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Identification of multiple constituents in the traditional Chinese medicine formula GuiZhiFuLing-Wan by HPLC–DAD–MS/MS

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ABSTRACT

GuiZhiFuLing-Wan (GFW) has been used in China for centuries to improve blood stagnation. In this paper, a HPLC–DAD–MS/MS method was established for the efficient and rapid identification of the chemical constituents in extract of GuiZhiFuLing-Wan. Separation was performed on an Alltima C_{18} analytical column by gradient elution with CH_3CN/H_2O–CH_3COOH as mobile phase at a flow rate 1.0 ml/min. 27 potentially bioactive compounds including monoterpene glycosides, galloyl glucoses, acetophenones, phenylallyl compounds and triterpenoids were identified or tentatively characterized by online ESI/MS/MS and the comparison with literature data and authentic compounds. After the identification, six different brands of GFW commercial products in various dosage forms were evaluated. The results demonstrated that capsule of GFW was superior to the other two dosage forms, honeyed pill and concentrated pill in administration. The points that should be paid more attention during the manufacturing process of GFW were also analyzed. The method can be the basis for the quality control of this commonly used herbal formula.

1. Introduction

Chinese herbal prescriptions contain several crude drugs at an intrinsic mass ratio to obtain beneficial bioactivities for clinical indications. These have attracted considerable attention worldwide in recent years due to their high effectiveness against many diseases with low toxicity [1–3]. However, the quality control and clarification of therapeutical mechanism of herbal prescriptions have always been bottleneck hindering its development since hundreds of various chemical constituents are present in these complex systems. Sensitive and reliable analytical methods are required to ensure the safety, efficiency and stability of herbal prescriptions.

GuiZhiFuLing-Wan (GFW) is an important multiherbal formula in traditional Chinese medicine (TCM), first described by an eminent Chinese physician Zhang Zhongjing in Han Dynasty. This prescription, consisting of five herbs of *Ramulus Cinnamomi*, *Poria Cocos*, *Cortex Moutan*, *Radix Paeoniae* and *Semen Persicae*, has been extensively used to improve syndrome of blood stasis in China, Japan and Korea [4]. A clinical report indicates that GFW extract is very effective in treating thrombosis in those patients who have difficulties with more conventional antithrombotic drugs [5]. It has been used in invigorating blood circulation, normalizing menstruation, eliminating blood stasis, to relieve pain and many other diseases. In pharmaceutical research, GFW was reported to prevent the progression of atheromatous plaque by creating a sounder antioxidant defense system than vitamin E to protect against NO-mediated neuronal death in cultured rat cerebellar granule cells, to reduce oxidative stress by hyperglycemia and to inhibit the growth of cancer cell lines such as HepG2 cell and Hep3B cell [6–8]. It was also known that the nonpolar extracts of GFW containing some antioxidative substances, could inhibit platelet aggregation and protect the myocardium against ischemia-induced derangement, while an aqueous extracts of GFW containing phenolic compounds were effective in protecting liver microsomes, hepatocytes and erythrocytes against oxidative damage [9]. Although so many beneficial effects have been shown, the actual bioactive components of GFW are still not well understood.

The constituents of each comprising herbal drugs of GFW have been well studied in previous reports [10–14] while the special investigation on the profile constituents of the formula has not been reported yet. Therefore, it is necessary to develop a rapid and sensitive on-line method to identify and characterize the compounds in GFW.

GFW has been widely manufactured in several dosage forms such as concentrated pill, honeyed pill and capsule. The current quality control standards of GFW products are only based on the quantification of marker compounds, cinnamic acid, cinnamic aldehyde, paeonol, paeoniflorin and amygdalin by GC and RP-HPLC [15–16]. These methods are not sufficient for the quality control

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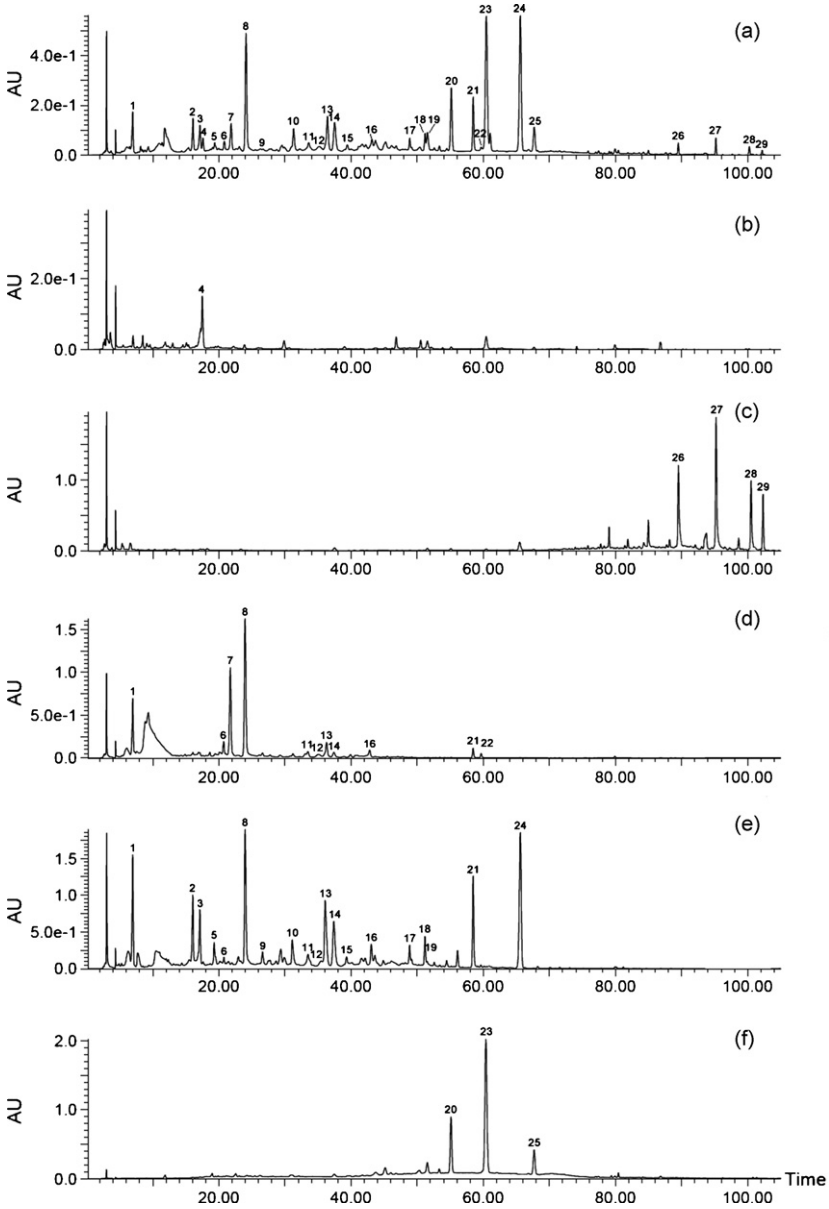


