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Analysis of the Analogues of Aristolochic Acid and Aristolactam in the Plant of *Aristolochia* Genus by HPLC

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ABSTRACT

A facile reversed-phase HPLC method for the analysis of aristolochic acid (AA) and aristolactam (AL) analogues was developed and used for the quantitative determination and quality control of the traditional Chinese medicine. Homonymic Chinese crude drugs such as *Stephania tetrandra* (粉防己) and *Aristolochia fanchi* (廣防己), *Clematis armandii* (川木通) and *A. manshuriensis* (關木通) can be easily identified by this method. The quantitative determination of AAs and ALs in 12 species of *Aristolochia* (*A. elegans, A. zollingeriana, A. cucurbitifolia, A. mollis, A. kaempferi, A. shimadii, A. heterophylla, A. debilis, A. foveolata, A. contorta, A. trilobata and A. odoratissima*) has been investigated. The chromatograms obtained in this study can serve as fingerprints to identify plant species in the *Aristolochia* genus, thus avoiding incorrect identification of herbal ingredients in manufacturing traditional Chinese medicine.

Key words: HPLC, aristolochic acid, aristolactam, *Stephania tetrandra, Aristolochia fanchi, Clematis armandii, A. manshuriensis*

INTRODUCTION

Aristolochia species has been used extensively in the traditional Chinese medicine. Its diverse biological functions include hypertension relief, leukocyte enhancement, rheumatism relief, edema therapy, as well as analgesic and diuretic effects[1-4]. Numerous *Aristolochia* species such as *A. debilis, A. fanchi, A. manshuriensis, A. contorta*, etc. have been used in the traditional Chinese medicine. It is noteworthy that the Chinese name for *Aristolochia fanchi* is "fangji", which is very similar to "fenfangji", the name of *Stephania tetrandra*, and confusion may thus arise in the manufacture of herbal medicines. Both of them have similar activities for edema therapy and pain relief. But "fenfangji", a species of *Stephania*, belongs to Menispermaceae (not Aristolochiaceae) and contains no aristolochic acids (AAs) or aristolactams (ALs). The AAs were reported to have some toxic properties such as mutagenicity, carcinogenicity in Wistar mice and nephrotoxicity in human. Cases of terminal or pre-terminal renal failure in patients who take weight-reducing pills with Chinese herbs containing ingredients of AAs[5,6] have also been reported in a clinical study. The occurrence might be caused by a manufacturing error in which *Stephania tetrandra* was inadvertently replaced by *A. fanchi*, which is nephrotoxic and carcinogenic. In some cases, patients developed severe nephrotoxicity and required kidney transplant[7]. In England, two similar cases of renal failure linked to AAs were reported[8,9]. The cause of disease was believed to be the preferential binding of AAs to the exocyclic amino group of purine nucleotides in DNA[10].

The striking relation between a specific type of fibrosing interstitial nephritis in young women and a slimming treatment involving Chinese herbs further supports the arguments against uncontrolled herbal therapy. The US Food and Drug Administration (FDA) issued a warning to consumers to discontinue the use of these Chinese herbal products in 2001 due to potential serious health hazard. Thus, routine analysis in quality control and quantity measurements of these active ingredients was considered important and necessary in Good Manufacturing Practice (GMP) and received much attention. Simultaneous resolution of analogues of AAs and ALs (compound 1 to 17, Figure 1 and Table 1) with a reversed-phase high performance liquid chromatography has been developed by us recently[11]. In this sturdy, we analyzed the AAs and ALs compositions in "fangji" and "fenfangji", thereby developed a way to differentiate between the two.

