

Method Article

## Determination of Aristolochic Acid Using Isocratic RP-HPLC Method

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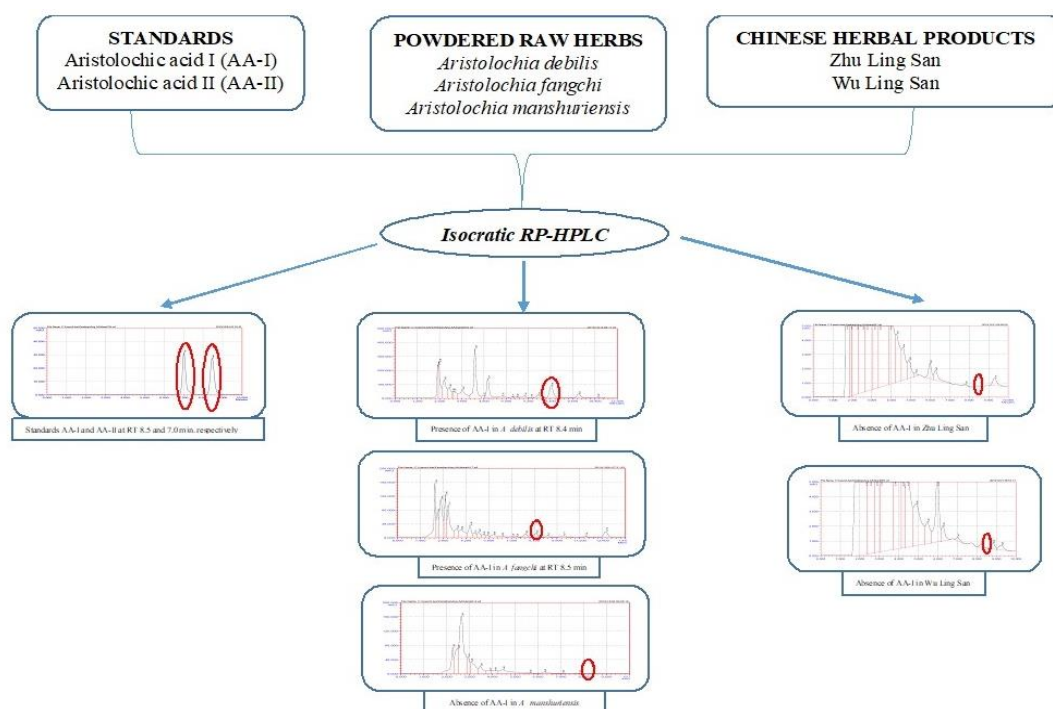
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**Abstract:** Aristolochic acid (AA) is an abundant chemical substance commonly present in the genus, *Aristolochia*. In terms of stereoisomerism, Aristolochic acid I (AA-I) is known to cause nephrotoxicity, genotoxicity, and carcinogenicity. Therefore, the determination of AA-I in herbal products is imperative to avoid unwanted adverse drug reactions. Hence, this study was undertaken to develop a low-cost isocratic HPLC method to detect AA-I and AA-II in two selected products. RP-HPLC instrument (Cyberlab LC-100) equipped with a photodiode-array UV detector with a Capcell Pak C<sub>18</sub> - column (5µm, 25 x 0.46 cm), and a mobile phase, methanol-water (75:25) added with 0.1% glacial acetic acid (pH 4.14) with a flow rate of 1.0 mL/min. at 254 nm was used. The total run time was 10 min. The results indicate that the isocratic RP-HPLC method developed in the present study is a reproducible and precise method with an optimum resolution for the separation of peaks. The presence of AA was determined in three herbal plants, *Aristolochia debilis*, *Aristolochia fangchi* and *Aristolochia manshuriensis* and two different Chinese herbal products. The retention time of the standards, AA-II and AA-I was found at 7.0 min and 8.35 min respectively, AA-I was also identified in raw herbs, *A. debilis*, and *A. fangchi* at 8.4 and 8.5 min. respectively; but there was no peak found for AA-I in *A. manshuriensis*. The HPLC analyses of methanol extracts of the two selected Chinese herbal products, Zhu Ling San and Wu Ling San indicated the absence of AA-I peaks at 8.4 min. The results conclude that the isocratic RP-HPLC method developed is simple and precise to determine the presence or absence of aristolochic acid.

**Keywords:** Herbal products; Aristolochic acids; High performance liquid chromatography; Retention time

## GRAPHICAL ABSTRACT



## 1. Introduction

Herbal products are utilized all over the world as a natural alternative to modern pharmaceuticals for their health benefits. Herbal products can be beneficial, but they can also be harmful in some cases<sup>[1]</sup>. Aristolochic acid (AA) is a chemical found in *Aristolochia* plants and other herbs in abundance. Previous research has found that AAs have anti-tumor, anti-infective, anti-inflammatory, analgesic, antifertility, and blood pressure regulating properties. As a result, AAs have long been used to treat various ailments, including eczema, pneumonia, stroke, hepatitis, snake bites, arthritis, gout, and coronary artery disease<sup>[2]</sup>.

