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# Phytochemical analysis of an antiviral fraction of Radix astragali using HPLC–DAD–ESI–MS/MS

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**Abstract** Radix astragali (*Huangqi* in Chinese) is a well-known traditional Chinese medicinal herb that has been used clinically in China for centuries to cure various diseases. To profile the antiviral constituents of this herb, a high-performance liquid chromatography–diode array detector–electrospray ionization–tandem mass spectrometry (HPLC–DAD–ESI–MS/MS) analytical method was developed to separate and determinate the active part of the extract of Radix astragali, which showed potent inhibition of several viruses. By comparing their retention time and MS data with those obtained from the authentic compounds and the published data, a total of 18 compounds, comprising 11 flavonoids and 7 saponins, were identified. This study provides an approach to rapidly characterize bioactive constituents in traditional Chinese medicines (TCMs).

**Keywords** Radix astragali · Antiviral activity · Flavonoid · Saponin · HPLC–DAD–ESI–MS/MS

## Introduction

Traditional Chinese medicines (TCMs) have been widely used for disease treatment and have made essential contributions to growth and prosperity in China for centuries; however, their remedial mechanisms are still not well understood. Until now, it is widely accepted that multiple ingredients are responsible for therapeutic effects of TCMs. Thus, it is necessary to clarify and analyze the bioactive constituents in TCMs to ensure their reliability in clinical application, to better understand the pharmacological basis of their action, and, moreover, to enhance product quality control [1].

Research in this field presents significant challenges due to the complex chemical composition of TCMs. Therefore, methods to identify the effective substances are in great demand [2]. Recently, high-performance liquid chromatography–mass spectrometry (HPLC–MS$^n$) has proved to be a modern, powerful tool for the identification of substances in TCM extracts [3–11]. This approach has an advantage in terms of sensitivity and specificity.

Radix astragali, the dried root of *Astragalus membranaceus* (Fisch.) Bge. or *Astragalus membranaceus* var. *mongholicus* (Bge.) Hsiao (family Leguminosae), known as *Huangqi* in China, is one of the most popular herbal medicines known worldwide to reinforce "Qi" (vital energy). Radix astragali possesses many biological functions, including hepatoprotective, antioxidative, antiviral, antihypertensive, and immunostimulant properties [12]. The roots have been reported to contain triterpene saponins, isoflavonoids, and polysaccharides [13, 14].

In the present research, we describe a study on phytochemical constituents of a bioactive fraction of Radix astragali, which showed antiviral activity. Using the developed HPLC–DAD–ESI–MS/MS technique, a total of 18 compounds, comprising 11 flavonoids and 7 saponins, were identified in the fraction (Fig. 1), based on the comparison of their UV and MS data with those of authentic compounds and published data. These results may help to

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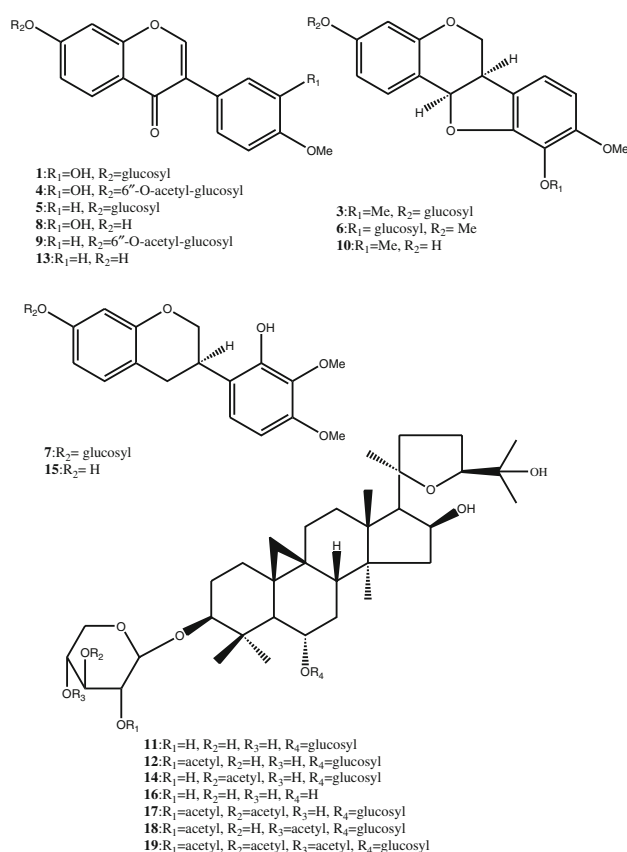650204 Kunming, People's Republic of China

**Fig. 1** Chemical structures of compounds identified from 70%F

gain a better understanding of the therapeutic basis of Radix astragali and establish a better quality control method for this age-old herbal medicine.

