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•Research article•

Systematic chemical characterization of Xiexin decoctions using high performance liquid chromatography coupled with electrospray ionization mass spectrometry

SHEN Li-Juan[1], LUO Ke-Di[1], WEN Xue-Xun[1], WANG Shu-Fang[1], FAN Xiao-Hui[1, 2*]

[1] *Pharmaceutical Informatics Institute, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China;*
[2] *State Key Laboratory of Component Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China*

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[ABSTRACT] Xiexin decoctions (XXDs) display beneficial anti-inflammatory and anti-diabetic effects, which raises interests on this group of formulae for broad clinical applications. However, there was no report about systematic analysis of XXDs to elucidate the constitution of chemical components, which hampers further investigations on the therapeutic values of XXDs. In this work, crude herbs were extracted and prepared to obtain the XXDs for systemic analysis on their chemical compositions, according to the information described in the ancient Zhang Zhongjing's herbal formulae. LC-MS analysis of five XXDs was carried out to facilitate recognition of the source herbs for compounds in the mixture. A total number of 93 compounds were identified through our methods and their chemical classes encompassed five major groups, including protoberberine alkaloids, flavonoids, stilbenes, anthraquinones and saponins. Our current work provided important information about material basis for pharmacological studies on XXDs and would help shed light on relationships between chemical compositions and therapeutic effects.

[KEY WORDS] Traditional Chinese Medicine; Analogous formulae; HPLC-MS; Xiexin decoctions; Systematic chemical characterization

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Introduction

Traditional Chinese Medicine (TCM), which has a robust history of medical usage that could date back thousands of years, has received increasing acceptance as a promising complementary and alternative medicine among the Western countries [1]. Xiexin decoctions (XXDs), the analogous formulae originating from ancient Zhang Zhongjing's herbal formulae (*Treatise on Cold Damage* and *Concise Essential of the Golden Cabinet*), include Shengjiang-Xiexin decoction (SJ), Ganchao-Xiexin decoction (GC), Banxia-Xiexin decoction (BX), Dahuang-Huanglian-Xiexin decoction (DHHL) and Fuzi-Xiexin decoction (FZ). XXDs have been practiced in traditional Chinese healthcare system for a long history [2, 3] and are still widely used to treat inflammation-related diseases such as upper respiratory tract infection and inflammation of alimentary canal. Consistent with clinical observations, experimental data indicated that XXDs could prevent lipopolysaccharide (LPS)-induced lung injury in rats by inhibiting the expression of the iNOS, TGF-β, p38MAPK and ICAM-1 [4]. Recent research suggested that downregulation of the nuclear factor-κB pathway was one of the key mechanisms involved in the anti-diabetic effects by XXDs [5]. Potential *ex vivo* immunomodulatory effects of XXDs also have been showed on mice and humans [6]. It was also been reported that XXDs could protect rat hearts from ischemia/reperfusion-induced apoptosis through eNOS and MAPK Pathways [7]. Clearly, XXDs exhibit their therapeutic values by mediating several important biological pathways involved in pathological processes of diseases.

XXDs are composed of Rhei Radix et Rhizoma, Coptidis Rhizoma, Scutellariae Radix, Aconiti Lateralis Praeparata Radix, Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle, Zingiberis Rhizoma, Zingiberis Rhizoma Recens, Pinelliae Rhizoma, Ginseng Radix et Rhazoma and Jujubae Fructus. Previous chemical analysis of XXDs focused on

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[*]**Corresponding author]** E-mails: fanxh@zju.edu.cn
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quantification of the bio-active substances from individual herbs. High-performance liquid chromatography (HPLC) [8, 9] and liquid chromatography/multistage mass spectrometry (LC-MS/MS) methods [3, 10, 11] have been utilized to determine alkaloids from Coptidis Rhizoma, flavonoids from Scutellariae Radix, and anthraquinones from Rhei Radix et Rhizoma [2, 3, 8-12]. However, there was no report about systematic analysis of XXDs to elucidate the constitution of chemical components in the context of formulae. Due to the difference in extraction methods and potential interactions among the crude herbs during preparation process of decoctions [13], chemical components in XXDs may vary significantly from those reported previously for each crude herb. Furthermore, individual formula of XXDs displays diverse clinical profiles in term of medical usage and chemical basis for this phenomenon remains elusive. Therefore, it is important to describe the chemical composition of XXDs in a systemic manner, which would help to uncover the relationships between chemical compositions and therapeutic effects of different XXDs.

TCM formula, one of the most remarkable characteristics of TCM, is a rational combination of multiple TCMs together under the guidance of TCM theories. Analogous formulae are a group of TCM formulae that are established from a basic formula by adding or removing TCMs and the purpose of this modification is to treat the same ailments based on different or severity of TCM syndromes exhibited by individual patient, in another word, to prescribe personalized medicine. Clearly, it is important to study chemical compositions of analogous formulae to identify active components for their pharmacological effects and correlate differences in therapeutic effects of analogous formulae with variations on their chemical constitutions. Furthermore, disappearance of peaks or observations of new peaks between spectrums of two formulae could relate to modification on involved TCMs in the formulae, which would significantly help to allocate those peaks to source TCM. With previous knowledge on these source TCMs, systemic identification of these chemicals are greatly facilitated by analyzing analogous formulae at the same time.

