CHAPTER - 2

Pharmacognosy of *Ixora johnsonii*

2.1. Introduction

Pharmacognosy which literally means a discourse on drugs is an essential component of herbal science. The relevance of pharmacognosy in standardization of herbal drugs has long been stressed. Many monographs on pharmacognosy have emerged as an aid in the pharmacognostic investigation (Claus, 1956; Wallis, 1985). The Wealth of India compiled by CSIR, New Delhi, is a monumental treatise compiling exhaustive data on thousands of Indian herbal drugs (Anon., 1997; 2003). Special mention may be made of the monographic glossaries on phytochemical and pharmacological compilation of Indian medicinal plants by several authors (Chopra *et al.*, 1956; Kirtikar and Basu, 1975; Nadkarini, 1976; Asolkar *et al.*, 1992). Unfortunately, pharmacognosy is not given due accent in the present day scenario, perhaps due to ignorance of the themes of the study or due to lack of expertise in the techniques involved in the study.

Pharmacognostic standardization is based upon the tenet that certain microscopic characters are specific and restricted in distribution (Metcalf and Chalk, 1979). Anatomical studies of phytodrugs form a major aspect of pharmacognosy. Anatomical data has proved to be very useful in discerning evolutionary trends and interrelationships of taxa at and above the species level and at higher taxonomic categories (Mondal, 2005). Microscopic parameters,
though limited in their application under certain circumstances, have still highly reliable diagnostic values and play appreciable role in the herbal drugs. It has the advantage of requiring only small quantities of material, simple, economical and can provide a rapid tentative identification, prior to confirmation by analytical techniques. Microscopical methods of diagnosis of drugs are indispensable while handling the fragmentary materials or powdered drugs. It has been established by plant anatomists beyond any ambiguity that certain anatomical traits are specific and fixed at species level, genus level or even at family level. Such anatomical character states are of considerable application value in botanical identification of crude drugs of doubtful origin.

After selecting a plant for its pharmacological studies, next step is to correctly identify the plant. Identification of the phytodrugs can be done with the help of floral and foliar characters, habits and habitat data. If the plant specimens are fragmentary and incomplete, such as bark, leaf, root, wood or stem, the only method of identification is through the anatomical studies. When the anatomical features are specific at the species level, they can be well employed for taxonomic identity of the species during pharmacognostic studies. The midrib structure, petiolar anatomy, periderm structure, vascular tissues, cell inclusions and foliar epidermis (stomata and trichomes) are potential sources of diagnostic values. These facts prompted to undertake the microscopic analysis of *I. johnsonii*. 
Problems in establishing identity and genuineness of fragmentary herbal drugs, which cannot be easily resolved through chemical procedures, can be cleared out through quick and simple micro techniques, like sectioning, clearing, maceration and histochemistry (Jayaraman, 2010). Histochemistry is a biological technique developed parallel to staining of cells and tissues. The technique involves localization of chemical compounds within the cells under the microscope. To localize the compounds, specific dyes are employed which will selectively stain the compounds depending upon the reaction affinities between the compound and the dye. The relevance of histochemical studies in biological sciences is gaining momentum because of the usefulness of the technique not only in retrieving information on the biochemical pool of the cells, but also for diagnostic purpose of the organism studied. Histochemical stains which were already established for their staining reaction of specific substances were employed to detect the compounds in the cell types and tissues, in particular, the presence of starch, alkaloids, tannin, protein, phenolics and flavonoids in the plant parts such as leaf, petiole, stem, root etc.

SEM studies on comparative morphology of the leaf epidermis have so far received very little attention in Rubiaceae. Importance of epidermal and anatomical characters in systematic evaluation has been emphasized by Fritsch (1903), Stace (1965), Heywood (1971), Soladoye (1982) and others. The various surface structures such as epicuticular, cuticular sculpturing on leaf surface as well as on the surface of trichomes, elevation of stomata are taken into consideration for providing base line information for classifying the Indian *Ixora* to establish species relationships. A literature survey and screening of scientific
data revealed that few species of *Ixora* have already been investigated as regards their botany and chemistry is concerned, however a systematic standardization including pharmacognostical and physicochemical study is still lacking in many species of *Ixora*.

Review of literature on *I. johnsonii* seems to be fragmentary; especially pharmacognostic studies are lacking. The present investigation of *I. johnsonii* Hook. f is therefore taken up to establish certain botanical and chemical standards which would help in crude drug identification as well as in checking adulteration, if any. In the present study the time renowned microtechnical procedures were employed and data pertaining to morphological and anatomical characteristics of the selected taxon was retrieved. Since the entire plant is taken for various analyses, all parts of the plant, from root to the stem including leaf petiole, have to be studied for providing a protocol of distinguishing features of the plant. Importance was given to customary parameters of pharmacognosy such as powder drug analysis and powder microscopy. Since the plant part is used in the tribal medicine to treat / cure healing of wound, it is desirable to decipher the bioactive compounds present in the plant parts by analytical methods such as fluorescence analysis, ash analysis and extractive values. Different extracts were phytochemically screened to detect the secondary metabolites. These studies provided referential information for identification of this crude drug. Further, the study will greatly help in avoiding taxonomic confusion of *I. johnsonii* with *I. polyantha*. 
2.2. Materials and Methods

2.2.1. Collection of specimens

The plant specimens (*I. johnsonii*) for the proposed study were collected from Pathanamthitta and Kottayam districts of Kerala. Care was taken to select healthy plants and normal organs.

2.2.2. Methods

2.2.2.1. Exomorphology

For recording morphological observations, ten matured plants with uniform size and spread were selected randomly from one accession in two districts of Pathanamthitta (Vazhoor East) and Kottayam (Maniyar check-dam) separately. Observations on vegetative and few floral characters and their variations in two accessions were recorded. The macroscopic characters of the plant such as colour, odour, nature, texture were also studied for morphological investigation.

The systematic position of *I. johnsonii* is based on Bentham and Hookers Classification (Gamble, 1957) and Wikipedia.

**Scientific classification**

- **Kingdom**: Plantae
- **Cladus**: Angiospermae
- **Class**: Dicotyledonae
- **Order**: Gentianales
- **Family**: Rubiaceae
- **Sub- family**: Ixoroideae
- **Tribe**: Ixoreae
- **Genus**: *Ixora*
- **Species**: *johnsonii*
2.2.2.2. Anatomy

For comparing the anatomical variations, various parts of the selected plant such as the leaf, petiole, stem and root from two accessions, one from each district were used for anatomical studies. Different steps involved in microscopic studies followed are:

a. Fixing

The required samples of different parts such as leaf, stem and root were cut and removed from the plant and individually fixed in FAA (Formalin-5ml + Acetic acid-5ml +70% Ethyl alcohol-90ml). After 24 h of fixing, the specimens were dehydrated with graded series of tertiary-Butyl alcohol (TBA) as per the schedule proposed by Sass (1940). Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. Then the individual specimens were cast into paraffin blocks.

b. Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the section was 10-12 µm. Dewaxing of the sections was following by the customary procedure (Johansen, 1940). The sections were stained with Toluidine blue as per the method described by O’Brien et al. (1964), and wherever necessary sections were also stained with safranin and Fast – green and Iodine Potassium iodide (IKI 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 fluid were prepared (Sass, 1940). Glycerin mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerin medium after staining. Different cell components were studied and measured.

c. Photomicrographs

Microscopic descriptions of tissues were supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic Unit and Nikon digital camera. For normal observations, bright field was used. For the study of crystals, 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 were indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard anatomy book (Esau, 1964).

2.2.2.3. Histochemical localization

Histochemical studies were carried out to localize proteins, starch, phenolics, alkaloids, tannins and flavonoids using standard procedures (Krishnamoorthy, 1988). The leaf, petiole, stem and root sections embedded in paraffin were used for histochemical studies. The sections were deparaffinised
and hydrated before staining them for the analysis. The following aspects of secondary metabolites were localized in the leaf, petiole, stem and root of *I. johnsonii* and the observations were recorded as well as photomicrographs were also taken.