Fig. 1. HPLC–DAD (245 nm) chromatograms of extracts of GFW (a), *Semen Persicae* (b), *Poria Cocos* (c), *Radix Paeoniae* (d), *Cortex Moutan* (e) and *Ramulus Cinnamomi* (f).

since the curative effects of TCM result from the integrated effects of a number of multiple compounds. Full evaluation of GFW products depends not only on the analysis of major compounds but also on the other active principles.

Direct coupling of high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) is an important method in TCM research [17–19], since it has been proven to be a suitable tool for the rapid on-line analysis of the constituents. The aim of this study was to develop a HPLC–DAD–MS/MS method that is capable of separating, identifying and characterizing 27 compounds in one chromatographic run and its application to the constituents in commercial products of GFW.

2. Experimental

2.1. Chemicals and materials

Standards of gallic acid, paeonol, amygdalin, paeoniflorin, cinnamic aldehyde, cinnamic acid, (+)-catechin, benzoic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China). Benzoylpaeoniflorin, albiflorin, pachymic acid, polyporenic acid C, trametenolic acid and dehydrotrametenolic acid were isolated by the authors. Their structures were unambiguously identified by NMR techniques, and their purities were above 98% as determined by HPLC.

HPLC-grade acetonitrile was purchased from Tedia (Fairfield, OH, USA); AR-grade acetic acid, ethanol and methanol were obtained from Jiangsu Hanbon (Jiangsu, China); Water was purified by a Milli-Q academic water purification system (Milford, MA, USA). Twice distilled water was used for the extraction and preparation of samples.

Ramulus Cinnamomi, *Poria Cocos*, *Cortex Moutan*, *Radix Paeoniae* and *Semen Persicae* were purchased from Fengyuan Tongling crude drug company (Anhui, China) and were identified by Professor Boyang Yu. The voucher specimens were deposited in our laboratory.

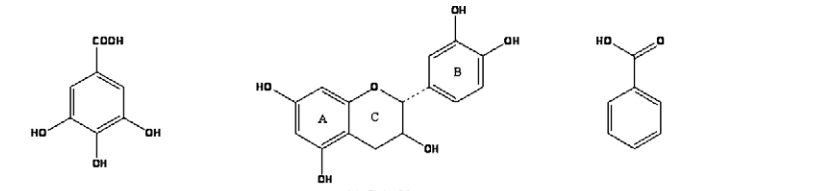
GFW products were purchased from six different brand manufacturers in China. The names of these manufacturers have been abbreviated to preserve commercial confidentiality.

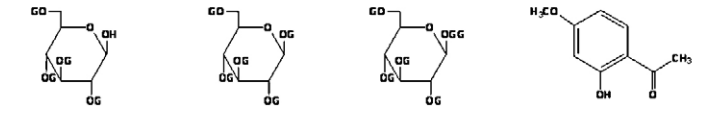
Table 1
Characterization of compounds in extract of GFW by HPLC–DAD–MS/MS.

