CHAPTER 4

PHARMACOGNOSY

4.1 GENERAL INTRODUCTION

4.1.1 Pharmacognosy -overview

Pharmacognosy, the modern science of natural medicines, is based on traditional medicines used in different parts of the world. Traditional medical heritages of Ayurveda, Traditional Chinese Medicine, Greco European Medicine, Egyptian Medicine, Kampo medicine and others are important precursors for the development of Pharmacognosy and Pharma Sciences. Etymologically, the term Pharmacognosy is composed of two Greek words, i.e. pharmakon (a drug) and gignosco (to acquire knowledge of) or gnosis (knowledge) Thus, literally Pharmacognosy is “to acquire knowledge of drugs.” [60, 61].

In the past, most of the drugs were derived from herbs and conventional Pharmacognostical studies were focused on the systematic study of medicinal plants and medicines derived from plants. However, the interests and scope of Pharmacognosy were gradually expanded, by incorporating applied domains of botany, chemistry, biology and pharmaceutical science.

As mentioned by [62, 63] Verpoorte and Tschirch, Pharmacognosy is defined as the “science which has the task to learn everything about drugs originating from plants or animals in all aspects, except the physiological effect.” In the modern pharmaceutical domain, animals, bacteria, fungi, marine organisms and minerals are also used as the promising sources of traditional and modern medicines [64]. Wallis explained pharmacognosy as a “Distinct department of pharmaceutical science dealing with the systematic study of structural, physical, chemical and sensory characters of crude drugs of animal, vegetable and mineral origin including their history, cultivation and collection”. Whereas, Kinghorn [65] defined Pharmacognosy, as the “pharmaceutical science, that deals with the discovery, characterization, production and standardization of drugs of natural
According to Heinrich et al. [66], contemporary Pharmacognosy deals with medicinal plants, crude drugs, extracts, pure compounds and foods having health benefits and it is, in fact, the “science of biogenic or nature derived pharmaceuticals or poison”. Furthermore, Pharmacognosy is also defined as “a molecular science that explores naturally occurring structures and activity relationships with a drug potential” [67]. The American Society of Pharmacognosy defines Pharmacognosy as “the study of physical, chemical, biochemical and biological properties of drugs, drug substances or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources” [68]. Thus, in a contemporary context, Pharmacognosy has become a multidisciplinary science of natural drugs and drug substances and it deals with medicinal plant cultivation, crude drug production, chemical; biological; pharmacological and molecular analysis of crude drugs and drug substances to assure their production, potency, purity and safety as well as to assist new drug discoveries.

4.1.2 Standardization and quality control of herbal crude drugs – Processes and procedures

According to WHO, standardization and quality control of herbals is the process involved in the physicochemical evaluation of crude drug covering aspects, such as selection and handling of crude material, safety, efficacy and stability assessment of finished product, documentation of safety and risk based on experience, provision of product information to consumer and product promotion. Herbal materials are categorized according to sensory, macroscopic and microscopic characteristics. An examination to determine these characteristics is the first step towards establishing the identity and the degree of purity of herbal materials. They are carried out before any further tests are undertaken. Visual inspection provides the simplest and quickest means by which to establish identity, purity and possibly quality. If a sample is found to be significantly different from the specifications in terms of colour, consistency, odour or taste, it is considered as not fulfilling the requirements.

4.1.2.1 Macroscopic and microscopic evaluation

The macroscopic identity of herbal materials is based on shape, size, colour, surface characteristics, texture, fracture characteristics and appearance of the cut surface. However,
since these characteristics are judged subjectively and substitutes or adulterants may closely resemble the genuine material, it is often necessary to substantiate the findings by microscopy and/or physicochemical analysis. Microscopic inspection of herbal materials is indispensable for the identification of broken or powdered materials; the specimens are treated with chemical reagents. An examination by microscopy alone cannot always provide complete identification, though when used in association with other analytical methods it can frequently supply invaluable supporting evidence. Comparison with a reference material reveals, characteristics not described in the requirements which might otherwise have been attributed to foreign matter, rather than normal constituents.

4.1.2.2 DNA Barcoding

The traditional system of medicine utilizes medicinal plants to cure various ailments but the herbal industry suffers from substitution and adulteration of medicinal herbs with closely related species [69]. The efficacy of the drug decreases if it is adulterated, and in some cases, can be lethal if it is substituted with toxic adulterants. Authentication using DNA barcodes overcomes these problems [70]. Authentication at the DNA level provides more reliability because, in contrast to RNA, DNA is a stable macromolecule that is not affected by external factors and is found in all tissues. Therefore, development of DNA-based marker is important for authentication of medicinal plants. The novel technique of identifying biological specimens using short DNA sequences from either nuclear or organelle genome is called DNA barcoding Figure 4.1. The term ‘DNA barcode’ as taxon identifiers was first proposed by Paul Hebert of University of Guelph in 2003 [71]. Following initial in-silico and laboratory-based assessment of different loci from chloroplast and nuclear genomes led to the conclusion that no single locus plant barcode exist, and soon it was realized that, multi-locus barcodes are requisite for plant barcoding.

Subsequently a number of loci were being tested for their suitability as plant barcodes and many multi-locus combinations were suggested. The Consortium for the Barcode of Life Plant Working Group (CBOL) [72] evaluated seven chloroplast genomic regions across the plant kingdom and proposed a combination of \textit{matK} and \textit{rbcL} as plant barcodes. High universality but less species resolution is provided by \textit{rbcL} whereas \textit{matK} affords high resolution but less universality. A combination of these two can help to achieve maximum species discrimination. Nevertheless, in closely related species, the discriminating
ability of these two markers is low [73, 74]. Therefore, the China Plant BOL Group [75] proposed the addition of nuclear ITS (Internal Transcribed Spacer) to the \textit{matK}+\textit{rbcL} combination as plant barcode in order to achieve maximum identification rates even in closely related species.

\textbf{Figure 4.1} Major steps in DNA barcoding
4.1.2.3 Proximate analysis

Ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in an ash residue consisting of an inorganic material (metallic salts and silica). A high ash value is an indication of contamination, substitution, adulteration in the crude drug. The ash remaining following ignition of herbal materials is determined by three different methods which measure total ash, acid-insoluble ash and water-soluble ash. The total ash method is designed to measure the total amount of material remains after ignition. The total ash usually consists of carbonates, phosphates, silicates and silica which includes both physiological (ash-derived from plant tissue) and non-physiological ash (residue of the adhering material to the plant surface, eg. sand and soil). Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth. Water-soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water.

Determination of water soluble and alcohol soluble extractive is used as a means of evaluation of the crude drugs for the presence of active constituents in the plant material. This method determines the amount of active constituents extracted with solvents from a given amount of herbal material. It is employed for materials for which as yet no suitable chemical or biological assay exists. Extraction of any crude drug with a particular solvent yields a solution containing different phyto-constituents. The composition of these phytoconstituents in the particular solvent depends upon the nature of the drug and the solvent used.

An excess of water in herbal materials will encourage microbial growth, the presence of fungi or insects and deterioration following hydrolysis. Limits for water content should therefore be set for every given herbal material. This is especially important for materials that absorb moisture easily or deteriorate quickly in the presence of water. Moisture is an inevitable component of crude drugs, which must be eliminated as far as practicable, to aid in their preservation. The test for loss on drying determines both water and volatile matter. Drying can be carried out either by heating to 100–105 °C or in a desiccator over phosphorus pentoxide under atmospheric or reduced pressure at room temperature for a
specified period of time. The desiccation method is especially useful for materials that melt to a sticky mass at elevated temperatures. Many medicinal plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant materials and their extracts is measured in terms of foaming index.

### 4.1.2.4 Elemental analysis

Elements play a remarkable role in varied illness. It has been archived that modification of trace elemental equilibrium in an organism has undeviating correlation with different pathological conditions [76]. Trace elements play a vital role in the evolution of active constituents in medicinal plants [77]. These elements are imperative in less concentration, but are toxic at higher levels; accumulation of heavy metals stimulates persistent destruction to ecosystems and should be cautiously examined [78, 79]. Existence of toxic metals in lakes, ponds, brackish water and in plants, chiefly edible is of significant concern, since it affects the existence of local people sustaining upon these wetlands for their every-day needs [80]. WHO has strongly highlighted on the need for safety evaluation and quality assurance of traditional medicines. Thus it becomes compulsory that all herbal preparations and raw materials acquired from the wild and cultivated source has to be screened for the presence of heavy metals to guarantee quality, efficacy and safety of herbal preparation [81].