## Materials and methods

### Chemicals and reagents

Calycosin-7-O-β-D-glucopyranoside, calycosin, and formononetin were isolated from *Astragalus membranaceus* var. *mongholicus* (Bge.) Hsiao in our laboratory. The structures were elucidated by ^{1}H and ^{13}C NMR (a Varian Mercury-Vx BB instrument, Palo Alto, USA, operating at 300 MHz for ^{1}H, 75 MHz for ^{13}C). Purities were higher than 95% checked by HPLC methods, and astragaloside IV was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P. R. China). HPLC-grade acetonitrile was purchased from Merck Company (Darmstadt, Germany); ethanol and acetic acid were analytical grade from Beijing Reagent Co. Ltd. (Beijing, P. R. China). The $D_{101}$ macroporous resin (Tianjin Resin Factory, Tianjin, P. R. China). Water was doubly distilled in the laboratory.

### Material and sample preparation

The plant material of *Astragalus membranaceus* var. *mongholicus* was purchased from Beijing Tongrentang Traditional Chinese Medicine Co. Ltd. (Beijing, P. R. China) in December 2007 and identified by one of the authors (Prof. Chunlin Long) based on the morphological characteristics of the plant. The plant was extracted by refluxing with ethanol for 3 times (for 2 h each time). After filtration and concentration, the dried extract was redissolved in water and separated on $D_{101}$ porous resin columns. After eluting with $H_2O$ and 30% EtOH, then washing with 70% EtOH, the three fractions were concentrated by rotary vaporization under reduced pressure and, together with the initial EtOH extract, were prepared for the antiviral test.

### HPLC–MS instrument and conditions

Analyses were performed on an Agilent 1100 Series LC/MSD Trap system (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment, a diode array detector (DAD) and an ion-trap mass spectrometer with electrospray ionization interface, controlled by Agilent LC/MSD Trap Software. Zorbax SB-C18 (150 mm × 4.6 mm i.d., 5 μm) and a Zorbax ODS-C18 guard column (12.5 mm × 4.6 mm i.d., 5 μm) were used for separation. Solvents for the mobile phase were water–0.3% acetic acid (A) and acetonitrile (B). The gradient elution was: 0–30 min, linear gradient 20–60% B; 30–35 min, linear gradient 60–20% B. The flow rate was 0.8 ml/min and the column was operated at 25°C. Peaks were detected with the DAD and positive ion mode MS. Mass spectrometry was carried out in the scan mode from $m/z$ 100 to 1500. To obtain the maximum signal for each analyte, the MS conditions were optimized as follows: drying gas $N_2$, 8 l/min; capillary temperature, 325°C; pressure of nebulizer, 30 psi; capillary voltage, 15 V; and ion spray voltage, 3.5 kV. Samples for assay were dissolved in MeOH as 5 mg/ml solutions and centrifuged at 12000 rpm for 15 min to remove particles before injection.

### Cell and virus

The Hep-2 cells were incubated in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) containing 2 mmol/l glutamine, 100 U/ml penicillin and streptomycin, supplemented with 10% fetal calf serum (FCS) (Sino-American Biotech Co.) and maintained at 37°C and 5% $CO_2$ in a humidified incubator.

Parainfluenza virus, respiratory syncytial virus, herpes simplex virus-1, herpes simplex virus-2, adenovirus-3, and

adenovirus-7 were generously provided by the Institute of Traditional Chinese Medicine, China Academy of Traditional Chinese Medicine (Beijing, P. R. China). Viruses were propagated in Hep-2 cell monolayers and stored at −70°C in a freezer.

