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Original Article

Finger Printing of the Anatomical Markers, HPTLC Profile and Heavy Metals Content in the Leaves of *Aristolochia indica* Linn.

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ABSTRACT

Background: *Aristolochia indica* Linn is now an endangered medicinal plant belonging to the family *Aristolochiaceae*. Many ethono-botanically important species of *Aristolochia* were found used in the traditional forms of medicine for the treatment of various illnesses. **Aim**: The present study concerns the microscopic, fluorescent, powder, quantitative microscopic characteristics of the leaves of *Aristolichia indica* Linn and its physico-chemical standards. **Materials and Methods**: Morpho-histological profile, High-performance thin-layer chromatographic (HPTLC) finger print profile, and heavy metals content of the leaves of *Aristolichia indica* Linn. **Results**: The presence of camptodromous pinnate venation with pentagonal shaped areoles with linear veinlet endings, non-glandular hooked trichomes, amphistomatic and anomocytic stomata, small palisade ratio, small stomatal index were the anatomical features characteristics of the taxon. Physico-chemical evaluation of the leaves gave moisture content of 10.5%, total ash 12.7%, acid insoluble ash 1.9%, acid soluble ash 10.8%. High-performance thin-layer chromatographic (HPTLC) identification of marker compound (Aristolochic acid I or (AAI)) in methanol extract of leaves was carried out. The developed HPTLC Chromatogram had revealed nine phyto-constutents in extract of leaf sample. The Rf value for Aristolochic acid I ($C_{17}H_{11}NO_7$) was found to be 0.41 and densitometric scanning had shown λ_{max} at 318 nm for the marker compound. The content of marker constituent (AA I) present in shade-dried leaves of *Aristochia indica* Linn was estimated as 0.049%. The Flame atomic absorption spectrometric determination of elements had shown appreciable amounts of the elements such as Fe (0.5442 mg/g), Zn (0.026.82 mg/g), Ni (0.008 mg/g,) and Cu (0.002 mg/g) by dry weight of the leaves of *Aristolichia indica*. **Conclusion:** The present study suggests that the delineated characteristics of the leaves of *A. indica*, could tag as the identifying parameters to substantiate and authenticate the raw drugs from the spurious/adulterants materials and could also be effectively used for the regulatory perspectives and quality assessment of Aristololic acid I in the medicinal formulation/finished products. The presence of important mineral elements inside this plant showed that it could be a nutritious plant and important to the human health.

Key words : Aristolic acid, Physico-chemical parameters, Stomatal index, Areoles, HPTLC finger print, Heavy metals.

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INTRODUCTION

The use of botanical and other natural products as traditional medicine and dietary supplements is increasingly popular owing to the perception that they are safer than prescription drugs. The long-history of use in traditional systems of medicine at the expense of using human body as the experimental screening model, accorded herbals the reputation of being looked upon as the gentle form of medicine, devoid of conspicuous side effects. *Aristolochia indica* Linn is now an endangered medicinal plant belongs to the family *Aristolochiaceae*. *Aristolochia* is an important genus both numerically (consisted of about 500 species) as well as medicinally. The genus has attracted great interest among investigators because of their multiple uses in traditional forms of medicine, and various bioactivity reports on its crude extracts. According to Kuo *et al.*[1] Aristolochic acid I (AAI) is the most potent fraction of *Aristolochia*'s constituents, and the members of this genus have been used for medicinal purposes since the Graeco-Roman period[1]. The name *Aristolochia* (Greek *cristos* = noblest + *locheia* = childbirth) is believed to have originated from the ancient Greek, which means "the best delivery or birth"; itself guarantors the centuries of its use in obstetrics. In Asian traditional medicine, many species of *Aristolochia* were found used as oxytocic agent to aid in child birth, menstrual cramps, and prevent infections in women after childbirth.[2-3] It is also found used as medicaments for the treatment of edema, arthritis, gout, rheumatism, as analgesic, anti-inflammatory, diuretic agents and tonics[4-5] in the traditional Chinese medicine.[6-7] Various *Aristolochia* species in the form of crude drugs as the anodynes, antiphlogistics, and detoxicants were reported to have been used in China[8] and the mature fruits of *A. debilis* Sieb *et al.* Zucc for the treatment of snakebite, tuberculosis and as antihypertensive agents.[9] The rhizomes of *A. brevipes* Benth were found used against snakebites, arthritis, and diarrhea by the local Tarasc people in Mexico.[10] *A. albida* Duch a native West African species was found used for the ailment of skin diseases, dysentery, gastrointestinal colic's, and snakebites.[11] In Indian System of medicine, the root and leaves of *Aristolochia* species were found used in the treatment for snakebite, scorpion stings, diarrhea, inflammation and abortifacient[9,12-13] and to alleviate insomnia, ease bowel obstruction, relieve symptoms of edema, cures Syphilis, Gonorrhea, and in the treatment for microbial infections.[14] However, some of the contemporary *Aristolochia*–containing Chinese herbal preparations, mostly used for weight loss in women were reported to have found nephrotoxic and carcinogenic effects.[15] Following which the use of *Aristolochia*-containing Chinese herbal preparations is the subject of warnings from the international regulatory agencies.[16]

Since *Aristolochia indica* Linn is an important herbal remedy in the Indian traditional medicine, and it has extensively been investigated by various workers for its phytochemical, pharmacological, Morpho-taxonomical, cytological, and organogenesis and micro-propagation studies.[17] However, the studies on the botanical pharmacognosy seem to be rare in literature, barring a few isolated studies on the aspects of ethono-biotanical,[18] folial stomatal development,[19] root morphology,[20] and root anatomy.[21-3] Therefore, the present study was undertaken with the objectives of delineating the anatomical markers, and develop active constituent based on High-performance thin-layer chromatographic (HPTLC) profile of the leaves of *Aristolochia indica* Linn. It also aims to

analyze the physico-chemical, fluorescent, quantitative microscopy, and contents of metal elements of the leaves of *Aristolochia indica* Linn.

MATERIALS AND METHODS

Plant Material

Aristolochia indica Linn (Figure 1) 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 are ovate and winged. The dried-leaves are extremely bitter in taste.

