Ultrasonication Extraction and Solid Phase Extraction Clean-up for Quantification of Aristolochic Acid I in the Thai Herbal Antitussive 3 Formula by High Performance Liquid Chromatography with Photodiode Array Detection

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ABSTRACT

Objective: To establish a reliable High Performance Liquid Chromatography method with photodiode array detection (HPLC-PDA) and a suitable extraction method for identification and quantification of Aristolochic acid I (AA-I) in 3 different sample types, i.e. *Aristolochia tagala* Cham.(ATC), Thai Herbal Antitussive 3 Formula (ATF3), and Thai Herbal Antitussive 3 Formula containing ATC (ATF3+ATC).

Methods: Two extraction methods, ultrasonication extraction (UE) and solid phase extraction (SPE), were used in the present study. Reversed phase separations were performed with a SunFire C18 column and HPLC-PDA. Mobile phase was performed by 1 mL/min infusion of mixture of 0.1% o-phosphoric acid and methanol (35:65).

Results: Calibration curve showed a good linearity with R2 equal to 0.9986. Every sample type has been analyzed. While the concentration of AA-I in ATF3 cannot be determined due to the absence of the AA-I peak, the AA-I concentrations in ATC and ATF3+ATC were 7.50-11.34 and 6.78-11.77 mg/mL respectively. In comparison with UE, SPE is more effective in eradication of the interfering compounds and improves the absorbance spectrum of the peak and increases extraction efficiency.

Conclusion: A reliable HPLC-PDA and SPE methods for identification and quantification of AA-I in ATF3+ATC, ATF3, and ATC were established in the present study. The SPE was more effective than UE in the pretreatment step.

Keywords: Aristolochic acid; *Aristolochia tagala* Cham.; Thai Herbal Antitussive 3 Formula; High Performance Liquid Chromatography (HPLC) (Siriraj Med J 2018;70: 227-232)

INTRODUCTION

The widespread occurrence of aristolochic acids (AA) toxicity in Aristolochia plants has greatly restricted their usefulness as dietary supplements or medicines. With the reports that virtually all of plants in genus *Aristolochia* contain AA[1], botanical products containing *Aristolochia* spp are not allowed in many countries including Thailand. In 2011, the National Drug Committee removed Krai-Krue, identified as *Aristolochia pierrei* Lec. and *Aristolochia tagala* Cham. (ATC)[2], from every Thai traditional herbal recipe. Testing for contamination and adulteration of AA in herbal products and medicines is now the focus of attention. These include many Thai traditional herbal recipes in the National Drug list

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previously containing Krai-Krue such as original Ammaruekkhawatee, Homnawakod, Homintrajak, and Thartbanjob. The Herbal Antitussive 3 Formula (ATF3), used in this study, contains the herbal ingredient as Ammaruekkhawatee. ATF3 has been used to relieve cough and reduce mucus in respiratory syndrome patients. It contains six herbal components which are *Terminalia chebula* Retz., *Glycyrrhiza glabra* L., *Cuminum cyminum* L., *Coriandrum sativum* L., *Phyllanthus emblica* L., and *Terminalia bellirica* (Gaertn.) Roxb.

AA is a mixture of structurally related nitrophenanthrene carboxylic acids compounds. AA and its derivatives are known to be nephrotoxic[3-7] and carcinogenic agents.[6-9] Aristolochic acid I (AA-I) is the most abundant of these compounds.[10] Although many assays can detect AA-I, a procedure based on High Performance Liquid Chromatography with photodiode array detector (HPLC-PDA) is a feasible choice, because of its ease of use and low cost of operation and maintenance, especially when compared to the other hyphenation procedures such as Liquid Chromatography with Mass Spectrometry (LC-MS). Previous studies revealed many extraction methods for detection of AA-I from Aristolochia plants such as ultrasonication extraction (UE)[11-16], Solid phase extraction (SPE)[17-18], Soxhlet extraction[19] and other techniques.[19,20] Among these, UE is an easy and efficient method for AA-I quantification when involving single herbal components. However, with complex herbal recipes, multiple peak separation still remains a significant problem. Concurrently, SPE has demonstrated efficacy when used in crude drugs and Kampo fomulations.[17] Therefore, testing of such SPE condition is needed in order to demonstrate similar efficacy for Krai-Krue containing ATF3 (ATF3+ATC). The aim of this study was to establish a reliable HPLC-PDA and a suitable extraction method for identification and quantification of AA-I in 3 different sample types, i.e. ATC, ATF3, and ATF3+ATC.

