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Rapid discovery and identification of the anti-inflammatory constituents in Zhi-Shi-Zhi-Zi-Chi-Tang

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[ABSTRACT] The anti-inflammatory active ingredients of Zhi-Shi-Zhi-Zi-Chi-Tang (ZSZZCT), a traditional Chinese medicine formula, were predicted and identified using an approach based on activity index, LC-MS, semi-preparative LC and NMR. Firstly, the whole extract of ZSZZCT was analyzed using liquid chromatography-quadrupole time of flight-mass spectrometry (LC-Q-TOF-MS) and liquid chromatography - ion trap mass spectrometry (LC-IT-MS), 79 constituents were detected and 39 constituents were identified unambiguously or tentatively. Subsequently, the whole extract of the formula was separated into multiple components and the activity index method was used to calculate index values of the 79 constituents by integrating the chemical and pharmacological information of multiple components. Four polymethoxyl flavones were predicted as the major active constituents according to the activity index values. Furthermore, three polymethoxyl flavones were prepared using the strategy with semi-preparative LC guided by LC-MS, and their anti-inflammatory activities were validated. The results show that three polymethoxyl flavones with higher positive index values, i.e., 3, 5, 6, 7, 8, 3', 4'-heptamethoxyflavone, 3-hydroxynobiletein and tangeretin had significant anti-inflammatory effects. In conclusion, the predicted results indicated that the activity index method is feasible for the accurate prediction of active constituents in TCM formulae.

[KEY WORDS] Zhi-Shi-Zhi-Zi-Chi-Tang; LC-MS; Semi-preparative LC; NMR; Activity index; Anti-inflammatory activity

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Introduction

Traditional Chinese medicine (TCM) has been widely applied for the treatment of diseases in China for thousands of years and is regarded as a valuable resource for the discovery of lead compounds or new drugs. But it is laborious and time-consuming to screen bioactive constituents by the conventional phytochemical approach, and sometimes it ignores the trace constituents in the process of systematical separation by the column chromatography. In an attempt to rapidly discover active compounds from TCM, several methods have been developed by combining liquid chromatography - mass spectrometry (LC-MS) with various screening strategies, such as cell membrane chromatography [1], affinity ultrafiltration [2], magnetic beads/sepharose beads with immobilized enzyme [3-4], hollow fiber based affinity selection and on-line biochemical detection [4-5]. Han *et al*. [6] screened an anaphylactic constituent, harpagoside, in MaiLuoNing injection by rat basophilic leukemia-2H3 cell membrane chromatography coupled with LC-MS. These methods can rapidly screen active constituents from complex samples, but generally ignore the trace constituents existing in TCM. It has been reported that some trace constituents in natural products have significant pharmacological activities [7-8]. Therefore, there is a critical need for the development of a rapid discovery, identification and preparation of the active trace constituents from TCM.

Bioassay-guided techniques combined with phytochemical approaches are known as the main strategies to discover active constituents. However, in general, there are several compounds found in one active component. So, it is still blindness to separate the compounds from active component. To attempt to resolve the problem, we have developed an activity index method that has been previously reported [9]. The activity index method can predict the potential active constituents in TCM and has been successfully used to discover the active compounds in TCM formulae [9-10]. Not only can this approach evaluate the activity of major constituents

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in TCM, but can also predict the activity of trace constituents which can be concentrated by separating the whole extract of TCM into fractions. Trace constituents can be detected in fractions and their contribution to the activity could be evaluated by activity indices.

ZSZZCT, a TCM formulae from Shang-Han-Lun, is made from *Gardeniae fructus* (Zhi-Zi in Chinese), *Citrus aurantium L.*(Zhi-Shi in Chinese), and *Sojae semen praeparatum* (Dan-Dou-Chi in Chinese). It is mainly applied to treat abdominal fullness and distention in clinic [11]. The chemical constituents of ZSZZCT have not been reported, and the effective constituents of ZSZZCT remain to be determined.

In this work, ZSZZCT was firstly analyzed by liquid chromatography-ion trap mass spectrometry (LC-IT-MS) and liquid chromatography-quadrupole time of flight mass spectrometry (LC-Q-TOF-MS) to characterize the main chemical constituents in ZSZZCT. LC-IT-MS can provide the MS^n fragment ions and correlate the fragment ions to their precursor ions of the constituents, which are very helpful to speculate the functional groups and molecular fragments in their structures. LC-Q-TOF-MS offers the accurate mass, which can be used to generate molecular formula of the constituents. Then, the whole extract of ZSZZCT was dissected into multiple components by macroporous resin column chromatography and preparative LC, which decreases the chemical complexity and concentrates the trace constituents. The anti-inflammatory activities of ZSZZCT and its components were then evaluated using LPS-induced NO production in RAW 264.7 macrophages. The activity index method was used to predict the active compounds in ZSZZCT, and the strategy [12] based on semi-preparative LC guided by LC-MS was used to prepare the potential active compounds. Finally, three compounds with high activity index values were validated with strong anti-inflammatory activity *in vitro*.

Experimental

Materials and reagents

The experimental materials of *Citrus aurantium L.*, *Gardeniae fructus*, *Sojae semen praeparatum* were purchased from the local market in Hangzhou. Reference standards of hesperidin, neohesperidin, tangeretin, were obtained from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China). Narirutin and naringin were purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). Geniposide, synephrine were acquired from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Citric acid from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Genipin-1-*β*-D-gentiobioside were purchased from Zhongxin Innova Laboratories (Tianjin, China), Succinic acid was purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) and didymin from Shanghai Pure One Biotechnology (Shanghai, China). Epijasminoside A, jasminoside A, shanzhiside, deacetyl asperulosidic acid methyl ester and scandoside methyl ester were isolated from *Gardeniae fructus* by our laboratory and their structure were identified by ^{1}H NMR and ^{13}C NMR.

Acetonitrile and methanol (both HPLC grade) were purchased from Merck KGaA (Darmstadt, Germany). Formic acid of HPLC grade was purchased from ROE Scientific Inc. (Newark, USA). Deionized water was prepared through a Milli-Q system (Millipore, Milford, MA, USA). Methanol-d_4 was acquired from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile for preparative HPLC was purchased from Amethyst Chemicals J&K Scientific Ltd. (Beijing, China). The 95% ethanol was bought from Zhejiang Changqing Chemical Co. Ltd. (Hangzhou, China) and D101 macroporous resin was purchased from Tianjing Haiguang Chemical Co. Ltd. (Tianjin, China).

Dulbecco's modified Eagle's medium (DMEM, 4.5 g·L^{-1} glucose), fetal bovine serum (FBS) were purchased from Corning.trypsin–EDTA and the penicillin–streptomycin were obtained from Gibico BRL (Grand Island, NY, USA). Indometacin, dimethylsulfoxide (DMSO, purity ≥99.5%), Thiazolyl Blue Tetrazolium Bromide (MTT), and lipopolysaccharides (LPS) were acquired from Sigma–Aldrich (St. Louis, MO, USA). RAW 264.7 cells were acquired from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). NO Assay Kit was purchased from Beyotime Biotechnology Co. Ltd. (Shanghai, China). NaCl, KH_2PO_4, KCl and $Na_2HPO_4{\cdot}12H_2O$ were all obtained from Sangon Biotech Co. Ltd. (Shanghai, China).

Sample preparation

Stock solutions of the 16 substances were prepared with a certain amount in 50% methanol and stored at 4 °C. A mixed solution (about 200 μg·mL^{-1} for each compound) was acquired by mixing the store solutions and diluted with 50% methanol. The solution was filtered with 0.22 μm membranes before LC-IT-MS analysis.