Methods

Aristolochia indica Linn was collected from its natural habitat from the Pathanamthitta District of the State of Kerala, India. The leaves 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. The plant was identified and authenticated with the help of the Flora of Presidency of Madras.[24] The shade-dried leaves were pulverized and passed through an 85–mesh sieve. The pulverized powder was kept in a labeled, air tight glass container. Fine hand sections of fresh leaves 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 (DP2). Images were concomitantly viewed and analyzed for anatomic characteristics, and quantitative measurements were taken using Olympus Image-Pro Plus (version 5.1). The number of epidermal cells, stomatal number, stomatal index were calculated per square millimeter of leaf area from intercostals areas of fresh leaves and vein islet number and vein termination number were calculated from cleared leaves and palisade ratio was determined based on Wallis[25] and size of Guard Cell Area (GCA) was estimated following Franco's formula.[26] The leaves powder was cleared with absolute alcohol and mounted on glass slides for powder analysis. The descriptive terms of the anatomical features used here as per Hickey,[27] Metcalfe and Chalk,[28] and Sudhakaran.[23,28-9] Fluorescence analysis of the leaves powder was carried out in daylight and UV light (254 nm & 366 nm) using Camang UV apparatus.

Physico-chemical parameters

An accurately weighed 3.387 g of shade-dried, powdered leaves were packed in a packing paper. This packed material was used for successive extraction in soxhlet apparatus for 24 h (approximately) with a series of solvents with increasing polarity as follows: Petroleum ether, Cyclohexane, Accetone, Ethanol and Water. Each time before extracting with the next solvent, the material was dried. After successive extractions, the extracts obtained were filtered, content were distilled, evaporated, concentrated, dried in desiccators and weighed. The respective percentage yield was calculated in w/w. The estimation of the total residual ash, acid insoluble ash, acid soluble ash, water soluble ash, and moisture content of the shade-dried powdered leaves were carried out using the standard procedure of the Ayurvedic Pharmacopeia.[30]

Qualitative phyto-chemical analysis

The shade-dried powdered leaves extracted with ethanol and aqueous medium were subjected to qualitative tests to identify the organic chemical constituents present in the drug.

High-performance thin-layer chromatographic (HPTLC) Finger print profile

Chemicals and reagents

Aluminum plate (20×10 cm) pre-coated with silica gel 60 F254 (Merck) of uniform thickness was used as adsorbent. Analytically pure standard Aristolochic acid I procured from Sigma and solvents of HPLC/ Chromatographic Grade procured from Merck and Qualigens Fine Chemicals, India were used. Analytical reagent grade of concentrated acids (HCI and HNO3) were also used.

Optimization of Chromatographic Conditions

The chromatographic separation was achieved using different ratios of solvents of varying polarity and the mobile phase consisted of chloroform:methanol (6:2 v/v) was found suitable for resolved separation of the analytes and quantification of marker compound.

Standard stock solution

The standard stock solution 0.5 mg/mL of Aristoloic acid I was prepared in HPTLC-grade methanol. One ml of this stock solution was diluted with 10 ml of methanol and a working solution of 0.05 mg/mL concentration was prepared and used for the HPTLC analysis.

Sample preparation

Accurately weighed 2.028 gms of leaves powder of *Aritolochia indica* Linn was refluxed in 25 ml of Methanol. Extract obtained was filtered using Whatman filter paper and transferred to a volumetric flask, and volume was made upto 50 ml with methanol.

Instrumentation

CAMAG HPTLC System (Switzerland) equipped with CAMAG Linomat V Automatic Sample Spotter with syringe (100 **µl**), Automatic development chamber (ADC2), UV cabinet with dual wavelength, and the densitometer consisted of TLC scanner 3 linked to WINCATS software were used for HPTLC method.

For the HPTL analyses, 4 different application volumes of the working solution were used in 4 different tracks on the pre-coated plate of Silica Gel and methanol extract of the test sample in one track (track no.12). The standard working solution and extract of leaves were applied on the plate using CAMAG LINOMAT V Applicator, the semi-automatic spotting device and Hamilton 100 µl syringe. The spotting volume of standard working solution on the plate were 2,4,6 and 8 µl with a concentration ranged from 100 to 400 ng/band respectively and 5 **µl** for the test sample (*4 Standards plus one methanol leaves sample*). The plate (20×10cm) was developed in Automatic development chamber (ADC2), using the mobile phase chloroform: methanol (6:2 v/v) at 26.7°C temperature and relative humidity 78.3. Densitometric scanning of the plate at 254 nm and 320 nm were performed using CAMAG TLC Scanner 3, and operated by the WINCATS software. Calibration graph was plotted using the peak area vs concentration of the standard marker compound. The content of marker compound (AAI) in the methanol extract of the leaves was estimated using linear regression equation. The developed method was validated for the parameters like specificity, linearity, limit of detection (LOD) and limit of quantitation (LOQ).

Flame Atomic Absorption Spectroscopic Determination of the metals

Flame Atomic Absorption Spectroscopy was used to determine the concentration of metal elements in the powdered drug. Thermo Electron Corporation Atomic Absorption Spectrometer (M series FS 95) equipped with auto-sampler and with Deuterium background corrector was used for the metals determination. The flames used were air/acetylene. The hollow-cathode lamps for Fe, Zn, Ni, Pb, Cu, and Cd were employed

Table 1: Quantitative Microscopy of *Aristolochia indica* Linn

Parameters	Mean value	Range
Stomata length (µm)	26.1	20.9 -27.2
Stomata width (µm)	18.1	17.4 -18.6
*Guard cell area GCA (µm²)	306.9	285.6 - 397.3
Stomatal density / mm²	121.0	20.3 - 138.6
Epidermal cell density / mm²	916.4	898.5 - 921.4
Stomatal index	11.7	11.8 - 13.1
Vein-islet number/mm²	5.0	3.9 - 5.2
Vein- termination/ mm²	7.2	6.1 - 9.9
Palisade ratio	2.75	2.5 - 2.75
Size of areoles (mm)	0.08	0.07- 0.09

** Franco's formula.*

Table 2: Physico-chemical parameters of the leaves of *A. indica* Linn

Parameters	Percentage w/w (Mean + SE)
Total Ash	12.72±.004
Acid insoluble ash	1.89±.04
Acid soluble ash	10.83±.02
Water soluble ash	1.92±.002
Moisture	10.5±.02
Successive extractives	
Pet-ether	4.19±.02
Cyclohexane	0.77±.006
Acetone	3.62±.005
Ethanol	6.51±.01