MATERIALS AND METHODS

Materials and solid phase

All plant materials were prepared by the GMP certified Manufacturing Unit of Herbal Medicine and Products, Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Krai-Krue used in the present study was identified as *Aristolochia tagala* Cham. (ATC). Three batches of herbs were used, with each batch comprising of 3 sample types, ATC, ATF3, and ATF3+ATC. HPLC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from LEDA (Spain). Analytical grade o-phosphoric acid was obtained from Merck (Germany). Standard AA-I was purchased from Sigma Chemical Co. (St. Louis, USA). HPLC quality water was purified using a Milli-Q water system from Millipore (France). Sep-Pak Vac 1cc (100 mg) Waters Accell Plus QMA cartridges were used.

Instrument and HPLC condition

Analyses were carried out using a Waters Alliance 2695 HPLC system with a 2998 photodiode array (PDA) detector and Empower Software. The separations were performed on a reverse phase column (Sunfire C18, 5µm, 4.6x150 mm, Water, Milford, MA), maintained at 25°C. The various percentages of mobile phase comprising of MeOH and 0.1% o-phosphoric acid were observed to optimize the separation and sensitivity. The experiments were performed under isocratic elution which consisted of 0.1% o-phosphoric acid: MeOH at 35:65 (v/v) at 1 mL/min flow rate. The injection volume for all samples was 10 µL. The PDA detector was scanned from 200 to 450 nm. The quantification wavelength was set at 393 nm to reduce interferences by other constituents.

Extraction of samples

According to the original formula of ATF3+ATC, there was 3.292 mg of ATC per 40 mg of ATF3+ATC. Therefore, the quantification of AA-I was performed with 3.292 mg/mL of ATC and 40 mg/mL of ATF3+ATC in order to maintain equal amounts of ATC in these 2 sample types.

Ultrasonication extraction (UE)

ATF3+ATC batch 1 and ATC batch 1 were used to optimize the number of UE. A sample from batch 1 of each sample type was divided into 3 groups which went through single, double and triple extraction cycles with 30 minutes ultrasonication for each extraction cycle in 100% MeOH. Supernates were separated from precipitates by centrifugation at 6,500 rpm for 10 minutes at 4˚C and filtered through the 0.2 µm PVDF (polyvinylidene fluoride) membrane before injection into the HPLC. The concentrations of AA-I for samples ATF3+ ATC and ATC alone are shown in Fig 1. For both sample types, double and triple UE yielded statistically higher concentration of AA-I compared to single extraction (Fig 1). However, when comparing double versus triple extraction, no differences in the yield of AA-I were seen. Therefore, the quantification experiment proceeded with only double extraction. (Table 1)

Solid phase extraction (SPE)

The SPE condition from Yamasaki K *et al*[17] was adapted, in order to obtain the appropriate procedure

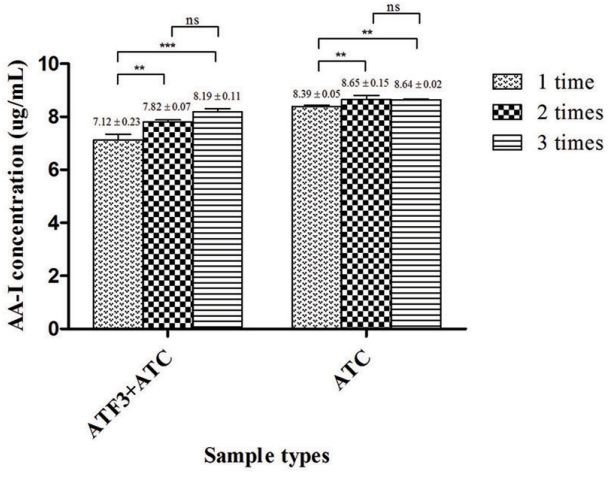

Fig 1. Test for the number of ultrasonication. The result expressed as mean±SD of triplicate experiments. One-way ANOVA followed by Bonferroni's *post hoc* test were applied for statistical study. ns = not significant, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

based on ATF3. First, all sample powders were extracted twice by mixing with 1% ammonia in MeOH over 10 minutes. Supernatants were separated from precipitates by centrifugation at 6,500 rpm for 10 minutes at 25°C. One milliliter of extraction solution was loaded onto the QMA cartridge, which was previously equilibrated with 1 mL of 1% ammonia in MeOH. In this step, ammonia ionized AA-I to negatively charged ion and adhered with an anion exchange resin. Next, washing was performed with 5 mL of MeOH in order to remove the interfering peaks which were seen to immediately precede the AA-I peak. The washing was done until the interfering peaks disappeared. Finally, cartridges were eluted with 1 mL of ACN:water:o-phosphoric acid (80:20:2) according to the best elution yield compared with other mixed ratios of ACN:water:o-phosphoric acid for elution solution. Each eluent was filtered through the 0.2 µm PVDF membrane before the analysis.