According to the composition recorded in Shang-Han-Lun, ZSZZCT was prepared by the following procedure: *Citrus aurantium* L. (45 g) and *Gardeniae fructus* (60 g) were mixed and immersed in 1 L distilled water (1/10, *W*/*V*) for 12 h and then extracted by reflux for 1 h. Then 1 L water containing 125 g of *Sojae semen praeparatum* (8/1, *V*/*W*) which had been immersed for 12 h, was added to the residue and extracted by reflux for 1 h. The two extracts were combined and then freeze-dried. 10 mg of freeze-dried powder of ZSZZCT was dissolved into 1 mL distilled water. Then the solution was centrifuged for 10 min and the resulting supernatant was filtered by a 0.22 μm membrane before introduced to the LC-MS analysis.

The extractions of *Citrus aurantium* L., *Gardeniae fructus* and *Sojae semen praeparatum* were carried out as following: the raw material immersed (10 g) in 100 mL distilled water (1/10, *W*/*V*) for 12 h and then extracted by reflux for twice (1 h for each time). These two extracts were mixed and freeze-dried. The following steps were the same as that for ZSZZCT before LC-MS analysis.

The preparation of ZSZZCT components was conducted through the following steps: (1): the extract of ZSZZCT was loaded in a glass column (4.6 cm × 30 cm) packed with the macroporous resin D101. After the extract was absorbed for 3 h, the column was firstly rinsed with H_2O to give aqueous component **B01**, and then in turn eluted with 20% (*V*/*V*), 40% (*V*/*V*) and 95% (*V*/*V*) aqueous ethanol solution to give components **B02**, **B03**, and **B04**, respectively; (2): components **B02**, **B03**, and **B04** were further separated by an Agilent 1200 equipped with a G1362A Prep pump, a G1365D Multi-wave length detector, a Preparative manual sampler and a Zorbax SB-C_{18} column (21.2 mm × 250 mm, 7 μm, Agilent) with the mobile phase consisted of phase A (H_2O) and phase B (Acetonitrile) at a flow rate of 10 mL·min^{-1}. The injection volume was 1 mL and UV spectra were recorded at 210, 230, 254, 280 and 310 nm. All the subcomponents were obtained every three minutes from the fourth minute to the sixty-fourth minute. However, the elute gradients were varied due to the difference between the polarity of the components. The elute gradient was used for component **B02** as follows: 0 min, 3% B; 50 min, 20% B; 64 min, 50% B; 67 min, 100% B; 70 min, 100% B. And the collected subcomponents were named as components **C01**–**C21**. For component **B03**, the elute gradient was set as follows: 0 min, 5% B; 5 min, 10% B; 55 min, 20% B; 60 min, 50% B; 63 min, 70% B; 65 min, 100% B; 70 min, 100% B. The collected subcomponents were named as components **D01**–**D21**. Components **E01**–**E21** collected from **B04** was set at the following gradient program: 0 min, 20% B; 20 min, 30% B; 40 min, 45% B; 55 min, 90% B; 60 min, 100% B; 70 min, 100% B. The treatment of the above 63 subcomponents and four components before LC-MS analysis was the same with that of the extract of ZZZSCT.

The *Citrus aurantium* L. (5 kg), was extracted with ethanol for two times (1 h for each time). The two extracts were combined and condensed to about 1.5 L, then the concentrate was loaded onto a glass column (112 mm × 180 cm) packed with the macroporous resin D101. After the concentrate was absorbed for 3 h, the column was rinsed with 60% aqueous ethanol solution and deserted the eluent, then only collected the fraction eluted by 95% ethanol aqueous solution. The obtained solution was freeze-dried after being concentrated in vacuum at 60 °C. 100 mg of freeze-dried powder was dissolved into 1 mL methanol solution. Then the solution was centrifuged for 10 min and the resulting supernatant was filtered through a 0.22 μm membrane before Semi-preparative HPLC and LC-IT-MS analysis.

LC-IT-MS system

Liquid chromatography was performed on an Agilent 1100 HPLC (Agilent, Waldbronn, Germany) equipped with a binary pump, photo-diode array detector, an auto plate-sampler, and a thermostatically controlled column compartment. The sample was separated on a Zorbax SB-C_{18} Rapid Resolution HT column (4.6 mm × 50 mm, 1.8 μm, Agilent) at a flow rate of 0.6 mL·min^{-1} with the injection volume of 3 μL. The mobile phases were 0.05% formic acid-water (A) and acetonitrile (B), respectively. A gradient program was applied according to the following profile: 0 min, 3% B; 35 min, 41% B; 40 min, 100% B; 50 min, 100% B. The column temperature was set at 30 °C and the PDA detector scanned from 190 to 400 nm.

IT-MS analysis was performed by a Finnigan LCQ Deca XP^{plus} ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) interface and an ion trap mass (IT-MS) analyzer, using the following operating parameters: auxiliary/sweep gas (high-purity N_2) flow rate, 20 arb; sheath gas (high-purity N_2) flow rate, 60 arb; collision gas, high-purity helium (He); capillary temperature, 350 °C; ESI spray voltage, ± 4 kV; capillary voltage, ± 19 V; tube lens offset voltage, ± 25 V. The sample collision energy for CID was set at 25–55 V. Each sample was analyzed in both positive and negative modes. The mass spectra were recorded within the range *m/z* 100–1500, while for the MS^n, the collision energy was adjust to its appropriate range.

LC-Q-TOF-MS system

An Agilent 6230 mass spectrometer was used for the accurate mass determination. (Agilent Corp., USA). The chromatographic separation conditions were the same with LC-IT- MS mentioned above. The Q-TOF-MS analysis was performed in positive and negative ion modes using a full scan mode with an electrospray ionization source, respectively. The mass range was set at 100–1500 Da. The parameters of the ESI source as set: drying gas temperature, 325 °C; drying gas (N_2) flow rate, 11 L·min^{-1}; capillary voltage, 4000 V; nebulizer pressure, 45 psig.

Semi-preparative LC and NMR system

The semi-preparative LC was conducted on an Agilent 1100 HPLC equipped with a quaternary pump, an ultraviolet detector, an auto plate-sampler, and a thermostatically controlled column compartment. The Zorbax SB-C_{18} column (Semi-Preparative, 9.4 mm × 250 mm, 5 μm, Agilent) was eluted with the gradient profile of A (0.1% formic acid–water) and B (acetonitrile) at a column temperature of 30 °C with a flow rate of 3.0 mL·min^{-1}: 0 min, 5% B; 60 min, 55% B; 110 min, 55% B; 150 min, 75% B; 155 min, 100% B; 165 min, 100% B. The injection volume was 100 μL and detected at 254 nm. NMR spectra were obtained on a Bruker AVANCE III-500 spectrometer (^{1}H: 500 MHz, ^{13}C: 125 MHz; Bruker Corporation, Billerica, MA, USA) in methanol-d_4 or $CDCl_3$ with TMS as the references.

Cytotoxic evaluation of the test sample

RAW 264.7 macrophages (5 × 10^3 cells per well) plated in a 96-well plates were pre-incubated and then treated with DMSO-dissolved samples for 24 h, which were diluted with DMEM supplemented with 10% inactivated FBS, 1% penicillin G (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}). MTT stock solution (5 mg·mL^{-1} in PBS) was diluted to 0.5 mg·mL^{-1} in culture medium before adding to each well to a volume of 100μL. Four hours later, the culture supernatants were removed and DMSO (100 μL per well) was added to dissolve the formazan crystalsin. The absorbance was detected at

580 nm by a microplate reader (Bio-Tek ELX800, Winooski, VT, USA). The experiments were finished for three times in parallel, and fresh culture medium was carried out as a blank in all experiments.

Anti-inflammatory activity evaluation of the test sample

RAW 264.7 macrophages (2×10^4 cells per well) plated in a 96-well plates were pre-incubated and then treated with lipopolysaccharide (LPS, 200 ng·mL^{-1}) plus samples in fresh medium (140 μL per well). The cells were then incubated for an additional 24 hours. The quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. Firstly, culture supernatant (100 μL) were collected to react with Griess reagent I and II (50 μL) at room temperature for 10 min away from light. Then, the absorbance at 535 nm was measured in a microplate reader (Bio-Tek ELX800, Winooski, VT, USA). Indometacin (50 μmol·L^{-1}) was adopted as a positive control. The results were presented as means ± standard division of triplicate experiment.