## Measurement of antiviral activity in vitro

The tested fractions were dissolved in DMSO to a concentration of 10 mg/ml. The working solution was prepared by twofold serial dilution (from 1/10 to 1/1280) with MEM and added into four wells at 200 μl/well. The normal cell was used as control. During 4 days' culture, cytopathic effects (CPE) in these cells were observed under a light microscope every day. The concentrations of the drug showing 50% cytotoxicity ($CC_{50}$) were determined by using the Reed–Muench method.

Hep-2 cells were grown to confluence, and then infected by the virus at the titer of $100 \times CCID_{50}$ (cell culture infectious dose 50%). Virus-containing media were exchanged to fresh media following a 1.5-h-long virus absorption period, and the tested fractions were added into culture media at serial dilutions. Infected cells receiving no drugs served as the virus control. When 100% CPE was observed at 48–96 h postinfection in the cells of the virus control, the drug concentration that achieved 50% inhibition to the observed CPEs was determined as $IC_{50}$, and calculated from three independent assays. The selectivity index (SI), or the ratio of $CC_{50}$ to $IC_{50}$, was also calculated to show the antiviral activity of the test fractions. Ribavirin was used as positive control.

## Results and discussion

### Bioassays for antiviral activity

Antiparainfluenza virus activity test was measured using the EtOH extract and fractions eluted from $D_{101}$ column chromatography using water, 30% EtOH, and 70% EtOH. The $IC_{50}$ values of these four parts were $20.7 \pm 5.2$, $135.2 \pm 4.6$, $48.4 \pm 3.9$, and $15.8 \pm 1.2$ μg/ml, respectively. Accordingly, the SI of the four parts were $5.8 \pm 0.6$, $1.5 \pm 0.4$, $3.1 \pm 0.3$, and $8.5 \pm 0.6$, respectively. This indicated that the 70% EtOH-eluted fraction from $D_{101}$ column chromatography (70%F) is the most active part of the herb drug. The successive antiviral activities of 70%F are presented in Table 1. The results indicated that 70%F showed a moderate activity against respiratory syncytical virus, herpes simplex virus-1, and herpes simplex virus-2, a significantly inhibitory activity to the adenovirus-3, adenovirus-7, and parainfluenza virus. These results suggested that 70%F is the antiviral principle of the herb medicine.

### HPLC–DAD–ESI–MS/MS analysis of 70%F

An HPLC–DAD–ESI–MS/MS method was developed to determine the phytochemical constituents of 70%F. As shown in Fig. 2a, 12 major peaks were separated under the HPLC conditions with UV detection at 280 nm. The chromatogram of MS total ion current (TIC) in positive mode is shown in Fig. 2b. Not all compounds were detected at 280 nm in the UV detector, e.g., due to a lack of conjugated systems in the saponins in the extract; however, in the TIC chromatogram, 19 major peaks were detected. Compounds **1**, **8**, **11**, and **13** were unequivocally determined to be calycosin-7-$O$-$\beta$-$D$-glucopyranoside, calycosin, astragaloside IV, and formononetin, respectively, by comparison with authentic compounds. Compound **2** was believed to be an unidentified minor flavonoid due to its low concentration in the extract and poor separation from other compounds. The other compounds were identified based on the UV adsorption, $m/z$ value, MS/MS characteristics, and retention time rule of each peak compared with the published data.

Compounds **3** and **6** are a pair of isomers. Both of them gave a $[M + H]^+$ ion at $m/z$ 463, and their $MS^2$ spectra yielded an ion at $m/z$ 301, which suggested the presence of

