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# Screening and analyzing potential hepatotoxic compounds in the ethanol extract of Asteris Radix by HPLC/DAD/ESI-MS$^n$ technique

Xiaodong Liu, Peipei Cao, Chaofeng Zhang*, Xianghong Xu, Mian Zhang*

*State Key Laboratory of Natural Medicines, Research Department of Pharmacognosy, China Pharmaceutical University, Longmian Road 639, Nanjing 211198, PR China*

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ABSTRACT

A refined toxic fraction, Fr-2, from the ethanol extract of *Aster tataricus* roots was obtained by a toxic tracing isolation. The hepatotoxic effects of Fr-2 were proved by the level elevation of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in mice serum and further confirmed by the histopathological examination on mice liver. A simple method was developed for qualitative analysis of constituents in Fr-2 fraction by high-performance liquid chromatography with diode array detection (HPLC-DAD) and electrospray ionization tandem mass spectrometry (ESI-MS$^n$). As a result, 35 compounds were detected and identified in Fr-2. Among them, 28 compounds are pentapeptides, including 18 cyclic and 10 linear ones, and the sum of their peak area occupied about 87% (71.2% for cyclic and 15.8% for linear) of total area of chromatographic peak. Other 7 compounds are phenols, occupied about 5.9% of total area of chromatographic peak. Moreover, the structures of 10 cyclic and 2 linear peptides were reported for the first time. The results suggested that the peptides, especially the cyclic ones, are probably the principal toxic substances in Fr-2 also in *A. tataricus* roots.



## 1. Introduction

Asteris Radix (AR), derived from the root of *Aster tataricus* L. f. (Compositae), is one of the traditional Chinese medicines (TCMs) with function of dispelling phlegm and relieving coughs. As an effective therapy, it has been widely used for more than 2000 years in China and included in all editions of Chinese Pharmacopoeia. Pharmacological researches revealed that it also has diuretic, antitumor, antibacterial, antiviral and antiulcer activities [1–4]. AR has been considered to be safe in clinic according to ancient herbal works. There were also no reports on side effects of AR in literatures. However, our previous researches discovered the potent toxicity of AR decoction especially for liver in mice [5]. The results of acute toxicity tests indicated that the toxic effects of AR were increased with the concentration of ethanol increased [6]. The 90% ethanol extract of AR was partitioned by petroleum ether, EtOAc (ethyl acetate) and *n*-butanol in order and their toxic effects were examined by acute toxicity test in mice. The results revealed that the EtOAc soluble portion was the major and the most poisonous portion [6,7] with a $LD_{50}$ value at 190 mg/kg in mice (i.g.), but the potential toxic constituents remained unclear.

There are many classes of chemical constituents in AR such as triterpenoids, flavonoids, peptides, anthraquinones and phenolic acids [8–12]. Astins are a class of cyclic pentapeptides contained in AR. Astin C (also called asterin), one halogenated compound of astins, has been considered as a hepatotoxic substance [13] owing to its structure is similar to cyclochlorotine. Cyclochlorotine is a toxic metabolite of *Penicillium islandicum* Sopp., the mold of islandia yellow rice [14], and can cause severe liver damage in a very short time [15–18]. Both astin C and cyclochlorotine are dichlorinated cyclopentapeptides with same amino acids in same sequence, the key difference is a Abu residue and a *cis* configuration in the proline peptide bond in astin C replaced by Ser and a *trans* one in cyclochlorotine [19]. From this point of view, astins are probably the major toxic constituents of AR. Therefore, it is important for the establishment of a rapid and selective analytical method to characterize and identify them, and furthermore, to monitor these hepatotoxic compounds in plasma or other pharmacology study. However, there is no sensitive method for detecting cyclopeptides in plants because of lacking free amino groups (NH or $NH_2$) in structures in most cyclopeptides. A new TLC method has been developed for detecting cyclopeptides in plants [20,21], but which is not sensitive enough and relatively time-consuming, and also absent of structural information. The combination of HPLC-DAD and HPLC–ESI-MS$^n$ has made it possible to detect and identify cyclopeptides and other constituents in AR.

Therefore, in order to find out the hepatotoxic components, a further investigation was performed in the present study and aimed

* Corresponding authors. Tel.: +86 25 8618 5137; fax: +86 25 8618 5137.
*E-mail addresses:* njchaofeng@126.com (C. Zhang), mianzhang@hotmail.com (M. Zhang).

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at: (1) getting a more refined toxic fraction from the EtOAc extract and verifying its hepatotoxic effect; (2) developing an HPLC/DAD method that can separate the most compounds contained in the refined toxic fraction; (3) charactering and analyzing the structures of components through the ESI/$MS^n$ method. The peptides contained in the toxic fraction would be as the key constituents for analysis.

## 2. Experimental

### 2.1. Materials, chemicals and reagents

The roots and rhizomes of *A. tataricus* (AR) were purchased from Anguo Medicinal Material Co. (Hebei Province, China) and authenticated by authors. Voucher specimens were deposited in the Research Department of Pharmacognosy, China Pharmaceutical University, Nanjing, PR China.

Authentic standards of astin B, astin C, astin D and asterinin D were isolated and characterized at Research Department of Pharmacognosy, China Pharmaceutical University. The purity of the reference standards was determined to be more than 98% by HPLC/DAD analysis.

Acetonitrile and formic acid were of HPLC grade (Merck, Darmstadt, Germany). Ultrapure water was prepared by Milli-Q SP Reagent water system (Millipore Corporation, MA, USA). Other reagents were of analytical grade.

