

Development of random amplified polymorphic DNA markers for authentication of *Baliospermum montanum* Willd. leaf with its pharmacognostical evaluation

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Abstract

Introduction: *Baliospermum montanum* Willd. (*Euphorbiaceae*) is an undershrub, distributed in outer range of Himalayas from Kashmir to Assam and in moist deciduous forests elsewhere in India. Leaves, seeds, and roots of this plant are being used for medicinal purpose. **Aim:** The aim of this study was to carry out detailed pharmacognostical study of *B. montanum* Willd. (*Euphorbiaceae*) leaf and its molecular characterization by random amplified polymorphic DNA (RAPD) markers. **Materials and Methods:** Fresh young leaves of *Baliospermum montanum* Willd., after proper botanical authentication, were subjected for macro and microscopic study and were also used for molecular characterization and DNA fingerprints, by standard and most convenient RAPD method. **Results and Discussion:** Microscopic study of leaf shows the presence of single layer of epidermis made up of single-layered barrel-shaped cells interrupted by unicellular trichomes. Mesophyll made up of single-layered compactly arranged cells filled with chlorophyll pigment. Transverse section of petiole shows single-layered barrel-shaped epidermal cells without any intracellular space, hypodermis consists of 8-10 layers of collenchymatous cells filled with rosette crystals and oil globules, reduced cortex filled with chlorophyll pigments. Vascular bundles consist of radially and circularly arranged open and collateral phloems. DNA RAPD analysis reveals that all the primers showed amplification. In primer 1, range of band size was observed from 400 to 1200 bp; in primer 2, band size was observed at 250 bp to around 1500 bp. **Conclusion:** These microscopic observations and the unique bright and light bands obtained in polymerase chain reaction amplification could serve as a measure for authentication and standardization of the plant.

Key words: *Baliospermum montanum*, *Danti*, pharmacognosy, randomly amplified polymorphic DNA

INTRODUCTION

Baliospermum montanum Willd. is a well-known plant of *Euphorbiaceae* family. It has been considered as the botanical source of *Danti*, a well-known purgative drug in Ayurveda and is being incorporated as an important ingredient of many Ayurvedic formulations such as *Dantyaristha*, *Dantiharitaki*, and *Arshakuthararasa*.^[1-3] Although *B. montanum* is a less controversial drug, in some part of the country, *Jatropha glandulifera* Roxb. (*Euphorbiaceae*). – *Ratan jot* (Gujarati),

Jangli erandi in Marathi, and those of *Ricinus communis* Linn. (*Euphorbiaceae*) are being mistaken as *Danti*.^[4] Detailing of Ayurvedic literatures reveals that two other drugs, i.e. *Dravanti* and *Hastidanti* are also considered as

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variety of *Danti*.^[5] Botanical source of *Dravanti* is reported as *Jatropha curcas* Linn. (Euphorbiaceae), while that of *Hastidanti* is reported as *Croton oblongifolius* Roxb. (Euphorbiaceae).^[6] However, *Bhavaprakasha*, a frequently referred lexicon of Ayurveda, has clearly stated two varieties of *Danti*, i.e. *Laghudanti* and *Vrihatadanti* where the recent commentator has clearly equated that *Laghudanti* as *B. montanum*.^[7] In spite of all these, it is observed that different botanical sources as the source plant of *Danti* are being sold in market. Thus, some advance differentiating tools and techniques for the identification of plant species at the molecular levels are the need of the hour. It will prevent the rampant emergence of substitution or adulteration and help establish the standardization of the medicinal plants.

Molecular techniques have found to be more useful and accurate for determination of both intra- and inter-specific variations in plants. Randomly amplified polymorphic DNA (RAPD) markers, in particular, have been successfully employed for determination of intraspecific genetic diversity in several species.^[8] Although few scientific studies have been carried out on the detailed pharmacognostical studies of *B. montanum*, evaluation through DNA RAPD study is still lacking. Hence, the present work has been undertaken to establish a definite botanical standards for identification and standardization of *B. montanum* through pharmacognostical evaluation of its transverse section (T.S.) of leaf with powder microscopy with its RAPD analysis.

