.19 | 1177| 1175   | RI, MS   |
| 29  | α-Terpinol                     | 0.60 | 1191| 1172   | RI, MS   |
| 30  | cis-Piperitol                   | 0.07 | 1204| 1190   | RI, MS   |
| 31  | (R)(+)-β-Citronellol            | 0.29 | 1225| 1220   | RI, MS   |
| 32  | Citral                          | 0.86 | 1235| 1241   | RI, MS   |
| No. | Components                        | %   | RI   | RI lit. | Identif. |
|-----|-----------------------------------|-----|------|--------|----------|
| 33  | Geraniol                          | 0.25| 1248 | 1238   | RI, MS   |
| 34  | α-Citral                          | 1.23| 1266 | 1250   | RI, MS   |
| 35  | *trans*-Cinnamaldehyde            | 2.63| 1268 | 1243   | RI, MS, S|
| 36  | Phellandral                       | 0.03| 1273 | 1252   | RI, MS   |
| 37  | (−)-Bornyl acetate                | 0.22| 1281 | 1273   | RI, MS   |
| 38  | 2-Undecanone                      | 0.35| 1291 | 1274   | RI, MS   |
| 39  | 2-Undecanol                       | 0.03| 1301 | 1294   | RI, MS   |
| 40  | Myrtenyl acetate                  | 0.01| 1319 | 1306   | RI, MS   |
| 41  | δ-Elemene                         | 0.05| 1332 | 1334   | RI, MS   |
| 42  | Cepheine                          | 0.15| 1348 | 1331   | RI, MS   |
| 43  | Ylangene                          | 0.08| 1363 | 1360   | RI, MS   |
| 44  | Copaene                           | 2.26| 1371 | 1376   | RI, MS, S|
| 45  | Berkheyaradulene                  | 0.22| 1382 | 1416   | RI, MS   |
| 46  | β-Elemene                         | 0.38| 1385 | 1387   | RI, MS   |
| 47  | Cyperene                          | 0.14| 1397 | 1390   | RI, MS   |
| 48  | Caryophyllene                     | 1.24| 1414 | 1421   | RI, MS, S|
| 49  | γ-Elemene                         | 2.17| 1426 | 1425   | RI, MS   |
| 50  | Humulene                          | 0.53| 1449 | 1454   | RI, MS   |
| 51  | α-Guaiene                         | 0.08| 1454 | 1440   | RI, MS   |
| 52  | Calarene                          | 0.16| 1468 | 1463   | RI, MS   |
| 53  | γ-Murolene                        | 0.92| 1475 | 1471   | RI, MS   |
| 54  | Curcumene                         | 2.86| 1478 | 1472   | RI, MS, S|
| 55  | β-Selinene                        | 1.09| 1483 | 1483   | RI, MS   |
| 56  | Zingiberene                       | 13.96| 1494 | 1492   | RI, MS, S|
| 57  | α-Farnesene                       | 1.34| 1503 | 1499   | RI, MS   |
| 58  | β-Bisabolene                      | 1.30| 1505 | 1500   | RI, MS   |
| 59  | δ-Cadinene                        | 1.20| 1514 | 1514   | RI, MS   |
| 60  | Calamenene                        | 0.42| 1516 | 1517   | RI, MS   |
| 61  | β-Sesquiphellandrene              | 2.68| 1522 | 1516   | RI, MS   |
| 62  | Ledene                            | 0.16| 1524 | 1520   | RI, MS   |
| 63  | Selina-3,7(11)-diene              | 2.83| 1532 | 1533   | RI, MS   |
| 64  | γ-Selinene                        | 0.67| 1536 | 1531   | RI, MS   |
| 65  | α-Copaen-11-ol                    | 0.74| 1542 | 1541   | RI, MS   |
| 66  | Germancrene B                     | 2.75| 1553 | 1554   | RI, MS   |
| 67  | *trans*-Nerolidol                 | 0.42| 1558 | 1555   | RI, MS   |
| 68  | Caryophyllenyl alcohol            | 0.08| 1568 | 1569   | RI, MS   |
| 69  | Spathulenol                       | 0.19| 1569 | 1569   | RI, MS   |
| 70  | Caryophyllene oxide               | 0.56| 1574 | 1575   | RI, MS   |
| 71  | Carotol                           | 0.21| 1585 | 1594   | RI, MS   |
| 72  | Isoromadendrene epoxide           | 0.02| 1594 | 1590   | RI, MS   |
| 73  | Viridiflorol                      | 0.06| 1598 | 1594   | RI, MS   |
| 74  | Humulane-1,6-dien-3-ol            | 0.09| 1601 | 1606   | RI, MS   |
| 75  | α-acoreno1                        | 0.30| 1610 | 1598   | RI, MS   |
| 76  | γ-eudesmol                        | 0.15| 1614 | 1626   | RI, MS   |
| 77  | α-eudesmol                        | 0.23| 1619 | 1637   | RI, MS   |
| 78  | (−)-Spathulenol                   | 1.39| 1622 | 1619   | RI, MS   |
| 79  | Atractylone                       | 22.28| 1656 | 1652   | RI, MS, S|
| 80  | Bulnesol                          | 0.29| 1660 | 1652   | RI, MS   |
| 81  | β-bisabolol                       | 0.62| 1665 | 1619   | RI, MS   |
| 82  | α-Bisabolol                       | 0.58| 1681 | 1683   | RI, MS   |
| 83  | Eudesm-7(11)-en-4-ol              | 0.56| 1689 | 1681   | RI, MS   |
| 84  | 2,2,7,7-Tetramethyltricycloc[6.2.1.0(1,6)]undec-4-en-3-one | 1.63| 1724 | 1730 | RI, MS   |
| 85  | 2-[(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydro-naphthalen-2-yl)-prop-2-en-1-ol | 0.13| 1756 | 1732 | RI, MS   |
|     | Total                             | 95.36|      |        |          |
|     | Hydrocarbon monoterpenes          | 12.65|      |        |          |
|     | Oxygenated monoterpenes           | 8.27 |      |        |          |
|     | Sesquiterpene hydrocarbon         | 39.51|      |        |          |
|     | Oxygenated sesquiterpene          | 30.54|      |        |          |
|     | Others                            | 4.39 |      |        |          |