Calculation of the activity indices of constituents

The activity index values of constituents were given by the following formula of our previously published work [9]:

$$AI_j = \sum_i^m I_{s,i} A_{n,i,j} C_i$$

AI_j: activity index of constituent j; $I_{s,i}$: standardized value of NO inhibition rate of component i; $A_{n,i,j}$: normalized value of peak area of constituent j in component i, C_i: weight of the anti-inflammatory concentration of component i.

Results and Discussion

Identification of chemical constituents in ZSZZCT using LC-IT-MS^n and LC-Q-TOF-MS

The ZSZZCT extract was analyzed by LC-IT-MS and LC-Q-TOF-MS in both negative and positive ion modes. The base peak chromatograms of LC-IT-MS are shown in Fig. 1. The extracts of ZS, ZZ and DDC were also analyzed by LC-IT-MS. A total of 79 compounds were detected in ZSZZCT as listed in Table 1, and the sources of compounds were attributed to the corresponding TCMs by comparing the retention time and MS^n data of the peaks in ZSZZCT with that in ZS, ZZ and DDC.

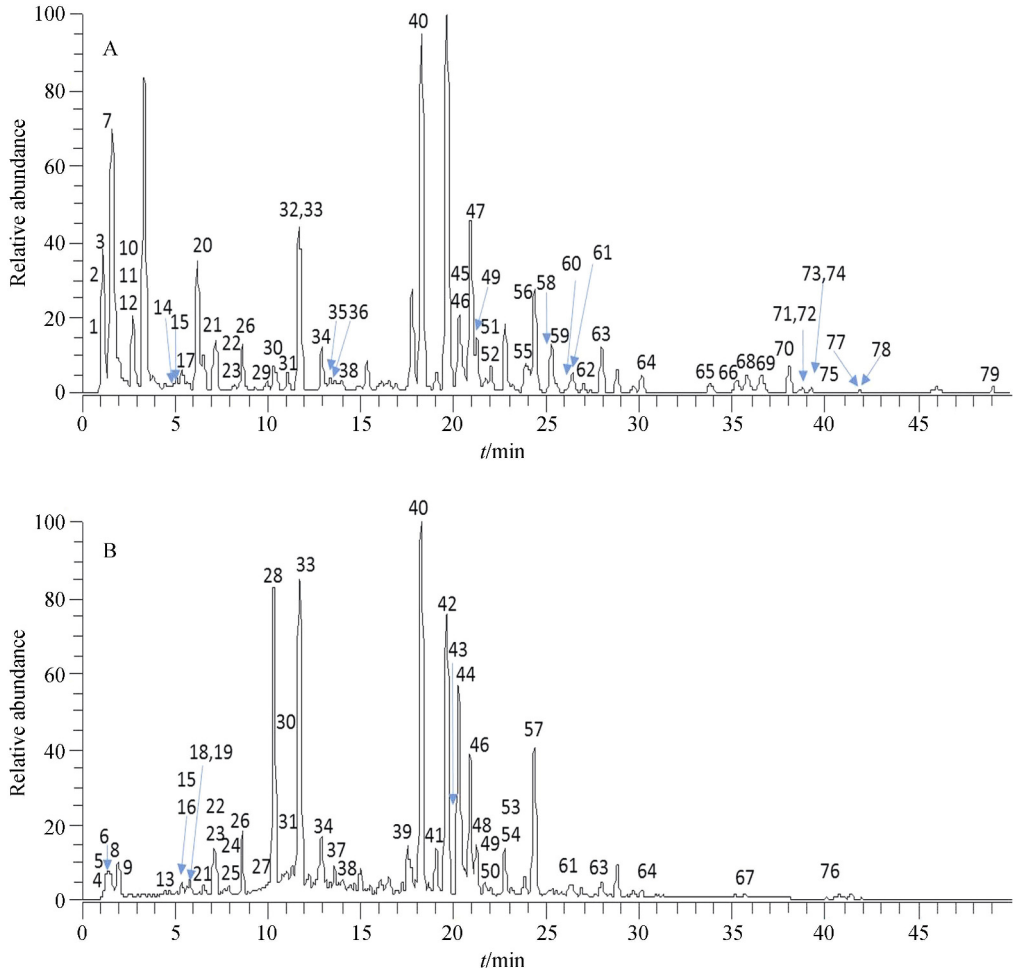


Fig. 1 The base peak chromatograms of ZSZZCT by LC-IT-MS in positive (A) and negative (B) mode

Table 1 Characterization of constituents of ZSZZCT by LC-IT-MS and LC-Q-TOF-MS

No.	t_R(–/+)	Identification	Formula	ESI-MS(–/+)		Source
				Mean measured mass (*m/z*)	Error (ppm)	
1	1.1	Unknown	-	145.1044 $[M + H]^+$	-	ZS
2[a]	1.17	Synephrine	$C_9H_{13}NO_2$	168.0839 $[M + H]^+$	–5.1	ZS
3	1.25	Unknown	-	150.0727 $[M + H]^+$	-	ZS
4	1.39	Unknown	-	135.0381 $[M – H]^–$	-	ZS
5[a]	1.5	Citric acid	$C_6H_8O_7$	191.0196 $[M – H]^–$	–1.0	ZZ, ZS
6	1.6	Unknown	-	174.0367 $[M – H]^–$	-	ZS
7	1.7	Unknown	-	132.0907 $[M + H]^+$	-	ZS
8[a]	1.96	Succinic acid	$C_4H_6O_4$	117.0174 $[M – H]^–$	5.44	ZZ, ZS
9	2.06	Unknown	-	257.0770 $[M + H]^-$	-	ZS
10	2.76	Unknown	-	254.1443 $[M + H]^+$	-	ZZ
11	3.38	Unknown	-	166.0737 $[M + H]^+$	-	ZZ, ZS
12	3.84	Unknown	-	328.1394 $[M + H]^+$	-	ZS
13	4.15	Unknown	-	515.1596 $[M – H]^–$	-	-
14	5.11	Unknown	-	227.0998 $[M + H]^+$	-	ZS
15	5.39	Villosolside	$C_{16}H_{26}O_9$	361.1367 $[M – H]^–$ 407.1464$[M + FA – H]^–$	–4.1 4.18	ZZ
16	5.65	Unknown	-	644.2018 $[M – H]^–$	-	-
17	5.76	Unknown	-	270.1882 $[M + NH_4]^+$	-	-
18[a]	5.85	Shanzhiside	$C_{16}H_{24}O_{11}$	391.1238 $[M – H]^–$	2.64	ZZ
19	5.97	Galactaric acid or glucaric acid derivatives	$C_{13}H_{14}O_9$	313.0556 $[M – H]^–$	2.15	ZS
20	6.26	Unknown	-	188.0686 $[M + H]^+$	-	ZS
21	6.6	Galioside	$C_{17}H_{24}O_{11}$	449.1270 $[M + FA – H]^–$ 422.1626 $[M + NH_4]^+$	6.03 6.47	ZZ
22[a]	7.09	Deacetylasperulosidic acid methyl ester	$C_{17}H_{24}O_{11}$	403.1227 $[M – H]^–$ 449.1284 $[M + FA – H]^–$ 422.1647 $[M + NH_4]^+$	3.55 3.69 3.15	ZZ
23	7.23	Picrocrocinic acid	$C_{16}H_{26}O_8$	345.1545 $[M – H]^–$ 364.2433 $[M + NH_4]^+$	–1.42 –4.38	ZZ
24	7.96	Galactaric acid or glucaric acid derivatives	$C_{13}H_{14}O_9$	313.0554 $[M-H]^-$	3.23	ZS
25[a]	7.98	Scandoside methyl ester	$C_{17}H_{24}O_{11}$	449.1274 $[M + FA – H]^–$	5.3	ZZ
26	8.66	Nomilinic acid	$C_{28}H_{36}O_{10}$	531.2291 $[M – H]^–$ 533.2433 $[M + H]^+$	–3.83 2.09	ZS
27	10.06	Galactaric acid or glucaric acid derivatives	$C_{13}H_{14}O_9$	313.0555 $[M – H]^–$	2.97	ZS
28[a]	10.33	Genipin 1-*O*-*β*-D-gentiobioside	$C_{23}H_{34}O_{15}$	549.1788$[M – H]^–$ 595.1850 $[M + FA – H]^–$	6.48 5.59	ZZ
29	10.35	Unknown	-	209.0717 $[M + H]^+$	-	ZZ
30	10.50	Unknown	-	513.2168 $[M – H]^–$ 515.2295 $[M + H]^+$	-	-
31	11.08	Unknown	-	513.2170 $[M – H]^–$ 515.2335 $[M + H]^+$	-	-
32	11.75	Unknown	-	209.0776 $[M + H]^+$	-	ZS
33[a]	11.74	Geniposide	$C_{17}H_{24}O_{10}$	387.1268 $[M – H]^–$ 433.1153 $[M + FA – H]^–$ 406.1708 $[M + NH_4]^+$	5.62 3.56 0.05	ZZ
34	12.91	Apigenin 6,8-di-*C*-glycoside	$C_{27}H_{30}O_{15}$	593.1473 $[M – H]^–$ 595.1643 $[M + H]^+$	–4.2 1.81	ZS
35[a]	13.14	Epijasminoside A	$C_{16}H_{26}O_7$	331.1725 $[M + H]^+$	5.83	ZZ
36[a]	13.40	Jasminoside A	$C_{16}H_{26}O_7$	331.1719 $[M + H]^+$	10.5	ZZ
37	13.62	Narirutin 4'-*O*-glucoside	$C_{33}H_{42}O_{19}$	787.2254 $[M + FA – H]^–$	–1.2	ZS
38	14.00/ 14.02	Diosmetin 6,8-di-*C*-glucoside	$C_{28}H_{32}O_{16}$	623.1579 $[M – H]^–$ 625.1746 $[M + H]^+$	4.57 1.1	ZS
39	17.59	Geniposide pentaacetate	$C_{27}H_{34}O_{15}$	597.1787 $[M – H]^–$	6.11	ZZ
40[a]	18.26/ 18.30	Narirutin	$C_{27}H_{32}O_{14}$	579.1517 $[M – H]^–$ 581.1875 $[M + H]^+$	5.51 –1.3	ZS