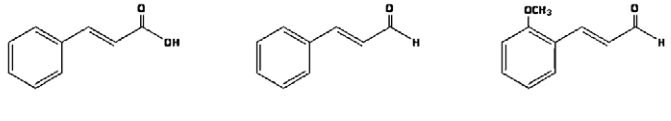
Peak no.	Retention time (min)	Compound	λ_{max} (nm)	$[M-H]^-$	$[M+Na]^+$	Plant material	Fragments ions *m/z*
1[a]	7.05	Gallic acid	213,270	169	–	CM, RP	$[M-H-COO]^-$: 125, $[M-H-COO-CO]^-$: 97, $[M-H-COO-CO-CO]^-$: 69
2	16.22	Oxypaeoniflorin	210,258	–	519	CM	$[M+Na-p\text{OHBA}]^+$: 381, $[M+Na-Glc]^+$: 357, $[M+Na-\text{aglycone}]^+$: 323, $[M+Na-C_{13}H_{12}O_5]^+$: 271, $[M+Na-p\text{OHBA-Glc}]^+$: 219
3[a]	16.85	(+)-Catechin	278	289	–	CM	$[M-H-C_7H_6O_3]^-$: 151, $[M-H-C_8H_8O_3]^-$: 137, $[C_6H_6O_2-H]^-$: 109
4[a]	17.54	Amygdalin	210	–	480	SP	$[M+Na-C_7H_8N]^+$: 374, $[M+Na-C_8H_7N]^+$: 363, $[M+Na-C_8H_7NO]^+$: 347, $[M+Na-Glc]^+$: 318
5	19.60	Apiopaeonoside	225,270	–	483	CM	$[M+Na-P]^+$: 317, $[P+Na]^+$: 189
6	20.87	Paeonilide	225,268	–	483	RP, CM	$[M+Na-A]^+$: 317, $[M+Na-C_2H_4O_2]^+$: 257, $[Ara+Na]^+$: 189
7[a]	21.83	Albiflorin	231,274	–	503	RP	$[M+Na-H_2O]^+$: 475, $[M+Na-BA]^+$: 381, $[M+Na-Glc]^+$: 341, $[M+Na-\text{aglycone}]^+$: 307, $[M+Na-C_{13}H_{12}O_5]^+$: 271, $[M+Na-BA-Glc]^+$: 219
8[a]	24.08	Paeoniflorin	232,274	–	503	RP, CM	$[M+Na-BA]^+$: 381, $[M+Na-Glc]^+$: 341, $[M+Na-\text{aglycone}]^+$: 307, $[M+Na-C_{13}H_{12}O_5]^+$: 271, $[M+Na-BA-Glc]^+$: 219
9	27.60	Suffruticoside B Suffruticoside D	220,275	–	635	CM	$[M+Na-Ara]^+$: 469, $[M+Na-Ara]^+$: 337, $[Ara+Na]^+$: 189
10	31.28	Unknown	258,365	–	–	CM	–
11	33.57	Galloylpaeoniflorin	220,275	–	655	CM, RP	$[M+Na-GA]^+$: 503, $[M+Na-GA-Glc]^+$: 317
12	35.27	Tetragalloylglucopyranose	217,279	787	–	CM, RP	$[M-H-GA]^-$: 617, $[M-H-GA-GA]^+$: 447, $[M-H-GA-GA-GA]^+$: 277
13	36.40	Pentagalloylglucopyranose	217,279	939	–	CM, RP	$[M-H-C_7H_4O_4]^-$: 769, $[M-H-C_7H_4O_4-GA]^+$: 599, $M-H-C_7H_4O_4-GA-GA]^+$: 429
14[a]	37.50	Benzoic acid	228,273	121	–	CM, RP	$[M-H-COO]^-$: 77
15	39.42	Mudanpioside H	258	–	639	CM	$[M+Na-p\text{OHBA}]^+$: 501, $[M+Na-p\text{OHBA-HCHO}]^+$: 471, $[M+Na-p\text{OHBA-HCHO-Glc}]^+$: 357, $[p\text{OHBA}+\text{Glc}+Na]^+$: 305
16	43.67	Hexagalloylglucopyranose	217,279	1091	–	CM, RP	$[M-H-C_7H_4O_4]^-$: 939, $[M-H-C_7H_4O_4-GA]^+$: 719, $[M-H-C_7H_4O_4-GA-GA]^+$: 599
17	48.90	Benzoyloxypaeoniflorin	227,259	–	623	CM	$[M+Na-BA]^+$: 501, $[M+Na-p\text{OHBA}]^+$: 485, $[p\text{OHBA}+\text{Glc}+Na]^+$: 323, $[\text{aglycone}+Na]^+$: 219
18	51.22	Mudanpioside C	233,258	–	623	CM	$[M+Na-BA]^+$: 501, $[M+Na-p\text{OHBA}]^+$: 485, $[\text{aglycone}+Na]^+$: 219
19	51.49	Unknown	243	–	768	CM	–
20[a]	55.18	Cinnamic acid	216,276	147	–	RC	$[M-H-COO]^-$: 103
21	58.50	Benzoylalbiflorin	230,274	–	607	RP, CM	$[M+Na-H_2O]^+$: 589, $[M+Na-BA]^+$: 485, $[M+Na-\text{aglycone}]^+$: 411, $[M+Na-BG]^+$: 341, $[M+Na-BA-BG]^+$: 219
22[a]	59.71	Benzoylpaeoniflorin	232,274	–	607	RP	$[M+Na-BA]^+$: 485, $[M+Na-\text{aglycone}]^+$: 411, $[M+Na-BG]^+$: 341, $[M+Na-BA-BG]^+$: 219
23[a]	60.48	Cinnamic aldehyde	227,296	131	–	RC	$[M-H-CO]^-$: 103
24[a]	65.62	Paeonol	228,274,312	165	–	CM	$[M-H-CH_3]^-$: 135, $[M-H-HCHO]^-$: 135, $[M-H-CH_3CO]^-$: 122
25	67.73	3-(2-Methoxyphenyl)-2-propenal	230,286	161	–	RC	$[M-H-CH_3]^-$: 147, $[M-H-OCH_3-CO]^-$: 103
26[a]	89.52	Polyporenic acid C	242	481	–	PC	$[M-H-H_2O-C_9H_{15}O_2]^-$: 308, $[M-H-H_2O-C_9H_{15}O_2-CO]^-$: 280, $[M-H-H_2O-C_9H_{15}O_2-CO-CH_2]^-$: 256
27[a]	95.23	Pachymic acid	242	–	–	PC	–
28[a]	100.45	Dehydrotrametenolic acid	242	–	–	PC	–
29[a]	102.32	Trametenolic acid	242	–	–	PC	–

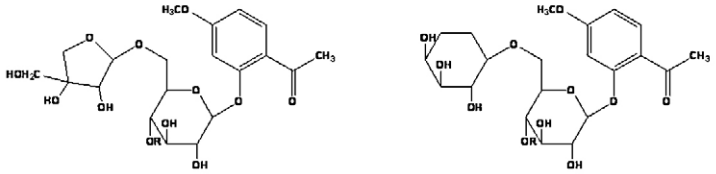
RC, *Ramulus Cinnamomi*; CM, *Cortex Moutan*; RP, *Radix Paeoniae*; PC, *Poria Cocos*; SP, *Semen Persicae*. BA, benzoic acid; GA, gallic acid; P, paeonol; Ara, arabinose; Glc, glucose; BG, glucosyl group with benzoate group on C-6′; aglycone: the mass of aglycone.