Another herb *Clematis armandii* (川木通) (Ranunculaceae) is very similar to "*A. manshuriensis*" (Aristolochiaceae) in terms of Chinese pronunciation and biological functions. We hope that they can be distinguished by the analysis of composition of AAs and ALs. We have collected roots and stems of 14 species of *Aristolochiaceae* and used our method to examine the AAs and ALs composition. The results obtained were useful for toxicity research and species identification and thus reduce

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Table 1. Names of 17 standards of aristolochic acid and aristolactam

Compound No.	Name
1	Aristolactam-C-*N*-β-glucoside
2	Aristolochic acid C
3	Aristolochic acid IVa
4	Aristolochic acid II
5	Aristolactam-*N*-β-D-glucoside
6	Aristolochic acid I
7	Aristolochic acid IV
8	Aristolactam AII
9	Ariskanin B
10	Cepharadione A
11	Ariskanin C
12	Aristolactam I
13	3-Hydroxy-4-methoxy-10-nitro-phenanthrene-1-carboxylic acid methyl ester
14	Ariskanin D
15	Aristolochic acid II methyl ether
16	Ariskanin A
17	Ariskanin E

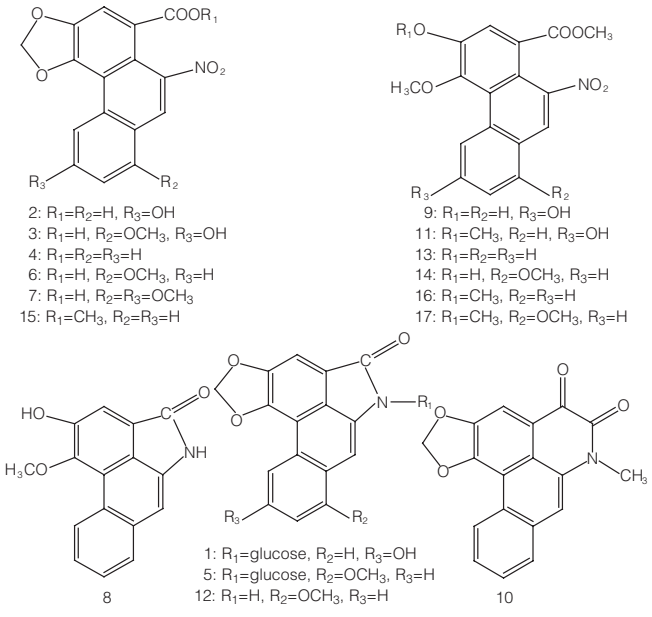

Figure 1. The structures of 17 analogues of aristolochic acid and aristolactam examined in this study.

inadvertent replacement of herbs in Chinese herbal preparations.

MATERIALS AND METHODS

I. *Plant Materials*

Stephania tetrandra, Aristolochia fanchi, Clematis armandii and *A. manshuriensis* were purchased in Taiwan market in July 2001. *A. elegans* was purchased in Kiangsi, Peoples Republic of China, in August 1997. *A. mollis, A. shimadii, A. debilis, A. contorta, A. trilobata, A. odoratissima, A. zollingeriana, A. cucurbitifolia, A. kaempferi, A. heterophylla* and *A. foveolata* were collected from Taiwan in April 1994. All plants were verified by Prof. C. S. Kuoh. A voucher specimen was deposited in the herbarium of Cheng Kung University, Tainan, Taiwan.

II. *Apparatus*

The HPLC system used in all analyses was a Gilson model 305 linked to a Gilson Model 115 UV detector with variable wavelengths. UV detection was set at 254 nm. All calculations based on the peak area were processed on a Hitachi D-2000 data integrating station. A 20 μL injection valve (Rheodyne) was used in all analyses. Separation was carried out on a C18 column (250 × 4.6 mm i.d., 5 μm particle diameter, supplied by E. Merck) at ambient temperature (~28˚C). A C18 column (250 × 10.0 mm, i.d.) was used to perform semi-preparative purification.

III. *Chemicals*

Solvents of HPLC grade such as methanol and acetonitrile were purchased from BDH (England). Dichloromethane, glacial acetic acid and sodium hydroxide were purchased from E. Merck (Germany). In all cases, water processed with a Millipore water purifying system was used.