Validation of the HPLC method

Stock solution of standard AA-I (100 µg/mL) was prepared by accurately weighing of standard AA-I and dissolving in MeOH using a volumetric flask. After filtering through 0.2 µm PVDF membrane filters, working standard solutions were prepared by further dilution of the stock solution with the appropriate volume of MeOH. Each concentration was analyzed in triplicate. The linearity was evaluated by analysis of the standard curve.

The method precision was evaluated from relative standard deviation (RSD) of the area under the curve of each standard concentration. Three standard concentration solutions were performed five times in one single day for intra-day precision. For inter-day precision, the measurements from three standard concentrations solutions were performed three times a day on three consecutive days.

Recovery of the method was performed by adding of three known amounts of the standard solution into the selected samples. Since, the chromatogram of UE of ATF3 batch 2 and 3 (Fig 3B, 3C) showed similar patterns which contained the interfering peak preceding that of AA-I peak. In order to test whether or not it effected the recovery of the method, ATC and ATF3 batch 2 were selected as the representatives for the recovery test of ATC and ATF3, respectively.

Statistical analysis

The concentrations of AA-I in different sample types

TABLE 1. Concentration of Aristolochic acid I in ATC and ATF3+ATC. The result expressed as mean±SD of six values. Two-way ANOVA followed by Bonferroni's *post hoc* test was applied for statistical study. ND = not determine, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Sample type	Batch	AA-I concentration (µg/mL)		P-value
		UE	SPE	
ATC	1	8.02±0.08	11.34±0.11	<0.001
	2	7.50±0.09	8.51±0.18	<0.001
	3	8.64±0.27	9.66±0.32	<0.001
ATF3+ATC	1	6.78±0.05	9.81±0.12	<0.001
	2	ND	6.94±0.30	-
	3	ND	11.77±0.51	-

and through various extractions were represented as mean±standard deviation of 3 and 6 measurements. One-way and two-way analysis of variance with Bonferroni post *hoc*-test were performed. This software used GraphPad Prism™ version 5.02 for Windows (GraphPad Software, www.graphpad.com); a *p*-value of less than 0.05 was considered to be significant.

RESULTS

The results of satisfactory precision and recovery rates were shown in Tables 2 and 3. The precision RSDs were 0.60-3.30 for intra-day and 2.15-3.83 for inter-day (Table 2). The recovery of AA-I of ATC and ATF3 were in range of 93.73-103.27%. Whereas, the recovery result from UE of ATF3 was not reported in the present study (Table 3) because of the interfering peak preceding that of AA-I peak after addition of AA-I standard solution. The calibration curve was investigated in the range of 2-18 µg/mL (2, 6, 10, 14, 16, 18 µg/mL). The regression equation and correlation coefficient (R^2) were as follows: $Y = 14408.942\ X - 6893.590$ and $R^2 = 0.9986$, respectively. Limit of detection (LOD) and limit of quantification (LOQ), calculated as the concentrations of AA-I needed to produce signal-to-noise ratios (S/N) of 3 and 10 were determined to be 0.7 and 2.0 µg/mL, respectively. All ATC samples from both UE and SPE methods represented similar chromatograms and UV spectrum patterns as ATC sample from batch 1 (Fig 2B and 2b). Based on retention times and UV spectra patterns, AA-I was found in all ATC samples. In contrast, the concentration of AA-I could not be determined due to the absence of the AA-I peak in all UE of ATF3 samples (Fig 2C-E). Therefore, the SPE experiment for all ATF3 samples were not needed to be performed and the amounts of AA-I in all ATF3 samples were not reported (Table 1). When compared to UE (Fig 3a-c), the absorbance spectrum of ATF3+ATC batch 1-3 using SPE (Fig 3d-f) showed more similar pattern to standard AA-I. (Fig 2a). SPE improved the absorbance spectrum of the peak in this case. UE of ATF3+ATC batches 2 and 3 (Fig 3B, 3C) showed interfering peaks which could not be distinguished from AA-I peaks, which lead to incorrect peak areas and amount calculation. In these particular cases, AA-I concentrations were reported as not determined (ND) as shown in Table 1. While, the SPEs of ATF3+ATC batches 2 and 3 (Fig 3E, 3F) show a valuable clean-up of the problematic peaks resulting in a proper quantification of AA-I.

