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Screening of immunomodulatory components in *Yu-ping-feng-san* using splenocyte binding and HPLC

Min Hong[a,b,c], Xin-zhi Wang[c], Liang Wang[b], Yong-qing Hua[a,b,c], Hong-mei Wen[c,*], Jin-ao Duan[a]

[a] *Jiangsu Key Laboratory for Traditional Chinese Medical Formulae Research, Nanjing University of Chinese Medicine, Nanjing 210046, China*
[b] *National Standard Laboratory of Pharmacology for Chinese Materia Medica, Nanjing University of Chinese Medicine, Nanjing 210029, China*
[c] *School of Pharmacy, Nanjing University of Chinese Medicine, 282 Hanzhong Road, Nanjing 210029, China*

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ABSTRACT

Yu-ping-feng-san (YPFS) is a widely used immunomodulatory herbal medication used in traditional Chinese medicine, but the active molecules remain obscure. To screen for bioactive components we combined splenocyte binding with high performance liquid chromatography (SB-HPLC). After enrichment by splenocyte binding, two YPFS components (C1 and C2) were analyzed by HPLC. Compound C2 was identified as linoleic acid (LA) based on UV absorption and mass spectrometry. Silica gel chromatography was used to purify compound C1 from *Radix Saposhnikoviae*, a major constituent of YPFS. This allowed identification of the molecule as panaxynol (PAN) based on EI-MS and NMR spectrometry. Bioassay *in vitro* demonstrated that PAN significantly inhibited splenocyte proliferation induced by concanavalin A (ConA) in a concentration-dependent manner, whereas LA had no significant effect on splenocyte proliferation. *In vivo*, PAN was found to attenuate allergic contact dermatitis in a mouse model of delayed-type hypersensitivity (DTH), a pharmacological activity not previously reported for this molecule. It is suggested that PAN contributes to the anti-DTH effects of YPFS. SB-HPLC provides a rapid and efficient method for the identification of potential immunomodulatory components in traditional Chinese medicines.

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1. Introduction

Yu-ping-feng-san (YPFS) is a herbal medication composed of *Radix Astragali* (RA, Huangqi), *Rhizoma Atractylodis Macrocephalae* (RAM, Baizhu) and *Radix Saposhnikoviae* (RS, Fangfeng). YPFS is widely used in the treatment of spontaneous sweating [1], and for the prevention or therapy of common colds with immune deficiency. It has been suggested that YPFS has beneficial immunomodulatory effects that can prevent viral infections including SARS [2] and enhance cellular immunity [3]. The medication is also widely used to treat allergic diseases, including asthma, allergic rhinitis, urticaria and allergic contact dermatitis, and the efficacy of YPFS in these conditions has been ascribed to its immunosuppressive effects. Although both clinical and experimental studies

attest to the prophylactic and/or therapeutic efficacy of YPFS, the bioactive molecules have not yet been identified. It was previously suggested that purified YPFS polysaccharides have immunostimulatory activity and could represent the active components of YPFS [4]. However, the molecules responsible for the immunosuppressive effect of YPFS have not been identified.

Because the active molecules are inferred to interact with target cells, cell-based affinity purification and assay techniques have been employed as a screen for bioactive components in Chinese medicines [5–7]. A screening method involving binding to human red blood cell membranes and HPLC analysis was developed previously by our group to identify active components of *Angelica sinensis* [5]. Hepatocyte extraction combined with HPLC was also applied for screening components with potential activity against liver disease [8]. In the present study we report on the development of a method based on splenocyte binding and HPLC (SB-HPLC) for screening potential active components in medicinal herbs.

2. Experimental

2.1. Materials and reagents

Radix Astragali (RA; Inner Mongolia, China), *Rhizoma Atractylodis Macrocephalae* (RAM; Zhejiang, China), and *Radix Saposhnikoviae* (RS; Heilongjiang, China) were purchased from the Herbal Decoc-

tion Slices division of Nanjing Pharmaceutical Company (Nanjing, China); botanic identification was confirmed by Professor Chungen Wang (Nanjing University of Chinese Medicine). YPFS was prepared by Nanjing University of Chinese Medicine. Linoleic acid was from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China); purity was 99% according to HPLC analysis.