MATERIALS AND METHODS

Collection and Preservation of the Sample

Fresh young leaves of *B. montanum* Willd. were collected from its natural habitat, Balangir, Odisha, during February 2016 and identified with the help of botanical texts and flora.^[9] A sample specimen was deposited to Pharmacognosy Laboratory (Specimen No. - PHM/6208/15-16) for the future references. The leaves were washed, shade dried, powdered, sieved through 60 no. mesh and preserved in an air-tight glass vessel for microscopical evaluation of its powder. Fresh leaf sample was preserved in a solution prepared from 70% ethyl alcohol: glacial acetic acid: formalin in the ratio of 90:5:5 and subjected for macro- and microscopic study. The study was conducted as per the guidelines of Ayurvedic Pharmacopoeia of India.^[10]

Morphological Study

Morphological characters were studied by observing the leaves of *B. montanum* as such and also with the help of the dissecting microscope systematically as per the standard textbook of botany as well as with the help of flora.^[11]

Microscopic Study

For detailed microscopical examination, free hand thin transverse section of leaf of *B. montanum* were taken. The sections were cleared with chloral hydrate and observed as such for the presence of any crystals and other inclusions, then were stained with phloroglucinol and hydrochloric acid to notice the lignified elements such as fibers and vessels of the meristele and other parts.^[12] Photographs of the sections were captured with the help of Canon digital camera attached to Trinocular Carl-Zeiss microscope.

Surface Study and Micrometry

The surface study of epidermis was carried out to determine type and distribution of stomata, epidermal cell, and trichomes. Quantitative microscopy was carried out to determine epidermal cell number, stomatal number, stomatal index and size of the stomata, etc. Mean values are taken by five successive readings.^[13]

The stomatal index was found using following formula

$$SI = \frac{S \times 100}{E + S}$$

SI = Stomatal index, S = Number of stomata per unit area, E = Number of epidermal cells in the same unit area.

Organoleptic Study

The dry powder *B. montanum* leaves were evaluated for their organoleptic characters including taste, odor, color, and touch.

Powder Microscopy

Powder of leaf of *B. montanum* was individually studied microscopically and photographed using Canon digital camera attached to Carl Zeiss trinocular microscope. Powder characters were studied as per the guidelines of Ayurvedic Pharmacopoeia of India.^[14]

Histochemical Tests

Histochemical tests of powder of *B. montanum* leaf were carried out to detect the presence of starch grains, calcium oxalate crystals, tannins, oil globules according to the standard guidelines.^[15]

DNA Isolation

Fresh young leaves of *B. montanum* from balangir forest area of Odisha were selected, cut into small pieces without damaging the veins. They were washed with distilled water

and ethanol, frozen with dry ice, and crushed. DNA was extracted using Doyle and Doyle (1990) method with minor modifications. DNA quantification was done using a Picodrop spectrophotometer and DNA sample was diluted using Tris ethylenediaminetetraacetic acid TE buffer up to 50 ng/μl. Quality of one sample of *B. montanum* leaf, DNA was checked by 0.8% agarose gel electrophoresis. RAPD-Intersimple sequence repeat (ISSR) polymerase chain reaction (PCR) was carried out in Veriti ABI thermal cycler. The resolved amplification products were visualized by illumination under ultraviolet light in Gel document system.

Procedure

Fresh leaves were used for molecular characterization and DNA fingerprints, by standard and most convenient RAPD method (Baum BR, Mechanda S, 2001). The RAPD reaction was performed following standard procedures at Food Testing Laboratory, Department of Biotechnology, Junagadh Agricultural University, Junagadh.

The high quality and purity of genomic DNA free from secondary metabolites were isolated from this species by modified cetyltrimethylammonium bromide method.^[16] PCR trials were undertaken with different concentrations of GCl_2 (0.5, 1 and 1.5 mM) keeping all other parameters constant. MgCl_2 of 1.5 mM concentration was proved best in 25 μl reaction volume. For most amplification reactions, 0.5-1.5 units of the enzyme were used. Initially, amplification reactions were carried out with 0.9 units of *Taq* polymerase; however, this was not found to work to generate better results. Therefore, the quantity was reduced to 0.5 units of *Taq* polymerase per 25 μl reaction volume, which gave better amplification. In all PCR trials, the annealing temperature 25°C has been used which was determined with gradient PCR. DNA denaturation is a critical step in DNA amplification reactions. For most DNA amplification reactions, incubation time for DNA denaturation is 1 min at 94°C.

DNA is unique to each individual like fingerprints. The specific arrangement of DNA base-pair sequences guides the production of proteins and enzymes.^[17] DNA fingerprinting in plants involves the extraction of DNA from plant cells, quantification, and quality assessment. While carrying out PCR-based duplication of DNA in RAPD, ISSR, or SSR, diluted DNA is mixed with a master mix.

