Journal of Food and Drug Analysis, Vol. 17, No. 4, 2009, Pages 274-281 藥物食品分析 第十七卷 第四期

Analysis of Aristolochic Acid in Asarum (Xixin) and Its Preparations by Liquid Chromatography/Tandem Mass Spectrometry

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(Received: October 24, 2008 ; Accepted: July 29, 2009)

ABSTRACT

A method for analysis of aristolochic acids (AAs: AA-I, AA-II) in Asarum (Xixin, named in Chinese) and its preparations by liquid chromatography coupled with tandem mass spectrometry has been developed. The chromatographic analysis was carried out using a C_{18} column (2.1×150 mm) and a mixture of 0.1% formic acid (containing 0.1% ammonium acetate) and acetonitrile (65:35, v/v) as the mobile phase at a flow rate of 0.3 mL/min. Piromidic acid was used as an internal standard. The mass detection was measured as the positive ion electrospray ionization mode. The precursor and daughter ions for quantitation were *m/z* 359 and 298 for AA-I, *m/z* 329 and 268 for AA-II. The instrument detection limit of AA-I and AA-II was 2 and 2.8 ng/mL, respectively. The result showed that Asarum contained AA-I while AA-II was not detected. AA-I distribution in different parts of Asarum was examined in detail and AA-I content in both methanol and water extracts was also compared in this study. In our twenty samples of Asarum, the average amounts of AA-I in 75% methanol extracts of roots, rhizomes, petioles and leaves were 1.23 ± 0.64, 1.72 ± 1.38, 7.81 ± 4.04 and 27.13 ± 25.31 ppm, respectively. The average amounts of AA-I in water extracts of roots, rhizomes, petioles and leaves were 0.33 ± 0.32, 0.45 ± 0.50, 3.98 ± 3.09 and 6.32 ± 9.75 ppm, respectively. The results indicated that the amounts of AA-I in roots were less than those in petioles and leaves, and the amounts of AA-I in water extracts were about 25 to 50 percent of those in 75% methanol extracts. For Asarum preparations, the amounts of AA-I in each six of Xi-xin-extract-powder and Ma-Huang-Fu-Zi-Xi-Xin-Tang ranged between 0.35-2.07 ppm and 0.21-1.00 ppm, respectively. Results of this study could be provided for the regulatory authority as references.

Key words: Asarum, Xixin, Aristolochic acid I, LC/MS/MS

INTRODUCTION

Aristolochic acids (AAs) are well known nephrotoxin and potential carcinogen found in *Aristolochia* spp., *Bragantia* spp. and *Asarum* spp.[1-3]. Herbal products containing AAs probably cause renal damage or renal failure, and have been associated with the development of nephropathy[4,5]. Therefore, those products have been banned in several countries, such as Canada, Australia, Germany, UK and USA[6].

Aristolochia spp. and *Asarum* spp. are widely used in traditional Chinese medicine (TCM), but those products are regulated as medicines in Taiwan. The genus *Aristolochia* includes numerous medicinal plants, such as *Aristolochia fangchi* Wu, *A. manshuriensis* Kom., *A. debilis* Sieb. et Zucc., and *A. contorta* Bge, etc.. Because *Aristolochia* contains AAs, herbs of this genus had been prohibited from manufacturing, dispensing, importing, exporting, selling and displaying in Taiwan[7]. In addition, Chinese herbal preparations (CHPs) contained those prohibited herbs were recalled from market immediately and drug permit licenses of those CHPs were revoked in 2003[8]. However, Asarum (Xixin, named in Chinese) which originated from a few *Asarum* spp. remained available for medicinal use[9].

Asarum is mainly derived from *Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* Kitag, *A. sieboldii* Miq. or *A. sieboldii* Miq var. *seoulense* Nakaihas[10]. Asarum is commonly used in TCM and has antipyretic, anti-inflammatory and analgesic effects[11]. Asarum could be used only when its manufacturing process follows the regulations[9] , i.e. the medicinal part shall be the roots instead of the whole plants; only water extracts

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of Asarum can be used for medicinal purpose; raw materials and finished products shall be examined by the official assay method (HPLC/UV) to ensure free of AAs before marketing.

