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Detection of aristolochic acid I, tetrandrine and fangchinoline in medicinal plants by high performance liquid chromatography and liquid chromatography/mass spectrometry

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Abstract

Problems with identification and labeling of medicinal plants, as well as substitution/adulteration of non-toxic plants by toxic ones have previously led to cancer, renal failure and even deaths. The non-toxic *Stephania tetrandra* (Fangji) has been known to be substituted by *Aristolochia fangchi* (Guang fangji), which contains the nephrotoxic and carcinogenic aristolochic acid (AA).

In this study, 10 samples of "Fangji" were bought from local medicinal shops. HPLC-DAD chromatographic fingerprints of each methanol extract were compared with those of *A. fangchi* and *S. tetrandra*, using aristolochic acid I (AAI), tetrandrine and fangchinoline as marker compounds. Nine of the samples were found to be similar to *A. fangchi*. The presence of AAI in the nine samples was confirmed using LC-MS/MS. Neither tetrandrine nor fangchinoline were detected in these samples. The methods developed in this study allow the simultaneous detection of AAI, fangchinoline and tetrandrine.

The results suggest possible substitution of *S. tetrandra* by *A. fangchi* at wholesale or retail level. This study highlights the importance of greater control of medicinal plants with toxic components as these may still be readily accessible to the public.

Keywords: Aristolochic acid I; Tetrandrine; Fangchinoline; *Stephania tetrandra*; *Aristolochia fangchi*; HPLC; LC-MS/MS

1. Introduction

Radix *Stephaniae tetrandrae*, generally known as "Fangji", is the dry roots of *Stephania tetrandra* S. Moore (Menispermaceae). Radix *Aristolochiae fangchi*, otherwise known as "Guangfangji", is the dry root of *Aristolochia fangchi* Y.C. Wu ex L.D. Chou et S.M. Hwang. Both herbs have been traditionally used as diuretics and for rheumatic conditions in China [1,2]. However, their chemical constituents are different. The main active chemical constituents in the roots of *S. tetrandra* are the alkaloids tetrandrine [3,4] and fangchinoline [4,5]; while aristolochic acid I (AAI) [6–8] is the major bioactive constituent of the root of *A. fangchi*. The structures of tetrandrine, fangchinoline and AAI are shown in Fig. 1.

Aristolochic acid (AA) is a mixture of structurally related nitrophenanthrene carboxylic acids, with AAI being the major component. AA was reported to be nephrotoxic and carcinogenic [6–9]. In February 1993, some young Belgium women, after taking a slimming regimen that included Chinese herbs, experienced renal failure [10] and the nephritic symptoms were referred to as Chinese Herbs Nephropathy (CHN) [11]. Suspicion that the condition was due to the introduction of Chinese herbs in the slimming regimen was reinforced by identification in the slimming pills of the nephrotoxic and carcinogenic aristolochic acid extracted from species of *Aristolochia* [12]. Pre-mutagenic AA-DNA adducts were subsequently identified in the kidney and ureteric tissues of CHN patients and successful induction of clinical features typical of CHN in rodents given AA alone confirmed the nephrotoxic agent to be AA. Hence, the original CHN is now known as aristolochic acid nephropathy

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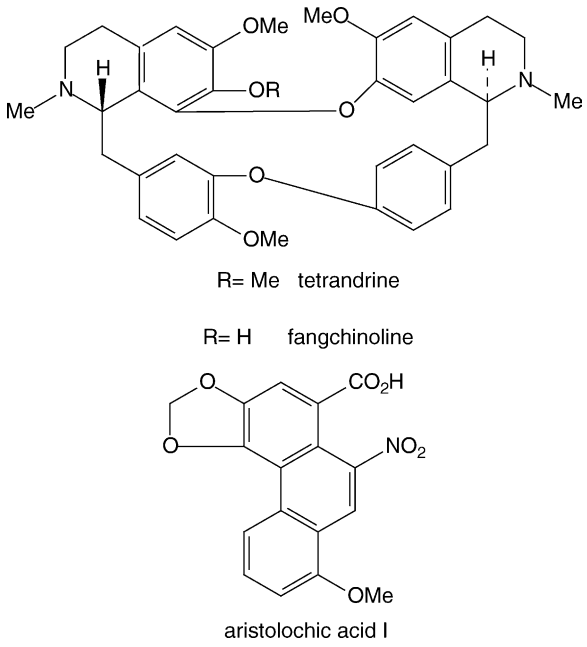

Fig. 1. Chemical structures of tetrandrine, fangchinoline and aristolochic acid I.

(AAN) [9]. Nortier et al. [13] also found cancer in 18 of 39 Belgiums who had been diagnosed with end-stage kidney failure caused by Radix *A. fangchi*. At least 65 cases of CHN have been reported worldwide and most of these cases could be classified as AAN patients [9]. Outbreaks of aristolochic acid-associated renal failure have been reported in several countries including France, Spain, Japan, Australia and the UK [14]. Several countries, including Canada, Australia, Germany and UK, banned the use of herbs containing AA [15].