**i. Alkaloids**

The sections were stained in Dragendorff’s reagent for 5 – 10 minutes. Orange red precipitate indicated the presence of alkaloids (Johansen, 1940).

**ii. Tannins (Ferric chloride method)**

The sections were placed in 10% formalin solution containing 2% ferric chloride. Tannins were stained with ferric chloride and were localized in tissue. Blue or blue green precipitate indicated the presence of tannins (Reeve, 1951; Mace, 1963).

**iii. Flavonoids**

The paraffin embedded sections were stained for two minutes in 1% diphenyl boric acid and 2- amino ethyl ester (Sigma) in ethanol (Natursoff reagent). Flavonoids appeared in yellow colouration (Tattini *et al.*, 2000).

**iv. Starch**

The deparaffinised sections were placed in Iodine-Potassium Iodide solution starch appeared blue or black in few seconds (Johansen, 1940).

**v. Protein**

The sections were immersed in 0.02% Coomassie brilliant blue R 250 in Clarke’s solution (pH 2.0) and rinsed in Clarke’s solution. Sections were
destained in fresh Clarke’s solution for 20 minutes and dehydrated in absolute alcohol and mounted. Protein stained blue colour (Fisher, 1968).

vi. Phenolics

Phenolic compounds were localized by a nitrosation histochemical method using nitrous acid. It was added to the fresh sections, followed by application of aqueous sodium hydroxide, to form red-colour (Reeve, 1951).

2.2.2.4. Quantitative Microscopic study

Clearing of leaf fragments were done by immersing the material in warm alcohol (to remove chlorophyll), followed by treating with 5 to 10% sodium hydroxide. Finally, the materials were rendered transparent due to loss of cell contents. The cleared materials were washed thoroughly, stained with safranin for quantitative microscopic studies. Quantitative microscopy was done as per the procedure given by Wallis (1985) and Evans (1996).

a. Stomatal Number

It is the average number of stomata per square mm of the epidermis of the leaf. A minimum of ten readings was taken from different locations of the leaflet and the average value was calculated.

b. Stomatal Index

Stomatal index was calculated using the equation \( I = \frac{S}{E+S} \times 100 \) where \( I = \) Stomatal index, \( S = \) No. of stomata per unit area, \( E = \) No. of epidermal cells per unit area.
c. Palisade ratio

It is the average number of palisade cells beneath particular number of epidermal cells. It is determined by counting the palisade cells beneath four continuous epidermal cells.

d. Vein-islet number

It is the average number of vein-islets per square millimeter of a leaf surface. It is determined by counting the number of vein-islets in an area of 4 sq. mm. of the central part of the leaf between the midrib and the margin.

2.2.2.5. Analytical studies

2.2.2.5.1. Quantitative determination

a. Physico-chemical characteristics

The following physic-chemical constituents were determined by standard methods.

Sample preparation

The plant parts such as leaf, stem and roots were separated and shade dried. Then the dried plant materials were milled individually into coarse powder by a mechanical grinder, sieved, packed in individual containers and used for further studies.

i. Determination of total ash

Total ash was determined by incinerating 20g of air-dried coarse powder in a tared silica crucible which was previously ignited and cooled before
weighing. The ignition was repeated until constant weight was obtained. The percentage of ash with reference to air-dried material was calculated (Anon., 1998).

ii. Determination of water soluble ash

A known weight of ash (about 200 mg) was boiled with 25 ml of distilled water. The insoluble matter was collected in a previously weighed sintered crucible, washed with hot water, dried to constant weight and weighed. The percentage of water soluble ash with reference to the air dried sample was calculated (Anon., 1996).

iii. Determination of acid insoluble ash

A known weight of ash (about 200mg) was boiled with 25 ml of 4 N Hydrochloric acid. The insoluble matter was collected in a previously weighed sintered crucible, washed with hot water, dried to constant weight and weighed. The percentage of acid–insoluble ash with reference to the air dried sample was calculated (Anon., 1996).

iv. Determination of loss of weight on drying

About 2 g of the powdered sample was weighed in a preweighed flat and thin porcelain dish. It was dried in the oven at 100°C and cooled in a desiccator. The loss in weight was recorded as loss of weight on drying (Anon., 1996).

v. Determination of extractive values

Extractive values of the powdered samples in petroleum ether (40 – 60°C), benzene, chloroform, methanol and water were determined individually.
About 5 g of the air-dried powdered sample was taken in a stoppered flask; 100 ml of the solvent was added, shaken well and allowed to stand for 24 h. Then the content was filtered. 50 ml of the filtrate was pipetted out into a clean previously weighed China dish and evaporated on a water bath. Finally, it was dried, cooled and weighed. The percentages of solvent soluble extractives with reference to the sample were calculated (Anon., 1966).

b. Fluorescence analysis

The powdered samples and the extract of the powder in various solvents such as petroleum ether (40 - 60°C), benzene, chloroform, methanol and water were examined individually under ordinary light and ultra violet light (365nm). These powder samples were also treated with 1 N NaOH (aqueous), 1N NaOH (ethanolic), 1 N HCl, 1:1 H₂SO₄ and 1:1 NNO₃ as per standard procedure (Pratt and Chase, 1949) and changes in colour were recorded.

2.2.2.5.2. Qualitative determination

a. Preliminary phytochemical screening

Preliminary phytochemical screening was carried out to assess the qualitative chemical composition of freshly prepared leaf, stem and root powders and various crude extracts by following standard procedures.

i. Test for alkaloids (Dragendorff's Test)

To 2ml extract, 1% HCl was added and steamed. To 1ml of this extract, 6 drops of Dragendorff's reagent was added, orange red precipitate indicated the presence of alkaloids (Sofowora, 1993).
ii. Test for glycosides

50 mg of extract was hydrolyzed with concentrated HCl for 2 h on a water bath, filtered and the hydrolysate was subjected to the following test (Trease and Evans, 1978).

**Borntrager’s test**

To 2 ml of filtrate hydrolysate, 3 ml of chloroform was added and shaken well. Chloroform layer was separated and 10% ammonia solution was added to it. Pink colour indicated the presence of glycosides.

iii. Test for saponins (Foam test)

The extract (50 mg) was diluted with distilled water and made upto 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes. A two cm layer of foam indicated the presence of saponins (Khandelwal, 2005).

iv. Test for terpenoids (Salkowski test)

To 5ml of extract, 2ml of chloroform was added and mixed well and a few drops of concentrated H$_2$SO$_4$ was carefully added and reddish brown colour layer was observed for terpenoid.

v. Test for tannins

A small quantity of the extract was mixed with water and then few drops of ferric chloride solution was added. Production of bluish black colour indicated the presence of tannins (Harborne, 1973).
vi. Test for phenolic compounds (Ferric chloride test)

The extract (50mg) was diluted to 5ml with distilled water. To this few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds (Mace, 1963).

vii. Test for flavonoids

Two different methods were followed for the determination of flavonoids, in the plant samples.

a) Ferric chloride test

0.2 ml of extract was added to 10% FeCl$_3$ and the mixture was shaken well. A woody brownish precipitate indicated the presence of flavonoids.

b) Lead acetate test

0.2 ml of extract was added to 0.2 ml 10% lead acetate and gently shaken. A dirty brownish precipitate indicated the presence of flavonoids (Evans, 1996).

viii. Test for steroids (Salkowski test)

0.5 g of extract was dissolved in 2.0 ml chloroform and 1.0 ml conc. H$_2$SO$_4$ was carefully added. A reddish brown colour indicated the presence of steroids (Evans, 1996).

ix. Test for resins

1 ml of extract was treated with few drops of concentrated nitric acid and a few drops of acetic anhydride solution, followed by 1 ml of concentrated sulphuric acid. Resins gave colorations ranging from orange to yellow.
x. **Test for gum and mucilage**

The extract (100 mg) was dissolved in 10 ml of distilled water and to this 25 ml of absolute alcohol was added with constant stirring. White or cloudy precipitate indicated the presence of gums and mucilage (Whistler and BeMiller, 1993 and Khandelwal, 2005).