Aristolochic Acid Nephropathy (AAN) is a chronic tubulointerstitial renal disease. It is linked to urothelial carcinoma of the upper tract (UTUC). In 1992, a group of Belgian patients developed quickly progressing interstitial nephritis after ingesting Chinese medicines used for slimming. Renal failure was a result of severe interstitial fibrosis, which included atrophy, tubule loss, and urothelial hyperplasia, mainly localized in the superficial cortex<sup>[3]</sup>. This nephropathy was once known as Chinese-herb nephropathy (CHN), and it was later renamed Aristolochic Acid Nephropathy (AAN). *Stephania tetrandra* (Han Fang Ji) was found to have been mistakenly swapped with AAs-rich *Aristolochia fangchi* (Guang Fang Ji). Both plants have the same common name in Chinese, and regardless of their botanical

classification, one might be used instead of the other in traditional Chinese medicine<sup>[4,5]</sup>. These patients reported the presence of AA-derived DNA adducts in their kidneys and ureteric tissues, confirming AAs as the primary cause of AAN. These patients reported finding AA-derived DNA adducts in their kidneys and ureteric tissues, suggesting AAs as the primary cause of AAN<sup>[6]</sup>. If patients consume products suspected of containing AAs for an extended time, they will be at a higher risk of nephrotoxicity. Although most authorities prohibit or restrict the sale and use of AA-containing products, AAN cases are continuously reported from all over the world<sup>[7,8]</sup>.

Unfortunately, AA consumption has been associated with kidney failure as well as urinary tract malignancies. The International Agency for Cancer Research (IARC) claims that (IARC) AAs are classed as group 1 because there is enough evidence that they cause cancer in humans. AAs have also been identified as one of the key contributors to the rapid progression of renal disorders<sup>[9,10]</sup>. Scientists discovered some clinical and morphological correlations between distinct phases of AAN and the patterns of Balkan-endemic nephropathy (BEN), suggesting that BEN could be linked to AAs as well. BEN is an endemic family disease related to upper urinary tract urothelial cancer. Countries such as Serbia, Bosnia and Herzegovina, Croatia, Bulgaria, and Romania are affected by this kind of nephropathy<sup>[11,12]</sup>. Ivic *et al.* proposed that ingestion of flour infected with seeds from *Aristolochia clematitis* has been suggested as a possible cause of BEN<sup>[13]</sup>. During the harvesting process, seeds from *A. clematitis*, which flourished abundantly in local wheat fields, mixed with wheat grains<sup>[11]</sup>. Long-term use of crops that uptake and bioaccumulate AAs from *Aristolochia* species-grown soil and water resulted in BEN. In recent times, definite correlations between BEN and AAs have been reported. AA-derived DNA adducts and typical AT transversions in the TP53 tumor suppressor gene mutational spectrum have been discovered in renal cortical and urothelial malignant tissues recovered from BEN patients<sup>[14,15]</sup>.

At least 100 cases of severe interstitial fibrosis of the kidneys were recorded in Belgium in the 1990s as a result of weight loss treatment. Because of the similar Chinese Pin Yin names, products containing active species like Han Fang Ji (*S. tetrandra*) were mistakenly exchanged with Guang Fang Ji (*A. fangchi*) in the treatment. Toxic aristolochic acids found in *A. fangchi* have been linked to kidney failure and malignancies of the urinary system. Uncontrolled usage and/or misidentification of therapeutic herbs that the general population believes to be harmless. It could be one of the factors that contribute to the occurrence of AAN in Western countries. AAN cases in Asia, on the other hand, are caused by the complicated pharmacopoeia of herbal therapies utilised in traditional medicine<sup>[16]</sup>.

AAN cases have been reported in several other countries. Twelve people have died in Taiwan as a result of using unidentified herbal medications to treat conditions like arthralgia, hepatitis, and hypertension. In Japan, twelve cases were reported, five of which were linked to the consumption of herbal medicines containing AAs, and seven of which were linked to the substitution of *Mokutsu* (*Akebia quinata*) for Kan-Mokutsu (*Aristolochia manshuriensis*), and *Boui* (*Sinomenium acutum*) for Kou Boui (*Aristolochia fangchi*) or *Kanchu-Boui* (*Aristolochia fang*) (*Aristolochia heterophylla*). In France, four cases have been documented and were linked to the use of slimming tablets. *S. tetrandra* was listed on the label, but it included *A. fangchi*<sup>[17]</sup>. Furthermore, there were two cases reported in the United Kingdom due to the consumption of herbal products containing *Mu Tong* for treating eczema. There was a case reported in Germany about the use of Chinese herbal medicine for hyperuricemia, and one case was reported in Australia involving the treatment of psoriasis using Chinese herbal medicine<sup>[16]</sup>. One case was reported in Spain due to the consumption of herbal tea consisting of *Aristolochia pistolochia* and one in the USA due to the consumption of herbal medicine for low back pain<sup>[17]</sup>.

Because of its significant nephrotoxic and carcinogenic potential, the use of *Aristolochia* plants has created global public health concerns. The usage of *Aristolochic* Acids in Malaysia has been restricted by the Malaysian Ministry of Health. The goal of this study was to use a simple HPLC approach to assess the safety of herbal products available in Malaysia for the presence of AAs. The current paper describes the invention of an isocratic RP-HPLC method for determining aristolochic acid in herbal products that is simple, precise, and accurate. The link between aristolochic acid levels in raw herbs and their products can be established using this method. We also claimed that the selected Chinese herbal products were free of aristolochic acid based on the findings.

