European Journal of Medicinal Plants
4(9): 1113-1124, 2014

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Pharmacognostic and HPTLC Fingerprint Profile of the Root of *Aristolochia indica* Linn. and Quantification of the Marker Compound

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Author's contribution

This whole work was carried out by author MVS.

Original Research Article

Received 17th February 2014
Accepted 3rd May 2014
Published 16th June 2014

ABSTRACT

Introduction: Root of *Aristolochia indica* Linn. has long been used as an oxytoxic agent to aid women in child birth and as abortifacient in Indian folk medicine. It is also one of the ingredients in some traditional Ayurveda medicinal preparations.
Aims: The present work has been designed to delineate the pharmacognostic profile of the root of *Aristolochia indica* Linn and the High-performance thin-layer chromatographic (HPTLC) identification of the active compound and its quantitative estimation in the herbal sample.
Materials and Methods: Macroscopic, microscopic evaluation, powder analysis, fluorescence standards of the root of *Aristolochia indica* Linn and its HPTLC fingerprint profile.
Results: Pharmacognostic profile of the root investigated revealed the transverse section possessing somewhat circular outline with tissue organization as outer thin walled cork layers, narrow cortex, and inner cortical cells with groups of stone cells. Secondary xylem tissues were fissured to form narrow strips, wide medullary rays with greater quantities of parenchyma, ray cells with rich deposition of starch. Vessels were solitary and occluded with tyloses and starch grains with 'Maltese cross' were the characteristic features of the taxon. HPTLC method was developed for the estimation of the marker constituent, Aristolochic Acid I (AAI) in dried root sample. Chloroform: Methanol (6:2v/v), was used as mobile phase to separate the analyte. The Rf value for Aristolochic Acid I (C17H11NO7) was found to be 0.53. Calibration plot was established showing the dependence of

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response on the amount chromatographed. Linearity was found to be in the concentration range of 100 to 500ng/spot for AAI with the correlation coefficient value r=0.998. The result showed that the content of marker compound (AAI) in dried root of *Aristochia india* Linn was 0.082%.

Conclusions: The results of the present study suggest that, the documented morphological descriptors, delineated anatomical markers and developed HPTLC methods are complementary characteristics, which could be effectively used for the identification and authentication of the root of *Aristolochia indica* Linn.

Keywords: Aristolochia indica Linn; microscopic determination; HPTLC finger print; active compound.

ABBREVIATIONS

Cam = cambium; ck, Cr = cork, Co = Cortex; Crl = Crystal; Fr= Fibre; Mdr = Medullary Rays; Ph= Phloem; S. Co= Secondary Cortex; S. Ph = Secondary Phloem; Stc, Stcl = Stone cell; Str = Starch Grain; S. XY = Secondary Xylem; Xy = Xylem; Xy. V= Xylem element; Tyl = tylosis.

1. INTRODUCTION

Aristolochia indica Linn (Indian birthwort) belongs to the family Aristolochiaceae and is now an endangered medicinal plant. The root of *Aristolochia indica* Linn has long been used in traditional medicine as an oxytoxic agent to aid women in child birth (Greek cristos = noblest + locheia = childbirth) and abortifacient in Indian folk medicine [1,2]. Roots and leaves of the plant also find diverse uses in certain types of fevers, as antidote to snakebite, to alleviate insomnia, ease bowel obstruction and relieve edema [3]. They were also being used to cure syphilis, gonorrhea and diarrhea and in the treatment of microbial infections [4,5]. Several members of the genus *Aristolochia* are being used as herbal ingredients in traditional Chinese medicines as anti-rheumatics, diuretics and in the treatment for edema [6]. However, recently it has been found that species of *Aristolochia* contain nitrophenanthrene carboxylic acid derivatives with perceived nephrotoxic and carcinogenic [7-9] effects, following which traditional Chinese medicines and herbal remedies are the subject of warnings from international (US FAD) regulatory agencies [10].