**Table 1** Antiviral activity of 70%F in Hep-2 cells

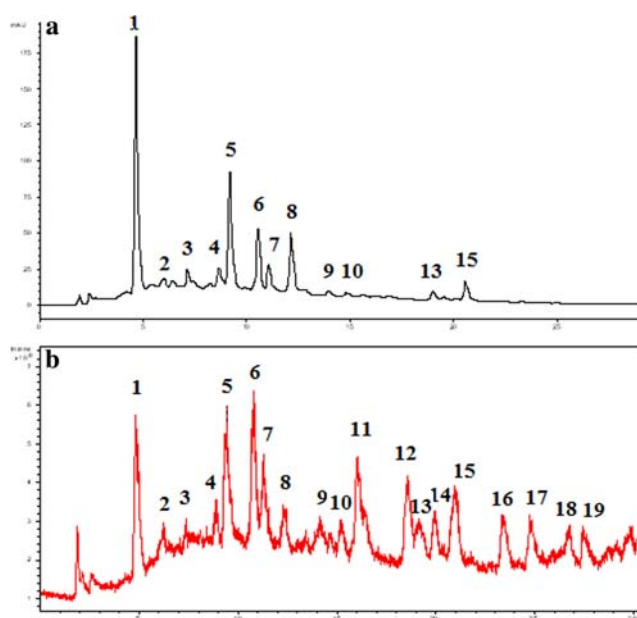| Virus | 70%F | | Ribavirin | |
|---|---|---|---|---|
| | $IC_{50}$ (μg/ml) | SI | $IC_{50}$ (μg/ml) | SI |
| Parainfluenza virus | $14.1 \pm 1.1$ | $8.5 \pm 0.6$ | $77.3 \pm 5.8$ | $9.4 \pm 0.7$ |
| Respiratory syncytial virus | $57.1 \pm 5.6$ | $2.1 \pm 0.2$ | $113.7 \pm 8.5$ | $6.4 \pm 0.4$ |
| Herpes simplex virus-1 | $41.4 \pm 4.7$ | $2.9 \pm 0.3$ | $143.3 \pm 5.8$ | $5.1 \pm 0.2$ |
| Herpes simplex virus-2 | $42.8 \pm 4.1$ | $2.8 \pm 0.3$ | $128.3 \pm 4.5$ | $5.7 \pm 0.2$ |
| Adenovirus-3 | $22.6 \pm 0.8$ | $5.3 \pm 0.2$ | $95.3 \pm 10.5$ | $7.7 \pm 0.7$ |
| Adenovirus-7 | $15.7 \pm 0.9$ | $7.6 \pm 0.5$ | $85.7 \pm 4.0$ | $8.5 \pm 0.4$ |

*SI* selectivity index

$n = 3$

**Fig. 2** HPLC–DAD–ESI–MS analysis of 70%F. **a** HPLC–UV chromatogram, monitored at 280 nm. **b** LC–positive ion ESI–MS total ion current (TIC) profile

a glucose losing 162 amu. The two compounds also had UV absorptions with maxima at 280 nm, which was consistent with the structure of pterocarpane. By examining the known pterocarpane glucosides in Radix astragali, only 9,10-dimethoxypterocarpane-3-*O*-β-D-glucopyranoside and 3,9-dimethoxypterocarpane-10-*O*-β-D-glucopyranoside were found and are consistent with the above data. According to the content difference and the retention time in HPLC reported before [3–10], compounds **3** and **6** were therefore tentatively identified as 3,9-dimethoxypterocarpane-10-*O*-β-D-glucopyranoside and 9,10-dimethoxypterocarpane-3-*O*-β-D-glucopyranoside, respectively.

Compound **4** exhibited a $[M + H]^+$ ion at *m/z* 489, i.e., 42 amu greater than that of compound **1**, and its MS/MS spectrum showed a prominent ion at *m/z* 285, which was the characteristic ion of calycosin. Additionally, **4**, just like compound **1**, had UV absorptions with maxima at 260 and 290 nm. Based on these data, the structure of **4** was proposed to be 6″-*O*-acetylcalycosin-7-*O*-β-D-glucopyranoside [5].

Compound **5** yielded a $[M + H]^+$ ion at *m/z* 431, and its MS/MS spectrum showed a prominent ion of $[M + H - Glu]^+$ at *m/z* 269, which was the characteristic ion of formononetin. Additionally, **5**, just like compound **13**, had UV absorptions with maxima at 250 and 301 nm. Based on these data [3–10], the structure of **5** was identified as ononin.

Compound **7** showed a $[M + NH_4]^+$ ion at *m/z* 482, and its MS/MS spectrum showed a prominent ion of

$[M + H - Glu]^+$ at *m/z* 303, which was the characteristic ion of 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane; thus, in agreement with the published data [3–10], the structure of **7** was identified as 2′-hydroxy-3′,4′-dimethoxyisoflavane-7-*O*-β-D-glucopyranoside.

