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## Original Article

# Development of validated high-performance thin layer chromatography for quantification of aristolochic acid in different species of the Aristolochiaceae family



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## ABSTRACT

This study was undertaken to isolate and quantify aristolochic acid in *Aristolochia indica* stem and *Apama siliquosa* root. Aristolochic acid is an important biomarker component present in the Aristolochiaceae family. The isolation method involved simple solvent extraction, precipitation and further purification, using recrystallization. The structure of the compound was confirmed using infrared spectroscopy, mass spectrometry and nuclear magnetic resonance. A specific and rapid high-performance thin layer chromatography (HPTLC) method was developed for analysis of aristolochic acid. The method involved separation on the silica gel 60 F<sub>254</sub> plates using the single solvent system of *n*-hexane: chloroform: methanol. The method showed good linear relationship in the range 0.4–2.0 µg/spot with  $r^2 = 0.998$ . The limit of detection and limit of quantification were 62.841 ng/spot and 209.47 ng/spot, respectively. The proposed validated HPTLC method was found to be an easy to use, accurate and convenient method that could be successfully used for standardization and quality assessment of herbal material as well as formulations containing different species of the Aristolochiaceae family.

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## 1. Introduction

*Aristolochia indica*, commonly known as Indian birthwort, is an important medicinal plant, which is a perennial climber, found growing throughout India, in plains and lower hills [1].

Traditionally, the plant has been used as an antidote to snake and insect bites [2,3]. The plant also shows significant antibacterial [4] and antifungal [5] activity. The leaf extract has been reported to inhibit drug-induced hyperuricemia [6]. Methanolic extract of *A. indica* leaf also shows antimalarial activity [7]. A preliminary chemical study of the plant revealed

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the presence of alkaloid and flavanoid. Results of phytochemical studies have shown that the plant mainly contains the phenanthrene alkaloid aristolochic acid (isoaristolochic acid) [8]. *Apama siliquosa* also known as *Bragantia wallichii* or *Thottea siliquosa*, is an erect, slender shrub commonly found in the evergreen forest of Western Ghats of Maharashtra southwards to Kerala, India [9]. The roots have been used since ancient times in treating diarrhea and dysentery in the indigenous system of medicine [10]. The methanolic extract of roots has been reported to show free radical and hydroxyl radical scavenging activity as well as cytotoxic activity [11]. This medicinally potent shrub mainly contains an important alkaloid, isoaristolochic acid I (aristolochic acid). Other constituents present are chakranine, palmitic, lignoceric, oleic, and linolic acids [12,13].

Aristolochic acid I (isoaristolochic acid), chemically 3,4-methylenedioxy-8-methoxy-10-nitro-1-phenanthrenecarboxylic acid (Figure 1), is the major alkaloid reported in the above two Aristolochiaceae family species. Aristolochic acid has been reported to have anti-tumor [8] and antimicrobial [14] activity.

Early attempts had been made to isolate aristolochic acid I by solvent extraction and subsequent column chromatography [8,15–17]. However, the reported method for isolation was found to be tedious as well as time consuming, since it involved use of a column that could not be used on a commercial scale. Also, the yield obtained was low. Thus, the objective of the present study was to isolate aristolochic acid on a commercial scale.

Also a high-performance thin layer chromatography (HPTLC) method was developed for identification, quantification and standardization of this biomarker compound. The HPTLC method has become a routine analytical technique due to its cost effectiveness and reduced analysis time. Also, HPTLC is feasible for fingerprinting and identifying the complex herbal constituents in extracts as well as formulations. Our literature survey revealed that aristolochic acid has been quantified in other species using HPTLC [17]. However, the reported methods did not show good resolution of bands in the same solvent system for both species. The established HPTLC method, thus, can be used in identification, quantification and standardization of raw materials as well as extracts of different species containing aristolochic acid.

## 2. Methods

### 2.1. Chemicals

*n*-Hexane, chloroform, acetone and methanol of laboratory grade were purchased from S.D. Fine Chemicals (Mumbai, India). Standard aristolochic acid (> 95%) was isolated in-house and its identity was confirmed by its spectral analysis.

### 2.2. Plant material

*Aristolochia indica* stem as well as *Apama siliquosa* root was collected from Chittoor District, India. A voucher specimen (ICT/MNPRL/AI/03 and ICT/MNPRL/AS/05 respectively) was

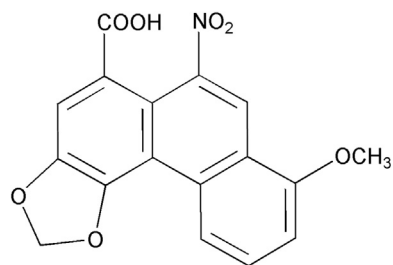


Figure 1 – Structure of aristolochic acid.

deposited in Medicinal Natural Products Research Laboratory, Institute of Chemical Technology, Mumbai, India.