In this work, we applied this strategy on five XXD analogous formulae and demonstrated it is effective for chemical analysis on TCM formulae systemically. Primary chemical composition of the five XXDs aforementioned was first identified in the context of a TCM formula rather than individual crude herbs. Results of this work would provide more information for mechanistic researches on XXDs.

Materials and Methods

Materials, chemicals and reagents

Crude herbs used in the Xiexin decoction were all obtained from the Hangzhou traditional Chinese herbal medicine factory (Hangzhou, Zhejiang Province, China). HPLC-grade Acetonitrile and methanol were purchased from Merck KGaA (Germany). HPLC-grade formic acid and acetic acid was purchased from Roe Scientific Inc. Water was prepared by Milli-Q synthesis (Millipore, USA). The reference compounds rhapontin (**27**), jatorrhizine (**38**), palmatine (**48**), berberine (**51**), baicalein (**74**) and rhein (**87**) were purchased from National Institute for the Control of Pharmaceutical and Biological Products. Notoginsenoside R1 (**29**) was obtained from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). Ginsenoside Rg1 (**40**), ginsenoside Re (**41**), ginsenoside Rb1 (**69**), ginsenoside Rg2 (**70**), ginsenoside Rd (**77**) and ginsenoside Rg3 (**86**) were purchased from Jilin University (Jilin, China). Scutellarin (**20**), Wogonoside (**60**), glycyrrhizic acid (**81**), wogonin (**89**) and emodin (**93**) were purchased from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China). 6-gingerol (**88**) was obtained from Aladdin Industrial Corporation (Shanghai, China).

Xiexin decoction samples preparation

The herbs compositions of the five Xiexin decoctions were shown in Table 1. These XXDs were prepared according to the information from ancient Zhang Zhongjing's herbal formulae. Different batches air-dried herbs of XXDs were mixed and extracted together with pure water for 1 hour under reflux for twice (the weight of the water were six and four times of the total weight of herbs for each time). Combined extracts were dried under 70 °C and then concentrated to total dryness by vacuum freeze-drying by step. Samples for LC-MS analysis were prepared by dissolving the extracts in 20% methanol-water to obtain a final concentration of 10 $mg \cdot mL^{-1}$. The solutions were centrifuged at 10 000 $r \cdot min^{-1}$ for 10 min before analysis.

Table 1 The herbs compositions of the five Xiexin decoctions (g)

	C	S	R	P	G	ZR	Z	GS	A	J
SJ	7.6	22.8	-	25.3	22.8	7.6	30.4	22.8	-	60.8
GC	9	26.9	-	29.9	35.8	26.9	-	22.8	-	71.6
BX	8.2	24.7	-	27.4	24.7	24.7	-	24.7	-	65.8
DHHL	66.7	-	133.3	-	-	-	-	-	-	-
FZ	35.3	35.3	70.6	-	-	-	-	-	58.8	-

Coptidis Rhizoma (C), *Scutellariae Radix* (S), *Rhei Radix et Rhizoma* (R), *Pinelliae Rhizoma* (P), *Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle* (G), *Zingiberis Rhizoma* (Z), *Zingiberis Rhizoma Recens* (ZR), *Ginseng Radix et Rhazoma* (GS), *Aconiti Lateralis Praeparata Radix* (A), *Jujubae Fructus* (J)

HPLC conditions

The HPLC analysis was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a binary solvent delivery system, a column temperature controller, and a diode-array detector (DAD). The analytical column (Zorbax SB-C_{18}, 5 μm, 4.6 mm × 250 mm; Agilent Technologies, Santa Clara, CA, USA) was maintained at room temperature. The mobile phase consisted of a mixture of water with 0.05% formic acid (A) and acetonitrile with 0.05% formic acid (B) with a gradient program of 5% B at 0 min, 15% B at 5 min, 35% B at 60 min, 95% B at 80 min and 95% B at 90 min. The injection volume was 10 μL and the flow rate was fixed at 0.6 mL·min^{-1}.

Mass spectrometry

The mass spectra were acquired for testing compounds using ThermoFinnigan model LCQ DECA XP^{plus} (Thermo Finnigan, San Jose, CA, US) ion-trap mass spectrometer equipped with an ESI source interface coupled to an Agilent 1100 HPLC device, which was controlled by Xcalibur 2.1 software (Thermo Fisher Scientific Inc., San Jose, CA, USA). The mass spectra were acquired in both positive and negative ion mode. The scan range was set at *m/z* 100−1600. The source voltage was −3.0 kV. Capillary temperature was 350 °C. The flow rate of sheath gas (N_2) was 60 arbitrary units (a.u) and 10 a.u for the auxiliary gas (N_2). Tube lens offset was −50 V.

