Fermentation of *Aristolochia debilis* by six different medicinal fungi and identification of aristolochic acid derivates in their products by HPLC-ESI-TOF-MS

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Abstract: To analyze the content change of the nephrotoxic substances, aristolochic acid derivates (AAs) in the roots of *Aristolochia debilis* and the products generated from the solid-state fermentation of six different medicinal fungi by HPLC-ESI-TOF-MS, the chromatographic separation was carried out on C_{18} column at 30°C with the DAD detector. The elution was performed using the mobile phase of acetonitrile (A) and 0.2% acetic acid (B). Several new peaks were found in the scale of 0-20 min elution of HPLC diagram in the fermentation products. The ESI-MS detection (negative ion mode) was carried out by post-column flow splitting method following the automatic injection. Seven AAs in the fermentation products and *A. debilis* were deduced, which were recognized as AAIa or IIIa (1), AAVIa (2), AAIVa, Va, VIIa or VIIIa (3); AAII (4); AAIII (5); AAI (6); AAIV or VII (7). The areas of almost all these seven components existing originally in the corresponding crude drug decreased after the fermentation process, suggesting that fermentation is an effective way of lowering the nephrotoxicity induced by AAs in Chinese medicines similar with *A. debilis*. In addition, Optimized HPLC-MS method is helpful to AAs content identification.

Keywords: Aristolochic acid derivatives; *Aristolochia debilis*; Fungal fermentation; Medicinal fungus; HPLC-ESI-TOF-MS.

INTRODUCTION

Aristolochia debilis (*A. debilis* called Qingmuxiang in Chinese) is a traditional Chinese herb widely used to lower blood pressure and relieve pain before. It has also been used as anti-diarrhoica or antidote. However, due to the nephropathy of aristolochic acids, which are the components in A. debilis, the herb has been banned for Chinese clinical use by the laws of the State Food and Drug Administration. This situation has some influence on the development of Chinese traditional medicine industry (Yang *et al.*, 2012; Luciano and Perazella, 2015). Studies have reported that aristolochic acid derivates (AAs) could induce tubular lesions and acute renal failure. And available evidences have showed the well-defined role of AAs in Chinese traditional medicine nephropathy. Besides, aristolochic acid I (AAI) was found to be the most toxic ingredient among the AAs, and the toxicity of AAII, AAVIII, and AAIa declined in turn (Balachandran *et al.*, 2005).

Typically, its renal toxicity can be reduced by means of Chinese processing techniques (Ma *et al.*, 2010; Liang *et al.*, 2012; Wu *et al.*, 2012) and a concerted application of complex prescription (Ruan *et al.*, 2012). But early in 1996, the Aristolochiaceae plants were reported to exhibit particular biological activity through the development of biotechnology. The study of suspension culture of *Aristolochia manshuriensis* by Ti-plasmids of Agrobacterium tumefaciens showed that in rat experiments the extract possessed antihypoxic activity and reduced approximately twice a zone of myocardial necrosis (Bulgakov *et al.*, 1996).

As one of the traditional Chinese processing techniques, fermentation has been specifically used to reduce the toxicity of different drugs (Madeira *et al.*, 2011; Wang *et al.*, 2013; Hanly and Henson, 2014; Najiar *et al*., 2014; Nowak *et al.*, 2014; Veerabhadrappa *et al.*, 2014). Previous studies have shown that *A. debilis* in solid state was fermented efficiently by thirteen medicinal fungi (Liu *et al.*, 2010a; Liu *et al.*, 2010b). The amounts of total AAs and AA I in the fermentation products all decreased compared with that in the original crude drug. In the current research, the effects of bidirectional fermentation of *A. debilis* were assessed with regards to both reducing toxicity and maintaining efficacy. The qualities and quantities of AAs in the products fermented by six fungi were analyzed by HPLC-ESI-TOF-MS.