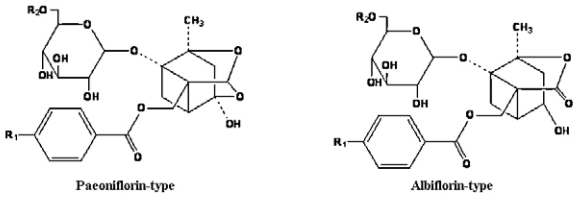
[a] Compared with authentic compounds.



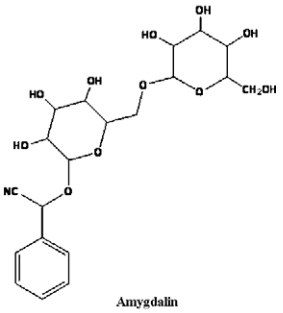


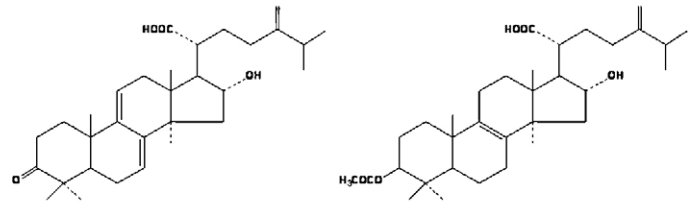






Peak NO.	Compound	Aglycone	R_1	R_2
2	Oxypaeoniflorin	Paeoniflorin-type	OH	H
7	Albiflorin	Albiflorin-type	H	H
8	Paeoniflorin	Paeoniflorin-type	H	H
11	Galloylpaeoniflorin	Paeoniflorin-type	H	Galloyl
15	Mudanpioside H	Paeoniflorin-type	OH	*p*-hydroxybenzoyl
17	Benzoyloxypaeoniflorin	Paeoniflorin-type	OH	benzoyl
18	Mudanpioside C	Paeoniflorin-type	H	*p*-hydroxybenzoyl
21	Benzoylalbiflorin	Albiflorin-type	H	benzoyl
22	Benzoylpaeoniflorin	Paeoniflorin-type	H	benzoyl





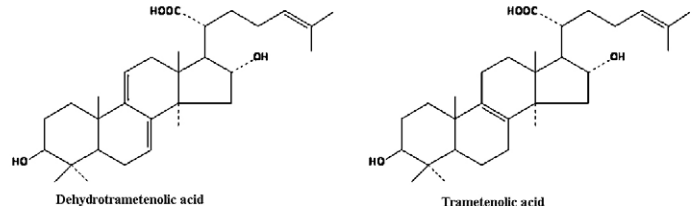


Fig. 2. Chemical structures of compounds identified in extract of GFW.

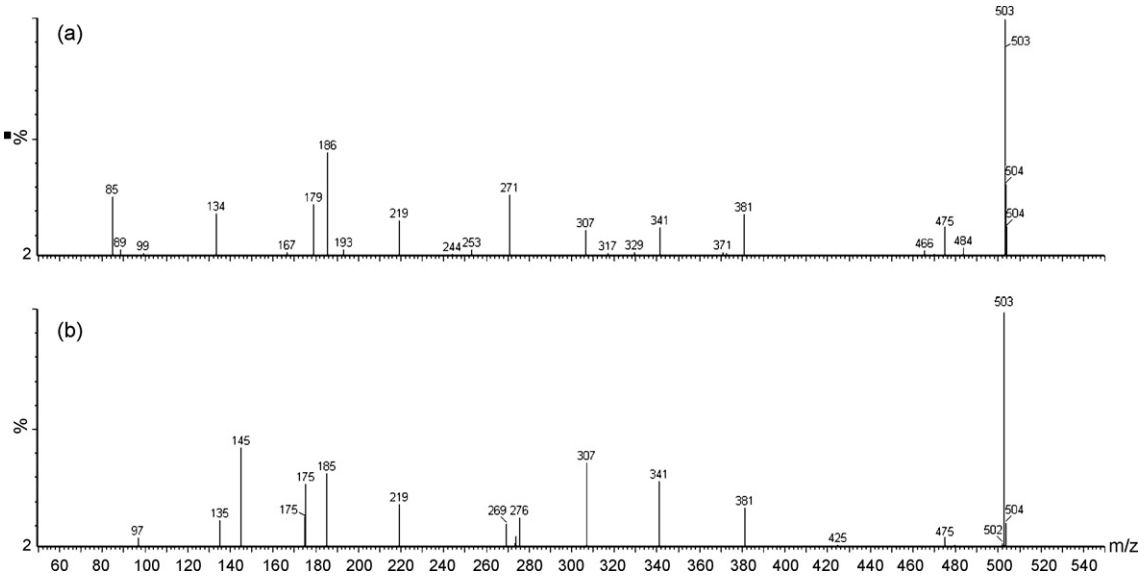


Fig. 3. The characteristic mass spectra of albiflorin (a) and paeoniflorin (b).

2.2. Sample preparation

The powdered sample of *Ramulus Cinnamomi* (20 g) was immersed in 200 ml 95% ethanol for 60 min and decocted by boiling for 60 min, and then extracted by 200 ml water for 60 min. The operations were repeated twice. The total extracts were combined and concentrated by a Buchi rotavapor (Flawil, Switzerland) to approximate 100 ml, and then the concentrated solution was freeze dried by a Labconco freeze dry system (Kansas City, MO, USA). The residue (0.1 g) was reconstituted with 1 ml methanol and filtered through a 0.45 μm filter before the HPLC analysis. The other four crude drugs samples were prepared using the same way as that for *Ramulus Cinnamomi*. The sample of GFW was prepared by combining *Ramulus Cinnamomi* (20 g), *Poria Cocos* (20 g), *Cortex Moutan* (20 g), *Radix Paeoniae* (20 g) and *Semen Persicae* (20 g). Then the mixture was immersed in 1000 ml 95% ethanol for 60 min and decocted by boiling for 60 min, and extracted by 1000 ml water for 60 min. The sample was prepared with the same procedure as that for *Ramulus Cinnamomi*. All sample solutions were stored at 4 °C and used at room temperature.