According to Chinese materia medica, the only medicinal part of Asarum is the roots because leaves are toxic[12]. Recent reports showed that Asarum contains trace amounts of aristolochic acid I (AA-I)[13,14], but researchers did not mention whether AA-I is extracted from whole plants or the roots only. More scientific evidences are needed to prove that Asarum roots are safer than whole plants. In addition, AAs could completely dissolve in organic solvents, but slightly dissolve in water[15]. Therefore, we predict that water extracts may contain less AAs. However, the amount of AAs that can be extracted by water from Asarum has not been evaluated yet. Asarum and its preparations shall be checked by the HPLC assay method to ensure those were free of AAs before marketing. Sensitive detection method still needs to be applied to Asarum to find out these were free of AAs or with trace AAs that were not detected by HPLC. Therefore, we used a sensitive and accurate instrument of LC/MS/MS to evaluate the amounts of AAs in roots, rhizomes, petioles and leaves of Asarum in this study, and compared the extraction ratio with different solvents between 75% methanol and water extracts.

MATERIALS AND METHODS

I. *Samples Identification*

Whole plant of Asarum samples were randomly collected from markets in Taiwan. Samples including 11 samples of *A. heterotropoidis* Fr. *var. mandshuricum* and 9 samples of *A. sieboldii* were identified by pharmacognosy exams. Each sample was subsequently divided into four parts including roots, rhizomes, petioles and leaves (Figure 1). Each part of samples was ground into powder for analysis. In addition, 6 samples of Xi-xin-extract-powder and 6 samples of Ma-Huang-Fu-Zi-Xi-Xin-Tang were purchased from pharmaceutical companies in Taiwan.

II. *Chemicals*

Aristolochic acids standard (Figure 2), a mixture of AA-I (40%) and aristolochic acid II (AA-II) (56%), was purchased from Sigma Co. (St. Louis, Mo, USA) (lot No. 092K1249). Piromidic acid was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA, USA) and used as the internal standard. LC grade methanol and reagent grade formic acid were purchased from Ridel-deHaën Co. (Seelze, Germany). LC grade acetonitrile was purchased from Labscan Co., Ltd. (Bangkok, Thailand). Reagent grade ammonium acetate was purchased from Merck & Co., Inc. (Darmstadt, Germany).

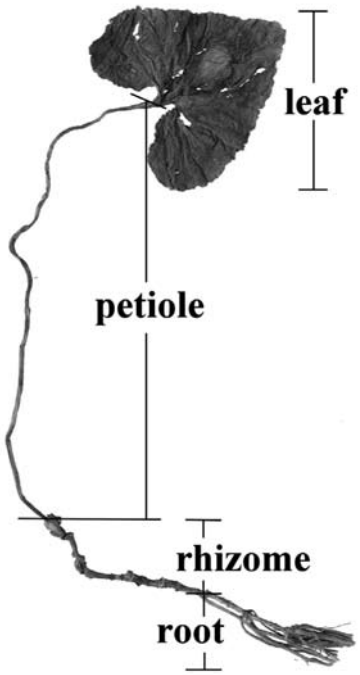
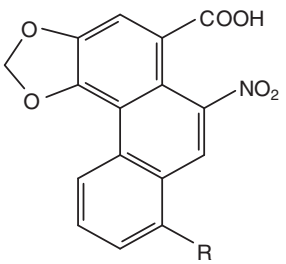


Figure 1. Portion of Asarum.



Aristolochic acid I: R = OC_3H
Aristolochic acid II: R = H

Figure 2. Structures of aristolochic acids.

III. *Instrumentation*

Analyses were carried out using a high performance liquid chromatograph (Waters 2690 Alliance, Milford, MA, USA) interfaced to a quadrupole mass spectrometer (Micromass Quattro Ultima) through a PEEK loop via a photodiode array detector (Waters 996) with split ratio adjusted as 1:1. Chromatographic separation was performed on a C18 column (Zorbax Extend-C18, 150 × 2.1 mm, i.d., 5 μm, Santa Clara, CA, USA) with an isocratic elution of mixed mobile phase solvents of 35% of acetonitrile and 65% of aqueous solutions (containing 0.1% ammonium acetate and 0.1% formic acid) at a flow rate of 0.3 mL/min. The photodiode array detector was set at 251 nm for chromatographic display. The mass spectrometer was fitted with an electrospray (ES) ioniza-

tion source, which was operated in positive mode. Identification of AA-I and AA-II was achieved using daughter ion scan mode. The parameters for daughter ion scan are listed in Table 1. Quantification was achieved by multiple reaction monitoring (MRM) mode. The pairs of ions selected as precursor and daughter ions were *m/z* 359 and 298 for AA-I, and *m/z* 329 and 268 for AA-II. The parameters for MRM analyses are listed in Table 2.