Recently, the structure activity relationship of AA analogues has been reported [16]. Nitro and methoxy groups are found to be critical determinants of nephrotoxicological potency of AA. Various medicinal plants have been reported to contain AA. These include Aristolochia species and some Asarum species. Due to its toxicity, the ability to detect or determine AA is crucial. Indeed, many methods are available, e.g. TLC [17,18], fluorometry [19], GLC [19], HPLC [20–23], CE [24], CZE [25,26], LC-MS [18,27] and LC-MS/MS [28–30]. An NMR method has also been reported [31]. However, this method was developed using synthetic mixtures containing the internal standard maleic acid with purified AAI or combined AAI and aristolochic acid II (AA II) sodium salts. The capability of this method to determine AA in herbal extracts is uncertain. An electrochemical method was also developed [32]. Alkaloids from *S. tetrandra* including tetrandrine and fangchinoline, have also been detected and determined by TLC [33], HPLC [34], CE [35,36] and CE-ESI-MS/MS [37]. As there are many methods available for the detection or determination of AA1, fangchinoline and tetrandrine, it is beyond the scope of this paper to review each paper in detail. However, to the authors' knowledge, there is no reported method that directly differentiates between *S. tetrandra* and *A. fangchi*, using the three active constituents (AA1, fangchinoline and tetrandrine) as markers.

The roots of *S. tetrandra* and *A. fangchi* are usually sold in the form of slices and look similar in appearance. Despite the similarity in appearance of both herbs and the presence of toxic AA in *A. fangchi*, to date, no method for the simultaneous detection of AAI, tetrandrine and fangchinoline is available for the purpose of differentiating between the two roots. The objective of this work is to develop a RP-HPLC method for the rapid identication and differentiation of both *A. fangchi* and *S. tetrandra*, using AAI, tetrandrine and fangchinoline as marker compounds. A LC-APCI-MS/MS method was also developed for the detection of AAI, tetrandrine and fangchinoline in medicinal plant extracts. Ten samples were bought under the name of "Fangji" from local medicinal shops and analysed.

2. Experimental

2.1. Materials and reagents

Aristolochic acid I, fangchinoline and tetrandrine were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China). Methanol and acetonitrile were of HPLC grade. Milli Q water (Millipore, France) was used.

2.2. Samples

Radix *S. tetrandrae* (Fangji) was bought from Tongrentang (Beijing, China) and Radix *A. fangchi* (Guangfangji) was purchased from NICPBP (Beijing, China). Ten different samples of dried and sliced roots were purchased as "Fangji" from local medicinal shops.

2.3. Sample preparation

Two grams of powdered herbs were accurately weighed and were ultrasonicated with 20 ml methanol for 20 min. The process was repeated three times for each herb. After filtration, the combined methanol extracts were evaporated to dryness by a rotary evaporator. The residue was dissolved in 10 ml methanol and filtered by 0.45 μm membrane before analysis.

2.4. HPLC-DAD

The analysis was performed using an Agilent 1100 HPLC series. The column used was an Inertsil ODS-3 column (4.6 mm × 250 mm, 5 μm), operated at 35 °C. The mobile phase was 0.01% H_3PO_4 (v/v) in Milli Q water (pH 3.0) (A) and acetonitrile (B) with a gradient program as follows: 5% B to 100% B (55 min), 100% B to 100% B (5 min), post-run (10 min) at a flow rate of 1 ml/min. The injection volume for

all samples was 5 μl. Detection wavelength was 210 nm. UV scan was set from 200 to 400 nm.

2.5. LC-MS/MS

A Spectra Series HPLC (Finnigan, San Jose, USA) equipped with an autosampler was used. Separation were performed using on a Luna 5u C18(2) (2.0 mm × 150 mm, 5 μm), at a flow rate of 0.3 ml/min. The mobile phase was 0.1% acetic acid (v/v) in Milli Q water (A) and methanol (B) with a gradient program: 5–100% (B) in 20 min.

Mass spectrometric analysis was performed on a Finnigan LCQ ion trap mass spectrometer (San Jose, USA) equipped with an atmospheric pressure chemical ionization (APCI) interface. Positive ionization mode was applied. The following APCI parameters was optimized for detection of AAI, tetrandrine and fangchinoline: vaporizer temperature 300 °C, capillary temperature 150 °C and tube lens offset 0 V. The HPLC fluid was nebulized using N_2 as both the sheath gas at a flow rate of 60 au (arbitrary units), and auxiliary gas at a flow rate of 5 au. Collision induced dissociation (CID) experiments, MS-MS, were conducted using helium as the

