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Metabolic profiling of the traditional Chinese medicine formulation Yu Ping Feng San for the identification of constituents relevant for effects on expression of TNF-α, IFN-γ, IL-1β and IL-4 in U937 cells

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ABSTRACT

Yu Ping Feng San (YPFS) is a classical TCM formulation which has been traditionally used for treatment of immune system related diseases such as chronic bronchitis, allergic rhinitis and asthma. The formula is a mixture of Radix Saposhnikoviae (Fangfeng), Radix Astragali (Huangqi), and Rhizoma Atractylodis macrocephalae (Baizhu). TLC- and LC-DAD-ESI-MS/MS methods have been developed for the analysis of the metabolic profiles of the single herbs and of the formula. Decoctions and ASE extracts were analyzed in order to trace components of the individual herbs in YPFS. Nine constituents of Radix Saposhnikoviae, ten constituents of Radix Astragali and five constituents of Rhizoma Atractylodis macrocephalae have been assigned in the chemical profiles of the formula, which now allow the standardisation of YPFS. The pharmacological testing showed that all extracts significantly inhibited expression of TNF-α, IFN-γ, and IL-1β in U937 cells, while the inhibition of IL-4 was consistently low. Compared to conventional analyses which are focused on a limited set of compounds, metabolomics approaches, together with novel data processing tools, enable a more holistic comparison of the herbal extracts. In order to identify the constituents which are relevant for the immunomodulatory effects of the formula, metabolomics studies (PCA, OPLS-DA) have been performed using UPLC/QTOF MS data.

1. Introduction

The quality control of herbal formulations has always been a great challenge due to the presence of hundreds of chemical constituents in such complex mixtures. However, in order to ensure consistent efficiency and safety of these preparations, standardisation by sensitive and reliable analytical methods is necessary. Yu Ping Feng San (YPFS), also known as Jade Windscreen Powder, is a classical Chinese medicinal formulation which is comprised of Radix Saposhnikoviae (RS; Fangfeng; the roots of *Saposhnikovia divaricata* (Turcz.) Schischk. [1]), Radix Astragali (RA; Huangqi; the roots of *Astragalus mongholicus* Bunge [1,2]) and Rhizoma Atractylodis macrocephalae (RAM; Baizhu; the rhizomes of *Atractylodes macrocephala* Koidz. [1]) in a weight ratio of 1:2:2. YPFS was first documented in Teachings of Zhu Dan-Xi (Dan Xi Xin Fa, 丹溪心法) in Song Dynasty 920 CE [3], and is mainly used in the treatment of respiratory diseases, such as recurrent respiratory tract infections, chronic bronchitis, nosocomial pneumonia, and allergic rhinitis [4–7]. Furthermore, it shall stimulate the appetite in end-stage cancer patients, and abate idiopathic sweating [8]. It also has been used for preventing viral infections and acute respiratory syndromes [9,10], and it has been reported to have immunoregulatory and positive effects in asthma, dermatitis and allergic rhinitis models [11–14].

Furocoumarins and furochromones have been found to be the major constituents of the roots of *Saposhnikovia divaricata* [15–18]. It has been reported that Radix Saposhnikoviae shows antiproliferative, antioxidant and anti-inflammatory activities *in vitro*. The extract of the roots also significantly reduced NO production and inhibited iNOS mRNA expression in LPS stimulated RAW

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264.7 murine macrophages [19]. More than 100 compounds, such as isoflavonoids, triterpene saponins, polysaccharides, and amino acids have been identified in RA so far [20,21]. The roots have been reported to have cardiotonic, hepatoprotective, hypotensive, immunostimulant, anti-aging, anti-oxidative, antidiabetic and anti-inflammatory activities [22–28].

Sesquiterpenes, with atractylon as major compound, are main constituents of the rhizomes of *Atractylodes macrocephala* [29]. The rhizomes are known to have immunomodulatory, antitumor, and anti-inflammatory properties [30–32].

A few methods have been already reported for the analysis of YPFS: A rapid LC–MS method was developed for the simultaneous identification of fifteen chemical markers deriving from the three single herbs in YPFS decoction [33]. A SPE-HPLC–MS method was described for simultaneous quantification of cycloastragenol, formononetin, calycosin, 4′-*O*-β-glucopyranosyl-5-*O*-methyvisamminol and cimifugin in rat plasma after oral administration of YPFS decoction [34]. Another study investigated the HPLC fingerprints of YPFS, Huangqi, Baizhu and Fangfeng ethanol extracts [35]. A HPLC method was developed for the determination of six bioactive components (cimifugin, *prim*-*O*-glucosylcimifugin, atractylon, atractylenolides I, II, III) in YPFS decoction [36].

In this study, we will present TLC- and HPLC-methods, including LC-DAD-ESI-MS/MS and UPLC-QTOF MS, which have been developed for the analysis of the metabolic profiles of the three single herbs and of the formula YPFS. Decoctions and ASE extracts have been investigated in order to compare the two methods in relation to the composition of their ingredients. The main aim of this analysis was to identify as many ingredients as possible in the three individual herbs in order to assign them in the chemical profiles of the formula YPFS. Furthermore, the immunomodulatory activities of YPFS should be studied by investigating the effects on the expression of TNF-α, IL-1β, IFN-γ and IL-4 in U937 human leukemic cells. Finally, the chemical profiles should be correlated with the pharmacological activities by multivariate data analysis (Principal Component Analysis, Orthogonal Partial Least Squares-Discriminant Analysis) in order to identify relevant constituents in YPFS.

2. Experimental

2.1. Samples, chemicals and reagents

RS, RA and RAM were purchased from Plantasia GmbH – Großhandel für asiatische Heilkräuter (Oberndorf bei Salzburg, Austria). Voucher specimens are held at the Institute of Pharmaceutical Sciences, Department of Pharmacognosy, University of Graz.

Reference compounds of astragaloside I and astragaloside II were purchased from PhytoLab GmbH & Co. KG (Germany). Astragaloside IV, *prim*-O-glucosylcimifugin and 5-O-methylvisammioside were purchased from NICPBP (Beijing, China). 5-Methoxypsoralen 99% and 8-methoxypsoralen were purchased from Sigma-Aldrich (MO, USA).