Compound **9** exhibited a $[M + H]^+$ ion at *m/z* 473, i.e., 42 amu greater than that of compound **5**, and its MS/MS spectrum showed a prominent ion at *m/z* 269, which was the characteristic ion of formononetin. Additionally, **9**, just like compound **5**, had UV absorptions with maxima at 250 and 301 nm. Thus, compound **9** was identified as 6″-*O*-acetylononin [5].

Compound **10** gave a $[M + H]^+$ ion at *m/z* 301 and had UV absorptions with maxima at 280 nm, which corresponded to the structure of pterocarpane. Thus, the structure of **10** was identified as 3-hydroxy-9,10-dimethoxyptero-carpane [3–10].

Compounds **12** and **14** are a pair of isomers. Both of them gave a $[M + Na]^+$ ion at *m/z* 849, and their MS/MS spectra showed a prominent ion of $[M + Na - Glu - H_2O]^+$ at *m/z* 669, together with other ions of $[M + Na - CH_3COOH]^+$ at *m/z* 789, $[M + Na - Glu - H_2O - CH_3COOH]^+$ at *m/z* 609, and $[M + Na - Glu - H_2O - acetylxyl]^+$ at *m/z* 477. The fragmentations supported the presence of a xylose moiety linked to an acetyl group and a glycose residue. By examining the known saponins in the Radix astragali, astragalosides II and isoastragalosides II were found and were consistent with the above data; in agreement with the content difference and the retention time in HPLC reported before [5–11], compounds **12** and **14** were plausibly identified as astragalosides II and isoastragalosides II, respectively.

Compound **15** gave a $[M + H]^+$ ion at *m/z* 303 and had UV absorptions with maxima at 282 nm; in agreement with the published literature [3–10], the structure of **15** was identified as 7,2′-dihydroxy-3′,4′-dimethoxyisoflavane.

Compound **16** gave a $[M + Na]^+$ ion at *m/z* 645, and its $MS^2$ spectrum showed a prominent ion of $[M + Na - Xyl]^+$ at *m/z* 513. By examining the known saponins in the Radix astragali, only astramembranin II was consistent with the above data [5–7]. Thus, compounds **16** was identified as astramembranin II.

Compounds **17** and **18** are a pair of isomers. Both of them gave a $[M + Na]^+$ ion at *m/z* 891, and their MS/MS spectrum showed a prominent ion of $[M + Na - Glu - H_2O]^+$ at *m/z* 711, together with other ions of $[M + Na - CH_3COOH]^+$ at *m/z* 831, $[M + Na - 2CH_3COOH]^+$ at *m/z* 771, $[M + Na - CH_3COOH - Glu - H_2O]^+$ at *m/z* 651, $[M + Na - 2CH_3COOH - Glu - H_2O]^+$ at *m/z* 591, and $[M + Na - Glu - H_2O - 2acetylxyl]^+$ at *m/z* 477; these fragmentations supported the presence of a xylose moiety linked to two acetyl groups and a glycose residue. By examining the known saponins in the Radix astragali,

astragalosides I and isoastragalosides I were found and were consistent with the above data. According to the content difference and the retention time in HPLC reported before [5–11]. Compounds **17** and **18** were plausibly identified as astragalosides I and isoastragalosides I, respectively.

Compound **19** gave a $[M + Na]^+$ ion at m/z 933, its MS/MS spectrum showed prominent ion of $[M + Na - Glu - H_2O]^+$ at m/z 753. By examining the known saponins in the Radix astragali, only acetylastragaloside I was consistent with the above data [5–11]. Thus, compound **19** was identified as acetylastragaloside I.

## Discussion

Although quality control has often represented a barrier to the development of plant drugs, a few strategies have been put forward and some progress achieved. The present HPLC–DAD–ESI–MS/MS analytical method was successfully used to separate and determinate various types of compounds in the complex mixture. Among the identified compounds, calycosin-7-O-β-D-glucopyranoside and astragaloside IV were reported to show the antiviral activity [15, 16]. Our findings suggested that multiple bioactive components were responsible for the antiviral activity of Radix astragali and may help to clarify the remedial mechanisms and develop a reliable quality control method for this herb drug. Moreover, we also showed that collective application of bioassays and HPLC–DAD–ESI–MS/MS analysis was an approach to rapidly characterize bioactive constituents of the plant drugs.

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