*RI: retention index; RI lit.: retention index of target compound in literature from database; MS: identify by comparing the mass spectrum fragments from the NIST11 database; S: identify by comparing with the standards.*
results showed that the addition of 5 μL of 2 mg mL⁻¹ aqueous ascorbic acid solution to 100 μL of plasma could maintain stable levels of zingiberene and curcumene under various storage and treatment conditions. To prevent the potential degradation in the sample collection stage, 10 μL of 8 mg mL⁻¹ aqueous ascorbic acid solution was immediately added to 200 μL of fresh blood samples, which were then placed on ice and immediately centrifuged to separate the plasma. In addition, all sample preparation procedures were performed under dark conditions.

3.3.1.2 Optimization of the SPDE conditions. The following types of coatings for solid phase dynamic microextraction were tested: the WAX coating (polyethylene glycol), CT-225 coating (25% cyanopropyl/25% phenylpolysiloxane/50% methylpolysiloxane), CT-1701 coating (14% cyanopropyl/86% dimethyldiphenylsiloxane), CT-5 coating (15% diphenyl/95% dimethyldiphenylsiloxane), PDMS coating (PDMS), and PDMS/AC coating (PDMS + 10% active charcoal). Our results showed that PDMS coating achieved the best extraction rate.

Because the salting-out effect usually results in an increase in recovery,⁹ different amounts of NaCl were added to 100 μL of the spiked plasma standard to optimize recovery. The maximum salting-out effect was achieved with 40 mg of NaCl per 100 μL of plasma.