IV. *Preparation of Standard Solution and Calibration Curve*

Accurately weighed 5 mg of aristolochic acids was transferred to a 100-mL volumetric flask and dissolved in 75% methanol. The solution was diluted with 75% methanol to give standard solutions with AA-I concentrations of 0.01, 0.02, 0.05, 0.1, 0.5, 1, 2 and 5 μg/mL, each containing 0.1 μg/mL of the Piromidic acid internal standard. The corresponding AA-II concentration was 0.014, 0.028, 0.07, 0.14, 0.7, 1.4, 2.8 and 7 μg/mL, respectively. Twenty microliters of the standard solution were injected and analyzed by LC/MS/MS with parameters as described in Table 2. Quantification was achieved using MRM method. Calibration curve was obtained from the plot of concentration versus peak area ratio of standard to internal standard. The linear regression equation and its correlation coefficient (r^2) were then calculated.

V. *Sample Preparation*

For root, rhizome, petiole or leaf part of Asarum, 2.5 g of powdered samples was extracted with 75% methanol or water (40 mL, 30 mL, 30 mL) thrice by sonication for 20 min, respectively. After filtration, the combined extract was evaporated to dryness. The residue of root or rhizome was dissolved in 2.5 mL of 75% methanol or water, and the residue of petiole or leaf was dissolved in 10 mL of 75% methanol or water. For Asarum preparations, an amount of the sample contained 2 g of Asarum was extracted with 50 mL of 75% methanol by sonication for 20 minutes. After filtration, the extract was evaporated to dryness. Then the residue was dissolved with 75% methanol and transferred into a 10 mL volumetric flask. If necessary, the sample was diluted to allow detection in the range of the calibration curve. Piromidic acid was added to each sample as the internal standard, giving a final concentration of 0.1 μg/mL. A volume of 20 μL was injected and analyzed by LC/MS/MS. Quantification of AA-I was achieved using MRM method.

VI. *Precision and Accuracy*

The intra- and inter-day assay for AA-I were assayed at 0.01, 0.1, 1 and 5 μg/mL on the same day and on 6 consecutive days, respectively. The coefficient of variation (C.V.) served as a measure of precision. Accuracy was assessed by calculating the relative error (R.E.), i.e. the magnitude of the difference between exact value and measured value divided by exact value.

Table 1. Parameters of daughter ion scan for AA-I, AA-II and Piromidic acid in the LC/MS/MS analysis

Parameters	AA-I	AA-II	Piromodic acid
Ionization mode	ESI^+	ESI^+	ESI^+
Selected precursor ion (*m/z*)	359	329	289
Carrier Flow (mL/min)	0.3	0.3	0.3
Capillary voltage (kV)	3	3	3
Cone voltage (V)	20	20	40
Collision energy (eV): Argon	10	12	20
Source Temp (°C)	120	120	120
Desolvation Temp (°C)	350	350	350
Scan range (*m/z*)	200-400	200-400	200-400

Table 2. Parameters of MRM set for quantitative analysis of AA-I, AA-II and Piromidic acid

Parameters	AA-I	AA-II	Piromodic acid
Precursor ion (m/z)	359	329	289
Daughter ion (m/z)	298	268	271
Dwell (secs)	0.25	0.25	0.25
Cone (Volt)	20	20	40
Collision (eV)	10	12	20

VII. *Recovery*

Three different amounts of AA-I were added to samples and prepared as described in sample preparation. Each spiked solution contained final concentrations of 0.2, 1, or 2 μg/mL of AA-I. Injection was repeated in triplicate. Recoveries of AA-I were calculated from calibration curves.

RESULTS AND DISCUSSION

There are many methods to analyze AAs, such as TLC, HPLC, UPLC[16], HPLC/MS[17], and HPLC/MS/MS[13]. However, only high sensitive instruments could detect Asarum due to its trace AA-I. In the preliminary test, HPLC/UV, an official method referred by the Japanese Pharmacopoeia[18], was used to detect AAs

