

Anticomplementary principles of a Chinese multiherb remedy for the treatment and prevention of SARS

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Received 20 August 2007; received in revised form 20 December 2007; accepted 2 February 2008

Available online 17 February 2008

Abstract

Aim of the study: To elucidate the anticomplementary principles of a Chinese multiherb remedy used for the treatment and prevention of SARS, which contains five ingredients of *Herba Houttuyniae*, *Flos Chrysanthemi Indici*, *Herba Artemisiae Scopariae*, *Herba Eupatorii* and *Fructus Tsaoko*.

Materials and methods: The anticomplementary activity was evaluated from hemolytic assays through the classical pathway (CP) and the alternative pathway (AP) of complement system *in vitro*. Compounds were isolated using bioactivity-guided fractionation and tested *in vitro* for their complement-inhibiting properties on the CP and AP. HPLC-DAD-ESI-MS was used to assign the isolated compounds in the five ingredient herbs.

Results: 15 compounds, including chlorogenic acid (**1**), rutin (**2**), hyperoside (**3**), *p*-hydroxyacephenone (**4**), scopoletin (**5**), quercitrin (**6**) (*3R,4R,6S*)-3,6-dihydroxy-1-menthene (**7**), acaciiin (**8**), scoparone (**9**), luteolin (**10**), quercetin (**11**), apigenin (**12**), acetin (**13**), aristolactam (**14**), and apigenin-7,4'-dimethyl ether (**15**) were isolated and identified. Nine flavonoids (**2**, **3**, **6**, **8**, **10–13**, **15**) were found to show inhibitory effects towards the CP and AP of complement system, luteolin (**10**) was the most potent with the CH_{50} and AP_{50} values of 0.19 and 0.17 mM. The bioactive flavonoids were mainly derived from *Herba Houttuyniae*, *Flos Chrysanthemi Indici* and *Herba Artemisiae Scopariae*.

Conclusions: A Chinese multiherb remedy used for the treatment and prevention of SARS has robust inhibitory effect on complement system through the CP and AP, and its anticomplementary activity could be attributed to the flavonoids present in some ingredient herbs. Luteolin could be a potential anticomplementary agent.

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Keywords: Anticomplementary activity; Traditional Chinese medicine; Flavonoid; Luteolin; SARS

1. Introduction

The complement system plays a crucial role in the elimination of invading pathogens and generation of an optimal host response. It can be activated by a cascade mechanism of the classical pathway (CP), alternative pathway (AP), or the lectin pathway (Carroll and Fischer, 1997). However, a wealth of data has clearly demonstrated that undesired activation of complement is responsible for the initiation, amplification or perpetuation of tissue damage and inflammation in a number of pathological situations (Morgan and Harris, 2003). Severe acute respiratory syndrome (SARS), an emerging infectious disease with severe mortality, affected many countries in 2003 and

the coronavirus is the etiologic agent (Peiris et al., 2003). In terms of pathogenesis of SARS, viral load in the first phase may in turn result in host inflammatory response during the second phase. The SARS complications are associated with severe inflammatory tissue destruction due to excessive activation of the innate immune system and a notable proportion of the patients progressed to septic shock, acute respiratory distress syndrome (ARDS) and multiple organ dysfunction (MODS) in the later phase of viral infection (Li et al., 2003). Considerable clinical and experimental evidence implicated that complement appears to be involved in the pathogenesis of these late post-viral complications, which could even be predicted by increased complement activations (Sahu and Lambris, 2000; Abe, 2006; Sarma et al., 2006). Hence, antiviral agents combined with inhibition of complement cascade reaction may represent an attractive and efficient therapeutic strategy for SARS.

Traditional Chinese medicine (TCM) played an important role in the fights to SARS and was reported by practitioners

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to be very effective (Lau et al., 2005). A multiterb remedy, which contains five ingredients of Herba Houttuyniae, Flos Chrysanthemi Indici, Herba Artemisiae Scopariae, Herba Eupatorii, and Fructus Tsaoko, was recommended for the treatment and prevention of SARS by State Administration of TCM of the People's Republic of China. According to TCM, Herba Houttuyniae, Flos Chrysanthemi Indici, and Herba Artemisiae Scopariae have functions of eliminating evil-heat and detoxification; Herba Eupatorii and Fructus Tsaoko are commonly used for dispersing dampness. Pharmacological experiments showed that these five-ingredient herbs possess direct inhibitory activity against virus, bacteria, and inflammation (Hayashi et al., 1995; Sun et al., 1995; Moon et al., 2004; Xie et al., 2004; Cheng et al., 2005).

Since complement inhibitory agents could be beneficial for the treatment and prevention of SARS, some medicinal plants and multiterb remedies were assessed *in vitro* for their complement-inhibiting properties in our preliminary study. The ethanolic extract of this five-herb remedy was found to possess reproducible activity against the CP and AP of complement system *in vitro*. Bioactivity-guided fractionation showed that the ethyl acetate-soluble fraction had significant anti-complementary activity. Further chromatographic separation of the active fraction led to the isolation of 15 compounds, including 9 flavonoids, 2 coumarins, 1 alkaloid, 1 monoterpene, 1 organic acid and 1 ketone. Flavonoids were found to show significant inhibitory effect towards the CP and AP of complement system. In addition, high performance liquid chromatography coupled with diode array detector and electrospray ionization-mass spectrometry (HPLC–DAD–ESI–MS) was applied to identify and track these compounds in the ingredient herbs. The isolation and anticomplementary activity of these compounds and the HPLC–DAD–ESI–MS analysis are reported herein.

2. Materials and methods

2.1. Instrumentation

Optical rotations were measured at 20 °C with a JASCO P-1020 polarimeter in methanol. ESI-MS was measured on an Agilent SL G1946D single quadrupole mass spectrometer (USA) equipped with an ESI source. ¹H (400 MHz) and ¹³C (100 MHz) NMR spectra were recorded on a Bruker DRX 400 spectrometer in CDCl₃, acetone-*d*₆ or DMSO-*d*₆ as solvents, the chemical shifts being represented as ppm. High-speed refrigerated centrifuge (Jouan Co., France) and a spectrophotometer (Wellscan MK3, Labsystems Dragon) were used in assay for anticomplementary activity. The HPLC system was an Agilent Technologies Series 1100 (USA), consisting of an automatic sampler injector, a binary pump, continuous vacuum degasser, a column heater-cooler, a variable wavelength diode array detector and an Agilent SL G1946D single quadrupole mass spectrometer (USA) equipped with an ESI source. All the operations, the acquiring and analysis of data were controlled by Chemstation software (Agilent Technologies, USA).