Dulbecco's phosphate buffered saline (PBS) was from Beijing Solarbio Science & Technology Corporation (Beijing, China). Gibco RPMI Medium 1640 and bovine serum albumin were from Invitrogen (Carlsbad, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was from AMRESCO (Cleveland, OH, USA), 2,4-dinitrochlorobenzene (DNCB) from Sigma (St. Louis, MO, USA), and HPLC-grade acetonitrile from Honeywell (Muskegon, USA). Concanavalin A (ConA) was a product of Sigma (St. Louis, MO, USA). Deionized water was obtained using a Milli-Q water system (Millipore, Bedford, MA, USA) and analytical grade methanol was from Hanbon Science & Technology Corporation (Nanjing, China). Dexamethasone sodium phosphate was from Zhengzhou Zhuofeng Pharmacy (Zhengzhou, China). All other chemicals were of analytical grade.

2.2. Animals

Male BALB/c mice were obtained from the Shanghai SLAC Laboratory Animal Company. All mice were maintained at Nanjing University of Chinese Medicine under specific pathogen-free conditions and were used at 6–10 weeks of age.

2.3. Preparation of YPFS extracts

Fifty grams of YPFS was immersed in 500 mL ethanol:water (95:5, v/v) for 1 h and then refluxed for 1 h. The extraction process was repeated twice and the extracts were combined, filtered, and evaporated to dryness using a vacuum concentrator system (Büchi R-114, Switzerland) at temperature of 60 °C. Residues were taken up in 50 mL of water.

2.4. HPLC analysis

YPFS extracts and splenocyte-binding extracts were analyzed by HPLC. Analysis used an Agilent Series 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a vacuum degasser, a quaternary pump, an autosampler and a diode-array detector (DAD) connected to Agilent ChemStation software. A Sepax GP-C$_{18}$ ODS column (4.6 mm × 250 mm, 5 μm) was used. The mobile phase was (A) water:phosphoric acid (100:0.1, v/v); (B) acetonitrile. The flow rate was 1 mL/min and elution conditions were: 0–10 min, linear gradient 1–15% (v/v) B in A; 10–20 min, linear gradient 15–20% B; 20–30 min, linear gradient 20–28% B; 30–40 min, linear gradient 28–40% B; 40–45 min, linear gradient 40–60% B; 45–50 min, 60% B isocratic; 50–85 min, linear gradient 60–95% B; column reconditioning involved 1% B isocratic for 10 min. The system operated at 30 °C and the injection volume was 20 μL. The DAD was set to scan from 200 to 400 nm; 203, 220, 238, 254 and 280 nm were used as detection wavelengths.

2.5. Mass spectrometry

2.5.1. HPLC–MS analysis

A Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA) and a Mariner 5140 TOF-MS system (Applied Biosystems, Foster City, CA) were used for HPLC–MS. HPLC conditions were as follows: a Sepax GP-C$_{18}$ ODS column (4.6 mm × 250 mm, 5 μm) was used as the analytical column, water/acetic acid (100:1, v/v) or acetonitrile (90:10) were used for the mobile phase, the detection wavelength was 203 nm and the flow rate was 1 mL/min. ESI-MS conditions were as follows: positive ion model, drying gas N$_2$, spray tip potential 5290 V, nozzle potential 140 V, nozzle temperature 140 °C, detector voltage 2100 V, scan range 50–1000 m/z.

2.5.2. GC–MS analysis

GC–MS was performed using an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer and Agilent ChemStation software (Agilent Technologies, Palo Alto, CA). An HP-5 column (30 m × 0.25 mm, 0.25 μm film thickness, Agilent Technologies) was used. The column temperature was 60 °C for injection, then programmed to rise at 5 °C/min to 260 °C. Split injection (1 μL) was conducted with a split ratio of 1:15 and helium was used as carrier gas with a flow rate of 1.5 mL/min. The mass spectrometer was operated in electron-impact (EI) mode, the scan range was 50–550 amu, the ionization energy was 70 eV and the scan rate was 0.2 s per scan. The inlet ionization source temperature was 230 °C.

2.6. Splenocyte binding

Mice were sacrificed and washed in 75% (v/v) ethanol. Spleens were removed aseptically and washed three times in PBS (pH 7.4). Using the plunger end of a syringe, spleens were dispersed in PBS and pressed through a cell strainer; cells were collected by centrifugation (1000 rpm, 10 min). The cell pellet was suspended into 0.83% NH$_4$Cl–Tris buffer (0.02 M) to lyse red blood cells. Splenocytes were then washed twice with PBS and resuspended in RPMI Medium 1640 containing 10% fetal calf serum (FCS).