2.3. **Results**

2.3.1. **Macroscopic observations**

**Description of *I. johnsonii* from two geographical regions**

A detailed description and photograph given in Plates 2.1 and 2.2 of this taxon are provided to facilitate easy identification.

**Taxonomic description of *I. johnsonii* Hook. f.**


2.3.1.1. ***I. johnsonii* from Pathanamthitta district (Plate 2.1)**

*I. johnsonii* Hook.f. is a perennial small shrub, with erect stem. Leaves opposite, decussate, simple, entire, petiolate; petiole 3-11 mm long, green to reddish-brown; lamina 8-26 x 4-14 cm, elliptic-ovate, cuneate at the base mucronate at apex, lateral veins 9-12 pairs, prominent on the adaxial side, greyish-white patches radiate from the midrib; stipules interpetiolar, 5-6.5 mm
long, laterally joined at the base forming a tube, broadly triangular with a central cusp ca.2-3.5 mm long with dense golden brownish hairs on the inner side. Inflorescence subsessile, peduncle 4-10 mm long, puberulous, bright red, with branchlets and pedicels articulated at ramifications; each unit a corymb of 9-12 flowers. Flowers sessile to shortly pedicelled; pedicel 1.5-5mm long, puberulous, reddish-brown; bracts to 5mm long, reddish brown, bracteoles to 4 mm long, reddish-brown; calyx lobes four, linear-triangular, acute at the tip, longer than ovary 5-6mm long, puberulous, reddish-brown; corolla, white, tubular, slender, glabrous, 15-35 mm long; lobes four, 4.5-9mm long, oblong, naked at the throat, buds with a reddish hue on the margins; stamens four attached at the mouth, alternating with the corolla lobes; filaments short 2.5-3mm long; anthers linear, 4.5-5 mm long beaked at the tip, bifid, mucronate at base, creamy-white; vary two celled. Ovules one in each locule, axillary; style slender 17-34 mm long bright red; stigma bifid, 3-4.5 mm long, bright red. Fruits a didymous berry, bright red when ripe 5.6-6.5 mm in diameter, calyx accrescent; seeds two.

**2.3.1.2. *I. johnsonii* from Kottayam district (Plate 2.2)**

*I. johnsonii* Hook.f. is a perennial undershrub, with erect stem. Leaves opposite, decussate, simple, entire, petiolate; petiole 2.5-10 mm long, green to reddish-brown; lamina 8-25 x 4-13 cm, elliptic-ovate, cuneate at the base, mucronate at apex, lateral veins 9-12 pairs, prominent on the adaxial side, grayish-white patches radiate from the midrib; stipules interpetiolar, 5-6.5 mm long, laterally joined at the base forming a tube, broadly triangular with a central cusp ca.2-3.5 mm long with dense golden brownish hairs on the inner side. Inflorescence subsessile, peduncle 4-10 mm long, puberulous, bright red, with
branchlets and pedicels articulated at ramifications; each unit a corymb of 6-9 flowers. Flowers sessile to shortly pedicelled; pedicel 1-5mm long, puberulous, reddish-brown; bracts to 5mm long, reddish brown, bracteoles to 4 mm long, reddish-brown; calyx lobes four, linear-triangular, acute at the tip, longer than ovary 5-6mm long, puberulous, reddish-brown; corolla, white, tubular, slender, glabrous, 15-30 mm long; lobes four, 4.5-9mm long, oblong, naked at the throat, buds with a reddish hue on the margins; stamens four attached at the mouth, alternating with the corolla lobes; filaments short 2.5-3mm long; anthers linear, 4.5-5 mm long beaked at the tip, bifid, mucronate at base, creamy-white; ovary two celled, ovules one in each locule, axillary; style slender 17-32 mm long bright red; stigma bifid, 3-4.5 mm long, bright red. Fruits a didymous berry, bright red when ripe 5.6-6.5 mm in diameter, calyx accrescent; seeds two.

Leaves are thick, leathery and dark green in colour. Stem is stiff, green and woody. Root is very hard and has a mild smell. Root bark is thick and brown in colour. These are some of the other organoleptic characters of the plant *I. johnsonii*.

### 2.3.2. Microscopic observations

#### 2.3.2.1. *I. johnsonii* from Pathanamthitta district

**2.3.2.1.1. Leaf**

The leaf consists of thick prominent midrib, lateral vein and thick dorsiventral lamina (Plate 2.3.1). The midrib consists of a thick, vertical pillar like adaxial cone and a wide, semicircular abaxial part. The midrib is 800µm in height; the adaxial cone is 250 µm high and 250µm wide. The abaxial part is
550µm wide. The epidermal layer of the midrib consists of fairly wide, squarish or oblong cells with prominent cuticle. The epidermis is 25 - 30 µm thick. The conical part has small, compact, thick walled cells (Plates 2.3.3 & 2.3.4). Similar type of thick walled cells are seen along the lower part of the midrib. The remaining part of the midrib, including the central part consists of angular thin walled parenchymatous cells (Plate 2.3.2).

The vascular system of the midrib comprises a hollow cylinder with thick flat plate of adaxial side and wide semicircular abaxial part (Plates 2.3.2 & 2.3.3). Two lateral parts of the cylinder extend into wide, long wings (Plate 2.3.1). The vascular cylinder consists of a continuous layer of phloem which is 100µm wide. The cylinder consists of thin and continuous line of sclerenchyma cells in the outer part and short parallel (radial) lines of thick walled, narrow, compact, angular xylem elements in the inner part (Plates 2.3.2 & 2.3.3). The vascular cylinder is 350µm in diameter (Plate 2.3.2).

Calcium oxalate druses are common in the ground parenchyma cells (Plates 2.3.5 & 2.3.6). The druses are solitary in the cells. The crystal bearing cells are not modified either in shape or size. The crystals are 20 - 30 µm thick.

**Lamina**

The lamina is smooth and even on both sides. It is distinctly dorsiventral and hypostomatic. It is 150µm thick. The adaxial epidermis has fairly wide and rectangular or vertically oblong, and thick cuticle. It is 30 µm in thickness. The abaxial epidermis is comparatively thin measuring 20µm in thickness (Plate 2.3.7). The cells are horizontally rectangular to squarish. The stomata occur at the
epidermal level. The guard cells have prominent stomatal ridges (Plate 2.3.10). The mesophyll tissue consists of short, cylindrical and conical loosely arranged single layer of palisade cells. The spongy parenchyma comprises 4 or 5 layers of lobed cells forming wide air-chambers (Plate 2.3.7).

**Leaf- margin**

The marginal part of the lamina is slightly thin and slightly bent down (Plate 2.3.8). It is about 140 µm thick. The epidermal cells are slightly reduced in size. The cells along the extreme marginal part are necrosed forming a thick mechanical barrier to the internal tissues. The differentiation of the palisade cells and spongy parenchyma cells is obscured (Compare plates 2. 3. 7 and 2. 3. 8). In the marginal parts, the mesophyll cells are in 5 or 6 horizontal rows and compactly arranged leaving no intercellular spaces (Plate 2.3.8).