2.2. Materials and reagents

The plant materials were all purchased from Shanghai Hua-Yu Chinese Materia Medica Co. Ltd. and identified as Herba Houttuyniae (the aerial part of *Houttuynia cordata* Thunb., Saururaceae), Flos Chrysanthemi Indici (the flowers of *Chrysanthemum indicum* L., Compositae), Herba Artemisiae Scopariae (the aerial part of *Artemisia scoparia* Waldst. et Kit., Compositae), Herba Eupatorii (the aerial part of *Eupatorium fortunei* Turcz., Compositae) and Fructus Tsaoko (the ripe fruits of *Amomum tsao-ko* Crevost et Lemaire, Zingiberaceae) by one of the authors (Dr. Ting Zhang). The voucher specimens have been deposited in the Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, PR China.

Silica gel and pre-coated TLC were obtained from Qingdao Haiyang Chemical Factory (Qingdao, China). Sephadex LH-20 and ODS were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Acetonitrile was of HPLC grade from Honeywell (USA), acetic acid and the other reagents were of analytical grade from Shanghai Chemical Factory (Shanghai, China). Water was purified using a Milli-Q water purification system (Millipore, USA).

Guinea pigs and New Zealand white rabbits were purchased from Laboratory Animals Research Institute of Fudan University (Shanghai, China). Animal experiments were conducted in accordance with current ethical regulations for animal care and use at Fudan University. Normal human sera (NHS) were obtained from three male donors of healthy blood, with an average age of 20 years ranging from 18 years to 22 years, after written informed consent to participate in this study. This study received approval from the ethics committee of Fudan University.

Sheep erythrocytes collected in Alsevers' solution were purchased from Shanghai Kang-Run Biotech Co. Ltd. Anti-sheep erythrocyte antibody was from Prof. Yunyi Zhang (Department of Pharmacology, Fudan University, Shanghai, China). Rabbit erythrocytes were obtained from the ear vein of New Zealand white rabbits. Serum from guinea pig and NHS were used as source of complement for the CP and AP, respectively. Heparin (sodium salt, 160 IU/mg) was purchased from Shanghai Aizite Biotech Co. Ltd. Veronal buffer saline (VBS), pH 7.4, VBS containing 0.5 mM Mg²⁺ and 0.15 mM Ca²⁺ (VBS²⁺), and VBS containing 5 mM Mg²⁺ and 8 mM EGTA (VBS–Mg–EGTA) were prepared as described in the literature for the CP and AP, respectively (Nelson et al., 1966).

2.3. Extraction and isolation

Herba Houttuyniae, Flos Chrysanthemi Indici, Herba Artemisiae Scopariae, Herba Eupatorii, and Fructus Tsaoko were pulverized and mixed well in a ratio of 15:6:15:10:3 (weight of dried herbs), according to the instructions of State Administration of TCM, PR China. The mixture (19.8 kg) was extracted with 95% EtOH (50 L 3×) at room temperature. Removal of EtOH under reduced pressure afforded a dark green residue (900 g). The residue was suspended in

Table 1
Anticomplementary activity of the different extracts and 15 compounds 1–15 through the CP and AP

Test samples	CH ₅₀ value (mM) ^a	AP ₅₀ value (mM) ^a
Ethanol extract (mg/mL)	0.83 ± 0.02	1.24 ± 0.07
petroleum ether fraction (mg/mL)	Haemolysis	Haemolysis
EtOAc fraction (mg/mL)	0.45 ± 0.02	0.96 ± 0.06
<i>n</i> -BuOH fraction (mg/mL)	2.88 ± 0.13	2.03 ± 0.27
1	NE ^b	NE
2	0.58 ± 0.01	0.42 ± 0.04
3	1.72 ± 0.01	0.25 ± 0.02
4	10.18 ± 1.51	NE
5	NE	NE
6	0.53 ± 0.02	0.32 ± 0.04
7	NE	NE
8	2.53 ± 0.05	NE
9	NE	NE
10	0.19 ± 0.02	0.17 ± 0.04
11	0.50 ± 0.02	1.02 ± 0.03
12	3.40 ± 0.06	2.02 ± 0.21
13	4.32 ± 0.86	NE
14	7.57 ± 0.52	NE
15	5.76 ± 0.91	NE
Heparin sodium salt (μg/mL)	38.50 ± 1.78	33.86 ± 2.28

^a Data were expressed as mean ± S.E. of three experiments.

^b This compound has no inhibitory effect at the maximal concentration.

water (1000 mL) and successively partitioned with petroleum ether (60–90 °C), EtOAc and *n*-BuOH (1000 mL 3×) to give three extracts (405 g, 200 g, and 100 g, respectively). The highest anticomplementary activity was observed in EtOAc extract according to the results of hemolytic assay on the CP and AP used as biological activity guide (Table 1). The EtOAc-soluble fraction (190 g) was subjected to column chromatography over silica gel (200–300 mesh, 1500 g), eluted with a gradient of petroleum ether–acetone (95:5, 90:10, 80:20, 70:30, 60:40, 50:50; 4500 mL for each eluent) to yield six fractions (I–VI). Fraction II (15 g) was subjected to column chromatography (silica gel, 300–400 mesh, petroleum ether–acetone, 8:2) and preparative TLC (silica GF₂₅₄, 10–40 μ, petroleum ether–acetone, 7:3) to give compounds **13** (50 mg), **14** (26 mg), **15** (5 mg). Fraction III (9 g) was subjected to column chromatography (silica gel, 300–400 mesh, petroleum ether–acetone, 7:3; Sephadex LH-20, chloroform–methanol, 1:1) to yield compounds **9** (12 mg), **10** (12 mg), **11** (24 mg), and **12** (28 mg). Fraction IV (11 g) was chromatographed on silica gel (300–400 mesh) column with chloroform–methanol (6:4), on Sephadex LH-20 column with chloroform–methanol (1:1) and on RP-18 column with methanol–water (8:2), to provide compounds **4** (28 mg), **7** (21 mg), and **8** (23 mg). Fraction V (16 g) was loaded to column chromatography (silica gel, 300–400 mesh, chloroform–methanol, 6:4; Sephadex LH-20, chloroform–methanol, 1:1; RP-18, methanol–water, 7:3), and preparative TLC (silica GF₂₅₄, 10–40 μ, chloroform–methanol, 7:3) to give compounds **5** (17 mg) and **6** (19 mg). Purification of fraction VI (21 g) on silica gel (300–400 mesh, chloroform–methanol, 1:1), Sephadex LH-20 (chloroform–methanol, 1:1; methanol–water, 1:1), RP-18 (methanol–water, 6:4) column chromatography and prepara-

tive TLC (silica GF₂₅₄, 10–40 μ, chloroform–methanol, 7:3) afforded compounds **1** (28 mg), **2** (14 mg), and **3** (30 mg).