IV. *Preparation of Standards and Sample Solutions*

Plant extracts of *A. cucurbitafolia and A. zollingeriana* were the source for all standards, examined in this study [12,13]. The crude extract was separated first by TLC and then purified with a semi-preparative column (250 × 10.0 mm, i.d.). Eluents were collected, concentrated and then dried under reduced pressure. The dried components were dissolved in a solvent mixture of dichloromethane and methanol (1:1, v/v) with the following concentrations: 10.0 μg/mL (compounds 1 and 11), 5.0 μg/mL (compounds **2-7**, **10**, **12** and **16**), 4.0 μg/mL (compound **13**) and 2.5 μg/mL (compounds **8**, **9**, **14**, **15** and **17**).

Roots and stems of the 14 species of *Aristolochia* were weighed 1.0 g of each and cut into small pieces before being refluxed in methanol of HPLC grade. The methanol extract was collected, filtered and diluted to 5 mL and was used as sample solution for HPLC analysis.

V. *Chromatographic Conditions and Calibration*

Separation of AAs and ALs was carried out on a 25 cm C18 column using gradient elution of a solvent mixture of sodium acetate (mobile phase A: 0.01M, pH5.0) and acetonitrile (mobile phase B) at ambient temperature (~28˚C). The gradient elution started with 20% B for 5 min. The percentage of mobile phase B was increased to 44% at 33 min, 50% at 43 min, 68% at 53 min and finally to 80% at 59 min.

Each standard was prepared in five different concen-

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trations (14-205 μg/mL), which were plotted against corresponding peak areas to create calibration curves. All measurements were in triplicate. Linear regression analysis was performed to calculate the value of R square. The method of external standard addition was applied to determine quantitatively the concentration of AA and AL analogues in the 14 species of *Aristolochia*.

RESULTS AND DISCUSSION

The resolution of 17 AA and AL analogues under gradient elution with a solvent mixture of sodium acetate and acetonitrile is shown in Figure 2. Unassigned peaks are impurities which failed to be removed by a semi-preparative column.

The tissue samples of renal failure patients who took weight-reducing pills containing *S. tetrandra* (fenfangji) were found to contain arislolactam-DNA adducts[7], which might come from the herb *A. fangchi* since we have not find any peaks of AAs or ALs in chromatogram of *S. tetrandra* (Figure 3A). Although *S. tetrandra* has similar biological activities as *A. fangchi*, it would not be contributed from AAs or ALs. The major component of AAs in *A. fangchi* is AA **2** (aristolochic acid C) with approximately 45% high (Figure 3B and Table 2B). According to NMR study, AAs are reduced to aristolactams (ALs) during metabolism under anaerobic conditions *in vitro* and then excreted *in vivo* in mammals including human beings[12]. Therefore, AA **2** may be reduced to AL **12** during the metabolic process thus forming the DNA-AL adduct in the kidney of patients.

The profile of *Clematis armandii* (Figure 3C) shows no AA or AL peaks, except the only doubtful peak (peak "a") at 55 min. In contrast, the profile of *A. manshuriensis* (Figure 3D) shows peaks 6 and 4 are the major components. Table 2 shows AA **15** is the biggest component of *A. manshuriensis* (30%). The pattern of chromatogram of *A. manshuriensis* is quite different from that of *A. fanchi* (Figure3B & 3D) and thus their compositions also differ from each other.