Splenocyte suspensions (9 mL; 2 × 10^7 cells) and 1 mL YPFS ethanol extract were incubated with gentle agitation for 30 min at 37 °C. The suspensions were then centrifuged at 2000 rpm for 8 min and the cell pellets were washed ten times to remove unbound components. The wash eluates were discarded except for the final eluate which was collected as a control for HPLC analysis (sample a). Finally, splenocytes were denatured by addition of 10 mL hydrochloric acid PBS solution (pH 4.0) to liberate components bound to the cells. The desorption eluate was clarified by centrifugation and retained for HPLC analysis (sample b). The blank desorption eluate, in which YPFS extract was replaced by PBS, was generated using the method described (sample c). Aliquots (1 mL) of samples a, b and c were separately mixed with 1 mL methanol, vortexed for 2 min, and centrifuged at 10,000 rpm for 3 min; supernatants were collected and dried under nitrogen at 45 °C. Residues were taken up into 200 μL methanol and filtered through a 0.45 μm nylon membrane filter before HPLC analysis.

2.7. Splenocyte proliferation assay

Splenocyte proliferation assays were performed by a modified MTT method. Briefly, cells were treated with concanavalin A (ConA) at 8 μg/mL and incubated at 37 °C under 5% CO$_2$ for 24 h in 96 well plates at a cell density of 2 × 10^6 in a final volume of 100 μL. MTT (5 mg/mL, 10 μL) was added to each well and incubation continued for 4 h at 37 °C. To prepare the 'triplex solution', 10 g sodium dodecyl sulfate (SDS), 5 mL isobutanol and 0.1 mL 10 M HCl were dissolved in 100 mL of distilled H$_2$O. Following incubation with MTT, 100 μL of triplex solution was added to each well and incubated at 37 °C overnight. Absorbance was measured at 570 nm with a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). All experiments were performed in triplicate.

2.8. Delayed-type hypersensitivity (DTH) assay

Allergic contact dermatitis, a model of DTH, was induced by topical application of DNCB. Briefly, mice were sensitized twice by
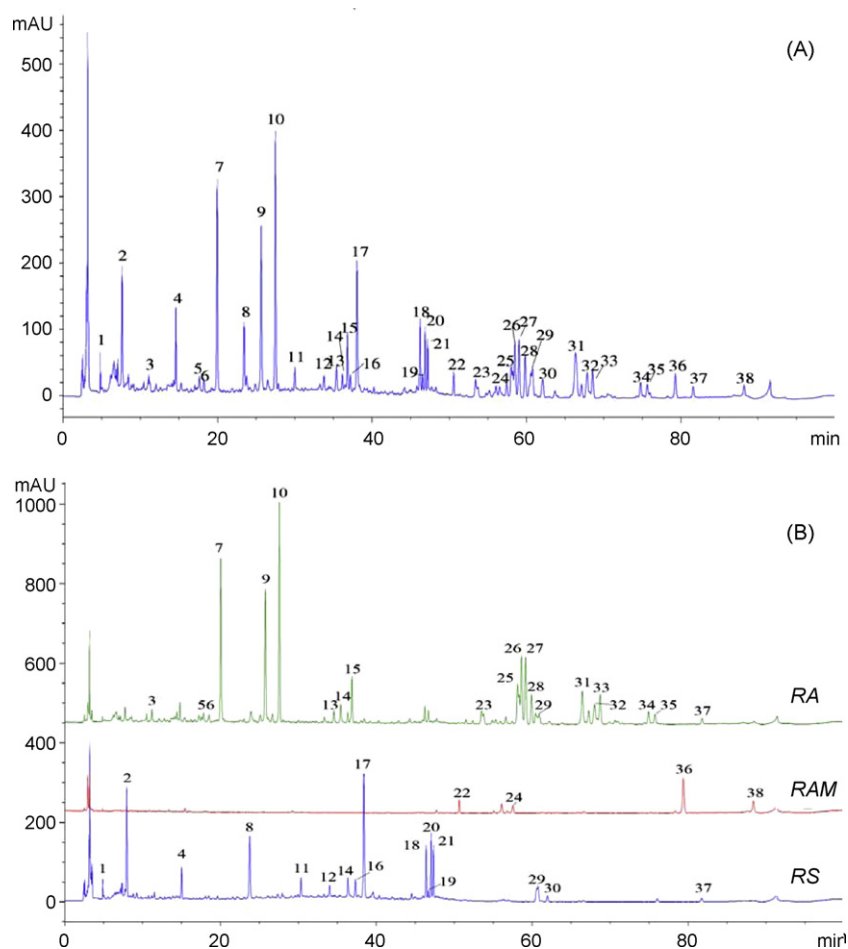
Fig. 1. HPLC fingerprint of YPFS ethanol extract and comparison with component herb extracts. (A) HPLC fingerprint of the YPFS ethanol extract. (B) HPLC chromatogram of *Radix Astragali* (RA), *Rhizoma Astractylodis Macrocephalae* (RAM) and *Radix Saposhnikoviae* (RS). Detection was at 220 nm.