The purities of these compounds were determined to be more than 98% by normalization of the peak areas detected by HPLC with DAD under chromatographic conditions described in Section 2.5.2.

2.4. Anticomplementary activity

2.4.1. Anticomplementary activity through the classical pathway

Assays were based on the haemolysis of erythrocytes by the membrane generated after complement activation. Based on Mayer's modified method (Kabat and Mayer, 1964), sensitized erythrocytes (EA) were prepared by incubation of sheep erythrocytes (4.0 × 10⁸ cells/mL) with rabbit anti-sheep erythrocyte antibody in VBS²⁺. Each compound was dissolved in DMSO then diluted with VBS²⁺ until the final concentration of DMSO was <1%, and then diluted to various concentrations for assay. Final concentration of DMSO (1%) showed no interference with the anticomplementary activity. The 1:80 diluted serum of guinea pig was chosen to give sub-maximal lysis in the absence of complement inhibitors. Various dilutions of tested samples in 1% DMSO (200 μL) were preincubated with 200 μL serum of guinea pig at 37 °C for 10 min. Then, 200 μL EA was added and the mixture was incubated at 37 °C for 30 min. In the same conditions, sample control, 200 μL dilution of each sample in 400 μL VBS²⁺, lysis control for extracts, 200 μL dilution of each extract and 200 μL EA in 200 μL VBS²⁺, all blanks, 200 μL EA in 400 μL VBS²⁺ containing 1% DMSO and 100% lysis, 200 μL EA in 400 μL water were incubated to provide different assay controls. The reaction mixture was centrifuged immediately and 200 μL of the supernatant was transferred to a flat-bottomed microplate and the optical density was measured at 405 nm. Anticomplementary activity was determined as a mean of triplicate tests per concentration and expressed as the 50% inhibitory concentration (CH₅₀ value) from complement-dependent haemolysis of the control. Heparin sodium salt, a polyanionic glycosaminoglycan, was used as the positive control.

2.4.2. Anticomplementary activity through the alternative pathway

The activation of complement through the AP was measured in the Ca²⁺ free condition according to the Klerx's method (Klerx et al., 1983), and VBS–Mg–EGTA was used as buffer medium for assay. Each compound was dissolved in DMSO then diluted with VBS–Mg–EGTA until the final concentration of DMSO was <1%, and then diluted to various concentrations for assay. The 1:10 diluted NHS was chosen to give sub-maximal lysis in the absence of complement inhibitors. After preincubation of dilutions of each sample (150 μL) with 1:10 diluted NHS (150 μL) at 37 °C for 10 min, the residual complement of the mixtures was measured in terms of the haemolysis of 200 μL rabbit erythrocytes (ER 3.0 × 10⁸ cells/mL). Controls for sample control, lysis control for extracts, all blanks, and 100% lysis were included. Anticomplementary activity was expressed as

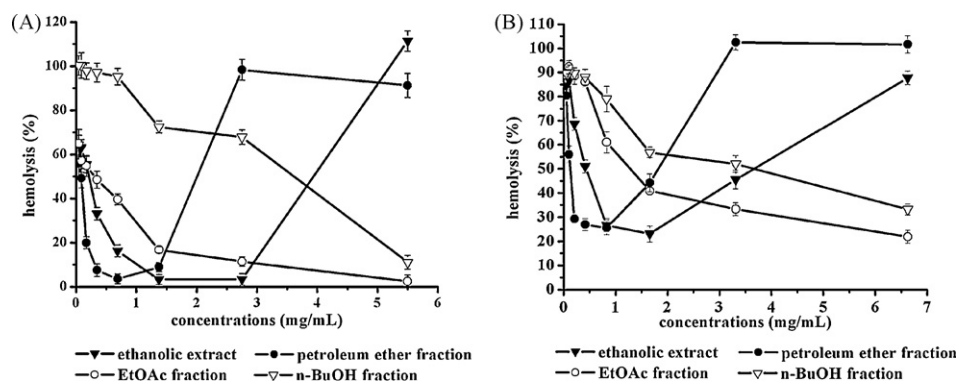


Fig. 1. Inhibitory effect of the extracts on the CP (A) and AP (B) of complement system.

the 50% inhibitory concentration (AP_{50} value). Heparin sodium salt was used as the positive control.

2.5. HPLC–ESI–MS analysis

2.5.1. Samples preparation

The five-ingredient herbs were individually dried and pulverized. Approximately, 1.0 g of each pulverized sample was accurately weighted and extracted twice for 30 min with 50 mL methanol in ultrasonic bath. The combined solution was evaporated to dryness and the residue was dissolved in a 10 mL flask with methanol. The resultant solution was filtered through a 0.45- μ m syringe filter and stored at -4°C , 20 μ L for HPLC–DAD–ESI–MS analysis.

2.5.2. Chromatographic conditions

LC separation was performed on a C-18 column (250 mm \times 4.6 mm \times 5 μ m, Phenomenex Luna, USA) at temperature of 30°C with binary mobile phase consisted of 0.1% aqueous acetic acid (A) and acetonitrile (B). All solvents were filtered through a 0.45- μ m nylon filter prior to use. The flow-rate was kept constantly at 1 mL/min for a total running time of 35 min and the outflows were firstly entered DAD, subsequently entered MS detector. A gradient elution program was used according to the following profile: 0–25 min, 5–55% B, 25–32 min 55–90% B, 32–35 min hold on 90% B. The sample injection volume was 20 μ L.