HPLC-grade acetonitrile (HiPerSolv CHROMANORM®) was from VWR International S.A.S (France). Water was purified by an EASYpure RF compact ultrapure water system (Barnstead, IA, USA). Formic acid (eluent additive for LC–MS) was purchased from Sigma-Aldrich (MO, USA).

n-Hexane (≥95%, for synthesis), dichloromethane (ROTIPURAN® ≥99.5% p.a., ACS, ISO), methanol (ROTIPURAN® ≥99.9%, p.a., ACS, ISO), acetic acid ethyl ester (≥99.5%, Ph. Eur., extra pure), *n*-butanol (ROTIPURAN® ≥99.5% p.a., ACS, ISO), chloroform (≥99%, for synthesis) and 4-anisaldehyde (≥97.5%) were bought from Carl Roth GmbH (Karlsruhe, Germany). Herba-Ethanol (S96% VG 1% MEK UVST K25l) was bought from BRENNTAG CEE GmbH (Wien, Austria), acetic acid (glacial) 100% (anhydrous for analysis, EMSURE® ACS, ISO, Reag. Ph. Eur.) and sulfuric acid (95–97%, for analysis EMSURE® ISO) were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Sample preparations

The reference compounds were dissolved in MeOH p.a. in a concentration of 1 mg/mL. The extracts of the three single herbs and of the formula YPFS were prepared by two different methods:

2.2.1. ASE

ASE (Accelerated Solvent Extraction) is a very fast technique for the extraction of plant material using common solvents at elevated temperatures and pressures (DIONEX ASE 200 Accelerated Solvent Extractor). The three single herbs were grinded into powders (0.25 mm sieve; ZM100 Retsch mill) and mixed in a ratio of 1:2:2 (RS, RA, RAM) to obtain the formula YPFS. 10 g powdered samples of the single herbs and of YPFS were extracted successively with *n*-hexane, dichloromethane (DCM) and methanol (MeOH) using standard methods with the following parameters: preheat 0 min, heat 5 min, static 5 min, purge 60 s, 3 cycles, pressure 69 bar, temperature 72 °C (*n*-hexane), 44 °C (DCM) and 68 °C (MeOH). The extracts were dried under nitrogen flow and stored at 4 °C until use. The yields of the ASE extraction of YPFS are 269. 8 mg (*n*-hexane), 91.8 mg (DCM) and 1838.5 mg (MeOH). For TLC and LC–MS analyses, the extracts were dissolved in MeOH p.a. in a concentration of 10 mg/mL and filtered through a 0.2 μm filter (Syringe Filter RC, Carl Roth).

2.2.2. Decoction

For preparing decoctions, 45 g dried plant material of the three single herbs and of the formula YPFS was immersed in 360 mL cold water for 30 min and decocted by boiling for 45 min. This operation was repeated once again. The total extracts were combined and concentrated by a Laborota 4000 rotavapor (Heidolph Instruments, Germany) to approximate 100 mL. The decoctions were fractionated three times with 30 mL *n*-hexane, DCM, ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) using a separation funnel. The fractions were concentrated by a Laborota 4000 rotavapor, dried under nitrogen flow and stored at 4 °C until use. The yields of the fractions of the decoction of YPFS are 31.8 mg (*n*-hexane), 70.5 mg (DCM), 68.2 mg (EtOAc), 438.3 mg (*n*-butanol) and 14.8 mg (water residue). For TLC and LC–MS analyses, the fractions were dissolved in MeOH p.a. in a concentration of 10 mg/mL and filtered through a 0.2 μm filter (Syringe Filter RC, Carl Roth).

2.3. HPLC-DAD-ESI-MS/MS analysis

HPLC-DAD-ESI-MS/MS analysis was carried out using a Thermo Scientific Dionex UltiMate 3000 liquid chromatography with an UltiMate 3000 RS pump and an UltiMate 3000 RS autosampler. It is interfaced with an UltiMate 3000 RS diode-array detector and a LTQ XL mass detector used in the ESI positive mode. The capillary temperature was 330 °C, source heater temperature 250 °C, sheath gas flow 50 arbitrary units, auxiliary gas flow 10 arbitrary units, source voltage 4.20 kV, source current 100 μA, capillary voltage 35 V and tube lens 110 V. The separation was carried out on a reversed-phase column (Merck LiChrospher® 100, RP-18 (5 μm), LiChroCART 250-4) at a temperature of 25 °C. It has been used a gradient system of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 1 mL/min and an injection volume of 5 μL. The gradient profile was optimized as follows: 0 min: A-B 95:5 (v/v); 25 min: A-B 80:20 (v/v); 40 min: A-B 60:40 (v/v); 55 min: A-B 45:55 (v/v); 60 min: A-B 45:55 (v/v); 85 min: A-B 20:80 (v/v);

87 min: A-B 5:95 (v/v); 95 min: A-B 5:95 (v/v); 96 min: A-B 95:5 (v/v); 106 min: A-B 95:5 (v/v). The DAD detector scanned from 190 to 400 nm, and the samples were detected at Total Scan PDA, at 254 nm and at 280 nm [37].

2.4. UPLC-QTOF MS analysis

UPLC/QTOF MS analysis was carried out using a Waters® ACQUITY UPLC® I-Class with a flow-through needle (FTN) system and a column heater. The instrument is interfaced with a Xevo® G2-S QTOF quadrupole time-of-flight mass spectrometer used in the ESI positive mode. The acquisition range was 100–1800 Da (scan time: 0.1 s), gas flow 10 L/hr (cone gas) and 800 L/hr (desolvation gas), collision energy 6 eV (low CE), 15–45 eV ramping (high CE), source heater temperature 120 °C, desolvation temperature 500 °C, capillary voltage 3.0 kV and cone voltage 30 V. The separation was carried out on a reversed-phase column (ACQUITY UPLC HSS T3, C18 (RP-18), 1.8 μm, 2.1 × 100 mm, Waters Corporation, Milford, USA) at a temperature of 40 °C. It has been used a gradient system of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.5 mL/min and an injection volume of 5 μL. The gradient profile was optimized as follows: 0 min: A-B 95:5 (v/v); 0.5 min: A-B 95:5 (v/v); 15 min: A-B 60:40 (v/v); 40 min: A-B 0:100 (v/v); 44 min: A-B 0:100 (v/v); 45 min: A-B 95:5 (v/v). Three replicate injections per sample were performed [37].

2.5. Pharmacological studies

All extracts were dissolved in DMSO and 20 mg/mL solutions were prepared. The solutions were diluted by phosphate buffered saline (PBS). U937 cells (human leukemic cells) were obtained from the American Type Culture Collection (Rockville, MD, USA). The leukemic cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco BRL, Gaithersburg, MD, USA) at 37 °C under 5% CO2. For cell viability assay, the U937 cells were seeded into 96 well plates (1.0×10^5cells/mL) and incubated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich Co., St. Louis, MO, USA) for 48 h at 37 °C in a 5% CO_2-humidified atmosphere to induce macrophages before the cells were washed three times with PBS to remove remaining PMA and non-adherent cells. Afterwards, the cells were incubated with different concentrations of the extracts (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) for 21 h at 5% CO2 and 37 °C. 10 μL of CCK-8 reagent (Dojindo, Tokyo, Japan) was added into each well and the cells were incubated for further three hours. Finally, the absorbance of each well was measured using a microplate reader at 450 nm. For performing ELISA, U937 cells were divided into six well plates (1.0×10^6cells/mL) and incubated with 10 ng/mL PMA for 48 h at 37 °C in a 5% CO_2-humidified atmosphere to induce macrophages. The medium was changed and the cells were pretreated with 25 μg/mL extracts for two hours, and then incubated for another 24 h with or without 1000 ng/mL of LPS (Sigma-Aldrich Co., St. Louis, MO, USA). Afterwards, the culture supernatants were collected and the levels of IL-4, IL-1β, TNF-α and IFN-γ were determined by the human IL-4, IL-1β, TNF-α, and IFN-γ Platinum ELISA's, following the manufacturer's instructions (eBioscience Inc., San Diego, CA, USA) [37].