application of DNCB (5% in acetone, 80 μL) to the shaved abdomen at day 1 and day 2. At day 7 of sensitization with DNCB the right ears of mice were treated topically with DNCB (1% in acetone, 50 μL); the control left ears were treated with acetone alone. After 24 h, ears were removed and weights were determined; ear swelling was calculated as the difference in the weights of the right and left ears.

3. Results and discussion

3.1. HPLC fingerprint of YPFS ethanol extract

In our previous study we used seven different methods to prepare YPFS extracts; these included water, 95% ethanol, ethyl acetate, ligarine and others. We found that the 95% ethanol

Table 1
^{1}H NMR and ^{13}C NMR 500 MHz CDCl$_3$ data of compound C1 compared with that of panaxynol.

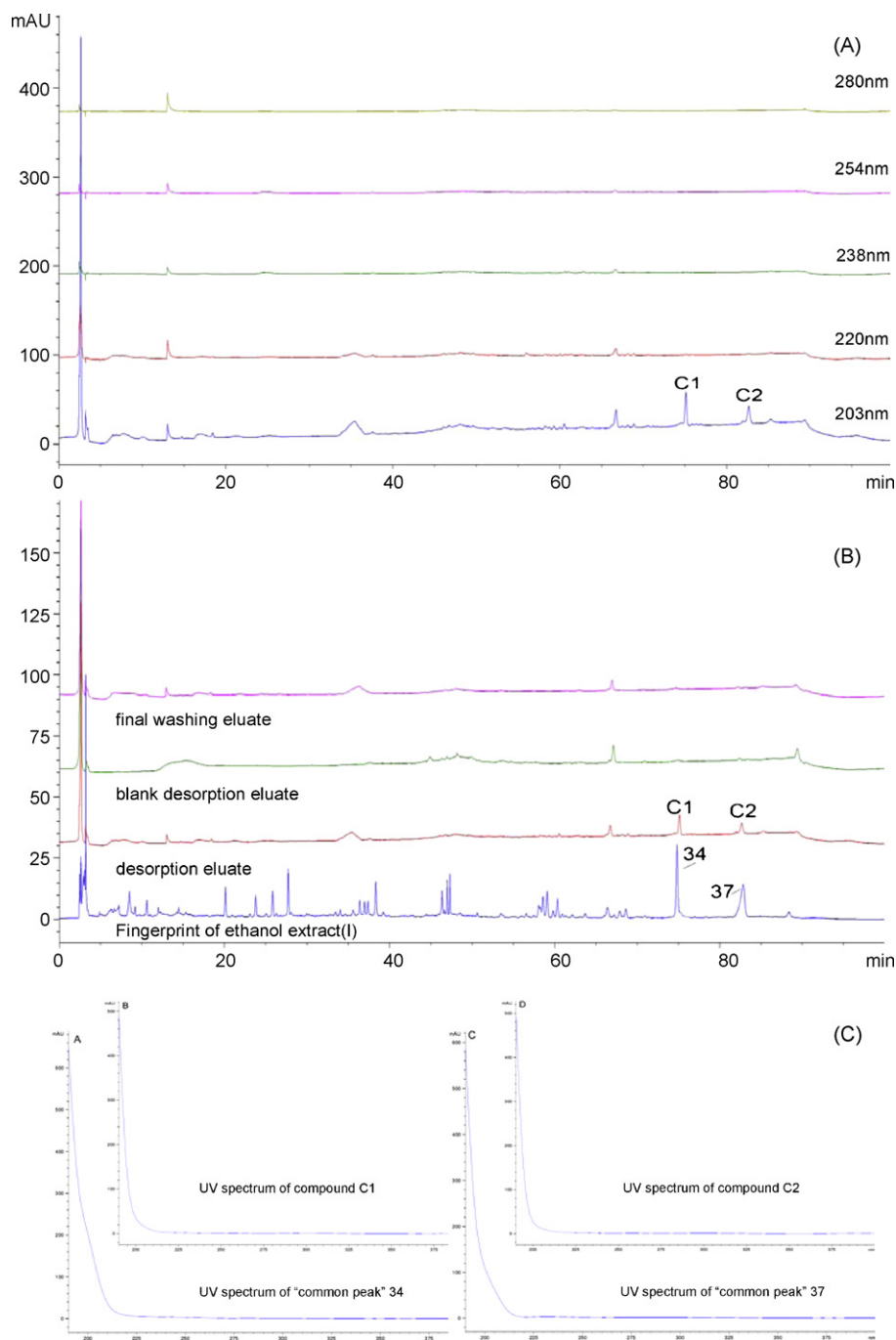| No.	^{1}H NMR		^{13}C NMR	
	Panaxynol [33]	Compound C1	Panaxynol [33]	Compound C1
1	a 5.24, *dt*, 1H (10.2, 1.1 Hz)	a 5.24, *dt*, 1H (10.1, 1.3 Hz)	116.9	117.0
	b 5.47, *dt*, 1H (16.8, 1.1 Hz)	b 5.46, *dt*, 1H (17.0, 1.3 Hz)		
2	5.94, *ddd*, 1H (17.4, 10.2, 5.3 Hz)	5.94, *ddd*, 1H (17.0, 10.0, 5.3 Hz)	136.1	136.3
3	4.91, *t*, 1H (5.3 Hz)	4.92, *t*, 1H (5.5 Hz)	63.5	63.6
4	–	–	74.4	74.3
5	–	–	71.2	71.2
6	–	–	63.9	64.1
7	–	–	80.2	80.3
8	3.03, *d*, 2H (7.1 Hz)	3.03, *d*, 2H (6.8 Hz)	17.7	17.7
9	5.37, *dtt*, 1H (10.3, 7.2, 1.3 Hz)	5.37, *dtt*, 1H (10.1, 7.4, 1.3 Hz)	122.1	122
10	5.51, *dtt*, 1H (10.3, 6.8, 1.3 Hz)	5.51, *dtt*, 1H (10.1, 6.9, 1.3 Hz)	133.1	133.2
11	2.02, *dt*, 2H (6.7, 6.7 Hz)	2.02, *dt*, 2H (6.4, 6.9 Hz)	27.2	27.2
12	1.27, *m*, 10 H	1.27, m, 10 H	29.2	29.2
13			29.3	29.3
14			29.1	29.1
15			31.9	31.9
16			22.6	22.7
17	0.88, *t*, 3H (6.7 Hz)	0.88, *t*, 3H (7.0 Hz)	14.1	14.1