### 4.2 MATERIALS AND METHODS

#### 4.2.1 Plant Material

Fresh, entire plants were collected from the marshy lake (*M. hastata*) and from the streams (*M. vaginalis*) of Ambalavayal, Wayanad District, Kerala, India, in December 2012 and were authenticated by Botanical Survey of India, Tamilnadu Agricultural University, Coimbatore. Voucher specimens of *M. vaginalis* (BSI SRC/5/23/2011-12) and *M. hastata* (BSI SRC/5/23/2012-2013) have been preserved in the laboratory for future reference. Three parts of two species namely *M. vaginalis* leaf (MVL), *M. vaginalis* stem (MVS), *M. vaginalis* rootstock (MVR) of *M. vaginalis* and *M. hastata* leaf (MHL), *M. hastata* stem (MHS), *M. hastata* rootstock (MHR) of *M. hastata* were used as plant materials. The plant parts were carefully separated and washed in running tap water to clean the foreign particles and cut
into small pieces with a sharp knife to facilitate drying and shade dried in room temperature for about three days, pulverized and passed through 60-mesh sieve and stored dry until use. All results were expressed by dry weight (DW).

4.2.2 Preparation of Extract

Cleaned plant parts were cut in to small pieces with a sharp knife to facilitate drying and shade dried in room temperature for about three days, pulverized and passed through 60-mesh sieve and stored dry until use. The air–dried plant parts were extracted with hydro-alcohol (30:70) by cold maceration. Extracts were then filtered through a paper filter (Whatmann, No.1) and concentrated under reduced pressure using rotovac. The dry mass (semisolid) was stored in glass vials for further analysis.

4.2.3 Macroscopy and Microscopy

(a) Preparation of specimens

Care was taken to select healthy plants and normal organs. Macroscopical characters were observed and the required samples of different organs were fixed in FAA (formaldehyde, acetic acid, 50% ethanol, 5:5:90, v/v/v). After 24 h fixing, the specimens were dehydrated with gradual series of TBA (tertiary- butyl alcohol). Infiltrations of the specimens were carried out by gradual addition of paraffin wax (m. p. 58 – 60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

(b) Sectioning

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thicknesses of the section were 10 - 12 µm. Dewaxing of the section was done by customary procedure [82]. The sections were stained with toluidine blue as per the method published by O’Brien et al [83]. Since toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also produced. The dye rendered pink color to the cellulose mass, 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 and 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 fluid [84] were prepared. Glycerin mounted temporary preparations were made for macerated / cleared material. Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell component were studied and measured.

(c) Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon, Japan, Labphoto 2 Microscopic unit. For normal observations bright field was used and 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 are indicated by the bars scales. Descriptive terms of the anatomical features are as given in the standard anatomy books [85].

4.2.4 DNA Barcoding

(a) DNA isolation

Genomic DNA was isolated by following the protocol of Saghai Maroof et al. [86] with minor modifications. About 100 mg of leaf tissue was taken for genomic DNA isolation and ground using mortar and pestle by adding 500 ml of CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 1% β-mercaptoethanol, 2% CTAB). The samples were transferred to 1.5 ml centrifuge tubes, incubated in water bath at 55°C for 30 minutes and then extracted with equal volume of chloroform. The samples were centrifuged at 10,000 rpm for 10 minutes and the aqueous phase was transferred to fresh 1.5 ml centrifuge tubes. The DNA was precipitated by adding equal volume of ice cold isopropanol and centrifuged at 10,000 rpm for 10 minutes. The DNA pellet was washed with 70% ethanol, air-dried at room temperature and dissolved in 100 ml TE buffer. In case of wood samples, genomic DNA was isolated by following the same protocol except that 2% PVP was included in the CTAB buffer and the samples were incubated at 55°C for 10 hours.
(b) PCR amplification and DNA sequencing

PCR amplification of DNA barcode marker was done using 50ng of total genomic DNA as template and the commonly used primers for matK (matK-1RKIM-F and matK-3FKIM-R, KiJoong Kim, School of Life Sciences and Biotechnology, Korea University, Korea, unpublished), and rbcL (rbcLa-F, rbcLajf634R) [86]. PCR reaction mixture (30 ml) contained 1X buffer with 1.5 mM MgCl₂, 200 mM dNTPs, 5 pmol primers and 1 unit Taq DNA polymerase. PCR was done in a thermal cycler (Eppendorf, Germany) using the following protocol: initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minutes, final extension at 72°C for 5 minutes, and hold at 16°C. The PCR products were checked by agarose gel electrophoresis, and purified using EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc. Ontario, Canada). The purified PCR products were sequenced from both the ends using the same PCR primers in 31306l Genetic analyzer (Applied Biosystems, CA, USA). The sequences were manually edited using Sequence Scanner Software v. 1.0 (Applied Biosystems, CA, USA) and full length sequences were assembled.

(c) Data analysis

Database search for species identification were done using Basic Local Alignment Search Tool (BLAST) against non-redundant nucleotide database at NCBI (www.blast.ncbi.nlm.nih.gov/Blast.cgi). DNA sequence obtained was matched with the existing database in the library.

4.2.5 Physiochemical Evaluation

Ash values, extractive values and foaming index were determined according to WHO guidelines [87] and moisture content was determined by drying at 105°C, until a constant weight was achieved.

4.2.5.1 Total ash

About 2–4g of ground air-dried material was accurately weighed, in a previously ignited and tared crucible (usually of platinum or silica). The material was spread in an even layer and ignited by gradually increasing the heat to 500–600 °C until it was white, indicating the absence of carbon. Cooled in a desiccator and weighed. The residue was
allowed to cool in a suitable desiccator for 30 minutes and weighed without delay. The content of total ash was calculated in mg per g of air-dried material.

4.2.5.2 Acid-insoluble ash

To the crucible containing the total ash, 25 ml of hydrochloric acid (~70 g/l) was added and covered with a watch-glass and boiled gently for 5 minutes. Filtered and the insoluble matter was collected on an ashless filter-paper and washed with hot water until the filtrate is neutral. Filter-paper containing the insoluble matter was transferred to the original crucible, dried on a hotplate and ignited to constant weight. The residue was allowed to cool in a suitable desiccator for 30 minutes and weighed without delay. The content of acid-insoluble ash was calculated in mg per g of air-dried material.

4.2.5.3 Water-soluble ash

To the crucible containing the total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble matter was collected in a sintered-glass crucible or on an ashless filter-paper. Washed with hot water and ignited in a crucible for 15 minutes at a temperature not exceeding 450 °C. The weight of this residue in mg was subtracted from the weight of total ash. The content of water-soluble ash was calculated as mg per g of air-dried material.

4.2.6 Extractive values

4.2.6.1 Hot extraction (Water soluble extractive)

4.0 g of coarsely powdered air-dried material was accurately weighed, in a glass-stoppered conical flask. 100 ml of water was added and weighed to obtain the total weight including the flask. Shaken well and allowed to stand for 1 hour. Reflux condenser was attached to the flask and boiled gently for 1 hour; cooled and weighed. Readjusted, with the solvent to the original total weight specified in the test procedure. Shaken well and filtered rapidly through a dry filter. 25 ml of the filtrate was transferred to a tared flat-bottomed dish and evaporated to dryness on a water-bath. Dried at 105 °C for 6 hours and cooled in a desiccator for 30 minutes, then weighed without delay. The content of extractable matter was calculated as mg per g of air-dried material.
4.2.6.2 Cold maceration (alcohol soluble extractive)

4.0 g of coarsely powdered air-dried material was accurately weighed, in a glass-stoppered conical flask and Macerated with 100 ml of the ethanol specified for the plant material selected for 6 hours, shaken frequently, allowed and kept for 18 hours. Filtered rapidly, taking care not to lose any solvent, 25 ml of the filtrate was transferred to a tared flat-bottomed dish and evaporated to dryness on a water-bath. Dried at 105 °C for 6 hours, and cooled in a desiccator for 30 minutes. The content of extractable matter was calculated in mg per g of air-dried material.

4.2.6.3 Loss on Drying

2–5 g of the prepared air-dried material, or the quantity specified in the test procedure for the herbal material concerned was accurately weighed, in a previously dried and tared flat weighing bottle. The sample was kept in an oven at 100–105°C; in a desiccator over phosphorus pentoxide under atmospheric pressure. The loss of weight was calculated in mg per g of air-dried material.

