Phytochemical Analysis of Anti-atherogenic Constituents of Xue-Fu-Zhu-Yu-Tang Using HPLC-DAD-ESI-MS

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Xue-Fu-Zhu-Yu-Tang (血府逐淤汤) is a famous traditional Chinese medicine (TCM) formula for treating cardiovascular disease and related ailments in China for centuries. To profile the phytochemical constituents of the formula, an HPLC-DAD-ESI-MS analytical method has been developed to separate and determinate the medium- or non-polar fraction of the decoction, which has been demonstrated potency to lower the serum total triglyceride concentration, strongly decrease the TXA_2/PGI_2 ratio and attenuate production of proinflammatory cytokines in high cholesterol-fed rats. By comparing their retention time, UV and MS data with those obtained from the authentic compounds, ferulic acid (1), naringin (2), neohesperidin (3), naringenin (8), marmin (13), senkyunolide A (14), dehydrosafynol (16), safynol (17) and *Z*-ligustlide (18) are unequivocally determined. Moreover, additional thirteen compounds are tentatively identified as senkyunolide I (4), senkyunolide H (5), poncirin (7), benzoylpaeoniflorin (10), (*Z*)-6,7-epoxyligustilide (11), senkyunolide G (12), 2-methoxy-safynol (15), cnidilide (19), tangeritin (20), saikosaponin b_2 (21), 2′-*O*-acetylsaikosaponin b_2 (22), saikosaponin b_1 (23) and auraptene (24), according to the comparison of their UV and MS data with the published data. The present study provides an approach to rapidly characterize bioactive constituents in TCM formulae.

Key words HPLC-DAD-MS; traditional Chinese medicine (TCM); atherosclerosis; herbal formula

In China, traditional Chinese medicines (TCMs) have already been used for centuries in the treatment of a wide variety of ailments successfully and have attracted more and more attention from industry and academia.[1—3] However, their remedial mechanisms are still not well understood. So far, it is widely accepted that multiple ingredients are responsible for therapeutic effects of TCMs. Thus, it is necessary to clarify and analyze the bioactive constituents in TCMs to ensure the reliability in clinical application, to better understand the pharmacological basis and moreover, to enhance products quality control.

However, research on TCMs presents significant challenges, especially for herbal formulae, due to the complexity of chemical composition. In recent years, the advent of HPLC coupled mass spectrometer has had tremendous impact upon the profile analysis of natural products and drug discovery.[4—7] HPLC-DAD-ESI-MS has been successfully applied to the determination of compounds present in material from a variety of natural product sources.[8—11] A significant number of reports have been published on Chinese medicinal herbs using this hyphenated technique,[12] although only few on TCM formulae.

Xue-Fu-Zhu-Yu-Tang is a famous Chinese TCM formula for treating cardiovascular disease with a history of several centuries. The formula mainly consists of six plant materials: *Paeonia lactiflora*, *Ligusticum chuanxiong*, *Citrus aurantium*, *Carthamus tinctorius*, *Prunus persicae* and *Bupleurum falcatum*. Satisfactory therapeutic effect has been achieved especially for atherosclerosis and hyperlipidemia.[13] But the bioactive principles of this formula are still not clarified as yet.

In this paper, we describe a study on phytochemical constituents of bioactive fraction of Xue-Fu-Zhu-Yu-Tang. Considering the criteria for drug-like compounds,[14] especially hydrophobic points and molecular weight range, we suppose that medium- or non-polar constituents of the formula might have significant bioactivity. It was demonstrated using a high cholesterol-fed animal model. And then, using an HPLC-DAD-ESI-MS method, 22 major constituents were identified in the fraction, based on the comparison of their UV and MS data with those of authentic compounds and published data. These results may help to gained better understanding of therapeutic basis of Xue-Fu-Zhu-Yu-Tang and establish a better quality control method for the old-age TCM formula.

Experimental

Chemicals and Reagents HPLC grade methanol (E. Merck, Darmstadt, Germany) was used for HPLC analysis. Reagent grade ethanol (Hangzhou Reagent Company, Hangzhou, China) was used for extraction and separation. The D101 macro-porous resin (Tianjing Resin Factory, Tianjing, China) was used for the chromatographic separation of the formulation and plant materials.

Materials and Sample Preparation The plant materials: *Paeonia lactiflora*, *Ligusticum chuanxiong*, *Citrus aurantium*, *Carthamus tinctorius*, *Prunus persicae* and *Bupleurum falcatum*, were provided and identified by China Academy of Traditional Chinese Medicine (Beijing, China). The six plant materials (the total weight of 20 kg) were mixed according to traditional formula, and then decocted for 3 times (for 2 h each time). After filtration and concentration, aqueous extract (AE) was evaporated to dryness. The yield of the preparation was 16.5%. 1 kg dried extract was re-dissolved in water and separated on D101 porous resin columns. A step gradient of (1) H_2O, (2) 90% H_2O, 10% EtOH, (3) 70% H_2O, 30% EtOH, (4) 50% H_2O, 50% EtOH, (5) 30% H_2O, 70% EtOH, (6) 5% H_2O, 95% EtOH was applied as mobile phase. The last fraction was collected as the medium-polar and non-polar fraction (MNPF). The yield of MNPF was about 1% of the aqueous extract. The corresponding extracts of individual plant material (each weight of 100 g) were also prepared with the same procedure.