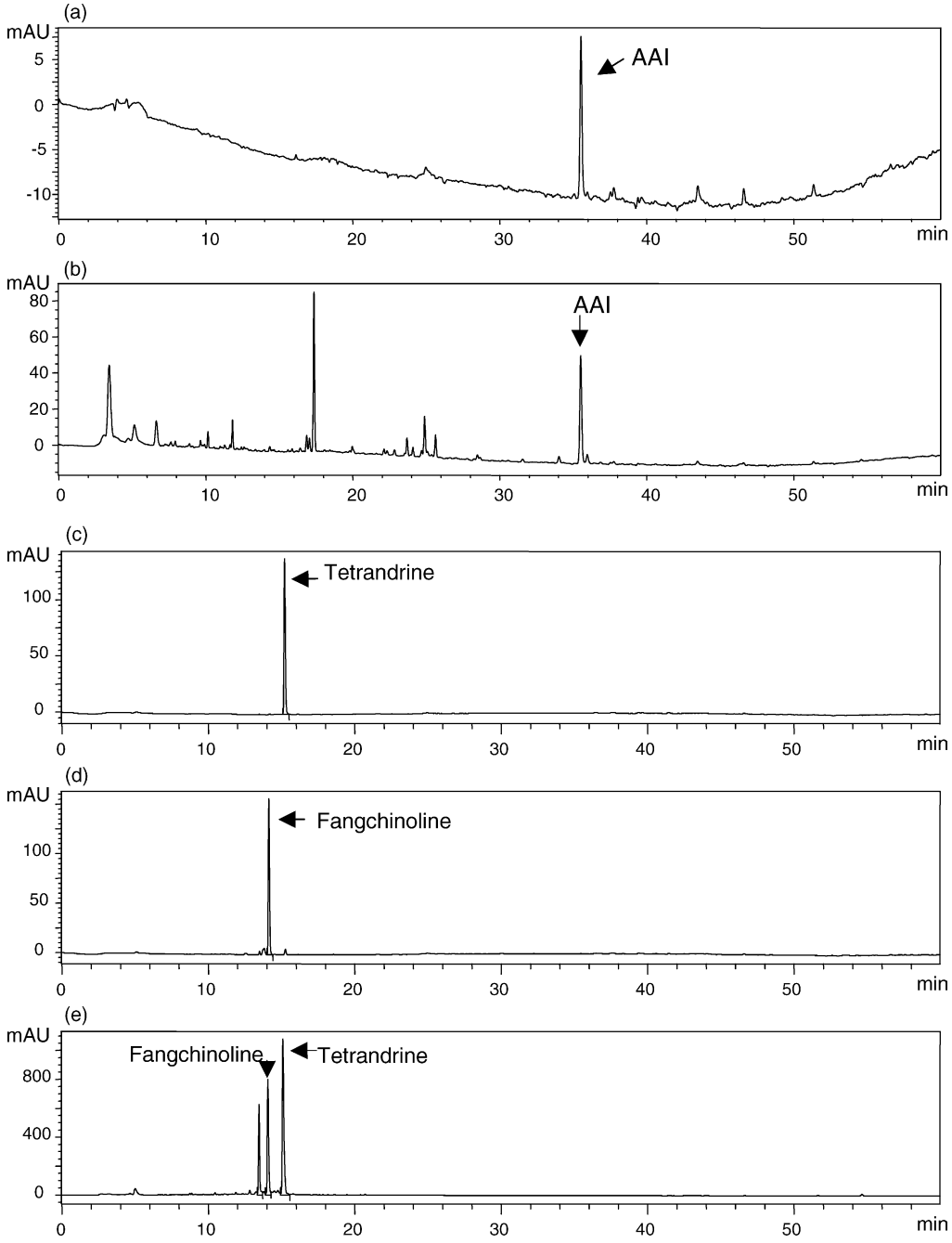

Fig. 2. HPLC chromatograms of: (a) AAI; (b) extract of *A. fangchi*; (c) tetrandrine; (d) fangchinoline; (e) extract of *S. tetrandra*.

collision gas, and the relative collision energy was set at 35–60%. Capillary voltage used was 10 V. The discharge current was 5 μA, while the relative collision energy was 35% for AAI, 40% for fangchinoline and 35% for tetrandrine.

3. Results and discussion

3.1. Chromatographic fingerprinting of A. fangchi and S. tetrandra

Fig. 2 a to e show the HPLC chromatograms of AAI, an extract of *A. fangchi*, tetrandrine, fangchinoline and an extract of *S. tetrandra* recorded at 210 nm, respectively. The HPLC chromatographic fingerprints of extracts of *A. fangchi* (from NICPBP) (Fig. 2b) and that of *S. tetrandra* (from Tongrentang) (Fig. 2e) were different. The marker compounds, tetrandrine, fangchinoline and AAI, eluted at different RT. AAI was detected in *A. fangchi* while tetrandrine and fangchinoline were detected in *S. tetrandra*.

3.2. HPLC analysis of local "Fangji" samples

The HPLC chromatographic fingerprints of the extracts of 10 locally bought "Fangji" samples were compared to those of *A. fangchi* and *S. tetrandra*. All but one of the samples resembled *A. fangchi*. The marker compounds, tetrandrine

