

Comparative Study on Pharmacognostical, Phytochemical Investigations and Quantification of Vasicine Content in the Extracts of *Adhatoda vasica* Nees and *Adhatoda beddomei* CB Clarke

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ABSTRACT

Background: *Adhatoda*, a perennial shrub of family Acanthaceae are well-known medicinal plant for the treatment and management of respiratory disorders such as asthma and bronchitis. *Adhatoda vasica* and *Adhatoda beddomei* are the species of *Adhatoda*, has been widely used in Indian system of medicine. Although, phytochemical and pharmacological investigations were reported on *A. vasica*, there has been comparative investigations on different *Adhatoda* species are lacking. **Objective:** The study was undertaken to compare the pharmacognostical and phytochemical parameters of two species of *Adhatoda* for rapid identification and authentication of the plants. **Materials and Methods:** Pharmacognostical features were studied by macroscopic, microscopic studies and physicochemical analysis such as determination of foreign matter, ash value, extractive value and loss on drying. Phytochemical investigations were analysed using phytochemical screening, bioactive content determination, HPTLC fingerprint analysis and estimation of vasicine content by HPLC analysis. **Results:** Microscopic study differentiated the pharmacognostical features between two species by demonstrating the anatomical characteristics. Powder microscopy of *A. vasica* revealed the presence of diacytic stomata, glandular and non-glandular trichomes whereas rod shaped crystals were seen only in *A. beddomei*. Qualitative and quantitative phytochemical investigations revealed the presence and estimation of various phytoconstituents in both the species. HPTLC fingerprint profiling evaluated the number of constituents present in the extracts and HPLC analysis revealed high content of vasicine in *A. vasica* extracts when compared to *A. beddomei*. **Conclusion:** The present study provides the useful information to differentiate the plant species and can serve as a diagnostic tool for the standardization and identification of adulterant in the crude drug market.

Key words: *Adhatoda vasica*, *Adhatoda beddomei*, Pharmacognosy, HPTLC fingerprint, Vasicine.

INTRODUCTION

Herbal medicine is the oldest form of healing and treating various diseases.¹ Herbal medicines have been widely used due to its more cost effective with no side effects when compared to synthetic drugs. About 80% of the world population rely mainly on herbal medicine for their primary health care.² The increasing demand in the use of herbal medicine gain an attention of the researchers interestingly to study the standardization and quality control of herbal drugs in order to avoid the erroneous medication and improve the quality of drugs. Standardization is a significant tool for ensuring the quality of herbal drugs. The drug identity, purity, content, chemical and other biological properties determine the quality of the drug.³

Tropical species of the genus *Adhatoda* (Acanthaceae) commonly known as 'Vasa' in India have long been used for the treatment of cold, cough, asthma, bronchitis and tuberculosis. *Adhatodavasica* Nees is a sub herbaceous widespread throughout the temperate regions of South Asia,⁴

used for more than 3000 years in Indian traditional medicine for the prevention, management and treatment of several illnesses and respiratory disorders viz., cold, severe cough, chronic bronchitis, asthma and tuberculosis⁵ etc. It is commonly known as vasaka or malabar nut tree. *Adhatoda beddomei* CB Clarke is one of the species of *Adhatoda* called as adalodakam in Tamil, rarely distributed in Western Ghats of Kerala and Tamil Nadu and used in Ayurveda, the ancient Indian System of Medicine (ISM).⁶ It has been the choice for the treatment and management of several diseases such as fever, cough, asthma, bronchitis, leprosy, blood disorders, heart troubles, inflammation, jaundice, tumors, and tuberculosis.^{7,8} Most of the pharmacognostic, phytochemical and pharmacological work of 'Vasa' carried out on *Adhatoda vasica* Nees.⁹⁻¹¹ It was reported as uterotonic, abortifacient,¹² oxytocic,¹³ anti-inflammatory,^{14,15} due to the presence of vasicine content. The ministry of Ayush recommended to consume Kabasura kudineer as an ayurvedic preparation and Adathodai manapagu, a siddha remedy which contain *A. vasica* as one of the main

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ingredients for the prevention and treatment of recently emerged respiratory infection, Covid-19. Earlier, these medications were used for the treatment of swine flu and dengue fever, respectively.^{16,17} Besides, *Adhatoda beddomei* also have been used for asthma by tribal in Kerala.

GC-MS fingerprint profiling of ethanolic extract of *A. beddomei* was reported and found to possess various volatile constituents⁸ and identification and quantification of alkaloids and flavonoids were also reported on these plants.^{18,19} Although, much phytochemical information about these plants were reported, there has been no evidence on pharmacognostical and comparative investigations with the plant *A. beddomei*, which can be a better alternative species for *A. vasica*. However, both the plants are closely related to each other and have similar morphological characteristics. The objectives of the present study were to evaluate the comparative pharmacognostical and phytochemical parameters of *Adhatoda vasica* and *Adhatoda beddomei* to elucidate the major differences between the two species of *Adhatoda*. Besides, an attempt has also been made to find out the presence or absence phytoconstituents and quantitative estimation of the pyroquinazoline alkaloid, vasicine in both the species.

MATERIALS AND METHODS

Collection and authentication of plant

The leaves of *Adhatoda vasica* and *Adhatoda beddomei* were collected from Chengalpattu and Gingee Hills, Villupuram (Dt), respectively in the month of October 2017. The plants were authenticated by Prof. P. Jayaraman (Botanist), Plant Anatomy Research Centre (PARC), Tambaram, Chennai, Tamil Nadu, India with a reference number of *A. vasica* and *A. beddomei* was PARC/2018/3653 and PARC/2018/3654 respectively. The plant specimens were preserved in the Herbarium at Interdisciplinary Institute of Indian System of Medicine (IIISM), SRM Institute of Science and Technology, Kattankulathur, Chengalpattu, Tamil Nadu, India. Collected leaves were cleaned with a running tap water, dried under shade and powdered using cutter mill, sieved through 60 mesh sieve and stored in an airtight container at room temperature.

Chemicals and reagents

Analytical grade hexane, chloroform, ethyl acetate and methanol used for extraction were purchased from Ranchem Private Limited. Formic acid (analytical grade), methanol (HPLC grade) were procured from Ranchem Private Limited. Chemicals of analytical grade and other reagents were obtained from Merck Specialities Private Limited, Mumbai.

Macroscopic analysis

Sensory and macroscopic analysis of both the leaves of *A. vasica* and *A. beddomei* were performed and the parameters evaluated for the fresh leaves were shape, size, colour, venation, apex, margin, base, texture, fracture, odour, taste, and petiole size.

Microscopic study

Collection of Specimens

The required samples of different organs were cut and removed from the plant and fixed in FAA (formaldehyde: alcohol: acetic acid, 10: 50: 5% with 35% water). After 24h of fixing, the specimens were dehydrated with graded series of tert-butyl alcohol as per the schedule given by Sass, 1940.²⁰ Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The embedded paraffin wax was sectioned with the help of Rotary

Microtome. The thickness of the section was 10-12µm. Dewaxing of the section was done by customary procedure.²¹ The sections were stained with Toluidine blue as per the method published by O'Brien et al 1964.²² Since Toluidine blue is a polychromatic stain. The staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. Wherever necessary sections were also stained with safranin and fast-green and iodine potassium iodide (for Starch).

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid²⁰ were prepared. Glycerin mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerin after staining. Different cell components were studied and measured.

Photomicrographs

Microscopic descriptions of tissues were supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Lab photo 2 microscopic Unit. For normal observations bright field was used. For the study of crystal, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property under polarized light, they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars.

Physicochemical analysis

Physicochemical analysis such as the percentage of total ash, acid-insoluble ash, water-soluble ash, water and alcohol soluble extractives and loss on drying were determined according to WHO guidelines.²³

Preparation of extracts

Each 5g of powdered *A. vasica* and *A. beddomei* were extracted in 50mL of hexane, chloroform, ethyl acetate and methanol for 24h at room temperature for non-successive extraction. The extracts were filtered and re-extracted thrice by following the same procedure. The filtrates were combined and concentrated under reduced pressure using a Buchi rotary evaporator.

Phytochemical screening

Phytochemical analysis was carried out for all the extracts according to standard methods²⁴⁻²⁶ (Table 1).