4.2.7 Foaming index

1 g of the herbal material was reduced to a coarse powder (sieve size no. 1250), weighed accurately and transferred to a 500ml conical flask containing 100ml of boiling water by maintaining at moderate boiling for 30minutes. Cooled and filtered into a 100-ml volumetric flask and sufficient water was added through the filter to dilute to volume. The decoction was poured into 10 stoppered test-tubes (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml, etc. up to 10ml and the volume of the liquid in each tube was adjusted with water to 10ml. The tubes were sealed and shaken in a lengthwise motion for 15 seconds (two shakes per second). Allowed to stand for 15 minutes and the height of the foam were measured. The results were assessed as follows:

If the height of the foam in every tube is less than 1cm, the foaming index is less than 100. If a height of foam of 1cm is measured in any tube, the volume of the herbal material decoction in this tube (a) is used to determine the index. If this tube is the first or second tube in a series, an intermediate dilution was prepared in a similar manner to obtain more precise result. Foaming index was calculated using the following formula:
1000/a, where a = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1 cm is observed.

4.2.8 Elemental analysis
4.2.8.1 Microwave digestion

A model CEM MARS 5 (CEM, Matthews, NC, USA) microwave oven was used. Each sample was precisely weighed in clean Teflon digestion vessel and 5mL of HNO₃ (65%) and 3mL of H₂O₂ (30%) was carefully added, after arranging in the rotor segment, it was placed in Microwave digestion system. Blank digest was carried out in similar way. Operating program of the microwave digestion system used for the sample is given in Table 4.1. Sample solutions were cooled to room temperature and quantitatively transferred to 50 mL polythene flasks. Digestion vessels were cleaned with 5 mL of concentrate HNO₃ in the microwave vessel at 800W for 15min and then at 0W for 10min cooling. Samples were diluted with Milli Q water to obtain desired volume.

**Table 4.1** The operating program for microwave digestion of the samples

<table>
<thead>
<tr>
<th>Power</th>
<th>Ramp time (mm:ss)</th>
<th>Pressure (Psi-limit)</th>
<th>Temperature (°C)</th>
<th>Stirring</th>
<th>Hold time (mm:ss)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum power (W)</td>
<td>Utilization rate (%)</td>
<td>15:00</td>
<td>800</td>
<td>200</td>
<td>off</td>
</tr>
</tbody>
</table>

4.2.8.2 Instrumentation

The metals Al, Cu, Zn, Ca, Co, Cr, Fe, Ni, Mg, Mn concentrations were determined using Flame atomic absorption spectrophotometry (FAAS) system (AA 7000 AAS, Lab India Instrument Pvt. Ltd). Cd and Pb were determined using Graphite furnace atomic absorption spectrometry (GF-AAS) system equipped with AA700 AAS. For graphite furnace measurements, argon was used as carrier gas. Pyrolytic coated graphite tubes with platform were used. As and Hg concentrations were determined by Hydride generator (HG-
01) associated with AA7000. HCl (5%v/v) was used as carrier liquid and 2% Sodium borohydride (NaBH₄) in 0.5 % (w/v) Sodium hydroxide (NaOH) was used as reducing agent. Instrument settings and optimal conditions are summarized in Table 4.2, 4.3. Instrument was calibrated and standardized with different working standards. After ensuring the calibration of instrument and the results of the standards were within the limit, concentration of individual metals in each samples were determined. Triplicate analyses were performed for each sample. Elemental concentrations were determined on natural weight basis and expressed as mg/kg.

**Table 4.2** Instrumental Analytical conditions for heavy metal determinations by Flame atomic absorption spectroscopy

<table>
<thead>
<tr>
<th>Elements</th>
<th>Instrumental analytical conditions</th>
<th>Wavelength (nm)</th>
<th>Lamp current (mA)</th>
<th>Slit width (nm)</th>
<th>Acetylene flow (L min⁻¹)</th>
<th>Air flow (L min⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>Al</td>
<td></td>
<td>309.30</td>
<td>3.0</td>
<td>0.2</td>
<td>2.0</td>
<td>6.0 (N₂O)</td>
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<tr>
<td>Ca</td>
<td></td>
<td>422.70</td>
<td>3.0</td>
<td>0.2</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Co</td>
<td></td>
<td>240.70</td>
<td>3.0</td>
<td>0.2</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Cr</td>
<td></td>
<td>357.90</td>
<td>3.0</td>
<td>0.2</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Cu</td>
<td></td>
<td>324.70</td>
<td>2.0</td>
<td>0.2</td>
<td>1.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Fe</td>
<td></td>
<td>248.30</td>
<td>3.0</td>
<td>0.2</td>
<td>1.5</td>
<td>5.0</td>
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<tr>
<td>Mg</td>
<td></td>
<td>285.20</td>
<td>3.0</td>
<td>0.2</td>
<td>2.0</td>
<td>5.0</td>
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<tr>
<td>Mn</td>
<td></td>
<td>279.5</td>
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<td>0.2</td>
<td>2.0</td>
<td>5.0</td>
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<td></td>
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<td>5.0</td>
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<tr>
<td>Zn</td>
<td></td>
<td>213.90</td>
<td>3.0</td>
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Table 4.3 Instrumental Analytical conditions for heavy metal determinations by Graphite and hydride generator atomic absorption spectroscopy

<table>
<thead>
<tr>
<th>Heavy metals</th>
<th>Analytical technique</th>
<th>Instrumental analytical conditions</th>
<th>Heating program temperature (°C) [ramp time (s) hold time (s)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GF-AAS</td>
<td>Wavelength (nm)</td>
<td>Drying Ashing Atomization Cleaning</td>
</tr>
<tr>
<td></td>
<td>HG-AAS</td>
<td>Negative high voltage (v)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>As</td>
<td>456.24</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Hg</td>
<td>172.64</td>
<td>400</td>
</tr>
</tbody>
</table>
4.2.8.3 Preparation of standard solutions

An aliquot (1mL) of AAS standard solutions (1000 ppm) were transferred using a micropipette in 500 mL volumetric flask and solutions were individually diluted to obtain desired working concentrations with 0.5 mol/L HNO₃.

4.2.8.4 Data Analysis

Results were expressed as mean ± S.D. of triplicate data. Correlations were analyzed using Pearson’s correlation in GraphPad Prism version 5.00 for windows (GraphPad Software, San Diego, CA). Level of significance was set at P <0.05.

4.3 RESULTS AND DISCUSSION

4.3.1 Morphology and Microscopy

Morphologically minor differences were observed like the shape of the leaf; *M. vaginalis* is cordate (heart shape) and *M. hastata* is sagittate (arrow shape), longer petioles, sub-umbellate inflorescence and denser rootstock of *M. hastata* and short petioles with solitary flowers and narrow rootstock with comparatively less root hairs, in case of *M. vaginalis*.

*M. vaginalis*-Leaves are variable in size, 5-10 by 3.2-5cm, from linear to ovate or ovate-cordate, usually acuminate (Figure 4.2 a, b); petioles of the lower leaves are long, stout and terete, the peduncles emerge from the channeled sheaths of the uppermost leaves. Inflorescence is centripetal; flowers are blue, usually spotted with red; in subspicate racemes with one large anther and 5 small anthers. Ovary is ellipsoid and glandular. Fruit is ellipsoid, glandular outside. Seeds are 0.8 mm long, ellipsoid, rounded at each end, pale, with many brown ribs. Root stock is short, sub-erect and spongy (Figure 4.2 c).

*M. hastata*-An emergent aquatic herb with stem approximately 0.7-1.2m long (Figure 4.2 d). Leaves long-petioled, sagittate, hastate or cordate; the basal leaves are arrow-shaped (Figure 4.2 e). The inflorescence of 25-60 flowers is in a dense spike 6-9cm long. The flowers are purplish blue or violet blue dotted with red, 13-16mm long in sub-umbellate racemes; One anther is coloured blue, 6mm long; the other 5 anthers are yellow and 4 mm long. The seed capsule is 7mm long and 5-6mm diameter. Rootstock is dense, long, sub-erect and spongy with numerous lateral roots (Figure 4.2 f).
Figure 4.2 Comparative morphology of *Monochoria* species- *Monochoria vaginalis* – plant with leaves & flowers, (a) Cordate leaf (b) rootstock (c). *Monochoria hastata* - whole plant (d) sagittate leaves & flowers (e) rootstock (f). Fl-Flower; Cle- Cordate leaf; SLe- Sagitate leaf; Rs-Rootstock; Pe- Petiole; Sr-Submerged rootstock, In-Inflorescence. Scale bars: (a, b, c, d, e & f) = 5mm

4.3.1.1 Anatomical Features of Leaf

(a) *M.vaginalis*

Surface view revealed squarish epidermal cells with a very thin cuticle. The leaf exhibits hydromorphic features with numerous wide air chambers, poorly developed xylem elements, reduced sclerenchyma cells and amphistomatic (stomata found on both upper and lower surfaces) lamina. The leaf has less prominent vein with distinct vascular bundle (Figure 4.3a) and the region of the midrib is 700µm thick with fairly large epidermis. The vascular bundle of the midrib is collateral consisting of few thin walled, angular xylem elements and thick mass of phloem elements (Figure 4.3b). Poorly lignified, thin layer of
sclerenchyma elements encloses the vascular bundle, which in turn is embedded in a thick sheath of parenchymatous ground tissue.