Continued

No.	t_R(–/+)	Identification	Formula	ESI-MS(–/+)		Source
				Mean measured mass (*m/z*)	Error (ppm)	
41[a]	19.08/ 19.12	Naringin	$C_{27}H_{32}O_{14}$	579.1689 $[M-H]^-$ 581.1855 $[M+H]^+$	1.01 1.0	ZS
42[a]	19.64	Hesperidin	$C_{28}H_{34}O_{15}$	609.1746 $[M-H]^-$	1.01	ZS
43	20.28	6"-*O*-[(*E*)-*p*-Coumaroyl]genipin gentiobioside	$C_{32}H_{40}O_{17}$	695.2154 $[M-H]^-$	5.83	ZZ
44[a]	20.44	Neohesperidin	$C_{28}H_{34}O_{15}$	609.1791 $[M-H]^-$	5.92	ZS
45	20.59	6"-*O*-*trans*-Sinapoyl genipin gentiobioside	$C_{34}H_{44}O_{19}$	757.2542 $[M+H]^+$ 774.2881 $[M+NH_4]^+$	2.1 2.2	ZZ
46	20.67	Limocitrin 3-*O*-(3-hydroxy-3-methylglutarate)- *β*-glucoside	$C_{29}H_{32}O_{17}$	651.1523 $[M-H]^-$ 653.1712 $[M+H]^+$	6.82 0.12	ZS
47[c]	20.91	Unknown	-	397.20 $[M+H]^+$	-	ZS
48	21.23	Unknown	-	533.1834 $[M-H]^-$	-	ZZ
49	21.67	6'-*O*-*trans*-Sinapoyljasminoside L	$C_{27}H_{36}O_{12}$	553.2273 $[M+H]^+$	1.17	ZZ
50	21.73	Unknown	-	533.1824 $[M-H]^-$	-	ZZ
51	21.75	Unknown	-	645.3261 $[M+H]^+$	-	-
52[c]	22.01	Unknown	-	453.56 $[M+H]^+$	-	ZS
53	22.77	Unknown	-	618.2832 $[M-H]^-$	-	-
54	22.77	Unknown	-	641.2860 $[M-H]^-$	-	ZZ
55	23.88	Unknown	-	663.3170 $[M+H]^+$	-	ZZ
56	24.08	Unknown	-	629.3332 $[M+H]^+$	-	ZZ
57[a]	24.34	Didymin	$C_{28}H_{34}O_{14}$	593.1842 $[M-H]^-$ 639.1886 $[M+FA-H]^-$	6.05 7.05	ZS
58	25.26	Unknown	-	663.3175	-	ZZ
59	25.56	Monohydroxy pentamethoxyflavonol 3-*O*-(3-hydroxy-3-methylglutarate)-*β*-glucoside	$C_{32}H_{38}O_{18}$	711.2125 $[M+H]^+$	0.25	ZS
60	26.23	7,4'-Dihydroxy-5,6,8,3'-tetramethoxyflavonol 3-*O*-(3-hydroxy-3-methylglutarate)-*β*-glucoside	$C_{31}H_{36}O_{18}$	697.1968 $[M+H]^+$	0.44	ZS
61	26.4	Citrusin III	$C_{36}H_{53}N_7O_9$	772.38291 $[M+FA-H]^-$ 728.3966 $[M+H]^+$	–1.0 2.35	ZS
62	27.00	Natsudaidain 3-*O*-*β*-D-glucoside	$C_{27}H_{32}O_{14}$	581.1853 $[M+H]^+$	1.30	ZS
63	27.98	Natsudaidain 3-*O*-[3-hydroxy-3-methylglutarate (1→6)]-β-glucoside.	$C_{33}H_{40}O_{18}$	723.2081 $[M-H]^-$ 725.2294 $[M+H]^+$	8.33 –0.33	ZS
64	30.10	Citrusin I	$C_{34}H_{53}N_7O_9$	702.3770 $[M-H]^-$ 704.3977 $[M+H]^+$	–5.1 0.82	ZS
65[a]	33.79	Sinensetin	$C_{20}H_{20}O_7$	373.1278 $[M+H]^+$	0.92	-
66	35.22	Unknown	-	274.2727 $[M+H]^+$	-	ZS
67	35.62	Unknown	-	274.2737 $[M+H]^+$	-	ZZ
68	35.78	Unknown	-	318.2995 $[M+H]^+$	-	ZZ
69	36.13	Unknown	-	274.2736 $[M+H]^+$	-	ZZ
70[b]	38.04	3,5,6,7,8,3',4'-Heptamethoxyflavone	$C_{22}H_{24}O_9$	433.1485 $[M+H]^+$	2.37	ZS
71[b]	38.62	3-hydroxynobiletein	$C_{21}H_{22}O_9$	419.1336 $[M+H]^+$	0.47	ZS
72[a]	38.78	Tangeretin	$C_{20}H_{20}O_7$	373.1287 $[M+H]^+$	–1.15	ZS
73	39.12	Unknown	-	302.3052 $[M+H]^+$	-	-
74	39.21	Unknown	-	346.3314 $[M+H]^+$	-	-
75	40.47	Unknown	-	374.3607 $[M+H]^+$	-	ZS
76	41.41	Unknown	-	595.2846 $[M+H]^-$	-	-
77	41.86	Unknown	-	294.9390 $[M+H]^+$	-	ZZ, ZS
78[c]	41.88	Unknown	-	457.36 $[M+H]^+$	-	ZS
79[c]	48.93	Unknown	-	188.81 $[M+H]^+$	-	-