## 2. Materials and Methods

### 2.1 Chemicals

25 mg of AA-I CRS was purchased from Aldrich-Sigma, China (batch number: WXBC8652V). 10 mg of AA-II standard was purchased from Phytolab, Germany (batch number: 77055940). Methanol AR grade was obtained from HmbG<sup>®</sup> Chemical (Selangor, Malaysia) and methanol HPLC grade was procured from Merck Millipore (Germany). Water was purchased from Ultrapure Water System (Elga, purelab Option-Q 7/15). Glacial acetic acid was procured from QRëC<sup>®</sup>. The above chemicals were used for the initial HPLC screening for the presence of aristolochic acids (AA). A HPLC/UV method was used to confirm the HPLC findings of AA. Other materials included PTFE syringe filters (0.45µm pore size). Plastic containers were used for storage of homogenized Chinese raw herbal materials.

## 2.2 Equipments

Detection and quantification of AAs were carried out using a Cyberlab LC-100 HPLC instrument equipped with a UV detector. The column used was Capcell Pak C18-reverse phase HPLC column, 5  $\mu\text{m}$ , 25 x 0.46 cm (Shiseido Co., Ltd., Japan). All Chinese raw herbal materials were homogenized with an electric stainless-steel grinder from Waring®. The mobile phase was filtered by using a 0.45  $\mu\text{m}$  membrane filter fitted to a Duran Schott filtration unit. A digital pH meter was used for pH adjustment.

## 2.3 Collection of Herbal Materials

Three dried raw herbs, *Aristolochia debilis* (Ma Dou Ling 马兜铃), *Aristolochia fangchi* (Guang Fang Ji 廣防己) and *Aristolochia manshuriensis* (Guan Mu Tong 關木通) were purchased from a local herbal wholesaler located in the state of Perak, Malaysia. Two Chinese herbal products, Zhu Ling San (茺苓) and Wu Ling San (五苓散) used for slimming purposes, were purchased from the local medical hall.

## 2.4 Homogenization of Herbal Materials

Three dried raw herbs *Aristolochia debilis* (Ma Dou Ling 马兜铃), *Aristolochia fangchi* (Guang Fang Ji 廣防己) and *Aristolochia manshuriensis* (Guan Mu Tong 關木通) were purchased from a local herbal wholesaler located in the state of Perak, Malaysia. Two Chinese herbal products Zhu Ling San (茺苓) and Wu Ling San (五苓散) used for slimming purposes were purchased from the local medical hall.

## 2.5 Extraction of Herbal Samples

An accurately weighed amount of 2 g of the respective powdered herbal drug was separately transferred into a 250 mL, brown, screw-cap bottle. To this, 100 mL of methanol was added and stirred for 30 min. at 300 rpm/min and filtered through a membrane filter (nominal pore size of 0.45 $\mu\text{m}$ ) to get the methanol extract of powdered herbal drug<sup>[23,24]</sup>.

## 2.6 Preparation of Mobile Phase for HPLC

The mobile phase used in HPLC analyses was methanol-water (75:25, pH 4.14) added with 0.1% glacial acetic acid. The mobile phase was prepared by mixing 750 mL of methanol (HPLC grade) with 250 mL of distilled water. The mixture was filtered through under a vacuum created by running a water tap and degassed by ultra-sonification before being used for HPLC analysis<sup>[24]</sup>.

### 2.7 Preparation of AAs Reference Solution

Reference solution (a) was prepared by dissolving the contents of a vial of AA-I in the solvent mixture to obtain a concentration of 0.04  $\mu\text{g/mL}$  of AA-I. Reference solution (b) was prepared by dissolving the contents of a vial of AA for system suitability (containing AA-I and II) in the solvent mixture and diluted to 20.0 mL with the solvent mixture.

### 2.8 Analysis of AA by HPLC

The amounts of AA-I and AA-II present in the herbal methanol extracts were determined by a modified HPLC method [18–20] using a Cyberlab LC-100 HPLC instrument equipped with a UV detector, under the following conditions: column, Capcell Pak C18-reverse phase HPLC column, 5  $\mu\text{m}$ , 25 x 0.46 cm, a mobile phase of methanol-water (75:25, pH 4.14) added with 0.1% glacial acetic acid; flow rate, 1.0 mL/min. The detector, a photodiode-array UV detector, was set at 254 nm. Aliquots of 25  $\mu\text{l}$  of AA-I and AA-II working standards were injected. The total run time was 10 min. For the test sample solution, three replicate extractions and duplicate HPLC analyses of standard, extracts of raw herbs and selected Chinese herbal products were carried out. The retention time (RT) for the AA was identified from the HPLC chromatogram. Identification of AA-I was established by comparison of the retention times with those of authentic standards.

### 2.9 Linearity, Limit of Detection and Quantification for AA-I

The range of working standard solutions (2 - 8  $\mu\text{g}/100\text{ mL}$ ) and methanol extracts of raw herbs (0.8–3.2  $\mu\text{g}/100\text{ mL}$ ) were chromatographed. The calibration curve for AA-I was plotted between the peak area and the concentration of the analyte. The calibration curves were calculated via least-square regression. The limit of detection and quantification were estimated as the minimum concentration giving a signal-to-noise ratio to measure the sensitivity of AA-I.