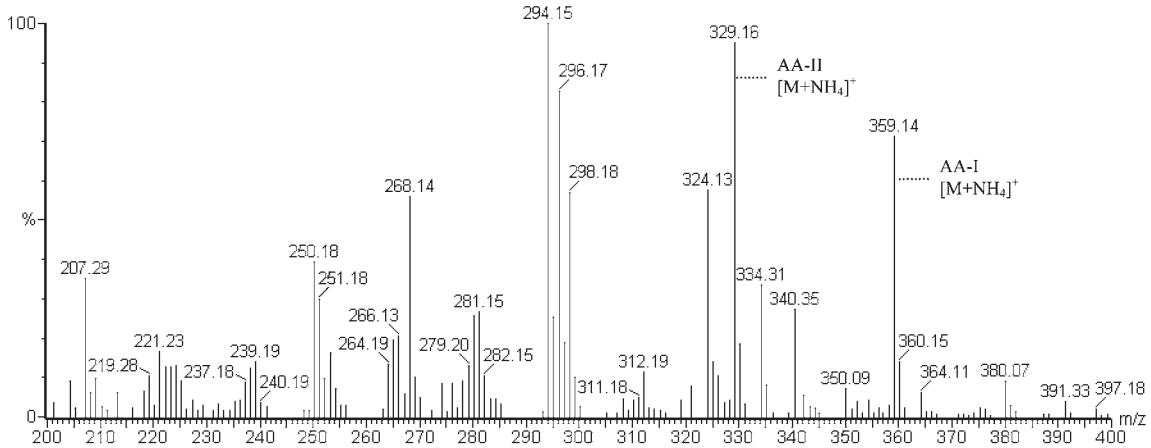


Figure 3. A full mass spectrum of AAs from *m/z* 200 to 400.

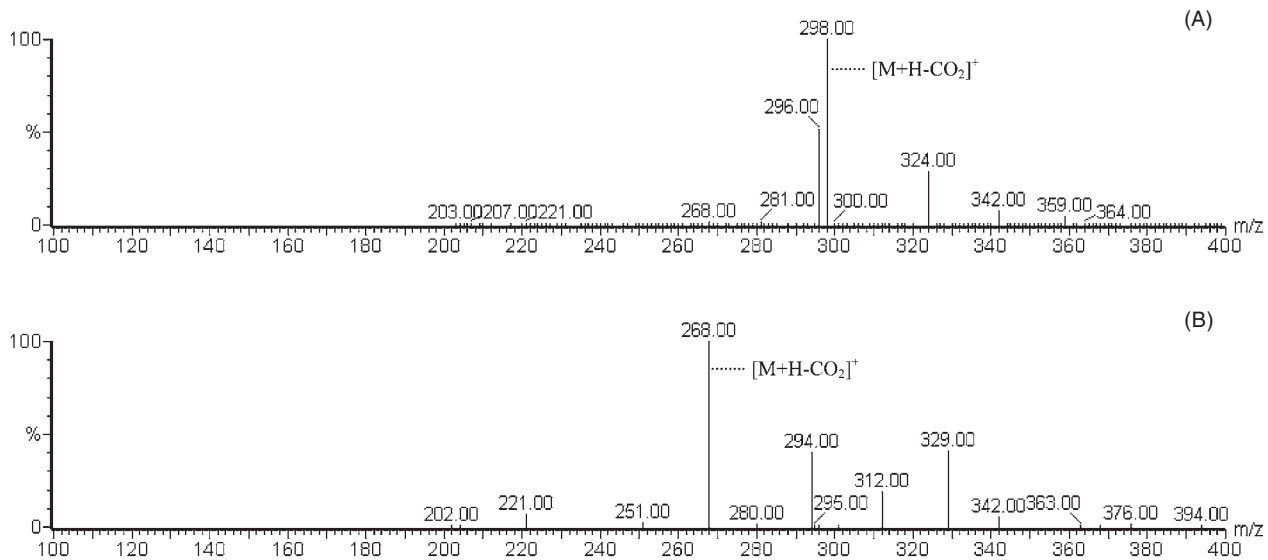


Figure 4. Daughter ion spectra of the $[M+NH_4]^+$ ions of (A) AA-I and (B) AA-II.

in Asarum. The result showed that AA-I was detected in leaf part of Asarum, but not in root part. In order to ensure accuracy of the above observation, the resulting test solution was further concentrated. AA-I was found in root part but could not be quantitatively determined due to matrix interferences. The preliminary data showed that HPLC/UV was not a suitable method for the analysis of trace AA-I in Asarum. Therefore, the present study applied LC/MS/MS to analyze AA contents in Asarum.

The analysis of AAs by LC/MS/MS method was developed in our previous report[19]. The reference standards AA-I and AA-II could be separated by LC. The positive ion electrospray mode used in MS/MS could yield a good spectra performance. High abundance $[M+NH_4]^+$ ion fragment of AA-I at *m/z* 359 and AA-II at *m/z* 329 occurred in the first stage of mass spectrum which was chosen as the precursor ion for the MS/MS analysis (Figure 3). The MS/MS product ions, *m/z* 298 and *m/z* 268, were used for quantitative measurement for AA-I and AA-II, respectively (Figure 4).