We also investigated the effect of pH on the extraction efficiency at five pH levels: initial plasma pH, two acidic pH values (achieved by adding 0.1 M HCl or 0.01 M HCl), and two basic pH values (achieved by adding 0.1 M NaOH or 0.01 M NaOH). As a result, the pH had no significant effect on the extraction efficiency of the other 8 components, and the acidic pH (by adding 0.1 M HCl) was used in the study.

According to the peak response, the number of extraction cycles of 50 was considered when the number of cycles ranged between 20 and 70 (Fig. 3A). The extraction temperature of 90 °C was chosen when the temperature ranged between 60 and 100 °C (Fig. 3B). The optimal preincubation time of 5 min was determined when preincubation time ranged between 5 and 20 min (Fig. 3C). The optimum desorption gas volume was considered to be 1 mL when desorption gas volume ranged between 0.25 and 1.00 mL (Fig. 3D), and the optimal desorption gas flow speed was 20 μL s⁻¹ when desorption gas flow speed ranged between 20 and 100 μL s⁻¹ (Fig. 3E).

Table 3 Contents of 11 volatile components in Yinchenzhuufu decoction (mean ± SD, n = 3)⁶

| Components          | Batch no. 180611 | Batch no. 180612 | Batch no. 180613 | Batch no. 180614 | Batch no. 180615 |
|---------------------|------------------|------------------|------------------|------------------|------------------|
| z-Pinene            | 13.8 ± 0.4       | 14.3 ± 0.3       | 13.5 ± 0.5       | 13.5 ± 0.4       | 13.3 ± 0.4       |
| Camphene            | 48.6 ± 3.0       | 47.7 ± 2.3       | 47.3 ± 1.0       | 49.4 ± 3.3       | 50.1 ± 2.3       |
| β-Phellandrene      | 117 ± 6          | 128 ± 5          | 121 ± 2          | 108 ± 2          | 129 ± 5          |
| Eucalyptol          | 23.0 ± 0.5       | 24.6 ± 1.2       | 23.4 ± 2.3       | 21.2 ± 1.8       | 23.7 ± 1.0       |
| Copaene             | 11.9 ± 0.5       | 12.8 ± 0.4       | 12.3 ± 1.0       | 11.9 ± 0.6       | 15.1 ± 0.4       |
| Caryophyllene       | 13.0 ± 0.4       | 14.2 ± 0.4       | 13.9 ± 0.3       | 12.9 ± 0.2       | 15.2 ± 0.4       |
| Borneol             | 21.6 ± 0.9       | 23.9 ± 0.5       | 24.3 ± 1.4       | 22.8 ± 0.9       | 25.4 ± 0.7       |
| Zingiberene         | 90.1 ± 2.9       | 112 ± 1          | 109 ± 2          | 85.3 ± 1.3       | 106 ± 1          |
| Curcumene           | 24.7 ± 1.0       | 27.2 ± 0.7       | 26.3 ± 0.8       | 23.0 ± 1.2       | 26.8 ± 0.8       |
| trans-Cinnamaldehyde| 67.7 ± 2.1       | 70.3 ± 3.3       | 72.2 ± 1.7       | 68.1 ± 3.3       | 71.6 ± 2.3       |
| Atractylon          | 458 ± 18         | 489 ± 18         | 434 ± 21         | 416 ± 17         | 504 ± 20         |

⁶ “n = 3” mean that three different samples were analyzed.