The high resolution MS (HR-MS) data were acquired on a Waters UPLC (Waters Corp. Milford, MA, USA) equipped with an AB Triple TOF 5600^{plus} System (AB SCIEX, Framingham, MA, USA). The MS conditions were as follows: the scan range was set at *m/z* 100−1600, both positive and negative ion mode, +5.5 kV or −4.5 kV ionization voltage, 600 °C source temperature, 60 psi Gas 1 (N_2) and Gas 2 (N_2). Both IT-MS and HR-MS used the same liquid chromatography conditions mentioned above, and MS data were acquired during 5−90 min.

Result and Discussion

To rapidly identify the origin source of the detected chemical constituents, the base peak chromatograms of five XXDs in negative and positive modes were compared with the mass spectrum of each 10 individual herb extract. The originality of a total number of 93 compounds were characterized in the five XXDs under the study of this work. These compounds belong to several chemical categories, including protoberberine alkaloids, flavonoids, stilbenes, anthraquinones, saponins and other types of structures. Protoberberine alkaloids came from *Coptidis Rhizoma*, while most flavonoids were from *Scutellariae Radix*. Table 2 summarized the identification results of all 93 compounds in XXDs.

Identification of protoberberine alkaloids

Totally nine protoberberine alkaloids were found in XXDs. They were the characteristic components of the *Coptidis Rhizoma*, the only common crude herb present in all five XXDs. Peak **23**, **26**, **28**, **33**, **35**, **38**, **39**, **48** and **51** were further confirmed to be protoberberine alkaloids according to the mass spectral characteristics.

HR-MS data of Peak **51** indicated its formula to be $C_{20}H_{18}NO_4^+$. It produced *m/z* 321 $[M - CH_3\cdot]^{+\cdot}$, *m/z* 320 $[M - CH_4]^+$, *m/z* 292 $[M - CH_4 - CO]^+$, *m/z* 306 $[M - CH_2O]^+$ and *m/z* 304 $[M - CH_2O - H_2]^+$ in MS^2. It was further characterized to be berberine according to the comparison with the reference literature [14]. Peak **35** displayed a similar fragmentation behavior as Peak **51** except for $[M - CO]^+$ at *m/z* 308 which led to a reasonable conclusion of epiberberine, the isomer of berberine [15]. HR-MS data of Peaks **33** and **38** confirmed the chemical formula to be $C_{20}H_{20}NO_4^+$. A vicinal methoxyl group was suggested based on main fragmentation pathways, including removal of methyl (–15) or methane (–16), – CH_2O group (–30) and – CH_2O – H_2 (–32). The MS^2 fragments at *m/z* 294 indicated a loss of *m/z* 28 from 322. It was a similar fragmentation pathway to that of berberine (Peak **51**), which suggested that ion at *m/z* 322 contained a methylenedioxy ring. Columbamine or jatrorrhizine could be the reasonable candidates. They were tentatively distinguished from each other according to the reported retention times in LC [15, 16]. The chemical formula of Peak **28** was $C_{20}H_{18}NO_5^+$. Its neutral loss fragment *m/z* 337 $([M - CH_3\cdot]^{+\cdot}$, *m/z* 336, $[M - CH_4]^+$ *m/z* 308 $[M - CH_4 - CO]^+$, *m/z* 322 $[M - CH_2O]^+$ and *m/z* 320 $[M - CH_2O - H_2]^+$ in MS^2) were identical to those for berberine. It was then temporarily characterized as 13-Hydroxyberberine according to the reported literature [11]. Peak **48**, whose fragmentation behavior was similar to Peak **28,** showed the formula of $C_{21}H_{22}NO_4^+$. It was characterized to be palmatine in the comparison with the literature [17]. The fragment pathway of Peak **39** (*m/z* 318 $[M - H_2]^+$, *m/z* 292 $[M - CO]^+$, *m/z* 290 $[M - CH_2O]^+$, *m/z* 277 $[M - CO - CH_3]^+$, *m/z* 275 $[M - CH_2O - CH_3]^+$, *m/z* 264 $[M - 2CO]^+$ *m/z* 262 $[M - CO - CH_2O]^+$ and other ions in MS^2) were in good agreements with the reported data of coptisine [14]. With the help of published results, other protoberberine alkaloids (Peaks **23** and **26**) were tentatively characterized to be demethyleneberberine and groenlandicine, respectively [14, 17].

According to the chemical structures of protoberberine alkaloids observed in this study, their MS/MS fragmentation pathways are proposed in Fig. 1. In the MS/MS spectra of these protoberberine alkaloids, diverse cleavage and rearrangement of methoxyl groups on precursor ion resulted in several fragments during the CID (collision induced dissociation) process. Notably, the loss of a methane molecule in alkaloids with vicinal methoxyl groups can lead to form a methylenedioxy ring, which then results in the dehydrogenated fragment ($[M - CH_2O]^+$ and $[M - CH_2O - H_2]^+$) through subsequent losses of CO or CH_2O ($[M - CO]^+$ and $[M - CH_2O]^+$) molecules. For the alkaloids with methylenedioxy groups, the characteristic ions were produced by the direct removals of CO or CH_2O molecules from the $[M]^+$ ion [14]. Furthermore, we proposed that the methylenedioxy ring extended from D-ring tend to lose the CO while the loss of CH_2O would be observed if methylenedioxy ring is connected to A-