MATERIALS AND METHODS

Instrumentation and reagents

Liquid chromatography separation was performed using a Waters 2695 HPLC system (Waters Corporation, USA), which is composed of an autosampler, a quaternary pump, a diode array detector, an on-line degasser and a column temperature controller. It was coupled to Micromass Q-Tof mass spectrometer (Waters-Micromass UK Ltd, Manchester, UK) with an ESI source for HPLC-ESI-TOF-MS analysis. AA I was obtained from National Institute for the Control of Pharmaceutical and Biological Products

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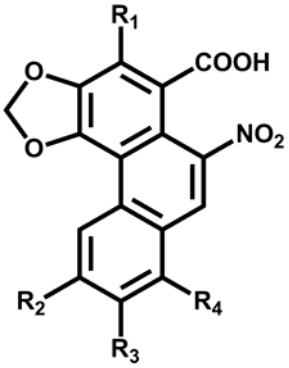
A. debilis fermentation and AAs identification

Table 1: HPLC-ESI-TOF-MS data of seven AAs in *A. debilis* and six kinds of products

No.	Identification	MW	$[M-H]^-$	$[2M-H]^-$
1	AAIa or IIIa	327	326 (s)	653 (w)
2	AAVIa	357	356 (m)	/*
3	AAIVa, Va, VIIa or VIIIa	357	356 (m)	/*
4	AAII	311	310 (w)	621 (w)
5	AAIII	341	340 (w)	681 (w)
6	AAI	341	340 (s)	681 (s)
7	AAIV or VII	371	370 (w)	/*

Note: * not detected

Table 2: Structural details of AAs identified in *A. debilis* and six products

Name	R_1	R_2	R_3	R_4	Structure formulae of AAs
AAI	H	H	H	OCH_3	
AAIa	H	H	H	OH	
AAII	H	H	H	H	
AAIII	H	OCH_3	H	H	
AAIIIa	H	OH	H	H	
AAIV	H	OCH_3	H	OCH_3	
AAIVa	H	OH	H	OCH_3	
AAVa	H	OH	OCH_3	H	
AAVIa	OH	H	H	OCH_3	
AAVII	H	H	OCH_3	OCH_3	
AAVIIa	H	H	OH	OCH_3	
AAVIIIa	H	H	OCH_3	OH	

(lot number: 110746-200305). The purity (higher than 98%) of AA I was identified through HPLC area normalization. Chromatographic grade acetonitrile was purchased from Tedia (USA). Distilled water was obtained from the Milli-Q Gradient A10 Water System (USA). Other solvents were all analytical grade.

Plant material and mother spawn

The roots of *A. debilis* were obstained from Nanjing Lujiang Prepared Pieces of Chinese Medicine Factory. The plant was identified by Prof. Jianwei Chen (Nanjing University of Chinese Medicine, NJUCM).