### 2.3. Isolation of aristolochic acid

About 100 g of powdered *Aristolochia indica* stem was defatted with 400 mL petroleum ether (60–80°C) using Soxhlet apparatus for 2 hours. The dried powder was further extracted with chloroform (500 mL) for 4 hours. The chloroform extract was concentrated. Petroleum ether was added to the chloroform layer to yield a yellow precipitate (138 mg). This yellow precipitate was repeatedly recrystallized using methanol to give pure aristolochic acid (100 mg). The isolated compound was further characterized using TLC, UV spectroscopy, infrared (IR) spectroscopy, mass spectrometry, and nuclear magnetic resonance spectroscopy. HPLC studies were carried out to determine the purity of the isolated compound.

### 2.4. Standard preparation for HPTLC analysis

Stock solution of aristolochic acid (1 mg/mL) was prepared using methanol. From the stock solution, 2 mL was transferred to a 10-mL volumetric flask and the volume was made up with methanol (200 µg/mL).

### 2.5. Sample preparation

Five grams of each powdered drug (*Aristolochia indica* stem and *Apama siliquosa* root) was extracted separately with 50 mL methanol in Soxhlet apparatus for 5 hours. The extract obtained was concentrated, transferred to a 25-mL volumetric flask, and the volume made up with methanol.

### 2.6. HPTLC analysis

Separation experiments were performed on silica-based HPTLC F<sub>254</sub> plates (Merck, Darmstadt, Germany). Before sample application, the silica plates (20 cm × 20 cm or 10 cm × 10 cm) were activated at 110°C for 15 minutes. Standard solution (1 mg/mL in methanol) was used for the preparation of a five-point calibration curve corresponding to an amount of 0.4–2 µg/spot. All the extracts obtained from the experimental runs were spotted in triplicate. Standard and sample solutions were applied in the form of a band using a 10-µL syringe (Hamilton, Bonaduz, Switzerland) by Desaga Applicator (Desaga, Burkhardtendorf, Germany). The linear ascending development was carried out in a twin trough

chamber previously saturated with 10 mL mobile phase (*n*-hexane: chloroform: methanol, 1: 8: 1 v/v) for 15 minutes at room temperature (25°C and 40% relative humidity). After development, plates were dried, and the components were visualized under 254 nm UV. Identification of aristolochic acid in the extract was performed on the basis of retention factor ( $R_f$ ) and chromatographic behavior with those of an authentic standard.

A TLC scanner controlled by proQuant software (Biostep; Desaga) was used for quantitative evaluation. The band was scanned and quantified densitometrically at 254 nm ( $\lambda_{\text{max}}$  of aristolochic acid). For quantification, the densitometric scanning was performed in the absorbance mode, slit width 8.0 mm × 0.20 mm, scanning speed 20 mm/s. Quantification was performed using linear regression equations of respective compounds. Precision of  $R_f$  values was based on 10 subsequent measurements. The HPTLC run of standard and sample track is shown in Figure 2.

## 2.7. Method validation

Validation of the developed HPTLC method was done according to International Council for Harmonisation guidelines [18,19].

The linearity was obtained by analyzing five standard aristolochic acid solutions with different concentration (0.4 µg/spot, 0.8 µg/spot, 1.2 µg/spot, 1.6 µg/spot and 2.0 µg/spot) of aristolochic acid. The calibration curve for the same was determined.

Accuracy was estimated by recovery studies using a standard addition method. A known amount of standard with three different concentrations was added to the pre-analyzed sample and each mixture was analyzed by the proposed method in triplicate. In order to detect the loss of analyte, benzoic acid was used as an internal standard. A specific amount of internal standard (100 µg/mL) was added to aristolochic acid

standard as well as samples with unknown concentrations of aristolochic acid.

Precision was estimated on the basis of repeatability and intermediate precision. Repeatability indicates the precision under the same operating conditions over a short interval time and interassay precision. Intermediate precision was carried out by intra-day and inter-day precision studies. In intraday studies, analysis was carried out on the same day with an interval of 1 hour, while in interday studies, the analysis was done on three different days. Precision was determined as intraday and interday variation. The intraday and interday precision analysis was carried out in triplicate at three different concentrations of 0.4 µg/spot, 0.8 µg/spot and 1.2 µg/spot.