However, in the Indian System of Medicine, species of *Aristolochia* have been extensively in use for more than 2500 years. The earliest Ayurveda classical text, the *Charakasamhita,* contains the description of *Gandhanakuli, Isvari and Nakuli,* later identified by scholars as *Aristolochia indica* Linn and *A. bracteolata* Lam. [2,11], used as antidotes to snake bites and for intermittent fevers. *Aristolochia indica* Linn is one of the ingredients used in traditional Ayurveda medicinal preparations namely *Mahavishagarbha taila* (medicated oil), *Puga Khanda* (medicated candy), *Maha Paisacika Ghrta* (medicated ghee) and *Agurvadya taila* (medicated oil*)*. *Mahavishagarbha tailam* is used as external application for the ailments of joint pains and stiffness. In *Puga Khanda, the herbal juices* are processed with ghee, sugar, and milk to be made as candy for internal use in treatment of debility, gastric disorders and *Maha paisacikaghrta* is for internal use in intermittent fever, insanity and epilepsy, and the *Agurvadya taila* is used as massage oil for patients suffering from fever according to Ayurveda Classical texts: 'Bhaishajya Ratnavali' [12], 'Astanga Hrdayam [13] and 'Charaka Samhita' [14].

Since it is an important herbal remedy in Indian traditional medicine, the present study was undertaken with the objectives of delineating the pharmacognostical and High-performance thin-layer chromatographic (HPTLC) fingerprint profile of the root of *Aristolochia indica* Linn and to estimate the marker compound (Aristolochic Acid I) in the sample. These may also assist in regulatory perspectives for the standardization of whole, cut or powdered plant material towards identifying the crude drug from the adulterated and spurious materials, and in fingerprinting by HPTLC for quality assessment in the formulation/ finished products.

2. MATERIALS AND METHODS

2.1 Plant Material

Aristolochia indica Linn is a perennial climber with greenish white long stem, found throughout India in low hills and plains. Leaves are petiolated, glabrous and very variable, usually obovate-oblong, entire, and acuminate. Flowers are few, in axillary racemes, pale-green, inflated and base narrowed into a cylindrical tube terminating in a funnel-shaped purple mouth. Capsules are oblong or globose, six chambered, seeds ovate and winged. Dried root of *Aristolochia indica* Linn is cylindrical, curved, tapers, 10-25cm long and 1.5–6 cm in diameter. Externally it is grayish brown, rough and longitudinally wrinkled and easily broken. When fractured, the inside is starchy, exhibiting alternately grayish brown and whitish radial lines. The dried root is extremely bitter in taste.

2.2 Methods

Aristolochia indica Linn was collected from its natural habitat, from the Pathanamthitta District of the State of Kerala, India. The plant was identified and authenticated with the help of the Flora of Presidency of Madras [15]. The root samples collected were brought to the laboratory and investigation was undertaken in the Drug Standardization Department of the Government Ayurveda College, Thiruvananthapuram, Kerala, where voucher specimens were deposited. For microscopic analyzes, the fresh root collected was cut into small pieces and fixed in a solution prepared from formalin, glacial acetic acid and alcohol following the standard procedure of Johansen [16]. The shade-dried roots were pulverized and passed through an 80 – mesh sieve. The pulverized powder was kept in a labeled, air tight glass container.

Fine hand sections of fresh root were taken using the razor blade and sections were stained with alcoholic Safranin (1%), and mounted on glass slides in glycerin. Microphotographs of sections and powder analysis were made by using Olympus Microscope (Japan, Model CX 41) with CCD camera (2 mega pixel). Images were concomitantly viewed and analyzed for pharmacognostic characteristics, and quantitative measurements were taken using Olympus Image-Pro Plus (version 5.1). Fluorescence analysis of the root powder was carried out in daylight and UV light (254 nm and 366nm) using Camang UV apparatus. The root powder was cleared with absolute alcohol and mounted on glass slides for powder analysis. The descriptive terms of the anatomical features were used here as per Metcalfe and Chalk [17] and Conquist [18] and Sudhakaran [19,20]. The solvents of HPLC/ Chromatographic Grade used were procured from Merck and Qualigens Fine Chemicals, India. Analytically pure sample of Aristolochic acid I (AAI) procured from Sigma was used as standard.

HPTLC studies were carried out on qualitative and quantitative analyses of methanol extract of root samples using CAMAG HPTLC System (Switzerland) equipped with CAMAG

Linomat V Automatic Sample Spotter with syringe (100µl), Automatic development chamber (ADC2), UV cabinet with dual wavelength, and the densitometer consisted of TLC scanner 3 linked to WINCATS software. Aluminum plate (20x10cm) pre-coated with silica gel 60 F254 (Merck) of uniform thickness was used as adsorbent and operated with settings of band length, 8 mm.