**Paradermal sections of the epidermal tissue**

In Paradermal (surface) sections, the epidermal cells and stomatal morphology were studied (Plate 2. 3. 9). The adaxial epidermal cells are polyhedral in outline with thick straight walls. Thin cuticular striations are seen in dense parallel lines within the boundary of the cells (Plate 2. 3. 10). The adaxial epidermis is apostomatic. The abaxial epidermis comprises fairly wider epidermal cells with thin, straight or slightly wavy anticlinal walls. Cuticular striations are seen even in the abaxial epidermal cells (2. 3.10). The stomata are paracytic type. Two subsidiary cells lie parallel to the guard cells (Plate 2.3.10). The guard cells are oblong elliptic with elongated narrow stomatal aperture (Plate 2.3.10). The guard cells are 15 x 30µm in size.
**Venation Pattern**

Main veins, minor veins and veinlets are thick and straight. The vein-islets are distinct; they are variable in size and shape; the outline is rectangular, squarish or polygonal. The vein terminations are either simple (unbranched), thick and straight or branched forming dendroid outline.

**2.3.2.1.2. Petiole**

The petiole is broadly semicircular on the abaxial side and broadly conical on the adaxial side; there are two wings directed adaxially or laterally (Plate 2.3.11). The petiole is 1.75 mm thick; the adaxial hump is 700µm wide and the abaxial part is 1-5 µm wide.

The midrib consists of a distinct epidermal layer of fairly wide, thick walled squarish cells with thick cuticle; the epidermal layer is 30µm thick. The ground tissue is homogeneous and parenchymatous; the cells are circular to angular and fairly compact (Plates 2.3.12 & 2.3.13).

The vascular strand is a hollow wide cyclinder and more or less circular in outline (Plate 2.3.11). It consists of several compact, parallel rows of xylem elements and a thin layer of phloem along the outer boundary of the xylem. Phloem occurs in continuous sheath around the xylem. The vascular strand is 600µm both along the vertical and horizontal planes.

**2.3.2.2.3. Stem**

Young internode with early stage of secondary growth was studied. It is circular and even in cross sectional outline measuring 2.2mm thick. It consists of
thin superficial and discontinuous periderm at isolated places; remaining regions are
devoid of periderm (Plate 2.3.15). It consists of well defined epidermal layer of
squarish thick walled cells. The cortex is 300 - 350 µm wide; it is homogeneous
and parenchymatous. The cells are angular, thin walled and compact (Plates
2.3.14 & 2.3.15). The vascular strand is closed hollow cylinder; it is uneven in
thickness ranging from 2 to 2.1mm. The vascular cylinder is slightly thicker on
the lateral parts than other two parts (Plate 2.3.14). The outer zone of the vascular
cylinder is the secondary phloem which comprises radial lines of fairly wider ray
cells and smaller, randomly disposed sieve elements (Plate 2.3.15). The phloem
zone is enclosed by discontinuous layer of sclerenchyma of one or two cells thick
(Plate 2.3.15).

Xylem cylinder consists of dense, radial rows of tracheary elements which
comprise xylem rays, fibres and thick walled angular vessels. The cells of the rays
and fibres are heavily thick walled, libriform and lignified (Plate 2.3.16). The
vessels are somewhat wider than the fibres (Plate 2.3.16). The vessels are 15 – 20 µm
wide. The widest vessel is less than 20µm in diameter. The pith is wide,
homogeneous and parenchymatous with circular, thin walled cells (Plate 2.3.14).

2.3.2.2.4. Root

Fairly thick root with considerable quantum of secondary vascular tissues
was studied. The root consists of a superficial darkly stained, tannin - filled
periderm of 100µm thickness. The cortical zone is wide and homogeneous
comprising tangentially oblong parenchyma cells (Plates 2.3.17 & 2.3.18). Starch
grains are abundant in almost all cells of the cortex (Plate 2.3.18). Fairly large
spherical bodies of unknown nature are seen in the cortical cells. Secondary phloem zone narrow and continuous around the xylem (Plate 2.3.17). It consists of short radial rows of phloem rays and small polyhedral sieve elements.

The xylem cylinder is dense, solid and circular in outline (Plate 2.3.17). It is 1-4 mm in diameter. It consists of vessels, fibres, parenchyma and rays (Plate 2.3.18). The vessels are mostly solitary or in radial multiples of two or three. They are thick walled and angular in outline (Plate 2.3.18). Diameter of the vessels is 40μm.

Xylem fibres are heavily thick walled and lignified with narrow lumen. The xylem fibres are libriform type. They have thick lignified walls. They are distributed in succeeding rings, cleaved radially by xylem rays (Plate 2.3.17). Alternating with the fibre cylinders are parenchyma cylinders, rendering the xylem cylinder as though having growth rings (Plate 2.3.17). Xylem rays are narrow and straight, 2-cells wide and run deviating from the centre towards periphery. Starch grains are abundant in the xylem parenchyma and ray cells.

2.3.2.2. *I. johnsonii* from Kottayam district

2.3.2.2.1. Leaf

The leaf is unique in having thick and prominent midrib and thick lamina. The midrib consists of a thick, vertical pillar like adaxial cone and a wide, semicircular abaxial part (Plate 2.4.1). The midrib is 800μm in height; the adaxial cone is 250 μm high and 250μm wide (Plate 2.4.2). The abaxial part is 550μm wide. The epidermal layer of the midrib consists of fairly wide, squarish or oblong cells with prominent cuticle. The epidermis is 25 - 30 μm thick. The
Ground tissue within the adaxial cone comprises angular, compact, thick walled cells (Plate 2.4.2). The abaxial part consists of outer zone of three or four layers of angular, compact, fairly thick walled cells and inner zone of thin walled ground tissue (Plate 2.4.3). The vascular strand is single, wide, closed circle with central core of parenchyma. The vascular cylinder consists of short, parallel, compact radial lines of xylem elements which are narrow and thick walled. Phloem occurs in thin continuous layer along the outer circumference; external to the phloem is again thin continuous line of sclerenchyma cells (Plates 2.4.4, 2.4.8 & 2.4.9). The vascular cylinder is 350µm in diameter (Plates 2.4.1 & 2.4.8).

Calcium oxalate druses are common in the ground parenchyma cells (Plate 2.4.8 & 2.4.9). The druses are solitary in the cells. The crystal bearing cells are not modified either in shape or size. The crystals are 20 - 30 µm thick.

Lamina

The lamina is uniform in thickness (plate 2.4.5). It is distinctly dorsiventral and hypostomatic. It is 150µm thick. The adaxial epidermis is thicker with large, vertically oblong cells and thick cuticle. It is 30 µm in thickness. The abaxial epidermis is comparatively thin measuring 20µm in thickness. The cells are horizontally rectangular to squarish. The stomata occur at the epidermal level (plate 2.4.6). The guard cells have prominent stomatal ridges (Plate 2.4.6). The mesophyll tissue consists of short, cylindrical and conical, loosely arranged single layer of palisade cells. The spongy parenchyma comprises 5 or 6 layers of lobed cells forming wide air-chambers (Plate 2.4.6).
Leaf margin

The marginal part of the lamina is slightly thin and slightly bent down (plate 2.4.7). It is about 140 µm thick. The epidermal cells are slightly reduced in size. The cells along the extreme marginal part are necrosed forming a thick mechanical barrier to the internal tissues. The differentiation of the palisade cells and spongy parenchyma cells is obscured (Compare Plate 2.4.6 and 2.4.7). In the marginal parts, the mesophyll cells are in 5 or 6 horizontal rows and compactly arranged leaving no intercellular spaces.

Paradermal sections of the epidermal tissue

In the paradermal (surface) sections, the adaxial epidermis appears in surface view (Plate 2.4.10). The cells apostomatic; they have straight, fairly thick anticlinal walls. Thin cuticular striations are seen in dense parallel lines within the boundary of the cells (Plate 2.4.10).

The abaxial epidermis comprises fairly wider epidermal cells with thin, straight or slightly wavy anticlinal walls (Plate 2.4.11). Cuticular striations are seen even in the abaxial epidermal cells (Plate 2.4.12). The stomata are paracytic type. There are two subsidiary cells, one on either side of the stoma and parallel to the guard cells. The two subsidiary cells are equal or unequal in size. The stomata are oblong elliptic in surface view; they are 15 x 30µm in size.