Table 4 Contents of 11 volatile components in Yinchenzhufu decoction volatile oil (mean ± SD, n = 3)⁶

| Components          | Batch no. 180611 | Batch no. 180612 | Batch no. 180613 | Batch no. 180614 | Batch no. 180615 |
|---------------------|------------------|------------------|------------------|------------------|------------------|
| z-Pinene            | 14.3 ± 0.4       | 14.8 ± 0.1       | 13.3 ± 0.4       | 13.7 ± 0.4       | 12.8 ± 0.3       |
| Camphene            | 47.5 ± 1.3       | 50.1 ± 0.4       | 49.1 ± 0.5       | 48.5 ± 3.1       | 48.9 ± 1.3       |
| β-Phellandrene      | 121 ± 4          | 131 ± 1          | 120 ± 2          | 104 ± 2          | 124 ± 4          |
| Eucalyptol          | 22.6 ± 0.7       | 24.2 ± 1.6       | 23.8 ± 0.5       | 22.2 ± 1.0       | 23.1 ± 0.4       |
| Copaene             | 11.7 ± 0.5       | 12.9 ± 0.3       | 12.0 ± 0.5       | 11.6 ± 0.2       | 14.7 ± 0.1       |
| Caryophyllene       | 13.4 ± 0.3       | 13.8 ± 0.7       | 13.9 ± 0.3       | 12.6 ± 0.2       | 15.9 ± 0.9       |
| Borneol             | 20.9 ± 0.7       | 24.7 ± 0.4       | 23.5 ± 0.9       | 22.4 ± 0.5       | 26.0 ± 0.7       |
| Zingiberene         | 89.1 ± 1.3       | 114 ± 1          | 106 ± 1          | 90.9 ± 0.5       | 107 ± 1          |
| Curcumene           | 23.7 ± 0.4       | 28.6 ± 1.8       | 26.3 ± 1.7       | 23.5 ± 0.8       | 27.4 ± 1.0       |
| trans-Cinnamaldehyde| 69.7 ± 1.0       | 72.2 ± 1.4       | 74.9 ± 2.7       | 70.3 ± 1.3       | 76.7 ± 0.4       |
| Atractylon          | 463 ± 16         | 504 ± 17         | 421 ± 10         | 438 ± 22         | 519 ± 9          |

⁶ “n = 3” mean that three different samples were analyzed.
3.3.2 Method validation

3.3.2.1 Specificity. Typical MRM chromatograms are shown in Fig. 4. The chromatogram of the blank plasma sample showed no interference peak for both the analytes and IS, and the analytical method displayed good specificity.

3.3.2.2 Linearity, accuracy, and precision. The calibration curves of the analytes showed good linearity (1/x2 weighted regression model) in rat plasma (Table 5). The intra- and inter-batch precision and accuracy are summarized in Table 6. The RE% was within the acceptable criteria of ±15% and the RSD% was less than 15%.

3.3.2.3 Recovery. The mean extraction recoveries of all analytes from rat plasma were 88.2–107% at three QC levels, and the RSD% was less than 15%.

3.3.2.4 Sample stability. All analytes in the rat plasma were stable at room temperature for 6 h, in an autosampler vial for 24 h, at −80 °C for 31 days, and after three freeze-thaw cycles (the RE% of all the analytes ranged from −10.7% to 4.11% and the RSD% was within 11.4%) (Table 7).

The above results showed that the established quantitative method of 8 volatile components from YCZFD in rat plasma met the requirements for biological sample determination.

In the present study, the simultaneous quantitation of eight volatile components in a traditional Chinese medicine formula in plasma using the GC-MS/MS method was reported for the first time. Of these eight components, five components (camphene, β-phellandrene, copaene, zingiberene, and curcumene) had not previously been quantified in the plasma. In the methods reported in the literature, only a small number of components such as borneol,20 atractylon,21 and eucalyptol22 were determined. Compared to the methods reported in the literature, this paper provided a sensitive and reliable method for the simultaneous determination of more volatile components in plasma with a simple, automatic SPDE.