## 3. Results

### 3.1 Selection of Ratio of Mobile Phase (Methanol: Distilled Water)

The optimum ratio of the mobile phase is important to separate the peaks clearly between AA-I and AA-II. Different ratios of mobile phase (methanol/water) exerts different HPLC chromatograms for the separation of AA-I and AA-II. A mobile phase consisting of methanol and distilled water was prepared at different ratios of 60:40, 70:30, 75:25, and

80:20, and the respective HPLC chromatogram was obtained. As per the distinct peaks in the chromatogram, the mobile phase, a mixture of methanol and distilled water in a ratio of 75:25, was fixed for the analysis (Table 1).

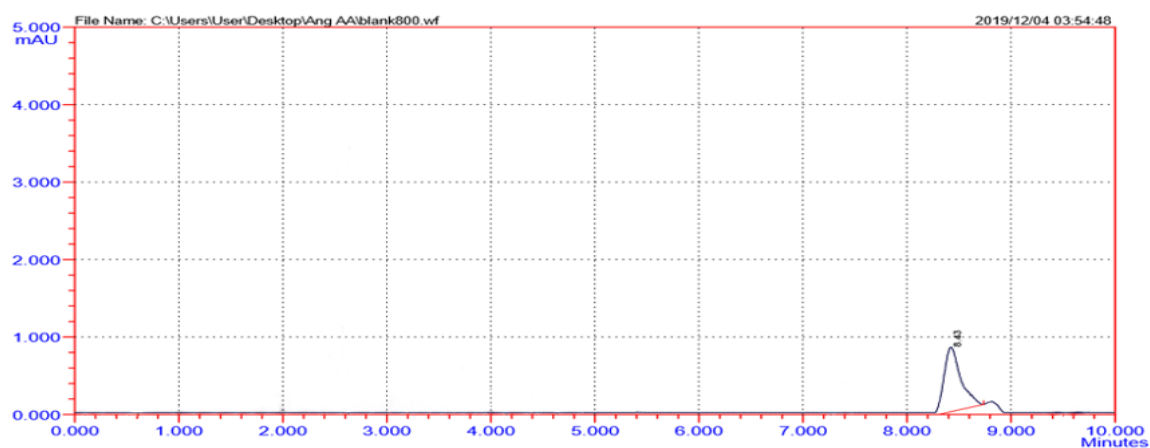
### 3.2 Selection of Concentration of Glacial Acetic Acid

The concentration of glacial acetic acid added into the mobile phase is important to aid the separation of AA-I and AA-II from peak tailing. Different concentrations of glacial acetic acid (0.1%, 0.2% and 0.3%) were used. Among these, 0.1% glacial acetic acid was fixed (Table 1) as it gave the HPLC chromatogram with high resolution for separation of AA-I and AA-II. The peaks for AA-I and AA-II were well separated with good peak sharpness, symmetry, and resolution.

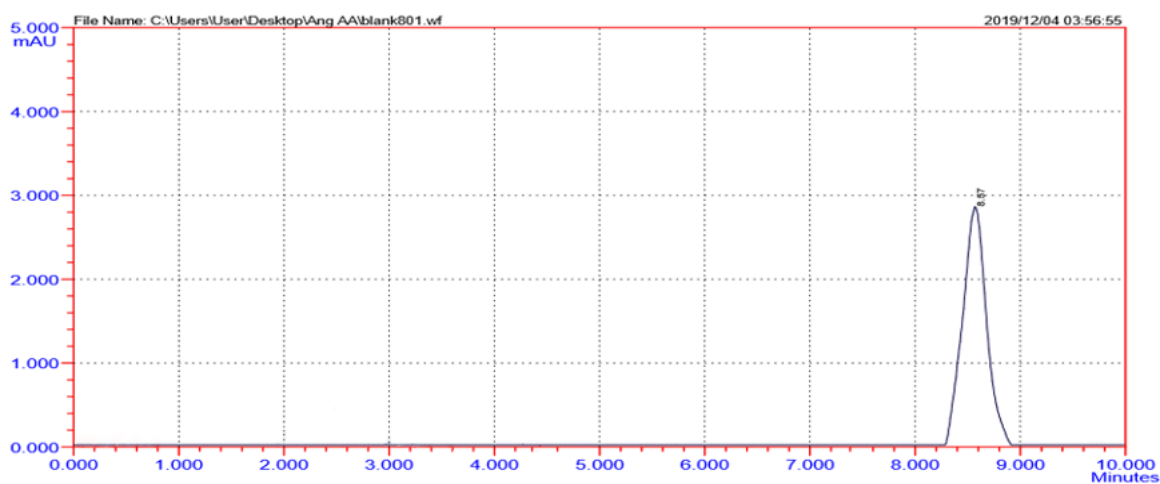
**Table 1.** RT for AA-I and AA-II in different ratio of mobile phase

Methanol: Water in 0.1% acetic acid	Retention time (mins)	
	AA-I	AA-II
60:40	33.4	22.3
70:30	12.4	9.6
75:25	8.4	7.0
80:20	6.2	5.4