Table 3: Qualitative phyto-chemical analysis of the leaves of *A .indica*

Type of compound	Reagents	Result
Alkaloid	Ethanol extract +Dragon dorff's	+
Carbohydrate	Aqueous extract +Fehling A & B	+
Phenolic compound	Ethanol extract + Neutral Ferric chloride	+
Saponin	Ethanol extract + Distilled water	+
Steroids	Ethanol extract + acetic anhydride + con. Sulfuric acid	+
Tannin	Aqueous extract + Lead acetate	+
Protein	Ethanol extract + con. Nitric acid	+
Glycosides	Ethanol extract + Benzene+ dil. Ammonia	-
Flavanoid	Ethanol extract + con. HCL+ Magnesium ribbon+ boiling	-
-	Present +	absent –

as radiation source. The operating parameters for the working elements were set as recommended by the manufacturer. The optimization wizards within SOLAAR software (version 11.01) contains the pre-set spectrometer parameters for these metals were made the optimum analytical conditions and the plotting of calibration curve.

Stock solution

Stock solutions were containing 1000 ppm of the elements such as; Cd, Ni, Zn, Cu, Pb and Fe procured from Merck (Germany). Appropriate dilutions of these stock solutions gave the working standard of each element for plotting the calibration graph.

Sample preparation

Accurately weighed 2.422 g of shade-dried powdered drug was burnt to ashes in a muffle furnace by gradually increasing the temperature from 25°C to 450°C, till the ashes turned to became white in color. The resulting ashes was digested in 24 ml of solution containing HCl and HNO3 (3:1 v/v) and digests were filtered and transferred to a standard volumetric flask and made up the volume as 100 ml with de-ionized water. Analyses for metal elements in the sample were carried out in triplicate by flame Atomic Absorption Spectroscopy.

Calibration curve

The 1000 ppm standard solutions of elements procured from Merck (Germany) were diluted in suitable four different working standard concentrations, and 2% w/v HNO3 solution as blank, prepared immediately before the use. Calibration graph was plotted using the absorbance vs concentration of the standard solutions and the method of segmented fit was used for quantification of the elements in the leaves sample.

RESULTS AND DISCUSSION

Macroscopic evaluation of Leaves

The leaf of *Aristolochia indica* Linn is microphyll, which consisted of an average of 9.5 cm in length and 3.2 cm. in width. The leaves were glabrous, very variable in size and green in color. Leaves were usually obovate-oblong, entire with undulate margins, cordate at the base, and having 3-nerved from base. When squeeze the fresh leaves produce a characteristic odor, and showed extreme bitter taste. Shade-dried leaves when pulverized to yield coarse power, and incinerated residue (ash) were appeared as white in color.

Microscopic evaluation of Leaves

Transverse sections (S.C) of the leaf showed dorsiventral differentiation. The midrib was broadly convex on the abaxial side and bluntly conical with short lump on the adaxial side (Figure 2). Midrib was consisted of three distinct tissue organizations such as the outer collenchyma, middle mesophyll, and inner vascular bundles. Collenchyma comprised of 6-7 layers. The vascular bundles appeared as crescent shaped and embedded in the middle of the ground tissues which were encircled by the sclerenchymatous bundle sheath. The mesophyll was consisted of the upper palisade tissues and lower spongy parenchyma (Figure 2a & 2b). Palisade appeared as single layered, cells were cylindrical and top shaped. The mean height and breadth of the palisade cell was found to be 45.7 µm (ranged 43.8-48.1 µm) and 20 µm (ranged 18.5-21.5 µm) respectively, and the palisade ratio was found to be 2.7. The spongy mesophyll cells were elongated, lying parallel to the surface of lamina and occupied more than a half of the areas of the mesophyll tissues. The cells of the spongy mesophyll were round or rectangular, cells were loosely arranged with large intercellular spaces, and formed a lobed network of 4-5 layers. Both the palisade and spongy cells were contained with plenty of chloroplasts (Figure 2c). Xylem vessels were arranged in radial rows of 4 or 5, and phloem lies on the abaxal side. The diameter of the vessel lumen ranged from 20 µm to 34 µm. The midrib was appeared much broader and wider than the lamina. The lamina appeared flat and much reduced in dimension (Figure 2). Both epidermises were one cell thick, cells were compactly arranged and rectangular in shape having a prominent layer of cuticle over the outer cell wall. The thickness of the cuticle varies and found to be ranged from 6.2 µm to 6.5 µm. The upper epidermal cells were larger in size (mean dimension 50.6 µm×27.8 µm) compared to the lower epidermal cells (mean dimension 34.5 µm×22.5 µm). The ground tissues of the lamina consisted of 3-4 layers of parenchymatous cells and cells were polygonal in shape.

Trichomes

The surface features of the epidermis showed non-glandular trichomes on both the adaxial and abaxial sides. Trichomes were uniseriate, having

Table 4: Calibration data of Aristolochic acid I at 320nm by HPTLC method

Track	Vial	Rf	Amount Fraction	Height	X(calc)	Area	X(calc)	Remark
5	1	0.40	100.00 ng	87.54		2125.75		Std 1
6	1	0.41	200.00 ng	146.55		3861.64		Std 2
7	1	0.41	300.00 ng	195.45		5268.33		Std 3
8	1	0.41	400.00 ng	235.15		6533.41		Std 4
12	2	0.42	-	97.11	0.00ng	2551.12	99.88 ng	Sample leaf

Table 5: Concentrations of metals in the shade-dried leaves of *A. indica* Linn

Metal elements	Reference levels [1, 2] (mg/Kg dry weight)	mg/Kg dry weight
Iron (Fe)[36-37]	-	544.20 mg/kg
Zinc (Zn)[36-37,40]	40.00 mg /kg	26.82 mg/kg
Copper (Cu)[36-37,40]	30.00 mg /kg	2.20 mg/kg
Lead (Pb)[39]	1.0 mg/kg	9.78 mg/kg
Nickel (Ni)	-	8.01 mg/kg
Cadmium (Cd)[39]	1.50 mg/kg	1.62 mg/kg

Source: *Joint Codex Alimentarius Commission*[36-39]; *Indian Standard*[40].

a short basal cell accompanied by an elongated terminal stalk cell (Figure 3b). In some of the trichomes, the terminal stalk cell appeared curved like a hook. Such hooked trichomes (Figure 3c) were found abundant on abaxial side of the lamina than the adaxial side. The outer epidermal layer of the mid rib was also provided with trichomes. The mean length of trichome was observed as 46.08 µm. The mean diameter of the bulbous basal cell of the trichome was found to be of 14.14 µm and that of terminal cell was 6.18 µm.