Table 2 Compounds of XXDs identified by HPLC-MS^n and HR-MS

Peak No.	t_R/min	M^+	$[M-H]^-$	Molecular Formula	Error (ppm)	Identification	Source
1	8.17		331.0674	$C_{13}H_{16}O_{10}$	1.0	Gallic acid 4-*O*-glucoside or Gallic acid 3-*O*-glucoside	R
2	8.85		331.0671	$C_{13}H_{16}O_{10}$	0.1	Isomer of Gallic acid-*O*-glucoside	R
3	9.15		169.0156	$C_7H_6O_5$	8.0	Gallic acid	R
4	14.91		367.1038	$C_{17}H_{20}O_9$	0.9	4-*O*-Feruloylquinic acid	C
5	15.29		567.1717	$C_{26}H_{32}O_{14}$	−0.4	Piceatannol-*O*-glucosylglucoside	R
6	18.13	342.1676		$C_{20}H_{24}NO_4^+$	−7.0	Magnoflorine	C
7	19.46		405.1191	$C_{20}H_{22}O_9$	0.0	Piceatannol glucoside	R
8	19.94		367.1038	$C_{17}H_{20}O_9$	0.9	3-*O*-Feruloylquinic acid or 5-*O*-Feruloylquinic acid	C
9	20.27		367.1038	$C_{17}H_{20}O_9$	0.9	3-*O*-Feruloylquinic acid or 5-*O*-Feruloylquinic acid	C
10	23.76		577.1565	$C_{27}H_{30}O_{14}$	0.4	Isoviolanthin	G
11	23.65		405.1194	$C_{20}H_{22}O_9$	0.7	Piceatannol glucoside	R
12	24.43		547.1456	$C_{26}H_{28}O_{13}$	−0.2	Chrysin-6-*C*-arabinosyl-8-*C*-glucoside or its isomer	S
13	24.63		549.1593	$C_{26}H_{30}O_{13}$	−3.8	Neolicuroside	G
14	25.01		547.1461	$C_{26}H_{28}O_{13}$	−0.2	Chrysin-6-*C*-arabinosyl-8-*C*-glucoside or its isomer	S
15	24.74		431.0983	$C_{21}H_{20}O_{10}$	−0.2	Aloe-emodin *O*-glucoside	R
16	25.67		547.1458	$C_{26}H_{28}O_{13}$	0.2	Chrysin-6-*C*-arabinosyl-8-*C*-glucoside or its isomer	S
17	25.63		549.1613	$C_{26}H_{30}O_{13}$	−0.1	Isomer of neolicuroside	G
18	26.13		389.1243	$C_{20}H_{22}O_8$	0.3	Resveratrol *O*-glucoside	R
19	26.70		417.1194	$C_{21}H_{22}O_9$	0.7	Liquiritin	G
20	26.97		547.1460	$C_{26}H_{28}O_{13}$	0.5	Chrysin-6-*C*-arabinosyl-8-*C*-glucoside or its isomer	S
21	27.18		461.0728	$C_{21}H_{18}O_{12}$	0.5	Scutellarin#	S
22	27.65		547.1460	$C_{26}H_{28}O_{13}$	0.5	Chrysin-6-*C*-arabinosyl-8-*C*-glucoside or its isomer	S
23	28.01	324.1208		$C_{19}H_{18}NO_4^+$	−6.9	Demethyleneberberine	C
24	28.95		547.1462	$C_{26}H_{28}O_{13}$	0.9	Chrysin-6-*C*-arabinosyl-8-*C*-glucoside or its isomer	S
25	29.82		547.1464	$C_{26}H_{28}O_{13}$	1.3	Chrysin-6-*C*-arabinosyl-8-*C*-glucoside or its isomer	S
26	30.31	322.1052		$C_{19}H_{16}NO_4^+$	−6.8	Groenlandicine	C
27	31.48		419.1347	$C_{21}H_{24}O_9$	−0.1	Rhapontin#	R
28	32.57	352.1153		$C_{20}H_{18}NO_5^+$	−7.5	13-Hydroxyberberine	C
29	37.01		977.5347*	$C_{47}H_{80}O_{18}$	2.1	Notoginsenoside R1#	GS
30	36.87		571.1463	$C_{28}H_{28}O_{13}$	1.0	Rhapontigenin *O*-galloylglucoside	R
31	36.83		475.0884	$C_{22}H_{20}O_{12}$	0.4	5,6,7-Trihydroxy-8-methoxy flavone-7-*O*-glucuronide	S
32	37.49		419.1351	$C_{21}H_{24}O_9$	0.8	Isorhapontin	R
33	37.81	338.1367		$C_{20}H_{20}NO_4^+$	−5.9	Columbamine	C
34	38.33		431.0987	$C_{21}H_{20}O_{10}$	0.8	Emodin-*O*-glucoside	R
35	38.72	336.1212		$C_{20}H_{18}NO_4^+$	−5.5	Epiberberine	C
36	38.97		571.1463	$C_{28}H_{28}O_{13}$	1.0	Rhapontigenin *O*-galloylglucoside	R
37	38.70		549.1618	$C_{26}H_{30}O_{13}$	0.8	Liquiritin apioside	G
38	39.08	338.1367		$C_{20}H_{20}NO_4^+$	−5.9	Jatrorrhizine#	C