Determination of bioactive contents

Determination of total alkaloid content (TAC)

The extracts were prepared with 1mg/mL of stock solution using 2N hydrochloric acid. 1mL of the solution from the stock was kept in a separating funnel and 1mL of phosphate buffer of pH 4.7 was added for the complex formation for 10min. The complex mixture was partitioned with 3mL of chloroform and the chloroform layer was separated and diluted to 10mL with chloroform. The absorbance of the mixture was measured at 415nm using UV spectrophotometer. Results were expressed as mg of atropine equivalent in g of dry weight of the extract.^{27,28}

Determination of total phenolic content (TPC)

The extract of 100µL solution were mixed with 3.5mL of distilled water and 1mL of Folin-Ciocalteu reagent and kept aside for 8min. To this, 7.5% of 5mL sodium carbonate was added and kept at room temperature for 30min and the absorbance was measured at 765nm. Gallic acid was

Table 1: Preliminary phytochemical screening test procedure.

S. No	Phytoconstituents	Test	Observation
1	Alkaloids	Mayer's Test	Yellow precipitate
2	Glycosides	Liebermann Burchard test	Blue to green coloration
3	Flavonoids	Lead acetate test	Yellow precipitate
4	Terpenoids	Copper acetate test	Emerald green coloration
5	Steroids	Salkowski test	Reddish brown at the junction
6	Saponins	Froth test	Foam formation
7	Amino acids	Ninhydrin test	Blue coloration
8	Carbohydrates	Molisch's test	Violet ring formation at the junction

used as standard and the samples were analysed in triplicate. Total phenolic content was expressed as mg of gallic acid equivalent in g of dry weight of the extract.²⁹

Determination of total flavonoid content (TFC)

Total flavonoid content was carried out using Aluminium chloride colorimetric assay with slight modifications.³⁰ The standard calibration curve was plotted with catechin solution (20-100µg/mL). The extract of 100µL was mixed with 4ml of distilled water and 0.3mL of 5% sodium nitrite. After 5min, 0.3mL 10% aluminium chloride solution was added and incubated for 5min at room temperature. Finally, 2mL of 0.1M sodium hydroxide was added and made upto 10mL with distilled water. The formation of pink colour indicated that the formation of complex which further absorbed at 510nm using UV spectrophotometer against blank.

Determination of total terpenoid content (TTC)

Determination of terpenoid content was performed based on the method Ni *et al* 2012, with ursolic acid as a standard.³¹ Calibration curve was constructed using standard concentration of 20-20µg/mL. Initially, 1.5mL of diluted extract was evaporated to dryness at 100°C in a water bath. To the dried residue, 2.5ml of vanillin in acetic acid and 4 mL of glacial acetic acid were added and cooled the solution at room temperature for 15min. The final mixture was absorbed at 548nm against a blank solution using UV spectrophotometer. The terpenoid content was expressed in mg of ursolic acid equivalent in g of dry weight of the extract.

Determination of total glycoside content (TGC)

The estimation of glycoside content was carried out using a Baljet reagent according to the method described in Solich *et al* 1992.³² 1mL of extract was added to the solution of Baljet reagent containing picric acid and 0.1N sodium hydroxide with the ratio of 95:5. The solution was allowed to incubate in a dark chamber for 60min and the solution was further diluted to 15mL with distilled water and absorbed at 495nm. Digitoxin was used as a standard for the determination of glycoside and the results were expressed in mg of digitoxin equivalent to g of dry weight of the extract.

Determination of total steroidal content (TSC)

The determination of steroidal content was carried out according to Liebermann-Burchard colorimetric assay method³³ with minor modification using cholesterol as a standard. The Liebermann-Burchard reagent was prepared by adding 5ml of concentrated sulphuric acid to 50mL of acetic anhydride solution. The extracts were diluted with chloroform and to the chloroform extract, freshly prepared Liebermann-Burchard reagent was added and measured at 650nm against a reagent blank. Steroidal content was expressed in mg of cholesterol equivalent to g of dry weight of the extract.

Determination of total saponin content (TSAC)

The total saponin content was determined based on Xu and Chang

2009, using diosgenin as a standard solution.³⁴ 1mL of 80% aqueous methanol was added to 1mL of diluted extracts followed by 1mL of Vanillin reagent and 1mL of 72% sulphuric acid were added to the sides of the tubes. The mixture was heated on a water bath 60°C for 10min and the absorbance was recorded at 544nm against 80% methanol as blank. The saponin content was calculated using standard calibration curve of diosgenin with a concentration range of 20-200µg/mL solution and the results were expressed in mg of diosgenin equivalent to g of dry weight of the extract.

Sample preparation

The extracts of leaves of *A. vasica* and *A. beddomei* (each 100mg) were dissolved in 1mL of methanol and sonicated for 15min. The extracts were filtered and made upto 1mL with methanol and stored in a refrigerator until further use.

HPTLC Fingerprinting Analysis

High performance thin layer chromatography (HPTLC) method was performed on pre-coated HPTLC aluminium sheets silica gel 60F 254 plate (10.0 X10.0 cm) of 250µm thickness (Merck, Darmstadt, Germany). The plates were pre-washed with methanol and then activated at 100°C for 15min before use. The prepared sample solutions of 2µL were spotted as 6.0mm narrow bands using 100µL syringe at a constant dosing speed of 150nL/s with a Linomat V semi-automatic applicator (Camag, Muttens, Switzerland). The HPTLC plate was developed at a distance of 70mm in a 10.0 X10.0 cm twin trough chamber using a pre saturated mobile phase of hexane: ethyl acetate: formic acid (6:4:0.1 v/v/v). The developed TLC plate was dried using hot air for a minute and visualized under 254nm, 366nm and white light using TLC Visualizer. Further, the plate was scanned at the wavelength of 366nm using a TLC Scanner 180712 (Camag) with winCATS Planar Chromatography Manager software.

Quantification of Vasicine by RP-HPLC-PDA

RP-HPLC-PDA method was developed for the quantification of vasicine using Shimadzu LC-20AD HPLC system equipped with CT0-20A controller and the column oven. A Rheodyne 7725 injection valve with 20 µL loop volume, an SPD-M20A photo-diode array detector and a Lab solution 7.1 version software was used for data acquisition and interpretation. Chromatographic separation was achieved on Agilent C₁₈ column (150mm x 4.6mm; 3µm pore size). The solvent mixture of 0.1%v/v trifluoroacetic acid (A) and methanol: acetonitrile (45:40) (B) with a ratio of (70:30%v/v) at a flow rate 0.6mL/min, the column was maintained at room temperature and the PDA detector was fixed at 280nm.

RESULTS AND DISCUSSION

Macroscopy

Adhatoda vasica and *Adhatoda beddomei* are sub-herbaceous shrub of family Acanthaceae. *Adhatoda vasica*, an evergreen, gregarious perennial shrub, 1.2 to 6m in height, distributed throughout India,

up to an altitude of 1300m. Leaves were elliptical lanceolate or ovate lanceolate in shape, entire, crenate margin and symmetric base, 5-30cm long, hairy, light green in colour at the upper surface and dark green in colour at the lower surface with characteristic odour. *A. beddomei*, a glabrous shrub of 1 to 3m in height rarely found in higher altitudes of Western Ghats of Kerala and Tamil Nadu. The leaf was simple, entire, wavy, ovate lanceolate in shape, attenuate at base and acuminate apex, 6 to 14cm long and 3 to 4.5mm broad with prominent midrib at the lower surface. Odour was not characteristic and slightly bitter in taste.

Macroscopic characters were studied, compiled and presented in Table 2.

Microscopy

Transverse section of midrib- entire view

In transectional view, the midrib of *A. vasica* showed bowl-shaped and thick in abaxial part with short thick two adaxial humps and a shallow median concavity (Figure 1a). In *A. beddomei*, the leaf appeared dorsiventral with thick midrib and bilateral lamina. The midrib was plano-convex in sectional view with semicircular abaxial part and slightly raised adaxial part with slightly wide and shallow adaxial cavity (Figure 1b). The midrib of *A. vasica* and *A. beddomei* were measured 1.3mm and 1.15mm in vertical plain and 1.5mm 1.75mm in horizontal plain, respectively. The adaxial epidermis of the midrib in *A. beddomei* consisted of small epidermal cells with minute papillate outer tangential walls which was similar to abaxial epidermis consisted of 3 or 4 layers of small thick-walled cells. The remaining ground tissue include, large thin walled, angular or circular, compact parenchyma cells. The lamina of *A. vasica* occurred in vertical plain on either side of the midrib. The midrib consisted of thin epidermal layer on both adaxial and abaxial sides. Inner to the epidermis is a thick layer of sclerenchyma cells on the abaxial part. The ground parenchyma in the midrib contained circular thin walled compact parenchyma cells.