Epidermal characters

Epidermes showed the amphistomatic and anomocytic stomata (Figure 3 & 3a). The density of the stomata was found to be at variance in both epidermses. The stomata were observed much higher in number on the abaxial epidermis than adaxial epidermis. Pataky[31] had classified the stomata with guard cell having measured a length of more than 38 µm as "large" and however, in the present study the mean length and breadth of stomata was found to be 26.1 µm and 18.1 µm respectively. According to Meltcafe and Chalk[26] the taxon having small sized stomata usually gave the high stomatal density, and their findings did hold true for *A. indica* Linn which gave a high mean stomatal density of 121 per square millimeter areas for the abaxial surface of the leaf. The stomatal index of *A .indica* Linn was found to be 11.7/mm^2, and the mean Guard cell area (GCA) was of 306.9 µm.^{2} Meltcafe and Chalk[26] have reported that the stomatal index could be used as a reliable taxonomic character for identifying the species, as the epidermal cell size is independent of the changes brought about by environmental factors. The epidermal cells were straight or wavy in nature, and the number of epidermal cells per square millimeter area of the leaf was found to be 916.4 (Table 1). The mean pore aperture length and breadth of the guard cell of *A.indica* was found to be 7.38 µm and 2.45 µm respectively.

Petiole

In cross sectional view the petiole was appeared somewhat spherical in shape with a deeply notched groove at the adaxial side (Figure 4). Epidermis was consisted of singled layered, elongated and rectangular shaped cells. Beneath the upper epidermis laid 3-4 layered collenchymatous hypodermis. The petiolar bundles were five in number and found to be arranged in the form of letter "v". The median three bundles were larger in size compared to the two laterals. The vascular bundles were widely separated from one another by parenchymatous ground tissues. Vascular bundles were collateral; the xylem vessels were arranged in radial rows of 3-4, and phloem situated in the abaxial side. The lumen of xylem vessels varied in size; its diameter ranged from 18 µm to 24 µm. Cells of the parenchymatous ground tissue had often contained the prismatic crystals of calcium oxalate.

Venation Pattern

The leaf architectural characters could also provide useful anatomical information for the characterization of the taxon. The terminology used for description of the leaf architecture in the present study was as per the Hickey.[27] The venation patterns of cleared leaves of *Aristolochia* indica Linn had observed the camptodromous pinnate venation under low (×2) magnification. Areolation was found to be well developed and pentagonal or polygonal in shape. The size of the areoles was small (> 0.30 mm). When critically analyzed microscopically, the number of areoles/ mm^2 was found to be 13.3. Within the areoles the terminal vein-ending was observed and the number of vein-endings per areola was found to be one, and appeared as linear (Figure 5). Veinlets of the lamina were observed twice branched and the marginal veins appeared fimbriate (Figure 5a). The minor venation pattern viz. the mean vein islet number/ mm^2 of leaf was found to be 5, and the veinlets termination number/mm^2 were 7.2. The mean size of the areole was found 0.08 mm.

Powder microscopy

Powdered drug under the microscope showed the presence of fragments of leaf epidermis with cell having straight wall and the cells appeared pentagonal or hexagonal in shape, and lamina comprised of having conspicuous venation (Figure 6 & 6a).

Fluorescence Analysis

Fluorescence analysis could be very useful adjunct to the botanical study, since it is an easy test to verify certain identification parametes of the crude drug. Hence, dried powdered leaves were taken in a series of solvent systems with increasing polarity as follows; petroleum ether, cyclohexane, toluene, benzene, ethylacetate, chloroform, acetone, ethyl alcohol and methanol. Their fluorescence properties were analyzed under the dual UV light (254 nm and 366 nm). Under the short wavelength (254 nm), leaves extracts in all the solvent systems were found to be non-florescent, whereas they observed florescent at 366 nm. All the extracts were appeared pink to orange color at 366 nm; except the extract of Toluene, where it appeared as cream white. The aqueous extract of the leaves was also found to be appeared as cream white. The leaf- powder in day light appeared green in color, and was highly bitter in taste.

Physico-chemical Evaluation

The evaluation of the physico-chemical constants of the drug is an important parameter for detecting the adulteration or improper handling of the drug. The values of the extractive yield of the drug obtained in a series of solvents were depicted in Table 2. The Table 2 showed that the percentage extractive yield obtained for ethanol was found to be higher

Figure 1: *Aristolochia indica* L.

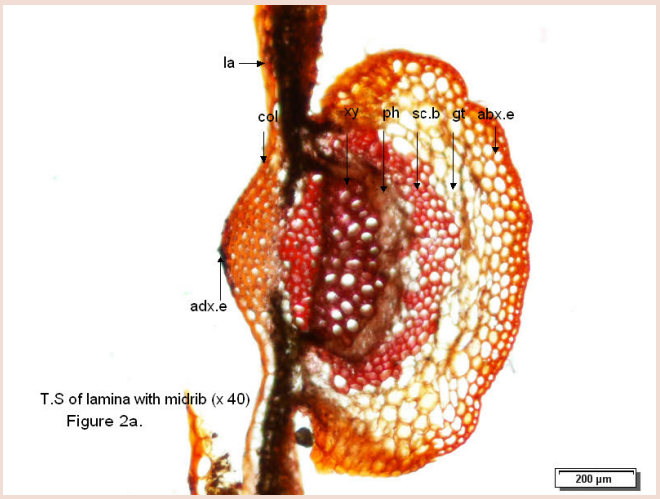

Figure 2b: T.S. of Lamina with midrib(x 40)