HPLC-DAD-ESI-MS Condition The analysis was performed using an Agilent 1100 series HPLC-DAD-MS system (Aglient Technologies, Willington, U.S.A.). A Zorbax StableBond C18 column (4.6 mm×250 mm, 5 μm, Aglient) with an C18 guard column (Hanbon Science & Technology Co., Ltd, Jiangsu, China) was utilized. Samples including MNPF from formula and individual plant material were dissolved in MeOH as 5 mg/ml solutions and centrifuged at 12000 rpm for 15 min to remove particles before injection. The mobile phase consisted of (A) 0.8% aqueous acetic acid and (B) methanol using a linear gradient (from 40% B to 90% B in 100 min). The flow rate was 0.5 ml/min. The temperature of column was 30°C. The UV spectrum was recorded from 190 to 400 nm. Two separate injections for each sample were performed in positive ion mode and negative ion mode respectively with electrospary ionization (ESI). The full scan mass spectrum

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Table 1. Hypolipidemia Effect of Aqueous Extract and MNPF in High Cholesterol-Fed Rats

Group	TC	TG	HDL-C	LDL-C	TC/HDL-C
Control	1.57±0.23**	0.41±0.14	1.02±0.19	0.48±0.25**	1.60±0.38**
Hyper	5.40±2.64	0.50±0.11	1.21±0.41	4.08±2.44	4.31±1.63
AE	3.00±1.80	0.34±0.14*	1.08±0.37	1.73±1.64*	2.45±1.25*
MNPF	3.71±2.17	0.31±0.08**	1.24±0.43	2.40±1.85	2.54±0.84*

Values are expressed as mean±S.D. for ten animals in each group. The values are expressed as mmol/l serum. * Different *vs.* hyper group ($p<0.05$); ** different *vs.* hyper group ($p<0.01$).

Table 2. Anti-coagulation Effect and Inhibition of Inflammatory Cytokines Release of Aqueous Extract and MNPF in High Cholesterol-Fed Rats

Group	6-keto-PGF_{1a}	TXB_2	TXB2/6-keto-PGF_{1a}	IL-6	IL-8
Control	626.45±166.20**	445.78±131.24	0.83±0.39**	0.102±0.059*	0.245±0.075
Hyper	365.94±80.95	589.42±172.01	1.68±0.55	0.183±0.052	0.289±0.044
AE	464.86±187.78	487.11±109.74	1.13±0.50*	0.177±0.103	0.243±0.012
MNPF	536.48±193.01*	442.45±200.96	0.79±0.20**	0.141±0.056	0.130±0.066**

Values are expressed as mean±S.D. for ten animals in each group. Lever of TXB_2 and 6-keto-PGF_{1a} are expressed as pg/ml serum. Lever of IL-8 and IL-6 are expressed as ng/ml serum. * Different *vs.* hyper group ($p<0.05$); ** different *vs.* hyper group ($p<0.01$).

was recorded over the range of *m/z* 100—1200. Temperature of drying gas was 350°C with gas flow rate of 10.0 l/min and a nebulising pressure of 45 psi. The value of fragmentor voltage was set at 120.

Reference Compounds Ferulic acid (**1**), naringin (**2**), neohesperidin (**3**), naringenin (**8**) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Senkyunolide A (**14**) and *Z*-ligustilide (**18**) were isolated from *Ligusticum chuanxiong* according to Kobayashi's description.[15] Marmin (**13**) was isolated from *Citrus aurantium* by the following procedures. The plant material (100 g) was refluxed with 70% EtOH (500 ml) for 3 times. The free solvent extract was subjected to preparative TLC and semi-preparative RP-HPLC for separation and purification. The developing solvents for preparative TLC were $CHCl_3$–MeOH (10 : 1, v/v). The mobile phase for semi-preparative HPLC was 60% methanol in water. Dehydrosafynol (**16**) and safynol (**17**) were isolated from *Carthamus tinctorius* according to Redl's discription.[16] The purity of each isolated compound was determined by HPLC analysis. And their identities were confirmed by the comparison of their ^{1}H-NMR spectrum with the published data.[15—23]

Animials and Treatment Male Wistar rats ($n=40$) weighing 160—190 g, obtained from the Chinese Academy of Sciences (Beijing, China) were randomly divided into two groups and housed in cages random, with water and food freely available. The first group (control group, $n=10$) received a control diet and another group (hyperlipidemia model group, $n=30$) was fed on a diet containing 4% cholesterol, 10% lard, 0.2% methylthiouracil and 0.8% cholic acid throughout the overall experimental period (21 d). After 7 d, the first hyperlipidemia group rats (AE group, $n=10$) were given AE orally (8 g/kg per day), the second hyperlipidemia group rats (MNPF group, $n=10$) were given MNPF orally (0.2 g/kg per day), the third hyperlipidemia group rats (hyper group, $n=10$) were given physiological saline, for 14 d.