2.3. HPLC–DAD–MS/MS instrumentation and conditions

HPLC–MS/MS analyses were carried out using a Waters Alliance HPLC instrument (Waters, Milford, MA, USA) linked simultaneously to both a PDA 2996 photo diode array detector (Waters, Milford, MA, USA) and a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionization (ESI) source operating in both negative and positive mode. MassLynx™ software (version 4.0, Waters, Milford, MA, USA) was used to control the instruments, and for data acquisition and processing.

Sample solutions were separated on an Alltima C18 column (4.6 mm × 250 mm, i.d. 5 μm, Serial No. 605051040.1, Alltech Company, Deerfield, IL, USA) with C18 guard column (Hanbon Science & Technology Co., Ltd., Jiangsu, China), which was maintained at 30 °C. A linear gradient elution of (A) CH_3CN and (B) H_2O–CH_3COOH (100:0.1, v/v) was used. A gradient programmer was used according the following profile: 0–15 min, 5–15% A; 15–35 min linear increase to 20% A; 35–65 min linear increase to 40% A; 55–100 min linear increase to 100% A. The solvent flow rate was 1 ml/min and 20 μl of sample solution was injected in each run. The effluent was introduced into a PDA detector (scanning range 210–400 nm, resolution 1.2 nm) and subsequently into an electrospray source (desolvation temperature 400 °C, capillary voltage 3.0 kV, cone voltage 30 V). The split ratio of HPLC flow between PAD detector and MS detector was 2:1. Helium was used as collision gas (collision energy 30 V) and nitrogen as desolvation gas (500 l/h).

2.4. Evaluations of the samples of GFW products

Six brands of GFW products including three different dosage forms were purchased from drugstores in China. They were concentrated pill, honeyed pill and capsule of the multiherb remedy prepared by various pharmaceutical manufactures. On the product labels, the manufactures claimed that the herbal remedies were composed of the ingredient herbs according to the recipe of GFW described in Zhang Zhongjing's works. The products were stored at 4 °C until used.

For HPLC analysis, each variety of GFW (0.1 g) was dissolved in 1 ml methanol. The solution was filtered through 0.45 μm filter. The resultant herbal solution was kept frozen at 4 °C before use.

3. Results and discussion

3.1. HPLC analysis

In order to obtain chromatograms with good separation and strong total ion current (TIC), low-gradient slope and CH_3CN/H_2O–CH_3COOH (100:0.1, v/v) was found to be the optimal mobile phase in both HPLC and MS analyses. CH_3CN remarkably improved separation of the major constituents in GFW as compared with CH_3OH. The addition of CH_3COOH has substantial effect on selectivity and efficiency.

245 nm was selected as the wavelength of monitoring since it is suitable to detect all constituents. 29 peaks from GFW were detected under the current HPLC condition. The peaks were characterized by the retention times and UV spectra and by comparing the chromatogram of GFW with those of single herb extracts. The representative HPLC–DAD chromatograms of the extract of GFW and single crude drugs are presented in Fig. 1.

3.2. Tandem mass spectrometry of authentic compounds

In order to obtain MS fragmentation patterns of constituents from GFW extract, 14 authentic compounds, including gallic

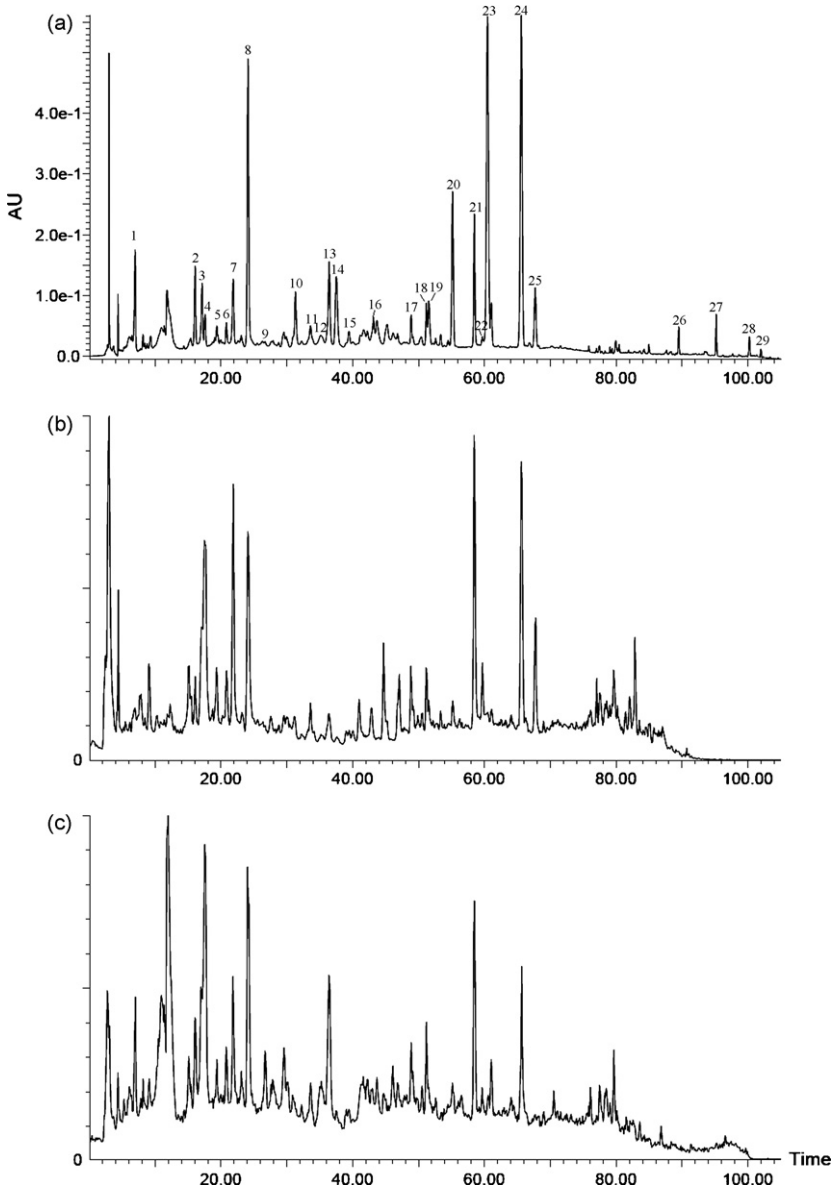


Fig. 4. HPLC–DAD–MS/MS analysis of extract of GFW. (a) HPLC–DAD chromatogram monitored at 245 nm. (b) Positive ion mode MS spectra. (c) Negative ion mode MS spectra.