Continued

Peak No.	t_R/min	M^+	$[M-H]^-$	Molecular Formula	Error (ppm)	Identification	Source
39	39.92	320.0898		$C_{19}H_{14}NO_4^+$	−6.0	Coptisine	C
40	40.16		845.4923*	$C_{42}H_{72}O_{14}$	2.2	Ginsenoside Rg1[#]	GS
41	40.33		991.5507*	$C_{48}H_{82}O_{18}$	2.4	Ginsenoside Re[#]	GS
42	40.29		549.1618	$C_{26}H_{30}O_{13}$	0.8	Isoliquiritin apioside	G
43	41.18		445.0783	$C_{21}H_{18}O_{11}$	1.5	Baicalin	S
44	44.01		695.1993	$C_{35}H_{36}O_{15}$	1.7	Licorice glycoside A	G
45	45.57		445.0792	$C_{21}H_{18}O_{11}$	3.5	Oroxylin A-7-*O*-glucoside	S
46	46.58		445.0783	$C_{21}H_{18}O_{11}$	1.5	norwogonin-7-*O*-glucuronide or its isomers	S
47	47.29		475.0890	$C_{22}H_{20}O_{12}$	1.7	5,7,8-Trihydroxy-6-methoxy flavone-7-*O*-glucuronide	S
48	47.68	352.1525		$C_{21}H_{22}NO_4^+$	−5.2	Palmatine[#]	C
49	49.20		445.0785	$C_{21}H_{18}O_{11}$	1.9	norwogonin-7-*O*-glucuronide or its isomers	S
50	49.34		459.0947	$C_{22}H_{20}O_{11}$	3.1	Oroxylin A-7-*O*-glucuronide	S
51	49.51	336.1214		$C_{20}H_{18}NO_4^+$	−4.9	Berberine[#]	C
52	49.67		429.0838	$C_{21}H_{18}O_{10}$	2.5	Chrysin-7-*O*-glucuronide	S
53	49.80		475.0895	$C_{22}H_{20}O_{12}$	2.7	5,7,2'-Trihydroxy-6-methoxy flavone-7-*O*-glucuronide	S
54	50.22		415.1043	$C_{21}H_{20}O_9$	2.0	Chrysophanol *O*-glucoside	R
55	50.43		431.0993	$C_{21}H_{20}O_{10}$	2.2	Emodin 1-*O*-glucoside	R
56	51.04		403.1407	$C_{21}H_{24}O_8$	2.1	Deoxyrhapontigenin *O*-glucoside	R
57	51.05		445.0784	$C_{21}H_{18}O_{11}$	1.7	Baicalein-6-*O*-glucuronide or Apigenin-7-*O*-glucuronide	S
58	51.54		431.0995	$C_{21}H_{20}O_{10}$	2.6	Emodin 8-*O*-glucoside	R
59	52.06		415.1045	$C_{21}H_{20}O_9$	2.5	Chrysophanol *O*-glucoside	R
60	52.68		459.0945	$C_{22}H_{20}O_{11}$	2.6	Wogonoside[#]	S
61	54.98		555.1526	$C_{28}H_{28}O_{12}$	3.2	Deoxyrhapontigenin *O*-galloylglucoside	R
62	57.12		555.1526	$C_{28}H_{28}O_{12}$	3.2	Deoxyrhapontigenin *O*-galloylglucoside	R
63	58.12		845.4940	$C_{42}H_{72}O_{14}$	4.2	Ginsenoside Rf	GS
64	58.74		445.1151	$C_{22}H_{22}O_{10}$	2.4	Physcion 8-*O*-glucoside	R
65	60.31		431.0995	$C_{21}H_{20}O_{10}$	2.6	Emodin *O*-glucoside	R
66	60.47		983.4539	$C_{48}H_{72}O_{21}$	4.6	Licorice saponin A3 isomer	G
67	60.80		445.1152	$C_{22}H_{22}O_{10}$	2.6	Physcion 1-*O*-glucoside	R
68	61.52		837.3958	$C_{42}H_{62}O_{17}$	5.2	Licorice saponin G2 or its isomer	G
69	62.18		1153.6064*	$C_{54}H_{92}O_{23}$	4.6	Gisenoside Rb1[#]	GS
70	64.07		829.4996*	$C_{42}H_{72}O_{13}$	4.9	Gisenoside Rg2[#]	GS
71	64.35		1123.5960	$C_{53}H_{90}O_{22}$	4.8	Ginsenoside Rc	GS
72	64.82		829.4995*	$C_{42}H_{72}O_{13}$	4.8	isomer of ginsenoside Rg2	GS
73	66.42		1123.5962*	$C_{53}H_{90}O_{22}$	5.0	Ginsenoside Rb2	GS
74	67.04		269.0467	$C_{15}H_{10}O_5$	4.3	Baicalein[#]	S
75	67.30		837.3965	$C_{42}H_{62}O_{17}$	6.1	Licorice saponin G2 or its isomer	G
76	67.80	616**			-	hypaconitine	A