3.3.3 Pharmacokinetic study. The developed method was successfully applied to quantify camphene, β-phellandrene, eucalyptol, copaene, borneol, zingiberene, curcumene, and atractylon in the plasma of rats after oral administration of YCZFD and YCZFD VO. The concentration–time curves of camphene, β-phellandrene, eucalyptol, copaene, zingiberene, curcumene, and atractylon are illustrated in Fig. 5. However, the plasma levels of borneol was below the lower limit of quantitation (LLOQ), and its concentration–time curves could not be drawn.
Fig. 4 Typical selected reaction monitoring chromatograms of 8 volatile components and their I.S in rat plasma samples. (I) Blank rat plasma; (II) blank plasma spiked with reference components (LLOQ); (III) plasma sample 1 h after oral administration of Yinchenzhufu decoction volatile oil in rats; (IV) plasma sample 1 h after oral administration of Yinchenzhufu decoction in rats (A: camphene, B: β-phellandrene, C: eucalyptol, D: copaene, E: borneol, F: zingiberene, G: curcumene, H: atractylon, I: naphthalene).
The results showed that after the oral administration of YCZFD and YCZFD VO, these volatile components had similar pharmacokinetic characteristics, such as rapid absorption \( t_{\text{max}} \leq 2 \) h, especially camphene, \( \beta \)-phellandrene, and eucalyptol \( t_{\text{max}} < 0.5 \) h, as well as rapid elimination (almost \( T_{1/2} \leq 4 \) h). However, their exposure levels varied widely. The AUC\(_0\) of atractylode was the highest, the AUC\(_0\) of camphene was approximately 13% that of atractylode, and the AUC\(_0\) of other components was less than 10% that of atractylode (Table 8). In the present study, atractylode and eucalyptol from YCZFD VO possessed a shorter \( T_{1/2} \) and a higher dose-normalized \( C_{\text{max}} \) and AUC\(_0\) than that of atractylode and \( C_{\text{max}} \) and AUC\(_0\) of other components in YCZFD VO group, the YCZFD group showed short \( T_{1/2} \) and increased \( C_{\text{max}} \) and AUC\(_0\) of camphene, \( \beta \)-phellandrene, copaene, and atractylode; and decreased \( t_{1/2} \) of \( \beta \)-phellandrene and copaene. The synergistic effect of traditional Chinese medicine formulae results from the interaction of multiple components in the formulae. The findings of this study showed that the WE of YCZFD increased the exposure of some volatile components, which may be beneficial to the overall effect of YCZFD.

In present study, the pharmacokinetic behaviors of multiple volatile components of YCZFD and the

| Components | Calibration curve | \( r^2 \) | Linear range (ng mL\(^{-1}\)) | LLOQ (ng mL\(^{-1}\)) |
|------------|------------------|--------|-----------------|-----------------|
| Camphene   | \( Y = 0.000248 \times X + 1.52 \times 10^{-5} \) | 0.9913 | 1.00–500 | 1.00 |
| \( \beta \)-Phellandrene | \( Y = 0.00105 \times X + 4.83 \times 10^{-5} \) | 0.9940 | 1.00–500 | 1.00 |
| Eucalyptol | \( Y = 0.000171 \times X + 4.8 \times 10^{-6} \) | 0.9943 | 1.00–500 | 1.00 |
| Copaene    | \( Y = 0.000195 \times X + 4.16 \times 10^{-7} \) | 0.9969 | 1.00–500 | 1.00 |
| Borneol    | \( Y = 0.0074 \times X + 8.79 \times 10^{-5} \) | 0.9943 | 0.500–250 | 0.500 |
| Zingiberene| \( Y = 0.000225 \times X - 3E - 05 \) | 0.9962 | 1.00–500 | 1.00 |
| Curcumene  | \( Y = 0.000334 \times X - 2.4E - 06 \) | 0.9945 | 1.00–500 | 1.00 |
| Atractylode| \( Y = 0.000485 \times X - 0.00057 \) | 0.9922 | 8.00–4000 | 8.00 |