Measurement of Biochemical Parameters At the end of the experimental trial the rats were fasted for 12 h. The blood samples were withdrawn from the abdominal aorta and then transferred directly into centrifuge tubes and allowed to clot at room temperature and centrifuged for 15 min at 3000 rpm. The supernatant clear serum concentration of total cholesterol (TC) was measured by CEH-COD-POD assay kit (Ningbo Asia-pacific Biotehnology Ltd., Ningbo, China), triglyceride (TG) was measured by GK-GPO-POD assay kit (Ningbo Asia-pacific Biotehnology Ltd., Ningbo, China). HDL-C was measured by PEG-modified enzyme HDL-C assay and LDL-C was measured by solubilization LDL-C assay (Roche Diagnostics, Switzerland). TXB_2 and 6-keto-PGF_{1a} were measured by EIA (Cayman Chemical Company, Ann Arbor, U.S.A.). IL-8 and IL-6 were measured by ELISA (Pierce Biotechnology Inc., Rockford, U.S.A.).

Statistical Analyses The results are expressed as mean±S.D. and they were evaluated statistically using the one-way analysis of variance (ANOVA) followed by the Dunnett *t*-test. In all cases, $p<0.05$ was regarded as significance and $p<0.01$ was regarded as very significance.

Results and Discussion

Bioassays for Anti-atherogenic Fraction A commonly used high cholesterol-fed rat model[24] was applied in the investigation. Three sectors of biochemical parameters were selected to evaluate anti-atherogenic effect of samples: serum lipid lever,[25] blood coagulation potential[26] and proinflammatory cytokine concentration,[27] which were well-known factors in pathogenesis of atherosclerosis. The results presented in Tables 1 and 2 indicated that AE of the formula significantly lowered triglyceride concentration and decreased TXA_2/PGI_2 ratio (measured by assays for TXB_2 and 6-keto-PGF_{1a}). And MNPF of the formula exhibited comparable effect on serum lipid lever. Furthermore, it showed stronger potency to increase the PGI_2 secretion and significantly inhibitory effect on IL-8 production. Considering that the treatment dose in MNPF group was 2.5% of that of AE group, we suggested that MNPF was the anti-atherogenic principle of the formula.

HPLC-DAD-ESI-MS Analysis of MNPF An HPLC-DAD-ESI-MS method was developed to determine the phytochemical constituents of the MNPF. As shown in Fig. 1A (overlapping chromatogram of 254, 280 and 320 nm), 24 major peaks were separated under the HPLC condition with UV detection. The chromatography of MS total ion current (TIC) in positive mode and negative mode was shown in Fig. 1B and Fig. 1C separately. The retention time, *m/z* value and UV maximum adsorption wavelength of each peak was summarized in Table 3.

Phenolic and Flavonoid Compounds Ferulic acid, naringin, neohesperidin and naringenin were unequivocally determined by comparison with authentic compounds. Peak 7 exhibited UV spectrum with maximum absorption at 226, 284 and 326 nm, indicating that the compound was a saturated flavanone (Fig. 2A). According to additional information about its plant origin and MS data, the peak was assigned as poncirin.[28] Naringin, neohesperidin and poncirin exhibited not only intense quasi-molecular ions $[M+H]^+$ and sodiated adduct ions $[M+Na]^+$, but also significant aglycone molecular ion (Figs. 3A—C) in positive mode. Peak 20 exhibited intense $[M+H]^+$ with adduct ion $[M+Na]^+$ and dimmer ion $[2M+Na]^+$ in positive mode, but weak response in