The DAD detector was set at 210, 254, 280, 327, 360 nm with 420 nm as reference wavelength, both at 4 nm bandwidth, with full spectral scanning 200–400 nm and 0.5 nm resolution.

The MS used two channels of detection for analysis in positive mode. The conditions of MS analysis were as follows: drying gas (N_2) flow-rate, 12 L/min; drying gas temperature, 350°C ; nebulizing gas (N_2) pressure, 45 psi; capillary voltage, 4 kV; full scan range, 100–1000 m/z ; fragment, 70 V. The standard compounds and extracted samples were analyzed in selective ion monitoring (SIM) mode using the ESI source by monitoring the protonated molecular ions. For the analytes, selected ions monitoring for each compound was restricted to specific molecular weights (m/z 354.9, 610.8, 464.8, 137.0, 193.0, 171.1, 592.9, 206.9, 286.9, 270.9, 284.9, 298.9 by channel 1, m/z 448.9, 302.9, 279.9 by channel 2).

3. Results and discussion

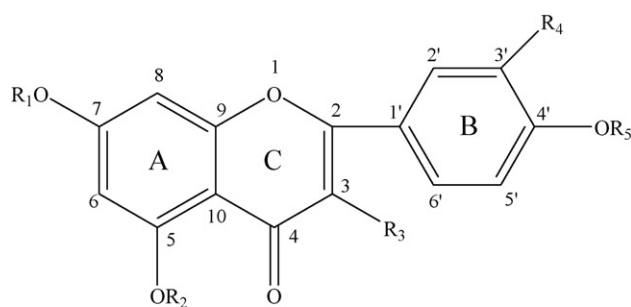
3.1. Bioactivity-guided fractionation and isolation of 15 compounds

The ethanolic extract showed anticomplementary activity with CH_{50} and AP_{50} values of 0.83 and 1.24 mg/mL, but resulted in haemolysis of the sheep and rabbit erythrocytes at higher concentrations of 2.75 and 1.66 mg/mL, respectively. The bioactivity-guided fractionation indicated that the petroleum ether fraction resulted in haemolysis of the sheep and rabbit erythrocytes at higher concentrations of 1.38 and 0.83 mg/mL, respectively. According to the results of lysis control without serum, both the ethanolic extract and the petroleum ether fraction definitely induced lysis when the doses for the assay were exceedingly large. The EtOAc and *n*-BuOH fractions inhibited both the CP and AP of complement system in a dose-dependent manner. The EtOAc fraction showed a more pronounced complement inhibition than the *n*-BuOH fraction, as measured by the diminished haemolysis of sensitized erythrocytes in the presence of complement factors (Fig. 1, Table 1). Thus, it was concluded that the main anticomplementary principles were enriched in the EtOAc fraction. Chromatographic separation of the EtOAc fraction yielded 15 compounds (1–15) and their structures were elucidated as chlorogenic acid (1) (Pauli et al., 1999), rutin (2) (Kazuma et al., 2003), hyperoside (3) (Wang et al., 2002), *p*-hydroxyacephenone (4) (Kai et al., 1999), scopoletin (5) (Sun et al., 2006), quercitrin (6) (Wang et al., 2002) (3*R*,4*R*,6*S*)-3,6-dihydroxy-1-menthene (7) (Maria and Cesar, 1991), acaciin (8) (Park et al., 1995), scoparone (9) (Tsukamoto et al., 1984), luteolin (10) (Miyazawa and Hisama, 2003), quercetin (11) (Miyazawa and Hisama, 2003), apigenin (12) (Miyazawa and Hisama, 2003), acetin (13) (Miyazawa and Hisama, 2003), aristolactam (14) (Kim et al., 2001) and apigenin-7,4'-dimethyl ether (15) (Li et al., 2001) on the basis of UV, MS, ^1H NMR and ^{13}C NMR spectral data (Fig. 2).

3.2. Anticomplementary activity of the compounds through the CP and AP

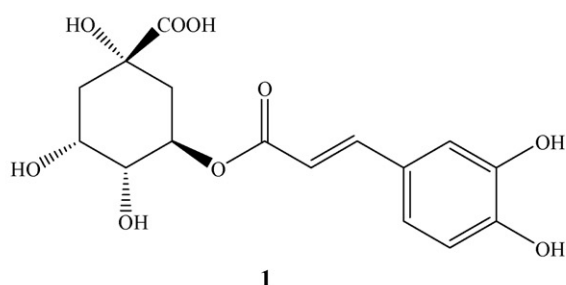
The 15 compounds were tested for their anticomplementary activity *in vitro* and the results obtained are summarized

Flavonoids

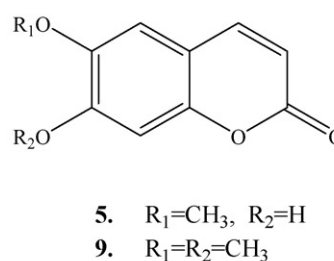


2. $R_1=R_2=R_5=H$, $R_3=O-glc-6-1$ rha, $R_4=OH$
3. $R_1=R_2=R_5=H$, $R_3=O-gla$, $R_4=OH$
6. $R_1=R_2=R_5=H$, $R_3=O-rha$, $R_4=OH$
8. $R_1=glc-6-1$ rha, $R_2=R_3=R_4=H$, $R_5=CH_3$
10. $R_1=R_2=R_3=R_5=H$, $R_4=OH$
11. $R_1=R_2=R_5=H$, $R_3=R_4=OH$
12. $R_1=R_2=R_3=R_4=R_5=H$
13. $R_1=R_2=R_3=R_4=H$, $R_5=CH_3$
15. $R_1=CH_3$, $R_2=R_3=R_4=H$, $R_5=CH_3$