The vascular system *A. vasica* and *A. beddomei* showed a wide bowl-shaped main vascular strand and one circular adaxial lateral vascular bundle on either side of the vascular arc (Figure 2) and a median wide cup-shaped main vascular strand, respectively. The main vascular arc consisted of several radial compact xylem elements each row comprising 4 or 5 xylem elements. The xylem elements were circular and thick walled. Along the lower surface of the arc occurs thick continuous layer of phloem elements in *A. vasica* (Figure 2a). The lateral margins of the main vascular strand of *A. beddomei* had separate vascular strands, (Figure 2b) there are 2 small circular independent vascular bundles located in the lower part of the adaxial ridge.

The adaxial lateral smaller bundles of *A. vasica* were circular with thick cluster of xylem elements surrounded by small distinct phloem elements. The xylem elements were angular wide and thick walled. The vascular strand was surrounded by thin layer of fibers (Figure 3a and Figure 3b). The vascular strands of *A. beddomei* were collateral with lower compact xylem elements and thick layer of continuous phloem elements which extend all along the lower surface of the vascular strand. The vascular bundles of the adaxial region were circular with compact mass of thick-walled xylem elements and a layer of phloem elements (Figure 3c). The vascular strands have a thin layer of bundle sheath sclerenchyma.

Lamina

The lamina of *A. vasica* was dorsiventral with distinction into adaxial and abaxial sides. The lamina was smooth and even on both surfaces of *A. beddomei*. The lamina was 270µm thick. Adaxial epidermal cells were larger, cubical and thin walled. The abaxial epidermal cells were smaller and rectangular in shape in *A. vasica* whereas the adaxial epidermal cells and abaxial epidermal cells were circular or squarish and thin walled in *A. beddomei*. The adaxial epidermal cells contained a prominent cuticle in *A. beddomei*.

Table 2: Comparative macroscopic features of the leaves of *Adhatoda vasica* and *Adhatoda beddomei*.

Particulars	<i>Adhatoda vasica</i>	<i>Adhatoda beddomei</i>
Colour	Dark green colour	Green colour
Odour	Characteristic	Not characteristic
Taste	Bitter	Slightly bitter
Habit	Dense shrub	Glabrous shrub
Shape	Ovate lanceolate	Elliptic lanceolate
Size	Length: 10-16cm Width: 3-4cm	Length: 6-14cm Width: 3-4.5cm
Margin	Crenate to entire	Entire
Base	Symmetric	Attenuate

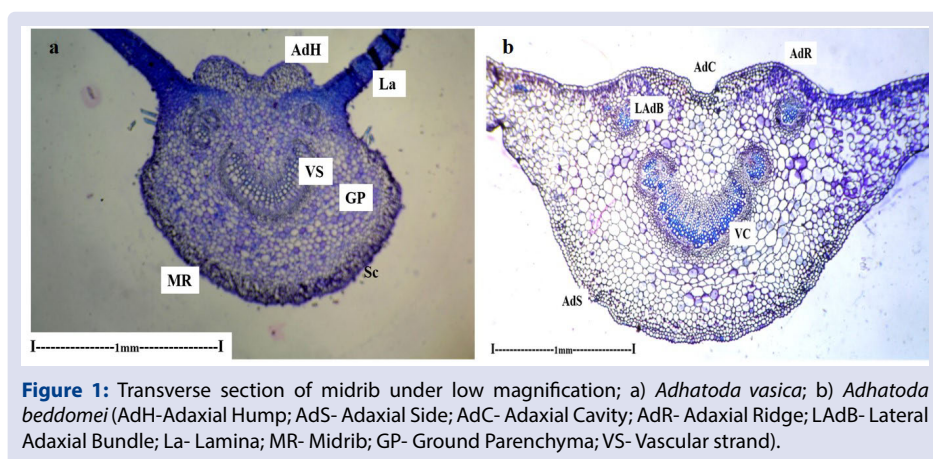


Figure 1: Transverse section of midrib under low magnification; a) *Adhatoda vasica*; b) *Adhatoda beddomei* (AdH-Adaxial Hump; AdS- Adaxial Side; AdC- Adaxial Cavity; AdR- Adaxial Ridge; LAdB- Lateral Adaxial Bundle; La- Lamina; MR- Midrib; GP- Ground Parenchyma; VS- Vascular strand).

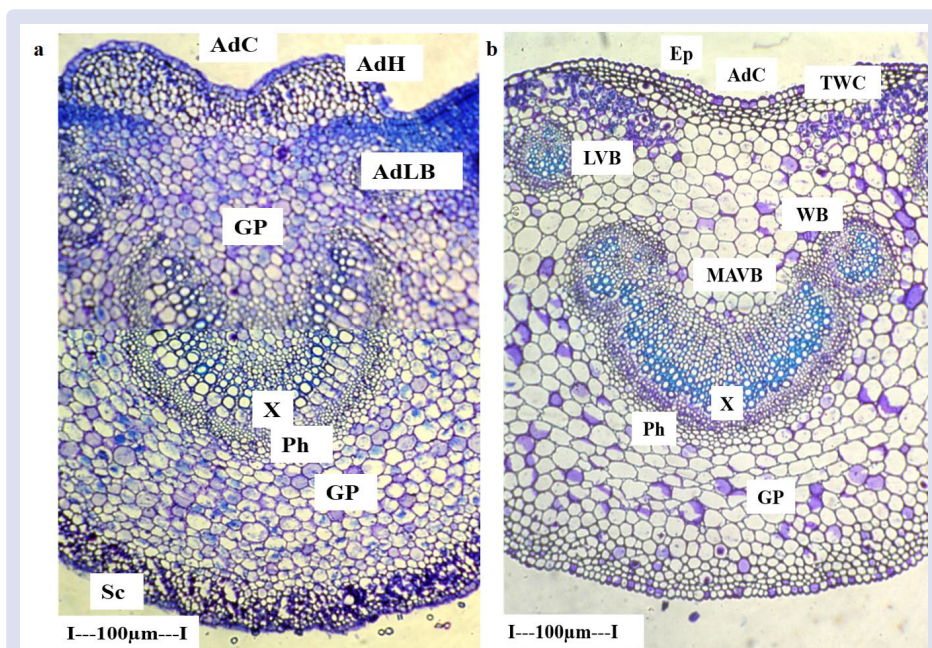


Figure 2: Transverse section midrib -Central portion enlarged; a) *Adhatoda vasica*; b) *Adhatoda beddomei* (AdH-Adaxial Hump; AdC- Adaxial Cavity; AdR- Adaxial Ridge; AdLB- Adaxial Lateral Bundle; MAVB- Median Adaxial Vascular Bundle; Ep- Epidermis; LVB- Lateral Vascular Bundle; TWC- Thick Walled Cells; WB- Wing Bundle; GP- Ground Parenchyma; Ph-Phloem; Sc-Sclerenchyma; X-Xylem).

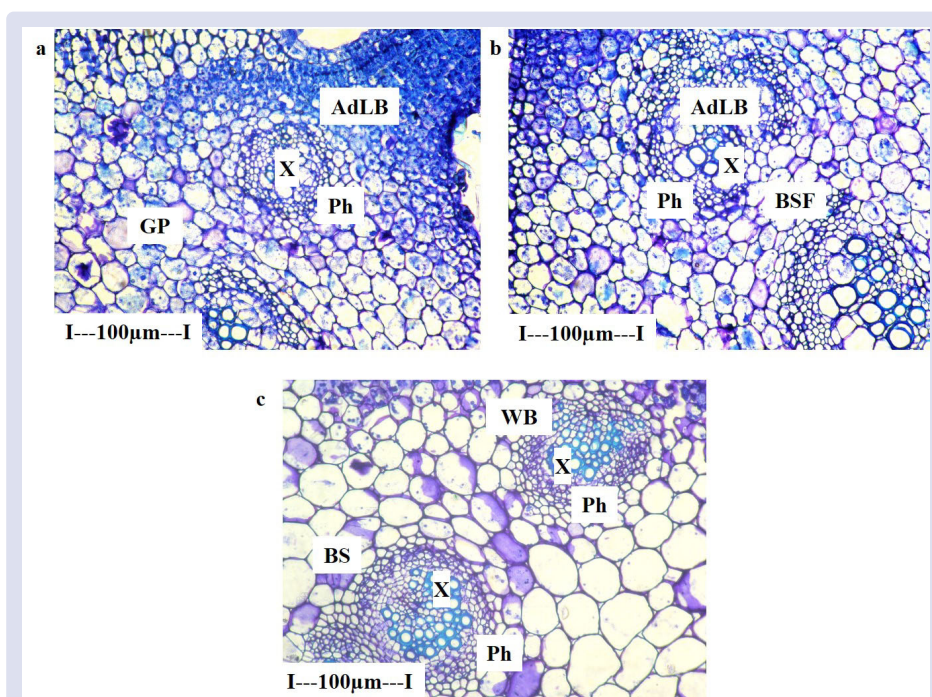


Figure 3: Transverse section of midrib showing adaxial ridge bundle. a) Adaxial lateral single bundle of *A. vasica*; b) Adaxial lateral double bundle of *A. vasica*; c) adaxial ridge bundle and lateral segment of median arc shaped strand of *A. beddomei* (AdLB- Adaxial lateral bundle; BSF- Bundle Sheath fibres; BS- Bundle Sheath; GP- Ground Parenchyma; Ph- Phloem; WB- Wing Bundle; X-Xylem).