Figure 4A is the chromatogram of *A. elegans* and

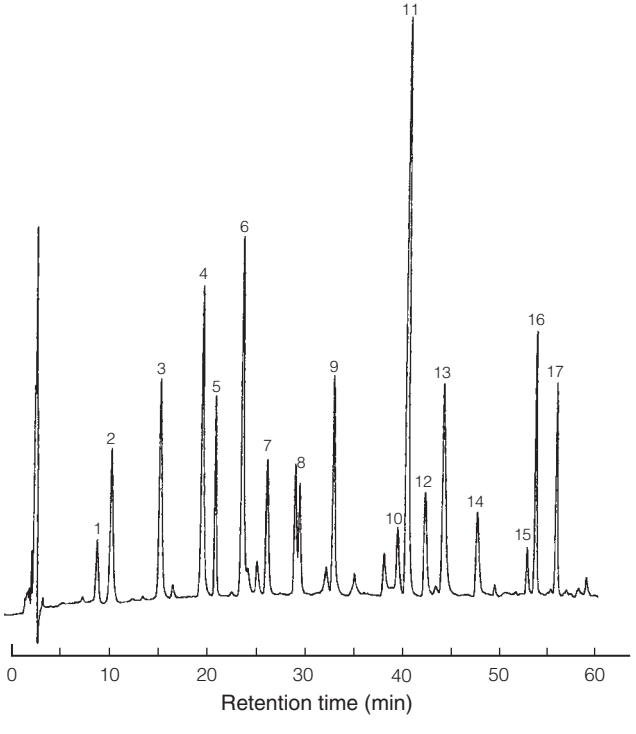

Figure 2. Chromatogram showing the resolution of 17 aristolochic acid and aristolactam analogues under gradient elution with a solvent mixture of sodium acetate and acetonitrile. Compound corresponding to the number designated in the elution order is described in Figure 1. Please refer to Experimental Section for the details of gradient elution. Unassigned peaks are impurities which fail to be removed by the semi-preparative column.

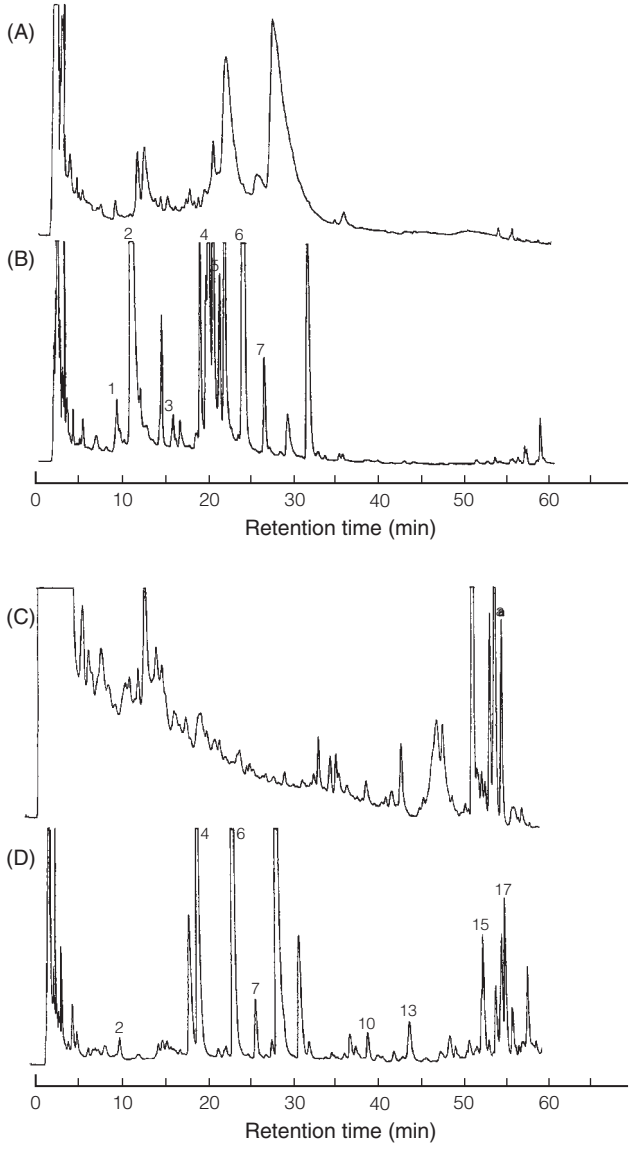

Figure 3. Chromatograms of aristolochic acid analogues in the methanol extract of 4 herbs under the same chromatographic conditions as described in Figure 2. (A) *S. tetrandra* (fenfangji); (B) *A. fanchi;* (C) *Clematis armandii*; (D) *A. Manshuriensis*.