Continued

Peak No.	t_R/min	M^+	$[M-H]^-$	Molecular Formula	Error (ppm)	Identification	Source
77	68.21		991.5533*	$C_{48}H_{82}O_{18}$	5.0	Ginsenoside Rd[#]	GS
78	68.43		837.3958	$C_{42}H_{62}O_{17}$	5.2	Licorice saponin G2 or its isomer	G
79	68.83		837.3962	$C_{42}H_{62}O_{17}$	5.7	Licorice saponin G2 or its isomer	G
80	68.86		299.0572	$C_{16}H_{12}O_6$	3.6	5,6,7-Trihydroxy-8-methoxy-flavone or 5,7,8-Trihydroxy-6-methoxy-flavone or 5, 7, 2-Trihydroxy-6-methoxy flavone	S
81	69.10		821.4007	$C_{42}H_{62}O_{16}$	5.1	Glycyrrhizic acid[#]	G
82	70.02		807.4216	$C_{42}H_{64}O_{15}$	5.4	licorice saponin B2 isomer	G
83	70.25		821.4010	$C_{42}H_{62}O_{16}$	5.5	Isomer of glycyrrhizic acid	G
84	70.65		821.4005	$C_{42}H6_2O_{16}$	4.9	Isomer of glycyrrhizic acid	G
85	72.12		807.4213	$C_{42}H_{64}O_{15}$	5.0	licorice saponin B2	G
86	73.52		829.5002*	$C_{42}H_{72}O_{13}$	5.7	Ginsenosid Rg3[#]	GS
87	73.43		283.0262	$C_{15}H_8O_6$	4.9	Rhein[#]	R
88	73.80	277**		$C_{17}H_{26}O_4$	-	6-gingerol[#]	ZR, Z
89	73.75		283.0624	$C_{16}H_{12}O_5$	4.2	Wogonin[#]	S
90	73.96		253.0515	$C_{15}H_{10}O_4$	3.4	Chrysin	S
91	74.20		373.0940	$C_{19}H_{18}O_8$	3.0	Skullcapflavon II	S
92	74.66		283.0624	$C_{16}H_{12}O_5$	4.2	Oroxylin A	S
93	78.22		269.0469	$C_{15}H_{10}O_5$	5.0	Emodin[#]	R

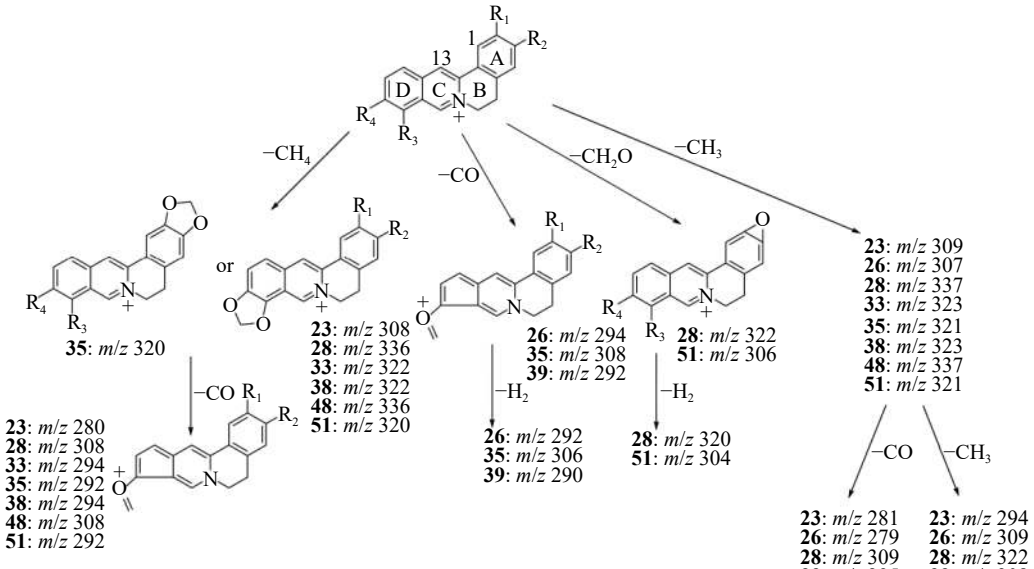
*: Peaks observed were $[M + HCOO]^-$. **: Peak observed was $[M + H]^+$ and HR-MS data didn't be observed. #: Compounds were identified by reference compounds.

ring (Fig. 1). Although further experiments are required to confirm this hypothesis, results from this study and data from other works [14, 18] both strongly support it, which provides useful assistance for accurate characterization of some structural isomers, such as berberine and epiberberine.