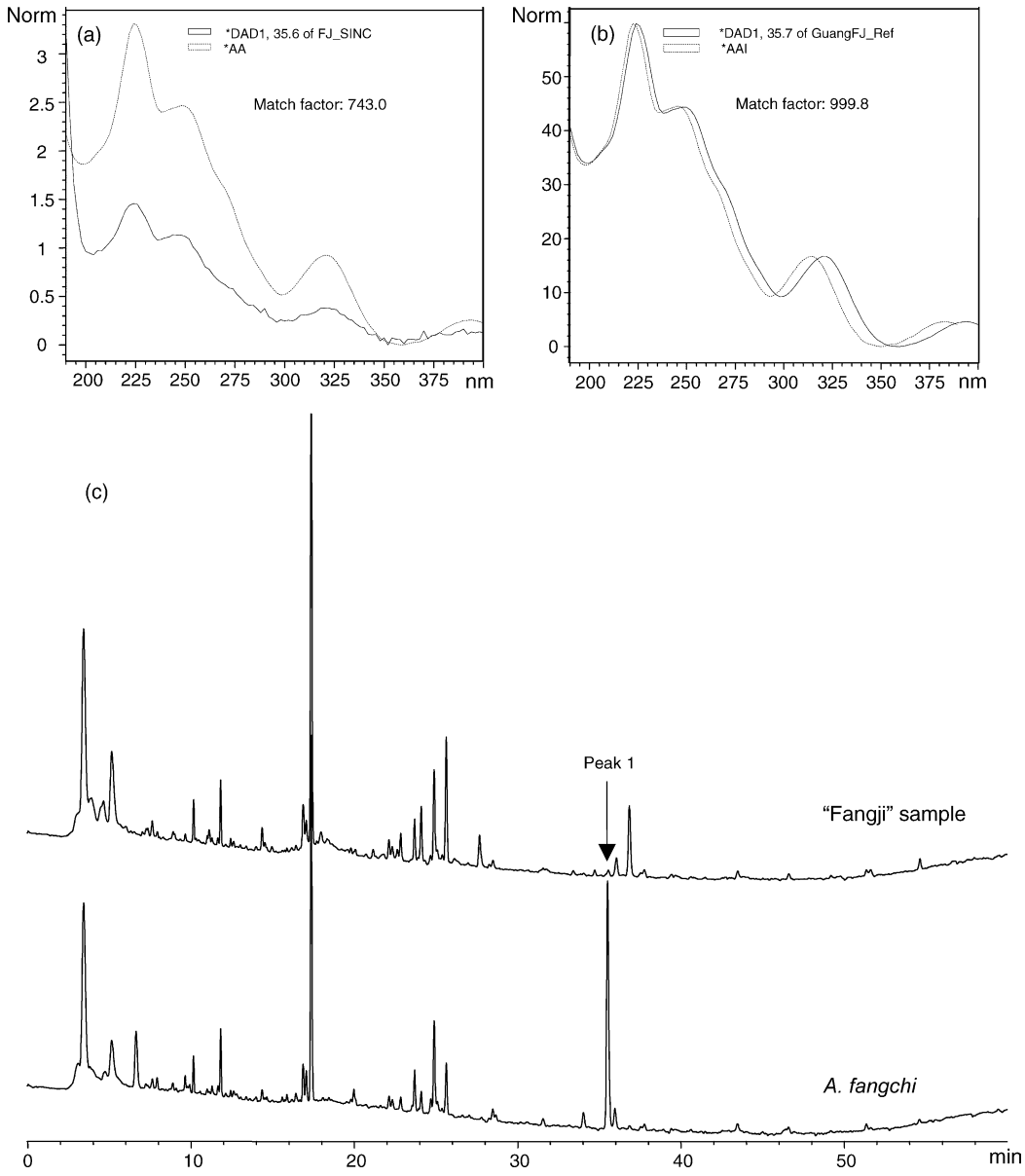

Fig. 3. (a) Overlaid UV spectra of peak 1 in Fangji sample from SINC (continuous line) and that of AAI (broken line); (b) overlaid UV spectra of peak at 35.7 min of reference *A. fangchi* (continuous line) and that of AAI (broken line); (c) overlaid chromatograms of the extracts of local Fangji sample from SINC and that of the reference *A. fangchi*.

and fangchinoline, could not be found in all the 10 herbal samples. Instead, AAI was found to be present in the nine samples that resembled *A. fangchi*. AAI was identified by comparisons of the RT and UV profiles, as well as spiking the samples with AAI. Fig. 3a shows an overlay of the UV profiles of AAI and the small peak (peak 1) at RT 35.6 min in one of the local "Fangji" samples. The profiles were similar but the match factor was only 743. Fig. 3b shows an overlay of the UV profiles of AAI and the peak at RT 35.7 min in the reference *A. fangchi*. An almost perfect match of the profiles was obtained, with a match factor of 1000. Fig. 3c shows an overlay of the chromatographic fingerprints of the Fangji sample in Fig. 3a and that of the reference *A. fangchi*. The fingerprints were very similar. The low concentration of peak 1, suspected to be AAI, may have given rise to the low match factor. To further confirm the presence of AAI in these samples, LC-MS/MS analyses were carried out. The on-line MS detector offers superiority over DAD detection in terms of specificity and sensitivity.

3.3. LC-MS/MS analysis

The LC/MS interface parameters were optimized in order to improve sensitivity and selectivity. This was done in the positive mode by taking into consideration of the vaporizer and the capillary temperature. The influence of the studied parameters on the response of the target analytes and the interaction of these parameters were evaluated based on the ammonium adduct molecular ion $[M+NH_4]^+$ m/z 359 for AAI, protonated molecular ion $[M+H]^+$ m/z 623 for tetran-