(b) *M. hastata*

The leaf exhibits hydromorphic features. Midrib is not prominent; it projects in to short, semicircular abaxial surface and a flat adaxial surface (Figure 4.3e, g). The midrib is 700µm thick, and the abaxial side of the midrib is 250 µm wide. The vascular bundle of the midrib consists of few xylem elements of which the meta-xylem element is the wide, circular and thin walled. Phloem occurs beneath xylem strand in the form of a circular mass. The entire vascular bundle is surrounded by two or three layers of fibres (Figure 4.3h).

4.3.1.2 Anatomical features of Lamina

(a) *M. vaginalis*

The lamina is dorsiventral, adaxial epidermis consists of thick rectangular cells, whereas the abaxial epidermis possesses cylindrical cells. Stomata are seen on both the sides. A narrow zone of thin, loosely packed palisade cells occurs below the abaxial epidermis (Figure 4.3c). The median part of the lamina contains a horizontal row of wide air-chambers, divided by vertical partitions. Vascular bundles are distributed both in the adaxial, abaxial regions and in the vertical partitions. The vascular bundles are collateral with a few wide, thin walled xylem elements and a small nest of phloem elements. The vertical partitions of the air-chambers possess prominent vascular strands with parenchymatous bundle sheath. The strands have two or more wide, thin walled Meta-xylem elements and small mass of phloem elements situated on the outer part, facing the palisade zone of the lamina (Figure 4.3g).

(b) *M. hastata*

Lamina is uniformly smooth, even and isobilateral, amphistomatic and hydromorphic. The lamina is 320 µm thick. The adaxial epidermis is thin and the cells are cylindrical; stomata occur at the level of the epidermis. The abaxial epidermis is also thin and the cells are squarish and thin walled. Mesophyll tissue is differentiated in to adaxial zone of vertically elongated pillar like, compact palisade cells and shorter, cylindrical palisade cells in the abaxial side. Median part of the lamina possesses wide circular air chambers divided by vertical partition filaments. Small or large vascular bundles are located
within the partitions and the vascular bundles are collateral with wide meta-xylem element and narrow protoxylem elements. The vascular bundles are enclosed in a sheath of parenchyma cells. The marginal portion of the leaf is conical, measuring 150 μm thick. The epidermis along the marginal part consists of squarish thick walled cells. Air-chambers are absent in the marginal part and the vascular bundles are reduced in size (Figure 4.3f).

4.3.1.3 Raphides

(a) *M.vaginalis*
Calcium oxalate crystals of raphides are frequently seen in the mesophyll tissue of the midrib. The raphide is a cylindrical bundle of many thin pointed needles (Figure 4.4a). Raphides are 20 μm thick and 50 μm long.

(b) *M.hastata*
Calcium oxalate, needle shaped crystals are seen in thick long cylindrical bundles. The needles are thin with pointed ends, they aggregate into compact bundles and these raphide bundles are diffusely distributed in the lamina and are varied in orientation, they appear as bright bundles when viewed under polarized light (Figure 4.4d). The raphides are 130 μm long and 20 μm thick.

4.3.1.4. Epidermal cells and stomata

(a) *M.vaginalis*
Para dermal sections were used for studying the stomatal type and epidermal cells. The stomata are present on both upper and lower sides of the lamina. The stomata are brachy- paratetracytic type (Figure 4.3d). A stoma has two lateral subsidiary cells and two larger polar subsidiary cells situated on the upper and lower poles of the guard cells. The guard cells are oblong, elliptic measuring 20×50 μm in size. The epidermal cells are fairly thick walled, angular and compact. (Figure 3e).

(b) *M.hastata*
Stomata occur on both the upper and lower surfaces of the lamina. They are diffuse and random in distribution. The stomata are either paracytic or cytocytic, the former being more in frequency (Figure 4.3i), the paracytic stoma has two wing like subsidiary cells, one on either side of the guard cells and parallel to the guard cells (Figure 4.3j). In the cytocytic
type, a stoma is surrounded by four subsidiary cells, two cells being polar and other two being lateral in position. The guard cells are 15×30 μm in size. The epidermal cells are polyhedral in outline; the anti-clinical walls are thick and straight.

4.3.1.5 Crystals

(a) *M. vaginalis*

Calcium oxalate crystals are abundant, especially, in the mesophyll tissues of the lamina (Figure 4.4c) the crystals are mostly raphides which are thick cylindrical bundles of several thin needles; occasionally spherical spiny balls of crystals called druses are also observed with the needle shaped bundles. The raphides are 40× 80 μmm in size (Figure 4.4b).

(b) *M. hastata*

Calcium oxalate crystals of prismatic type are randomly distributed in the mesophyll tissue of the leaf (Figure 4.4e), the crystals are solitary and sometimes occur in large aggregates (Figure 4.4f).
Figure 4.3 Comparative anatomy of leaf- *M. vaginalis* - Transverse section through midrib (a), Mid rib (b), Lamina (c), Paradermal peeling of epidermis (d), Stomata enlarged (e). *M. hastata* - Transverse section through midrib (f), Mid rib (g), Lamina (h), Paradermal peeling of epidermis(i), Stomata enlarged (j). Ab-Abaxial side; Ac-Air chamber; Ad-Adaxial side; Mr-Mid rib; Vb-Vascular bundle; Pf-Partition filament; Ph-Phloem; Xy-Xylem; Ade-Adaxial epidermis; ABe-Abaxial epidermis; Sl-Sclerenchyma; Ep-Epidermis; Pm-Palisade mesophyll; St-Stomata; La-Lamina; Lv-Lateral vein; Bs-Bundle sheath; Sc-Subsidiary cell. Scale bars: a, g-1mm; b-350µm; c, d, h, i, j-250 µm; e -100 µm.
4.3.1.6 Anatomical features of Petiole

(a) *M. vaginalis*

In sectional view, the proximal part of the petiole is circular. Petiole is 1.9mm thick continuous layer of small epidermal cells followed by two or three layers of compact parenchyma cells. The remaining major part of the petiole has a large number of circular or
polygonal air-chamber divided by thin uniseriate partition filaments (Figure 4.5a). Situated within the junctions of the partition filaments are circular vascular strands possessing, thin walled xylem elements and small clusters of phloem (Figure 4.5b). The vascular bundles in the central part of the petiole are larger than those in the peripheral part (Figure 4.5c). Raphide types of crystals were commonly observed in the cells of the partition filaments. Distal part of the petiole has a wide central canal surrounded by three or four layers of smaller air-chambers with uniseriate filaments; vascular bundles have well developed xylem and phloem elements. The air-chambers possess thin plate stellate parenchyma cells forming thin diaphragm (Figure 4.5b).

(b) *M. hastata*

Petiole is elliptical in sectional view, 2.6 mm in horizontal plane and 1.7 mm in vertical plane (Figure 4.5d). It consists of small squarish epidermal cells with thick cuticle. The ground tissue consists of outer dense zone of short palisade cells and with thin palisade cells in the inner zone, followed by a wide region of three layers of air-chambers, divided by thin uniseriate partition filaments. Located in the intercepts of the partition filaments are collateral vascular bundles (Figure 4.5f) with two or three wide circular xylem elements and a small mass of phloem elements. The vascular strand is enclosed by thick layer of fibres (Figure 4.5e). The central core of the petiole is occupied by wide circular air-canal (Figure 4.5d) the air-chambers have thin membranous plate of circular cells; these plates are called diaphragm.
Figure 4.5 *M. vaginalis*—Transverse section of petiole- Partial view (a), Marginal view (b), Central vascular bundle region (c), *M. hastata*-Transverse section of petiole- Entire view (d), Marginal view (e), Central vascular bundle region (f). Ep-Epidermis; Ac-Aerenchyma; Pf-Partition filament; CVb-Central vascular bundle; OVb-Outer vascular bundle; Ra-Raphides; Xy-Xylem; Ph-Phloem; CAc-Central aerenchyma; OAc-Outer aerenchyma; Dp-Diaphragm. Scale bars: a, d-1mm; b, c, e, f-350 μm.
4.3.1.7 Anatomical features of Stem

(a) *M.vaginalis*

Stem is circular or elliptical in sectional view, 3 mm thick consisting of wide aerenchymatous cortex with central stele of vascular elements (Figure 4.6a). Vascular elements are present in both peripheral and middle portions of the cortex (Figure 4.6b). The central stele has wide circular xylem elements and small group of phloem elements in the outer boundary of the stele. The central part of the stele has small air-chambers and parenchymatous ground tissue. Small vascular strands are distributed in the central ground tissue of the stele (Figure 4.6c). Starch grains are observed in the ground parenchyma of the stem.