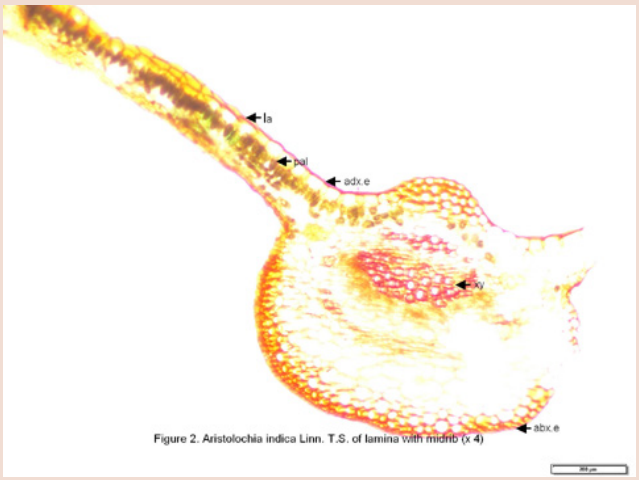

Figure 2: T.S. of Lamina with midrib(x 4)

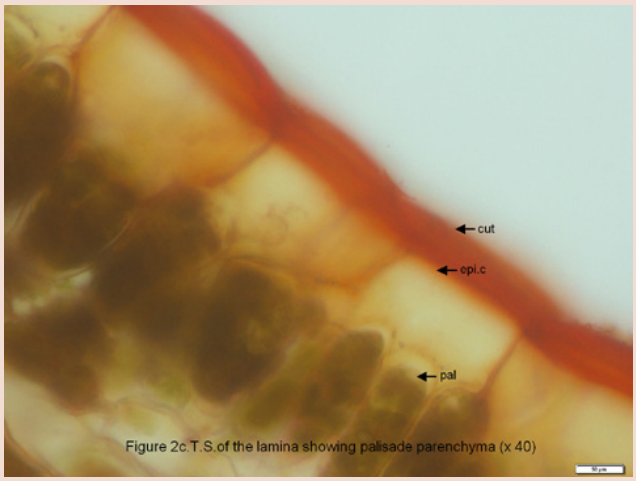

Figure 2c: T.S. of Lamina showing palisade (x 40)

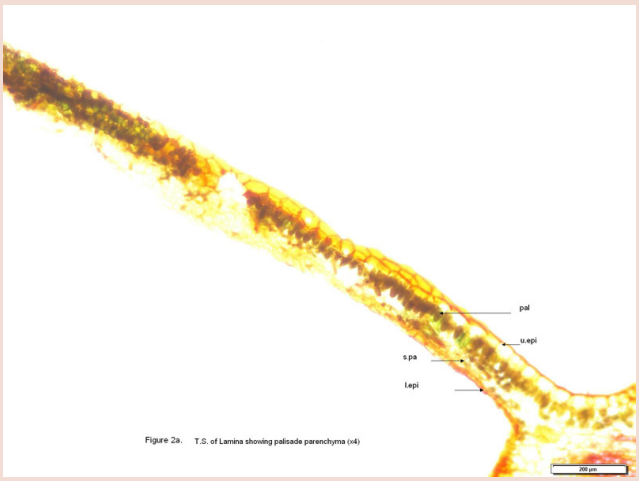

Figure 2a: T.S. of Lamina showing palisade (x 4)

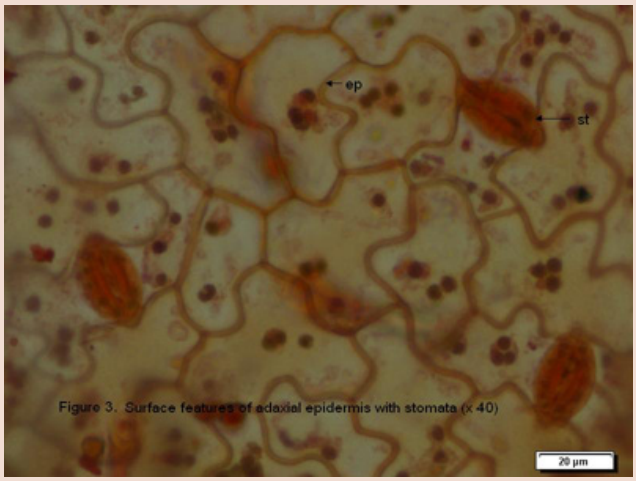

Figure 3: Surface features of adaxial epidermis with stomata(x 40)

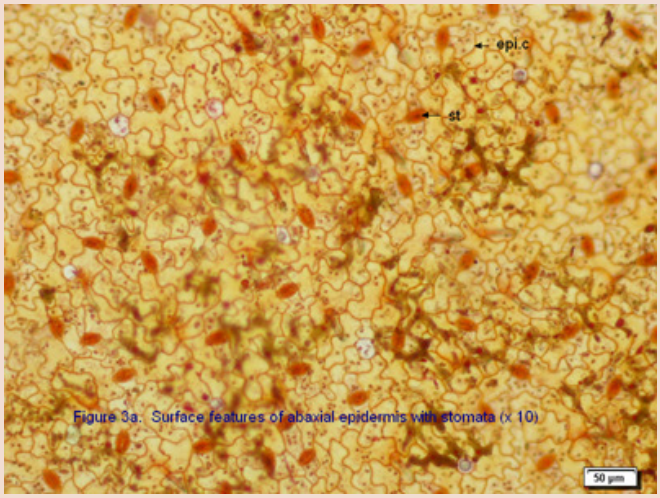

Figure 3a: Surface features of abaxial epidermis with stomata (x 10)

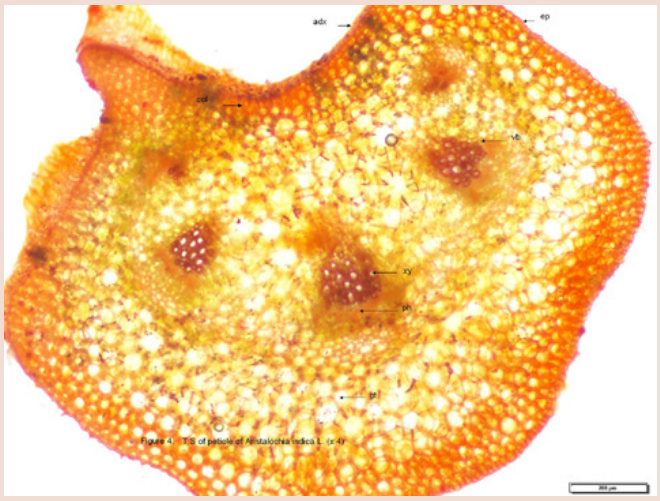

Figure 4: T.S of Petiole of *Aristolochia indica* L. (x 40)