Table 6  Intra- and inter-day variability for the assay of 8 volatile components in rat plasma

| Components | Conc. (ng mL\(^{-1}\)) | Intra-day \((n = 6)\) | Inter-day \((n = 18)\) |
|------------|------------------------|---------------------|---------------------|
|            | Mean (ng mL\(^{-1}\)) | RSD (%)             | Mean (ng mL\(^{-1}\)) | RSD (%)             |
| Camphene   | 3.00 3.0 400           | 3.17 ± 0.22 377 ± 29 | 3.04 ± 0.22 386 ± 20 | 7.0 7.5            |
|            | 30.0 30.0 400          | 30.5 ± 2.3 377 ± 29 | 30.1 ± 2.2 386 ± 27 | 7.7 6.9            |
|            | 300 400                | 28.8 ± 2.2 380 ± 28 | 29.5 ± 2.1 380 ± 20 | 7.5 5.2            |
| \( \beta \)-Phellandrene | 3.00 3.0 400 | 2.84 ± 0.08 383 ± 15 | 2.91 ± 0.18 388 ± 22 | 2.9 5.6            |
|            | 30.0 3.0 400          | 2.90 ± 0.18 383 ± 15 | 2.94 ± 0.20 388 ± 22 | 7.6 6.5            |
| Eucalyptol | 3.00 3.0 400           | 30.5 ± 2.3 383 ± 15 | 29.9 ± 2.0 396 ± 21 | 7.6 5.3            |
|            | 30.0 3.0 400          | 30.5 ± 2.3 383 ± 15 | 29.9 ± 2.0 396 ± 21 | 7.6 5.3            |
| Copaene    | 3.00 3.0 400           | 28.9 ± 1.8 404 ± 20 | 29.2 ± 1.6 404 ± 20 | 6.1 5.0            |
|            | 30.0 3.0 400          | 28.9 ± 1.8 404 ± 20 | 29.2 ± 1.6 404 ± 20 | 6.1 5.0            |
| Borneol    | 1.50 1.5 200           | 1.47 ± 0.13 195 ± 17 | 1.51 ± 0.11 199 ± 14 | 9.2 6.9            |
|            | 15.0 200               | 14.3 ± 1.2 195 ± 17 | 14.6 ± 1.0 199 ± 14 | 8.4 6.7            |
| Zingiberene| 3.00 3.0 400           | 2.86 ± 0.21 386 ± 27 | 2.92 ± 0.21 389 ± 27 | 7.5 6.9            |
|            | 30.0 3.0 400          | 29.2 ± 1.5 386 ± 27 | 30.2 ± 1.7 389 ± 27 | 5.2 6.9            |
| Cucumene   | 3.00 3.0 400           | 3.14 ± 0.28 423 ± 29 | 2.97 ± 0.25 404 ± 30 | 8.8 7.3            |
|            | 30.0 3.0 400          | 29.5 ± 2.0 423 ± 29 | 30.3 ± 2.1 404 ± 30 | 6.6 6.8            |
| Atractylone| 24.0 240               | 25.2 ± 1.6 228 ± 18 | 24.6 ± 1.6 238 ± 16 | 6.3 6.7            |
|            | 240 3200               | 228 ± 18 3110 ± 240 | 238 ± 16 3080 ± 170 | 8.1 7.8            |

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characteristics of the influence of water extract on the pharmacokinetics of volatile components were firstly clarified, which can provide valuable information for understanding the potential effect of YCZFD components. However, to date, to the best of our knowledge, there have been no studies on the CYP enzymes and transporters involved in these volatile

Table 7 Stability of 8 volatile components in rat plasma under different storage conditions (n = 6)