Fig. 2. Detection of YPFS splenocyte-binding molecules by HPLC. (A) HPLC chromatograms of the desorption eluate of the splenocyte-binding fraction of the YPFS ethanol extract. Peaks were detected at 280, 254, 238, 220 and 203 nm. (B) Comparative chromatograms of the ethanol extract, the final wash eluate, the blank desorption eluate, and the splenocyte-binding extract; detection was at 203 nm. (C) UV absorption profiles of YPFS components C1 and C2 and peaks 34 and 37 in the HPLC fingerprint of the YPFS ethanol extract.

extract had by far greater effects on DTH *in vivo* and on cytokine production *in vitro* than the other extracts [9]. So 95% ethanol was used for sample preparation. The HPLC fingerprint of the YPFS ethanol extract at 220 nm is shown in Fig. 1A. The three component herbs (*Radix Astragali*, RA; *Rhizoma Atractylodis Macro-cephalae*, RAM; *Radix Saposhnikoviae*, RS) were separately extracted and analyzed using the same procedures. This demonstrated that peaks 1, 2, 4, 8, 11, 12, 14, 16–21, 29, 30 and 37 originated from RA; peaks 22, 24, 36 and 38 were from RAM and peaks 3, 5–7, 9, 10, 13–15, 23, 25–35 and 37 were from RS (Fig. 1B).