2.3 Standard Stock Solution

Analytically pure standard Aristolochic acid I procured from Sigma was used. Accurately weighed 4.6mg of Aristolochic acid I was prepared in 10ml of Methanol in a volumetric flask. One ml of this stock solution was diluted to 10ml of methanol, (0.046mg/ml) and was used for the HPTLC analysis as standard.

2.4 Sample Preparation

Accurately weighed 2.016g of root powder of *Aritolochia indica* Linn. was refluxed in 25ml of Methanol. Extract obtained was filtered using Whatman filter paper and transferred to a volumetric flask, and volume was made upto 50ml with methanol.

2.5 Calibration Curve

From the stock solution 2µl, 4µl, 6µl, 8µl and 10µl per spot were applied on pre-coated plate of Silica Gel in track nos. 1 to 5; and 5µl and 10µl sample solution per spot were applied in track nos. 6 and 7 respectively using CAMAG LINOMAT V Applicator. Densitometric scanning was performed using CAMAG TLC Scanner 3 at λ max 319nm and operated by the WINCATS software.

3. RESULTS AND DISCUSSION

3.1 Microscopic Evaluation of Root

Transverse section (T.S) of the root showed somewhat circular outline with peripheral cork cells interrupted at places and margin appearing irregular (Fig. 1). Cork consisted of 7-9 layers of thin walled cells. Cork cells were empty and devoid of colored substances. Cortex forms a narrow zone, which consisted of 8-10 layers of cells. The outer cortex contained 2-3 layers of thick-walled parenchymatous cells, and the cells were filled with reddish brown content. Inner cortex consisted of 6-7 layers of thin walled, square or rectangular cells. The cells of the outer cortical layer contained numerous prismatic crystals of calcium oxalate. The crystals varied in size, and ranged from 25µm to 30µm in length and 11 to 14µm in diameter.

Three to four rows of stone cells (Figs. 2 and 3) aligned to form a more or less continuous ring, and a broad band of parenchyma tissues occurred in between the ring of sclerids and the secondary phloem. The stone cells were striated, pitted, and the lignified secondary cell wall with wide lumen (Fig. 3). Secondary xylem were fissured, and observed in the form of narrow strips (Figs. 1 and 4).

Fig. 1. *Aristolochia indica* Linn T.S. of root (x 4)

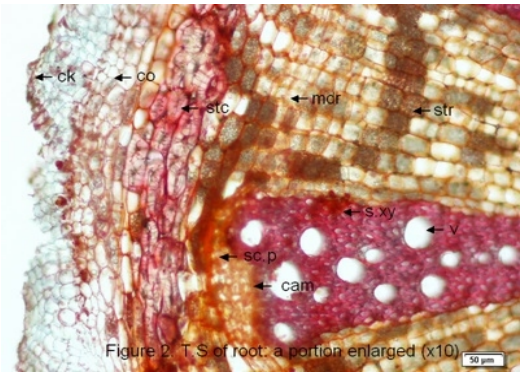

Fig. 2. T. S of root:a portion enlarged (x10)

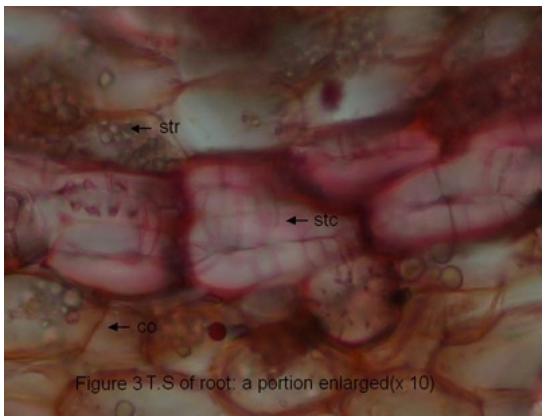

Fig. 3. T.S of root: A portion enlarged showing stone cells (x 10)

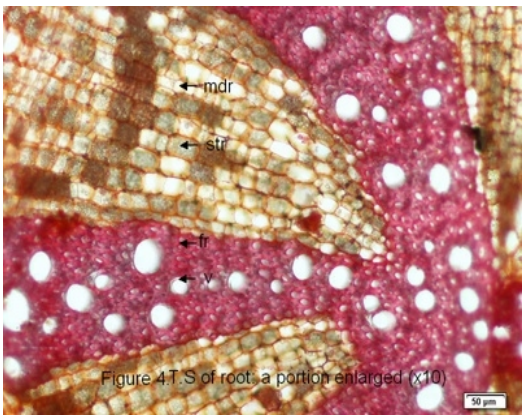

Fig. 4. T.S of root: A portion enlarged showing fissured xylem(x 10)