TABLE 2. Intra-day and inter-day precisions of Aristolochic acid I quantification.

Chemical compound	Concentration (µg/mL)	Intra-day (RSD)	Inter-day (RSD)
Aristolochic acid I	2	3.30	3.83
	10	2.64	3.28
	18	0.60	2.15

TABLE 3. Recovery tests of Aristolochic acid I in UE of ATC batch 2, SPE of ATC batch 2, and SPE of ATF3 batch 2. Each concentration was performed in triplicate.

Sample	Extraction method	Amount added (µg/mL)	Amount measured (µg/mL)	% recovery	Mean % recovery
ATC	UE	6.00	5.56	92.66	93.73±1.90
		7.50	7.05	93.98	
		9.00	8.51	94.54	
ATC	SPE	6.80	6.38	93.76	103.27±7.53
		8.50	9.06	106.62	
		10.20	11.16	109.44	
ATF3	SPE	5.50	5.70	103.67	100.59±3.83
		6.90	7.00	101.47	
		8.30	8.02	96.63	

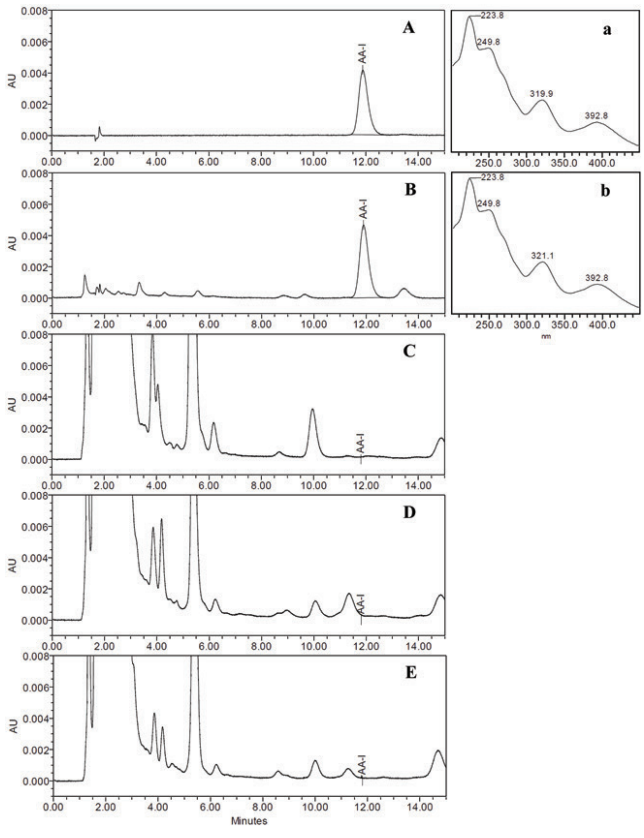

Fig 2. HPLC chromatograms (393 nm absorbance) of **(A)** standard AA-I; **(B)** UE of ATC batch 1; **(C)** UE of ATF3 batch 1; **(D)** UE of ATF3 batch 2; **(E)** UE of ATF3 batch 3; (a) Absorbance spectrum of standard AA-I recorded at 200-450 nm; (b) absorbance spectrum of the AA-I peak in UE of ATC batch 1.