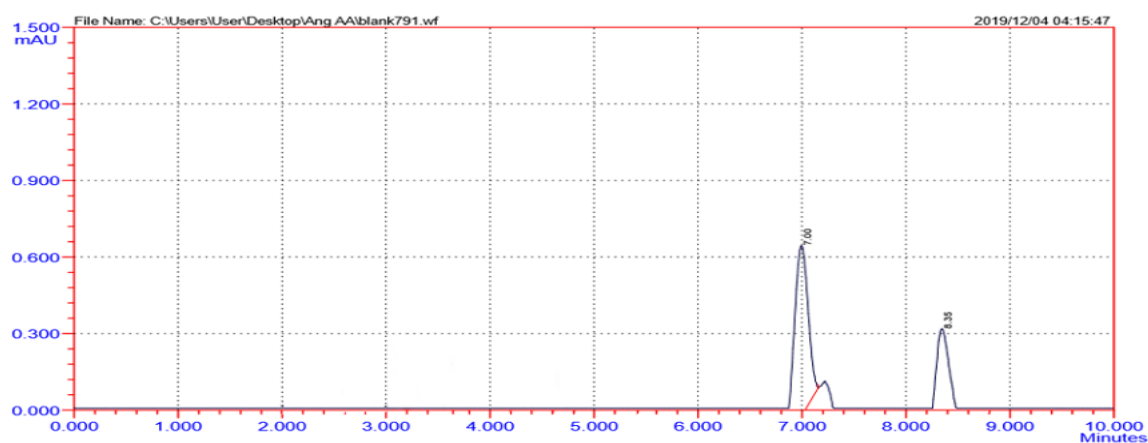
The HPLC chromatograms for the standards, AA-I and AA-II were obtained with and without spiking the standards using the mobile phase, methanol-water (75:25) with 0.1% glacial acetic acid (Figure 1–4)



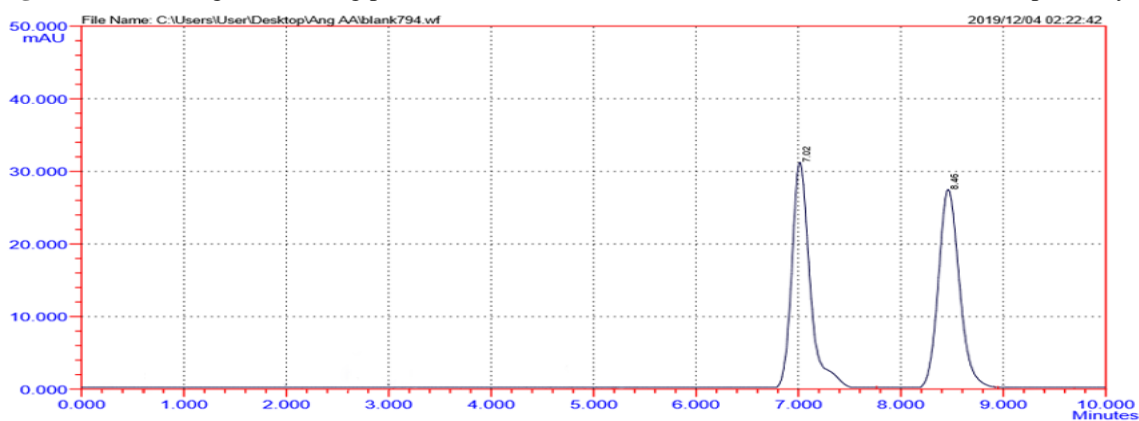
**Figure 1.** Chromatogram showing peaks of standards AA-I at RT 8.4 min.



**Figure 2.** Chromatogram for spike solution in mobile phase showing peaks of standards AA-I at RT 8.6 min.



**Figure 3.** Chromatogram showing peaks of standards AA-I and AA-II at RT 8.4 and 7.0 min. respectively.



**Figure 4.** Chromatogram for spike solution in mobile phase showing peaks of standards AA-I and AA-II at RT 8.5 and 7.0 min. respectively.

### 3.3 Linearity, Limit of Detection and Quantification for AA-I

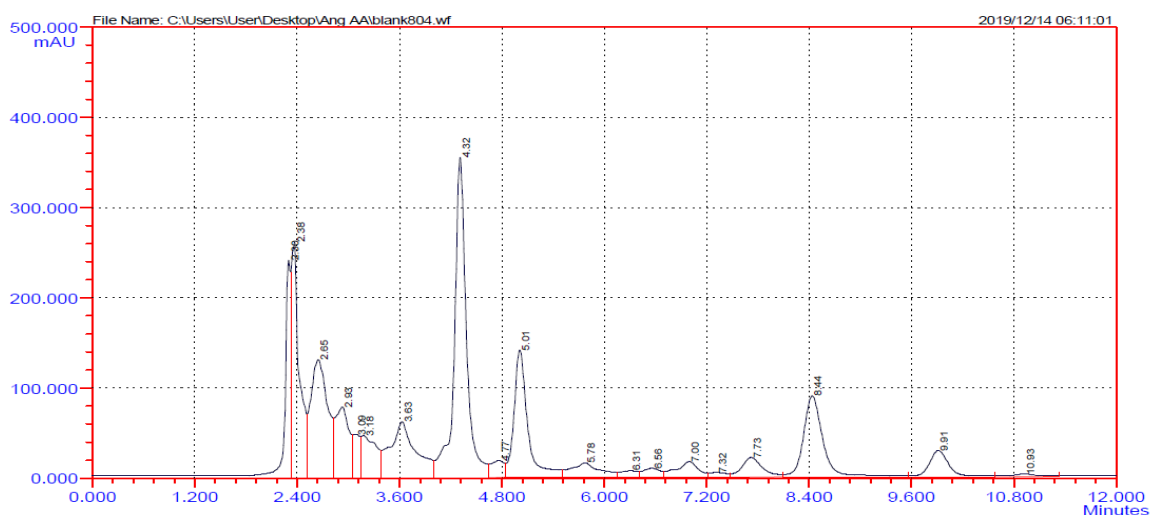
This simple HPLC method was modified from the BP method and served reasonably well as a screening procedure for AAs. The correlation coefficients for the calibration curves