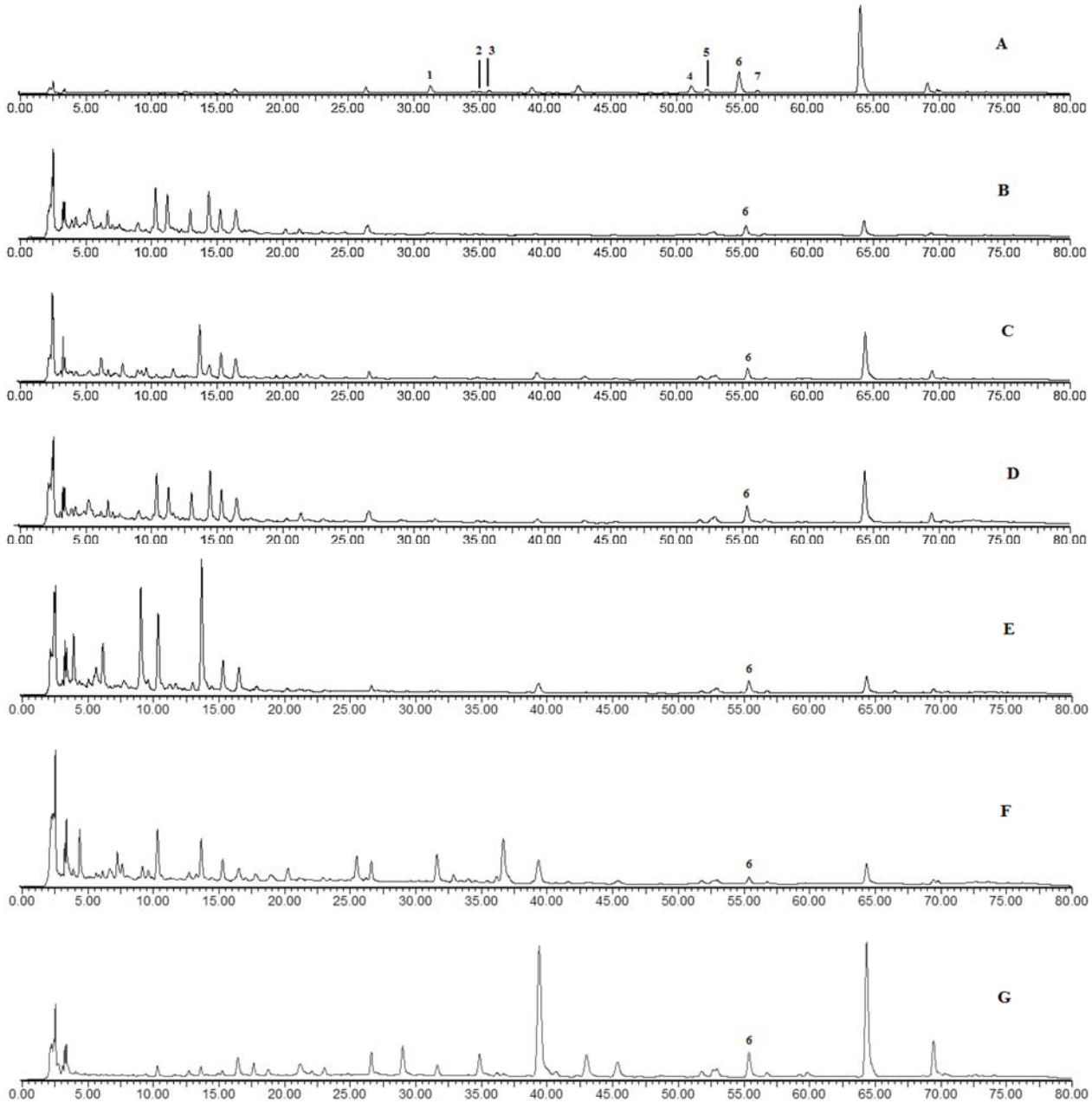
The young fruiting bodies of six fungi, Ganoderma lucidum (a), Coriolus versicolor (L.ex Fr.) Quel (b), Trametes robinpphila Murr. (c), Trametes cinnabarina (jacq.) Fr. (d), Poria cocos (Schiw.) Wolf (e) and Beauveria bassiana (Bals.) Vuill. (f), were picked in Purple Mountain located in Nanjing, Jiangsu, China. This kind of fruiting body was confirmed by Prof. Yang Pan (NJUCM). The mother spawn mainly included potato dextrose agar slants for aseptic processing, which consisted of 200g diced potato, 20g dextrose, 20g agar powder, 1L water. All samples mentioned above are kept in the laboratory refrigerator.

Solid-state fermentation of A. debilis

The mother spawn of the six fungi available for fermentation was regenerated using PDA medium slants for a seven-day incubation (Within three to four days, mycelium would cover the tissue and branch out on the agar.) at 26°C under 70% relative humidity condition. The powdered *A. debilis* substrate over a 10-mesh (to 2.0 mm) sieve was moistened. To determine if the substrate was moist enough (A few drops of water should be released under some pressure.), a squeeze test was applied. Before sterilization small substrate containers (usually heat-resistant 500mL-bottles) were filled with 200g *A. debilis* substrate and then sterilized by autoclaving for 60min under 0.105MP at 121°C. The substrate should be spawned as soon as it was cooled down below 30°C. Spawning was performed by taking off the cotton plugs from the bottles containing the substrate and putting in a small piece (5 mm × 5 mm was sufficient.) of the spawn in the middle of it with a heated scalpel in the biological safety cabinet with laminar air flow. Once the substrate was spawned, the plugs should be replaced right away. The newly inoculated *A. debilis* were placed in 26°C of incubation room under previous humidity condition. It should keep the room air fresh for solid-state fermentation. The mycelium grew on the substrate after 45 days. Then the fermented *A. debilis* substrate was taken out and dried at 60°C for about 5 h.