3.2. Splenocyte-binding components of YPFS

Because the bioactive components present in YPFS exert immunomodulatory properties we used immune cell binding as an enrichment method. As source tissue we used mouse spleen, the largest organ of the immune system, which contains diverse immune cell types including B and T lymphocytes, macrophages, dendritic cells, and natural killer cells as well red blood cells. The organ has been considered to represent an 'immunological conference center'. YPFS extracts were incubated with mouse splenocytes and bound components were collected by centrifu-
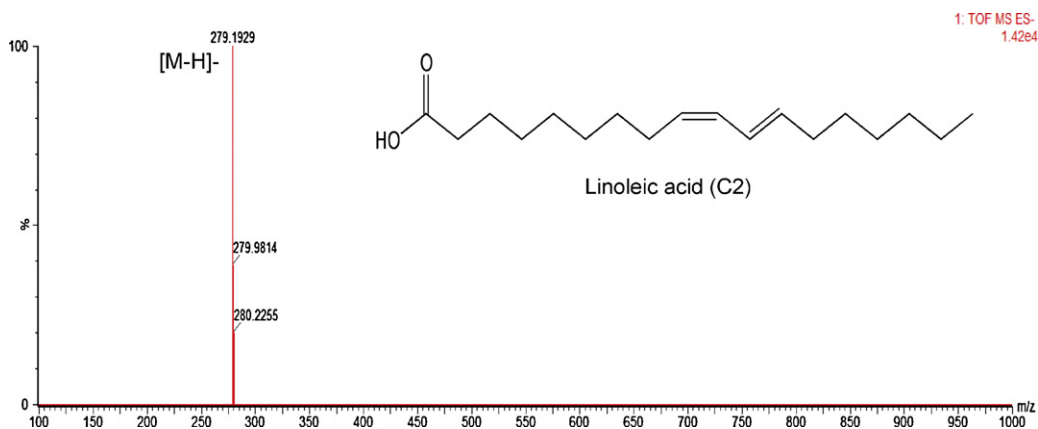
Fig. 3. ESI-MS negative ionization spectrum of compound C2. Inset: chemical structure of C2 (linoleic acid).

gation. After thorough washing and elution from the cell pellets, retained components were subjected to HPLC analysis using simultaneous monitoring at 203, 238, 254 and 280 nm to maximize the likelihood of successful detection. The profiles of the HPLC chromatograms at 220, 238, 254 and 280 nM were very similar, whereas at 203 nm the sensitivity appeared to be increased and a greater number of peaks were detected. As shown in Fig. 2A, two principal peaks (C1 and C2) were detected in the 203 nm chromatogram of the YPFS splenocyte-binding extract. No comparable peaks were detected in the chromatograms of the two control samples (sample a, final wash eluate; and sample b, blank desorption eluate) (Fig. 2B). By comparison of retention times and UV absorption profiles of C1 and C2 with the HPLC fingerprint of the YPFS extract, components C1 and C2 were identified as peaks 34 and 37; both peaks originated from a single constituent herb (RS and RA, respectively) (Fig. 2B and C). These findings identify splenocyte-binding molecules C1 and C2 as potential immunomodulatory components of YPFS.

3.3. Identification of C1 and C2 by LC–MS

The compound C2 was identified as linoleic acid (LA) by comparison of the retention times and molecular masses of compound C2 and reference LA in HPLC–ESI-MS negative ionization spectra (Fig. 3). Compound C1 could not be identified based on its UV absorption profile and MS data alone. We therefore sought to purify this molecule from YPFS or single herbs to permit further analysis using MS and NMR.

3.4. Purification of C1 by column chromatography

The splenocyte binding study identified component C1 as peak 34 in the HPLC fingerprint of the ethanol extract of RS. Accordingly, ~4.5 kg of RS were immersed in ethanol–water (95:5, v/v) for 24 h and the extraction process was repeated three times. The extracts were combined, filtered and evaporated to dryness in a vacuum concentrator at 60 °C. The residue was diluted with purified water and extracted three times with same volume of petroleum ether; the solvent was again evaporated at 60 °C. The petroleum ether extract was sequentially partitioned on a 200–300 mesh silica gel column using petroleum ether (60–90 °C), 1% (v/v) ethyl acetate/petroleum ether, 5% (v/v) ethyl acetate/petroleum ether and 10% (v/v) ethyl acetate/petroleum ether systems to give 50 fractions of 500 mL of each. All fractions were concentrated under reduced pressure and examined by silica gel thin-layer chromatography (TLC). Fractions containing compound C1 (fractions 74–94, monitored by HPLC) were combined and further purified on preparative TLC and eluted with 10% (v/v) ethyl

acetate/ether. The purity of compound C1 was 96.5% from HPLC analysis.

3.5. Identification of compound C1

Compound C1 gave a light yellow oil and GC–MS was used to identify the molecule. The retention time of compound C1 was 28.808 min in the total ion current (TIC) chromatogram (1 mg/mL in ether) (spectrum not shown). Data of the mass spectrum of compound C1 are as following: EI-MS m/z 244 (M$^+$), 229, 187, 159, 131, 115, 91 (base peak), 76, 55; molecular formula: $C_{17}H_{24}O$. Comparison with the NIST05a Database (Agilent Technologies, Palo Alto, CA) revealed that the mass spectrum of compound C1 was similar to that of panaxynol (PAN) [EI-MS m/z 244 (M$^+$), 217, 187, 159, 141, 117, 91 (base peak), 76, 55, 29; molecular formula $C_{17}H_{24}O$], and the matching rate was 96.8%.