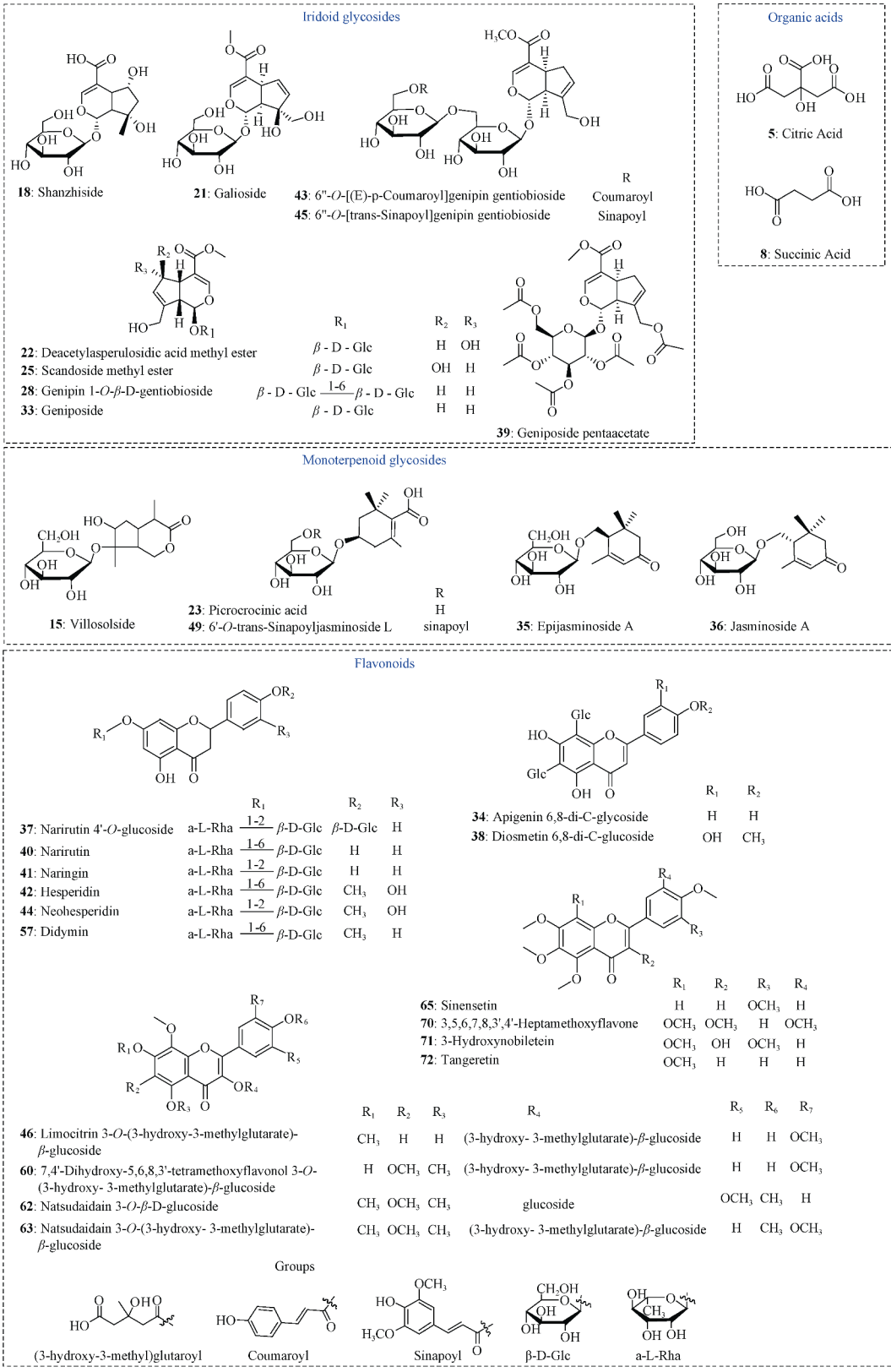
Note: t_R, retention time; FA: formic acid; ZS: Citrus aurantium L.; ZZ: Gardeniae fructus; DDC: Sojae semen praeparatum; - not detected in TCMs.

[a] compared with reference standards.

[b] prepared and identified by LC-MS and NMR.

[c] the MS data were from LC-IT-MS.

By analyzing the MS^n fragmentation behavior and molecular formula of the chromatographic peaks, and comparing with the reference substances, 39 compounds were identified tentatively or unambiguously, which included 9 iridoid glycosides, 5 monoterpenoids, 16 flavonoid glycosides, 2 organic acids, 2 cyclopeptides and 5 other types of compounds. The chemical structures identified in ZSZZCT are presented in Fig. 2.



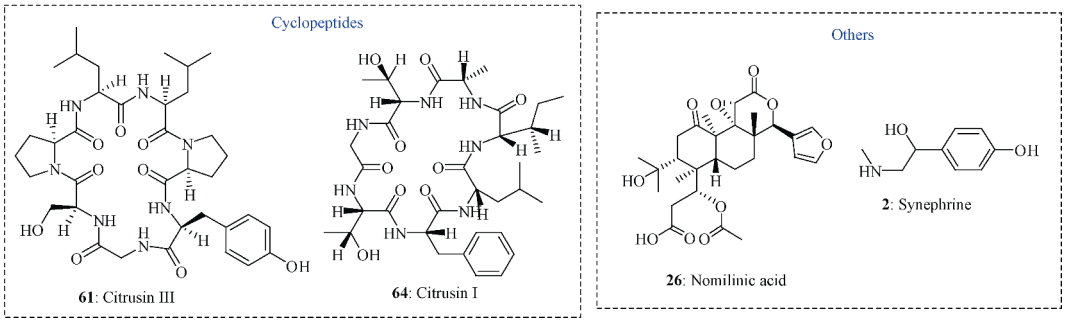


Fig. 2 The chemical structures of compounds identified in ZSZZCT

Characterization of iridoids

Compound **21** gave an adduct ion at *m/z* 449 [M – H + HCOOH]$^-$ and a molecular formula of $C_{17}H_{24}O_{11}$ by LC-Q-TOF-MS analysis. In the MS^2 spectrum, compound **21** produced an ion at *m/z* 241 [M – H – 162]$^-$. In the MS^3 spectrum, the base peak ion at *m/z* 193 [M – H – 162 – H_2O – CH_2O]$^-$, and other ions at *m/z* 223 [M – H – 162 – H_2O]$^-$ and *m/z* 211 [M – H – 162 – CH_2O]$^-$ were observed. Compared with the data reported in the literature [12], compound **21** was tentatively identified as galioside.

Compounds **39** and **43** exhibited the quasi-molecular ions at *m/z* 597 and *m/z* 695, respectively. In the MS^2 spectra, compound **39** produced the major fragment ions at *m/z* 553, *m/z* 391 and *m/z* 353 (base peak) as a result of the loss of C_2H_4O, $C_8H_{14}O_6$ and $C_{10}H_{12}O_7$, respectively, from the precursor ion at *m/z* 597. Compound **43** formed the base peak ion at *m/z* 663 [M – H – CH_3OH]$^-$ and typical ions at *m/z* 225 [genipin – H]$^-$ as well as *m/z* 469 [M – genipin – H]$^-$, *m/z* 307 [M – genipin – H – 162]$^-$. By comparing with fragment ions reported in the literatures [13-14], compounds **39** and **43** were deduced as geniposide pentaacetate and 6''-*O*-[(*E*)-*p*-coumaroyl]genipin gentiobioside, respectively.

Compound **45** displayed a [M + H]$^+$ ion at *m/z* 757 in positive ion mode. The diagnostic ion at *m/z* 226 [genipin + H]$^+$ in the MS^2 spectrum was observed, and a fragment ion at *m/z* 548 was detected due to the loss of a sinapoyl group and H·. Compared to the data in the literature [13], compound **45** was tentatively inferred to be 6''-*O*-*trans*-sinapoyl genipin gentiobioside.