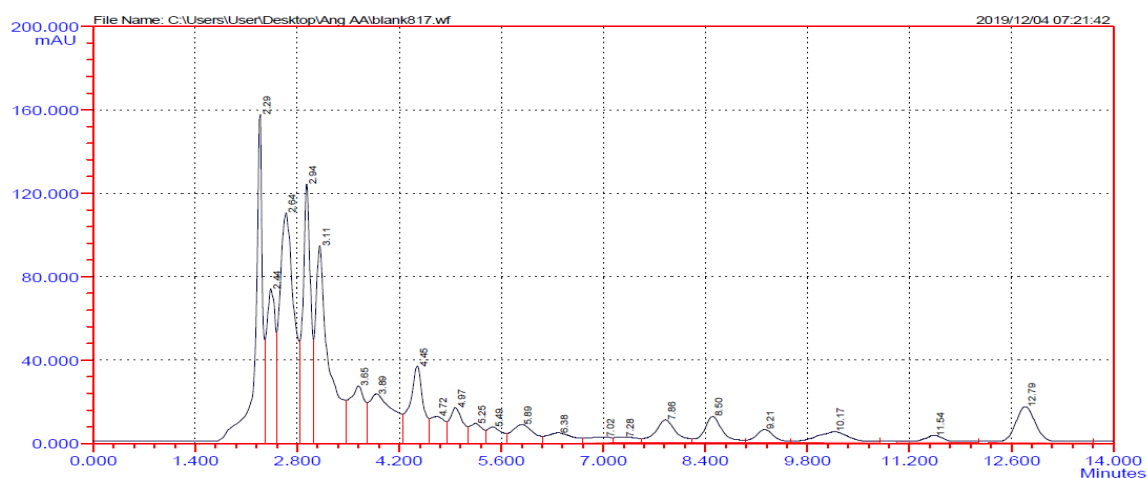
using a quadratic plot were 0.9914 for AA-I (Range: 2–8  $\mu\text{g}/100\text{mL}$ ). The equation for the calibration curve was  $y=66.86x + 324.35$ . The detection limit for AA-I was approximately 2  $\mu\text{g}/100\text{ mL}$ .

### 3.4 HPLC Analysis of AAs in Raw Herbs

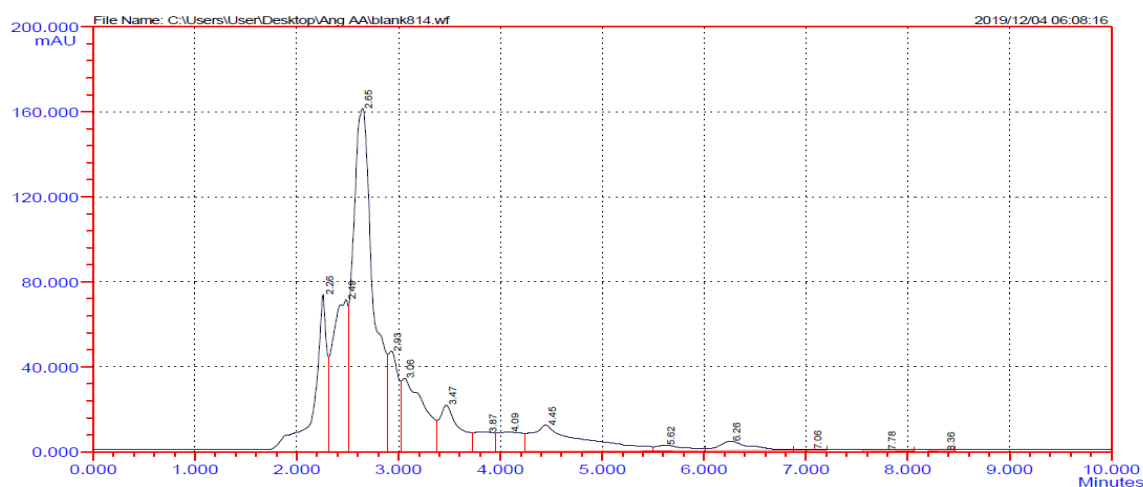
A HPLC analysis was carried out on a methanol extract of three raw herbs. Two raw herbs, *A. debilis* and *A. fangchi*, contained AAs, whereas, They were absent in *A. manshuriensis*. Figures 5 and 6 are the HPLC chromatograms of two samples, *A. debilis*, and *A. fangchi*, clearly showing the peak for the presence of AA-I at 8.4 and 8.5 minutes, respectively. Figure 7 shows the chromatograms of the herb, *A. manshuriensis*, that did not contain the peak for AA-I.