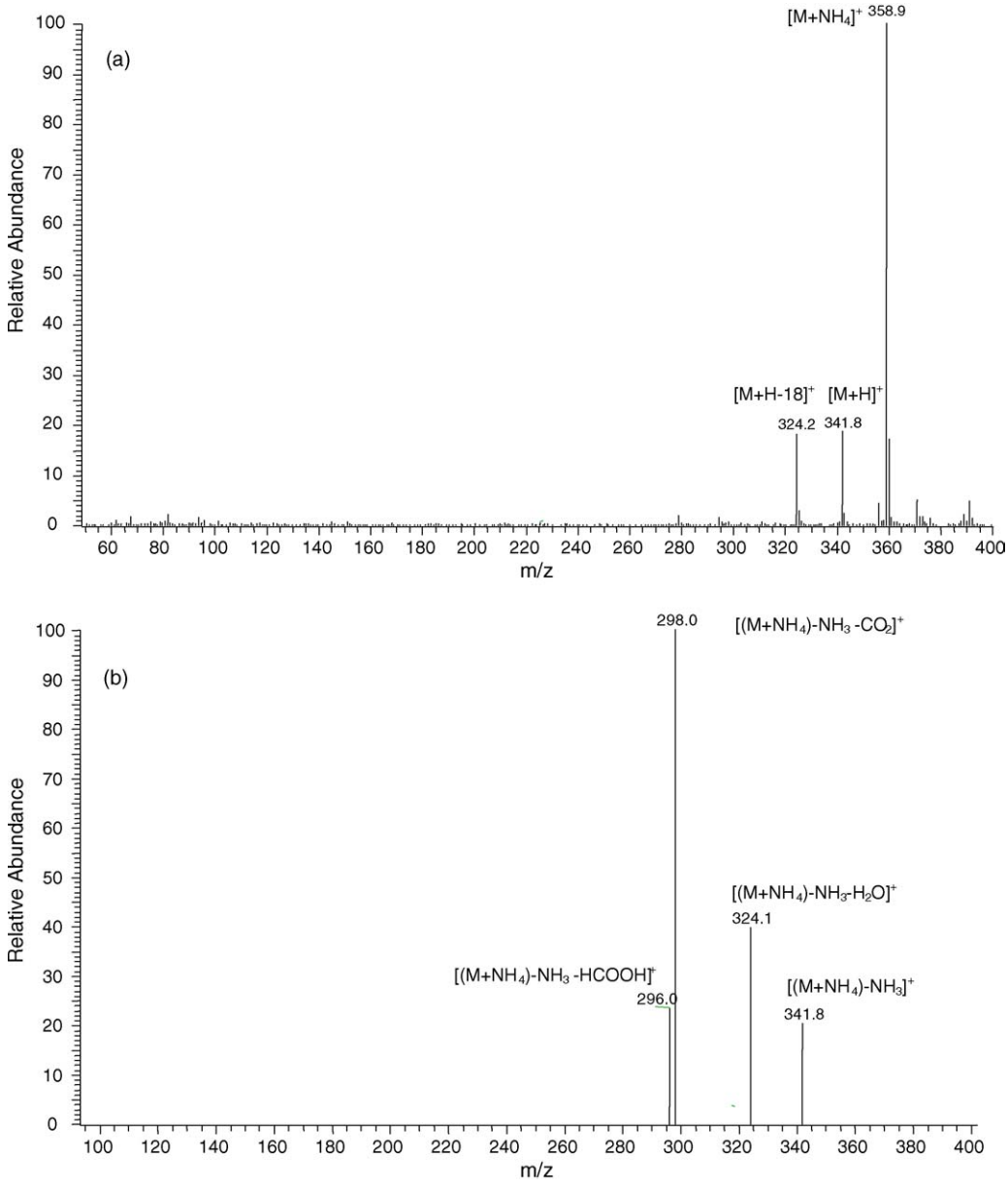

Fig. 4. (a) Full scan mode MS spectrum of AAI; (b) MS/MS spectrum of AAI, with precursor ion, $[M+NH_4]^+$ m/z 359.

drine and $[M + H]^+$ *m/z* 609 for fangchinoline in the positive mode.

3.3.1. Reference standards

In the full scan mode MS spectrum (Fig. 4a) of AAI, both protonated ($[M + H]^+$, *m/z* 342) and ammoniated ($[M + NH_4]^+$, *m/z* 359) molecules were generated together with a fragment ion, $[(M + H)\text{-}18]^+$ (*m/z* 324). The ammoniated molecular ion was the base peak. Although there is no ammonium additive in the mobile phase, however, it is not uncommon to find ammonium adducts in MS spectra. Possible sources of the ammonia include the ionization processes, from the atmosphere or from the herbal matrix. Further studies need to be carried out to determine the exact source. Full product ion spectrum of precursor ion at *m/z* 359 (the ammoniated molecular ion of AAI) was recorded (Fig. 4b). Protonated molecular ion, $[(M + NH_4)\text{-}NH_3]^+$ *m/z* 342, was generated by the loss of ammonia. Further loss of water from the precursor ion was very common, giving rise to $[(M + NH_4)\text{-}NH_3\text{-}H_2O]^+$ (*m/z* 324) ion. Another two specific product ions (*m/z* at 298 and 296) were also generated. The $[(M + NH_4)\text{-}44]^+$ ion due to the loss of carbon dioxide, while the $[(M + NH_4)\text{-}46]^+$ was due to the loss of formic acid (HCOOH).