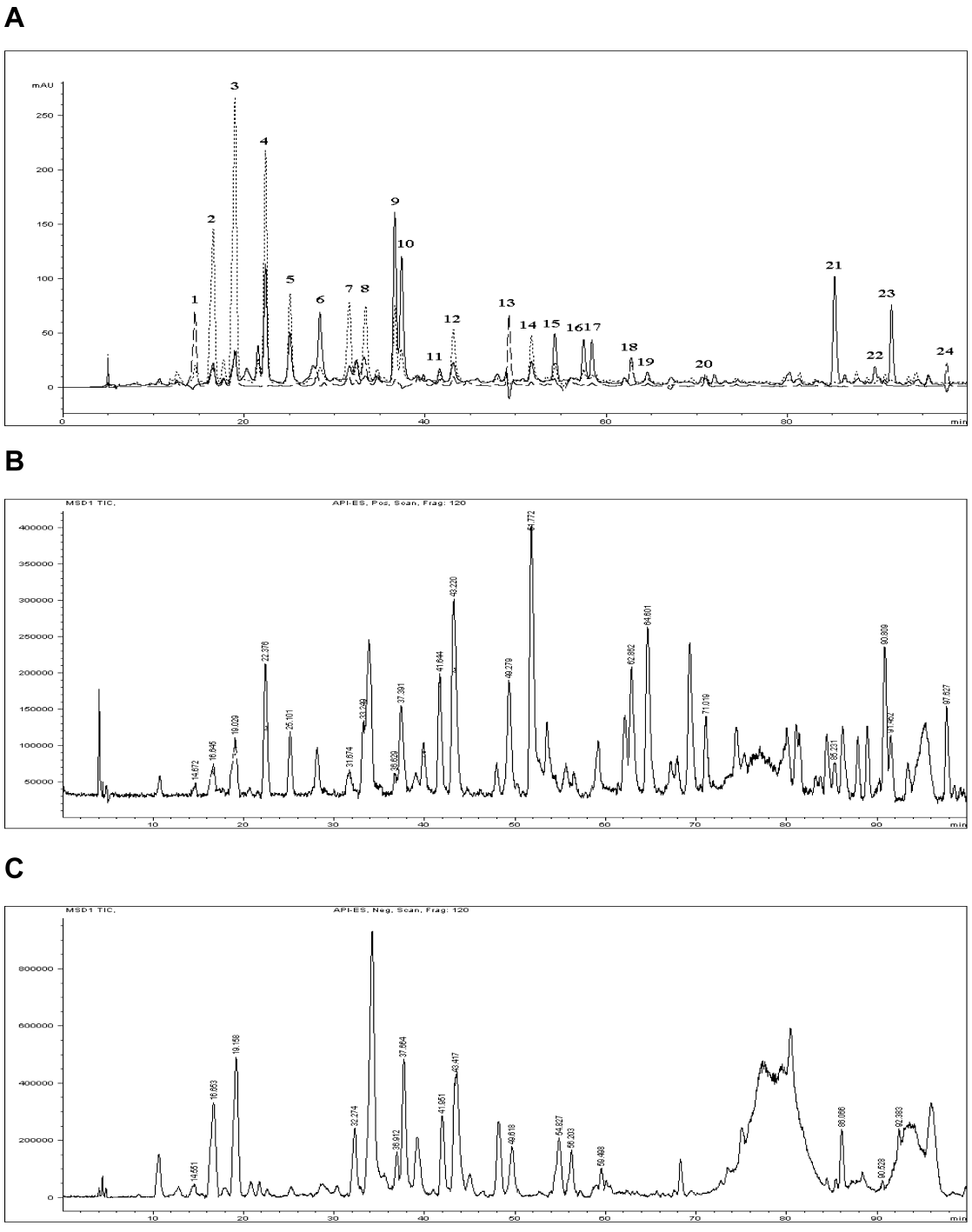

Fig. 1. Chromatogram of MNPF of Xue-Fu-Zhu-Yu-Tang

(A) Overlapping chromatogram of 254 nm (solid line), 280 nm (dot line) and 320 nm (dash line) of MNPF. **1**: ferulic acid, **2**: naringin, **3**: neohesperindin, **4** and **5**: senkyunolide I or senkyunolide H, **6**: unknow, **7**: poncirin, **8**: naringenin, **9**: unknow, **10**: benzoylpaeoniflorin, **11**: (*Z*)-6,7-epoxyligustilide, **12**: senkyunolide G, **13**: marmin, **14**: senkyunolide A, **15**: 2-methoxy-safynol, **16**: dehydrosafynol, **17**: safynol, **18**: *Z*-ligustlide, **19**: cnidilide, **20**: tangeritin, **21** and **23**: saikosaponin b_1 or saikosaponin b_2, **22**: 2′-*O*-acetylsaikosaponin b_2, **24**: auraptene. (B) TIC chromatogram in positive mode of MNPF (retention time of the identified peak was indicated). (C) TIC chromatogram in negative mode of MNPF (retention time of the identified peak was indicated).

negative mode. It was tentatively inferred that the peak was tangeritin based on its molecular mass and UV spectrum, which agreed with the published data.[9)]

Phthalides Seven phthalides found in MNPF of Xue-Fu-Zhu-Yu-Tang were assigned to *Ligusticum chuanxiong*. Except senkyunolide A and *Z*-ligustlide, the other five peaks were tentatively determined based on comparison their UV and MS data with published data.[15,17,29—31)] Peak 4 and peak 5 exhibited a dehydrating ion $[M+H-H_2O]^-$ indicated the structure dihydroxylated at C-6 and C-7 (Fig. 3D). UV and MS data of the two peaks were in good agreement with the published data for senkyunolide I and senkyunolide H.[10)] Since the UV and MS data could not provide stereochemistry information, the two compounds were not differentiated. (*Z*)-6,7-Epoxyligustilide and senkyunolide G showed protonated molecular ion $[M+H]^+$, sodiated monomer $[M+Na]^+$ and