Assay kits for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were purchased from Nanjing Jiancheng Technology Co., Ltd. (Nanjing Jiancheng Bioengineering Institute).

### 2.2. Extraction and isolation

AR (800 g) was crushed to coarse powder and extracted with water for three times. The combined extract was concentrated in vacuo to give a residue of aqueous extract, named as AE (480 g).

Coarse powder of AR (6 kg) was extracted three times with 90% EtOH under reflux. After concentrated in vacuo, the combined extract was partitioned with petroleum ether (PE) and EtOAc successively to yield PE extract (77 g) and EtOAc extract (132 g), respectively. The EtOAc extract (129 g) was subjected to silica gel column chromatography eluted with $CHCl_3$–MeOH system (30:1 to 5:1) to obtain 53 eluates which were combined to 3 fractions, named as Fr-1 (35 g), Fr-2 (33 g) and Fr-3 (55 g), on the basis of TLC comparison.

Another 3 kg of AR were extracted and divided according to the above methods to get the fraction Fr-2. Fr-2 (15 g) was further separated on Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) to yield astin B (180 mg), astin C (300 mg), astin D (10 mg) and asterinin D (8 mg), and their structures were confirmed by MS, $^1$H NMR and $^{13}$C NMR [13,22,23].

### 2.3. Evaluation of hepatotoxicity

#### 2.3.1. Animals

Male ICR mice (18–22 g), 6–8 weeks of age, were purchased from Yangzhou University Comparative Medicine Center of Jiangsu Province (Jiangsu, China). They were maintained with free access to pellet food and water in plastic cages at a constant temperature (21 ± 2 °C) and relative humidity (50 ± 5%) on a 12-h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and the related ethical regulations of China Pharmaceutical University.

#### 2.3.2. Acute toxic test on Fr-1, Fr-2 and Fr-3

Healthy male ICR mice were fasted overnight with free access to water. Animals were randomly divided in four groups with 10 animals per group. The fractions of Fr-1, Fr-2 and Fr-3 dissolved in 0.5% CMC-Na (sodium carboxymethylcellulose) solution were orally administered to 30 mice at doses of 416.6, 120.0 and 416.6 mg/kg (0.2 ml/10 g body weight), respectively, and an equal volume of 0.5% CMC-Na solution was orally administered to 10 mice as control. Animals were observed for general behavioral, hazardous symptoms and mortality [24] for a period of 14 days after treatment. Necropsies were performed on any animals that died during the 14-day period, and on the survivors at the end of the experimental protocol.

#### 2.3.3. Hepatotoxic experiment on AE and Fr-2 fraction

Fifty animals were divided into 5 groups of 10 per group and fasted for 12 h with free access to water before dosing. One group was orally administered AE at dose of 4.55 g/kg raw material (equivalent to a clinical large dose of 30 g per day raw materials for a 60 kg person), and three groups were orally administered Fr-2 fraction at dose of 10, 20, and 40 mg/kg, respectively. All drugs were suspended in 0.5% CMC-Na solution (0.2 ml/10 g body weight), respectively, and given to animals once daily for 7 consecutive days. The remained group received the same volume of 0.5% CMC-Na solution as control. One hour after the last administration, the mice were killed by cervical dislocation, blood was collected from the orbital sinus and centrifuged for 5 min (12,000 rpm, 4 °C) to obtain serum for the test of ALT, AST and ALP activities. Liver tissue was fixed with formalin for 24 h before making paraffin embedded sections for histopathological examination.

The levels of ALT, AST and ALP of serum were measured by Multiskan Spectrum (Thermo Electron Corporation) and UV2450 UV-VIS Spectro Photometer (UV-2450-prober 2.33 Workstation, Shimadzu Corporation). Liver tissue sections were stained with hematoxylin and eosin (H&E) and liver injuries were evaluated by morphological changes.

#### 2.3.4. Statistical analysis of bioassay data

Bioassay data are expressed as mean ± SEM from at least three independent experiments. Statistical analyzes were performed using one-way analysis of variance (ANOVA) (IBM SPSS Statistics 19.0 software), followed by Student's two-tailed *t* test. A *p*-value of $<0.05$ was considered as significant.

### 2.4. HPLC-DAD and LC–ESI-$MS^n$ analysis

#### 2.4.1. Sample preparation

An aliquot of 10.0 mg of Fr-2 was dissolved with 5 ml of methanol by ultrasonic for 15 min and centrifuged for 15 min (12,000 rpm), filtered through a 0.45 μm filter membrane before use. A volume of 10 μl was injected into the HPLC system for analysis. Authentic standards of astins B, C, D and asterinin D were dissolved in methanol with proper concentrations. All the stock solutions were stored at 4 °C until analysis.

#### 2.4.2. Instrumentation and analytical conditions

The HPLC system consisted of an Agilent 1100 HPLC instrument (Agilent Technologies, USA) with a quaternary pump, a diode array detector, an auto sampler and a column compartment. The samples were separated on a Zorbax SB-$C_{18}$ column (250 mm × 4.6 mm, I.D. 5 μm, Agilent) with a $C_{18}$ guard column (7.5 mm × 4.6 mm, 5 μm, Alltech). The mobile phase consisted of acetonitrile (A) and 0.01% formic acid (B). A gradient program was used as follows: initial 0–35 min, linear change from A–B (10:90, v/v) to A–B (17:83, v/v); 35–44 min, linear change to A–B (19:81, v/v); 44–55 min, linear change to A–B (21:79, v/v); 55–100 min, linear change to