2.6. Data processing and metabolite identification

The natural product application solution with UNIFI (Waters Corporation, Milford, USA) is a LC–MS data analysis software which allows a rapid, objective and reliable identification of metabolites in the analyzed samples. Compound identification was performed using Waters Traditional medicine library in UNIFI informatics platform which contains over 6000 compounds from more than 600 herbs of the Chinese Pharmacopoeia. The compound identification can be performed based on precursor exact mass, fragment ion mass, theoretical isotopic distribution, retention time and collision cross section when these parameters are available. In this analysis the identification is base only on precursor exact mass, fragment ion mass and theoretical isotopic distribution. The data matrix was mean centered and Pareto scaled with SIMCA 13.0.3 (Umetrics, Umea, Sweden) before multivariate data analysis. PCA gives a first overview of the information hidden in the data and it is used to classify the samples based on their chemical profiles. OPLS-DA is used to discriminate two classes of data to increase the class separation, simplify interpretation, and find potential biomarkers [38].

2.7. Thin-layer chromatography analysis

TLC analysis for quality control of the formula YPFS has been established using silica gel 60 F_{254} as stationary phase (Merck KGaA, Darmstadt, Germany) and chloroform-MeOH 4:1 (v/v; mobile phase A) for the detection of the main compounds of RS [37,39] and RA in the formula. Another mobile phase system comprising of *n*-hexane-EtOAc 95:5 (v/v; mobile phase B) was used for the detection of the non-polar constituents of RAM in YPFS [37,39]. 5 μL of the reference solutions and 10 μL of the extracts were applied on the TLC plates in a distance from 8 cm to the solvent front. While the chamber had to be saturated with mobile phase A 30 min before developing the TLC plates, with mobile phase B no chamber saturation was necessary. Compounds of RAM could be detected under UV 254 nm as well as in visible light after spraying with anisaldehyde-sulfuric acid reagent [40] and drying at 110 °C for 5 min. Constituents of RA could be detected after spraying with a 10% solution of sulfuric acid in ethanol and drying at 105 °C for 5 min in visible light as well as under UV 366 nm. Compounds of RS could be detected under ultraviolet light at 254 nm [37,39].

3. Results and discussion

3.1. Qualitative analysis of the formula YPFS by HPLC-DAD-ESI–MS/MS

For characterizing the chemical constituents in YPFS ASE extracts and decoction, an HPLC-DAD-ESI–MS/MS method has been established. The constituents in the formula have been identified by comparing their retention times and *m*/*z* values with reference compounds, and by comparing their *m*/*z* values and fragmentation patterns with data from literature [43–51]. Their origin was confirmed by comparing the base peak chromatograms of YPFS with those of the three individual herbs. Altogether, a total of 24 compounds from the three single herbs have been assigned in YPFS (Table 1). These constituents represent the major components in YPFS. Chemical structures of the compounds are shown in Fig. 2. Fig. 1A shows the base peak HPLC-ESI–MS/MS chromatograms of the *n*-hexane, DCM, EtOAc and *n*-BuOH fractions of YPFS decoction. Fig. 1B shows the base peak HPLC-ESI–MS/MS chromatograms of the *n*-hexane, DCM and MeOH ASE extracts of YPFS. The base peak HPLC-ESI–MS/MS chromatograms of RS, RA and RAM are shown in Fig. A1–7 Supplementary Material.

3.1.1. Identification of compounds from Radix Saposhnikoviae

Nine compounds of RS have been assigned in YPFS extracts, including furocoumarins and furochromones. RS seems to be very dominant because its ingredients were detected as the major compounds of the formula. *Prim*-O-glucosylcimifugin **(1)** ($C_{22}H_{28}O_{11}$), 5-O-methylvisammioside **(4)** ($C_{22}H_{28}O_{10}$), 8-methoxypsoralen **(10)** ($C_{12}H_8O_4$), and bergapten **(14)** ($C_{12}H_8O_4$) were confirmed by

Table 1
Characterization of chemical constituents detected and identified in YPFS by HPLC-DAD-ESI-MS/MS in positive ion mode. * Compared with a reference compound; RA: Radix Astragali; RS: Radix Saposhnikoviae; RAM: Rhizoma Atractylodis macrocephalae.

Peak No.	Retention time (min)	UV (nm)	Identification	Positive ion (*m*/*z*)	Fragment ions (*m*/*z*)	Plant material	Literature
1*	22.94	220, 285, 299	*prim*-O-Glucosylcimifugin	469 $[M+H]^+$	307, 289, 235	RS	–
2	26.91	200, 220, 250, 287	Calycosin-7-*O*-β-D-glucoside	447 $[M+H]^+$	285, 270, 253, 225	RA	[46]
3	28.45	230, 247, 301	Cimifugin	307 $[M+H]^+$	289, 259, 235, 221	RS	[43]
4	30.26	218, 232, 296	5-O-Methylvisammioside	453 $[M+H]^+$	291, 273, 219	RS	–
5	34.39	196, 252	Ononin	431 $[M+H]^+$	269, 254, 237, 213	RA	[46]
6	35.89	206, 285, 334	(6αR, 11αR)-9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside	301 $[M+H\text{-}Gluc]^+$, 463 $[M+H]^+$	286, 269, 191, 167, 152	RA	[46]
7	36.41	210, 229, 253, 300	*sec*-O-Glucosylhamaudol	439 $[M+H]^+$	277, 259, 205	RS	[43]
8	36.94	206, 289	5-O-Methylvisamminol	291 $[M+H]^+$	273, 243, 219, 205	RS	[43]
9	38.00	200, 220, 249, 291	Calycosin	285 $[M+H]^+$	270, 253, 225	RA	[46]
10*	43.08	220, 249, 303	8-Methoxypsoralen	217 $[M+H]^+$	202, 185, 174, 146	RS	–
11*	44.86	222	Astragaloside II	827 $[M+H]^+$, 1653 $[2\ M+H]^+$	1617, 895	RA	–
12	45.51	230, 300	Formononetin	269 $[M+H]^+$	254, 237, 213, 136	RA	[46]
13	45.96	210, 280	3-Hydroxy-9,10-dimethoxypterocarpan	301 $[M+H]^+$	269, 191, 167, 152	RA	[46]
14*	46.56	223, 269, 312	Bergapten	217 $[M+H]^+$	202, 173, 146, 118	RS	–
15	47.44	221, 290, 368	Astragaloside II isomer	827 $[M+H]^+$, 1653 $[2\ M+H]^+$	1617, 895	RA	–
16*	50.59	222	Astragaloside I	869 $[M+H]^+$, 1737 $[2\ M+H]^+$	805	RA	–
17	51.85	195, 223	Atractylenolide III	249 $[M+H]^+$	231, 213, 203, 175, 163, 149	RAM	[51]
18	54.07	225, 267	Astragaloside I isomer	869 $[M+H]^+$, 1737 $[2\ M+H]^+$	805	RA	–
19	57.39	224, 298	Ledebouriellol	375 $[M+H]^+$	275, 233, 221, 205	RS	[43]
20	60.39	224	Atractylenolide II isomer	233 $[M+H]^+$	215, 187, 159	RAM	–
21	60.90	224, 277, 335	Deltoin	329 $[M+H]^+$	229, 211, 183, 167	RS	[43]
22	61.44	223	Atractylenolide II	233 $[M+H]^+$	215, 187, 159	RAM	[51]
23	68.63	224, 278	Atractylenolide I	231 $[M+H]^+$	185, 157, 142, 129	RAM	[51]
24	86.20	223	Atractylon	217 $[M+H]^+$	199, 156, 141, 128	RAM	[40]