Compounds **18**, **22**, **25**, **28** and **33** were unambiguously identified by comparing data with the fragmentation patterns in the literature [12, 15-16] and with the reference standards.

Characterization of monoterpenoids

Compound **15** had an adduct ion at *m/z* 407 [M – H + HCOOH]$^-$ and a molecular formula of $C_{16}H_{26}O_9$. In the MS^2 spectrum, the base peak ion was at *m/z* 361 [M – H]$^-$. In the MS^3 spectrum, the precursor ion at *m/z* 361 produced a base peak ion at *m/z* 181 by losing one molecule of glucose, which further formed a fragment ion at *m/z* 137 [M – H – 180 – CO_2]$^-$. Thus, compound **15** was assigned as villosolside.

In positive ion mode, compounds **23** and **49** exhibited adduct ions at *m/z* 364 [M + NH_4]$^+$ and *m/z* 553 [M + H]$^+$, respectively. In the MS^2 spectra, compound **23** produced the base peak ion at *m/z* 167 [M + H – 180]$^+$, while compound **49** formed the base peak ion at *m/z* 207 [sinapic acid + H – H_2O]$^+$. In the MS^3 spectrum, compound **49** generated the base peak ion at *m/z* 175 through the loss of 2CH_3· and 2H· from the ion at *m/z* 207. Compounds **23** and **49** were tentatively characterized as picrocrocinic acid and 6'-*O*-*trans*-sinapoyl-jasminoside, respectively. The MS^n spectrum and fragment pattern of compound **49** are illustrated in Fig. S2.

Compounds **35** and **36** both gave ions at *m/z* 331 [M + H]$^+$ and shared identical molecular formula of $C_{16}H_{26}O_7$. In the MS^2 spectra, the compounds yielded the same base peak ions at *m/z* 169 by loss of a glucosyl moiety. Compounds **35** and **36** were unambiguously identified as epijasminoside A and jasminoside A, respectively, by comparing with reference standards.

Characterization of flavonoids

Compounds **34** and **38** were identified as C-glycoside of flavones. The compounds shared characteristic fragment ions at *m/z* [M – H – 120]$^-$ (base peak), *m/z* [M – H – 90]$^-$, *m/z* [M – H – 120 – 90]$^-$ and *m/z* [M – H – 120 – 120]$^-$ in the MS^2 spectra in negative ion mode. These fragment ions were in accordance with the literature [9]. Thus compounds **34** and **38** were assigned as apigenin 6, 8-di-*C*-glycoside and diosmetin 6, 8-di-*C*-glucoside, respectively.

Compounds **37**, **40**, **41**, **42**, **44**, **57** and **62** were identified as *O*-glycoside of flavones which all showed ions at *m/z* [aglycone – H]$^-$ in the MS^2 or MS^3 spectra in negative ion mode with the loss of a neutral rhamnosyl unit and glucosyl unit. Compound **62** produced ions at *m/z* [aglycone + H]$^+$ in positive ion mode by losing a glucosyl moiety. Finally, by comparing with the reference standards, compounds **40**, **41**, **42**, **44** and **57** were identified as narirutin, naringin, hesperindin, neohesperidin, and didymin, respectively. Compounds **37** and **62** was referred to be narirutin 4'-*O*-glucoside and natsudaidain 3-*O*-*β*-D-glucoside according to the MS^n data and literature [9, 12], respectively.

Compounds **46**, **60** and **63** were considered to have a (3-hydroxy-3-methyl) glutaroyl group in their structures. Compound **46** formed a base peak at *m/z* 507 [M – H – 144]$^-$ and the fragment ion at *m/z* 549 [M – H – 102]$^-$ in the MS^2 spectrum. In the MS^3 spectra, the precursor ion *m/z* 507 produced a base peak at *m/z* 345 [M – H – 144 – 162]$^-$, while the

base peak ions of compounds **60** and **63** were *m/z* 391 $[M - H - 144 - 162]^-$ and *m/z* 417 $[M - H - 144 - 162]^-$, respectively. In comparison to previous reports in the literature [9], compounds **46**, **60** and **63** were inferred as limocitrin 3-*O*-(3-hydroxy-3-methylglutarate)-*β*-glucoside, 7, 4'-dihydroxy-5,6,8,3'-tetramethoxy flavonol-3- *O*-(3-hydroxy-3-methylglutarate)-*β*-glucoside and natsudaidain 3-*O*-(3-hydroxy-3-methylglutarate)-*β*-glucoside, respectively.

Compounds **65**, **70**, **71** and **72** were all characterized as polymethoxy flavones. The typical ions in the MS^n were generated by the loss of 15 Da (CH_3), 18 Da (H_2O), 31 Da (CH_3O) and 28 Da (CO). Compound **65** was tentatively inferred as sinensetin. Compounds **70**, **71** and **72** would be separated using semi-preparative LC and identified by ^1H NMR and ^{13}C NMR.

Characterization of organic acids

Compounds **5** and **8** had the quasi-molecular ion at *m/z* 191 $[M - H]^-$ and *m/z* 117 $[M - H]^-$ in negative ion mode, respectively. They both had characteristic fragment ions generated by the neutral loss of H_2O and CO_2 in the MS^2, which indicated the existence of carboxyl in the molecular structures. Compounds **5** and **8** were identified as citric acid and succinic acid by comparing with the reference standards.

Characterization of cyclopeptides

Compounds **61** and **64** gave the quasi-molecular ion at *m/z* 728 $[M + H]^+$ and *m/z* 704 $[M + H]^+$ with the formulae $C_{36}H_{53}N_7O_9$ and $C_{34}H_{53}N_7O_9$, respectively. The typical loss of 17 Da (NH_3), 28 Da (CO), 18 Da (H_2O), as well as the elimination of amino residues, for example, prolyl, seryl, tyrosyl, glycyl, alanyl, phenyl, leucyl, and isoleucyl, were observed in the MS^n spectra. Based on the literature [17-18], compounds **61** and **64** were tentatively identified as citrusin III and citrusin I, respectively.

Characterization of other types of compounds

Compound **2** displayed the $[M + H]^+$ ion at *m/z* 168, and had molecular formula of $C_9H_{13}NO_2$. It produced the base peak ion at *m/z* 150 $[M + H - H_2O]^+$ in the MS^2 spectrum, the base peak ion at *m/z* 119 $[M + H - H_2O - CH_5N]^+$ and the fragment ion at *m/z* 135 $[M + H - H_2O - CH_3]^+$ in the MS^3 spectrum. By comparing with the literature [19] and reference standard, compound **2** was unambiguously identified as synephrine.

Compound **26** had an ion at *m/z* 531 $[M - H]^-$ and a molecular formula of $C_{28}H_{36}O_{10}$. It produced a base peak ion at *m/z* 513 $[M - H - H_2O]^-$ in the MS^2 spectrum. In the MS^3 spectrum, a fragment ion at *m/z* 495 $[M - H - 2H_2O]^-$ was generated by the loss of H_2O from the ion at *m/z* 513, which then lost CO_2 to generate the ion at *m/z* 451. It was inferred as nomilinic acid by referring to literature [15].

Compounds **19**, **24** and **27** all gave the same deprotonated molecule ions at *m/z* 313 $[M - H]^-$ and detected the same formula $C_{13}H_{14}O_9$. In the MS^2 spectra, all of the compounds generated the same base peaks at *m/z* 191. In the MS^3 spectra, ions at *m/z* 85 (base peak), *m/z* 173 and *m/z* 147 were observed. These fragmentation patterns were almost the same as the data previously reported in literature [9] and so were speculated as galactaric acid or glucaric acid derivatives.