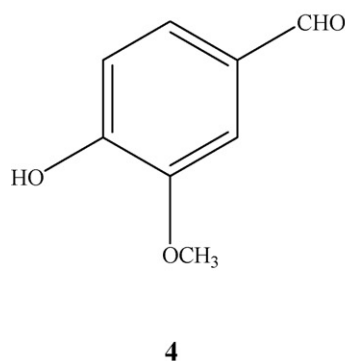
Organic acid



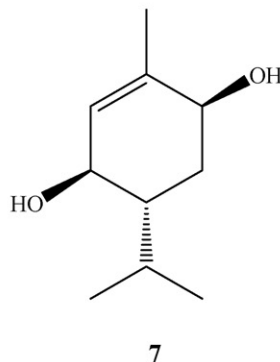
Coumarins



Ketone



Monoterpene



Alkaloid

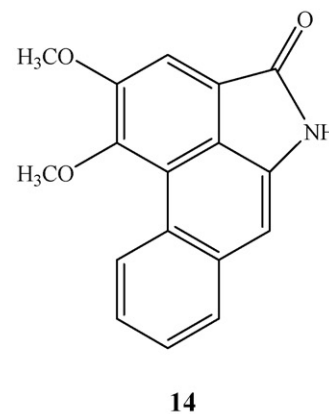


Fig. 2. The structure of 15 compounds 1–15.

in Table 1. Nine flavonoids (2, 3, 6, 8, 10–13, 15) showed a higher inhibitory effect towards the CP and AP of complement system than other compounds. *p*-Hydroxyacetophenone (4, ketone) and aristolactam (14, alkaloid) were weakly active on the CP but inactive on the AP. Chlorogenic acid (1, organic acid), scopoletin (5, coumarin), scoparone (9, coumarin) and (3*R*,4*R*,6*S*)-3,6-dihydroxy-1-menthene (7, monoterpene) had no inhibitory effect towards the CP and AP.

On the CP of complement system, luteolin (10) was found to have most strong anticomplementary activity with CH_{50} value of 0.19 mM. Rutin (2), quercitrin (6) and quercetin (11) were moderately active while hyperoside (3), acaciin (8), apigenin (12), acacetin (13) and apigenin-7,4'-dimethyl ether (15) exhibited

weak activity in this assay system. On the AP of complement system, rutin (2), hyperoside (3), quercitrin (6) and luteolin (10) showed higher activity with AP_{50} values of less than 1 mM, followed by quercetin (11) and apigenin (12). Acaciin (8), acacetin (13) and apigenin-7,4'-dimethyl ether (15) were completely inactive.

Flavonoids have been found to be the active principles of various medicinal plants against inflammatory diseases and considered as good sources for some kinds of inhibitors of the complement system, such as the flavonoids from *Morinda morindoides* (Cimanga et al., 1995), *Olea europaea* L. (Pieroni et al., 1996) and *Centaureum spicatum* (Shahat et al., 2003). In the present study, flavonoids were found to be the main anticomplementary constituents of a five-herb remedy for the treatment

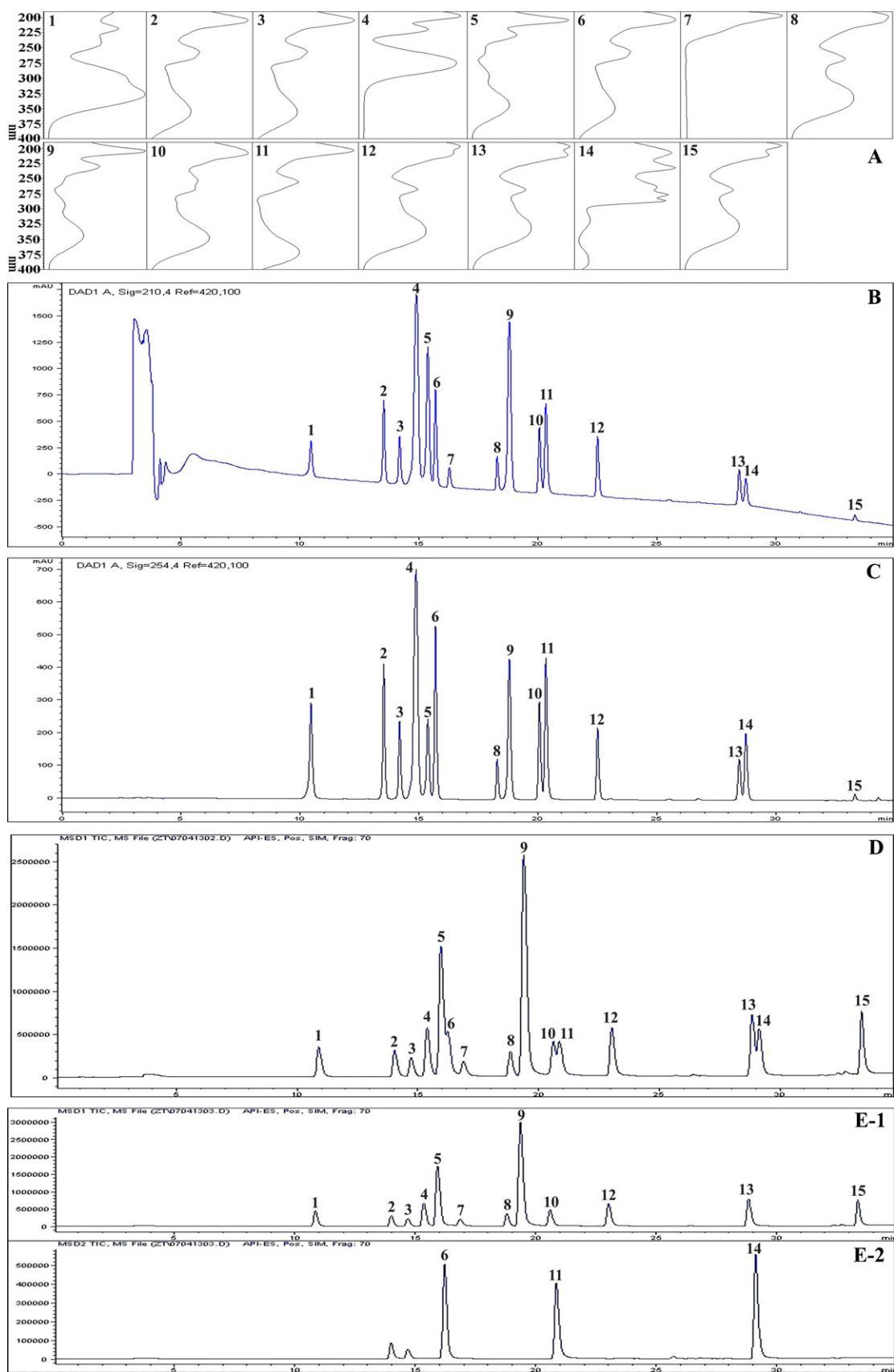


Fig. 3. The typical chromatographic profiles of the standard mixture solution. Peak numbers correspond to standard compounds presented in Fig. 2. (A) UV spectra of the target analytes; (B) HPLC chromatogram at 210 nm; (C) HPLC chromatogram at 254 nm; (D) HPLC-ESI-MS total ion current (TIC) chromatogram in positive ion mode; (E) HPLC-ESI-MS selected ion monitoring (SIM) chromatogram in positive ion mode using two channels: (E-1) channel 1; (E-2) channel 2.

and prevention of SARS, luteolin (**10**) was the most potent and could be served as a potential anticomplementary agent.