The GC–MS identification of compound C1 was further supported by comparison of NMR data obtained with compound C1 and PAN (Table 1). This allowed compound C1 to be unambiguously assigned as panaxynol (PAN, also known as Falcarinol).

3.6. Effects of PAN and LA on splenocyte proliferation in vitro

Because PAN and LA were both identified as splenocyte-binding molecules we studied their effects on splenocyte proliferation in response to the T cell mitogen concanavalin A (Con A) [10]. Splenocytes were incubated with ConA and different concentrations of the two purified molecules for 24 h, followed by measurement of the extent of splenocyte proliferation. All experiments were performed in duplicate. PAN and LA concentrations employed were 6.25–50 and 0.7–700 μM, respectively. DMSO (0.1%, v/v) was included as a vehicle control. Splenocyte proliferation was assessed by the modified MTT assay.

As shown in Fig. 4A, ConA-induced splenocyte proliferation was significantly attenuated by PAN in a concentration-dependent manner (Fig. 4A). PAN, first isolated from the lipophilic fractions of *Panax notoginseng*, was previously reported to exert anticancer effects and inhibit the growth of malignant cells [11–14]. Other studies reported antihypertensive effects [15], reduced NO production in macrophages [16,17], inhibition of 15-hydroxyprostaglandin dehydrogenase activity [18], protection against liver injury [16], neuroprotective effects [19–21], and inhibition of the proliferation of rat aortic vascular smooth muscle cells (RASMCs) [22]. Although some of these activities could reflect immunomodulation by PAN, no previous studies have addressed specific effects on splenocytes or T and B lymphocytes.

By contrast, LA treatment had no significant effect on splenocyte proliferation (Fig. 4B). Different LA isomers were previously
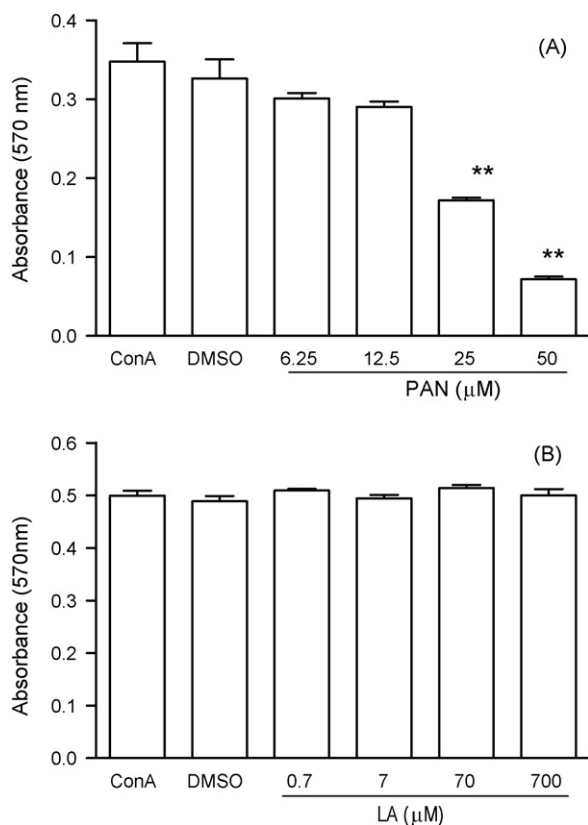
Fig. 5. Effects of panaxynol (PAN) on DTH induced by DNCB in mice. **$p < 0.01$ versus controls.

Fig. 4. Effects of compounds on *in vitro* splenocyte proliferation stimulated by ConA. Data are means ± SD from three independent experiments. (A) Effects of panaxynol (PAN) on splenocyte proliferation; **$p < 0.01$ versus the DMSO group. (B) Effects of linoleic acid (LA) on splenocyte proliferation.

reported to modulate gene expression involved in fatty acid metabolism and atherosclerosis [23,24], to have anti-tumor properties and inhibit NF-kappaB activity and COX2 [25], and to be negatively associated with age [26]. LA has also been reported to affect immune/inflammatory responses, with effects including LA-mediated upregulation PMN phagocytic responses [27], stimulation of specific immune responses [28], and modulation of oxidative stress and splenocyte IL-6 and TNF-alpha production induced by lipopolysaccharide in aged mice [29]. However, in the present study we found that LA had no significant effect on ConA-induced splenocyte proliferation, and further studies will be required to elucidate the immunomodulatory effects of LA.