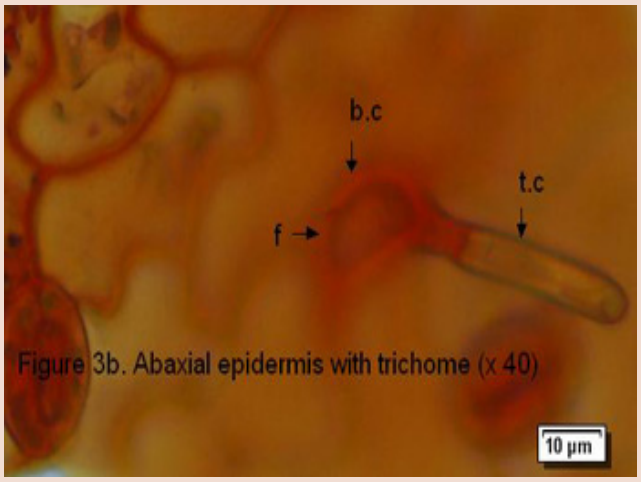

Figure 3b: Abaxial epidermis with trichome (x 40)

Figure 5: Cleared leaf of *Aristolochia indica* showing areoles,vein-islets and vein termination (x 2)

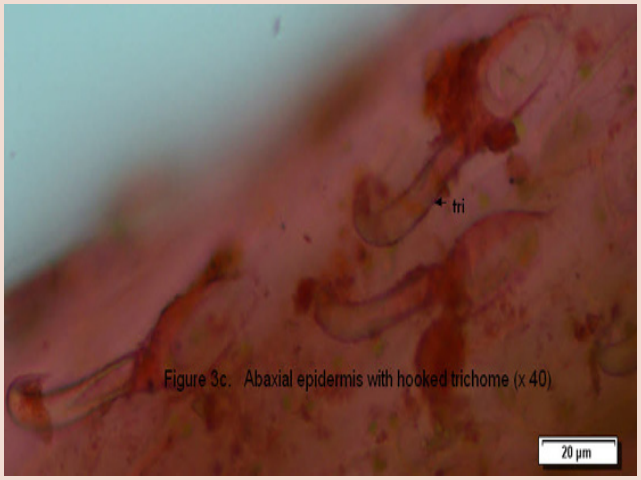

Figure 3c: Abaxial epidermis with hooked trichome (x 40)

Figure 5a: Cleared leaf showing leaf margin (x 2)

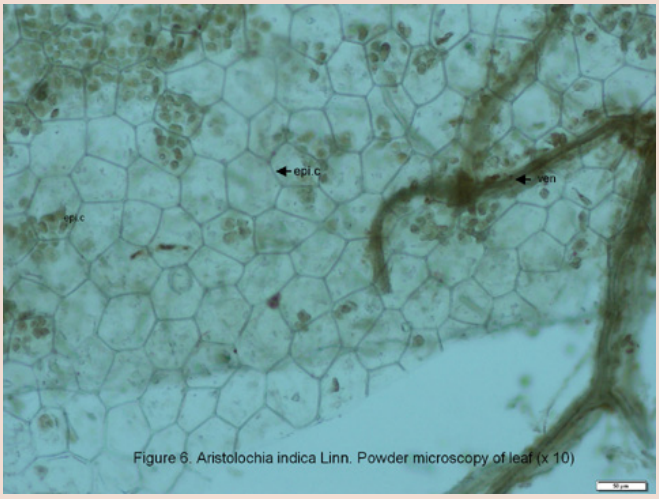

Figure 6: Powder microscopy of leaf (x 10)

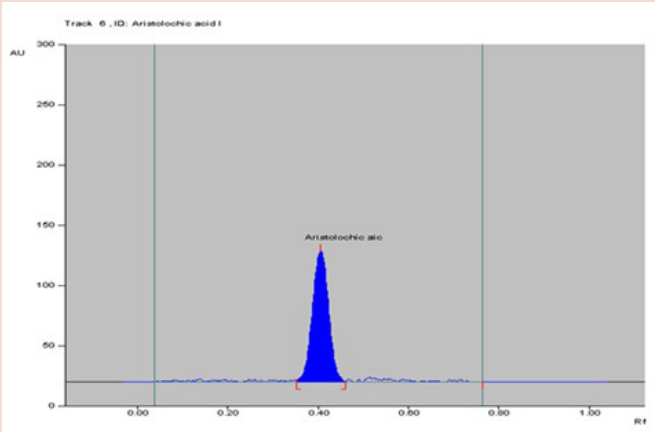

Figure 8: HPTLC chromatogram of Aristolochic acid I (200 ng/band) with R_f=0.41

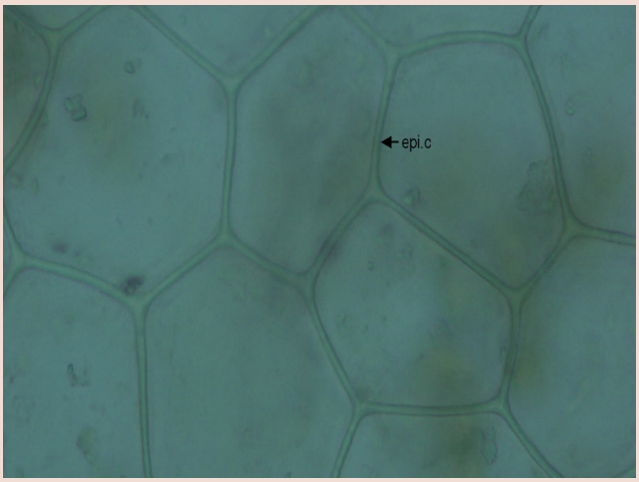

Figure 6a: Powder microscopy of leaf (x 40)