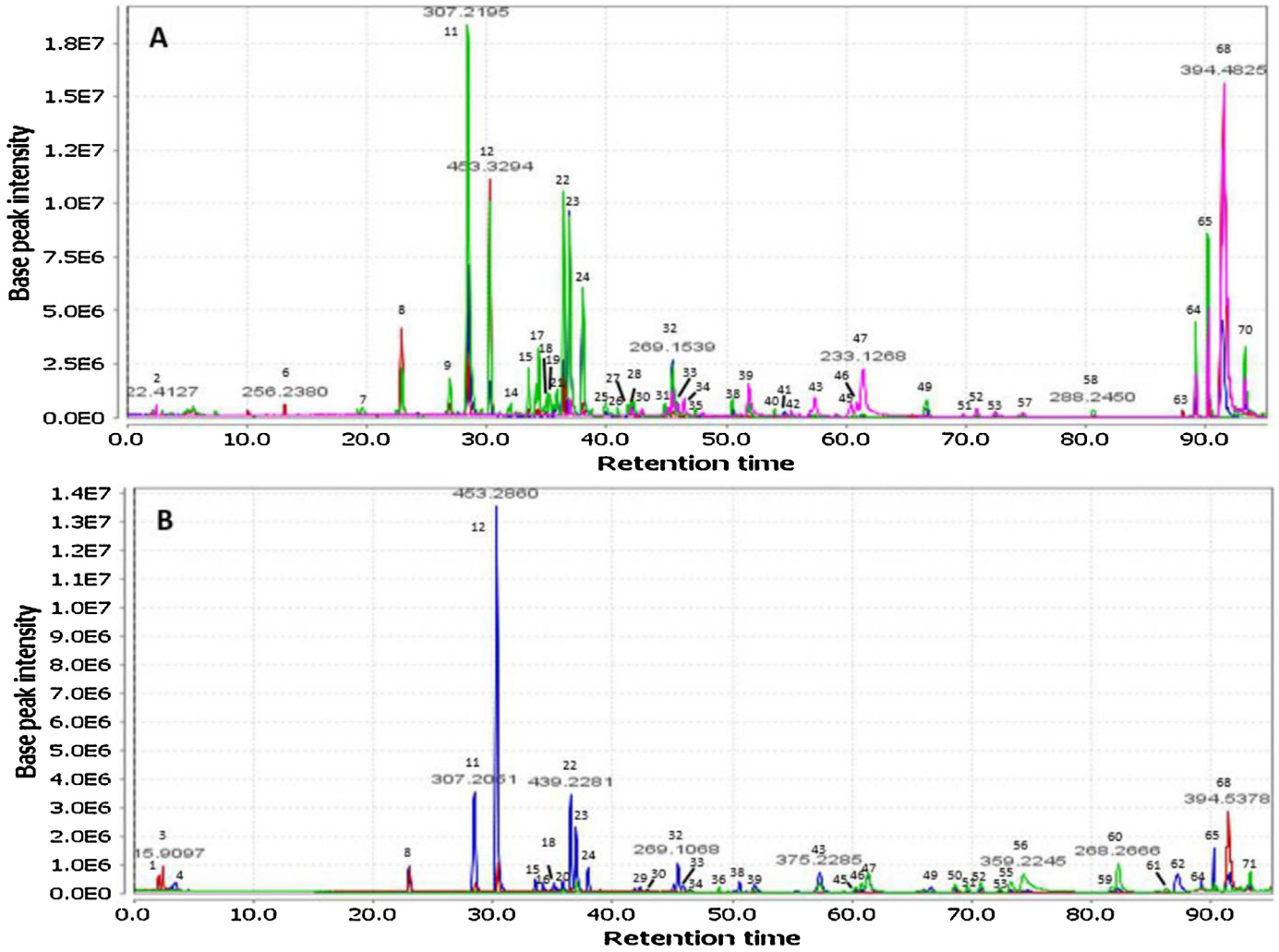

Fig 1. Base peak HPLC-ESI-MS/MS chromatograms of YPFS extracts in positive ion mode. Fractions of YPFS decoction: Pink = n-Hexane, Blue = DCM, Green = EtOAc, Red = n-BuOH (A); YPFS ASE extracts: Green = n-Hexane, Blue = DCM, Red = MeOH (B).

comparison of their retention times, UV and mass spectrometric data with reference standards. Compounds **3, 7, 8, 19** and **21** have been identified by comparing their *m/z* data and fragmentation patterns with literature data [36–38]. Cimifugin **(3)** ($C_{16}H_{18}O_6$) showed a $[M+H]^+$ ion at *m/z* 307, *sec*-O-glucosylhamaudol **(7)** ($C_{21}H_{26}O_{10}$) gave a $[M+H]^+$ ion at *m/z* 439, 5-O-methylvisamminol **(8)** ($C_{16}H_{18}O_5$) presented a $[M+H]^+$ ion at *m/z* 291, ledebouriellol **(19)** ($C_{20}H_{22}O_7$) generated a $[M+H]^+$ ion at *m/z* 375 and deltoin **(21)** ($C_{19}H_{20}O_5$) showed a $[M+H]^+$ ion at *m/z* 329 (Fig. 1, Table 1) [37].

3.1.2. Identification of compounds from Radix Astragali

Ten compounds of RA have been assigned in YPFS extracts, including isoflavonoids and triterpenoid saponins. The saponins astragaloside I **(16)** ($C_{45}H_{72}O_{16}$) and astragaloside II **(11)** ($C_{43}H_{70}O_{15}$) were identified by comparison of their retention times, UV and mass spectrometric data with reference standards. Furthermore, isomers of astragaloside I **(18)** and of astragaloside II **(20)** have been detected in the decoction of the formula. Based on *m/z* data and fragmentation patterns, further known compounds of RA could be assigned in YPFS by comparing with literature data [39–42]. Calycosin-7-*O*-β-D-glucoside **(2)** ($C_{22}H_{22}O_{10}$) generated a $[M+H]^+$ ion at *m/z* 447, ononin **(5)** ($C_{22}H_{22}O_9$) presented a $[M+H]^+$ ion at *m/z* 431, (6αR, 11αR)-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside **(6)** ($C_{23}H_{26}O_{10}$) showed a $[M+H]^+$ ion at *m/z* 462, calycosin **(9)** ($C_{16}H_{12}O_5$) gave a $[M+H]^+$ ion at *m/z* 285, formononetin **(12)** ($C_{16}H_{12}O_4$) presented a $[M+H]^+$ ion at *m/z* 269 and 3-hydroxy-9,10-dimethoxypterocarpan **(13)** ($C_{17}H_{16}O_5$) showed a $[M+H]^+$ ion at *m/z* 301 (Fig. 1, Table 1). Astragaloside IV ($C_{41}H_{68}O_{14}$), a major compound of RA could be identified only in traces in the single herb as well as in YPFS. However, it is not a genuine compound of the roots, but formed by hydrolysis from acylated astragalosides, like astragaloside I, astragaloside II and malonylastragaloside I during extraction with ammonia [37,48].