Venation Pattern

Main veins, minor veins and veinlets are thick and straight. The vein-islets are distinct; they are variable in size and shape; the outline is rectangular,
squarish or polygonal (Plates 2.4.13 & 2.4.14). The vein terminations are either simple (unbranched), thick and straight (Plate 2.4.13) or branched forming dendroid outline (Plate 2.4.14).

2.3.2.2.2. Petiole

The petiole is broadly semicircular on the abaxial side and broadly conical on the adaxial side; there are two wide wings directed adaxially or laterally (Plate 2.4.15). The petiole is 1.7 mm thick; the adaxial hump is 700µm wide and the abaxial part is 1.5 µm wide.

The midrib consists of a distinct epidermal layer of squarish cells with thick cuticle; the epidermal layer is 30µm thick. The ground tissue is homogeneous and parenchymatous; the cells are circular to angular and fairly compact.

The vascular system includes a broad bowl shaped abaxial part and a flat adaxial plate lying as a lid on the abaxial bowl (Plates 2.4.15 & 2.4.16). The xylem rows in the abaxial part of the cylinder are longer than those in the adaxial part. The vascular cylinder consists of several compact, parallel rows of xylem elements and a thin layer of phloem along the outer boundary of the xylem. No specific inclusions are seen in the ground cells of the petiole. The vascular strand is 600µm both along the vertical and horizontal planes.

2.3.2.2.3. Stem

Stem measuring 2.2mm was studied. It is circular and even in sectional view. It consists of thin superficial and discontinuous periderm at isolated places; remaining regions are devoid of periderm (Plates 2.4.17, 2.4.18 & 2.4.19).
cortex is 300 - 350 µm wide; it is homogeneous and parenchymatous. The cells are angular, thin walled and compact (Plate 2.4.18 & 2.4.19). The vascular strand is closed hollow cylinder; it is uneven in thickness ranging from 2 to 2.1 mm. The outer zone of the vascular cylinder is the secondary phloem which comprises radial lines of fairly wider ray cells and smaller, randomly disposed sieve elements (Plate 2.4.20). The phloem zone is enclosed by discontinuous layer of sclerenchyma of one or two cells thick (Plate 2.4.20).

Secondary xylem consists of dense, radial rows of tracheary elements which comprise xylem rays, fibres and vessels. The cells of the rays and fibres are heavily thick walled and lignified (Plate 2.4.21). The vessels are slightly wider than the fibres and not much distinct from them. The vessels are 15 - 20 µm wide.

Calcium oxalate druses are sparsely seen in the phloem parenchyma (Plate 2.4.22). The crystals are solitary and are located in the ordinary (unmodified) parenchyma cells.

2.3.2.2.4. Root

Thick root with well developed secondary vascular tissues was studied. The root consists of a superficial darkly stained, tannin - filled periderm of 100 µm thickness (Plates 2.4.23 & 2.4.24). The cortical zone is wide and homogeneous comprising tangentially oblong parenchyma cells. Fairly large spherical bodies of unknown nature are seen in almost all cells of the cortex (Plate 2.4.24 & 2.4.26). Secondary phloem zone narrow and continuous around the xylem. It consists of short radial rows of phloem rays and small polyhedral sieve elements (Plate 2.4.26).
Secondary xylem is dense, solid cylinder comprising vessels, fibres, parenchyma and rays (Plates 2.4.25 & 2.4.27). The vessels are solitary or in multiples of two or three. They are angular, thin walled and angular in outline (Plate 2.4.27). Diameter of the vessels is 40µm.

The xylem fibres are libriform type. They have thick lignified walls. They are distributed in successing rings, cleaved radially by xylem rays (Plates 2.4.23 & 2.4.25). Alternating with the fibre cylinders are parenchyma cylinders, rendering the xylem cylinder as though having growth rings (Plates 2.4.23 & 2.4.25). Xylem rays are narrow, two-cells wide and run deviating from the centre towards periphery. Starch grains are abundant in the xylem parenchyma and ray cells.

2.3.3. Cell inclusions

Two major types of cell inclusions are found in the plant. Calcium oxalate crystals of druses or sphaero-crystals are fairly abundant in the ground parenchyma cells of the midrib (Plates 2.3.5, 2.3.6 & 2.4.8, 2.4.9) and petiole. In the root, crystals are less abundant and are of prismatic type. This type of crystals is also seen in the vascular cylinder of the leaf midrib region (Plate 2.3.6). The druses occur in ordinary, unmodified cells (Plate 2.4.22). In the root, starch grains are abundant. They occur in the cortical parenchyma cells as well as xylem fibres (Plate 2.4.27). The starch grains are simple concentric type. The druses in the midrib are 20µm thick; those in the petiole are 30µm thick. The starch grains are 15µm in diameter.
2.3.4. Powder Microscopic observations

2.3.4.1. Stem Powder

The stem powder consists of fibres, vessel elements and parenchyma cells. Observation showed that there is no significant difference of diagnostic values of stem elements of the powder.

(i) Vessel elements

The vessel elements are very narrow, long and fiber-like (Plates 2.5.1 & 2.6.1). Some of them have very long tail (Plate 2.6.2). The perforation plate is simple and oblique in the vessel elements that possess long tails. The tailless element has horizontals perforation plate (Plate 2.6.3). The lateral walls have minute, circular multiseriate pits (Plates 2.6.2 and 2.6.3). The vessel elements are 300 to 700 µm long and 30 µm wide.

(ii) Fibers

The fibers are libriform type. They have thick, lignified secondary walls. Pits are simple-type; they are canal like and abundant. The fibers are 550 µm long and 20 µm wide (Plates 2.5.2, 2.5.3 & 2.6.1).

2.3.4.2. Root Powder

The root powder also consists of vessel elements and fibres. The parenchyma cells are also seen in the powder. Like the stem power, the root elements of the power also do not exhibit significant differences of diagnostic values.
(i) Vessel elements

The vessel elements are unique in being very long, much narrow and extremely long tailed (Plates 2.7.1, 2.7.2 & 2.7.4). Some of the elements are tailless (Plate 2.7.3). They have simple, oblique perforation plates. The tail is up to 300 µm long. The lateral wall pits are minute, circular and multiseriate. The vessel elements are 650-850 µm long and 40 µm wide.

(ii) Fibres

The fibres are libriform type with wide lumen and thick walls (2.7.5 & 2.7.6). Pits are not evident. Some cell inclusions are seen in the lumen of the fibre. The fibres are 950 µm long and 20 µm wide.

2.3.5. Histochemical localization

In the present study some of the compounds such as starch, alkaloids, tannin, protein, phenolics and flavonoids were histochemically identified and highlighted in different tissues of the leaf, petiole, stem and root of *I. johnsonii*. The results are given in Table 2.1.

(i) Alkaloids: In the root, alkaloids were localized in the cortex, secondary xylem parenchyma and secondary phloem (Fig 2.8.F). It also occurred in the peri-vascular fibers (Fig 2.8.K), and outer cortex of the stem (Fig 2.8.I). In the leaf, it was densely accumulated in the outer cortex region of midrib. In the petiole, it occurred in the ground tissue.
(ii) **Flavonoids**: Flavonoids occurred in the cuticle and outer cells inner to the epidermis of the stem (Fig 2.8.I) and phloem region of the midrib in the leaf (Fig 2.8.J). In the root, flavonoids were localized in the secondary xylem and secondary phloem (Fig. 2.8.F). Phloem and cortical parenchyma cells of the petiole possessed dense deposition of flavonoids (Fig 2.8.H).

(iii) **Tannins**: Tannin was localized in the periderm (Fig 2.8.G) and secondary xylem parenchyma cells of the root (Fig 2.8.L). In the stem, it was present in the cortex (Fig 2.8.D) and periderm region (Fig 2.8.C).

(iv) **Phenolics**: Phenolic compounds were found in the periderm of the root (Fig 2.8.A) and cortical cells of the stem (Fig 2.8.D). In the secondary phloem of the root, phenolic compounds were sparse.