The limit of detection (LOD) and limit of quantification (LOQ) was determined based on signal to noise ratio technique using the following equations:

$$\text{LOD} = 3.3 \sigma/S$$

$$\text{LOQ} = 10 \sigma/S$$

where  $\sigma$  is the standard deviation of the intercept and  $S$  is the slope of the calibration curve.

The specificity of the method was determined by comparing the retention factor values and spectra of the standard and sample. The peak purity of the standard was assessed by comparing the peak area and position in the spectrum.

## 2.8. Statistical analysis

The applications of each analyzed solution were performed in triplicate. The results were presented as mean ± standard

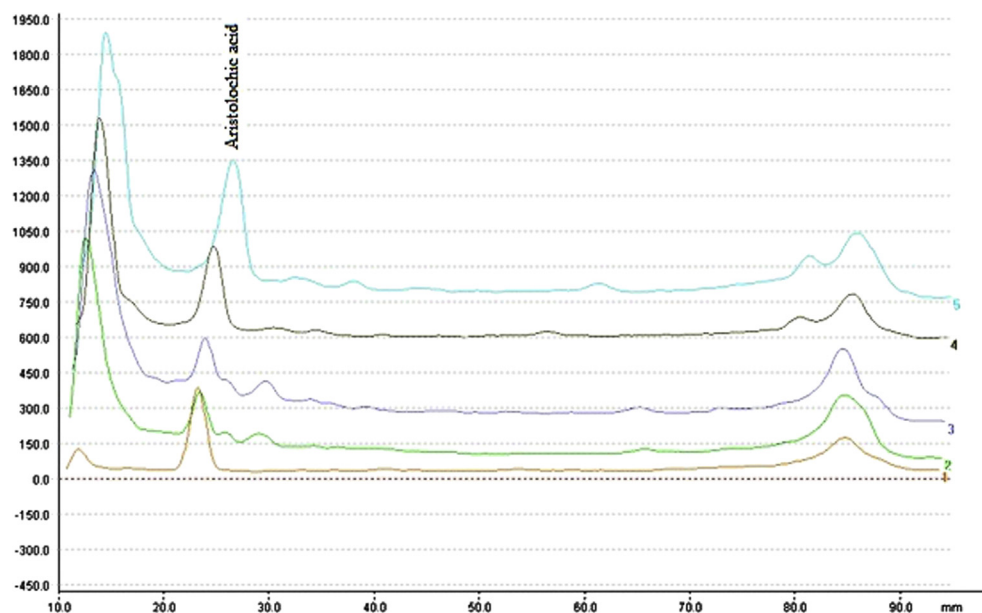


Figure 2 – HPTLC densitogram of standard aristolochic acid (1), *Aristolochia indica* extract (2–3) and *Apama siliquosa* extract (4–5). HPTLC = high-performance thin layer chromatography.

deviation (SD). Statistical comparisons of several mean values were done by using one-way analysis of variance, taking the appropriate condition as a single factor.

### 3. Results

#### 3.1. Isolation and characterization of aristolochic acid

Aristolochic acid was isolated from *Aristolochia indica* stem using a simple solvent extraction process, which was found to be relatively economical as well as less time consuming. Also, the yield obtained was about 0.13% of crude aristolochic acid (92% recovery of total aristolochic acid from the plant). After recrystallization, the compound was found to be 95% pure. The isolated compound was identified by characterization using UV, IR, mass spectrometry and nuclear magnetic resonance. The UV/Vis maxima in methanol were found to be at 221 nm, 318 nm and 386 nm. IR spectrum of the isolated compound showed peaks at 3445  $\text{cm}^{-1}$  (hydroxyl), 1349  $\text{cm}^{-1}$  (C–H bending), 1595  $\text{cm}^{-1}$  (nitro) and 1249  $\text{cm}^{-1}$  (nitro). Molecular ion peak at 341  $m/e$  gave the molecular weight of the compound. The structure of the compound was confirmed by comparing the spectra with those in the literature [15].

#### 3.2. HPTLC optimization

While developing the HPTLC method, different parameters like solvent system composition, wavelength, band separation and symmetry were taken into consideration to obtain good resolution. The final solvent system comprised *n*-hexane: chloroform: methanol, 1: 8: 1.

#### 3.3. Validation of HPTLC

##### 3.3.1. Linearity

The linear regression equation was found to be  $y = 628.911x + 93.636$  ( $r^2 = 0.998$ ). The result indicated a good linear relationship between the concentrations and peak areas.