(b) *M.hastata*

The cross sectional outline of the stem is circular and similar to that of the petiole excepting the absence of wide central canal in the stem. The stem is 3mm thick. The epidermal layer is intact, comprising thick walled squarish cells (Figure 4.6d). The epidermis is deeply sunken forming a wide cavity; within this cavity occurs the stoma (Figure 4.6e), along the inner part of the epidermis is seen a thick, continuous cylinder of chlorenchyma cells. Small circular vascular bundles are embedded in the chlorenchyma cylinder; these vascular bundles possess parenchymatous bundle sheath. The entire central part of the stem has dense reticulate, uniseriate partition filament which enclose wide circular air-chambers (Figure 4.6d). In the intersections of the partition filaments occur a vascular bundle, with wide one or two xylem elements and small cluster of phloem elements. The vascular bundles have sclerenchyma caps or parenchyma bundle sheath (Figure 4.6f)
Figure 4.6 Comparative anatomy of stem- *M. vaginalis*-Partial view (a), Central zone (b), Central bundle (c). *M. hastata* - Partial view (d), Marginal part (e), Central bundle (f) Ep-Epidermis; Cb-Cortical bundle; CVb-Central vascular bundle; OVB-Outer vascular bundle; SL-Sclerenchyma; St-Stomata; Ac-Aerenchyma; Pf-Partition filament; Xy-Xylem; Ph-Phloem; Bs-Bundle sheath. Scale bars: a, d- 1mm; b, e-350 μm; c, f- 250 μm.
4.3.1.8 Anatomical features of Rhizome

(a) *M. vaginalis*

The rhizome has thick epidermal layer of papillate cells with wide aerenchymatous cortex. The air-chambers of the cortex are divided by thick uniseriate large celled partitions; (Figure 4.7a) large and prominent vascular strands are sparsely distributed in the cortical region.

Lateral roots are seen emerging from the central stele of the rhizome (Figure 4.8a). The lateral roots are fully developed before they emerge from the cortex of the rhizome. The lateral roots observed in the cortex region are circular and have well developed epidermis with two layers of compact outer cortical cells, middle wide zone of radially elongated narrow air-chambers divided by radial uniseriate partition filaments and inner four or five layers of compact parenchyma cells (Figure 4.7d).

In the central core of the stele occur, wide, circular xylem elements and phloem elements in the outer zone (Figure 4.7b). The central stele consists of peripheral xylem strands and phloem strands (Figure 4.7c). Stellar lamelles are present in the central part, which include well developed angular xylem elements and prominent mass of phloem elements. Starch grains occur densely in the cortical cells and in the central ground parenchyma cells (Figure 4.7b). Raphide bundles of calcium oxalate needles are frequently seen in the rhizome.

(b) *M. hastata*

The rhizome has thin continuous epidermal layer of small squarish cells, wide aerenchymatous cortex and solid stellar cylinder. The cortex consists of about 15 layers of large polygonal air-chambers, separated from each other by thin uniseriate partition filaments. In the aerenchymatous cortical zone occur, many circular vascular bundles which are located in the place where the partition filaments unite (Figure 4.7d). Vascular bundles in the periphery of the rhizome are smaller and the size of the bundles increases progressively towards the centre with few vertical row of proto-xylem elements. Phloem occurs as circular mass xylem. Thick mass of bundle cap fibres encloses the vascular bundles (Figure 4.7f) Two layers of pericyclic fibers and endodermis is observed in the stellar region of the rhizome. Along the outer part of the stele and adjoining the pericycle
occur tangentially flat dense masses of xylem elements and phloem tissue in a collateral manner (Figure 4.7e). Small collateral vascular bundles are scattered in the ground tissue of the stele. Starch grains are densely filled in the partition filaments of stellar region.

4.3.1.9 Anatomical features of Root

(a) *M.vaginalis*

The roots that are seen in the surface of the rhizome are either thin or thick. The thin roots have quite wide, thin walled central meta-xylem elements and phloem strands in between the proto-xylem elements. The cortex is aerenchymatous (Figure 4.8a). The thick rows have three layers of compact inner cortical cells, prominent layer of endodermis, and thin layers of pericycle. The xylem elements have central wide meta-xylem cells and outer several protoxylem elements alternating with phloem elements (Figure 4.8b, c).

(b) *M.hastata*

Lateral roots emerge from the stele and run through the cortex, lateral roots have distinct and prominent epidermal layers with aerenchymatous cortex and dense circles of spherical parenchyma cells enclosing the stele (Figure 4.8d, e, f).
Figure 4.7 Transverse section of rhizome- *M. vaginalis* - Outer zone (a), Outer zone of stele portion (b), Central zone of stele portion (c), *M. hastata* - Cortical aerenchymatous region (d), Central stelar region (e), Central vascular bundle region (f), Central vascular bundle region. Sg-Starch grains; OVb- Outer vascular bundle; Xy- Xylem; Gt-Ground tissue; CVb- Central vascular bundle; Sl-Slerenchyma; Pf-Partition filament; En- Endodermis; Ep-Epidermis; Ac-Air chamber. Scale bars: a, e -250 μm; b, f- 100 μm; c-1mm; d-350 μm.
Figure 4.8 Comparative anatomy of root- *M. vaginalis*-Transverse section of rhizome showing origin of lateral roots (a), transverse section of the root embedded in the cortical zone of rhizome (b), Central vascular bundle region of the root (c). *M. hastata*-Transverse section of rhizome showing origin of lateral roots (d), Transverse section of the root embedded in the cortical zone of rhizome (e), Central vascular bundle region of the root (f). McO-Middle cortex; OC0-Outer cortex; Xy-Xylem; Ph-Phloem; En-Endodermis; Mxy-Metaxylem; Pxy-Protoxylem; Ro-Root; ACo-Aerenchymatous cortex. Scale bars: a, d- 250 μm; b, e-100 μm, c,f-1mm.
4.3.10 Anatomical features of Flower

(a) *M.vaginalis*

The flowers are bisexual and possess 6-perianth members, 6 stamens and tricarpellary, syncarpous ovary with many ovules. In cross sectional- view, the flower show 6 perianth members which include three outer and three inner members (Figure 4.9a) the perianth members are thick in the middle and generally thin on the lateral margins. Perianth has several vascular strands situated all along the median portions. Alternating with the vascular strands are wide chambers. Vascular strands are collateral having a small group of xylem elements and phloem elements. The ground tissue is parenchymatous and compact. The wall of the ovary is uniformly 100 µm thick, consisting of thin inner and outer epidermisis of small thin walled cells. Small vascular strands are seen in the median part of the ovary wall (Figure 4.9b). The ovary has three carpels and the axial placentum is divided in to many thin radial segments. The ovules are attached on the surface of lobed placentum.

(b) *M.hastata*

The flower is epigynous with inferior ovary. The ovary is tricarpellary, syncarpous and three celled. The axile placental tissue is forked and intrudes into carpel, the seeds are borne at tips of the forked placenta (Figure 4.9e,f)Numerous seeds are observed in each carpel.