The vessels were found to be solitary, and they were more or less radially aligned in the centre of the xylem strip. The fissured xylem strips were separated by broad medullary rays. Rays were exceptionally wide (18-20 cells); the mean length and breadth of the ray were found to be 1287µm and 56µm respectively. The mean diameter of the vessel was found to be 92µm (range 37-109µm). According to Carlquist [18], wide vessel diameter contributes to a greater conductive efficiency to the plant. Vessel density in the xylem was found to be 71 per mm^2 when medullary rays and fasicular areas were excluded. Xylem was scanned for vessel density measurement, the vessels together with the medullary rays and fasicular areas were found to be 40 per mm^2. The presence of Tylosis in the vessel (Fig. 5) was a common feature, and at places the accumulation of starch grains in tylosis were also observed. Radial longitudinal section (RLS) of the root had shown the vessels with bordered pits, and vessel to vessel pitting in alternate position (Fig. 6).

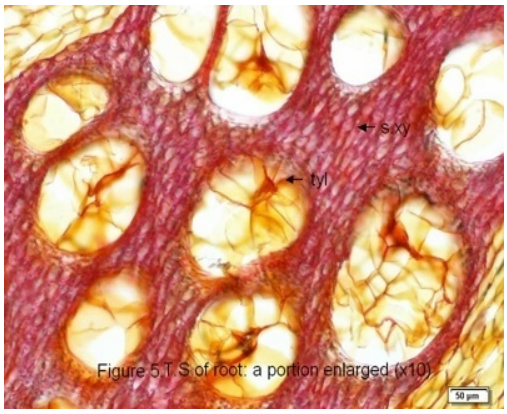

Fig. 5. T. S of root: Showing vessels with Tyloses (x 10)

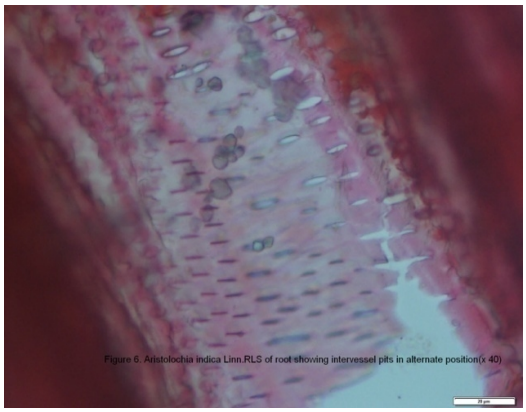

Fig. 6. R.L.S of root showing inter vessel pits in alternate position (x 40)

The pits were moderately sparse and the diameter of the pit apertures found to be about 8µm (Fig. 6). The length of the vessel element varied, and it measured 215µm to 514µm **(**Fig. 8**)**. Cells of the medullary rays were radially elongated and parenchymatous. Medullary ray cells contained prismatic calcium oxalate crystals and abundant starch grains. Cortical parenchyma cells and interfasicular parenchyma cells were also found to contain plenty of starch grains (Fig. 7). Both simple and compound starch grains were observed. Individual starch grain appeared to be ovoid in shape, and its size ranged from 6µm to 14µm in diameter.

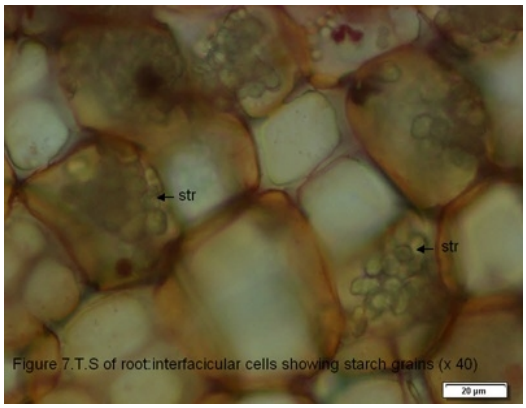

Fig. 7. T. S of root: Interfasicular cells showing starch grains (x 40)