In this study, the detection limit for AA-I and AA-II based on a signal-to-noise ratio of three was 2.0 and 2.8 ng/mL, respectively. Calibration curve ranged from 0.01 to 5 μg/mL for AA-I and 0.014 to 7 μg/mL for AA-II, and the correlation coefficient (r^2) was over 0.9995. This indicated a definite linear relationship between the concentrations and the detector responses. The precision and accuracy results are showed in Table 3. For 0.01 μg/mL of AA-I, intraday and interday coefficients of variation (C.V.) were 19.98% and 19.03%, respectively. For concentration of 0.1, 1, and 5 μg/mL, intraday and interday C.V. ranged from 0.20 to 5.54%. In addition, the relative error ranged from 0.08 to 8.48%. This showed that the reproducibility was acceptable for analyzing AA-I. The recov-

Table 3. Precision and accuracy tests for aristolochic acid I

Concentration (μg/mL)	Intra-day			Inter-day		
	Measured value (μg/mL) (mean ± S.D.)[a]	C.V. (%)	R.E.[b] (%)	Measured value (μg/mL) (mean ± S.D.)[a]	C.V. (%)	R.E.[b] (%)
0.01	0.009 ± 0.002	19.98	5.99	0.011 ± 0.002	19.03	8.48
0.1	0.094 ± 0.004	4.19	5.95	0.094 ± 0.005	5.54	5.74
1	1.012 ± 0.033	3.29	1.22	0.998 ± 0.021	2.10	0.23
5	4.996 ± 0.013	0.27	0.08	4.990 ± 0.010	0.20	0.21

[a]n = 6.

[b]Relative error (%) = |exact value – mean measured value|/exact value × 100

Table 4. Recovery tests of aristolochic acid I in Asarum

Spiked concentration (μg/mL)	Recovery of each part (%)				mean ± SD
	Roots	Rhizomes	Petioles	Leaves	
0.2	110.7	96.2	114.6	111.4	108.2 ± 8.2
1	117.0	114.4	90.6	113.9	109.0 ± 12.3
2	93.7	117.7	92.3	92.1	99.0 ± 12.5

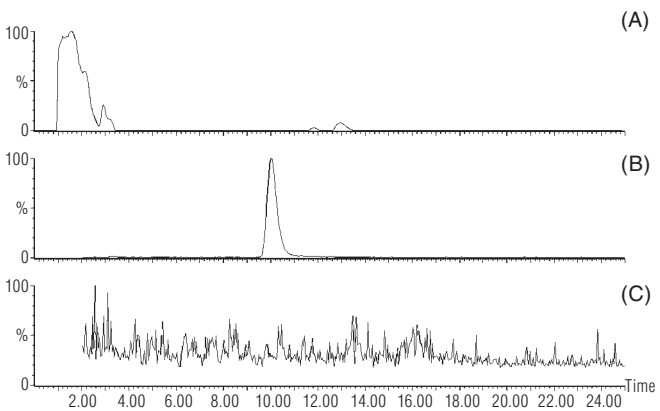


Figure 5. Chromatograms of the selected representative sample. (A) LC-UV chromatogram. (UV = 251 nm), (B) MRM chromatogram of AA-I (*m/z* 359>298), (C) MRM chromatogram of AA-II (*m/z* 329>268).

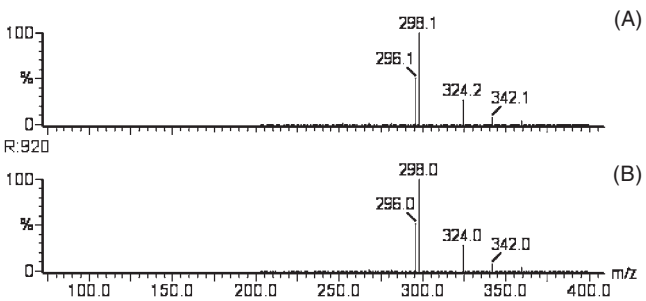


Figure 6. Daughter ion spectrum of the selected representative sample (A) was compared with the reference spectrum of AA-I (B).

eries of AA-I from methanol extracts of Asarum were determined by analyzing the extracts before and after spiking with three different concentrations of AA-I. The average recoveries of AA-I in root, rhizome, petiole and leaf parts of Asarum ranged from 99.0 to 109.0% (Table 4), suggesting the extraction procedure was suitable and the matrix of Asarum did not affect analyses. All data demonstrate LC/MS/MS method used in this study has good sensitivity, precision, accuracy and reproducibility for the determination of AA-I in Asarum.