acid, (+)-catechin, amygdalin, albiflorin, paeoniflorin, benzoic acid, cinnamic acid, benzoylpaeoniflorin, cinnamic aldehyde, paeonol, trametenolic acid, dehydrotrametenolic acid, pachymic acid and polyporenic acid C, were studied by means of HPLC–DAD–MS/MS. In the full scan mass spectra, most of the authentic compounds exhibited $[M+Na]^+$ ions in positive mode or their quasi-molecular $[M-H]^-$ in negative mode. Characteristic UV spectra of four triterpenoids were detected in *Poria cocos* and extracts of GFW. However, no ion peak attributed to them was found in the MS analysis via direct injection except polyporenic acid C. MS, MS/MS and UV data are summarized in Table 1. The structures are depicted in Fig. 2.

The characteristic fragmentation of three monoterpene glycosides was studied in the positive ion mode. This type of compounds exhibited $[M+Na]^+$ ions of sufficient abundance to be subjected to MS/MS analysis. The predominant fragment ions of monoterpene glycosides can be attributed to the loss of a benzoic acid (122 Da), a glucosyl group (162 Da) and their combined loss (284 Da). The product ion at m/z 307 was formed by a benzoate group and a glucosyl group connected via the newly formed bond. Some isomers of monoterpene glycosides could be distinguished according to the skeleton of their aglycones. Based on the different dehydration ability of the C4 hydroxyl, the hydroxyl carbon has more freedom in albiflorin-type. As examples, the MS/MS spectra of albiflorin and paeoniflorin are depicted in Fig. 3. Phenylallyl compounds generally displayed the $[M-H]^-$ ion as base peak and yielded the product ion by loss of CO and CO_2 in MS/MS spectra.

3.3. HPLC–MS/MS analysis of the GFW extract

The screening, identification and further characterizing of components in GFW extract were performed firstly by HPLC–MS in both the positive and negative ion mode to provide the elemental compositions of the molecular ions. Their TIC chromatograms are shown in Fig. 4. Diagnostic fragmentations were then checked by MS/MS to confirm the results of HPLC–MS. Compounds **1**, **3**, **4**, **7**, **8**, **14**, **20**, **22**, **23**, **26**, **27**, **28** and **29** were unambiguously identified by comparing with the authentic compounds. For unknown constituents, the structures were characterized based on their retention behav-