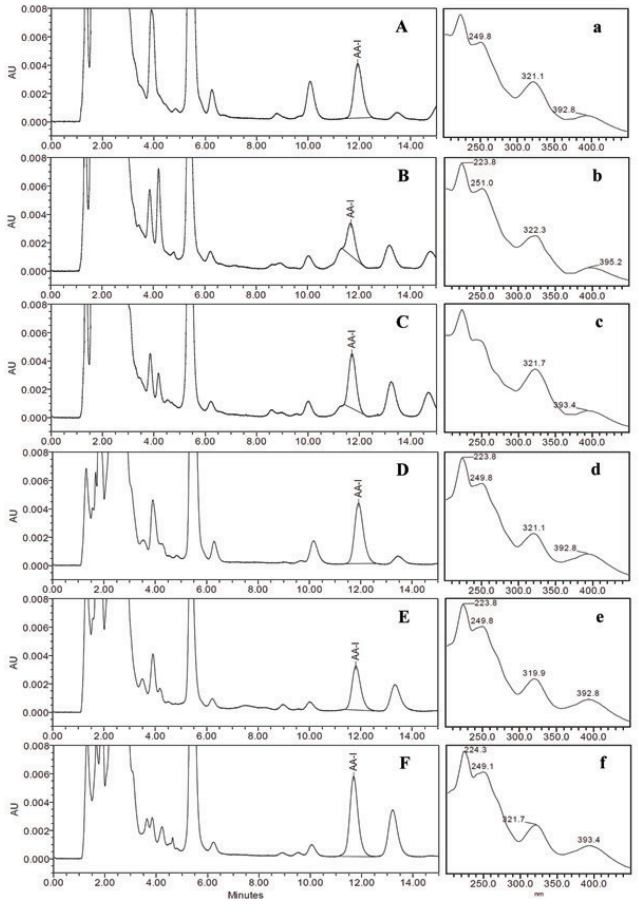

Fig 3. HPLC chromatograms (393 nm absorbance) of **(A)** UE of ATF3+ATC batch 1; **(B)** UE of ATF3+ATC batch 2; **(C)** UE of ATF3+ATC batch 3; **(D)** SPE of ATF3+ATC batch 1; **(E)** SPE of ATF3+ATC batch 2; **(F)** SPE of ATF3+ATC batch 3 **(a)**; Absorbance spectrum of the AA-I peak in UE of ATF3+ATC batch 1; **(b)** absorbance spectrum of the AA-I peak in UE of ATF3+ATC batch 2; **(c)** absorbance spectrum of the AA-I peak in UE of ATF3+ATC batch 3; **(d)** absorbance spectrum of the AA-I peak in SPE of ATF3+ATC batch 1; **(e)** absorbance spectrum of the AA-I peak in SPE of ATF3+ATC batch 2; **(f)** absorbance spectrum of the AA-I peak in SPE of ATF3+ATC batch 3.

DISCUSSION

There is no doubt that different batches of herbs can contain varying types and amount of compounds even in the same species of plant. A wide range of factors can modulate the active components of herb products, including physiological, environmental/cultural factors and genetic variation.[21] Therefore, the different chromatographic patterns from UE of ATF3+ATC batch 1 compared with batches 2 and 3 were not entirely unexpected. In addition, the good values of recovery from SPE of ATF3+ATC (Table 3) represented that SPE eliminated the interfering peaks without effect recovery of the method. When compared with other studies, there were 2 new findings in this study. Firstly, SPE showed a significant increase in AA-I concentrations compared to UE for all samples. This confirmed that SPE is a more efficient extraction method than UE alone. Secondly, it can be assumed that AA-I originates from the ATC component, since AA-I was found in ATF3+ATC, but not in ATF3 alone. In addition, the difference of AA-I concentrations in ATC compared with ATF3+ATC using SPE within the same batch indicated that the differences in type and amount of compound integrands in ATF3 in the sample matrix affected the efficiency of extraction. Overall, the ability of SPE to eradicate interfering compounds while improving the absorbance spectrum of AA-I peak and extraction efficiency makes it a more superior method for sample extraction of complex herbal mixtures.

CONCLUSION

This study provides reliable clarification method for the use of HPLC-PDA in analyzing AA-I compound in complex herbal mixtures. Since eradication of interfering peaks is crucial for accurate AA-I quantification using HPLC-PDA, the understanding of the role of SPE may contribute to the development of future methods for analyzing other complex herbal mixtures.