In order to investigate the distribution of AAs in whole plant of Asarum, each plant was divided into four parts, i.e. roots, rhizomes, petioles and leaves. In addition, methanol and water extracts were compared in this study. The LC/MS/MS chromatograms of a selected representative sample are shown in Figure 5. AA-I was not detected by PDA detector (Figure 5-A) but could be determined by MRM analysis (Figure 5-B). AA-II was not detected by either PDA detector or MRM analysis (Figure 5-C). AA-I was further confirmed by the NLFD3/LM library database, the matching quality of the background subtracted daughter ion spectra between the sample and standard was over 800 (Figure 6).

The amounts of AA-I in various parts of Asarum are summarized in Table 5. The average amount of AA-I in 75% methanol extracts of roots, rhizomes, petioles and leaves was 1.23 ± 0.64, 1.72 ± 1.38, 7.81 ± 4.04 and 27.13

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Table 5. The amounts of aristolochic acid I in each part of Asarum

	Concentration (ppm)							
	Roots		Rhizomes		Petioles		Leaves	
Samples	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
A. *heterotropoidis* Fr. var. *mandshuricum* (n = 11)								
75% methanol extract	0.82 – 2.90	1.35 ± 0.78	0.41– 4.89	2.14 ± 1.51	2.34 – 18.41	7.59 ± 4.40	8.01 – 53.76	18.23 ± 14.23
H_2O extract	0.08 – 1.34	0.37 ± 0.40	0.08 – 1.87	0.61 ± 0.59	0.79 – 9.36	3.66 ± 2.71	0.69 – 9.29	3.00 ± 2.62
H_2O/75% methanol		0.27		0.28		0.48		0.16
A. sieboldii (n = 9)								
75% methanol extract	0.48 – 1.70	1.07 ± 0.40	0.19 – 3.87	1.21 ± 1.07	1.89 – 14.05	8.08 ± 3.81	11.10 – 89.78	38.01 ± 32.04
H_2O extract	0.03 – 0.59	0.28 ± 0.18	0.04 – 0.97	0.26 ± 0.29	1.26 – 12.19	4.36 ± 3.62	0.93 – 40.42	10.39 ± 13.54
H_2O/75% methanol		0.26		0.21		0.54		0.27
Total samples (n = 20)								
75% methanol extract	0.48 – 2.90	1.23 ± 0.64	0.19 – 4.89	1.72 ± 1.38	1.89 – 18.41	7.81 ± 4.04	8.01 – 89.78	27.13 ± 25.31
H_2O extract	0.03 – 1.34	0.33 ± 0.32	0.04 – 1.87	0.45 ± 0.50	0.79 – 12.19	3.98 ± 3.09	0.69 – 40.42	6.32 ± 9.75
H_2O/75% methanol		0.27		0.26		0.51		0.23

± 25.31 ppm, respectively. This result showed that the leaf has the highest amounts of AA-I and the root instead of the whole plant indeed reduces AA intake. In addition, the average amount of AA-I in water extracts of roots, rhizomes, petioles and leaves was 0.33 ± 0.32, 0.45 ± 0.50, 3.98 ± 3.09 and 6.32 ± 9.75 ppm, respectively. This result showed that the leaf and petiole contained higher amounts of AA-I than the root and rhizome. Furthermore, the AA-I contents in water extracts were one-fourth of that in 75% methanol extracts of roots, rhizomes or leaves and half of that in 75% methanol extracts of petioles. This showed that water extracts indeed contain reduced quantity of AAs. In conclusion, it unambiguously reduces AA intake by using water extracts of Asarum roots for medicinal purpose.

AA-II was not detected in any samples. This result corresponded with report by Jong *et al.*[13] who have analyzed the AA-I content in methanol extracts of 9 *Asarum* species by LC/APCI/MS. However, the authors did not mention which part of Asarum samples were analyzed. On the other hand, Xeu *et al.*[16] analyzed AA-I from the aerial and underground parts of Asarum by UPLC/UV. Their result showed that aerial part contains higher level of AA-I (ranged of 5.95-71.19 ppm) than the underground part (ranged of 0.48-5.0 ppm). In our study, the amounts of AA-I in roots, rhizomes, petioles and leaves of Asarum were evaluated separately. Our study showed that leaves have the highest amount of AAs followed by petioles, rhizomes and roots. This result corresponded with the report of Xeu *et al.*, but AA-I distribution in Asarum was surveyed in more details in our study.