Sample preparation

The roots of *A. debilis* and their fermentation products were air-dried and ground into powder passing through 40 mesh sieve (0.45mm), respectively. An aliquot (2.0g) of the powder was weighed into a conical flask and 20ml



Peaks: 1. AA Ia or IIIa; 2. AAVIa; 3. AAIVa,Va,VIIa or VIIIa; 4. AAII; 5. AAIII; 6. AA I; 7. AAIV or VII.

Fig. 1: Comparison of the HPLC/ UV of total AAs in *A. debilis* and six fermentation products: (A), (B), (C), (D), (E), (F) and (G) corresponding to *A. debilis*, product fermented by a, b, c, d, e, f.

60% methanol (HPLC grade) was added. After immersing for 30min at room temperature, the mixtures were reflux-extracted for an hour. The solutions were centrifuged at 2 500r·min^{-1} for 10min at room temperature and the supernatants were evaporated to dryness. The residues were dissolved with methanol R and then diluted to 50 mL with the same solvent in a volumetric flask. The solutions were filtered through a syringe filter (0.45μm) before HPLC analysis.

HPLC-ESI-TOF-MS analysis

The chromatographic separation was carried out on a Hanbon C_{18} column with 250mm long and 4.6mm in internal diameter at 30°C, packed with ODS-3 for chromatography R (5μm) (Kong *et al.*, 2015). The wavelength was recorded at 250nm for UV detection. The mobile phase used were acetonitrile (A) and aqueous solution of acetic acid (0.2%, B) at a flow rate of 1.0 ml·min^{-1}. All solvents were filtered by 0.45μm nylon filters and then degassed by sonication in an ultrasonic bath prior to use. Gradient was as follows: 15%-30% A (0-25 min), 30%-50% A (25-45min), 50%-60% A (45-55 min), 60%-80% A (55-75min), 80%-15% A (75-80 min).