Table 3. HPLC-DAD-ESI-MS Identification

Peak	t_R [a] (min)	$[M+H]^+$	Other ions (ESI^+)	$[M-1]^-$	Other ions (ESI^-)	λ_{max} (nm)	Plant material	Identify
1	14.53	197	217	193		236, 323	LC	Ferulic acid[b]
2	16.53	581	603	579	615	283, 328	CA	Naringin[b]
3	18.95	611	633	609	645	284, 328	CA	Neohesperindin[b]
4	22.31	225	207, 247, 471	223		275	LC	Senkyunolide I[c]
5	25.02	225	207, 247, 471	223	259, 283	276	LC	Senkyunolide H[c]
6	28.33	317		315		252, 372	N	Unknown
7	31.57	595	617	593	629, 653	226, 284, 326	CA	Poncirin
8	33.37	273		271		286, 328	CA	Naringenin[b]
9	36.60	331	353, 683	329		250	PL	Unknown
10	37.36	585	607	583	643, 1167	234	PL	Benzoylpaeoniflorin
11	41.93	207	229, 435	205		276	LC	(*Z*)-6,7-Epoxyligustilide
12	43.07	209	231, 439	207		284	LC	Senkyunolide G
13	49.21	333	315, 355		367, 391	324	CA	Marmin[b]
14	51.69	193	215, 407			281	LC	Senkyunolide A[b]
15	54.29		237		249, 273	258, 268, 324, 348	CT	2-Methoxy-safynol
16	57.46		181, 221	197	233, 257	240, 256, 270, 304	CT	Dehydrosafynol[b]
17	58.38		223	199	235, 259	246, 254, 268, 308	CT	Safynol[b]
18	62.72	191	213, 403			280, 328	CT	*Z*-Ligustlide[b]
19	64.52	195	217, 411			234, 315	LC	Cnidilide
20	71.92	373	395, 767			272, 323	CA	Tangeritin
21	85.19		763, 803	779	815	244, 252, 262	BF	Saikosaponin b_2[c]
22	89.64		845	821		244, 252, 262	BF	2′-*O*-Acetylsaikosaponin b_2
23	91.47		763, 803	779		244, 250, 260	BF	Saikosaponin b_1[c]
24	97.57	299	321, 619	297		325	CA	Auraptene

a) Retention time of peaks in Fig. 1A. *b*) Unequivocally determined by comparison with authentic compounds. *c*) Not able to differentiate absolute configuration. The abbreviation for plant material: PL *vs. Paeonia lactiflora*; LC *vs. Ligusticum chuanxiong*; CT *vs. Carthamus tinctorius*; CA *vs. Citrus aurantium*; SP *vs. Prunus persicae*; BF *vs. Bupleurum falcatum;* N means not found in any plant material.