Identification of flavonoids

Flavonoids broadly exist in many species of plants. The main sources of flavonoids in XXDs were *Scutellariae Radix* and *Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle*. *Scutellariae Radix* is present in FZ and BX while it is not in DHHL. Similarly, *Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle* is only present in BX of three formulae. *i.e.* peaks that exist in both FZ and BX while vanished in the DHHL would come from *Scutellariae Radix and* peaks only observed in BX may have the source of *Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle.* Then the fragmentation behavior of mass spectrometry was taken into consideration to identify these flavonoids. Flavonoids glucuronides or glucosides are the most abundant form of flavonoids in XXDs.

Peaks **43**, **45**, **46**, **49** and **57** shared the same molecular weight of 446 Da and HR-MS showed a common molecular formula of $C_{21}H_{18}O_{11}$. In MS^2 spectrum, Peak **45** yielded a unique fragment with *m*/*z* 283 compared with other peaks produced *m*/*z* 269 fragment. With the observation of *m*/*z* 268, we concluded a methoxyl group was removed from the fragment of *m*/*z* 283. Peak **45** was then identified as oroxylin A-7-*O*-glucoside in combination with the data of the literature [18]. Peak **43** was identified as baicalin referring to the fragment behavior of baicalein (Peak **74**) reported before. Peak **57** was temporarily identified to be baicalein-6-*O*-glucuronide as its MS^3 fragment showed the existence of baicalein skeleton. Peaks **46** and **49** could be norwogonin-7-*O*-glucuronide or its isomers. More information was needed before accurate identification could be made. Peaks **31**, **47** and **52** had the same molecular formula of $C_{22}H_{20}O_{12}$. These isomers displayed similar fragmentation pathway through the appearance of *m*/*z* 299, 284 and 175 fragments in MS^2. The retention time was then employed to compare among these isomers and these peaks were identified as 5,6,7-Trihydroxy-8-methoxy-flavone-7-*O*-glucuronide, 5, 7, 8-Trihydroxy-6-methoxy-flavone-7-*O*-glucuronide and 5, 7, 2′-Trihydroxy-6-methoxy-flavone-7-*O*-glucuronide or their isomer, respectively [19, 20]. Peaks **12**, **14**, **16**, **20**, **22**, **24** and **25** present a common molecular formula of $C_{25}H_{26}O_{11}$. According to their similar fragmentation pathways, Chrysin-6-*C*-glucosyl-8-*C*-arabonoside and its isomers were suggested as possible compounds corresponding to these peaks and more information is needed to distinguish them [19, 20]. The same core fragment with *m*/*z* 255 was observed for Peaks **13**, **17**, **19**, **37** and **42** in the negative ion mode MS/MS and two main fragments with *m*/*z* 135 and 119



Compound	MW
23: Demethyleneberberine ($R_1 - R_2 = -OH$, $R_3 = R_4 = OCH_3$)	324
26: Groenlandicine ($R_1 = OCH_3$, $R_2 = OH$, $R_3 = R_4 = -OCH_2-$)	322
28: 13−Hydroxyberberine ($R_1 - R_2 = -OCH_2O-$, $R_3 = R_4 = OCH_3$, $C_{13} = C - OH$)	352
33: Columbamine ($R_1 = OH$, $R_2 = R_3 = R_4 = OCH_3$)	338
35: Epiberberine ($R_1 = R_2 = OCH_3$, $R_3 = R_4 = -OCH_2O-$)	336
38: Jatrorrhizine ($R_1 = R_3 = R_4 = OCH$, $R_2 = OH$)	338
39: Coptisine ($R_1 - R_2 = -OCH_2O-$, $R_3 - R_4 = -OCH_2O-$)	320
48: Palmatine ($R_1 = R_2 = R_3 = R_4 = OCH$)	352
51: Berberine ($R_1 - R2 = -OCH_2O-$, $R_3 = R_4 = OCH_3$)	336

Fig. 1 Proposed fragmentation pathway of protoberberine alkaloids observed in XXDs

were yielded for these peaks at the same time. Referring to the published results, they are determined as Neolicuroside, isomer of Neolicuroside, Liquiritin, Liquiritin apioside and Isoliquiritin apioside, respectively [21, 22].

Fig.2 showed the proposed fragmentation pathway of baicalin as an example for flavonoids in negative ion mode. Lose of small neutral molecules such as CO, CO_2 and H_2O was common for flavonoids.

Identification of stilbenes and anthraquinones

As the characteristic compounds in *Rhei Radix et Rhizoma*, stilbenes and anthraquinones only exist in the DH-HL and FZ. Peak **27** was characterized as rhapontin using the fragmentation pattern of reported reference [23]. It displayed MS^2 fragment at *m/z* 401 $[M - H - H_2O]^-$, 299 $[M - H - C_4H_8O_4]^-$, 257 $[M - H - C_6H_{10}O_5]^-$ and MS^3 fragments at *m/z* 242 $[257 - CH_3]^-$, 241 $[257 - CH_4]^-$, 224 $[257 - CH_3 - H_2O]^-$ and 213 $[257 - CH_4 - CO]^-$. Fig. 3 showed the proposed fragmentation pathway of rhapontin as an example for stilbenes. Peak **32** performed the same spectral signature with Peak **27**. It was then identified to be the isomer of rhapontin.