**Figure 5.** Chromatogram for the presence of AA-I in *A. debilis* at RT 8.4 min.



**Figure 6.** Chromatogram for the presence of AA-I in *A. fangchi* at RT 8.5 min.



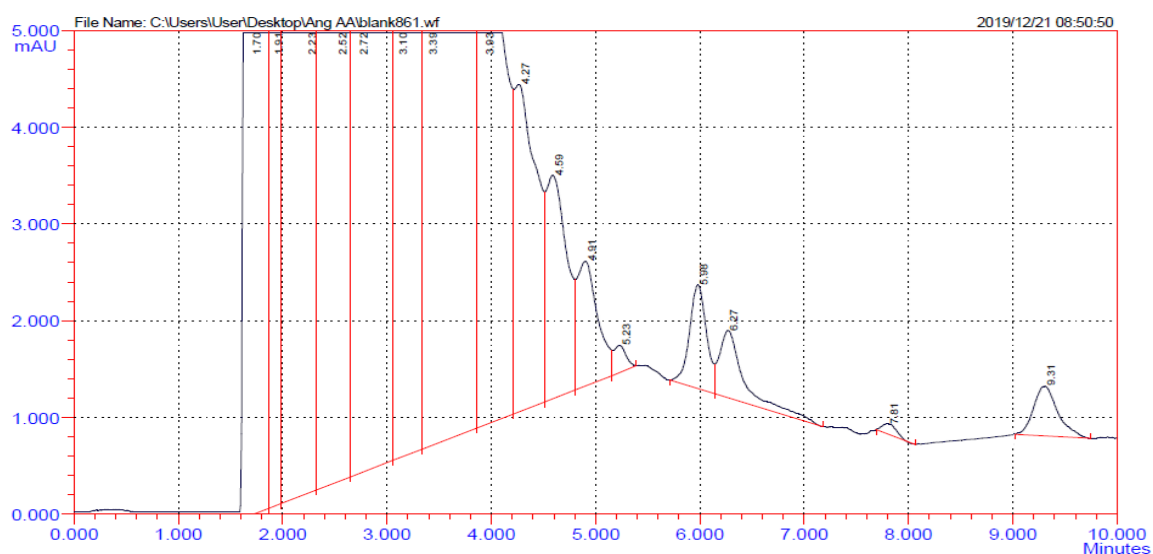
**Figure 7.** Chromatogram for the absence of AA-I in *A. manshuriensis*.

### 3.5 Linearity, Limit of Detection and Quantification for AA-I in Raw Herbs

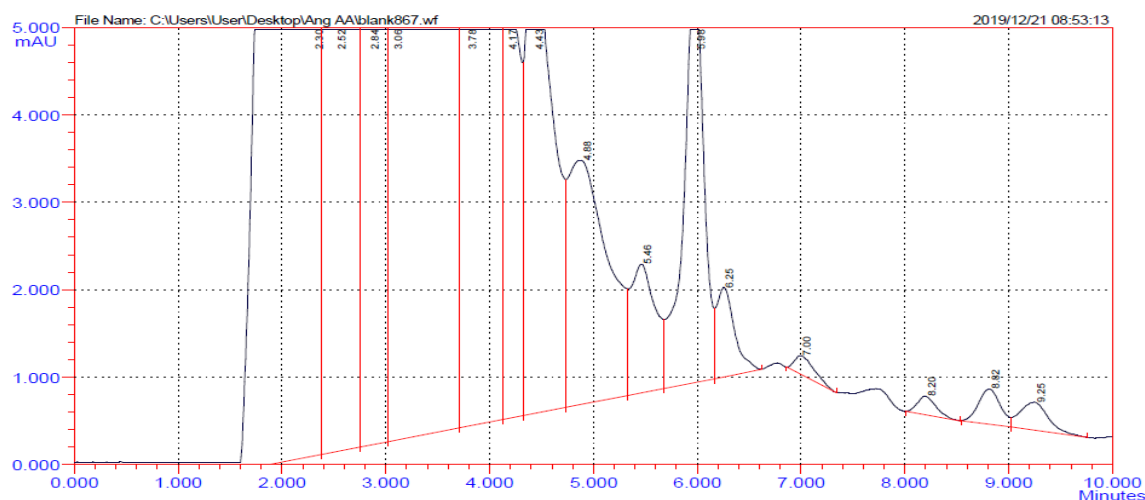
Different concentrations of *A. debilis* were prepared at 0.4, 0.8, 1.6, 2.4, and 3.2  $\mu\text{g}/100\text{ mL}$ , respectively. A linear curve with peak area against concentration was constructed with the equation of  $y = 68116x + 3218.8$  and  $R^2 = 0.9958$ . The limit of quantification of AA-I present in *A. debilis* was 1.7  $\mu\text{g}/100\text{ mL}$ .

### 3.6 HPLC Analysis of AAs in Chinese Herbal Products

A HPLC analysis was carried out on a methanol extract of two Chinese herbal products. HPLC Chromatograms (Figure 8 and 9) obtained for the selected Chinese herbal products, Zhu Ling San (茺苓散) and Wu Ling San (五苓散) respectively, indicated the absence of AA-I peaks at 8.4 minutes.