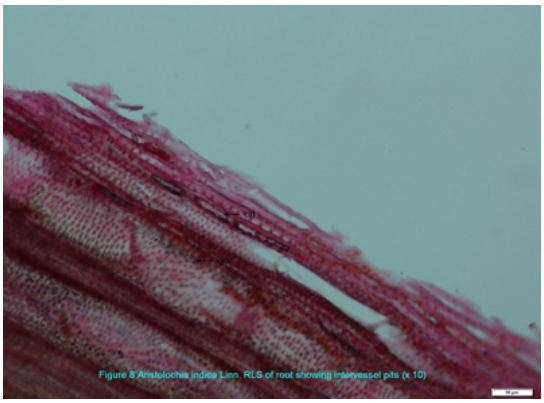

Fig. 8. RLS of root showing inter vessel pits (x 40)

When viewed under crossed polarized light, the starch grain showed centered extinction cross or 'Maltese cross' (Fig. 10), which indicates the hilum centrally in the grain [21]. Secondary phloem tissues, including the cells of phloem rays were found to contain prismatic calcium oxalate crystals. Calcium oxalate crystals were also observed in vessel elements and in xylem fibers (Fig. 9). Tangential section of the root showed diffuse axial parenchyma and absence of vessel contacts with rays. According to Metcalfe and Chalk [17], Rao et al. [22] and Carlquist [23], rays are remarkably wide in most members of the

family Aristolochiaceae, and the vessel density roughly inversely proportional to the diameter of the lumen of the vessel. Species with the widest vessels had the fewest vessels per rnm^2.

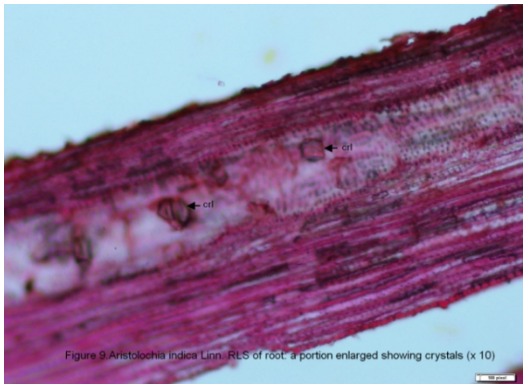

Fig. 9. RLS of root showing crystals in vessel elements; Polarised microscopic view (x10)

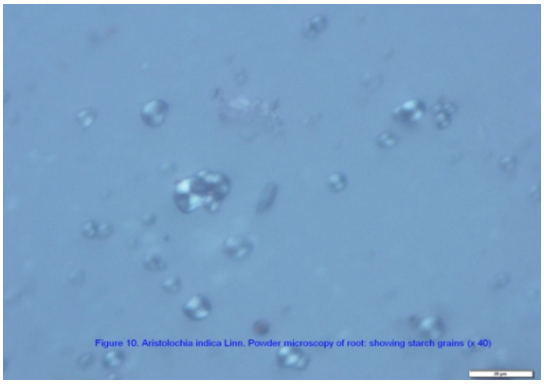

Fig. 10. Powder microscopy of root showing starch grains; Polarised microscopic view (x 40)

3.1.1 Powder microscopy and fluorescence analysis

The dried root of *Aristalochia indica* Linn. was analyzed for powder characteristics. Light microscopic and polarized microscopic examinations showed abundant pyramidal calcium oxalate crystals (Fig. 11) and many grape-like congregates of starch grains. Under the microscope illuminated with polarized light, unstained starch grain exhibited a distinctive dark birefringence cross or 'Maltese cross' (Fig. 10), indicating the radial or circumferential crystalline macromolecule organization inside each granule [21]. Root powder was also found to contain fragments of parenchymatous medullary ray tissues with aggregation of starch grains and fragments of cortical parenchyma tissues with groups of stone cells**.**

Fluorescence property of the powdered drug is often considered as an adjunct to botanical study. Hence, the root extract of *Aristolochia indica* Linn. was taken in different solvent systems with increasing polarity such as Cyclohexane, Toluene, Benzene, Ethyl acetate, Chloroform, Acetone, and Ethyl alcohol. Their fluorescence properties were analyzed under the dual UV light (254nm and 366nm). Under UV (254nm), root extracts appeared green in all solvent systems. Whereas, under UV (366nm) it appeared yellow green in Ethyl alcohol and Acetone, pink in Benzene, and cream white in others. The root powder in day light appeared dark brown in color and highly bitter in taste.

3.1.1.1 HPTLC finger print

The plates were developed in Automatic development chamber (ADC2) with the solvent system Chloroform: Ethanol 22.5:7.5 (v/v) to a distance of 8 cm and scanned at 254nm. The Rf values and the resolved bands were noted. Chromatogram of the drug extracted from the root had revealed 11 phytoconstituents. Data of peak area and peak height of each band recorded in track no 7 were depicted in (Fig. 12). Of these, the resolved band at Rf 0.54 corresponded to that of the marker constituent (ie., AA I).