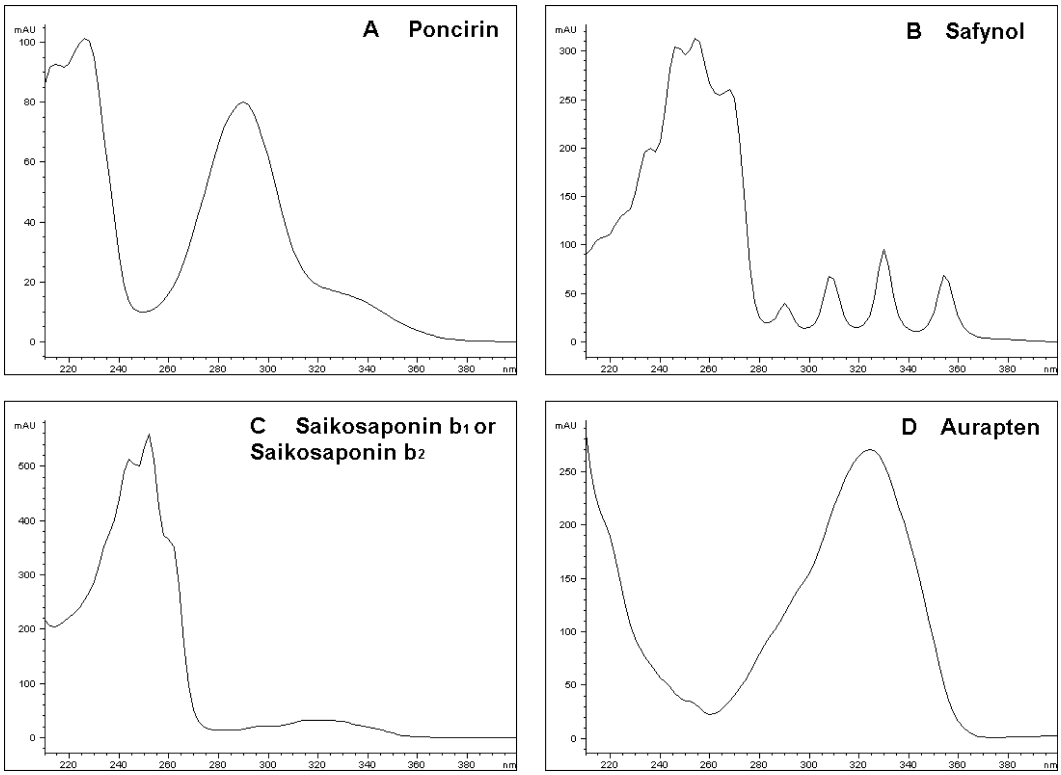

Fig. 2. Typical UV Spectrum of Saturated Flavanone, Polyacetylene, Saponin and Coumarins

dimer ions $[2M+Na]^+$ in positive mode, and intense quasi-molecular ions $[M-H]^-$ (in negative mode. But for senkyunolide A, *Z*-ligustlide and cnidilide, the responses in positive mode were much stronger than those in negative mode.

Monoterpene Glycoside One monoterpene was found in MNPF of the formula, namely peak 10, which was from *Paeonia lactiflora*. The peak exhibited very intense quasi-molecular ions $[M-H]^-$, adduct ion $[M+AcO]^-$ and dimmer ion $[2M-H]^-$ in negative mode. The molecular mass was inferred to be 584, which was confirmed by MS data ob-