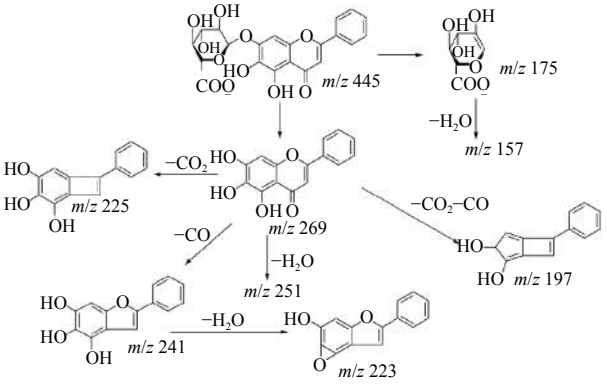


Fig. 2 Proposed fragmentation pathway of baicalin in negative ion mode

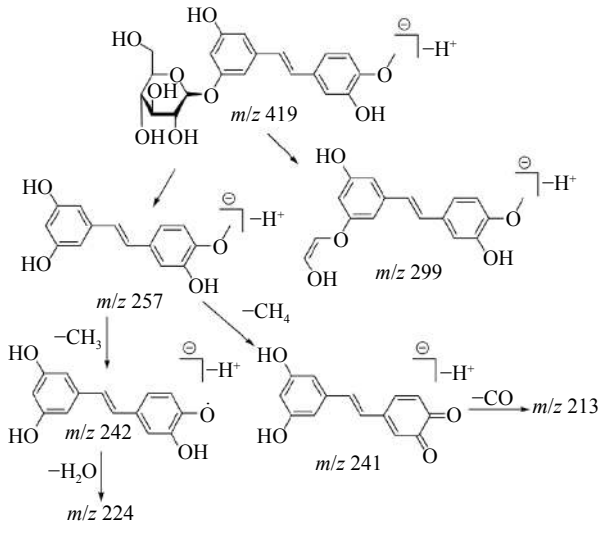


Fig. 3 Proposed fragmentation pathway of rhapontin

Peaks **5**, **7** and **11** displayed a common core fragment of *m*/*z* 243 in MS^2. It could then be inferred as piceatannol based on the MS^3 fragments at *m*/*z* 225, 201, 199, 175, 159 and 157. Thus these peaks were identified as piceatannol glucoside [23]. According to their molecular weight of 572 and fragment of *m*/*z* 313 in MS^2 and *m*/*z* 169 in MS^3, Peaks **30** and **36** were then identified to be rhapontigenin *O*-galloylglucoside or its isomer. Peaks **15**, **34**, **55**, **58** and **65** had the same molecular weight of 432 Da. Peak **15** had a fragment of *m*/*z* 293, 311, 283 which fit the reported literature of aloe-emodin [23]. Relative retention time also supported this conclusion. Peaks **34**, **58** and **65** had the aglycone of emodin. Peak **55** showed a base peak of *m*/*z* 268 rather than *m*/*z* 269 indicated that it was emodin 1-*O*-glucoside [24]. Peak **67** with a negative MS peak at *m*/*z* 491 $[M + HCOOH - H]^-$ and 445 $[M - H]^-$ indicated its molecular weight (m.w.) was 446. The HR-MS data also confirmed that this compound had its molecular ion peak of 445.1151, corresponding to molecular formula of $C_{22}H_{22}O_{10}$ (2.6 ppm). Main fragmentation pathways for this compound would be to lose glucoside (−162). The MS^2 fragment at *m*/*z* 283 indicated its aglycone structure. It was temporarily characterized to be Physcion 1-*O*-glucoside.

Identification of saponins

Saponins were another common type of compounds wide spread in plants. The main sources of saponin in these decoctions were *Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle* and *Ginseng Radix et Rhizoma*. It could be observed in BX, SJ and GC. Although saponins lacked characteristic UV absorption, their mass spectrum could be distinctive from each other. Ginsenoside, for example, could dissociate to protopanoxadiol (PPD, at *m*/*z* 459 in negative ion mode) or protopanaxatriol (PPT, at *m*/*z* 475 in negative ion mode) by eliminating several monosaccharides. Similar patterns would occur to saponins from *Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle*. The identification of these saponins has been showed in Table 2.

Identification of other compounds

Peak **76** only existed in FZ, which indicated that it came from *Aconiti Lateralis Radix Praeparata* and further identified to be hypaconitine by comparing the retention time and fragmentation pathway with previous report [25]. Identification of other compounds was listed in Table 2 as well.

Conclusion

An LC-MS method was applied to characterize five XXDs that were prepared following the instructions of ancient Zhang Zhongjing's herbal formulae. Tentatively, 93 compounds from different chemical categories, including protoberberine alkaloids, flavonoids, stilbenes, anthraquinones, saponins and others, were described here. To our knowledge, it is the first systematic analysis of XXDs conducted to illustrate the constitution of their chemical components and fragment pathways were summarized for each chemical category. This work laid the important foundation for mechanistic studies on biological activities of XXDs, which also would provide valuable information on relationship between chemical compositions and therapeutic effects of XXDs.

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