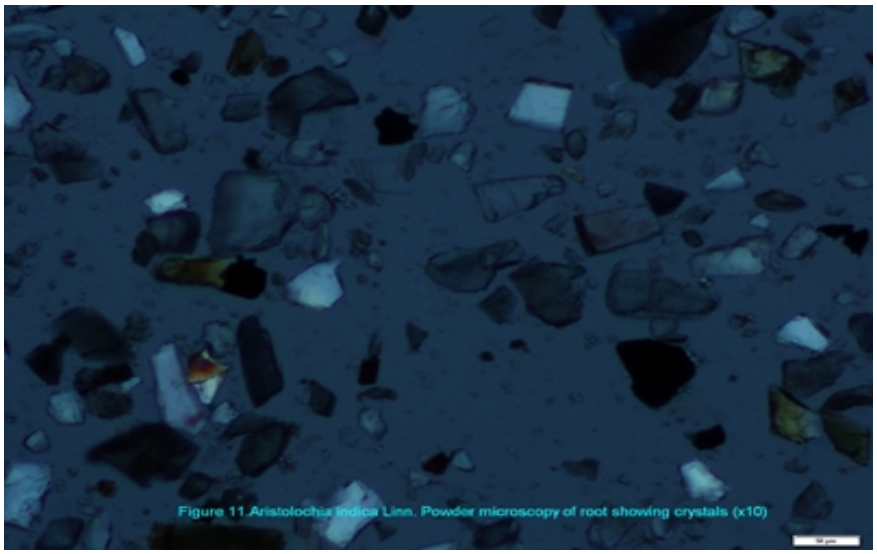

Fig. 11. Powder microscopy of root showing calcium oxalate crystals; Polarised microscopic view (x10)

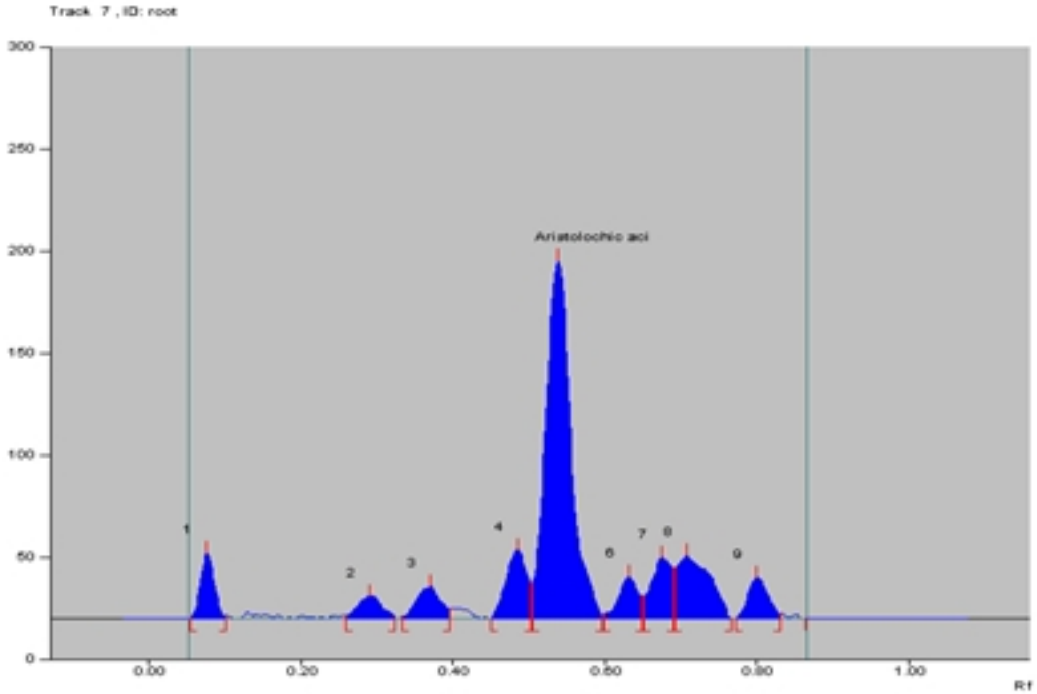

Fig. 12. HPTLC chromatogram of methanol extract of root of *Aristolochia indica* Linn

3.2 Quantitative Estimation

In the chromatogram of the drug extracted from the root, many well resolved spots were observed. Out of these spots, one spot (Rf = 0.54) matched with the Rf 0.53 value, and had the same λ max (319nm) shown by Standard Aristolochic Acid I (Fig. 13).