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Table 2A. The AAs and ALs composition of 7 species of *Aritolochia*

Botany	*A. elegans*	*A. zollingeriana*	*A. cucurbitifolia*	*A. mollis*	*A. kaempferi*	*A. shimadi*	*A. manshuriensis*
1	19.1%	Na	N	N	N	14.3%	N
2	N	20.1%	2.7%	2.4%	N	43.1%	2.9%
3	12.5%	7.9%	2.7%	4.3%	N	3.5%	N
4	30.5%	7.0%	13.6%	16.0%	30.1%	0.5%	21.5%
5	4.0%	N	N	11.4%	2.9%	N	N
6	1.9%	12.6%	64.5%	51.4%	41.1%	20.8%	24.1%
7	8.8%	22.5%	16.6%	12.5%	25.0%	14.7%	6.8%
8	8.9%	1.8%	N	N	N	N	N
9	N	N	N	1.9%	N	N	N
10	N	3.7%	N	N	N	N	5.4%
11	14.4%	3.0%	N	N	N	N	N
12	N	2.3%	N	N	N	N	N
13	N	5.7%	N	N	N	N	2.7%
14	N	1.8%	N	N	0.9%	N	N
15	N	1.8%	N	N	N	3.1%	30.3%
16	N	9.0%	N	N	N	N	N
17	N	0.7%	N	N	N	N	6.3%

[a]Not detected: "N" means that the concentration bellow 2.5 μg/mL.
All experiments were based on triplicate measurement.

Table 2B. The AAs and ALs composition of 7 species of *Aritolochia*

Botany	*A. trilobata*	*A. odoratissima*	*A. heterophylla*	*A. debilis*	*A. foveolata*	*A. contorta*	*A. fangchi*
1	N	27.5%	N	12.0%	N	N	16.2%
2	29.9%	3.2%	6.2%	34.0%	0.6%	N	44.8%
3	36.0%	2.1%	N	1.4%	6.1%	19.1%	1.9%
4	N	N	7.8%	25.9%	12.0%	16.3%	7.7%
5	1.7%	N	N	N	12.2%	4.8%	8.1%
6	21.0%	11.1%	64.4%	12.5%	29.0%	24.0%	14.5%
7	11.5%	3.0%	12.8%	0.9%	21.5%	28.8%	6.9%
8	N		8.8%	N	N	N	N
9	N	0.9%	N	11.6%	N	0.7%	N
10	N		N	N	N	N	N
11	N	49.3%	N	N	N	N	N
12	N	N	N	N	18.5%	N	N
13	N	N	N	N	N	N	N
14	N	3.1%	N	N	N	N	N
15	N	N	N	N	N	N	N
16	N	N	N	1.6%	N	5.1%	N
17	N	N	N		N	1.2%	N

All experiments were based on triplicate measurement.

Table 2A shows compound **4** and **1** (30 and 19%) are the major components of AAs. The special profile in Figure 4B (*A. zollingeriana*) shows 14 peaks out of 17. *A. zollingeriana* is the only plant with so many AAs and ALs.

Figure 4C, 4D and 4E are chromatograms of *A. cucurbitifolia, A. mollis and A. kaempferi*, respectively. Their common major components of AAs are compound 4, 6 and 7 (Table 2A). Their chromatogram profiles are similar to each other. The profile of *A. mollis* with a special peak 9 at 32 min can be distinguished from the profile of *A. cucurbitifolia* which has two small peaks of AA **2** and **3** but no AA **9**. The profile of *A. kaempferi* has a special big unknown peak at 10 min. The plants can be identified easily from their chromatograms and compositions.

A. shimadii (Figure 4F) with major components of compound **2** and **6** (Table 2A) is similar to *A. fangchi* (Table 2B), but the latter has two different peaks of 4 and 5 (Figure 4F and 3B).

The chromatogram of *A. trilobata* (Figure 5A) has three major peaks of compound **3**, **2** and **6** (Table 2B). It is one of the two profiles that have no AA or AL peaks after peak 7. The chromatogram of *A. odoratissima* in Figure 5B has two major peaks of 11 and 6 and a peak 14 which is very special among these profiles in this study. The chromatogram of *A. heterophylla* (Figure 5C) has only one major component of AA **6** (Table 2B, 64% high). Its composition is similar with *A. cucurbitifolia*, but the latter has no peak 8. Figure 5D is the chromatogram of *A. debilis* with major peaks of 2, 4, 6 and 9, and AA 9 is the biggest one in all 14 Aristolochia plants. Chromatogram of *A. foveolata* in Figure 5E has the largest AA **12** peak (about 18%, Table 2B) whereas *A. contorta* (Figure 5F) shows its major components as compound **7**, **6** and **3** (Table 2B) and has a big unknown peak at 11 min.