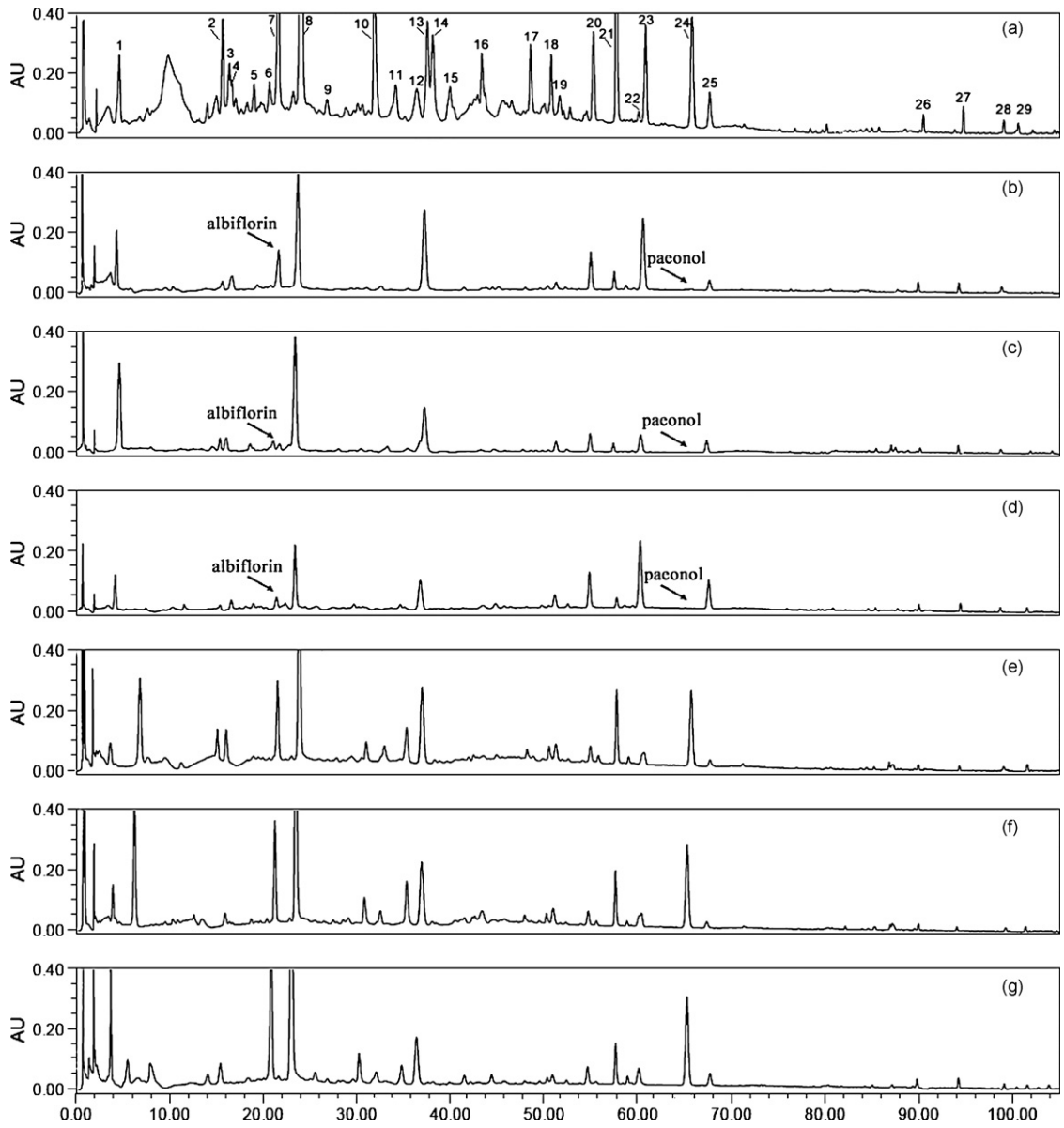


Fig. 5. HPLC–DAD chromatograms of six brands of GFW products. (a) GFW extract, (b) ZYSB, (c) JZT, (d) WRSJ, (e) TS, (f) HK and (g) KY.

ior and UV spectra and their MS spectra obtained on-line (Table 1 and Fig. 2).

Among these 27 compounds, there were nine monoterpene glycosides, three galloyl glucoses and four acetophenones from *Cortex Moutan* and *Radix Paeoniae*, three phenylallyl compounds from *Ramulus Cinnamomi*, four triterpenoids from *Poria Cocos* and four other types of compounds. It is notable that several identified compounds in GFW extract have been reported to have beneficial effects in blood stagnation and related ailments. Gallic acid, (+)-catechin and paeonol were reported to be anti-artherosclerosis [20–22]. Phenylallyl compounds, such as cinnamic acid and cinnamic aldehyde were found to have antioxidative activity [13,23]. Monoterpene glycosides, such as paeoniflorin, albiflorin and related derivatives possess antithrombotic activity [24]. Triterpenoids in *Poria cocos* can significantly inhibit tumor promotion and are anti-inflammatory agents [25,26]. These active components may just be the basic substances contributing to various biological effects of GFW. Consequently, the quality control of GFW commercial products should be focused on these compounds.

3.3.1. Identification of monoterpene glycosides

Cortex Moutan and *Radix Paeoniae* representing the species *Paeonia*, are rich sources of monoterpenes possessing a "cage-like" pinane skeleton. According to the MS/MS analysis of authentic compounds, the major fragmentation mechanisms of monoterpene glycosides were concluded. In the positive MS experiments, all of monoterpene glycosides were ionized as sodiated molecules. The diagnostic ions of this type of compounds were the loss of glucosyl group, aglycone group, benzoyl group, etc., and leading to the occurrence of ions at m/z 185, 219 or 121. Based on fragmentation patterns, the chemical structures of compounds **2**, **11**, **15**, **17**, **18** and **21** in the complex mixtures were identified, and some isomers were distinguished.

Structures of compound **2**, **11**and **15** were deduced from their characteristic UV and MS spectra and fragmentation patterns due to lack of reference compounds. The fragmentation patterns of compound **11** and **15** were similar with those of monoterpene glycosides. By referring to the literature data [27,28], compound **2**, **11** and **15** were tentatively identified as oxypaeoniflorin, galloylpaeoniflorin and mudanpioside H, respectively.

Table 2
Compounds in six different brands of GFW commercial products.

Peak no.	Compound	Samples of GFW products					
		concentrated pill			Honeyed pill		Capsule
		ZYSB	JZT	WRSJ	TS	HK	KY
1	Gallic acid	+	+	+	+	+	+
2	Oxypaeoniflorin	+	+	+	+	+	+
3	(+)-Catechin	+	+	+	+	+	+
4	Amygdalin	+	+	+	+	+	+
5	Apiopaeonoside	+	+	+	–	+	–
6	Paeonilide	+	+	–	–	+	–
7	Albiflorin	+	+	+	+	+	+
8	Paeoniflorin	+	+	+	+	+	+
9	Suffruticoside B Suffruticoside D	–	+	–	+	+	+
10	Unknown	–	–	–	+	+	+
11	Galloylpaeoniflorin	–	–	+	+	+	+
12	Tetragalloylglucopyranose	–	+	+	+	+	+
13	Pentagalloylglucopyranose	–	+	–	–	+	+
14	Benzoic acid	+	+	+	+	+	+
15	Mudanpioside H	–	–	–	–	–	+
16	Hexagalloylglucopyranose	–	+	–	–	–	–
17	Benzoyloxypaeoniflorin	+	+	+	+	+	+
18	Mudanpioside C	–	+	+	+	+	+
19	Unknown	–	+	+	+	+	+
20	Cinnamic acid	+	+	+	+	+	+
21	Benzoylalbiflorin	+	+	+	+	+	+
22	Benzoylpaeoniflorin	+	+	+	+	+	+
23	Cinnamic aldehyde	+	+	+	+	+	+
24	Paeonol	+	+	–	+	+	+
25	3-(2-Methoxyphenyl)-2-propenal	+	+	+	+	+	+
26	Polyporenic acid C	+	+	+	+	+	+
27	Pachymic acid	+	+	+	+	+	+
28	Dehydrotrametenolic acid	+	+	+	+	+	+
29	Trametenolic acid	+	+	+	+	+	+

+, means the product containing this compound; –, means the product not containing this compound.

Compound **17** and **18** were a pair of isomers. Both exhibited the same $[M+Na]^+$ ion at *m*/*z* 623. However, the characteristic presence of a [*p*-hydroxybenzoyl glucose + Na]$^+$ (323 Da) suggested the hydroxybenzoyl group connected with the glucosyl group, which could differentiate compound **18** from compound **17**. Thus, compound **17** and **18** were plausibly identified as benzoyloxypaeoniflorin and mudanpioside C, respectively.