4.3.11 Anatomical features of Fruits and seeds

(a) *M.vaginalis*

The ovary develops into a capsule in which numerous seeds are attached on the placentum with long funicle (Figure 4.9c) the seeds are cylindrical measuring 800 µm long and 350 µm thick. Seed is densely endospermous, the endosperm is nuclear type lacking cell walls and cells. The seed has thin seed coat with small lignified cells and 10 short conical ridges found along the circumference. The embryo is cylindrical with long thick cotyledon (Figure 4.9d)
Figure 4.9 Comparative anatomy of flower and fruit- *M. vaginalis* - Transverse section of flower showing perianth and ovary (a), Transverse section of ovary (b), Longitudinal section of fruit (c), Transverse section of seed (d). *M. hastata* - Transverse section of gynoecium showing central axile placenta and branched intruding placental tissues with seeds attached on the branches (e), Transverse section of ovary- one carpel with seeds (f), Longitudinal section of ovary (g). Transverse section of seed (h). Vs-vascular strand; Ip-Inner perianth; Ov-Ovule; Op-outer perianth; Pl-Placentum; Em-Embryo; En-Endodermis; OVw-Ovary wall; OpE-Outer epidermis; IPE-Inner epidermis; OSE-Outer seed coat; Se-Seed; Sc-Seed coat; Ri- Ridge; Vs-Vascular strand; Pc-Pericarp. Scale bars: a, b, c, d, e, f, g-1mm; d, h-350μm.
(b) *M. hastata*

Seeds are cylindrical and thick. They are 500×800µm in size. They are endospermous. The seed coat consists of an epidermal layer of narrow thin cells, a middle layer of osteosclereids and inner thin layer of thick walled, darkly stained cells. The outer epidermal layer has about 14 ridges and shallow furrows. The seeds contain copious endosperm with outer thin layer of small thick walled cells, which become vertically elongated in the region of ridges (Figure 4.9g, h)

4.3.2 Powder microscopy

4.3.2.1 *Monochoria vaginalis*

(a) Veination pattern

The lamina exhibits several straight vertical, parallel and thick major veins. The major vertical veins are interconnected by several thinner horizontal and vertical veins (Figure 4.10a), thus a reticulate system comprising several rows of regular squarish vein islets (Figure 4.10b) the islets are fairly wide and open; but no vein terminations are evident. The powder preparation of *M. vaginalis* exhibits the following inclusions.

(b) Fibres (Figure 4.10c)

Long fibres with thick and narrow wall were observed. The cell lumen is wide, pits were not evident, and some granular inclusions were observed in the lumen. The fibre is 630 µm long and 30 µm wide (Figure 4.10d).

(c) Xylem elements

The veins of the leaf possess thick bundles of several vascular elements. The elements are xylem cells with annular, spiral and reticulate lateral wall thickenings, (Figure 4.10d) these are also isolated vessel elements, which possess distinct scalariform thickenings on the lateral walls (Figure 4.10e).

(d) Parenchyma cells

Two types of parenchymal cells were observed in the powder. Some of the cells are spherical in shape, possessing darkly stained cell contents and occur in groups. The cells are 100 µm in diameter. Parenchyma cells were vertically elongated, rectangular and thin walled occurring in vertical rows, (Figure 4.10f) aggregated in to thick bundles. The cells have prominent nuclei and granular cytoplasm.
(e) *Brachysclereids*

Semicircular two cells, attached with each other are common in the powder the dual cells are highly thick walled and are scleroids; they are half spherical in shape and hence they are called brachysclereids. The cell walls are lignified, the cell lumen is wide. They have wide circular simple pits (Figure 4.10g, h, i) the cells are 70 µm in diameter.

4.3.2.2 *M. hastata*

(a) Abaxial epidermis

The abaxial lower epidermis is stomatiferous. The epidermal cells are polyhedral is out line with thin and straight anticlinal walls. The stomata are fairly dense and diffused in distribution (Figure 4.11a). The stomata are paracytic type. These are two rectangular subsidiary cells which occur parallel to the long axis of the guard cells. (Figure 4.11b, c). Stomatal opening is normal and slit like. The guard cells are 20 ×40 µm in size.

(b) Adaxial epidermis

The adaxial epidermis consists of vertically elongated straight, compact rectangular thin walled cells. The epidermis is apostomatic (Figure 4.11f). The cells are up to 260 µm long and 40 µm wide.

(c) Veination pattern

The leaf exhibits several straight thin, unbranched veins. The distance between two adjacent veins is uniform (Figure 4.11d). In the powder preparation of the leaf, thick bundles of xylem elements are observed. The xylem elements has spiral, annular and scalariform lateral wall thickenings (Figure 4.11i, j). The powder exhibits leaf epidermal layer in lateral view and a layer of vertically elongated columnar palisade cells (Figure 4.11g). The epidermal cells are 50 µm thick and palisade cells are 220 µm in height.

(d) Calcium oxalate crystals

Calcium oxalate crystals of thin needles are seen in bundles. The bundles are elongated and uniform in thickness. The bundle is also seen as broken individual needles. The bundles are called raphides and they are scattered in the powder (Figure 4.11k, l). Spherical thin walled parenchyma cells are seen scattered in the powder. Some of the parenchymal cells have tannin content and others have granular protoplast (Figure 4.11h).
Figure 4.10 Powder microscopy of *M. vaginalis*- Lamina showing parallel vein and squarish vein islets (a), Lamina enlarged (b), Fibre enlarged (c), Bundle of xylem elements (d), Xylem with scalariform thickenings (e), Spherical parenchyma (f), Double brachysclereids (g, h, i) Vv-vertical major veins; Hv- Horizontal veins; Fi-Fibre; Ve-Vessel; Xe-Xylem element; Pa-Parenchyma; Spa- Spherical parenchyma; SCI-Sclereid; CLu-Cell Lumen; Cw-Cell wall. Scale bars: a-1mm; b, f-350 μm; c, d, e, g, h, i- 100 μm.
Figure 4.11 Powder microscopy of *M. hastata* - Abaxial epidermis-surface view (a), Stomata and epidermal cells enlarged (b), Single stomata enlarged (c), Lamina showing veination pattern-unbranched parallel veins (d), Veins under polarized light (e), adaxial epidermis-surface view (f), Epidermal cells with palisade cells (g), Spherical parenchyma (h), Vascular strands of veins (i), Lateral wall thickenings of xylem vessel elements (j), Raphides under polarized light (k), Scattered raphides (l). Ec- Epidermal cells; Ge-Guard cells; Sc-Subsidiary cells; St-Stomata; Ve-Veins; AdEc-Adaxial epidermal cells; Xe-Xylem elements; Ep-epidermis; Pe-Palisade cells; Pa-Parenchyma; Ta-Tannins; Ra-Raphides. Scale bars: a, g, h, j, k- 250μm; b-100 μm, c-70 μm; d, f, j, l- 350 μm; e- 1mm.
4.3.3 DNA barcoding

4.3.2.1 PCR amplification and bidirectional sequencing of rbcl

Identification of two species was further confirmed by DNA barcoding of both the plants. Success of PCR amplification and sequence recoverability is an important criterion for assessing the utility of DNA barcodes. rbcL barcode marker was amplified using universal primer pairs and standard protocols. There was no variation in sequence length for rbcL; bidirectional sequencing recovered the 365 base pair target sequence for all the PCR amplifications. The rbcl fragment was successfully amplified for both *M. vaginalis* and *M. hastata*. High bidirectional sequences obtained from rbcl for both the plants confirmed its identity at genus level (Figure 4.12, 4.13). NCBI search confirmed their identity by giving 100% match for *M. hastata* and 99% match for *M. vaginalis*.

4.3.4 Proximate Analysis

The proximate composition of different parts of *M. vaginalis* and *M. hastata* was presented in Table 4.6, 4.7. Ash values and moisture content were high in the stem of both the species of *M. vaginalis* and *M. hastata*, whereas high water soluble extractive values of rootstocks indicate the presence of polar compounds in the rootstock of both the species. Foaming index represents the presence of saponins in all the parts.
**Figure 4.12** DNA barcoding of *M. vaginalis*—rbcl sequence of *M. vaginalis* (a), DNA chromatogram (b), BLAST report of rbcl sequence (c).
Figure 4.13 DNA barcoding of *M.hastata* - rbcl sequence of *M.hastata* (a), DNA chromatogram (b), BLAST report of rbcl sequence (c).
### Table 4.4 Proximate composition of *Monochoria vaginalis*

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<td>Moisture content (%w/w)</td>
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### Table 4.5 Proximate composition of *Monochoria hastata*

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4.3.5 Elemental analysis

Medicinal plants growing in nature accumulates poisonous elements based on their individual properties, concentration of metals in air, soil and water, plant species, and other environmental factors [88]; Chemical elements, both essential and non-essential may have toxic effects on plants and to humans, if present in higher concentrations [89]. The variability in elemental composition of various parts of *Monochoria hastata* and *Monochoria vaginalis* was presented in Table 4.8, Figure 4.14.

Aluminium (Al) with an atomic number of 13 is a member of boron group of chemical elements; it is the most inexhaustible metallic element in earth crust [90]. Al is present in water, soil and air but most of it are merged in to alumino-silicate soil minerals and at sub-micro molar levels appears in soluble forms competent to influence the biological systems [91] plants are classified as Al accumulators (≥1000 mg kg\(^{-1}\)) and Al non-accumulators (<1000 mg kg\(^{-1}\)), based on concentration of Al in the leaves of different species [92]. Al content was high in roots of both the species followed by stem and leaf. This may be due to poor transport of metal to the aerial parts. Among the parts of the two species, Al content was ranged in the following order MHR>MVR>MVS>MHS>MVL>MHL. From the data observed that, both the species are Al non-accumulators and their Al content was within permissible limits.