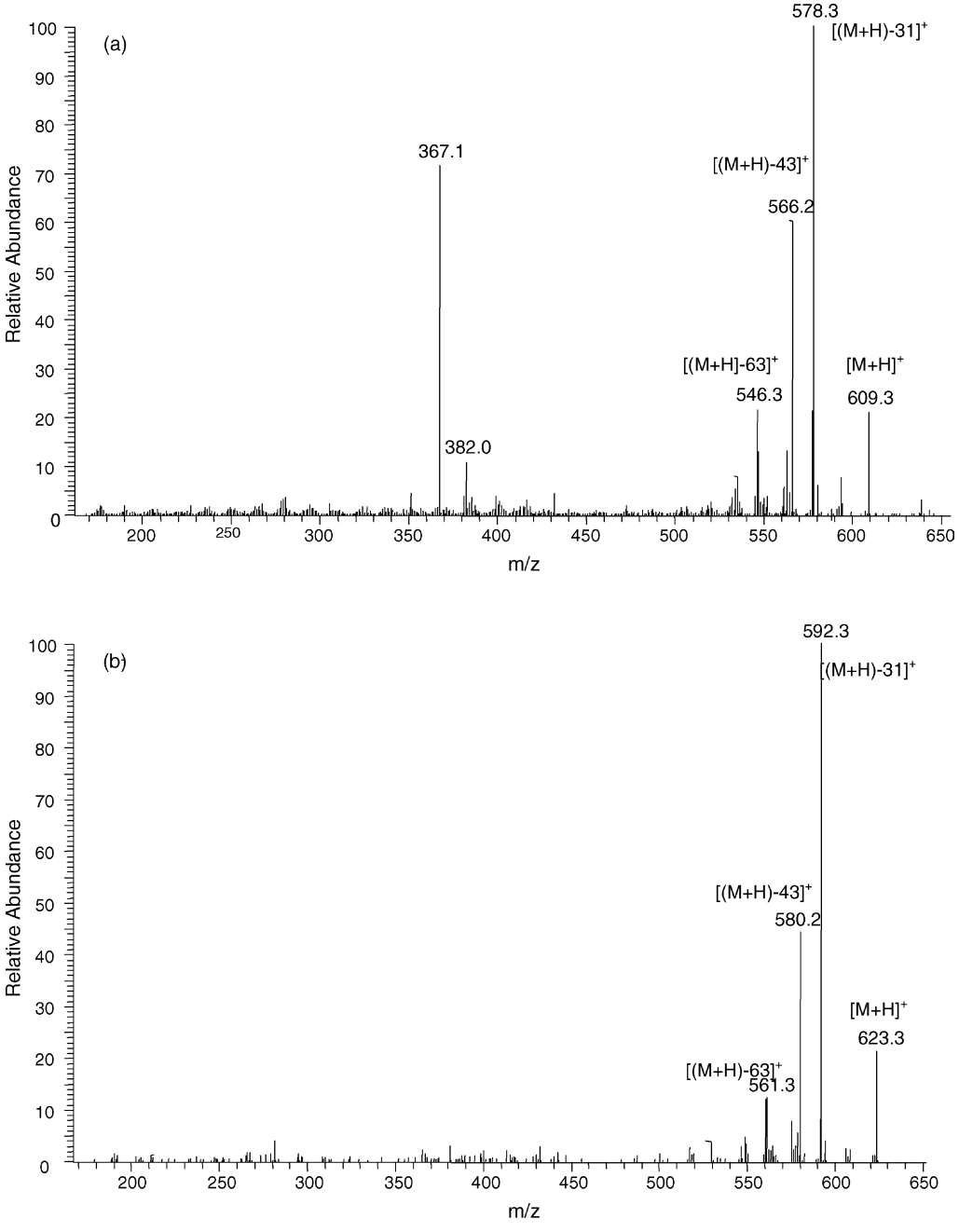

Fig. 5. MS/MS spectra of (a) fangchinoline with precursor ion *m/z* 609; (b) tetrandrine with precursor ion *m/z* 623.

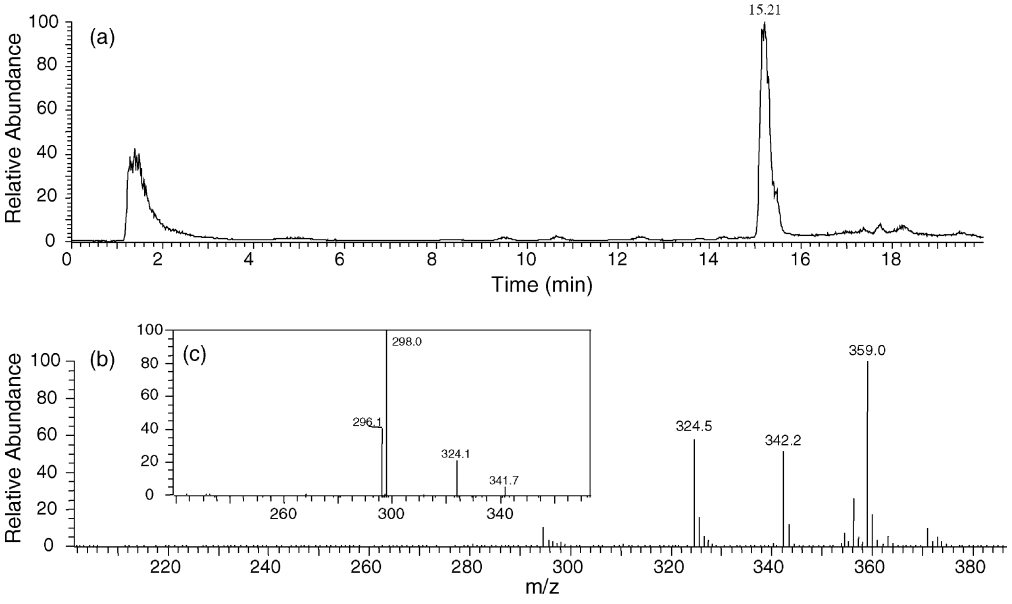

Fig. 6. LC-MS/MS analysis of *A. fangchi*: (a) total ion chromatogram (TIC); (b) full scan mode mass spectrum of the peak at 15.21 min; (c) MS/MS spectrum with parent ion *m*/*z* 359.