Quantitative investigation was performed by densitometry at its λ max (319nm). The specificity was confirmed by overlaying the spectra of standard AA I (λ max 319nm) with the absorption spectrum of the sample (Fig. 14). Linearity was justified by calibration curve (polynomial regression) by plotting peak area versus concentration.

When the concentrations of Aristolochic Acid I ($C_{17}H_{11}NO_7$) and their respective peak areas were subjected to regression analysis by least squares method, linearity was found to be in the concentration range of 100 to 500ng/spot with the calibration equation Y=347.29+14.18X and regression coefficient, r=0.998 and sdv=3.16%. The results revealed that 162.59ng of Aristolochic acid I in 5µL (Table 1) of root extract and the content of marker constituent (AA I) present in dried root sample of *Aristochia india* Linn (a Kerala habitant) was estimated as 0.082%.

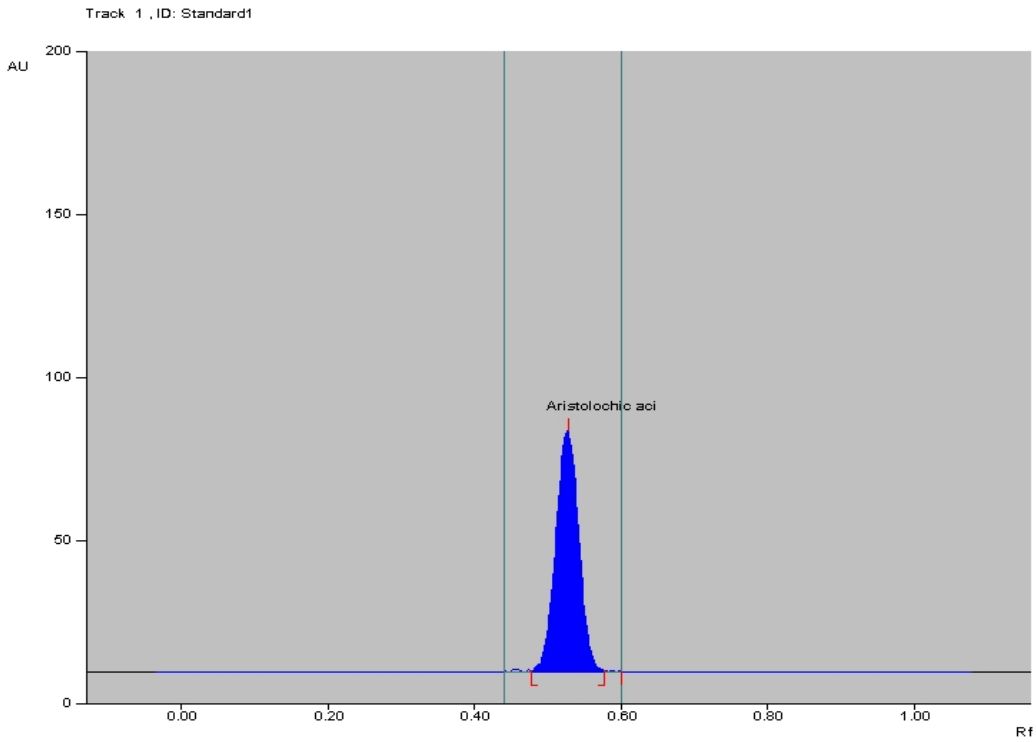

Fig. 13. HPTLC chromatogram of aristolochic acid I (92 ng/spot) with Rf=0.53

Since plant raw materials are traded mostly in a worn out, crumbled, granulated or powdered form with their botanical morphology largely spoiled, microscopic examination may lead to erroneous identification of botanical species. In such a scenario, the biological fingerprint by chromatography and/or spectroscopy (is)/are the most commonly used methods in

identification of herbal sample. But herbal system is not easy to analyze because of the inherent complexity of the chemical composition. Therefore, according to WHO [24], for herbal medicine with a well-documented history of traditional use, the bioactive extract should be standardized on the basis of the active principle or major compound(s) along with chromatographic fingerprint. If identification of an active principle is not possible, it may be sufficient to identify a characteristic substance or mixture of substances. Thus the morphological descriptors, anatomical markers and the developed HPTLC method have the advantage of rapid and easy identification of *Aristolochia indica* Linn. and quantitative estimation of the content of the marker constituent.