| Components   | Nominal conc. (ng mL⁻¹) | Room temperature for 6 h | In autosampler vials for 24 h | Three freeze–thaw cycles | −80 °C for 31 days |
|--------------|-------------------------|--------------------------|-------------------------------|--------------------------|-------------------|
|              | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) |
| Camphene     |           |         |         |         |         |         |         |         |         |         |       |
| 3.00         | 10.6     | −0.9    | 4.3     | −2.9    | 4.0     | −0.1    | 6.6     | −4.4    |       |         |
| 400          | 4.1      | −4.1    | 7.1     | −1.8    | 2.5     | −2.2    | 7.2     | 1.5     |       |         |
| β-Phellandrene| 3.00    |         |         |         |         |         |         |         |         |         |       |
| 400          | 2.9      | −9.2    | 9.3     | −2.4    | 2.0     | −6.8    | 7.3     | −3.6    |       |         |
| Eucalyptol   |           |         |         |         |         |         |         |         |         |         |       |
| 3.00         | 7.0      | −2.4    | 10.1    | 0.0     | 2.7     | 0.3     | 11.4    | −2.2    |       |         |
| 400          | 2.9      | −6.6    | 7.6     | −3.9    | 9.1     | −4.2    | 2.0     | −0.3    |       |         |
| Copaene      |           |         |         |         |         |         |         |         |         |         |       |
| 3.00         | 5.1      | −6.9    | 5.4     | −5.5    | 8.8     | −5.0    | 3.3     | −7.9    |       |         |
| 400          | 2.4      | −9.8    | 9.4     | −2.7    | 6.6     | 0.1     | 7.3     | −3.7    |       |         |
| Borneol      | 1.50     | 6.6     | −6.0    | 3.4     | −6.2    | 5.9     | −3.8    | 3.3     | 2.9     |       |         |
| 200          | 5.3      | −8.0    | 7.9     | −6.0    | 4.6     | −6.4    | 6.7     | 2.7     |       |         |
| Zingiberene  | 3.00     | 3.9     | −0.7    | 11.0    | −0.3    | 7.5     | −1.3    | 7.4     | 2.1     |       |         |
| 400          | 5.7      | −2.2    | 3.1     | −5.3    | 3.3     | −3.0    | 8.6     | −0.5    |       |         |
| Curcumene    | 3.00     | 2.6     | −8.8    | 5.6     | −10.7   | 9.7     | −4.8    | 3.7     | −5.5    |       |         |
| 400          | 2.8      | −9.2    | 3.0     | −9.4    | 2.9     | −4.5    | 7.0     | −3.4    |       |         |
| Atractylon   | 24.0     | 6.0     | −2.8    | 5.7     | −5.6    | 5.8     | 4.1     | 4.0     | −2.2    |       |         |
| 3200         | 3.5      | −6.0    | 4.3     | −3.9    | 3.1     | −2.2    | 6.5     | −3.3    |       |         |

Fig. 5 Profiles of mean plasma concentration–time of (A) camphene, (B) β-phellandrene, (C) eucalyptol, (D) zingiberene, (E) curcumene, (F) copaene and (G) atractylon after a single oral dose of 4.8 g kg⁻¹ Yinchenzhufu decoction (YCZFD) and 29.2 mg kg⁻¹ YCZFD volatile oil (VO) in rats (mean ± SD, n = 6).
Table 8 Pharmacokinetic parameters of volatile components in rat plasma after oral administration of 4.8 g kg⁻¹ Yinchenzhufu decoction (YCZFD) and 29.2 mg kg⁻¹ YCZFD volatile oil (VO) (n = 6, mean ± SD)⁴