Evaluation of anti-inflammatory activity and LC-MS analysis of ZSZZCT components

The whole extract of ZSZZCT was divided into 62 components (**B01**–**B04**, **C01**–**C21**, **D01**–**D21**, **and E01**–**E16**) according to the procedure in section 2.2. The anti-inflammatory activities of the 62 components were evaluated on LPS-induced RAW 264.7 macrophages at the concentrations that were not cytotoxic. The maxim non-cytotoxic concentrations were 25.0 $\mu g \cdot mL^{-1}$ for component **D16,** 12.5 $\mu g \cdot mL^{-1}$ for components **D12**, **D19**, **D20**, **E02**, **E10**, **E14**, **E15**, and **E16**, 6.25 $\mu g \cdot mL^{-1}$ for **D10** and **E13**, and 50 $\mu g \cdot mL^{-1}$ for other components.

As shown in Fig. 3, components **B02**, **B03**, **B04**, **C06-C12**, **E07**, **E09**, **E12**, **E13**, **E15**, and **E16** could inhibit the release of NO production at a rate of around 25%–38%. While components **B04** and **E13** exerted better inhibitory rates than that of indomethacin (positive control). In particular, components **E13** had an inhibitory rate against NO production of more than 72% at a concentration of 6.25 $\mu g \cdot mL^{-1}$.

In addition, the constituents in 62 components were analyzed using the LC-IT-MS method. The chromatographic peak areas of 79 constituents in the 62 components were obtained. The peak areas of the 79 compounds and the NO inhibitory rate of 62 components were used to calculate the activity indices of 79 constituents.

Prediction of the effective compounds in ZSZZCT

The Activity index method [9] proposed by our research group was used to evaluate the contribution of constituents to the pharmacological activity of formula. The constituents with positive activity index value suggested the constituent might be active, and the higher the activity index value, the more likely the constituent will be active. According to the mathematical formulae of section 2.8, the activity indices of 79 constituents in ZSZZCT were calculated, and the results are shown in Fig. 4. As shown in Fig. 4, 11 constituents had positive activity indices including compounds **13** (unknown), **21** (galioside), **22** (deacetyl asperulosidic acid methyl ester), **25** (scandoside methyl ester), **28** (genipin-1-*β*-D-gentiobioside), **29** (unknown), **35** (epijasminoside A), **65** (sinensetin), **70** (3,5,6,7,8,3',4'-heptamethoxyflavone), **71** (3-hydroxynobiletein), **72** (tangeretin). Among them, the four polymethoxy flavones (compounds **65**, **70**, **71**, and **72**) had the higher activity index values, suggesting these four compounds were the potential constituents with anti-inflammatory activities.

Preparation and identification of potential active compounds

To verify the predicted results from activity index method, the potential polymethoxy flavones with high positive values were isolated from *Citrus aurantium* L. with semi-preparative LC guided by LC-MS, and their structures were elucidated by off-line NMR analysis [12].

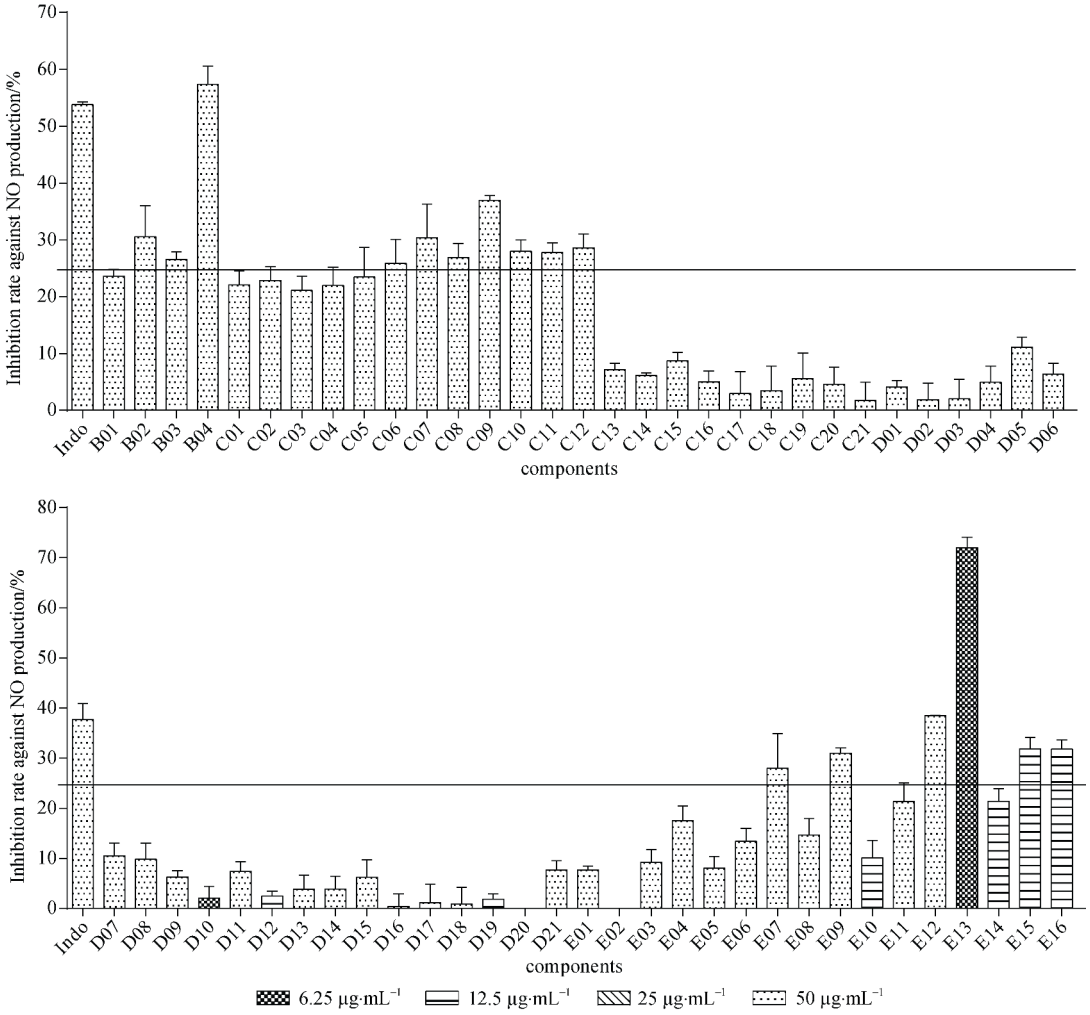


Fig. 3 The inhibition rate of NO of 62 ZSZZCT components

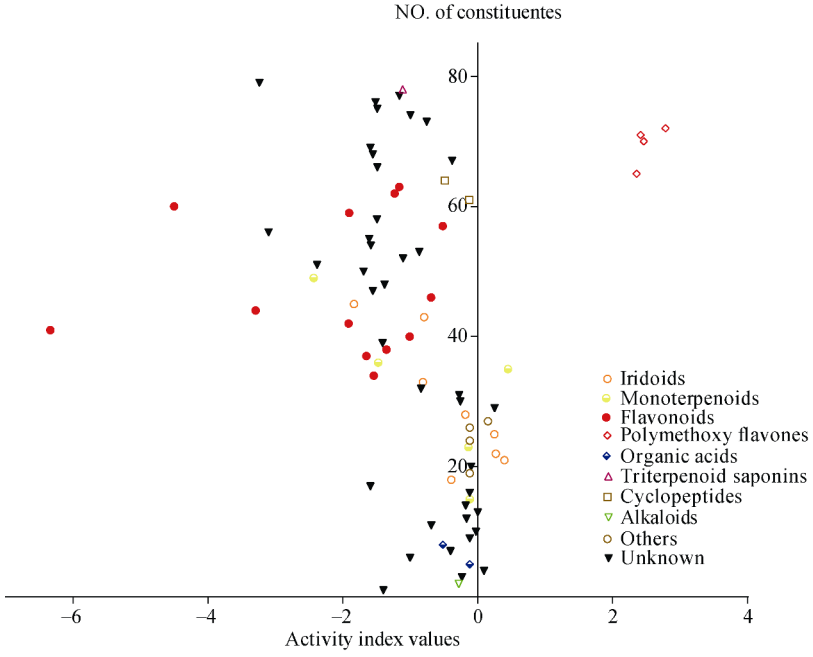


Fig. 4 The activity index vaules of 79 constituents

Preparation of the target compounds

The chromatograms of *Citrus aurantium* L. by analytical LC and semi-preparative LC are shown in Fig. 5. It was seen that the separation effect of semi-preparative LC was almost the same as that obtained by analytical LC with the exception of compound **65**. Then compounds **70**, **71**, and **72** were prepared and identified by semi-preparative LC and NMR.