Arsenic (As) is an environmental noxious substance that is found in most soils [93]. It enters food chain through contaminated crops and fodder. The metalloid invades in the farming systems through various means, which include natural geochemical process. Due to its cancer-causing properties, its exposure is of great concern [94]. As a human toxin, it has conceivably influenced human history, more than any other noxious element [95]. A recent finding state that, rice can efficiently accumulate arsenic from the paddy fields, which is of great concern, since rice plays a major role in the food chain [96]. Contamination of medicinal plants with toxic metals varies between plant species and in our study, As was not in detectable limit in any of the parts.

Cadmium (Cd) in soils is acquired from both natural and anthropogenic sources. Large amount of Cd is spread in the environment (about 25,000 ton a year) by various means, like, weathering of rocks, forest fires and volcanoes. 0.1 to 0.5 ppm is the average
natural abundance of Cd in the earth’s crust, Cd is a naturally prevailing component of soil and therefore humans are exposed to natural levels of Cd. It has been reported that Cd is accumulated in leafy vegetables and potato tubers at higher levels than cereals and fruits [97]. Due to its solubility in water, it is promptly adsorbed in tissues and greatly influence the metabolism of plants. Homeostasis in plant shoot, mineral nutrition, root growth and development is greatly affected due to the transport of Cd to the vegetative and reproductive parts of the plants [98]. Our study revealed that, Cd was not in detectable limit in any parts.

Calcium (Ca) is an essential plant nutrient, playing a major role in cell wall formation, stability, maintenance of membrane structure and permeability; it triggers enzymes and tunes the response of cells to stimuli [99]. Plants germinating with sufficient Ca in their natural habitats will have shoot Ca concentrations between 0.1 and 5% dry weight. Ca deficiency is infrequent in nature, but can occur on acidic soils with low base saturation [100]. Ca content among the various parts of two Monochoria species was in the following range, MVR > MHS > MHR > MVS > MVL > MHL.

Chromium (Cr), a transition metal is a major source of environmental pollution. The compounds of hexavalent chromium are relatively more toxic than trivalent chromium; due to its subsequent interactions with cellular proteins, nucleic acids and its rapid permeability through biological membranes in plant systems [101]. Cr is widely diffused in soil, water and biological material and prevails in the range of 5 to 1000 ppm in soil [102]. Cr is linked with glucose tolerance factor and is connected with carbohydrate metabolism in humans and animals. Cr supplement improves and normalize the impaired glucose tolerance in some diabetic people. Present study reveals that Cr content was more in rootstock, when compared to other parts M. hastata and in case of M. vaginalis, leaf contains more amount of Cr, the content varied in the following order, MHR > MVL > MHL > MVS > MVR > MHS.

Cobalt (Co) is an indispensable element for humans, animals and prokaryotes, but its physiological role in plants has not been revealed. Normal Co concentration in plants are quoted to be as low as 0.1-10 mg Kg$^{-1}$ dry weight[92]. Co content in the different parts of Monochoria species ranged as MHL > MVR > MVS > MVL > MHR > MHS.

Copper (Cu) is a vital micronutrient for normal plant growth and development, it involves in photosynthetic electron transport, hormone signaling, mitochondrial respiration
Cu ion plays an important role as Cofactors in many enzymes, such as cytochrome c oxidase, Cu/Zn superoxide dismutase (SOD), amino oxidase, polyphenol oxidase and plastocyanin [104]. Rootstock of M. vaginalis and M. hastata showed greater amount of Cu content than other parts, Cu content was found to be more in the stem than leaves. MHR> MVR > MVS> MHS > MHL > MVL.

Iron (Fe) is an essential element for plant growth, it is highly reactive and at the same time toxic via Fenton reaction. Accordingly, plants strictly control iron homeostasis and react to iron deficiency and to its overload. The capability of plants to respond to Fe availability eventually affects the nutrition of human in crop yield and its concentration in edible tissues [105]. Whilst Fe itself is not considered to be toxic, it is environmentally significant due to its interaction with other toxic metals. Iron oxides adsorb many elements and engage in the attenuation of most trace and heavy elements [105]. Fe was almost present in all parts of the plants; in particular the rootstocks showed more Fe content when compared to other parts. Fe content ranged in the following order, MVR > MVL>MHL >MHS >MVS > MHR

Lead (Pb) is one of the crucial element since antiquity and has acquired considerable significance as environmental pollutant, as it is most toxic and frequently encountered [106]. The consequence of environmental Pb contamination results both from its perseverance and from its current and past numerous sources [107]. In spite of its long history of its advantageous use for humans, it does not possess any biological function for living organisms [108]. Approximately 70 % of the total Pb intake by humans results from food and water. Infants and young children are more vulnerable than adults to the toxic effects of Pb, since it can easily cross the placental barrier [109, 110]. Pb was found to be present in all the analyzed samples ranging from 0.29 to 0.16 mg kg^{-1}. The highest content was in M. vaginalis leaf and the lowest in M. hastata stem. The decreasing order of Pb content is MVL>MVR>MHR > MHL > MVS > MHS.

Magnesium (Mg) is an essential element in biological systems, it occurs usually as Mg\(^{+}\) ion [111]. Most of the physiological functions of plants demand adequate Mg supply, the most noticable, being its role in chlorophyll, root formation and photosynthesis.
content of Mg in *Monochoria* species varies in the following order MHL > MVR > MHS > MVS > MVL > MHR.

Manganese (Mn) is a crucial micronutrient in most organisms. Mn plays a major role in photosynthesis as it is required for the water-splitting system of photosystem-II (PS-II), which provides electrons necessary for photosynthetic electron transport [112]. Mn is required for the biosynthesis of aromatic amino acids (tyrosine), chlorophyll, secondary products, like lignin and flavonoids [113]. High concentration of Mn was found in certain foods of plant origin, especially rice and wheat, with concentrations ranging between 10 mg Kg$^{-1}$ to 100 mg kg$^{-1}$. The chief recipients of Mn toxicity are the brain and central nervous system. When deposited in important parts of the brain and central nervous system, it shows symptoms of impaired neurological and neuromuscular control [114]. Content of Mn among the two species vary in following order, MHR>MVL>MHL>MVS>MVR>MHS.

Among metals Mercury (Hg) is unique and is present in the environment in several physical and chemical forms. Hg metal is different than other transition metals, two important properties of this heavy metal is that, high solubility in water and easiness in shifting to gaseous state. These properties explain the potential and effectiveness of Hg to advance in varied ecosystems and to remain in the atmosphere for prolonged periods, being slowly deposited in the soil or water bodies [115]. The binding of Hg to DNA results in potential toxic effects; chronic intake of methyl-Hg$^{2+}$ at subtoxic levels result in chromosomal damage in humans, persumably due to its direct interaction with DNA. Since mercury can cross blood brain barrier and affect brain development, its effects are of special concern to pregnant or lactating women and young children [116,117]. Hg was not in detectable limit in any of the parts of *Monochoria* species.

Nickel (Ni) holds an exceptional place among the heavy metals. Unlike other heavy metals (Pb, Cd, Hg, As) which are not the constituents of plant enzymes, Ni plays an integral part of urease and small quantity (0.01 to 5µg/g) is essential for some plant species [118]. Normal heavy metal contents of terrestrial plants growing in uncontaminated soils were found to be in the range of 0.1 to 3.7mg kg$^{-1}$ for Ni. Weathering of rocks, inflow of particulate matter and precipitation are the major sources of Ni in water. Anthropogenic sources of Ni correspond from the burning of coal and other fossil fuels and discharges from
industries as electroplating and smelting [119]. Ni is an allergen and a potential immunomodulatory and immunotoxic agent in humans [119]. Ni content among the parts of *Monochoria* species was in the following order, MHR>MVS>MVL>MHS>MVR>MHL.

Zinc (Zn) is mandatory for the growth in animals, human beings and plants, it plays a vital role in crop nutrition and required in various metabolic processes enzymatic reactions and oxidation-reduction reactions. Zn deficiency is common in humans, animals and plants. More than 30% world ‘population suffers from Zn deficiency [120]. Zn is extraordinarily useful in biological systems, it is involved in many biochemical processes, which support life and required for a host of physiological functions including normal immune function, neurosensory function such as cognition and vision [121]. Although Zn is relatively non-toxic, current studies increasingly show that free ion zinc (Zn$^{2+}$) is a potent killer of neurons and other cell types. Zn concentration in the brain is perpetuated within narrow range of 600-800ng/L with deviations considerably above and below this range being pro-convulsive and cyclolethal respectively [122]. Zn was observed in all parts of the *Monochoria* species in the following order, MVR>MHR>MHL>MVL>MVS>MHS.