The mesophyll tissue of *A. vasica* was differentiated into adaxial band of thick rectangular palisade mesophyll cell and abaxial zone of 4 or 5 layers of large spherical spongy mesophyll cells. The mesophyll tissue consisted of an abaxial band of 2 short, cylindrical palisade mesophylls were seen in *A. beddomei*. Small air chambers were present in the spongy mesophyll zone of *A. vasica* (Figure 4a) and the spongy mesophyll was circular, intercellular air space with stomata occurred on the abaxial epidermal layer of *A. beddomei* (Figure 4b).

Epidermal trichomes

In *A. vasica*, two types of epidermal trichomes were arised from epidermal cells. Some are non-glandular type which are multicellular, uniseriate and unbranched. The cells of the trichome were vertically elongated and thin walled. These trichomes were 150µm long and 15µm thick (Figure 5a) and glandular trichomes have short stalk cell with which it was attached on the epidermis (Figure 5b). The upper secretory body was two celled with dense accumulation of cell inclusions. The glandular trichomes was 10 µm broad and 15µm in height.

In *A. beddomei*, short prominent glandular trichomes were seen on the abaxial epidermal layer. The trichome was buried in the shallow epidermal cavity which were multicellular and funnel shaped. The cells were darkly stained and the gland was at the level of epidermal layer (Figure 5c).

Crystal distribution

In the mesophyll tissues of *A. beddomei*, calcium oxalate crystals were seen which was absence in *A. vasica*. The crystals may be raphide type consisting of several thin needles (Figure 6a) or it could be rectangular long crystals were aggregated in the form of circular cluster (Figure 6b).

Powder microscopy

Trichomes

Adhatoda vasica were observed with more non glandular covering type of trichomes on the leaf, stem and petiole surfaces and less frequent glandular trichomes. The non-glandular trichomes were long,

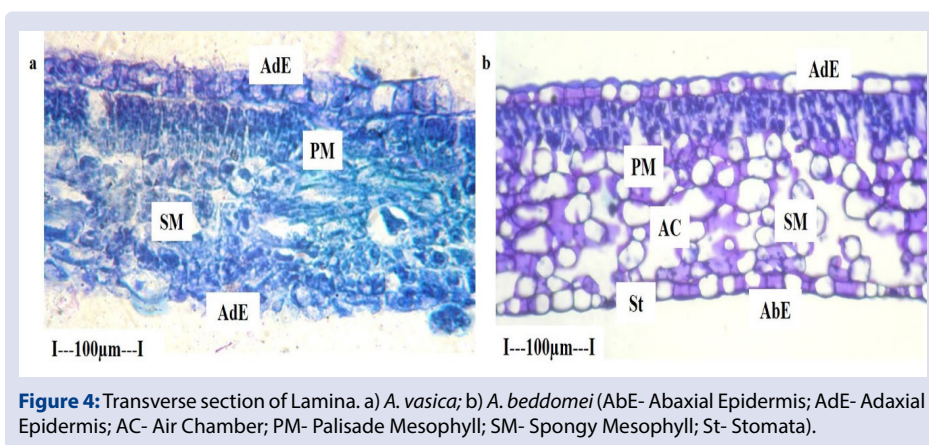


Figure 4: Transverse section of Lamina. a) *A. vasica*; b) *A. beddomei* (AbE- Abaxial Epidermis; AdE- Adaxial Epidermis; AC- Air Chamber; PM- Palisade Mesophyll; SM- Spongy Mesophyll; St- Stomata).

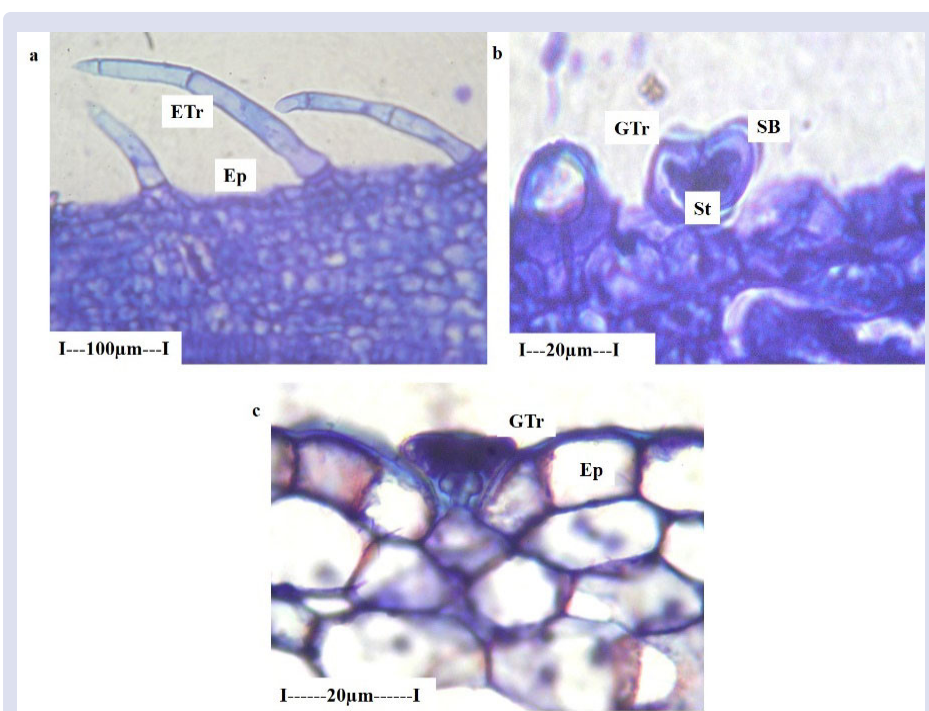


Figure 5: Transverse section of lamina with glandular and non-glandular trichomes; a) Non-glandular trichome of *A. vasica*; b) Glandular trichome of *A. vasica* and c) Glandular trichome buried in the epidermal cavity of *A. beddomei* (Ep- Epidermis; GTr- Glandular Trichomes; ETr- Epidermal Trichomes; SB- Secretory Body; St- Stalk).

multicellular, unbranched with thick walls and tapering ends (Figure 7a). The cells of the trichomes are vertically elongated with wide lumen. The trichomes were 130µm long and 10µm thick (Figures 7a and 7c). The glandular trichomes were large, spherical, multicellular (usually 4 celled) secretory head with dense cytoplasm. The gland was subsessile with very short, non-glandular stalk. The glandular head may be 80µm in diameter and capitate type (Figure 7d).

In *A. beddomei*, short prominent glandular trichomes were seen on the abaxial epidermal layer with multicellular and funnel shaped. The cells were darkly stained and the gland was at the level of epidermal layer (Figure 8).

Stomata

The adaxial epidermis of both *A. vasica* and *A. beddomei* were apostomatic (without stomata). The abaxial epidermis were densely stomatiferous in nature. The stomata of both the species were diacytic type and the guard cells were enclosed with two unequal subsidiary

cells with their common walls situated at right angles to long axis of the stoma (Figures 7b and 8c). The guard cells were levelly elliptical in shape and measured 10x25µm and 35x60µm size in *A. vasica* and *A. beddomei*, respectively. The abaxial epidermal cells were lobed with their anticlinal walls with thin or thick and slightly wavy. Stomata were densely distributed and they are random in orientation.