3.1.3. Identification of compounds from Rhizoma Atractylodis macrocephalae

Five constituents of RAM have been detected in small amounts in YPFS extracts, mainly in the non-polar extracts. By referring to literature data [44–46], compounds **17, 22** and **23** were identified as atractylenolide III, II and I, three characteristic sesquiterpene lactones of RAM. Atractylenolide I ($C_{15}H_{18}O_2$) generated a $[M+H]^+$ ion at *m/z* 231, atractylenolide II ($C_{15}H_{20}O_2$) gave a $[M+H]^+$ ion at *m/z* 233, and atractylenolide III ($C_{15}H_{20}O_3$) showed a $[M+H]^+$ ion at *m/z* 249. Compound **20** has been assigned in YPFS as an isomer of atractylenolide II. The sesquiterpene atractylon **(24)** ($C_{15}H_{20}O$) is a very non-polar and unstable component which could be detected in small amount in the *n*-hexane ASE extract of YPFS with a $[M+H]^+$ ion at *m/z* 217 [33] (Fig. 1, Table 1) [37].

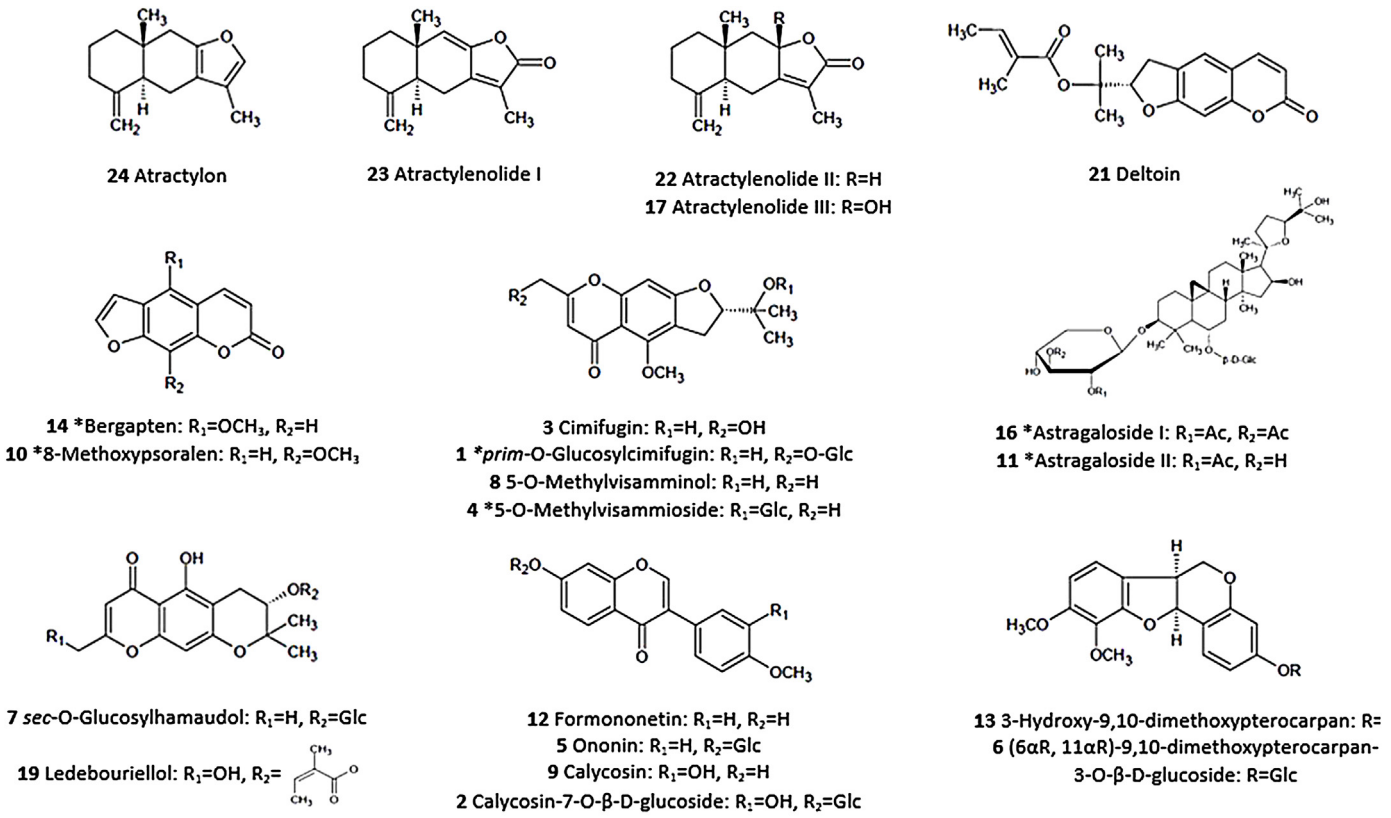

Fig. 2. Chemical structures of compounds identified in YPFS extracts; *: Chemical structures of reference compounds; Glc = Glucose; Ac = Acetyl.

3.1.4. Comparison of the two extraction methods

Decoctions are the traditional application form of TCM in China. Decoction is an extraction method mainly for the water soluble polar compounds. In order to extract the polar as well as the non-polar components of YPFS, Accelerated Solvent Extraction (ASE) was used. Differences in the metabolic profiles could be detected when comparing the two different extraction methods. Interestingly, more compounds could be detected in the decoction compared to the ASE extracts. Calycosin-7-*O*-β-D-glucoside **(2)**, (6αR, 11αR)-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside **(6)**, astragaloside II **(11)**, astragaloside II isomer **(15),** and astragaloside I isomer **(18)** could be detected only in the decoction of YPFS, while atractylenolide I **(23)** and atractylon **(24)** were contained only in the ASE extracts of the formula [37].

3.2. Qualitative analysis of the formula YPFS by TLC

Methods for thin-layer chromatographic analysis have been established for the qualitative assessment of YPFS, using silica gel F_{254} as stationary phase and various mobile phases, depending on the major constituents of the three individual herbs. Chromatograms are available in Fig. A8–10 Supplementary Material.

3.2.1. Identification of compounds from RS

The reference compounds *prim*-O-glucosylcimifugin and 5-O-methylvisammioside with Rf values of 0.24 and 0.43 are two main constituents of RS, which could be detected mainly in the DCM and in smaller amounts also in the MeOH ASE extracts of RS as well as of YPFS. Both compounds could also be identified in the decoction of RS and YPFS, mainly in the EtOAc and *n*-BuOH fractions (Fig. A.8, Supplementary Material) [37].

3.2.2. Identification of compounds from RA

Astragaloside I, II, and IV are main compounds of RA. Astragaloside I and II with Rf values of 0.47 and 0.19 are major constituents of RA which could be detected mainly in the DCM, as well as in smaller amounts in the MeOH ASE extracts of RA and YPFS. In the decoctions, both compounds could also be identified mainly in the DCM and EtOAc fractions. Astragaloside IV could not be detected in any extract by TLC, because it is an artifact formed only under special conditions (Fig. A9, Supplementary Material) [37,48].

3.2.3. Identification of compounds from RAM

Atractylon, a major component of RAM, could be identified only in the *n*-hexane ASE extract of the single herb and of YPFS by comparison with literature data [34]. Atractylon is a non-polar constituent. Therefore, it could not be detected in decoctions (Fig. A10, Supplementary Material) [37].