There are several CHPs containing Asarum, such as Ma-Huang-Fu-Zi-Xi-Xin-Tang (麻黃附子細辛湯), Da-Qin-Jiao-Tang (大秦艽湯), Chuan-Chiong-Char-Tyau-Sann (川芎茶調散), Qing-Shang-Juan-Tong-Tang (清上蠲痛湯), Yeh-Gan-Ma-Hwang-Tang (射干麻黃湯), Jiu-Wei-Qiang-Huo-Tang (九味羌活湯), Dun-Hwa-Jih-Sheng-Tang (獨活寄生湯), and Sheau-Qing-Long-Tang (小青龍湯), etc.. Among these preparations, Ma-Huang-Fu-Zi-Xi-Xin-Tang contained 38% Asarum while the others contained less than 6% Asarum. Therefore, six samples of Xi-xin-extract-powder, a single-ingredient formula, and six samples of Ma-Huang-Fu-Zi-Xi-Xin-Tang, a multiple-ingredients formula, were selected as samples to evaluate the amounts of AAs in CHPs. The 12 samples were first analyzed by HPLC/UV and no AAs were found in them. We suspected that the samples either were free of AAs or contained trace AAs under detected limitation. To clarify whether those CHPs contain AAs, LC/MS/MS was then applied to analyze the samples. The amounts of AA-I in 12 CHPs are shown in Table 6. The amounts of AA-I in Xi-xin-extract-powder and Ma-Huang-Fu-Zi-Xi-Xin-Tang ranged from 0.35 to 2.07 ppm and 0.13 to 1.00 ppm, respectively. We estimated raw material Asarum contained 0.09-0.75 ppm of AA-I. This result indicated that the medicinal part of Asarum in these 12 CHPs might be roots or roots with

rhizomes. Furthermore, we estimated that daily doses of AA-I intake in those CHPs ranged from 0.79 to 7.44 μg. For renal toxicity of AAs in rats, it was reported that the non-toxic level was 0.2 mg/kg[20]. We predicted that short-term treatment with Xi-xin-extract-powder or Ma-Huang-Fu-Zi-Xi-Xin-Tang may not cause obvious renal damage. However, AAs are associated with nephropathy, and so the package of Asarum and its preparations should state potential risk of nephropathy of AAs to remind practitioners and customers.

CONCLUSIONS

This study has surveyed AA-I distribution in different parts of Asarum and compared AA-I contents in methanol and water extracts. The amounts of AA-I in leaves were the highest followed by petioles, rhizomes and roots. No AA-II was detected in Asarum. Furthermore, the amounts of AA-I in water extracts were about 25 to 50% of those in 75% methanol extracts. This result proved that the root and water extracts of Asarum definitely reduce AA intake. In addition, no AAs were found in the 12 Asarum preparation samples by the HPLC/UV method, but trace AA-I was found using the LC/MS/MS method.

Table 6. The amounts of AA-I in Xi-xin-extract-powder and Ma-Huang- Fu-Zi-Xi-Xin-Tang

	AA-I in preparation (ppm)	AA-I in Asarum[a] (ppm)	AA-I intake[b] (μg/day)
Xi-Xin-extract-powders			
1	0.90	0.15	0.81
2	0.88	0.19	3.15
3	0.36	0.09	1.96
4	2.07	0.75	7.44
5	0.35	0.11	3.16
6	0.59	0.18	2.13
Ma-Huang-Fu-Zi-Xi-Xin-Tang			
1	0.60	0.45	3.60
2	0.73	0.55	4.39
3	0.13	0.10	0.79
4	1.00	0.75	6.01
5	0.37	0.41	3.31
6	0.21	0.12	0.95

[a]AA-I in Asarum (ppm) = AA-I in preparation (ppm) ÷ the amount of Asarum in 1 g of preparation

[b]AA-I intake (μg/day) = labeled daily dose (g/day) × AA-I in preparation (ppm)