Physicochemical analysis

Physicochemical analysis was studied and represented with standard deviation in Table 3. Different physicochemical parameters studied were within permissible limits according to the Pharmacopoeial standards. The total, water soluble and acid insoluble ash of *A. vasica* and *A. beddomei* was found to be 12.85, 3.17, 0.95%w/w and 13.20, 2.68, 0.62%w/w, respectively. The water soluble and alcohol soluble extractive were found to be 28.53 and 23.99%w/w for *A. vasica* and 22.17 and 6.12%w/w for *A. beddomei*. 5.77 and 7.80% of loss on drying was found in *A. vasica* and *A. beddomei*, correspondingly.

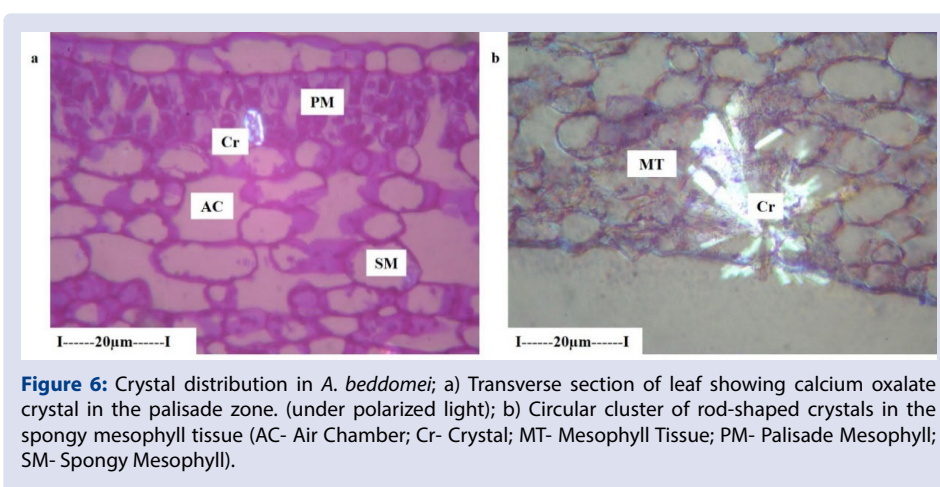


Figure 6: Crystal distribution in *A. beddomei*; a) Transverse section of leaf showing calcium oxalate crystal in the palisade zone. (under polarized light); b) Circular cluster of rod-shaped crystals in the spongy mesophyll tissue (AC- Air Chamber; Cr- Crystal; MT- Mesophyll Tissue; PM- Palisade Mesophyll; SM- Spongy Mesophyll).

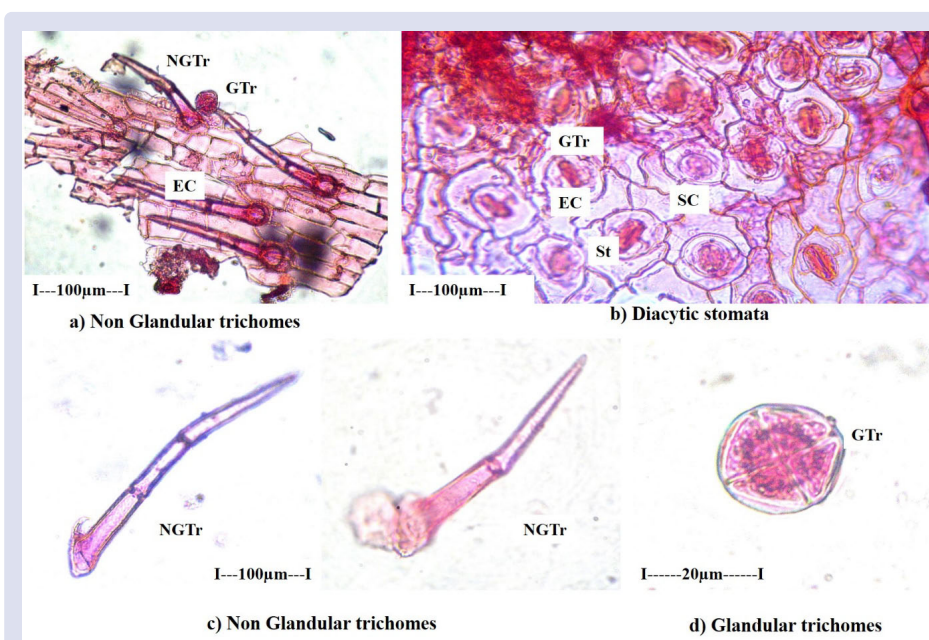


Figure 7. a) Non glandular epidermal trichomes on the surface of the stem; b) Abaxial epidermis showing diacytic type of stomata; c) Non glandular Trichomes; d) Glandular Trichomes- the Secretory head (EC- Epidermal Cells; Gtr- Glandular Trichomes; NGTr- Non-glandular Trichomes; SC- Subsidiary Cells; St- Stoma).

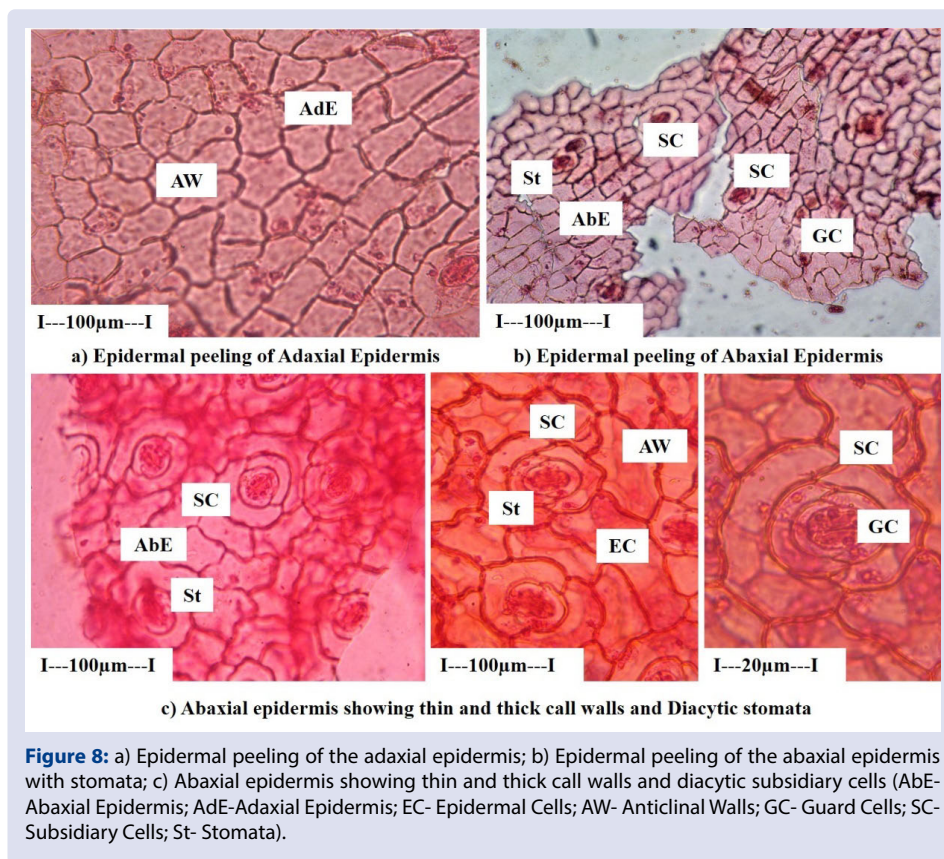


Table 3: Proximate analysis of *Adhatoda vasica* and *Adhatoda beddomei*.

Parameters	<i>A. vasica</i>	<i>A. beddomei</i>
Foreign Organic Matter (%)	0.53	0.20
Total Ash (%w/w)	12.85 ± 0.52	13.20 ± 0.31
Acid-insoluble ash (%w/w)	0.95 ± 0.06	0.62 ± 0.03
Water soluble ash (%w/w)	3.17 ± 0.49	2.68 ± 0.25
Water soluble extractive value (%w/w)	28.53 ± 0.42	22.17 ± 0.36
Alcohol soluble extractive value (%w/w)	23.99 ± 0.29	6.12 ± 0.21
Loss on drying at 105°C	5.77 ± 0.37	7.80 ± 0.40

Values were in mean ± standard deviation, n=3

Phytochemical screening

The phytochemical examination revealed the presence of various phytoconstituents such as alkaloids, flavonoids, steroids, terpenoids and glycosides in *A. vasica* and *A. beddomei* (Table 4).