3.7. Effects of PAN on delayed-type hypersensitivity (DTH)

Allergic contact dermatitis (ACD) was induced in mice by treatment with DNCB. Mice were injected daily with PAN (10 mg kg^{-1}) intraperitoneally (i.p.) during the induction phase (days 1–6). Dexamethasone sodium phosphate (DEX, 0.65 mg kg^{-1}) was injected into ACD mice (i.p.) as a positive control from day 1 to day 7. Negative control animals received normal saline (NS). Following sensitization, ACD was induced by topical application of DNCB to the ear; the extent of swelling was measured by comparing the weights of treated and control tissues.

As shown in Fig. 5, topical DNCB induced significant ear swelling. Importantly, in animals treated with PAN during sensitization the amount of swelling induced by application of DNCB was significantly reduced.

These findings demonstrate that PAN is effective against DTH induction. It was previously reported, in contrast to our results, that PAN is an allergen and can induce pro-allergic effects in skin [30].
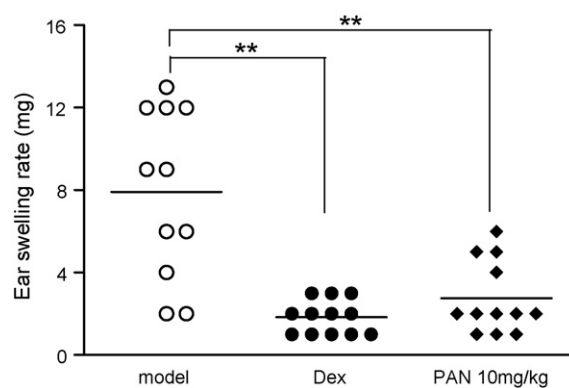
However, it is possible that the differences between our findings and the report of pro-allergic activity of PAN reflect the different experimental models used in the two studies. Notably, our study addressed the effects of PAN in a type IV hypersensitivity (DTH) model, whereas the other study [29] addressed type I hypersensitivity. Another possible difference between the two studies is that PAN was applied i.p. in our work whereas the molecule was applied topically in the contrasting study. Further investigations will be required to clarify the type and mechanism of immunomodulation exerted by PAN in different model systems.

YPFS is commonly used against cold and flu, but is also used widely to treat allergic diseases such as asthma, allergic rhinitis, urticaria and allergic contact dermatitis. Previous studies focused on saponins and polysaccharides as the biologically active components of YPFS in relation to the immune system [4]. In the present study, PAN, previously detected in the essential oils of RS and RA [31,32], was found to bind to splenocytes. Moreover, PAN was found to inhibit ConA-induced splenocyte proliferation *in vitro* and attenuate DTH *in vivo*. We previously reported that extracts of YPFS can both inhibit DTH *in vivo* and inhibit spleen T cell proliferation and IFN-γ production induced by ConA *in vitro* [9], and the bioactivity of PAN on splenocyte proliferation and DTH correlates with that of YPFS. This implies that PAN is likely to play an important role in YPFS therapy of allergic diseases related to DTH, and argues that, in addition to polysaccharides and saponins, YPFS contains other potent immunomodulatory compounds.

In contrast to traditional procedures, that involve the extraction and purification of different compounds contained in medicinal herbs followed by conventional pharmacologic screening, the approach used here of combined splenocyte binding and HPLC (SB-HPLC) avoids the need for systematic purification and assay of a large number of different molecules. Although it remains necessary to assay the bioactivity of the compounds identified by SB-HPLC, the number of compounds to be assayed is markedly reduced. The combined method used here therefore provides a rapid and efficient technique for screening for immunomodulatory components in extracts of medicinal herbs.

4. Conclusions

A combined SB and HPLC method was used to screen for potential active components in YPFS. PAN, a compound identified by this method, was shown to inhibit ConA-induced splenocyte proliferation *in vitro* and attenuate DTH *in vivo*. These findings argue that the anti-DTH effect of YPFS is at least partially mediated by PAN. Although the second compound identified, LA, was not observed to affect splenocyte proliferation in this study, several other reports have suggested that LA might modulate immune/inflammatory

responses in different experimental systems. We conclude that SB-HPLC provides a rapid and efficient method for the identification of potential immunomodulatory components in complex mixtures derived from medicinal herbs used in traditional Chinese medicine.

Role of the funding sources

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