3.3. Immunomodulatory effects of YPFS

Cell viability of the ASE extracts and of the fractions of the decoction of YPFS was measured using the CCK-8 assay. The samples were tested in concentrations between 3 and 100 μg/mL in order to find that concentration which did not show any cellular toxicity against U937 human leukemic cells. A concentration of 25 μg/mL was chosen for the pharmacological assays on the expression of TNF-α, IL-1β, IFN-γ and IL-4 in U937 cells, because at this concentration the extracts did not show any cellular toxicity over 24 h. As shown in Fig. 3, the LPS stimulation increased the expression of the four cytokines and almost all tested extracts significantly inhibited the expression of TNF-α, IL-1β, IFN-γ and IL-4. YPFS showed a significant inhibition of the expression of TNF-α, with ASE *n*-hexane extract showing the best inhibition rate with 97%. The expression of IL-1β was also significantly reduced by the

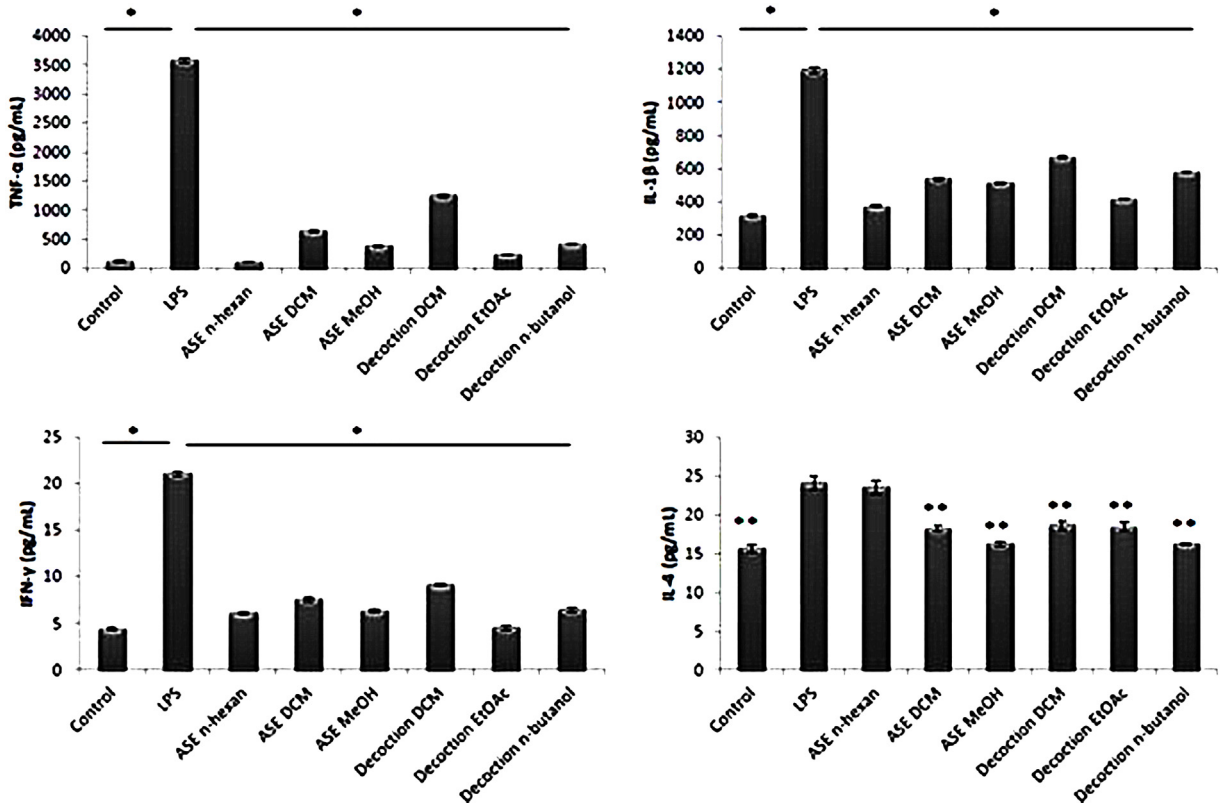

Fig. 3. Effects of YPFS on the expression of TNF-α, IL-1β, IFN-γ and IL-4 in U937 cells; concentration of the extracts: 25 μg/mL; n = 3; mean ± SD; control: unstimulated cells; LPS: LPS-stimulated cells (1000 ng/mL); * ($p < 0.05$); ** ($p < 0.01$).

formula extracts. The ASE *n*-hexane extract showed the best inhibition with 69%, while the DCM fraction of the decoction reduced the expression moderately by 44%. The expression of IFN-γ was also significantly inhibited by all tested extracts and fractions of YPFS with an inhibition range of 57%–79%. The inhibition of the expression of IL-4 by YPFS extracts was consistently low compared to the other cytokines. While the ASE *n*-hexane extract did not show any influence on IL-4 expression, all the other extracts and fractions had a low inhibitory effect of 23%–33%. These results revealed that YPFS has immunomodulatory properties by inhibiting TNF-α, IFN-γ, IL-1β and IL-4 expression in U937 cells at a concentration of 25 μg/mL [37].

3.4. Multivariate data analysis of UPLC-QTOF MS data of YPFS

Untargeted metabolomics provides unique chemical fingerprints of natural products for potential marker discovery. It focuses on all detectable compounds in a biological system, including compounds which are currently unknown or at least unidentified. Therefore, data processing, accurate quantification and reliable identification of metabolites are major challenges in metabolomics [52,53]. The processed UPLC-QTOF MS data matrix was imported into SIMCA 13.0.3 (Umetrics, Sweden) for multivariate data analysis, including PCA and OPLS-DA. In order to perform OPLS-DA, the pharmacological data need to be classified into active, moderately active and inactive groups (Table 2). As shown in PCA score plot (Fig. 4), an obvious separation between the ASE extracts and the fractions of the decoction of YPFS could be registered. These results indicated that the extraction method has a rather great influence on the chemical profile of herbs. To find potential biomarkers which contribute most to the immunomodulatory activities of YPFS, the UPLC-QTOF MS data and the classified pharmacological data were further analyzed by OPLS-DA. The OPLS-DA score plots (Fig. 5) demonstrated that the YPFS extracts could be separated according to their pharmacological activities. These results indicated that the metabolic profiles of the active (1), moderately active (2) and inactive (3) extracts are different. The S-plot (Fig. 6) provides information about peaks which contribute most to the pharmacological activity. For the S-plot, two different pharmacological classes were compared: For TNF-α and IFN-γ, active and moderately active extracts were compared, while for IL-4 moderately active and inactive extracts were compared. Compounds that contribute most to the different pharmacological activities are called “hits”. These compounds were matched against over 6000 compounds in the traditional medicine library in UNIFI informatics platform and against known compounds from the three individual herbs. Two of the “hits” which seem to be most likely relevant for the inhibition of the expression of TNF-α in U937 cells could be identified as 5-*O*-methylvisammioside (Rt 7.85 min, neutral mass 452.1693) and *prim*-*O*-glucosylcimifugin (Rt 5.90, neutral mass 468.1639) by comparing their mass spectra and fragmentation patterns with reference compounds and with literature data (Fig. 2). Both constituents are known constituents of RS. In the literature it has been already been reported that a 70% ethanol extract of RS, which contained *prim*-*O*-glucosylcimifugin and 5-*O*-methylvisammioside in a high amount, inhibited the production of TNF-α in LPS-induced RAW 264.7 cells [54]. Two of the compounds which seem to be mainly responsible for the inhibitory activity on the expression of IL-4 in U937 cells could be tentatively identified by UNIFI as astragaloside V (Rt 12.47 min, *m*/*z* 969.5032) and blestritin B (Rt 5.21 min, *m*/*z* 487.2142). Astragaloside V is a minor component of RA, which has not been investigated very well yet. Blestritin B is a constituent of *Monomeria barbata* [55] which has not yet been identified in any of the three single herbs, and it is not known whether it has an inhibitory effect on the expression of IL-4 in U937 cells. Therefore, further investigations are necessary to

Table 2
Class assignment for the individual pharmacological activities.