Determination of bioactive contents

The bioactive content of total alkaloid, phenolic, flavonoid, terpenoid, glycoside, sterol, and saponin were determined in the extracts of *A. vasica* and *A. beddomei* and the results were depicted in Table 5. The content of alkaloid, phenol, flavonoid, glycoside, sterol and saponins were present in high concentration in *A. vasica* extracts whereas the terpenoid content were approximately similar in both the plant species.

The alkaloid content of the extracts was expressed as atropine equivalent and it was varied from 3.48mg/g to 238.05mg/g in *A. vasica* extracts. *A. vasica* extracts was found to contain a value of total phenolic (6.15 to 19.25mg/g), flavonoid (13.12 to 48.33mg/g), terpenoid (4.85 to 20.27mg/g), glycoside (7.74 to 195.19mg/g), saponin (0.26 to 2.97mg/g). The steroidal content was found to be high concentration in hexane extract as 51.98mg/g whereas 42.10mg/g in methanolic extract of *A. vasica*. In case of *A. beddomei*, the alkaloid content was found to

be 171.84mg/g and 195.72mg/g in ethyl acetate and methanolic extract which was lesser than *A. vasica* extracts and in hexane and chloroform extract not in detectable limit. The other contents such as phenolic, flavonoid, terpenoid, glycoside and saponin contents for *A. beddomei* extracts were found to be in the range from 4.73 to 10.13mg/g, 6.98 to 26.62mg/g, 10.83 to 19.48mg/g, 15.34 to 160.13mg/g and 0.16 to 0.87mg/g respectively. The steroidal content of *A. beddomei* was found to be high concentration in hexane extract as 27.69mg/g whereas 22.09mg/g in methanolic extract. High concentration of bioactive compounds was found in *A. vasica* when compared to *A. beddomei* extracts.

HPTLC fingerprinting analysis

HPTLC fingerprint profiling of *Adhatoda* species were developed with different mobile phases in order to obtain high resolution and peak reproducibility. The phytochemicals were eluted with a mobile phase consisting of hexane, ethyl acetate and formic acid with a ratio of 6:4:0.1v/v/v. Visual examination of obtained chromatogram (Figure 9) revealed a difference in the chemical composition of various extract of *Adhatoda*. The fingerprinting analysis revealed the presence of 3, 9, 7 and 5 spots in hexane, chloroform, ethyl acetate and methanolic extracts respectively in *A. vasica* extract. In case of *A. beddomei*, 1, 5, 7

and 4 spots were obtained. The extracts of *A. vasica* consisted of more phytochemicals when compared to *A. beddomei* extracts (Figure 10).

Quantification of Vasicine by RP-HPLC-PDA

Vasicine, a principal bioactive marker has extensively used as a potential bronchodilator. HPLC-PDA method was developed for the estimation of vasicine using a mobile phase of 0.1%v/v trifluoroacetic acid (A) and methanol: acetonitrile (45:40)(B) with a ratio of (70:30%v/v). Linearity graph (Figure 11) was plotted from the concentration range from 20-120µg/mL with $y=29487x-88941$ and regression coefficient of 0.998. The retention time of vasicine was found to be 3.946min. The HPLC chromatogram of vasicine standard and different extracts of *Adhatoda* species were shown in Figure 12.

The hexane, chloroform, ethyl acetate and methanolic extract of *A. vasica* and *A. beddomei* revealed that the content of vasicine as 0.143, 0.907, 1.672, 2.051mg/g and 0.042, 0.077, 0.165 and 0.475mg/g, respectively (Table 6).The high amount of vasicine (2.051mg/g) was

found in methanolic extract of *A. vasica* when compared to other extracts, whereas in methanolic extract of *A. beddomei*, was found to be 0.475mg/g which was lesser than *A. vasica* extracts.

The comparative pharmacognostical studies revealed that, the morphological and microscopical differences in the *Adhatoda* species viz, *A. vasica* and *A. beddomei*. There were no crystals seen in *A. vasica*, where as rod shaped crystals were observed in the leaves of *A. beddomei*. Powder microscopic study showed the difference in epidermal trichomes. The presence of non-glandular trichomes were seen in the leaves of *A. vasica*, which was absent in *A. beddomei*. Diacytic stomata were seen in both the species of *Adhatoda*. The total, water soluble and acid insoluble ash of *A. vasica* was found to be 12.85, 3.17, 0.95%w/w, respectively. The water soluble and alcohol soluble extractive were found to be 28.53 and 23.99%w/w respectively for *A. vasica* and loss on drying was found to be 5.77%. *A. vasica* was found to contain more water soluble, acid insoluble ash and extractive values and *A. beddomei* was found to contain more total ash and loss on drying.

Table 4: Preliminary phytochemical screening of *A. vasica* and *A. beddomei*.

Phytochemical Tests	<i>Adhatoda vasica</i>				<i>Adhatoda beddomei</i>			
	Hexane	Chloroform	Ethyl acetate	Methanol	Hexane	Chloroform	Ethyl acetate	Methanol
Alkaloids	-	-	+	+++	-	-	+	++
Glycosides	-	-	-	+	-	-	-	+
Saponins	-	-	+	++	-	-	+	+
Phytosterols	+++	+	+	+	+++	+	+	+
Flavonoids	+	+	++	++	+	+	++	++
Terpenoids	++	+	+	++	++	+	+	+
Phenols	+	+	++	++	+	+	++	++

(+)- Low; (++)- Medium; (+++)- Strong; (-)- Absence

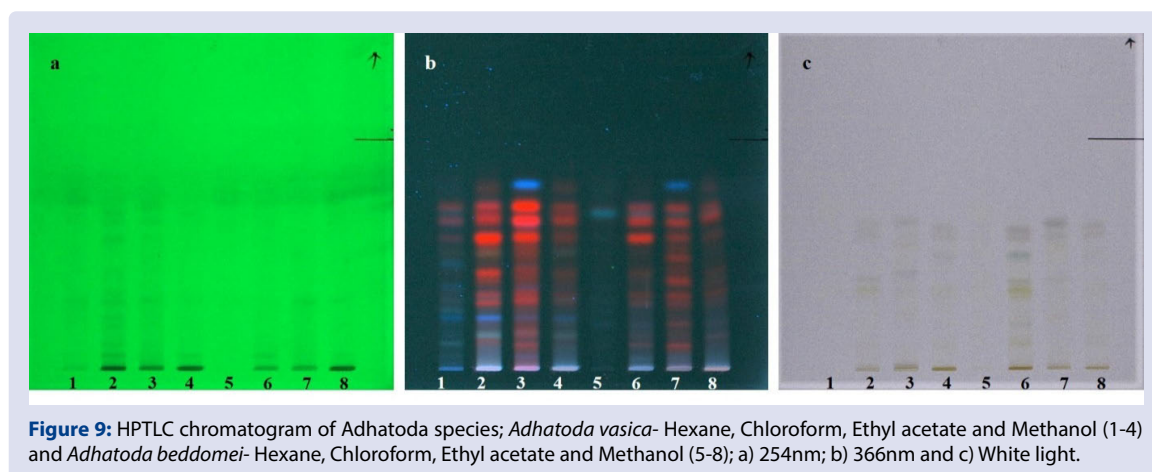
Table 5: Estimation of bioactive content (mg/g) in *A. vasica* and *A. beddomei* by UV Spectrophotometer.

Quantitative Parameters	Alkaloids	Phenolics	Flavonoids	Terpenoids	Glycosides	Steroids	Saponins
AV-Hexane	3.48 ± 0.002	6.15 ± 0.0002	13.12 ± 0.0003	4.85 ± 0.005	7.74 ± 0.004	51.98 ± 0.0004	0.26 ± 0.001
AV-Chloroform	42.92 ± 0.001	7.96 ± 0.0002	15.42 ± 0.0004	5.21 ± 0.01	18.68 ± 0.01	49.51 ± 0.0005	0.60 ± 0.001
AV-Ethyl acetate	176.4 ± 0.005	18.56 ± .0003	39.27 ± 0.0002	12.19 ± 0.01	112.27 ± 0.001	46.00 ± 0.0004	2.08 ± 0.004
AV-Methanol	238.05 ± 0.003	19.25 ± 0.0003	48.33 ± 0.0003	20.27 ± 0.003	195.19 ± 0.004	42.10 ± 0.0003	2.97 ± 0.004
AB-Hexane	ND	4.73 ± 0.0004	6.98 ± 0.0004	10.83 ± 0.01	15.34 ± 0.02	27.96 ± 0.0004	ND
AB-Chloroform	ND	6.18 ± 0.0004	11.87 ± 0.0006	15.54 ± 0.01	16.66 ± 0.0004	25.36 ± 0.0003	0.16 ± 0.006
AB-Ethyl acetate	171.84 ± 0.001	8.64 ± 0.0002	15.78 ± 0.0005	18.04 ± 0.005	119.78 ± 0.007	24.14 ± 0.0007	0.48 ± 0.01
AB-Methanol	195.72 ± 0.002	10.13 ± 0.0004	26.62 ± 0.0001	19.48 ± 0.005	160.13 ± 0.01	22.09 ± 0.0002	0.87 ± 0.006

Values were in mean ± standard deviation, n=3, ND- Not Detected

Alkaloids equivalent to Atropine, phenolics equivalent to Gallic acid, flavonoids equivalent to Catechin, terpenoids equivalent to Ursolic acid, glycosides equivalent to Digitoxin, steroids equivalent to Cholesterol and saponins equivalent to Diosgenin.