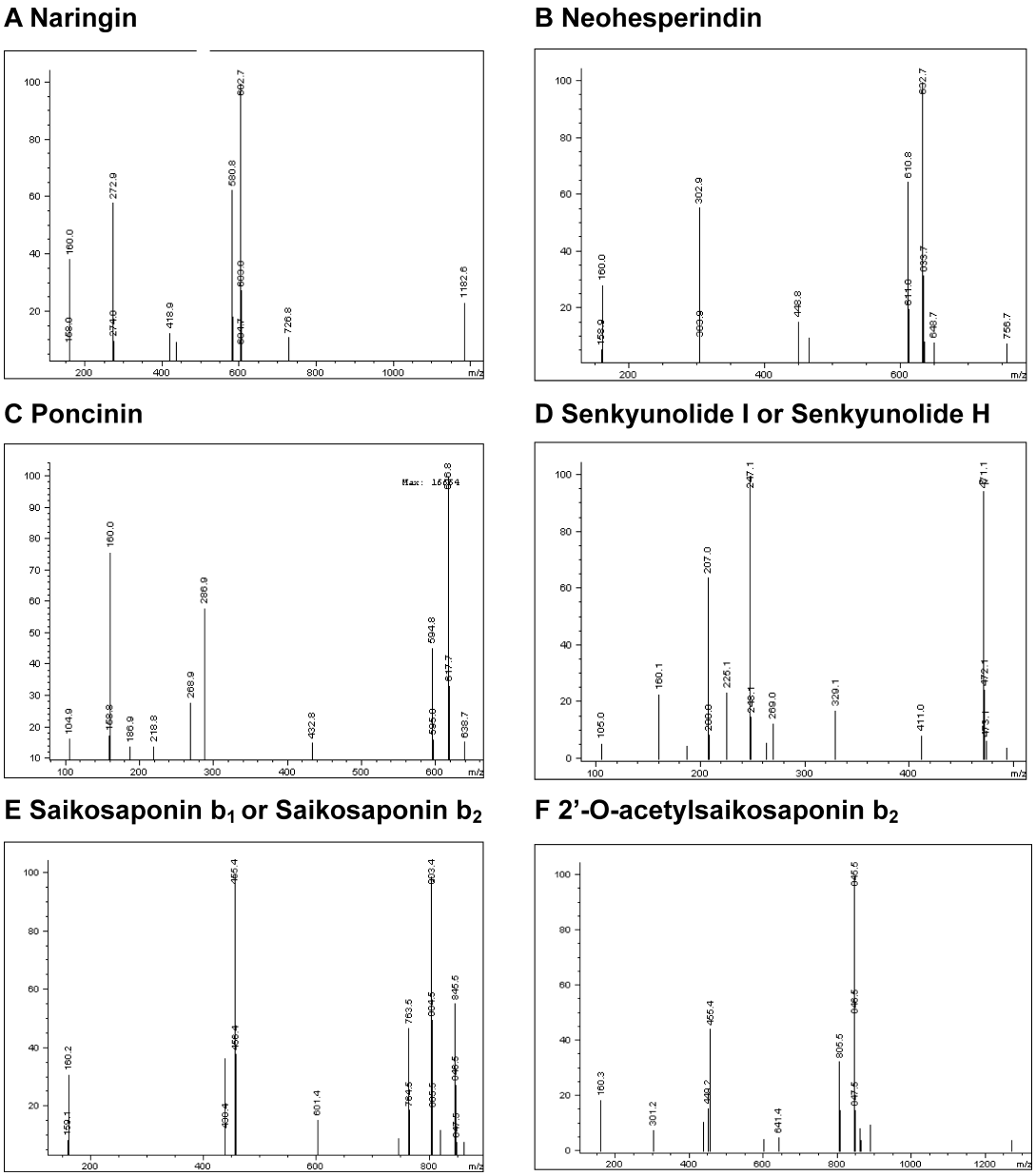

Fig. 3. MS Spectrum of Several Identified Compounds in Positive Mode

tained in positive mode. The typical UV spectrum with maximum absorption at 236 nm corresponded to the structure of benzoylpaeoniflorin.[32)]

Polyacetylenes Typical characters of polyacetylene UV spectrum were observed in peak 15, 16 and 17 (Fig. 2B). According to comparison with individual plant material, the three peaks were assigned to *Carthamus tinctorius*. Peak 16 and 17 were unequivocally determined to be dehydrosafynol and safynol respectively by comparison with authentic compounds. From the presence of $[M+AcO]^-$ in negative mode and $[M+Na]^+$ in positive mode, the molecular mass of peak 15 was inferred to be 214. The UV and MS data corresponded to 2-methoxylsafynol, which was found in *Centaurea ruthenica* previously.[33)] The polyacetylenes gave significant adduct ion $[M+AcO]^-$ and weak quasi-molecular ions $[M-H]^-$ in negative mode, whereas the responses in positive mode were very weak.

Saponins According to comparison of retention time, UV and MS data with those of individual plant material, peak 21, 22 and 23 were assigned to *Bupleurum falcatum*. The UV spectrum of the three peaks with absorption maximum at 244, 252 (250) and 262 (260) nm (Fig. 2C), indicated the existence of conjugated heteroannular diene. Peak 21 and 23 exhibited intense $[M+Na]^+$ and significant $[M+H-H_2O]^+$ together with fragments of $[M+H-H_2O-Glc]^+$ at *m*/*z* of 601 and $[M+H-H_2O-Glc-Fuc]^+$ at *m*/*z* of 455 in positive mode (Fig. 3E). These data were in agreement with those of saikosaponin b_1 and saikosaponin b_2, which had been found in *Bupleurum falcatum*.[34)] But we could not differentiate the two compounds due to insufficient stereochemistry information provided. Peak 22 exhibited $[M+Na]^+$ at *m*/*z* of 845, $[M+H-H_2O]^+$ at *m*/*z* of 805, and a dehydrating aglycone ions at *m*/*z* of 455 (Fig. 3F), which corresponded to the structure of 2′-*O*-acetylsaikosaponin b_2.[35)]

Coumarins Peak 13 and 24 were assigned to *Citrus aurantium*. The two peaks exhibited very similar UV spectrum