Compound **21** has the same molecular weight as benzoylpaeoniflorin. In the ESI-MS/MS experiment there was an extra ion at *m*/*z* 589 that was not found in benzoylpaeoniflorin, which may correspond to the loss of a molecule of water from the aglycone. Hence compound **21** was tentatively identified as benzoylalbiflorin.

3.3.2. Identification of galloyl glucoses

In this work all the galloyl glucoses observed in GFW belong to *Cortex Moutan*. Due to the lack of reference compounds, this type of constituents was tentatively identified according to their HPLC–MS/MS analysis. All of them contain a glucose moiety and gallic acid moieties. The MS/MS spectra of the $[M-H]^-$ ions generally yielded a series of product ions originating from the successive losses of gallic acid and a galloyl group.

Compound **12** showed the quasi-molecular ion at *m*/*z* 787 and the MS/MS spectrum of afforded ions at *m*/*z* 617, 447, and 277, originating from the successive loss of three gallic acids from the $[M-H]^-$ ion. By examining the known compounds in *Cortex Moutan*, compound **12** was tentatively identified as tetragalloylglucopyranose isomer. Compounds **13** and **16** exhibited the $[M-H]^-$ ion at *m*/*z* 939 and *m*/*z* 1091 in the ESI mass spectra and they showed similar fragmentations with tetragalloylglucopyranose. The product ions were resulted from successive neutral losses of four gallic acids and galloyl radicals. Based on the above observations, compounds **13** and **16** was characterized as pentagalloylglucopyranose and hexagalloylglucopyranose.

3.3.3. Identification of acetophenones

A number of acetophenones have been reported from *Cortex Moutan* previously [27]. In the positive MS experiments, all of acetophenones were ionized as sodiated molecules. The MS/MS spectra of the $[M+Na]^+$ ions displayed ions arising from the readily eliminated sugar moieties.

Compounds **5** and **6** displayed the same $[M+Na]^+$ ions at *m*/*z* 483. They both gave the product ion at *m*/*z* 317, resulting from the loss of paeonol unit (166 Da). But the MS/MS spectrum of compound **6** afforded a significant $[M+Na-166-60]^+$ ion at *m*/*z* 257, originating from $^{1,3}X$ cleavage of the arabinose ring of the disaccharide ion. The presence of a product ion at *m*/*z* 257 allowed the differentiation between these two isomers. Based on the above observations, compounds **5** and **6** were identified as apiopaeonoside and paeonilide, respectively. Compounds **9** exhibited $[M+Na]^+$ ions at *m*/*z* 635 in the ESI mass spectra. The fragmentation pattern of this compound was the same as acetophenones contained in *Cortex Moutan*. After comparison with the literature data [27], it was identified as suffruticoside B or D.Identification of phenylallyl compoundsPhenylallyl compounds have been previously reported as the main components of *Ramulus Cinnamomi*, owing to its pharmaceutical effects as well as their high content in the herb. This type of compounds has small molecular mass and displayed deprotonated molecular ions in the MS/MS experiment. The molecular mass of compound **25** was by 30 Da greater than that of cinnamic aldehyde, consistent with the additional substitution of a methoxyl group. The MS/MS spectrum of the deprotonated molecular ion was very similar to that of cinnamic aldehyde. The product ion at *m*/*z* 147 indicated the methoxyl group was at C_2 of benzene. Thus this

compound was tentatively identified as 3-(2-methoxyphenyl)-2-propenal.

3.3.5. Application to analysis of samples of GFW commercial products

The results obtained from the six brands GFW commercial products are presented in Fig. 5 and Table 2. Most constituents of GFW extract were comprised in the six preparations, while the contents of compounds were various among three dosage forms. Honeyed pill is the original dosage form of GFW dating back to Zhang Zhongjing's works. Besides gallic acid, (+)-catechin and benzoic acid, the monoterpene glycosides, the acetophenones, the triterpenoids and phenylallyl compounds were also found in the two samples of the honeyed pill, suggesting that the manufacture process of this dosage form could retain the major constituents of the five crude drugs. In the case of concentrated pill, the amounts of constituents from *Radix Paeoniae* and *Cortex Moutan*, especially the most abundant and effective component albiflorin and paeonol were quite low, probably as a result of the discrepancy in raw materials used and during the herb extraction process. The capsule is a modern improved dosage form of this traditional formula. The main components of this preparation could be well preserved during the manufacturing course. Phenylallyl compounds as the considered bioactive constituents of *Ramulus Cinnamomi* were detected at a low amount in three dosage forms of GFW products. The reason of the loss was assumed that the essential oil in *Ramulus Cinnamomi* could be easily volatilized during the manufacturing process. By comparing the daily ingested amount of the three dosage forms of GFW products, the honeyed pill manufacturers used a greater amount of excipients in their GFW preparation than the other two manufacturers, resulting in a bigger size of the products for daily administration. Owing to the smaller daily ingested amount and maximal remaining of the major compounds, the capsule dosage form of GFW takes advantage among the three dosage forms.

4. Conclusions

In the present study, a reliable and simple analytical method by using a HPLC–DAD–MS/MS for rapid identifying multiple components in the extract of GFW was established. As a result, 27 components including monoterpene glycosides, acetophenones, galloyl glucoses, even some isomers in the complex system were separated and characterized by HPLC–DAD–MS/MS technique. According to the literature, most of the identified compounds in GFW possess pharmacological activities related to the clinical application of this formula. The real bioactive components in GFW and their remedial mechanism could be well understood based on the elucidation of these compounds. Meanwhile, the application of the method to the commercial products of GFW also provided the chemical support for the chromatographic fingerprint technology and facilitates to improve the quality control standard of this age-old TCM formula. It indicated that the manufacture of GFW prescription should focus on their production process in order to keep the lipid-soluble and water-soluble constituents totally in this formula to ensure the therapeutic effects.

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