The fragmentation patterns are in good agreement with those previously reported using LC/ESI-MS [28]. These product ions provide useful confirmatory information for AAI.

In the full scan mode MS spectra of fangchinoline and tetrandrine, only protonated ions $[M+H]^+$ (*m*/*z* 609 for fangchinoline and *m*/*z* 623 for tetrandrine, respectively) were generated. With these protonated molecular ions as precursor

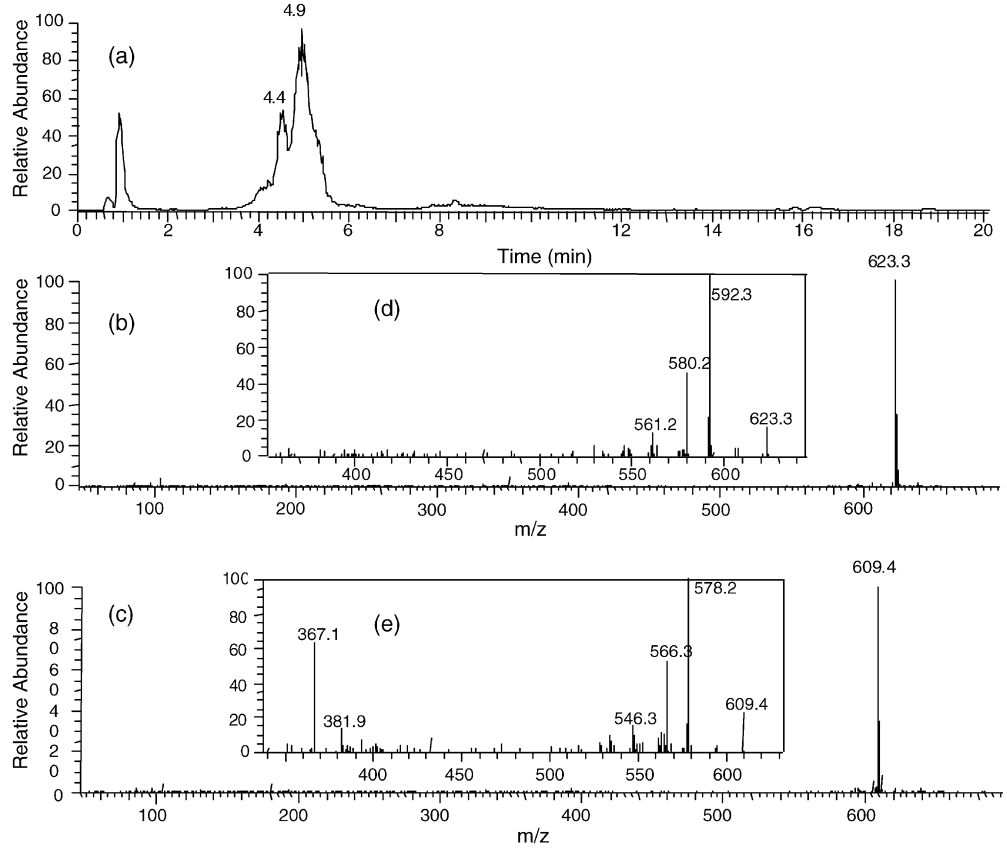

Fig. 7. LC-MS analysis of Fangji (Tongrentang): (a) total ion chromatogram, (b) mass spectrum of peak at 4.9 min, (c) mass spectrum of peak at 4.4 min; (d) MS/MS spectrum in full scan mode, precursor ion m/z 623.3; and (e) MS/MS spectrum in full scan mode, precursor ion *m*/*z* 609.4.

ions, the CID tandem spectra (MS/MS) yielded characteristic products ions (Fig. 5).

Under the optimized APCI parameters, *A. fangchi* and *S. tetrandra* were analyzed (Fig. 6). Fig. 6a shows a typical total ion chromatogram (TIC) of *A. fangchi* and related on-line mass spectrum of the peak of AAI with retention time at 15.21 min, obtained in positive ion mode (Fig. 6a). The major ions in the full scan mode are the ammoniated ($[M + NH_4]^+$, m/z 359) and protonated ($[M + H]^+$, m/z 342) molecules, and a fragment ion, $[(M + H)\text{-}18]^+$ (m/z 324). The ammoniated molecule was further selected. MS/MS experiment was performed. The secondary spectrum (Fig. 6c) shows all the characteristic product ions: m/z 342 for protonated molecule ion, m/z 324 for the ion due to further loss of water, m/z 298 for the ion further loss of CO_2 and m/z 296 for the ion further loss of HCOOH. The full scan mode mass spectrum and MS/MS spectrum are consistent with those of AAI standard (Fig. 4).

A typical TIC of an extract of *S. tetrandra* and related full scan mass spectra of the peaks of tetrandrine and fangchinoline with retention time at 4.9 and 4.4 min, respectively, were both obtained in positive ion mode (Fig. 7a). Only protonated molecular ions were detected, m/z 623.3 for tetrandrine (Fig. 7b) and m/z 609.4 for fangchinoline (Fig. 7c). The protonated molecules were further fragmented to give characteristic product ions (Fig. 7d and e). The full scan mode mass spectra and MS/MS spectra are consistent with those of tetrandrine and fangchinoline standards (Fig. 5).