(v) **Protein**: Protein was another compound predominant in the cortical cells (Fig 2.8.B) and secondary phloem region of the root.

(vi) **Starch**: In the leaf, starch was found localized in spongy cells of the mesophyll and inner cortical cells. It was also present in the ray cells, parenchyma cells in the cortex (Fig 2.8.B), secondary xylem (Fig 2.8.E) and secondary phloem of the root.
Table 2.1. Histochemical localization in various parts of *I. johnsonii*.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Phytochemicals</th>
<th>Localization in tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>Root - cortex, Secondary xylem parenchyma and secondary phloem</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stem - peri- vascular fibers, cuticle, outer cortex.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leaf - outer cortex.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Petiole - ground tissue.</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</td>
<td>Root bark - Periderm, Secondary phloem.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leaf - phloem in midrib, outer cells inner to the epidermis.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Petiole - phloem, cortex parenchyma.</td>
</tr>
<tr>
<td>3</td>
<td>Tannin</td>
<td>Root - periderm, Secondary xylem parenchyma.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leaf - phloem in midrib.</td>
</tr>
<tr>
<td>4</td>
<td>Starch</td>
<td>Root - Secondary phloem, cortical parenchyma, secondary xylem parenchyma and ray cells.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leaf - Inner cortex, spongy mesophyll tissues.</td>
</tr>
<tr>
<td>5</td>
<td>Phenolics</td>
<td>Root - Periderm, Secondary phloem.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stem - Peridrm</td>
</tr>
<tr>
<td>6</td>
<td>Protein</td>
<td>Root - cortex, Secondary phloem.</td>
</tr>
</tbody>
</table>

2.3.6. Analytical studies

2.3.6.1. Quantitative microscopy

Quantitative microscopic data are found to be constant for a species. These values are especially useful for identifying the different species of genus and also helpful in the determination of the authenticity of the plant. The study of the leaf constants showed that the average stomatal number was 28 mm²; the vein islet number was 16mm². The stomatal index and palisade ratio were 14% and 9, respectively. The findings are presented in Table 2.2.
Table 2.2. Quantitative microscopic data of *I. johnsonii*

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Parameter</th>
<th>Mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stomatal number</td>
<td>28mm²</td>
</tr>
<tr>
<td>2</td>
<td>Stomatol index</td>
<td>14%</td>
</tr>
<tr>
<td>3</td>
<td>Palisade ratio</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>Vein-islet number</td>
<td>16mm²</td>
</tr>
</tbody>
</table>

2.3.6.2. Physico-chemical characteristics

The results on physico-chemical analysis such as the percentage loss of weight on drying, total ash, water-soluble ash, acid-insoluble ash and extractive values in various solvents for three different parts such as leaf, stem and root of *I. johnsonii* are presented in Table 2.3. Proximate analysis showed that total ash value of 3.96% in root, 3.66% in stem and 3.46% in leaf. In root sample, acid insoluble ash content recorded was 0.71% and water soluble ash content was about 2.28%, whereas they were 0.41% and 1.78% in stem but in leaf, they were 0.31 and 1.28%, respectively. The loss on drying revealed the percentage of moisture present in the samples and its value was higher in root (89.70%), than in stem (88.20%) and leaf (83.20%). The extractive values of leaf, stem and root samples in different solvents were ranged from 2.32 to 3.22% in petroleum ether, 2.87 to 3.67% in benzene, 2.92 to 3.76% in chloroform, 2.97 to 3.84% in methanol and 2.84 to 3.74% in aqueous form. Among the three samples, maximum extractive value was recorded in root sample in all the tested solvents, however the least value was recorded in leaf samples of all the tested solvents except aqueous form.
Table 2.3. Physicochemical analysis of leaf, stem and root of *I. johnsonii*

<table>
<thead>
<tr>
<th>SI. No.</th>
<th>Parameters</th>
<th>Leaf (%)</th>
<th>Stem (%)</th>
<th>Root (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Loss of weight on drying</td>
<td>83.20 ± 1.36</td>
<td>88.20 ± 1.84</td>
<td>89.70 ± 2.12</td>
</tr>
<tr>
<td>2</td>
<td>Total ash</td>
<td>3.46 ± 0.08</td>
<td>3.66 ± 0.03</td>
<td>3.96 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>Acid – insoluble ash</td>
<td>0.31 ± 0.001</td>
<td>0.41 ± 0.002</td>
<td>0.71 ± 0.001</td>
</tr>
<tr>
<td>4</td>
<td>Water-soluble ash</td>
<td>1.28 ± 0.04</td>
<td>1.78 ± 0.02</td>
<td>2.28 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td><strong>Extractive values</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a) Petroleum ether (40 – 60°C)</td>
<td>2.32 ± 0.06</td>
<td>2.62 ± 0.04</td>
<td>3.22 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>b) Benzene</td>
<td>2.87 ± 0.01</td>
<td>2.87 ± 0.04</td>
<td>3.67 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>c) Chloroform</td>
<td>2.92 ± 0.05</td>
<td>2.96 ± 0.03</td>
<td>3.76 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>d) Methanol</td>
<td>2.97 ± 0.03</td>
<td>2.99 ± 0.06</td>
<td>3.84 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>e) Water</td>
<td>3.24 ± 0.04</td>
<td>2.84 ± 0.02</td>
<td>3.74 ± 0.03</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD of triplicate analysis
2.3.6.3. Fluorescence analysis

Fluorescence analysis of the leaf, stem and root powder and extracts of *I. johnsonii* in various solvents can be used as a diagnostic tool for testing adulteration. Many phytodrugs when suitably illuminated emit light of a different wavelength or colour from that which falls on them. The fluorescence colour is specific for each compound. The fluorescence analysis of drug extract helps to identify the drug with specific fluorescence colour and also to find out the fluorescent impurities. The results of the fluorescence analysis of the powdered samples and the extract of the powder are presented in Table 2.4. The leaf, stem and root powders of *I. johnsonii* showed dark brown colour when treated with HNO₃, but treatments with HCl, H₂SO₄, ethanol and aqueous gave brown colour under long UV light (365 nm). The leaf powder as such under ordinary light showed characteristic grey colour, but stem and root powders showed brownish black colour.

In various extracts, the root powder alone showed some variation in colour change when compared to leaf and stem. Root powder gave greenish brown in petroleum ether extract, light yellow in benzene extract, pinkish orange in chloroform extract, bluish brown in methanol extract and brown colour in aqueous extract under UV light (365 nm). At the same time, under ordinary light, the high polar solvent extracts (water and methanol) showed light brown colour, petroleum ether extract showed brown colour, benzene extract showed colourless and chloroform extract showed brownish green colour.
<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Particulars of treatment</th>
<th></th>
<th>Leaf</th>
<th></th>
<th>Stem</th>
<th></th>
<th>Root</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Powder as such (drug powder)</td>
<td></td>
<td>Grey</td>
<td>Black</td>
<td>Brownish black</td>
<td>Black</td>
<td>Brownish black</td>
<td>Black</td>
</tr>
<tr>
<td>2</td>
<td>Powder + 1N NaOH (aqueous)</td>
<td></td>
<td>Yellowish green</td>
<td>Brown</td>
<td>Yellowish green</td>
<td>Brown</td>
<td>Yellowish green</td>
<td>Brown</td>
</tr>
<tr>
<td>3</td>
<td>Powder + 1N NaOH (ethanolic)</td>
<td></td>
<td>Yellowish green</td>
<td>Brown</td>
<td>Yellowish green</td>
<td>Brown</td>
<td>Dark brown</td>
<td>Brown</td>
</tr>
<tr>
<td>4</td>
<td>Powder + 1N HCl</td>
<td></td>
<td>Dark green</td>
<td>Brown</td>
<td>Dark green</td>
<td>Brown</td>
<td>Dark brown</td>
<td>Brown</td>
</tr>
<tr>
<td>5</td>
<td>Powder + H₂SO₄ (1:1)</td>
<td></td>
<td>Green</td>
<td>Brown</td>
<td>Green</td>
<td>Brown</td>
<td>Green</td>
<td>Brown</td>
</tr>
<tr>
<td>6</td>
<td>Powder + HNO₃ (1:1)</td>
<td></td>
<td>Brown at the centre and yellow at the edge</td>
<td>Dark Brown</td>
<td>Brown at the centre and yellow at the edge</td>
<td>Dark Brown</td>
<td>Dark brown</td>
<td>Dark Brown</td>
</tr>
<tr>
<td>7</td>
<td>Extracts</td>
<td></td>
<td>a. Petroleum ether</td>
<td>Yellowish green</td>
<td>Pinkish orange</td>
<td>Yellowish green</td>
<td>Pinkish orange</td>
<td>Brown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b. Benzene</td>
<td>Brownish green</td>
<td>Pinkish orange</td>
<td>Brownish green</td>
<td>Pinkish orange</td>
<td>Colourless</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c. Chloroform</td>
<td>Brownish green</td>
<td>Pinkish orange</td>
<td>Brownish green</td>
<td>Pinkish orange</td>
<td>Brownish green</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>d. Methanol</td>
<td>Green</td>
<td>Pinkish orange</td>
<td>Green</td>
<td>Pinkish orange</td>
<td>Light Brown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>e. Water</td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Light Brown</td>
</tr>
</tbody>
</table>
2.3.7. Preliminary phytochemical screening