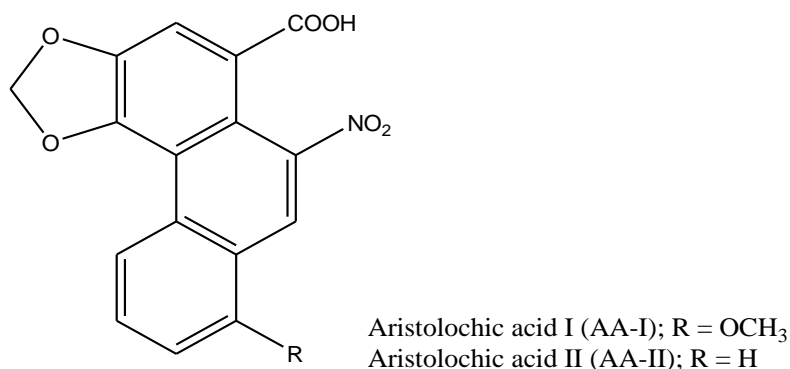
**Figure 8.** Chromatogram for the absence of AA-I in Zhu Ling San (茺苓散).



**Figure 9.** Chromatogram for the absence of AA-I in Wu Ling San (五苓散).

#### 4. Discussion

The genus, *Aristolochia* has various biological activities and one of the important compounds present is aristolochic acids (AAs), which is a mixture of structurally related nitrophenanthrene carboxylic acids. AAs include AA-I and AA-II, chemically known as 8-methoxy-6-nitro-phenanthro (3,4-d)-1,3-dioxolo-5-carboxylic acid and 6-nitro-phenanthro (3,4-d)-1,3-dioxolo-5-carboxylic acid, respectively (Figure 10). But the concentration of AAs in these medicinal plants is varied and depends upon the species, regions, and seasons. Also, the concentration is varied to some extent in medicinal plants due to the various metabolic pathways and environmental factors. The pharmacological activity of the plants in the aristolochiaceae is attributed to the AAs that are found in the *Aristolochia* species worldwide. These compounds are used in traditional folk medicine, particularly as a part of weight-loss regimens in Chinese herbal medicine<sup>[21]</sup>.



**Figure 10.** Chemical structure of Aristolochic acids.

AAs were found in various Chinese herbal products used for the treatment of eczema, psoriasis, arthralgia, hepatitis, and hypertension, etc., and identified as one of the main causative agents for rapid progression of renal disease. The supplements formulated using herbal plants containing AAs as part of traditional Phytotherapies might cause Aristolochic acid Nephropathy. Therefore, it is important to identify the presence of AAs in herbal products before their usage. Almost all countries have their own legislative and regulatory actions on AAs in herbal medicinal products, and they have the right to cancel or disapprove the registration of products that are suspected to contain AAs<sup>[22]</sup>.

Chan *et al.* reported the use of HPLC-FLD and HPLC MS/MS methods to detect AA from herbal medicines<sup>[25]</sup>. In their study, powdered plant materials were used as samples. The results indicated significant similarity between the HPLC-FLD and HPLC MS/MS methods. However, HPLC-FLD was found to be more sensitive. Similarly, Yuan *et al.* also reported the use of the gradient HPLC technique for simultaneous detection of AA from herbs and herbal preparations<sup>[26]</sup>. In the present study, isocratic RP-HPLC methods were used. The isocratic technique is the simplest where an object constituent is to be detected. It simplifies the detection process and saves the time and cost of analysis.

The results found that the RP-HPLC method developed in the present study is a simple and precise method, as evident from the observed resolution for the separation of peaks. The RP-HPLC method was employed to identify the presence of AA in three herbal plants, *A. debilis*, *A. fangchi*, and *A. manshuriensis*, and two Chinese herbal products. The calibration curve was plotted between the peak area of the analyte and the concentration of the analyte using a least-squares linear regression method. The calibration curves had a correlation coefficient ranging from 0.9914 to 0.9958 for the AA-I standard and AA-I in *A. debilis*, respectively. The detection limit for AA-I standard and AA-I in *A. debilis* was found to be approximately 2 µg/100 mL and the limit of quantification was found to be 1.7 µg/100 mL respectively. The retention time of the standards AA-I and AA-II was identified at 8.35 min and 7.0 min respectively from the HPLC chromatogram (Figure 4).

In addition, HPLC analyses on methanol extract of three raw herbs revealed a peak for the presence of AA in two of the raw herbs, *A. debilis* and *A. fangchi*, at 8.4 and 8.5 minutes, respectively, whereas the peak was absent in *A. manshuriensis* (Figure 5, 6 and 7). The HPLC analyses on the methanol extract of the selected two Chinese herbal products, Zhu Ling San (茺菀散) and Wu Ling San (五苓散) indicated the absence of AA-I as there was no peak found at 8.4 minutes. However, the limitation of this method is the use of UV detectors, and the isocratic method. Thus, this method will be more useful to detect AA, and spectroscopic methods such as mass spectrometry can be conducted further as this method

may not be significant where the detection of other complex derivatives that are structurally or chemically related to AA is required.

## 5. Conclusions

The findings may be used to interpret the relationship between the levels of AAs in raw herbs and their products. Also, it concludes that the selected Chinese herbal products are free from aristolochic acid. The present study is a simple and precise isocratic RP-HPLC method to facilitate the accurate determination of the aristolochic acid in herbal products.

**Conflicts of Interest:** The authors declare that there is no conflict of interest.

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