REFERENCES

1. Liu, M. C., Maruyama, S., Mizuno, M., Morita, Y., Hanaki, S., Yuzawa, Y. and Matsuo, S. 2003. The nephrotoxicity of *Aristolochia manshuriensis* in rats is attributable to its aristolochic acids. Clin. Exp. Nephrol. 7: 186-194.
2. Sato, N., Takahashi, D., Chen, S. M., Tsuchiya, R., Mukoyama, T., Yamagata, S., Ogawa, M., Yoshida, M., Kondo, S., Satoh, N. and Ueda, S. 2004. Acute nephrotoxicity of aristolochic acids in mice. J. Pharm. Pharmacol. 56: 221-229.
3. Nortier, J. L., Martinez, M. C., Schmeiser, H. H., Arlt, V. M., Bieler, C. A., Petein, M., Depierreux, M. F., De Pauw, L., Abramowicz, D., Vereerstraeten, P. and Vanherweghem, J. L. 2000. Urothelial carcinoma associated with the use of a Chinese herb (*Aristolochia fangchi*). N. Engl. J. Med. 342: 1686-1692.
4. Vanherweghem, J. L., Depierreux, M., Tielemans, C., Abramowitz, D., Dratwa, M., Jadoul, M., Richard, C., Vandervelde, D., Verbeelen, D. and Vanhaelen-Fraste, R. 1993. Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. Lancet 341: 387-391.
5. Vanherweghem, J. L. 1998. Missue of herbal remedies: the case of an outbreak of terminal renal failure in Belgium (Chinese herbs nephropathy). J. Altern. Complement. Med. 19: 15-20.
6. Cosyns, J. P. 2003. Aristolochic acid and 'Chinese herbs nephropathy': a review of the evidence to date. Drug Saf. 26: 33-48.
7. Department of Health, Executive Yuan. 2003. Prohibiting the sale of raw materials of *Aristolochia fangchi* Wu, *A. manshuriensis* Kom., *A. debilis* Sieb. et Zucc. and *A. contorta* Bge.. Announcement No. 0920002350, Taiwan, ROC. http://www.ccmp.gov.tw/public/public.asp?selno=816&relno=816&level=C
8. Department of Health, Executive Yuan. 2003. Revoking drug licenses of Chinese herbal preparation containing *Aristolochia fangchi* Wu, *A. manshuriensis* Kom., *A. debilis* Sieb. et Zucc. and *A. contorta* Bge.. Announcement No. 0920002349, Taiwan, ROC. http://www.ccmp.gov.tw/public/public.asp?selno=815&relno=815&level=C
9. Department of Health, Executive Yuan. 2004. Regulations for Asarum and its preparations. Announcement No. 0930000756, Taiwan, ROC. http://www.ccmp.gov.tw/public/public.asp?selno=601&relno=601&level=C
10. Commission of Chinese Herbal Medicine Pharmacopoeia. 2004. Chinese Herbal Medicine Pharmacopoeia. pp. 145-147. Department of Health, Executive Yuan. Taiwan, ROC.
11. Hsu, H. Y., Chen, Y. P., Shen, S. J., Hsu, C. S., Chen, C. C. and Chang, H. C. 1986. Oriental materia medica-a concise guide. pp. 47-48. Oriental Healing Arts

Institute. Long Beach, U.S.A.
12. Lee, S. Z. 1979. Ben Cao Gang Mu. pp. 816-817. People's Medical Publishing House. Beijing, China.
13. Jong, T. T., Lee, M. R., Hsiao, S. S., Hsai, J. L., Wu, T. S., Chiang, S. T. and Cai, S. Q. 2003. Analysis of aristolochic acid in nine sources of Xixin, a traditional Chinese medicine, by liquid chromatography/ atmospheric pressure chemical ionization/tandem mass spectrometry. J. Pharm. Biomed. Anal. 33: 831-837.
14. Hashimoto, K., Higuchi, M., Makino, B., Sakakibara, I., Kubo, M., Komatsu, Y., Maruno, M. and Okada, M. 1999. Quantitative analysis of aristolochic acids, toxic compounds, contained in some medicinal plants. J. Ethnopharmacol. 64: 185-189.
15. Budavari, S., O'Neil, M. J., Smith, A., Heckelman, P. E. 1989. The Merck Index. 11th ed. pp.124. Merk & CO., Inc. New York, U.S.A.
16. Xue, Y., Tong, X. H., Wang, F. and Zhao, W. G. 2008. Analysis of aristolochic acid A from the aerial and underground parts of Asarum by UPLC-UV. Acta Pharmacol. Sin. 43: 221-223.
17. Ioset, J. R., Raoelison, G. E. and Hostettmann, K. 2003. Detection of aristolochic acid in Chinese phytomedicines and dietary supplements used as slimming regimens. Food Chem. Toxicol. 41: 29-36.
18. Society of Japanese Pharmacopoeia. 2001. The Japanese Pharmacopoeia (English version) 14th. pp. 1303. Society of Japanese Pharmacopoeia. Tokyo, Japan.
19. Huang, C. Y., Tseng, M. C. and Lin, J. H. 2005. Analyzing aristolochic acids in Chinese herbal preparations using LC/MS/MS. 13: 125-131.
20. Mengs, U. and Stotzem, C. D. 1992. Toxicity of aristolochic acid-a subacute study in male rats. Med. Sci. Res. 20: 223-224.