AV- *Adhatoda vasica*; AB- *Adhatoda beddomei*



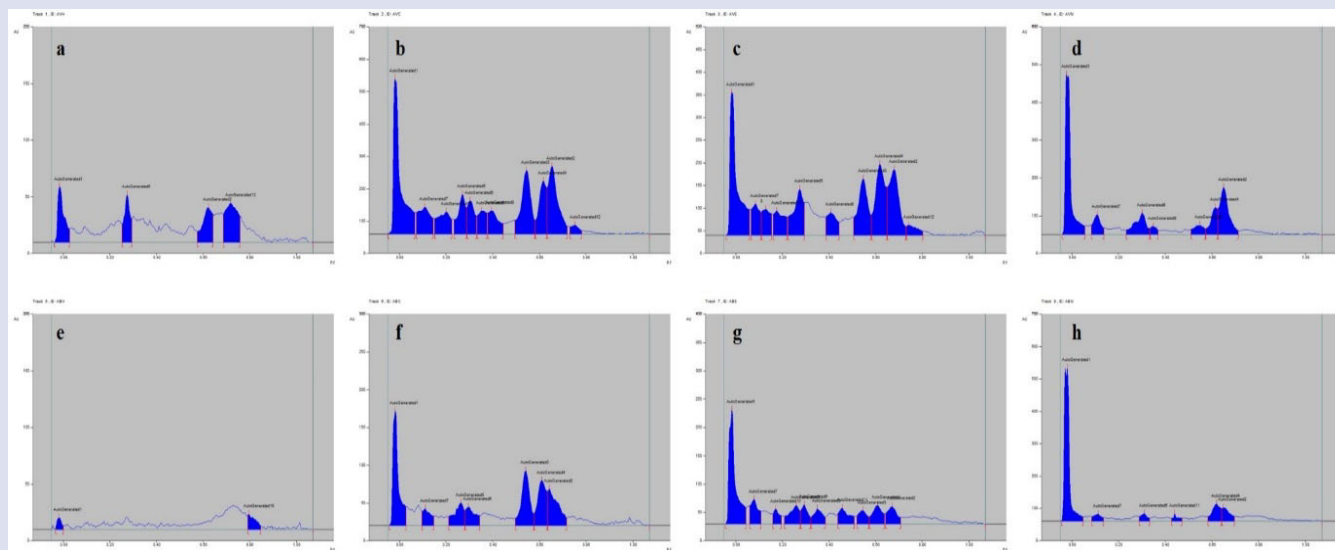


Figure 10: HPTLC densitometric chromatogram of *Adhatoda* species at 366nm; *Adhatoda vasica*- Hexane, Chloroform, Ethyl acetate and Methanol (a-d) and *Adhatoda beddomei*- Hexane, Chloroform, Ethyl acetate and Methanol (e-h).

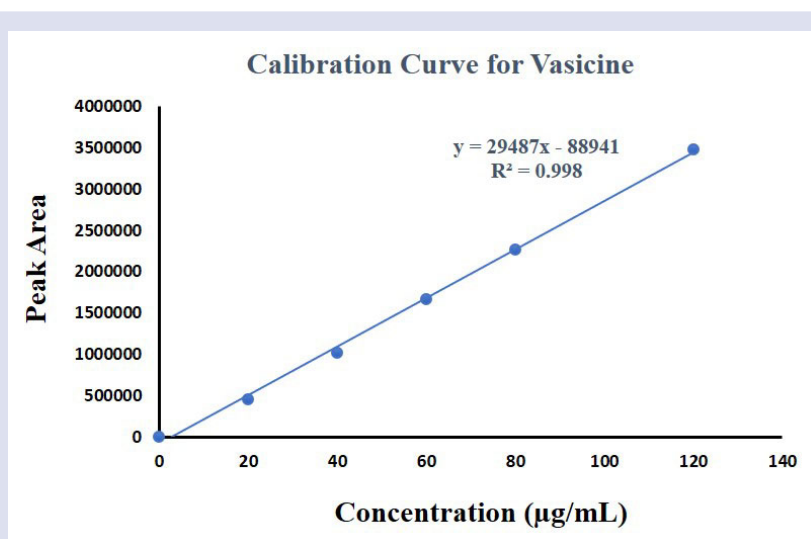


Figure 11: Calibration curve of vasicine standard in the concentration range 20-120µg/mL.

Table 6: Quantification of Vasicine in *Adhatoda* species.

Marker compound	<i>Adhatoda vasica</i>				<i>Adhatoda beddomei</i>			
	Hexane	Chloroform	Ethyl acetate	Methanol	Hexane	Chloroform	Ethyl acetate	Methanol
Vasicine (mg/g)	0.143	0.907	1.672	2.051	0.042	0.077	0.165	0.475

The preliminary phytochemical screening revealed the presence of more number of phytoconstituents in methanolic extracts of leaves of *A. vasica*, which was further confirmed by estimation of crude bioactive content. The total alkaloid, phenolic, flavonoid, terpenoid, glycoside and saponin contents were found to be more concentration in polar when compared to non-polar solvents with the order methanol> ethyl acetate> chloroform> hexane, whereas high concentration of steroids was found in non-polar solvents. HPTLC fingerprint profiling revealed that, the chloroform extracts of *A. vasica* showed a larger number of phytoconstituents when compared to other extracts and also in *A. beddomei*. HPLC analysis revealed the presence of high content of vasicine in methanolic extracts of *A. vasica* (2.051mg/g) whereas *A.*

beddomei contains only 0.475mg/g, which was found to be 5 times lesser than the methanolic extracts of *A. vasica*.

CONCLUSION

Standardization of herbal medicine is a key importance in establishing its proper identity, purity, quality and therapeutic efficacy. The macroscopic, microscopic, physicochemical and phytochemical analysis are the confirmatory test for standardization and quality control. Microscopic, pharmacognostical, preliminary phytochemical screening provides relevant information which may be helpful in authentication of crude drug and checking the adulteration of raw material. Microscopic studies differentiate the difference in the anatomical features and