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REFERENCES

1. U.S. Department of Health and Human Services Public Health Services National Toxicology Program Research Triangle Park, NC27709. FINAL Report on Carcinogens Background Document for Aristolochic Acids. September 2, 2008. [Cited Oct 29, 2015; Available from:http://ntp.niehs.nih.gov/files/aristolochic_acids_%28final-02sep08%29_redo2%5B3%5D.pdf]
2. Sathornviriyapong S, Picheansoonthon C, Tiasakul R, Tiyaworanant S, Reutrakul V. Botanical Origin and Identification of Krai-Krue Herbal Plant. Kasetsart J (Nat Sci). 2007;41:420-32.
3. Vanherweghem JL, Depierreux M, Tielemans C, Abramowicz D, Dratwa M, Jadoul M, et al. Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. Lancet. 1993;341(8842): 387-91.
4. Wojcikowski K, Johnson DW, Gobe G. Medicinal herbal extracts - renal friend or foe? Part one: the toxicities of medicinal herbs. Nephrology (Carlton). 2004;9(5):313-8.
5. Li B, Li XM, Zhang CY, Wang X, Cai SQ. Injury in renal proximal tubular epithelial cells induced by aristololactam I. China J Chin Materia Medica.2004;29(1):78-83.
6. Nortier JL, Vanherweghem JL. Renal interstitial fibrosis and urothelial carcinoma associated with the use of a Chinese herb (Aristolochia fangchi). Toxicology. 2002;181-182:577-80.
7. Cheng CL, Chen KJ, Shih PH, Lu LY, Hung CF, Lin WC. Chronic renal failure rats are highly sensitive to aristolochic acids, which are nephrotoxic and carcinogenic agents. Cancer Lett. 2006; 232(2):236-42.
8. Nortier JL, Martinez MC, Schmeiser HH, Arlt VM, Bieler CA, Petein M, et al. Urothelial carcinoma associated with the use of a Chinese herb (Aristolochia fangchi). N Engl J Med. 2000; 342(23):1686-92.
9. Arlt VM, Stiborova M, Schmeiser HH. Aristolochic acid as a probable human cancer hazard in herbal remedies: a review. Mutagenesis. 2002;17(4):265-77.
10. Kuo PC, Li YC, Wu TS. Chemical constituents and pharmacology of Aristolochia species. J Tradit Complement Med. 2012;2(4): 249-66.
11. Chang Y-S, Deng J-S, Ku Y-R. Determination of Aristolochic acid in Traditional Chinese medicinal prescriptions, containing Radix Aristolochiae fangchi, by HPLC. J Liquid Chromatography & Related Technologies. 2002;25(6):961-975.
12. Chan W, Hui KM, Poon WT, Lee KC, Cai Z. Differentiation of herbs linked to "Chinese herb nephropathy" from the liquid chromatographic determination of aristolochic acids. Anal Chim Acta. 2006;576(1):112-6.
13. Yuan J, Nie L, Zeng D, Luo X, Tang F, Ding L, et al. Simultaneous determination of nine aristolochic acid analogues in medicinal plants and preparations by high-performance liquid chromatography. Talanta. 2007;73(4):644-50.
14. Zhao ZZ, Liang ZT, Jiang ZH, Leung KS, Chan CL, Chan HY, et al. Comparative study on the aristolochic acid I content of Herba Asarifor safe use. Phytomedicine. 2008;15(9):741-8.
15. Huang CY, Tseng MC, Lin JH. Analyzing Aristolochic acids in Chinese Herbal Preparations Using LC/MS/MS J Food Drug Anal. 2005;13(2):125-131.
16. Wattanarangsan J, Akarasereenont P, Lumlerdkij N, Chotewuttakorn S, Piwtong M, Laohapand T. Development of Ultra-High Performance Liquid Chromatography Technique for Detection of Aristolochic Acid I in Dried Root of Aristolochia tagala Cham. Siriraj Med J. 2012;64:110-3.
17. Yamasaki K, Tagami T, Kawaguchi M, Okihashi M, Takatori S, Sakagami Y, et al. Simple and rapid analysis of aristolochic acid contained in crude drugs and Kampo formulations with solid-phase extraction and HPLC photodiode-array detection. J Nat Med. 2009;63(4):451-8.
18. Ioset JR, Raoelison GE, Hostettmann K. Detection of aristolochic acid in Chinese phytomedicines and dietary supplements used as slimming regimens. Food Chem Toxicol. 2003;41(1):29-36.
19. Zhou T, Xiao XH, Wang JY, Chen JL, Xu XF, He ZF, et al. Evaluation of microwave-assisted extraction for aristolochic acid from Aristolochiae Fructus by chromatographic analysis coupled with nephrotoxicity studies. Biomed Chromatogr. 2012;26(2):166-71.
20. Ong ES, Woo SO, Yong YL. Pressurized liquid extraction of berberine and aristolochic acids in medicinal plants. J Chromatogr A. 2000;904(1):57-64.
21. Kays SJ. Preharvest factors affecting appearance. Postharvest Biology and Technology. 1999;15(3):233-47.