It is worthy to mention that the pathogenesis and symptoms of avian influenza A are similar to those of SARS (Ku and Chan, 1999; Jong and Hien, 2006). Hence, inhibition of complement system could also be an efficient therapeutic strategy for human avian influenza A. The studies on naturally occurring inhibitors of complement system would provide a great prospect of developing novel drugs for the treatment and prevention of SARS and avian influenza A, particularly from the traditional Chinese medicines.

3.3. Assignment of 15 compounds in the five-ingredient herbs

Owing to the chemical complexity and diversity of multiherb remedies, an analytical method of HPLC coupled with DAD and ESI-MS was used for simultaneous identification of the 15 compounds in the five-ingredient herbs.

Acetic acid was used as a mobile phase modifier as it significantly restrained the peak tailing of phenol compounds. The baseline separation of the target components was achieved within 35 min using a simple gradient elution programme (Fig. 3B and C). The positive mode was selected because MS spectra obtained from ESI single stage mass were simple, stable and easy to interpret, in which the quasi-molecular ions typically correspond to the base peak when a lower fragment voltage of 70 V was selected to generate rare fragmentation patterns. Under the chromatographic condition as described, protonated molecular ions $[M+H]^+$ could be detected for each compound. Selected ion monitoring (SIM), which had more sensitivity and specificity than full scanning, was further employed for detecting 15 compounds. The typical positive ESI mass spectra of compounds **1–15** were shown in Fig. 3D and E, as well as the retention times (t_R) and protonated molecular ions were summarized in Table 2.

Table 2

The retention times (t_R) and ESI-MS data in the positive ion mode (m/z) of compounds **1–15**

Compound	t_R (min) ^a	$[M+H]^+$ (m/z) ^b
1	10.844	354.9
2	14.003	610.8
3	14.687	464.8
4	15.352	137.0
5	15.919	193.0
6	16.212	448.9
7	16.859	171.1
8	18.798	592.9
9	19.366	206.9
10	20.596	286.9
11	20.852	302.9
12	23.016	270.9
13	28.833	284.9
14	29.124	279.9
15	33.369	298.9

^a Retention times in MS spectrum.

^b Protonated molecular ions.

The newly developed HPLC–DAD–MS assay was subsequently applied for simultaneous identification of compounds **1–15** in five-ingredient herbs. The representative chromatograms of the five herbs were shown in Fig. 4. The 15 compounds of the analyzed samples were identified by comparing the retention times, UV spectra and MS data with those obtained from the reference substances (Fig. 3, Table 2), as well as by spiking samples with the reference compounds. The analysis confirmed the presence of compounds **1–3**, **6**, **8**, **10–15** in Herba Houttuyniae with different contents. Compounds **1**, **3**, **8**, **10–13**, and **15** were detected in Flos Chrysanthemi Indici and compounds **1**, **4**, **5**, **6**, **9–13**, and **15** were found in Herba Artemisiae Scopariae. Meanwhile, compounds **4**, **7**, and compounds **1**, **3**, **4**, **8**, **13**, and **15** could be detected in Herba Eupatorii and Fructus Tsaoko, respectively (Table 3). According to the literatures (Haruna et al., 1986; Chu et al., 2004; Xie et al., 2004; Jiang and Gao, 2005; Xu et al., 2006), a plenty of flavonoids were present in Herba Houttuyniae, Flos Chrysanthemi Indici, and Herba Artemisiae Scopariae while sesquiterpenes and monoterpenes were distributed in Herba Eupatorii, which were in good agreement with the results of HPLC–DAD–MS analysis. Although there were few reports on flavonoids obtained from Fructus Tsaoko, several flavonoids were detected in the methanolic extract of Fructus Tsaoko with MS detector in the SIM mode in our investigation. Therefore, further isolation and NMR elucidation would be very helpful to confirm the presence of these flavonoids in Fructus Tsaoko.

As a result of HPLC–DAD–MS analysis, it was found that the flavonoids derived from Herba Houttuyniae, Flos Chrysanthemi Indici, and Herba Artemisiae Scopariae were mainly responsible for the inhibitory effect of the investigated multiherb remedy on the complement system.

Table 3

The assignment of 15 compounds **1–15** in five-ingredient herbs by HPLC–DAD–ESI–MS analysis

Compound	Herb ^a				
	HH	FCI	HAS	HE	FT
1	+	+	+	ND ^c	+
2	+	ND	ND	ND	ND
3	+	+	ND	ND	+
4	ND	ND	+	+	+
5	ND	ND	+	ND	ND
6	+	ND	+	ND	ND
7	ND	ND	ND	+	ND
8	+	+	ND	ND	+
9	ND	ND	+	ND	ND
10	+	+	+	ND	ND
11	+	+	+	ND	ND
12	+	+	+	ND	ND
13	+	+	+	ND	+
14	+	ND	ND	ND	ND
15	+	+	+	ND	+

^a Herbs: HH, Herba Houttuyniae; FCI, Flos Chrysanthemi Indici; HAS, Herba Artemisiae Scopariae; HE, Herba Eupatorii; FT, Fructus Tsaoko.

^b (+): Detected.

^c Not detected.