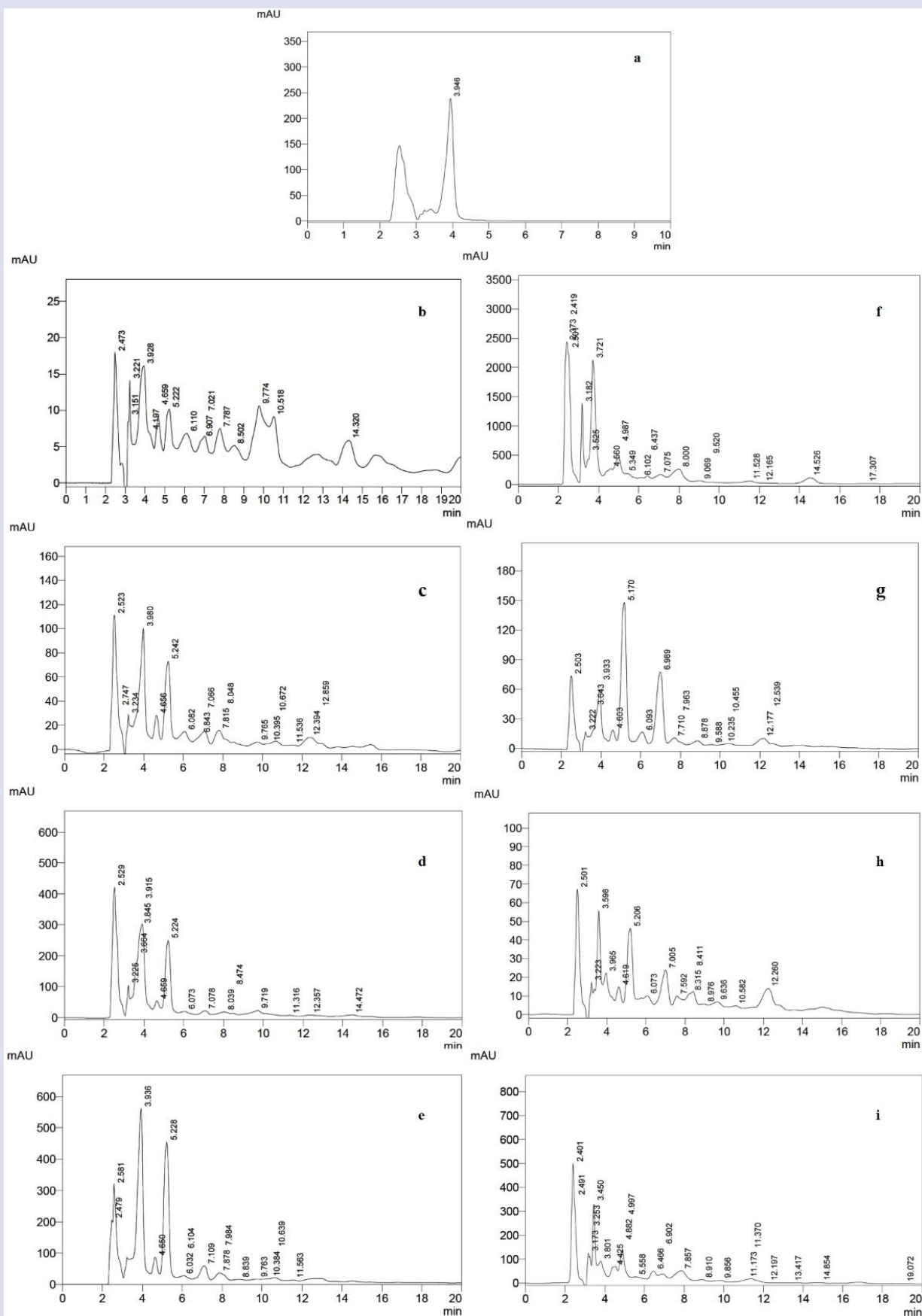


Figure 12: HPLC chromatogram of *Adhatoda* species; Vasicine standard (a); *Adhatoda vasica*- Hexane, Chloroform, Ethyl acetate and Methanol (b-e) and *Adhatoda beddomei*- Hexane, Chloroform, Ethyl acetate and Methanol (f-i).

physicochemical parameters showed a major difference between the two plants. Phytochemical screening revealed the presence of various constituents and estimation of bio active compounds confirmed the high concentration of all the bioactive contents in *A. vasica* when compared to *A. beddomei*. HPTLC serves as an important and powerful tool for standardization which confirms the species of the herbal drugs and HPLC analysis provides a useful information on vasicine content of *Adhatoda* species. Vasicine estimation also revealed that high concentration in *A. vasica* extracts when compared to *A. beddomei* extracts which enables the differentiation between two species. Both HPTLC fingerprint analysis and HPLC quantification report further confirmed the availability of more amounts of phytoconstituents in *A. vasica*. The pharmacognostical study of *A. beddomei* and the comparative study of both *A. vasica* and *A. beddomei* were reported for the first time which helps in differentiating the plant from other species and useful for the identification and standardization. Although, the plant *A. beddomei* could be used in Kerala for medicinal purpose, the high content of phytoconstituents was observed in *A. vasica*. In conclusion, the present study can be used as a reference information for proper identification, authentication, collection and investigations of *Adhatoda* species to enhance the therapeutic efficacy in the treatment.

CONFLICTS OF INTEREST

None.

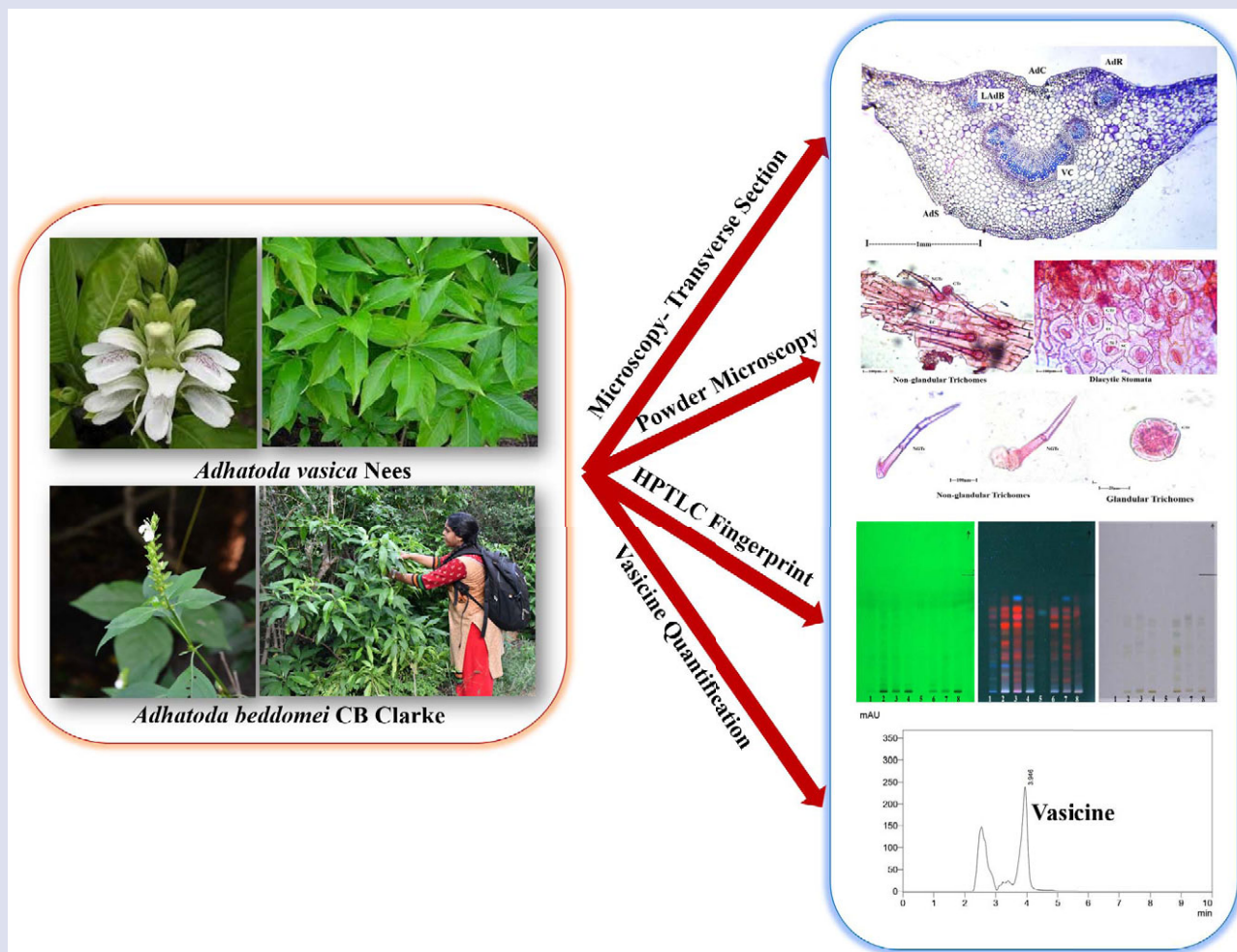
ABBREVIATIONS

A. vasica and AV- *Adhatoda vasica*; *A. beddomei* and AB- *Adhatoda beddomei*; FAA- Formaldehyde, Alcohol, Acetic acid; HPTLC- High performance thin layer chromatography; ISM- Indian System of Medicine;PARC- Plant anatomy research centre; RP-HPLC-PDA-Reverse phase high performance liquid chromatography-photodiode array; TBA- tert-butyl alcohol; TAC- Total alkaloidal content; TPC- Total phenolic content; TFC- Total flavonoid content; TTC- Total terpenoid content; TGC- Total glycoside content; TSC- Total steroidal content; TSAC- Total saponin content.

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GRAPHICAL ABSTRACT



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