##### 3.3.2. Accuracy

The accuracy of the developed method was calculated by recovery studies. Accordingly, it was clear that the method was accurate for the quantitative estimation of aristolochic acid as the value for relative SD (RSD) was found to be within

the acceptance criteria (i.e. RSD, 3.0%). The percentage recovery of aristolochic acid in *Aristolochia indica* stem and *Apama siliquosa* root extract was found to be 100.02 and 101.93, respectively (Table 1). The recovery was also calibrated using an internal standard. The RSD was found to be < 5%. The percentage recovery of aristolochic acid in *Aristolochia indica* stem and *Apama siliquosa* root extract as compared to internal standard was found to be in the range 100–105%.

##### 3.3.3. Precision

The reproducibility of the method was expressed in terms of % RSD, which was found to be < 3%, signifying good precision (Table 2).

##### 3.3.4. LOD and LOQ

The LOD and LOQ were determined using SD of response and slope estimated from the calibration curve of a standard solution of aristolochic acid. The LOD and LOQ were 62.84 ng/spot and 209.47 ng/spot, respectively.

##### 3.3.5. Specificity

The specificity was determined by comparing the chromatogram of the standard and the sample. The sample solution was spiked with standard solution in order to observe the interference. No interference was observed with the peak of aristolochic acid in the sample, therefore, the method was found to be specific.

The results indicated that the method may be suitable for the estimation of aristolochic acid in different species of the Aristolochiaceae family. The percentage content in *Aristolochia indica* stem and *Apama siliquosa* roots was 0.15% and 0.186%, respectively.

## 4. Discussion

The aim of the study was to isolate an important proto-alkaloid, aristolochic acid from *Aristolochia indica* stem, with application of minimum time and solvent. Different parameters such as solubility, temperature and stability were taken into consideration. The powdered sample initially was extracted with methanol and then isolated using the conventional acid–base extraction used for alkaloids. This method was time consuming and the yield obtained was low. Another method used for isolation involved defatting and further extraction with chloroform. The

**Table 1 – Recovery studies of aristolochic acid ( $n = 3$ ).**

Amount added ( $\mu\text{g}$ )	Amount present ( $\mu\text{g}$ )	Total amount ( $\mu\text{g}$ )	Amount recovered ( $\mu\text{g}$ )	Recovery (%)	Average recovery (%)	RSD (%)	
<i>Aristolochia indica</i> stem	10	9.25	19.25	19.8	102.86	100.02	1.66
	20	9.25	29.25	28.99	99.11		2.93
	30	9.25	39.25	38.5	98.089		2.89
<i>Apama siliquosa</i> root	50	43.4	93.4	94.8	101.49	101.93	1.18
	60	43.4	103.4	104.1	100.67		1.31
	70	43.4	113.4	117.5	103.61		1.73

RSD = relative standard deviation.

**Table 2 – Intra- and interday precision study of aristolochic acid (n = 3).**

Amount (µg/spot)	Intraday precision		Interday precision	
	Mean area	% RSD	Mean area	% RSD
0.4	391.57	0.541	363.73	1.07
0.8	587.01	1.364	581.09	2.84
1.2	881.77	2.525	837.16	2.16

RSD = relative standard deviation.

chloroform layer was concentrated and acid–base treatment was performed [15]. The isolated compound was impure as well having a low yield, hence, the method was rejected. Aristolochic acid was also isolated using column chromatography as reported previously but the yield was low [16]. Finally, since aristolochic acid was insoluble in petroleum ether and soluble in chloroform, the powder was first defatted and then extracted with chloroform. The chloroform layer was concentrated and petroleum ether was added to give a yellow precipitate of crude aristolochic acid. The advantage of this method was that it was less time consuming, and gave a better yield with good purity. The isolated compound was further recrystallized using methanol.

HPTLC was developed for estimation of aristolochic acid in plant samples. Different trials were carried out using acetone, chloroform, ethyl acetate, methanol and *n*-hexane. The solvent system comprising *n*-hexane: ethyl acetate in different ratios (1: 9, 5:5), resulted in poor resolution of the bands with low  $R_f$ . Addition of methanol separated the bands but the method was not reproducible. When chloroform and methanol in different proportions were mixed, the  $R_f$  was low. Finally, *n*-hexane was added, which gave better resolution of the bands with comparatively good peak symmetry and  $R_f$  of 0.5. Also, no interference was observed with the other constituents in the extract. Thus, the final system comprised *n*-hexane: chloroform: methanol, 1: 8: 1. The method was validated according to International Council for Harmonisation guidelines. The developed method has advantages such as high sensitivity, selectivity and ease of identification of various species in the Aristolochiaceae family. Thus, the developed method can be used to standardize the extract and plant material containing aristolochic acid as a biomarker.

### Conflicts of interest

The authors have no conflicts of interest to declare.

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