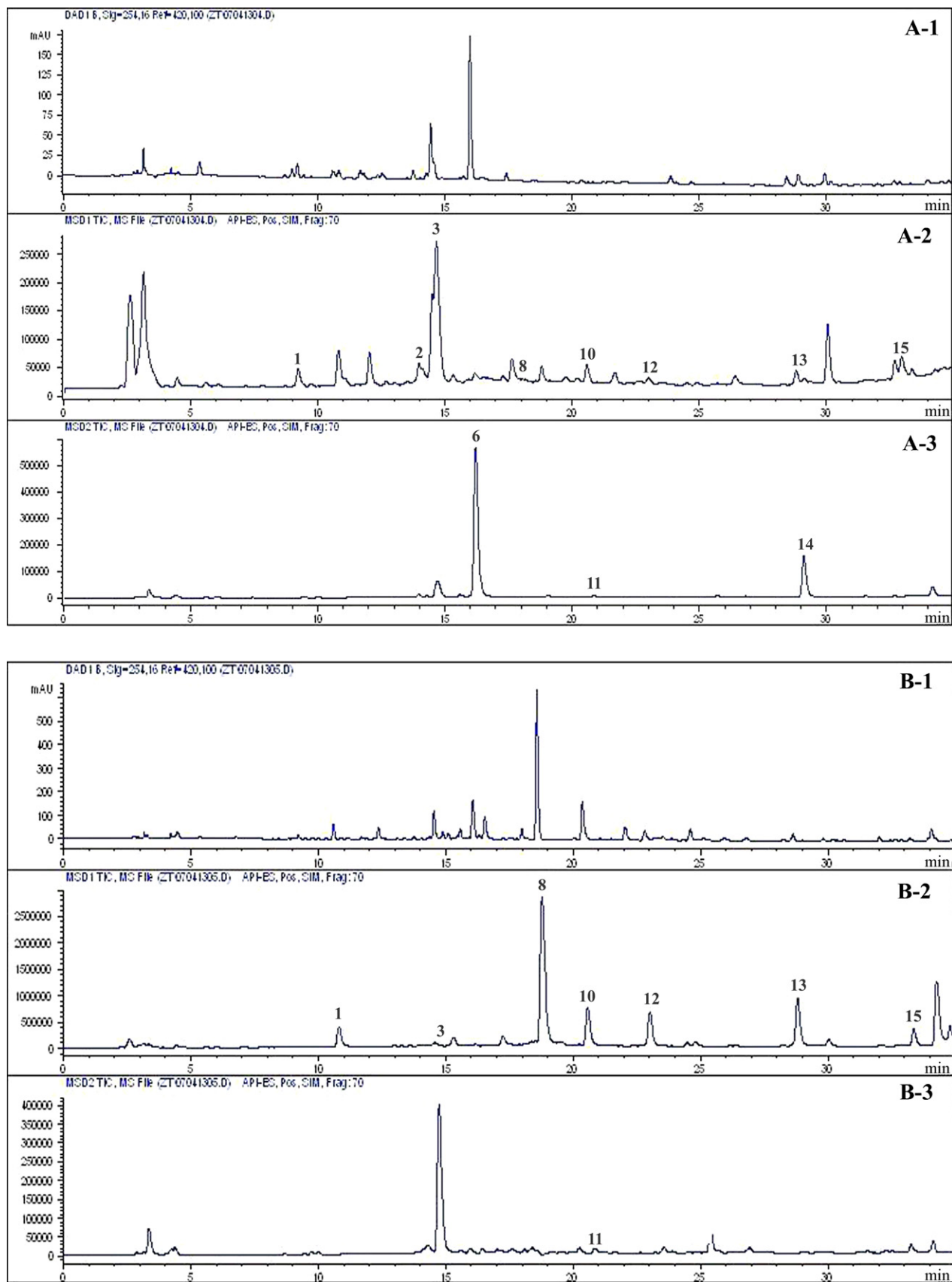


Fig. 4. The chromatographic profiles of the five-ingredient herbs. Peak numbers correspond to standard compounds presented in Fig. 2. (A) Herba Houttuyniae; (B) Flos Chrysanthemi Indici; (C) Herba Artemisiae Scopariae; (D) Herba Eupatorii; (E) Fructus Tsaoko. (1) HPLC chromatogram at 254 nm; (2) HPLC-ESI-MS SIM chromatogram using channel 1; (3) HPLC-ESI-MS SIM chromatogram using channel 2.