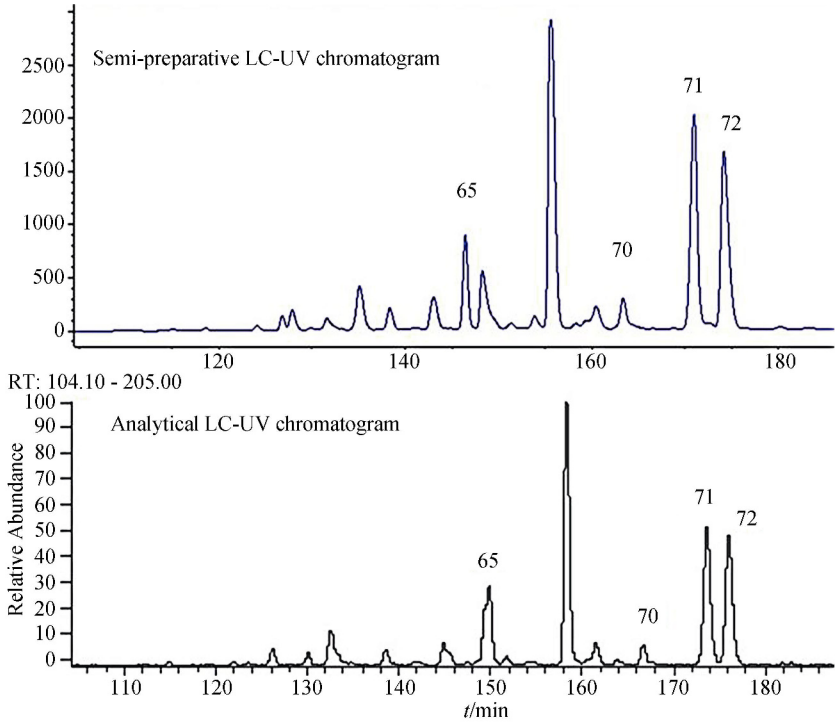


Fig. 5 The UV chromatograms of *Citrus aurantium* L. by semi-preparative LC and analytical LC

Structure confirmation of the target compounds

The ^{1}H NMR spectrum of compound **70** showed characteristic signals of the ABX coupling system of B ring of flavones at δ_H 7.80 (1H, d, J = 1.7 Hz, H-2'), 6.96 (1H, d, J = 8.6 Hz, H-5') and 7.86 (1H, dd, J = 8.5, 1.5 Hz, H-6'); seven methoxyl signals at 3.94 (3H, s), 3.93 (3H, s), 3.92(3H, s), 3.92 (9H, s) and 3.83 (3H, s) can also be seen. By comparing with the spectroscopic data in the literature [21], compound **70** was confirmed as 3, 5, 6, 7, 8, 3', 4'-heptamethoxyflavone.

For compound **71**, the characteristic signals of the ABX coupling system of B ring of flavone appeared at δ_H 7.90 (1H, d, J = 2.1 Hz, H-2'), 7.03 (1H, d, J = 8.5 Hz, H-5') and 7.92 (1H, dd, J = 8.5, 2.0 Hz, H-6'), the signal at δ_H 7.27 (1H, s) demonstrated the presence of hydroxyl linked to C-3; six methoxyl signals at 4.12 (3H, s), 4.04 (3H, s), 4.00 (6H, s), 3.98 (3H, s) and 3.96 (3H, s) were also detected. By comparison with data in the literature [21], compound **71** was identified as 3-hydroxynobiletein.

In the ^{1}H NMR spectrum of compound **72**, the diagnostic signals of AA'BB' coupling system of B ring of flavone were observed at δ_H 7.96 (2H, d, J = 8.3 Hz, H-2', 6') and 7.09 (2H, d, J = 8.3 Hz, H-3', 5'); the characteristic proton signal of H-3 at C-ring was observed at δ_H 6.65 (1H, s); four methoxyl signals at δ_H 4.02 (3H, s), 3.91(3H, s) and 3.88 (6H, s) were also observed. These data were in accordance with the literature [22] and so compound **72** was identified as tangeretin.

Validation of the anti-inflammatory activities of potential effective compounds

The anti-inflammatory activities of three prepared compounds (compounds **70**, **71** and **72)** and two reference standards (compounds **28** and **35)** were tested in LPS-induced RAW 264.7 macrophages. It was found that compounds **70**, **71** and **72** with greater positive index values exhibited obvious inhibitory effects on NO production with 46% at 100 μmol·L^{-1} for **70**, 68% at 200 μmol·L^{-1} for **71** and 28% at 200 μmol·L^{-1} for **72**. However, compounds **28** and **35** which had positive indices close to zero, showed no inhibitory effects at the concentration without cytotoxicity (compound **28** at 100 μmol·L^{-1}, and compound **35** at 200 μmol·L^{-1}). The dose-effect relationship of the three potentially active compounds investigated in Fig. 6 shows that compounds **70**, **71** and **72** had IC_{50} values of 233.1 ± 38.1, 38.9 ± 12.2 and 27.9 ± 1.7 μmol·L^{-1}, respectively. Noticeably, compound **72** had a higher inhibitory rate against NO production than indomethacine (positive control). These results indicated that compounds structurally containing polymethoxyl flavones may be the main effective compounds responsible for the anti-inflammatory activity of ZSZZCT.

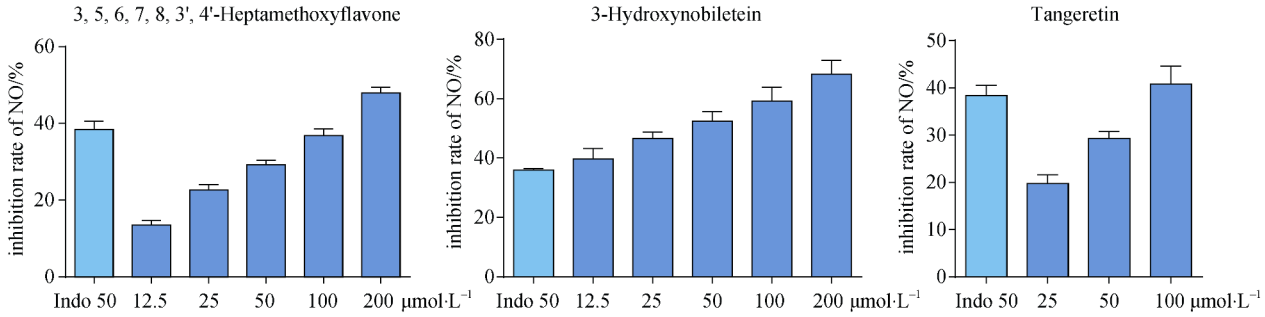


Fig. 6 The dose-effect relationship of three polymethoxy flavones. 70 (3, 5, 6, 7, 8, 3', 4'-Heptamethoxyflavone), 71 (3-Hydroxynobiletein), 72 (Tangeretin)

Conclusions

The activity index and the strategy using semi-preparative LC guided by LC-MS were developed to predict and prepare the anti-inflammatory active constituents from ZSZZCT in this study. A total of 79 compounds were rapidly characterized by LC-MS. The predicted results using the activity index method showed that the four polymethoxyl flavones with higher activity values were likely to have anti-inflammatory activities. Three of the compounds prepared and identified using semi-preparative LC guided by LC-MS and NMR were validated to possess remarkable anti-inflammatory activities in LPS-induced RAW 264.7 macrophages. These results suggest that the approach developed in this study is a rapid and efficient method for the discovery of the effective constituents from TCM formulae *in vitro*.

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