The present study is a first attempt of phytochemical screening and identification of different chemical constituents present in the root, stem and leaf of *I. johnsonii*. Screening of plant extracts using five solvents of increasing polarity from hexane to water indicated the presence of all major phytochemical constituents like alkaloids, terpenoids, flavonoids, phenolics and saponins (Table 2.5). Methanol extract showed more efficiency in the recovery of phytochemicals than all other extracts. Methanolic extracts of leaves, stem and root showed the presence of alkaloids, flavonoids, phenolic compounds, tannins, saponins, glycosides and terpenoids. Resins occurred in methanolic extracts of stem, leaves and also in aqueous extract of root. Except methanolic extracts of various parts of the selected plant, tannin was absent in all other solvent extracts. Like methanolic extract, saponin was present in acetone extract of all parts of the plant as well as in hexane extract of root.

Glycosides occurred in acetone and methanol extracts of root and stem, but it was absent in aqueous extract of leaf and stem, instead it was in ethyl acetate extract of leaf and stem. Terpenoids were present in various extracts of different parts of the plant, but gum/mucilage was completely absent in all the solvent extracts. Flavonoids were present in mid and high polar solvent extracts of leaf, stem and root, except hexane extract of root. Steroids were present only in methanolic extracts of stem and root. Except aqueous extract of root and stem, all other extracts showed the presence of alkaloids.
Table 2.5. Qualitative phytochemical constituents present in various extracts of different parts of *I. johnsonii*

<table>
<thead>
<tr>
<th>SL No</th>
<th>Phytochemical constituents</th>
<th>Plant parts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leaf</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
</tr>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Phenolic compounds</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Tannins</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Gums/Mucilage</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Steroids</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Glycosides</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Resins</td>
<td>-</td>
</tr>
</tbody>
</table>

H-Hexane, EA-Ethyl Acetate, A-Acetone, M-Methanol, Aq-Aqueous
+ = Present, - = Absent.
2.4. Discussion

*I. johnsonii* is a small shrub, with erect unbranched stem. During summer, sometimes the shoot apex die-back, in the next coming seasons, the lateral buds will sprout and produce branches and gives the appearance of a branched stem. Macro and micro morphological standards discussed here can be considered as identifying parameters to authenticate the fragmentary sample. Structural variations related to the environment or locations within a plant and between individual plants of the same species have well recognized by many investigators as early as the beginning of 20th century. Changes in the mesophyll tissue, as result of shading, variability venation, areole size, for example due to intensity of light and differences in the size of adaxial and abaxial epidermal cells of leaf are some of the common instances to indicate the effects of environment of the plant structures. Morrison (1953) has shown that in a given species significant differences in vessel diameter, vessel - element length, fibre length, fibre wall thickness, ray height and width, vessel concentration in root may be related to relative depth at which the roots are formed.

Variability can be so great as to negate the use of features for taxonomic purposes, unless an investigator can discriminate heritable characteristics and those that are environmentally modified. Unfortunately the fact that some features may be influenced by environment or location within a plant has led to skepticism as to the value for study of anatomical features at large. Such skepticism has been adequately answered by Metcalfe (1959).
During the present investigation, two populations of *I. johnsonii* were found inhabiting, at two different localities. The two localities were found to exhibit different climatic conditions (Table 1.8). With an inquisition to probe into any structural differences among the individual of the two different populations, a study was carried out. The morphological characters showed very few differences among the plants of *I. johnsonii* in two accessions. Meager differences were noticed in the length of the petiole, lamina, pedicel, corolla tube and style. Variation was found out regarding the number of flowers in each unit of a corymb.

Of the leaf characters, the character states of the midrib and lamina express some reliable structural features of comparative values. The midrib of the leaf of *I. johnsonii* collected from Pathanamthitta area is curious as seen in cross sectional outline. It possesses a thick, vertical adaxial cone comprising collenchymatous tissue. The abaxial part is semicircular, with outer narrow zone of collenchymas. The cuticle of the epidermis is highly thick. The vascular strand has a very thick flat plate of adaxial side. The xylem elements are thick walled. The vascular cylinder is encircled by a thin, two layered sclerenchymatous cylinder. The mesophyll tissue consists of a single short, layer of conical palisade cells; the spongy mesophyll zone is wider with 6 layers of cells having small intercellular spaces. The area from which the sample was studied seems to be xeric in climatic conditions. The xeric conditions are well expressed in the xeromorphic structure of the midrib and lamina. The midrib of the leaf of *I. johnsonii* collected from Kottayam area has thick, vertical adaxial cone comprising collenchymatous tissue. The abaxial part is semicircular, with few layers of collenchyma. The cuticle of the epidermis is thick. The vascular cylinder is encircled by a thin continuous line of
sclerenchyma cells. The mesophyll tissue consists of a single short, layer of palisade cells; the spongy mesophyll zone is with 5-6 layers of lobed cells forming wide air-chambers. The area from which the sample was studied seems to be mesic in climatic conditions. The mesic conditions are well expressed in the mesomorphic structure of the lamina.

Thickness, wall characteristics and nature of sculpturing on the wall as seen in surface view have often been used as taxonomic criteria. Epidermal wall sculpturing may result from ridges in the epidermal wall itself or it may be formed wholly from cuticle, resulting in cuticular relief. These may be close relation, as shown by Cooper (1922), between thickness of the cuticle and ecological situation. Thick cuticle is attributed to be an ecological adaptation in xeric condition. In the leaf of Pathanamthitta sample, the cuticle of the leaf epidermis, especially along the margins is very thick as compared to the samples from Kottayam, the former locality as inferred from the ecological data, seems to be xeric which renders the lamina to evolve a thick cuticle to protect the mesophyll tissue from excess of transpiration.

The vascular strand in the midrib of the leaf is closed cylinder, ensheathed externally by a thin sheath of fairly thick walled cells or sclerenchyma layer. Such sclerenchyma sheath is also seen around the vascular cylinder of stem in both Kottayam and Pathanamthitta samples. Of these two cases, sclerenchyma sheath is more expressed in the Pathanamthitta sample than the Kottayam sample. Well developed sclerenchyma sheath around the vascular tissues is obviously are adaptive value for the protection of the conducting elements.
Vessel elements both in the stem and root are quite unique in being very narrow, long and long-tailed. These vessel attributes are of high diagnostic values of the fragmentary samples of the taxon. Distribution of vessels as in cross sectional view shows that the vessels are mostly solitary. Solitary vessels are believed to be vulnerable for air-embolism during dry seasons. As a safety measure, the wood has evolved narrow vessels which do not suffer from air-embolism. Thus narrow vessels in *Ixora* render adaptive strategy to overcome air-blocking in the vessels. Unusually long, narrow thick walled tails of the vessel elements is not only useful character of identification, may also provide some mechanical strength to the vessel lines.