	TNF-α inhibition	IL-1β inhibition	IFN-γ inhibition	IL-4 inhibition
class 1 (active)	>70%	>60%	>60%	>38%
class 2 (moderately active)	50–69%	45–59%	45–59%	30–37%
class 3 (inactive)	<49%	<44%	<44%	<29%

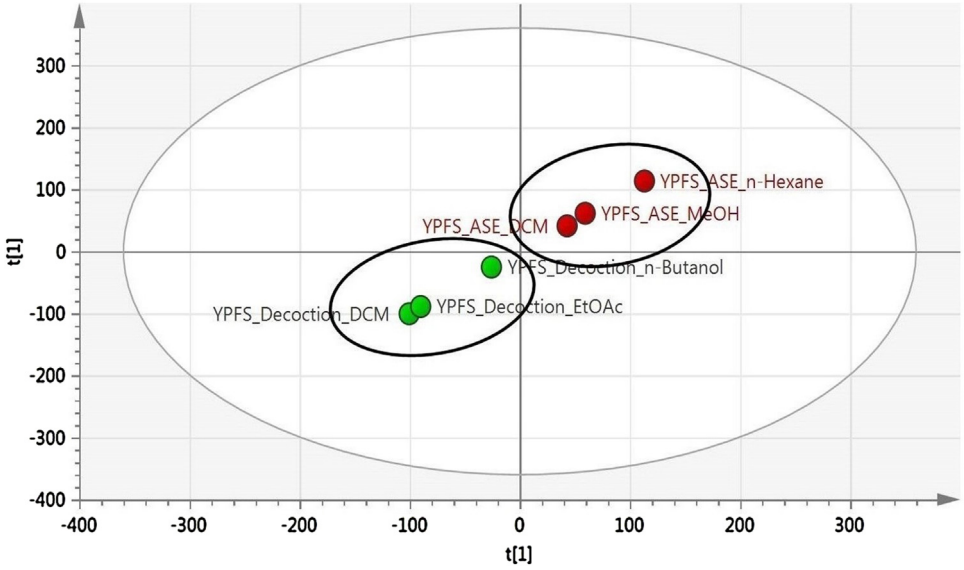

Fig. 4. PCA t_1/t_1 score plot of the LC-QTOF MS data of YPFS; Red: ASE extracts; Green: Fractions of the decoction; R2X = 0.413; Ellipse: Hotelling's T2 (95%).

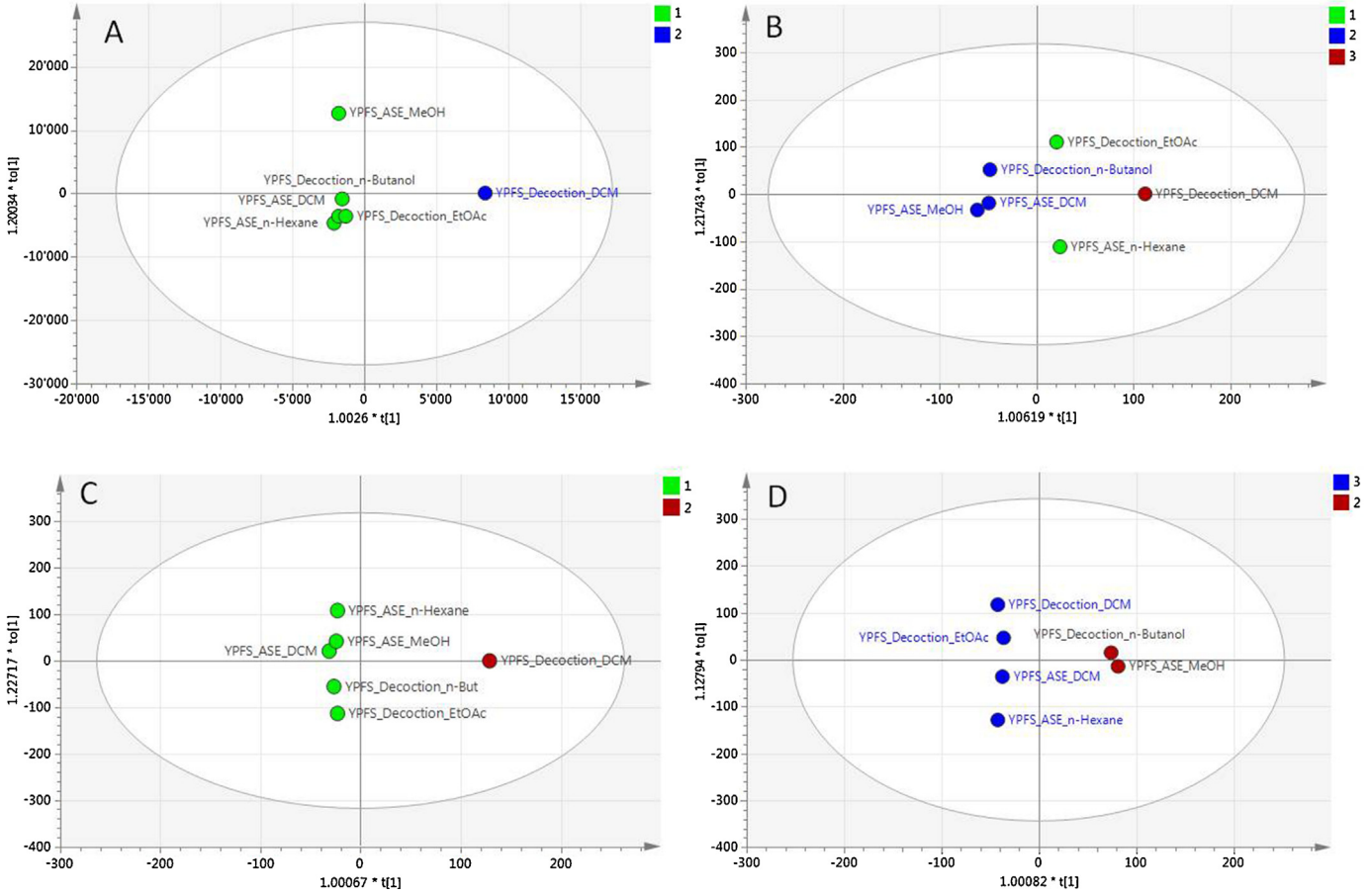

Fig. 5. t_1/to_1 Score plots of the OPLS-DA of UPLC-QTOF MS data of YPFS in correlation with the pharmacological effects; A: TNF-α expression (R2(cum) = 0.996, Q2(cum) = 0.607); B: IL-1β expression (R2(cum) = 0.993, Q2(cum) = 0.588); C: IFN-γ expression (R2(cum) = 0.998, Q2(cum) = 0.815); D: IL-4 expression (R2(cum) = 0.998, Q2(cum) = 0.789); 1: active extracts; 2: moderately active extracts; 3: inactive extracts; Ellipse: Hotelling's T2 (95%).