A. debilis fermentation and AAs identification

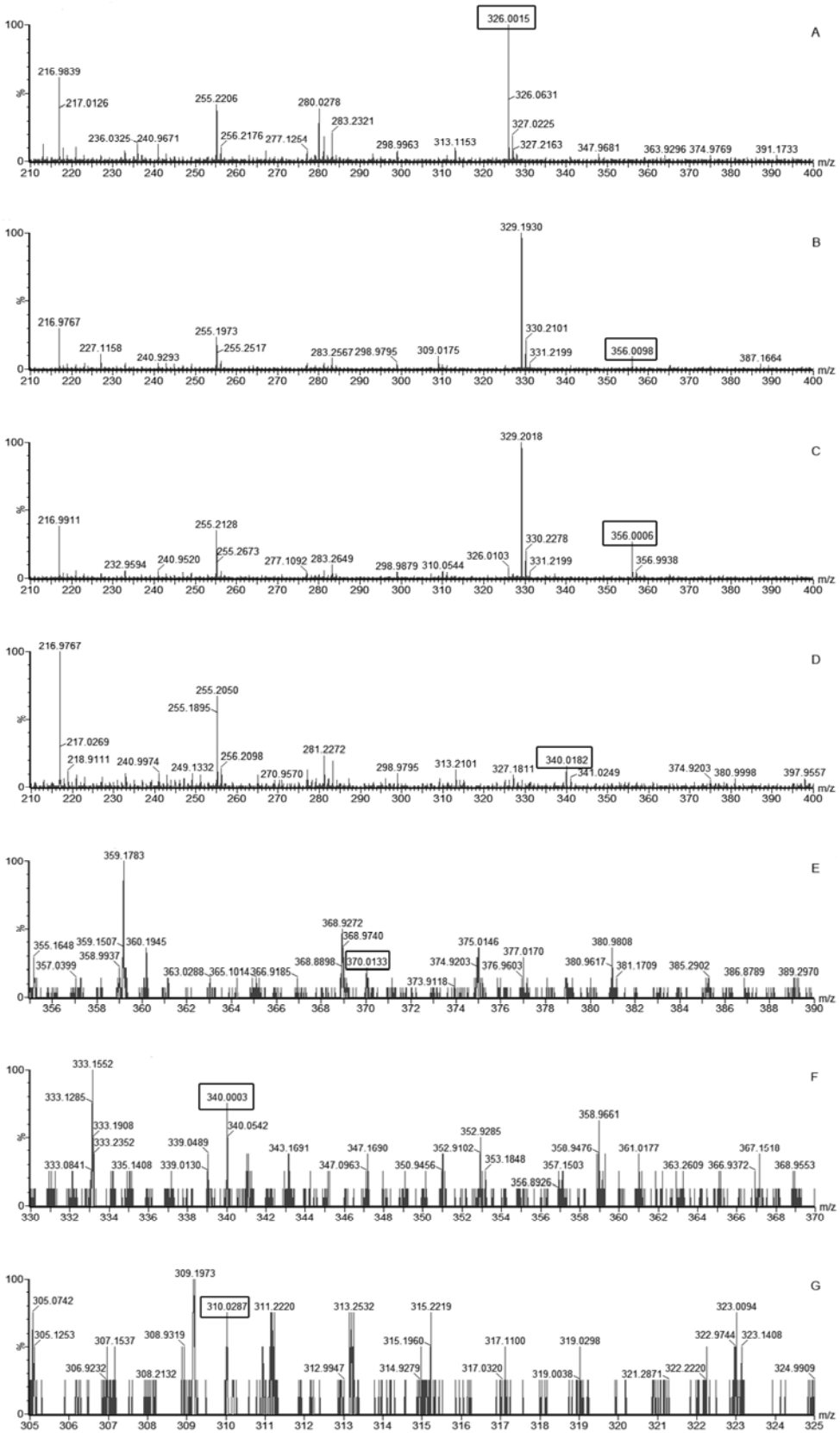


Fig. 2: MS spectra of seven aristolochic acid derivatives: (A) AA Ia or IIIa; (B) AAVIa; (C) AAIVa,Va,VIIa or VIIIa; (D) AAI; (E) AAIV or VII; (F) AAIII; (G) AA II.