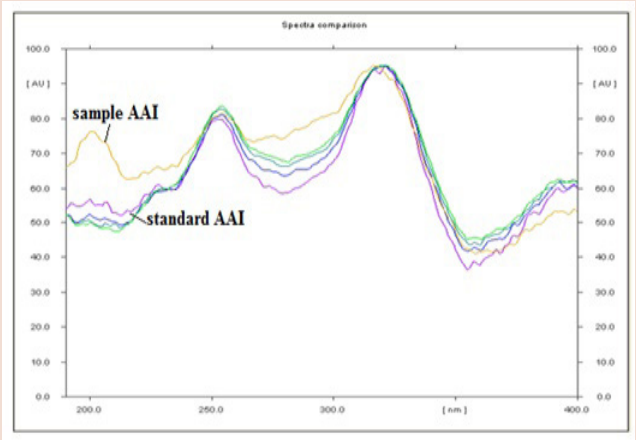

Figure 9: Overlay of spectra (λ_{max} 318 nm) with standard AAI and methanol extract of leaves

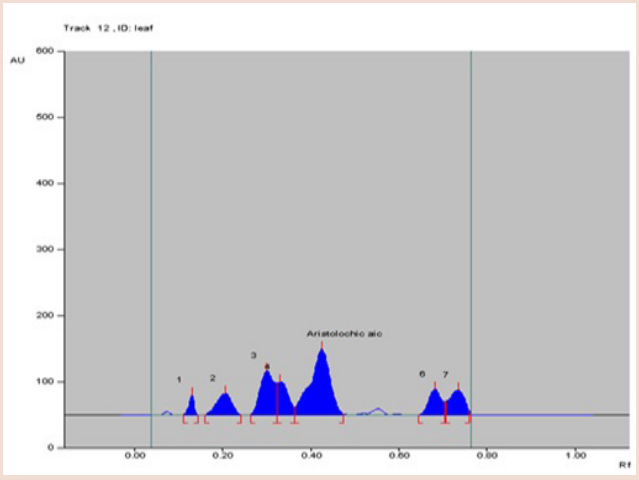

Figure 7: HPTLC profile of methanol extract of the leaf of *Aritolochia indica*

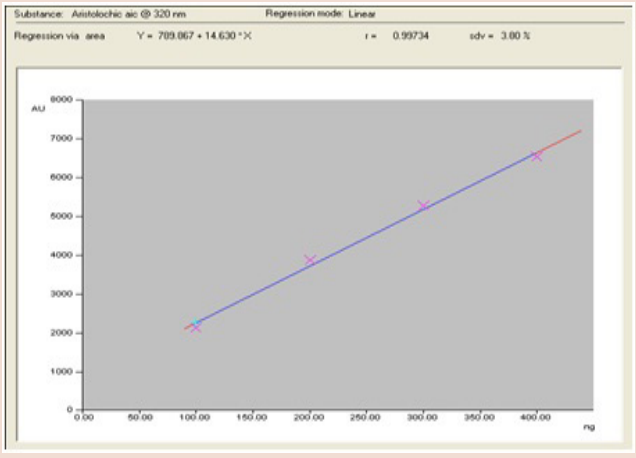

Figure 10: Calibration curve of Aristolochic acid I (concentration Vs peak area)

and relatively low value obtained for Cyclohexane. The moisture content of the shade-dried powdered drug was found be not more than 10.5 %, which often discourages the growth of microbes. The total residual ash value was found to be higher than that of the acid soluble ash. The value of 1.89% obtained for the acid insoluble ash in the present study may be owing to the incidence of siliceous matters in the crude drug.

Qualitative phyto-chemical analysis

The preliminary screening of the methanol leaves extract of *Aristolochia india* Linn had showed the presence of alkaloid, carbohydrate, phenols, steroid and saponin and the results obtained were depicted in Table 3.

HPTLC Finger print

The spotted samples of methanol extract of leaves and standard marker compound (AAI) containing on the silica gel plates were developed in the Automatic development chamber (ADC2) with the solvent system Chloroform: Ethanol 22.5:7.5 (v/v) to a distance of 8 cm. All tracks in the plate were scanned at 254 nm and individual Rf values and the resolved bands were recorded. Chromatogram of the drug extracted from the leaves had revealed 9 phyto-constituents. Data of peak area, peak height, Rf value of each band were recorded in track no.12 and the expressed pattern of chemical ingredient's distribution were also depicted in Figure 7. Out of these bands, one band (R_f= 0.42) generated matched with the R_f value (R_f= 0.41) of the marker compound (Figure 8) and the densitometric scanning had the same λ_{max} (318 nm) as that shown by Standard Aristolochic Acid I.

Quantitative Estimation

Quantitative investigation of the marker compound (AAI) was carried out by densitometric reflection/absorption mode at 320 nm (Figure 9). Spectral matching by overlaying the absorption spectra of standard marker compound (100-400 ng/band) with the absorption spectrum of marker compound in leaves sample (ie., *AAI in the leaves-sample*) was confirmed the specificity of $\boldsymbol{\lambda}_{max}$ at 318 nm (Figure 9).

Calibration curve

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, calibration curve was found to be linear in the concentration range of 100 to 400 ng/band with the calibration equation Y=789.867+14.63X and regression coefficient, r=0.997 and sdv=3.8% (Figure 10). Calibration graph showing the acceptable correlation (r=0.99) between the marker compound in both the methanol extract of leaves and standard in the track had revealed a good linearity response for the method developed (Figure 10). The calibration data depicted in Table 4 had revealed that 5 µL of methanol extract of leaves contained 99.88 ng of Aristolochic acid I and the content of marker constituent (AA I) present in dried leaves sample of *Aristochia india* Linn (*a Kerala habitant*) was estimated as 0.049%.

A validated HPTLC method for the quantification of the Aristolochic acid I present in the dried root samples of *Aristolochia indica* Linn had already been developed by this author[23] and results obtained suggests that the concentration of bioactive compound (AAI) is very high in the shade-dried roots (0.08%)[23] when compared to that of the shade-dried leaves (0.049%). However, the study fails to obtain any detectable amount of Aristolochic acid I from the dried stem of *Aristolochia india* Linn by HPTLC method, which further justifies the privileged use of its roots and leaves for the preparations of Indian traditional herbal medicine.