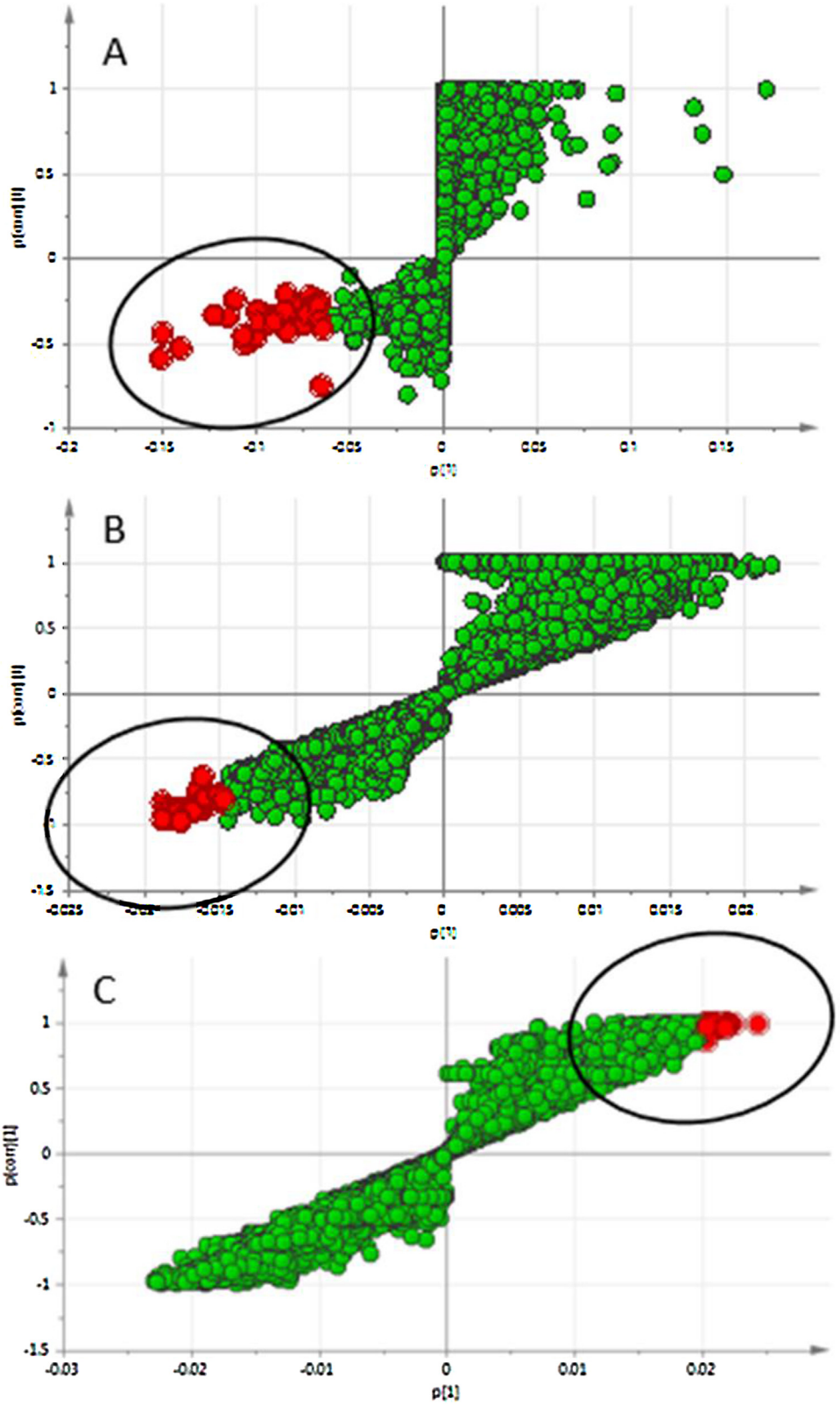

Fig. 6. S-plot from the OPLS-DA model using LC-QTOF MS data and pharmacological data from the evaluation of the inhibition of the expression of TNF-α [active and moderately active extracts] (A), IFN-γ [active and moderately active extracts] (B) and IL-4 [moderately active and inactive extracts] (C); interesting compounds ("hits") were marked in red.

isolate these compounds and to verify these results. Mass spectra of the components are shown in Fig. A11–14 in the Supplementary Material. It was not possible to identify components which contribute to the inhibitory effect on the expression of IL-1β in U937 cells, because based on the low data number the software calculated not significant values. Furthermore, none of the "hits" which

seem to be relevant for the inhibition of IFN-γ expression could be identified by UNIFI [37].

4. Conclusion

In the present study, chemical profiles of YPFS were obtained by a HPLC-DAD-ESI-MS/MS method in positive ion mode. As a result, 24 compounds including furocoumarins and furochromones of RS, isoflavonoids and triterpenoid saponins of RA, and sesquiterpene lactones of RAM were characterized based on their *m*/*z* data and fragmentation patterns, and assigned in the chemical profiles of YPFS. Nine constituents of RS, ten constituents of RA, and five constituents of RAM have been assigned in the formula. The traditional decoction of YPFS contained more components than the ASE extracts which indicates that the boiling process is an efficient method for extracting compounds in YPFS. The HPLC-DAD-ESI-MS/MS method can be used for quality control (QC) of YPFS and provides a reference method for QC of the three individual herbs. The immunomodulatory data revealed that all extracts and fractions of YPFS significantly inhibited the expression of TNF-α, IFN-γ and IL-1β in U937 cells, while inhibition of IL-4 was consistently low. For metabolomics, UPLC/QTOF MS analysis was performed. PCA of the processed data showed a clear separation between the ASE extracts and the fractions of the decoction of YPFS. These results indicated that the extraction method has a great influence on the chemical profiles of the herbs. The abundance of the peaks was linked to the pharmacological activities by OPLS-DA which led to the identification of compounds ("hits") that are predicted to be highly relevant for the immunomodulatory effects of YPFS. Two of the constituents in YPFS which seem to be mainly responsible for the inhibition of TNF-α expression were identified as 5-O-methylvisammioside and *prim*-O-glucosylcimifugin. Two of the "hits" which seem to be most likely relevant for the inhibitory activity on IL-4 expression could be tentatively identified as astragaloside V and blestritin B. To conclude, multivariate data analysis by means of OPLS-DA is a promising strategy for the tentative identification of bioactive components in herbs as well as in formulations. However, further investigations, particularly the isolation and unambiguous identification of the "hits", as well as the pharmacological testing of these compounds are necessary to verify the results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at **http://dx.doi.org/10.1016/j.jpba.2017.03.049**.

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