Figure 4.14 shows the comparative elemental profile of different parts (Leaf, Stem, and Rootstock) of *Monochoria vaginalis* and *Monochoria hastata* respectively. It was observed that Cu and Fe was the most abundant trace element in all the parts. Mg, Co, Al, Pb, Mn, Cr were found to be distributed more in the leaf of both the species, whereas Ca, Cu, & Zn were predominant in the rootstock. Trace element distribution in the stem was in consistent with leaf and rootstock.

A linear correlation test was performed to investigate the correlation between metal concentrations. The values of correlation coefficient between metal concentrations among the two species are given in Table 4.9. There was a good correlations between copper and zinc (r=0.8843), there were positive correlations between cobalt-chromium, lead-copper, zinc-calcium, cobalt-zinc, lead-iron with corresponding r values of 0.9071, 0.08709, 0.6739, 0.8156, 0.8325 respectively. The negative correlations between iron-nickel, chromium-magnesium, cobalt-nickel were found to be – 0.6185, - 0.7562, -0.1175 respectively.
Table 4.6 Elemental levels (mg/kg) in different parts of *M. vaginalis* and *M.hastata*

<table>
<thead>
<tr>
<th>Elements</th>
<th>MHL (mgkg⁻¹)</th>
<th>MHS (mgkg⁻¹)</th>
<th>MHR (mgkg⁻¹)</th>
<th>MVL (mgkg⁻¹)</th>
<th>MVS (mgkg⁻¹)</th>
<th>MVR (mgkg⁻¹)</th>
<th>Normal range in plants (mgkg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminium (Al)</td>
<td>0.7876±0.003</td>
<td>0.2406±0.001</td>
<td>0.3568±0.003</td>
<td>0.6688±0.001</td>
<td>0.3276±0.002</td>
<td>0.4437±0.002</td>
<td>200–≥1000</td>
</tr>
<tr>
<td>Arsenic (As)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>0.6253±0.464</td>
<td>1.8367±0.008</td>
<td>1.2967±0.001</td>
<td>1.0130±0.001</td>
<td>0.9191±0.000</td>
<td>3.352±0.006</td>
<td>1830.2–2042.5</td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>Cobalt (Co)</td>
<td>0.2201±0.002</td>
<td>0.0515±0.003</td>
<td>0.0682±0.004</td>
<td>0.0712±0.002</td>
<td>0.0807±0.002</td>
<td>0.1076±0.003</td>
<td>0.1–10</td>
</tr>
<tr>
<td>Chromium (Cr)</td>
<td>0.1190±0.005</td>
<td>0.0417±0.019</td>
<td>0.2500±0.032</td>
<td>0.1488±0.040</td>
<td>0.1071±0.015</td>
<td>0.0744±0.014</td>
<td>0.006–18</td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>8.9585±0.002</td>
<td>8.1347±0.002</td>
<td>7.2408±0.002</td>
<td>10.1050±0.007</td>
<td>8.1208±0.003</td>
<td>10.1564±0.003</td>
<td>640–2486</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>0.2121±0.039</td>
<td>0.1667±0.033</td>
<td>0.2140±0.013</td>
<td>0.2992±0.022</td>
<td>0.1951±0.013</td>
<td>0.2973±0.047</td>
<td>3</td>
</tr>
<tr>
<td>Mercury (Hg)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>3.1149±0.230</td>
<td>2.7816±0.190</td>
<td>1.9080±0.144</td>
<td>2.2529±0.105</td>
<td>2.3103±0.069</td>
<td>3.000±0.103</td>
<td>0.73–1.41</td>
</tr>
<tr>
<td>Manganese (Mn)</td>
<td>0.1843±0.004</td>
<td>0.0513±0.008</td>
<td>0.2756±0.005</td>
<td>0.1907±0.003</td>
<td>0.1389±0.006</td>
<td>0.2388±0.003</td>
<td>15–100</td>
</tr>
<tr>
<td>Nickel (Ni)</td>
<td>1.4870±0.029</td>
<td>1.9651±0.021</td>
<td>3.6857±0.040</td>
<td>2.3725±0.036</td>
<td>2.6587±0.050</td>
<td>1.8203±0.015</td>
<td>0.1–3.7</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>2.0373±0.003</td>
<td>2.00349±0.001</td>
<td>3.0368±0.009</td>
<td>2.0368±0.002</td>
<td>2.0331±0.002</td>
<td>3.1611±0.001</td>
<td>1–160</td>
</tr>
</tbody>
</table>

Results are expressed as mean ±S.D. ND-Not under detectable limit
Table 4.7 Correlations between metal concentrations of different parts of *Monochoria* Species

<table>
<thead>
<tr>
<th>Traits</th>
<th>Mg</th>
<th>Ni</th>
<th>Ca</th>
<th>Cu</th>
<th>Co</th>
<th>Al</th>
<th>Zn</th>
<th>Pb</th>
<th>Fe</th>
<th>Mn</th>
<th>Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>1.0000</td>
<td>-0.9328**</td>
<td>0.3535</td>
<td>-0.3563</td>
<td>0.6380</td>
<td>0.3085</td>
<td>-0.1175</td>
<td>0.01447</td>
<td>0.4638</td>
<td>-0.2963</td>
<td>-0.7462</td>
</tr>
<tr>
<td>Ni</td>
<td>1.0000</td>
<td>-0.1884</td>
<td>0.6287</td>
<td>-0.5758</td>
<td>-0.4398</td>
<td>0.3837</td>
<td>-0.1080</td>
<td>-0.6185</td>
<td>0.4355</td>
<td>0.7999</td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>1.0000</td>
<td>0.5054</td>
<td>-0.2492</td>
<td>-0.3759</td>
<td>0.6739</td>
<td>0.3970</td>
<td>0.3758</td>
<td>0.1545</td>
<td>-0.3997</td>
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<td></td>
</tr>
<tr>
<td>Cu</td>
<td>1.0000</td>
<td>-0.3210</td>
<td>-0.5354</td>
<td>0.8843*</td>
<td>0.08709</td>
<td>-0.3372</td>
<td>0.6065</td>
<td>0.4111</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>1.0000</td>
<td>0.0820</td>
<td>0.8156</td>
<td>0.9451</td>
<td>0.6360</td>
<td>0.7164</td>
<td>0.9071</td>
<td></td>
<td></td>
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<tr>
<td>Al</td>
<td>1.0000</td>
<td>-0.2305</td>
<td>0.4714</td>
<td>0.5658</td>
<td>0.3161</td>
<td>0.1519</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>1.0000</td>
<td>0.3960</td>
<td>0.3972</td>
<td>0.7613</td>
<td>0.3690</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pb</td>
<td>1.0000</td>
<td>0.8325</td>
<td>0.5856</td>
<td>0.1136</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>1.0000</td>
<td>0.1275</td>
<td>-0.3916</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>1.0000</td>
<td>0.6435</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Cr</td>
<td>1.0000</td>
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</tbody>
</table>

**Significant at p< 0.01, respectively
*Significant at p < 0.05, respectively
Figure 4.14 Level of elements in different parts of *Monochoria* Species
CHAPTER 5
PHYTOCHEMISTRY

5.1 INTRODUCTION

The whole plant or organism serves as an active laboratory for the production of natural products from primary metabolites such as proteins, amino acids, carbohydrates, fats and oils, which are mostly obtained from food items. The primary metabolites are basic biological molecules also called biochemicals, which are functional compounds found virtually in all plants and organisms. Secondary metabolites are varieties of simple to sophisticated bizarre molecules, also called natural products. They are fascinating chemical molecules, very useful and of great importance in nature, as well as highly diversified in structures, properties, uses, chemistry etc. These varied properties and characters emerge from their biological generation, production and formation from basic primary metabolite sources and origin. Natural products are in restricted taxonomic groups and species of organisms. They are from secondary metabolic processes and express individualities of organisms [123].

5.1.1 Phytoconstituents

Phytochemicals are chemicals derived from plants and the term is often used to describe the large number of secondary metabolites found in plants. Phytochemical compounds usually exert peculiar, unique and specific active physiological effects responsible for their therapeutic and pharmacological functions. Activities of such naturally occurring compounds are generally responsible for changes, which are utilized to satisfy human being’s desires. These complex substances of diverse nature occur mostly in plant based foods; they are in very small amounts in gms or mg or µg/Kg of samples. They do not add to body calorie and are numerous in types. These phytochemicals are applied mostly for preventive and healing purposes.