Resolution and quantitative determination of 17 analogues of aristolochic acids and aristolactams under gradient elution with a solvent mixture of sodium acetate and acetonitrile are demonstrated. Analysis of Chinese herbs such as *S. tetrandra* vs. *A. fangchi* and *Clematis armandii* vs. *A. manshuriensis* has proven this method to be facile and reliable. This method provides fingerprints to

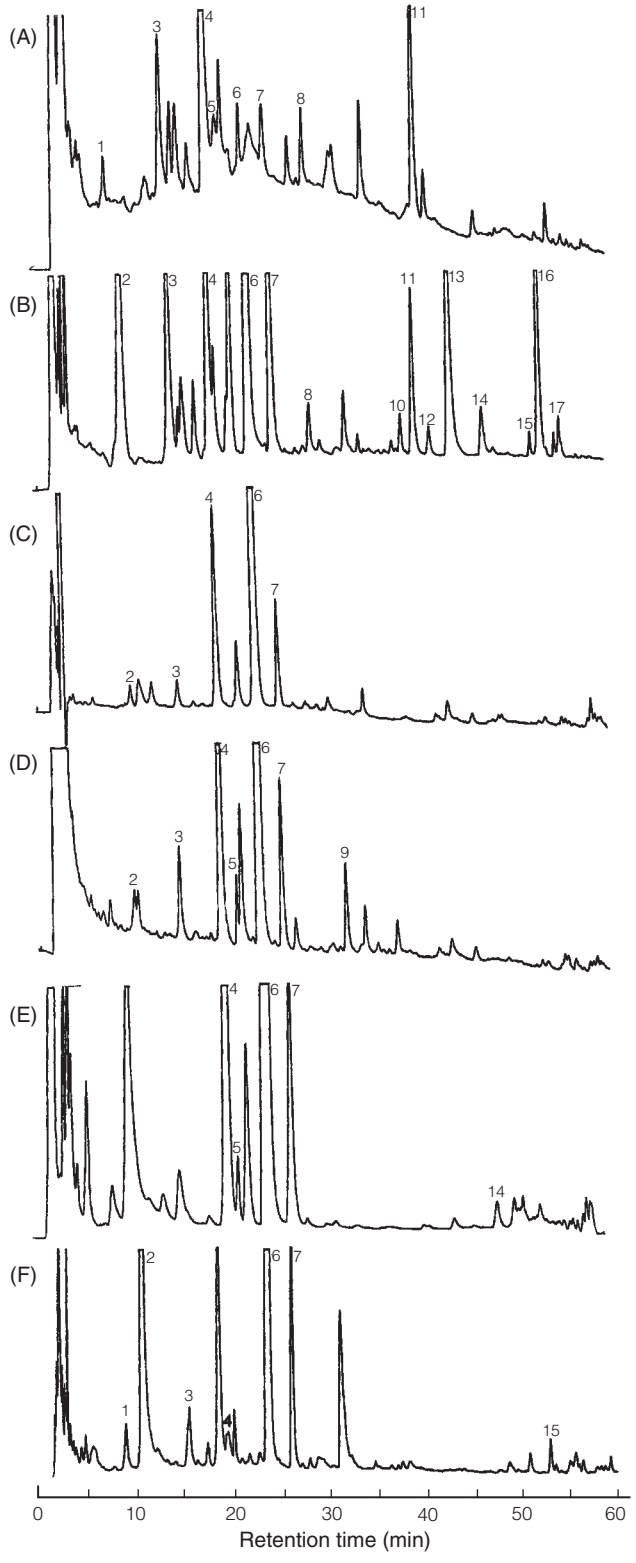

Figure 4. Chromatograms of aristolochic acid analogues in the methanol extract of 6 species of Aristolochia under the same chromatographic conditions as described in Figure 2. (A) *A. elegans;* (B) *A. zollingeriana;* (C) *A. cucurbitifolia;* (D) *A. mollis;* (E) *A. kaempferi;* (F) *A. shimadii*.