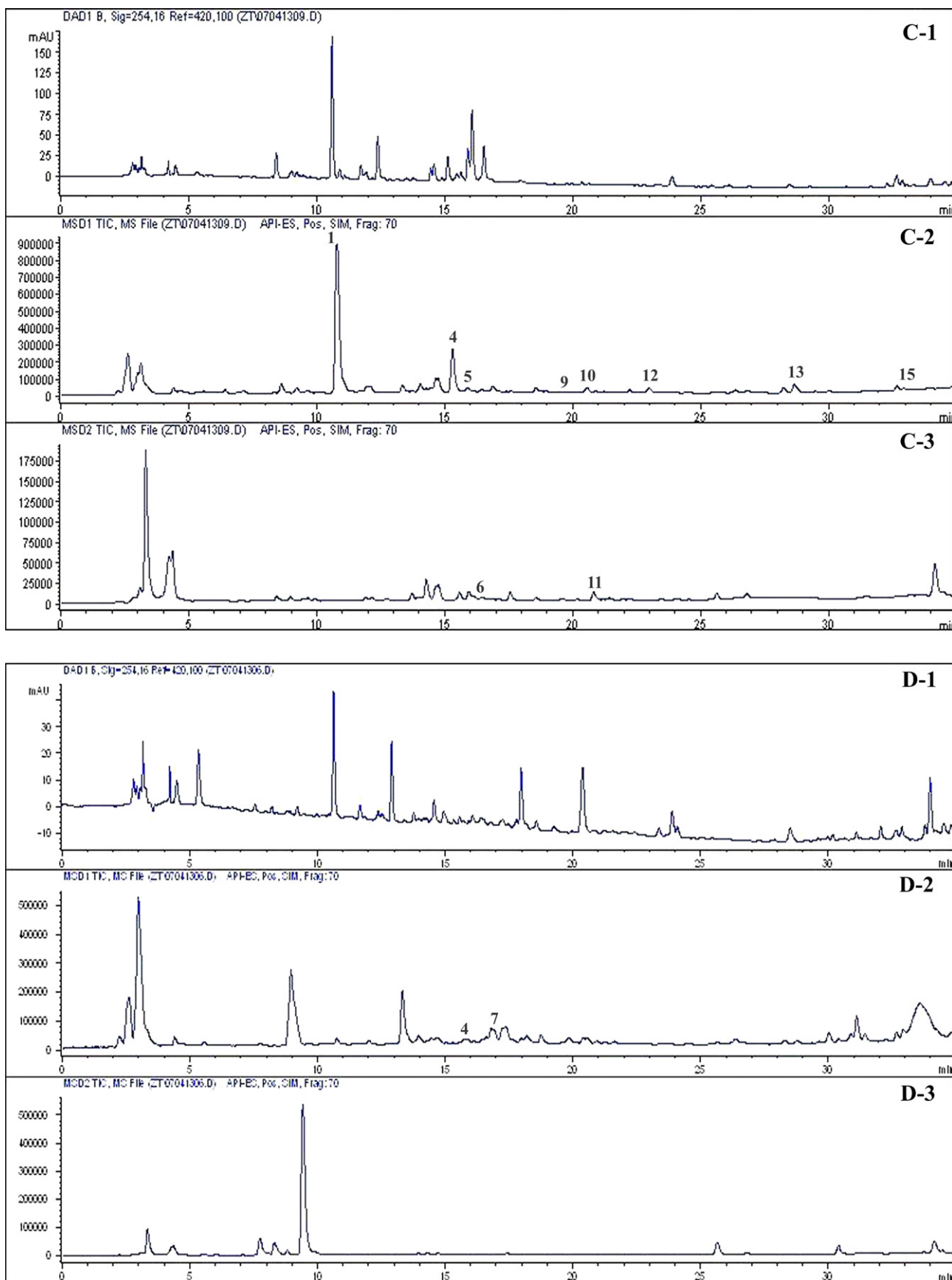


Fig. 4. (Continued)

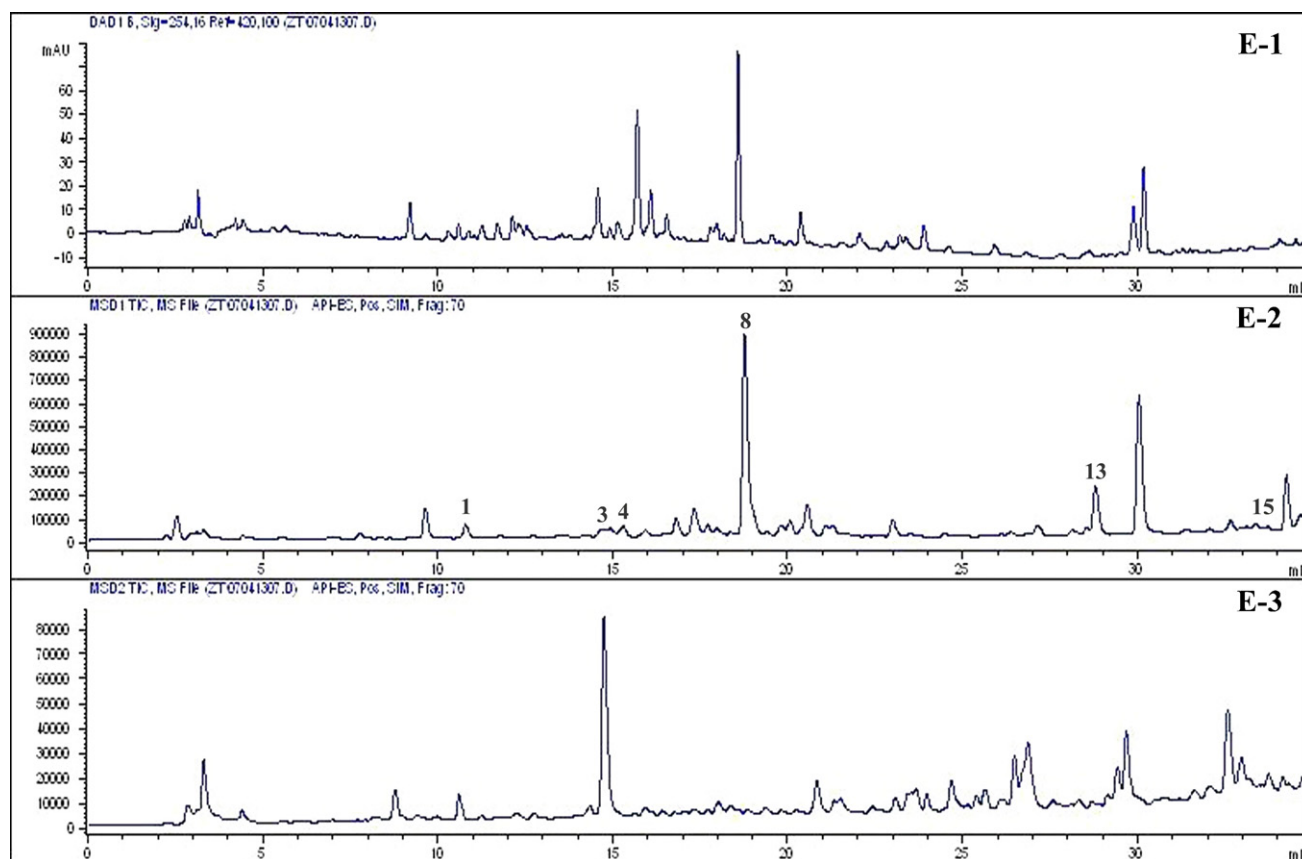


Fig. 4. (Continued).

4. Conclusions

Our study has demonstrated that the five-herb remedy, which was used for the treatment and prevention of SARS, has robust inhibitory effect on complement system through the CP and AP. The anticomplementary activity of this remedy could be attributed to the flavonoids present in some ingredient herbs. Luteolin could be a potential anticomplementary agent for its high activity potency and extensive abundance in medicinal plants. The present study provides an important basis for the medical application of the bioactive extract and compounds for the treatment of inflammatory diseases associated with inappropriate activation of complement system. However, further pharmacological and toxicological studies will be necessary to confirm this speculation.

Acknowledgements

This work was supported by grant from China Postdoctoral Science Foundation (20060390609). The authors are grateful to Prof. Yunyi Zhang at Fudan University for the helps in bioassay.

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