In the leaf of *I. johnsonii*, a single peristomatal rim is found parallel to the stomata and the outer stomatal ledge is not conspicuous. This is an exception as in all the other species peristomial rims if present surrounds the stomata and are in rows of 2 or 3 and the outer stomatal ledge is quite conspicuous. In *I. subsessilis*, stomata are found on both the abaxial and adaxial leaf surface (amphistomatic).

In the root of *I. johnsonii*, xylem rays are narrow, 2-celled wide and seen deviating from the center towards from the periphery. Similarly, Sudhakaran Nair (2001) reported the anatomy of the root of *I. coccinea* and he revealed that in *I. coccinia* medullary ray is uniseriate and phloem fibres are distributed either isolated or in small groups of 2 or 3.

The stem of different species of *Ixora* can be differentiated from each other on the basis of distribution pattern of sclereids, bast–fibres and presence /absence
of sclerized pith in the stem. Sharma (1970) made a comparative study of sclereids in four species of *Ixora* and found that Brachysclereids are present in pith, cortex and stem bark of *I. barbata* in a diffuse manner and as groups in pith of the stem in *I. fulgens* and both pith and cortex regions of the stem in *I. javonica*. There is no sclereids in *I. coccinea*, but possess sclerized pith. Bast-fibres are present in *I. barbata* and *I. coccinea*. *I. coccinea* differs from *I. barbata* by having a ring made up of bast fibres, outside the secondary phloem. In *I. brunnescens*, axial parenchyma is apotracheal, and scandy in the xylem region of the wood. Xylem rays are uniseriate. Growth ring absent and vessels are diffuse porous (Jayakumar, 2003). In *I. johnsonii* there is a thin layer of fibres encircling the vascular cylinder. Xylem cylinder consists of highly thick walled libriform fibres and the pith is wide, homogeneous and parenchymatous with circular, thin walled cells. The differences observed are important in separating the secondary woods of different species of *Ixora*. The above noted pronounced differences in the distribution and form of the sclereids help not only in distinguishing them anatomically, but also emphasize their diagnostic importance.

The petiole is of considerable taxonomic importance, since its structure appears to be little affected by the environmental changes (Metcalf and Chalk, 1957). There is no difference in the petiole region of *I. johnsonii* from Pathanamthitta and Kottayam districts.

Histochemistry is an ingenious technique that accomplishes dual purpose. It highlights the chemical nature of substance in tissues and concomitantly, it
locates the types of tissues where the compounds are deposited. So gross microscopic studies are complemented with the cytochemical analyses so that one can get a full insight into the histology of the plants. Histochemical studies are also complementary to the qualitative analysis of the compounds, where in plant extracts are subjected to detection of presence or absence of various compounds. The results so obtained will be of application value in diagnosis of the crude phytodrugs. Alkaloids, flavonoids, phenolics, protein, starch and tannin are compounds which come under the preview of the present investigation. The pharmacological actions of alkaloids vary widely. Detecting the presence and localization of the alkaloids in plants will be of mud’s inventory importance in the herbal science. Tannins are said to be antimicrobial and their presence in the root, stem and leaves seems to be strengthen the pharmacological potentials of the plant.

In *I. johnsonii* calcium oxalate crystals of druses or sphaero-crystals are fairly abundant in the ground parenchyma cells of the midrib and petiole. In the root, crystals are less abundant and are of prismatic type but in *I. brunnescens*, the calcium oxalate crystals are unique in that they are extensively long, narrow and oblong with wedge shaped ends occurring in the axial parenchyma cells in the collapsed phloem region of the inner bark. Tannin is abundant in the ray cells as well as axial parenchyma cells of the wood (Jayakumar, 2003). In *I. johnsonii* tannin is present in the phloem cells of the midrib and in the periderm as well as the secondary xylem parenchyma cells of the root.
Quantitative microscopy includes certain leaf measurements to distinguish some closely related species, which are not easily characterized by general microscopy. This technique has been effectively employed by Zoring and Weiss (1925) in their studies on compositae and by George (1943) to distinguish Senna and Belladonna. Thus, this parameter seems to be a valid measure to distinguish closely resembling species of a genus. The stomatal number (the number of stomata per square millimeter) is another old technique, but a simple device for diagnosis of fragmentary leaf specimens. According to Timmerman (1927; 1927a), the stomatal numbers of epidermal cells of *Datura inoxia* enabled him to distinguish it from other species of *Datura*. Rowsan (1943) advocates the relevance of stomatal index in botanical diagnosis of herbal drugs; his axion is based on the studies of different species of *Atropa* spp., *Cassia* spp., *Datura* spp., *Digitalis* spp., *Erythroxylon* spp., and *Phytolacca* spp. Palisade ratio refers to the average number of cells beneath particular number of epidermal cells, is another old technique but a simple device for diagnosis of fragmentary leaf specimens. Vein-islet number is another simple technique for distinguishing fragmentary specimens at species levels, for eg., Levin (1929) determined the vein islet numbers of a number of species of *Senna*, *Digitalis* and *Buchu*. In those cases, if palisade ratio is not dependable for diagnosis, vein-islet numbers have proved to be a reliable diagnostic feature. From the above mention examples, the quantitative microscopic values of especially fragmentary drugs may be relied upon with some confidence. In *I. johnsonii*, the study of the leaf constants showed that the average stomatal number is 28/ mm$^2$; the vein islet number is 16/mm$^2$. The stomatal index and palisade ratio are 14% and 9
respectively. Similar type of observation found in *I. coccinea* showed the stomatal index is 12.2% the average stomatal number is 24mm², vein islet number 13.5mm² and the palisade ratio is 9.3. This distinguishes *I. johnsonii* from other species. The data given for *I. johnsonii* for its quantitative values may be taken to supplement other microscopic and macroscopic features for its identity.

Since the ash values are constant for a given drug, these values are also one of the diagnostic parameters of the drug. In *I. johnsonii*, all the drug samples have more water soluble ash than acid insoluble ash. The results of various types of ash and extractive values may provide a basis to identify the quality and purity of the drug. These values also indicate the value of the constituents present in a crude drug. Extractive values of crude drugs are useful for their evaluation especially when the constituents of drug cannot be readily estimated by any other means. The results of proximate composition of *I. johnsonii* leaves showed high moisture content (83.20%w/w). This is within the reported range (81.4-90.3%) in Nigerian green leafy vegetables. Proximate analysis showed that total ash value of leaf of *I. johnsonii* is 3.46%. Ash content, which is an index of mineral contents in biota, is high in *Amaranthus hybridus* (13.8% DW), *Talium triangulare* (20.05% DW) (Ladan *et al.*, 1996); *Ipomea batatas* (11.10%), *Vernonia colorata* (15.86%) and *Moringa oleifera* (15.09%) (Lockeett *et al.*, 2000; Antia *et al.*, 2006) and a leafy vegetable *Hibiscus esculentus* (8.00%) (Akindahunsi and Salawu, 2005) when compared to the leaf ash value of *I. johnsonii*. 
Since the plant *I. johnsonii* is useful in traditional medicine for the treatment of wound healing, it is important to standardize for use as a drug (Binu, 1999). The pharmacognostic constants for the leaf, stem and root of this plant, the diagnostic microscopic features and the numerical standards reported in the work could be useful for the compilation of a suitable monograph for proper identification. Thus, the anatomical characters coupled with preliminary phytochemical results are specific for the plant *I. johnsonii*. 