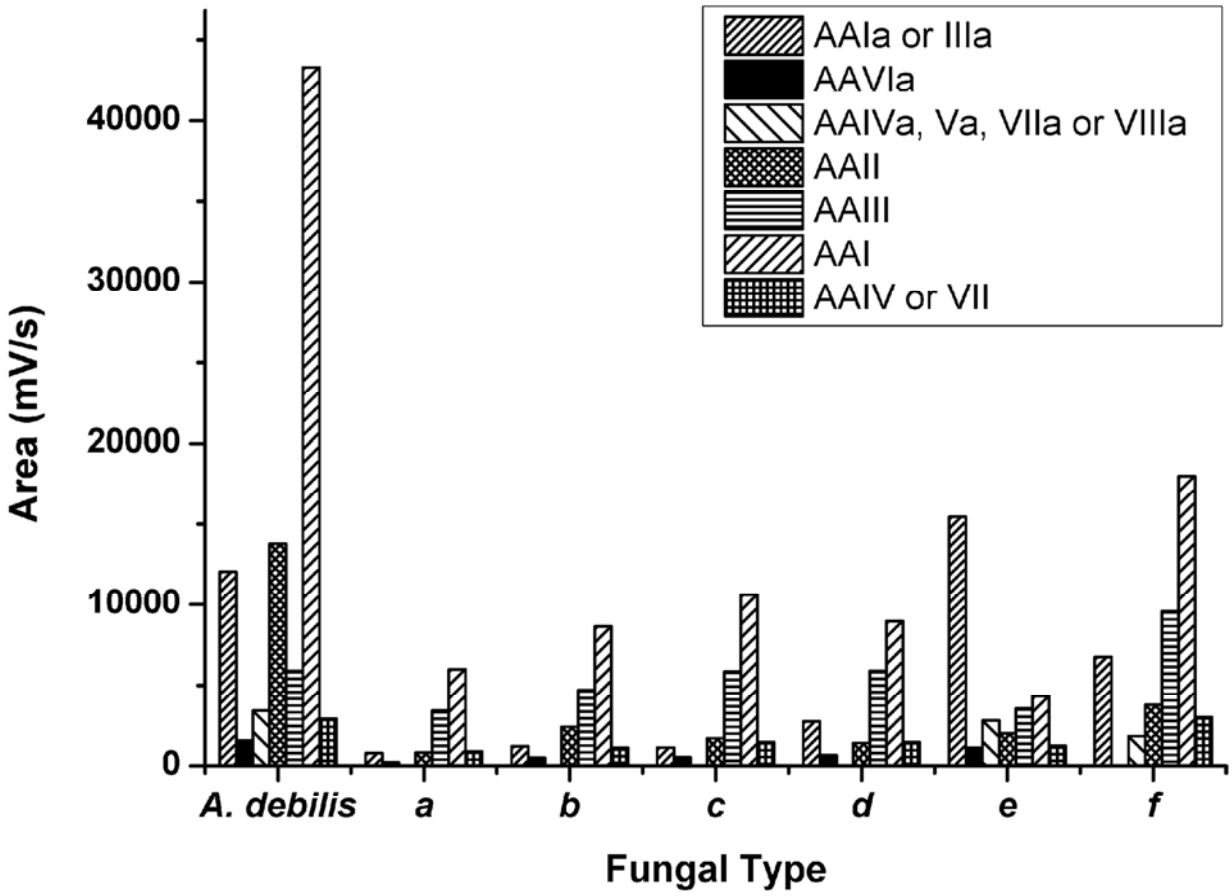


Fig. 3: Comparison of peak area changes of seven AAs in *A. debilis* and the six fermentation products (a-f).

After the injection by autosampler into the mass detector, the ESI-MS detection was carried out by post-column flow splitting method. The mass spectrometry detector (MSD) parameters were as follows: negative ionization, mass scan range from m/z 100 to 1000, 3.0 kV capillary voltage, 25 V cone voltage. Source temperature was set to 100°C, with desolvent gas temperature to 200°C. High purity of nitrogen (99.99%) was used as nebulizing gas and the flow rate was adjusted to 400L·h^{-1}. HPLC/ UV and TIC diagrams were all recorded. All the operations, data acquisition and analysis were through MassLynx Ver. 4.0 software (Waters Corporation).

RESULTS

HPLC-UV chromatograms

The previously optimized solvent gradients (Liu *et al.*, 2010b) were used for separation of AAs by HPLC. The HPLC/UV chromatograms of *A. debilis* and six fermentation products were illustrated in fig. 1. By comparing the MS data and retention times of each component from the roots of *A. debilis* and the fermention products with those of authentic standard AA I, seven peaks were unambiguously identified as AAI (6), AAII(4), AAIII(5), AA Ia or IIIa (1); AAIVa,Va, VIIa or VIIIa (3), AAIV or VII (7), AAVIa (2) (fig. 1A).

Mass spectra

The ESI-MS spectra of seven AAs were shown in fig. 2. All authentic compounds exhibited molecule ion $[M-H]^-$ and dimeric ion $[2M-H]^-$ in negative ion mode with different levels of abundance. Based on the mass law and molecular weights, the tentative assignments of seven AAs in *A. debilis* before and after the fermentation by six fungi, respectively, were deduced. The molecular weights of each AA were shown in table 1.

DISCUSSION

The chemical structures of the compounds from peak 1 to 7 were shown in table 2. The peak areas from fig. 1 were calculated and plotted in fig. 3. According to fig .3, the peak areas of 1-7 nearly all decreased after the fermentation, suggesting that the content of seven AAs in *A. debilis* basically dropped in the fermentation products. In addition, the content decreasing rate of AA I, which was recognized as the major and the most toxic component in *A. debilis*, was very obvious, especially in the fermentation product of fungus (e) (90.0%). Furthermore, a lot of new peaks were found in the HPLC diagrams of the fermentation products in the scale of retention time of 0-20 min, indicating the formation of

polar substances during the biotransformation of fermentation.

HPLC-MS technique was successfully used to identify seven AAs in *A. debilis* and the fermentation products of six medicinal fungi with high resolution and sensitivity. They were recognized as AAIa or IIIa, AAVIa, AAIVa, Va, VIIa or VIIIa, AAII, AAIII, AAI and AAIV or VII. The content of seven AAs existing originally in *A. debilis* nearly all decreased after the fermentation. And the decline percent of the most toxic component (AAI) in the products fermented by six different fungi was 58.5%-90.0% compared with the corresponding crude drugs. It was worth mentioning that several new peaks were found in the scale of retention time of 0-20 min, which deserves further study.

CONCLUSION

Although the efficacy and renal toxicity of the fermentation products should be extensively investigated, the results have indicated that fermentation is an effective way of lowering the nephrotoxicity induced by aristolochic acids in *A. debilis* and may be in other similar Chinese medicines.

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