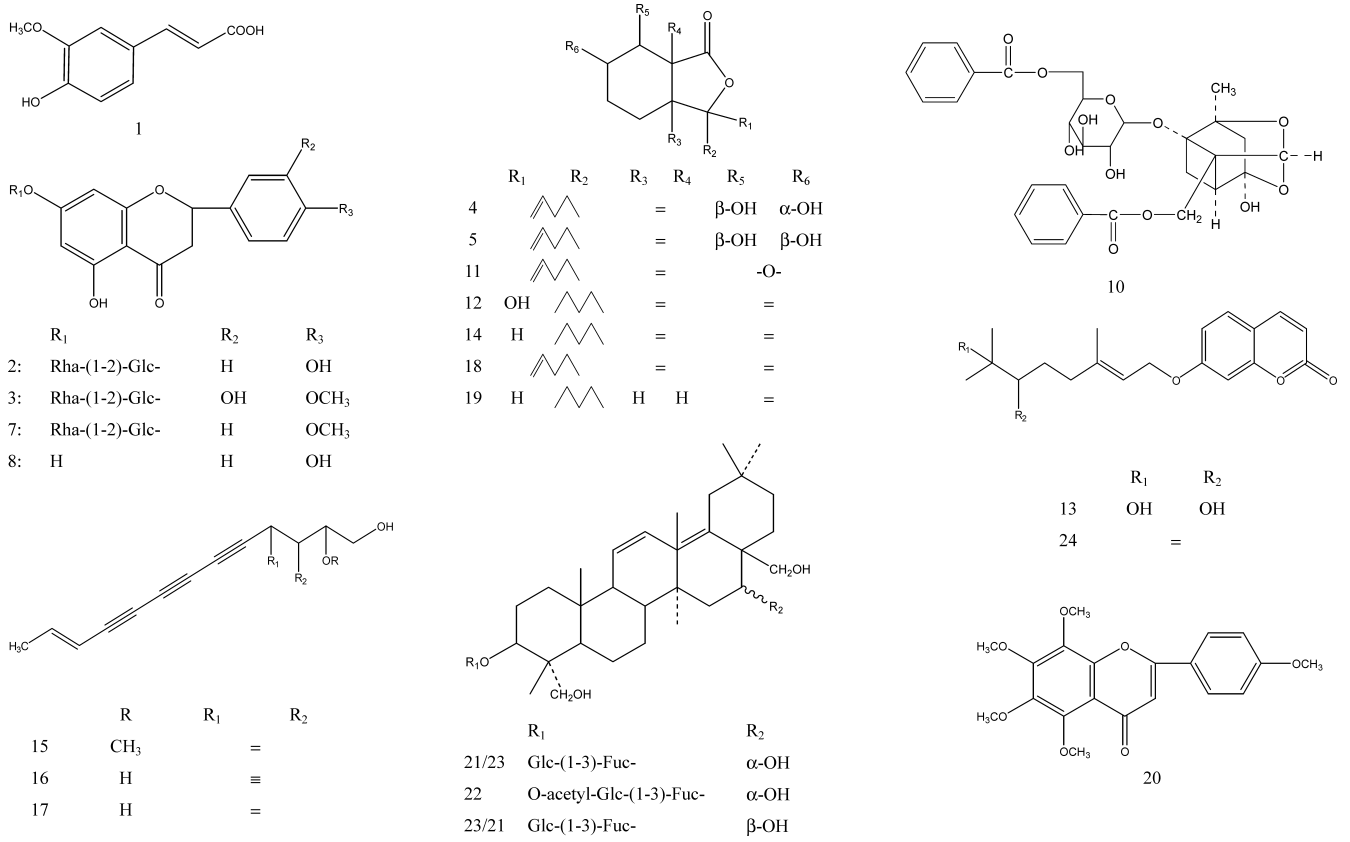

Fig. 4. Structure of Compounds Identified

Ferulic acid (**1**), naringin (**2**), neohesperidin (**3**), senkyunolide I (**4**), senkyunolide H (**5**), Poncirin (**7**), naringenin (**8**), mudanoside (**9**), benzoylpaeoniflorin (**10**), (*Z*)-6,7-epoxyligustilide (**11**), senkyunolide G (**12**), marmin (**13**), senkyunolide A (**14**), 2-methoxy-safynol (**15**), dehydrosafynol (**16**), safynol (**17**), *Z*-ligustlide (**18**), cnidilide (**19**), tangeritin (**20**), saikosaponin b_2 (**21**), 2′-*O*-acetylsaikosaponin b_2 (**22**), saikosaponin b_1 (**23**), auraptene (**24**).

with absorption maximum at 324 nm (Fig. 2D), suggested the presence of coumarin moiety. Peak 13 was unequivocally determined as marmin by comparing with authentic compounds. Peak 24 showed significant response in both positive mode and negative mode. The molecular mass was inferred to be 298. We tentatively assagened it as aurapten, considering that its UV and MS data was in agreement with published literature.[36)]

As a result, 22 compounds showed in Fig. 4 were determined and assigned to plant materials: the flavonoids and coumarins were mainly from *Citrus aurantium*, phthalides were mainly from *Ligusticum chuanxiong*, polyacetylenes were from *Carthamus tinctorius*, saponins were from *Bupleurum falcatum*. Moreover, a monoterpene glycoside was assigned to *Paeonia lactiflora*. The present HPLC-DAD-ESI-MS analytical method was successfully used to separate and determinate various types of compounds in the complex mixture. Moreover, it is notable that several identified compounds in this fraction have been reported beneficial effect in atherosclerosis and related ailments. Ferulic acid,[37,38)] auraptene,[39)] saikosaponins[40)] were reported to be anti-inflammatory. Flavonoids, such as naringenin, hersperetin and related derivatives were found hypolipidemia[41,42)] and phytoestrogen[43)] activity. Several phthalides were reported inhibitory effect on proliferation of aorta smooth muscle cells.[44)] Taken together, we suggested that multiple bioactive components were responsible for the therapeutical effect of Xue-Fu-Zhu-Yu-Tang. Our finding may help to clarify the remedial mechanisms and develop a reliable quality control method for the age-old TCM formula. Moreover, we also showed that collective application of bioassays and HPLC-DAD-ESI-MS analysis was an approach to rapidly characterize bioactive constituents in TCM formulae.

Acknowledgements This study was supported by Chinese National Basic Research Priorities Program-973 (No. G1999054405) and Key Research Program of National Science Foundation of China (No. 90209005). The author would like to thank Beijing Xiyuan Hospital for the animal experiments and Mr. Yecheng Xiao for ^{1}H-NMR analysis and his valuable advise.

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