Fig. 8a shows a typical total ion chromatogram of a local "Fangji" sample. The full scan mode mass spectrum of peak at 15.14 min (Fig. 8b) exhibited the characteristic ammoniated molecular ion, m/z 359 ($[M + NH_4]^+$), protonated ion, m/z 342 ($[M + H]^+$) and a fragment ion at m/z 324 ($[(M + H)\text{-}H_2O]^+$). The secondary spectrum shows the characteristic product ions, m/z 342 ($[(M + NH_4)\text{-}NH_3]^+$), m/z324 ($[(M + NH_4)\text{-}NH_3\text{-}H_2O]^+$), m/z 198 ($[(M + NH_4)\text{-}NH_3\text{-}CO_2]^+$), and m/z 296 ($[(M + NH_4)\text{-}NH_3\text{-}HCOOH]^+$) (Fig. 8c). The content of AAI in these samples was much lower than that in the reference *A. fangchi* (NICPBP, Beijing) (Fig. 6). These results are consistent with those of HPLC-DAD analysis (Fig. 3).

A case of unexplained nephropathy was reported [38] in a patient who had taken Herba *A. mollissemae*. This plant was found to contain AA. The patient had originally been prescribed a non-nephrotoxic plant, which unfortunately had been substituted by *A. mollissemae* at wholesaler level. Many countries have banned the sale of herbs or products containing AA [14,15]. Likewise in Singapore, to safeguard public health, products (including Chinese Proprietary Medicine) and herbs sold and supplied in Singapore are not allowed to contain aristolochic acids and their salts with effect from 1 January 2004 [39]. However, in this study, 9 out of 10 samples analysed were found to contain AAI. These samples are freely available for sale. There is a need for greater control of medicinal plants containing toxic components. Perhaps the usage of such plants can be restricted to trained and qualified practitioners, e.g. qualified traditional Chinese Medicine practitioners. The use of pharmaceutical names [40] with Latin scientific names according to established or official references, as well as more research into establishing the safety, quality and efficacy of medicinal plants will be important steps forward.

In summary, 9 out of 10 local samples bought as "Fangji" had similar chromatographic fingerprints as that of *A. fangchi*, and the presence of toxic AAI in all these samples were confirmed by LC-MS/MS analysis. This case has been reported to Health Sciences Authority. Interestingly, the sample that

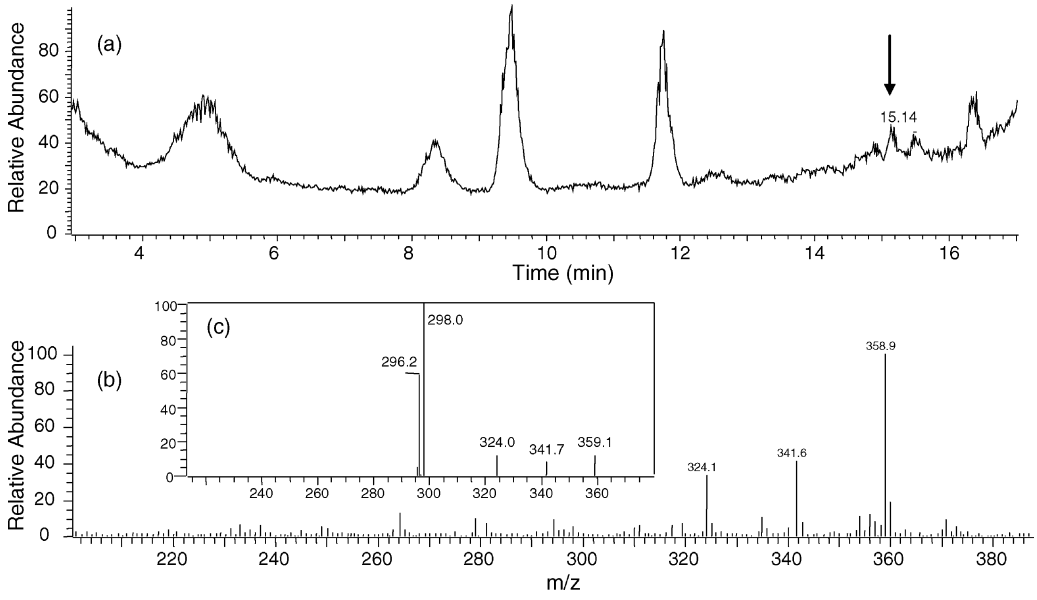

Fig. 8. LC-MS/MS analysis of a local "Fangji" sample: (a) total ion chromatogram; (b) full scan mode mass spectrum; (c) MS/MS spectrum, precursor ion m/z 359.

did not resemble *A. fangchi* did not resemble *S. tetrandra* either. Its identity has to be further elucidated.

4. Conclusion

This study is the first report of chromatographic fingerprinting of *A. fangchi* and *S. tetrandra* and locally available dried and sliced medicinal roots sold as "Fangji", using active constituents of both herbs as chemical markers. The methods developed in this study allow the simultaneous detection of AAI, fangchinoline and tetrandrine. The results suggest possible substitution of *S. tetrandra* by *A. fangchi* at wholesale or retail level. This study highlights the importance of greater control of medicinal plants with toxic components as these may still be readily accessible to the public. Additional measures to ensure the correct identification and labeling of medicinal plants are necessary to minimize risks to consumers and to maximize potential benefits from medicinal plants.

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