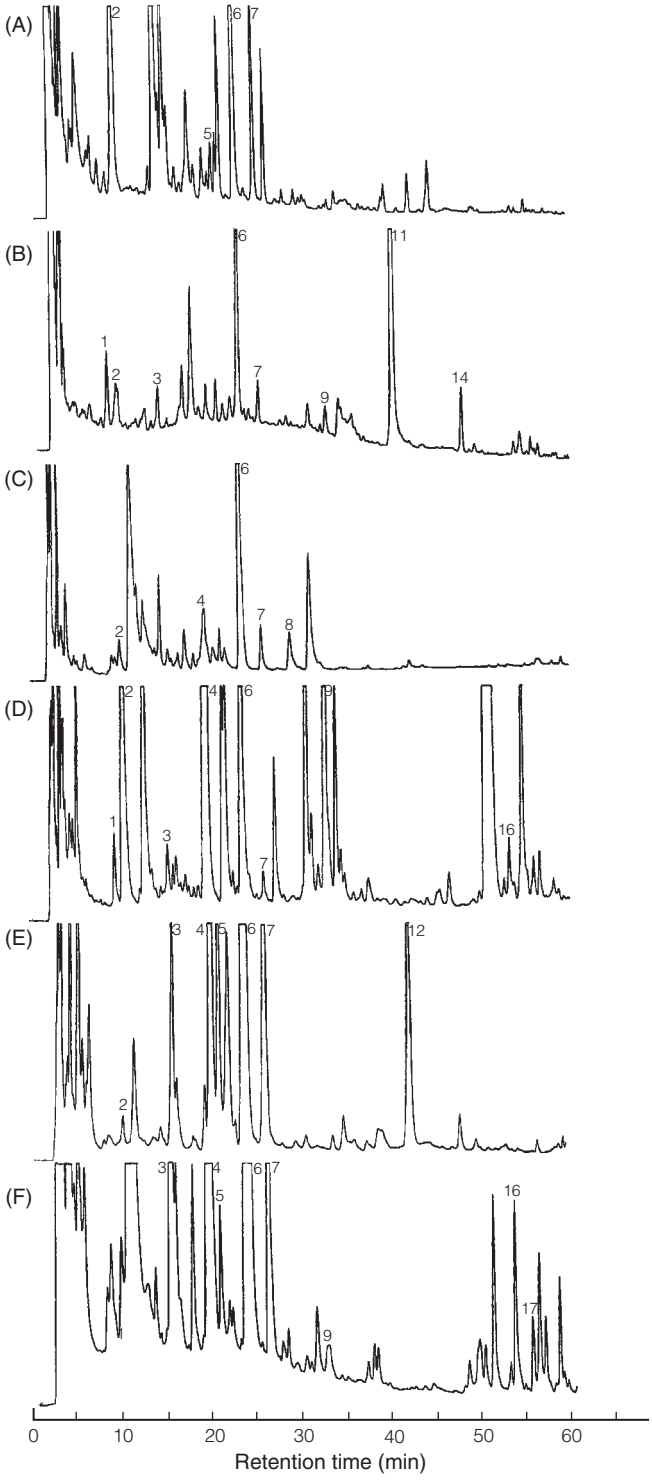

Figure 5. Chromatograms of aristolochic acid analogues in the methanol extract of another 6 species of Aristolochia under the same chromatographic conditions as described in Figure 2. (A) *A. trilobata;*(B) *A. odoratissima;* (C) *A. heterophylla;* (D) *A. debilis;* (E) *A. foveolata;* (F) *A. contorta*.

easily identify the origin or species of plants in the genus *Aristolochia* in order to avoid the inadvertent replacement of herbs in the manufacture of Chinese herbal drugs and thus prevent health hazards.

ACKNOWLEDGMENTS

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