Sensitivity of the HPTLC method

The sensitivity of an instrument is a measure of its ability to discriminate between small differences in analyte concentrations in an analytical procedure. According to Srivastava[32] a signal-to-noise ratio (S/N) of 3 and 10 were generally be considered as limits of detection (LOD) and limits of quantitation (LOQ) respectively for HPTLC. The LOD and LOQ values of AAI calculated from the calibration regression equation were found to be 0.779 ng and 2.597 ng respectively. This suggests that the developed HPTLC method exhibits a good sensitivity for the quantitation of AAI. The developed active constituent based on HPTLC method has the advantage of simple, specific and easy identification of Aristolochic acid I in the leaves samples and could be applied for the regulatory perspective as routine quality analysis for the *Aristolochia* species in the formulation/finished products.

Flame Atomic Absorption Spectroscopic evaluation of metal elements

Analyses of the flame atomic absorption spectrometry after acid digestion of the leaves extract showed the mean concentrations of metals in the leaf-sample were in the order of Fe(544.2 ± 0.02)>Zn(26.82 ± 0.004)>Pb(9.78 ± 0.005)>Ni(8.01 ± 0.005)>Cu(2.2 ± 0.002)>Cd(1.62 ± 0.001). The results showed that (Table 5), iron had the highest concentration in the investigated samples followed by zinc and the least encountered were cadmium.

Since, the dietary intakes of fruits, vegetables and crude drugs are known the most affordable source of minerals and vitamins important to the human health. Exceeding the maximum tolerable levels or the excessive uptake of dietary heavy metals may cause serious health problems, owing to their long biological half-lives, non-biodegradable nature and their potential to accumulate in most systemic parts of the body.[33-4] Therefore, many standards and a great deal of recommendations exist all over the world regarding the amount of heavy metals tolerable levels for the animals and humans, including the limit for the level of heavy metals concentration in different plant types such as vegetables, cereal crops and medicinal plants.[35] The standard values set out by the Codex Alimentarius Commission (FAO/WHO)[36] for the daily intakes for Fe is 0.767 mg/kg/day for both children and adults. The international standard limits for Pb, Cd, Cr, and Cu for an average person (*70 kg body weight*); for daily intakes through the consumption of root vegetables are limited to 250 µg, 70 µg, 50–200 µg and 35 mg respectively.[37-9] In Prevention of Food Adulteration Acts & Rules of India,[40] the permitted limits for Cu, Zn, Pb and Cd levels for the fruits and vegetables are found to be 30, 50, 1 and 1.5 parts per million by weight respectively. Ajasa *et al.*,[41] have reported that micronutrient elements Cu and Zn in agricultural products may be ranged between level 4-15 mg/L and 15-20 mg/L respectively and concentration of iron may be ranged between 261 ppm to 1230 ppm for some medicinal plants. The WHO[42] prescribed limit for lead contents in herbal medicine is 10 ppm while the dietary intake limit for lead is 3 mg/week. However, for medicinal plants the WHO limits yet to be established for Fe and Ni.

The estimated values for the mineral elements in shade-dried leaves of *A. indica* Linn (Table 4) in the present study suggests that the use of this plant's leaves in moderate quantities would provide the range of amounts of Fe, Zn, Cu and Ni necessary for the healthy body. The presence of some important mineral elements inside this plant also showed that it could be a nutritious plant and important to the human health. However, the high concentration of toxic element Pb in the leaves may pose risk to human health and hence be cautious for the indiscriminate use of the leaves.

CONCLUSION

The present study suggests that the delineated characteristics of the leaves of *A. indica*, could tag as the identifying parameters to substantiate and authenticate the raw drugs from the spurious/adulterants materials and which could also be effectively used for the regulatory perspectives and quality assessment of Aristololic acid I (AAI) in the medicinal formulation/finished products. The presence of important mineral elements inside this plant showed that it could be a nutritious plant and important to the human health.

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CONFLICT OF INTEREST

Author has declared that no conflict of interest exists

Abbreviations used in the figures are

A: Areola; **Abx:** Abaxial; **Abx.E:** Abaxial Epidermis; **Adx:** Adaxial, **Adax.E:** Adaxial Epidermis; **B.C:** Basal Cell; **Col:** Collenchyma; **Crl :** Crystal; **Cut:** Cuticle; **Ep, Epi.C:** Epidermal Cell; **Epi:** Epidermis; **F:** Foot Cell; **Gu.C:** Guard Cell; **Gt:** Ground Tissue; **La:** Lamina; **L. Up**: Lower Epidermis; **Pal:** Palisade Cell; **Ph:** Phloem; **Pi:** Pith; **Scb:** Sclerenchymatous Bundle Sheath; **Spa,Spo:** Spongy Parenchyma; **St:** Stomata; **Stcl:** Stone Cell; **Str:** Starch Grain; **S.Xy:** Secondary Xylem; **Tri:** Trichome; **T.C:** Stalk Cell; **U.Epi:** Upper Epidermis; **Vb:** Vascular Bundle; **VI:** Vein Islet; **VT:** Veinlet Termination.

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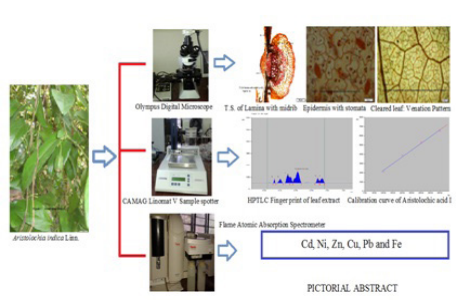
PICTORIAL ABSTRACT



SUMMARY

- Aristolochic acid I (AAI) is the most potent fraction of Aristolochia's constituents, and the members of this genus have been used for medicinal purposes since the Graeco-Roman period, and the root and leaves of Aristolochia species were found used in Indian System of medicine.
- High-performance thin-layer chromatographic (HPTLC) method developed had revealed nine phyto-constituents in methanol extract of leaves and the content of marker compound, AAI was found to be 0.049% by dry weight of the leaves.
- The Flame atomic absorption spectrometric determination of elements had shown appreciable amounts of the elements such as Fe, Zn, Ni and Cu.
- The leaf anatomical markers, quantitative microscopy, chemoprofiling and HPTLC profile of Aristolochia india delineated in this study could effectively be used for the regulatory perspectives of the quality assessment of the raw drugs and the medicinal formulation/finished products.

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