| Components | Group | T_{max} (h) | C_{max} (ng mL⁻¹) | C_{max}/dose (µg mL⁻¹) | T_{1/2} (h) | AUC_{0-4} (ng h mL⁻¹) | AUC_{0-dose} (µg h mL⁻¹) |
|------------|-------|-------------|-------------------|------------------------|------------|------------------------|-------------------------|
| Camphene   | VO    | 0.50 ± 0.00 | 14.4 ± 4.1        | 305 ± 86               | 2.20 ± 0.35| 38.5 ± 9.0             | 811 ± 189               |
|            | YCZFD | 0.42 ± 0.13 | 26.0 ± 8.0⁴       | 535 ± 16³             | 1.78 ± 0.21| 66.5 ± 17.9⁴          | 1370 ± 377³             |
| β-Phellandrene | VO    | 0.25 ± 0.00 | 40.2 ± 4.1        | 332 ± 34               | 3.59 ± 1.48| 63.7 ± 10.4            | 526 ± 86                |
|            | YCZFD | 0.29 ± 0.10 | 68.0 ± 23.9⁵      | 581 ± 204⁶            | 1.77 ± 0.19| 99.2 ± 22.5⁶          | 848 ± 192⁵              |
| Eucalyptol | VO    | 0.42 ± 0.30 | 81.7 ± 19.9       | 3620 ± 881            | 1.84 ± 0.30| 216 ± 98               | 9560 ± 4340             |
|            | YCZFD | 0.46 ± 0.10 | 101 ± 27          | 4390 ± 1170           | 1.69 ± 0.23| 305 ± 43               | 13 300 ± 1870           |
| Copaene    | VO    | 2.00 ± 0.63 | 17.7 ± 4.2        | 1510 ± 359            | 2.39 ± 0.65| 64.0 ± 19              | 5470 ± 1620             |
|            | YCZFD | 1.83 ± 0.41 | 25.1 ± 4.5⁵       | 2110 ± 378⁶           | 1.72 ± 0.17| 82.2 ± 16.2            | 6910 ± 1360             |
| Zingiberene| VO    | 1.00 ± 0.00 | 63.8 ± 9.6        | 716 ± 108             | 1.76 ± 0.81| 171 ± 38               | 1920 ± 426              |
|            | YCZFD | 1.00 ± 0.00 | 75.0 ± 18.3       | 823 ± 203             | 1.59 ± 0.28| 191 ± 41               | 2120 ± 455              |
| Curcumene  | VO    | 1.00 ± 0.00 | 7.66 ± 1.26       | 323 ± 53              | 2.57 ± 0.57| 21.8 ± 4.2             | 920 ± 177               |
|            | YCZFD | 0.58 ± 0.20 | 7.94 ± 1.66       | 321 ± 67              | 4.14 ± 0.89| 22.3 ± 4.7             | 903 ± 190               |
| Atractylon | VO    | 0.92 ± 0.20 | 495 ± 105         | 1070 ± 227            | 1.86 ± 0.34| 1610 ± 330            | 3480 ± 713              |
|            | YCZFD | 1.08 ± 0.49 | 721 ± 153⁴        | 1570 ± 334¹           | 1.84 ± 0.20| 2230 ± 460⁴           | 4870 ± 1000⁴            |

⁴ Data are expressed as the mean ± SD, n = 6. ⁵ p < 0.05 vs. VO group. ⁶ p < 0.01 vs. VO group. ⁷ The observed value. ⁸ Dose-normalized value.

compounds. The mechanism of the pharmacokinetic interactions between the components of the WE and volatile components of YCZFD is still unclear and requires further research.

4. Conclusions

In the present study, 85 volatile compounds were identified in the VO of YCZFD, and 11 highly abundant volatile components in YCZFD and YCZFD VO were quantified using an established GC-MS/MS method. An HS-SPDE-GC-MS/MS method for the simultaneous quantification of camphene, β-phellandrene, eucalyptol, copaene, borneol, zingiberene, curcumene, and atractylol in rat plasma was developed, which provides a methodological basis for the pharmacokinetic study of volatile components in YCZFD and other related Chinese materia medica. The pharmacokinetic laws of camphene, β-phellandrene, eucalyptol, copaene, zingiberene, curcumene, and atractylol from YCZFD, as well as the characteristics of exposure of volatile components increased by WE of YCZFD, were established. These results elucidate the potentially effective components of YCZFD that can be used to promote progression in the development of YCZFD.

Author contributions

Bin Zan performed the experiments and completed this manuscript. Yuanyuan Li guided the experiment. Xiaoshu Sun and Tianming Wang assisted the experiments. Rong Shi guided the experiment and revised the article. Yueming Ma designed the experiment, guided and revised the article.

Conflicts of interest

The authors declare no conflict of interests, financial or otherwise.

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