Table 1. Caliberation data of aristolochic acid I by HPTLC method

Track	Vial	Rf value	Amount fraction	Height	Area	X(calc)	Remark
1	1	0.53	92.00ng	73.98	1661.78		Std 1
2	1	0.53	184.00ng	125.60	2953.57		Std 2
3	1	0.53	276.00ng	176.53	4344.71		Std 3
4	1	0.54	368.00ng	216.22	5374.98		Std 4
5	1	0.54	460.00ng	281.48	6975.04		Std 5
6	2	0.54		116.43	2653.59	162.59ng	Sample root

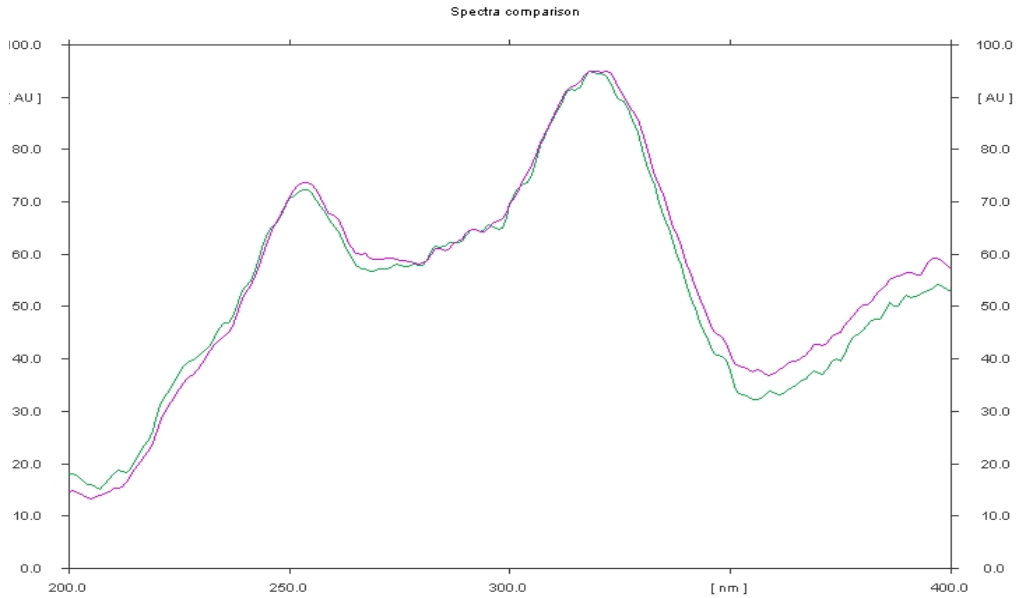

Fig. 14. Overlay of spectra (λ max 319nm) with standard AA I (in orange) and methanol extract of root (in green)

4. CONCLUSION

Aristolochic acids are constituents characteristically occurring in most members of the genus, *Aristolochia*. The principal components are Aristolochic acid I (AAI) and its demethoxylated derivative, Aristolochic acid II (AAII). The content of Aristolochic acids in plants or traditional herbal preparations varies depending on the plant species. Some of the

Ethno-botanically important species of Aristolochia found used in traditional Chinese medicine are *Aristolochia debilis* Sieb et al. Zucc., *A. contorta* Bge., *A. manshuriensis* Kom. and *A. fangchi* Wu. According to WHO [6], they are often considered interchangeable, and substitutes of one plant species for another is the established practice in traditional Chinese medicine. In India, three species of Aristolochia are found used in traditional medicine [25], namely; *A. indica* Linn, *A. bracteata* Retz and *A. tagala* Cham. Substitute of one plant species for another is also the established practice in the Ayurveda system of medicine. This is mainly because, most traditional forms of medicine are likely to use a mixture of substances, and the curative properties of the traditional herbal medicine principally based on the synergic effects of their multi-ingredients /poly herbal preparations.

The results of the present study suggest that, the documented morphological descriptors, delineated anatomical markers and developed HPTLC methods are complementary characteristics, which could be effectively used for the identification and authentication of the root of *Aristolochia indica* Linn. The developed active constituent based on HPTLC finger print profile may also supplement the regulatory perspective of routine quality control analysis for *Aristolochia* species in the formulation/ finished products of the traditional herbal medicine.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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