PCR Amplification Method

Among the PCR-based molecular techniques, RAPD is convenient in performance and does not require any information about the DNA sequence to be amplified.^[18] Due to its procedural simplicity, the use of RAPD as molecular markers for taxonomic and systematic analyses of plants.^[19]

RESULTS

Morphological Findings

Leaf

Leaves simple, alternate, upper leaves are 10 cm × 15 cm with a pair of glands at the base; upper leaves smaller; lanceolate, or ovate-lanceolate with toothed margins, the lower ones broadly ovate-oblong, entire and 3-5 lobed with toothed margins and rounded base. Apex is acute, while lower ones reach up to 15-30 cm in length and 10-20 cm breadth. Base is rounded. The details of macroscopic and micrometric findings, surface study, and list of RAPD primers used for the analysis of DNA samples of *B. montanum*. Willd has been presented in Tables 1 and 2, respectively. The detailed figures have been presented in Plates 1 and 2.

Microscopic Study

T.S. of petiole

T.S. of petiole measures about 16.6 mm × 5.1 mm in length (×10), circular to oval in shape. Outer epidermis followed by cortex and centrally located long ground tissue [Plate 3].

Table 1: Macroscopic and micrometric findings

Characters	Findings	
Color	Upper surface: Dark green, lower surface: Light green	
Odor	Characteristic	
Taste	Bitter	
Size	Length: 20.3±1.4 cm, Breadth: 9.5 cm±0.8 Petiole length - 6.6 cm×5.1 cm	
Shape	Upper leaf: Ovate-oblong Lower leaf: Ovate to lanceolate	
Touch	Upper: Glabrous	
Extra features	Margin: Serrated, apex: Acute Base: Cordate with 2 special notches Main node - 7 pair Petiole: 6.5 cm	
Venation	Dorsiventral with reticulate venation	
Characters	Upper surface	Lower surface
Nature of stomata	Parasitic	Parasitic
Length	0.7±0.1 mm	0.9±0.2
Breadth	0.5±0.2 mm	0.8±0.2
Stomatal cells	14±2	17±2
Epidermal cells	60±5	70±3
Stomatal index	20.6±6.24	18.2±1.8
Palisade ratio	07±01	

Mean±SD (n=5), SD: Standard deviation

Epidermis

Outermost epidermis single-layered barrel-shaped cells without any intracellular space; some of the epidermal cells bear warty trichomes.

Hypodermis

8-10 layered collenchyma just beneath epidermis forms hypodermis. Collenchyma with oil globules. Rosette crystal.

Cortex

Cortex reduced made up of parenchyma cells heavily loaded with chlorophyll pigment, rosette crystals, cluster crystals, oil globules, and brown content.

Endodermis

Single-layered circularly arranged endodermis just beneath the cortical region.

10-12 packets vascular bundle protosomal toward pith. Large space oil globule. Rosette and cluster crystals. The presence of starch grains.

Vascular bundles

Circularly arranged open and collateral vascular bundles. Xylem made up of xylem parenchyma and xylem fibers. Phloem present above the xylem made up of phloem fibers and sieve elements. Central large pith made up off parenchyma cells.

T.S. Through midrib

- Epidermis: Leaf is dorsiventral. Epidermis made up of single-layered barrel-shaped cells interrupted by unicellular trichomes. Some of the cells interrupted by stomata in both sides of epidermis. Epidermis is covered with cuticle.
- Mesophyll: Dorsiventral, mesophyll differentiating upper palisade, lower spongy cells. Mesophyll made up of single-layered compactly arranged cells filled with chlorophyll pigment. 6-8 palisade cells present beneath the epidermal cells. Lower spongy parenchyma cells consist of round to oval parenchymatous cells with intercellular space. Some of the cells contain oil globules, rosette crystal, and cluster crystal. Palisade and spongy parenchyma separated by running vascular strands. Simple layer of warty trichome is present. Palisade ratio in upper epidermis, 1:4.

Table 2: List of RAPD primers used for the analysis of DNA samples of *B. montanum* Willd

S. No.	Primer	Sequence 5'-3'	Temperature (0°C)
01	OPO-07	CAGCACTGAC	25.0
02	OPP-06	GTGGGCTGAC	27.0
03	OPR-04	CCCGTAGCAC	27.0
04	OPS-03	CAGAGGTCCC	27.0
05	OPQ-03	GGTCACCTCA	25.0
06	OPS-10	ACCGTTCCAG	25.0
07	OPT-09	CACCCCTGAG	27.0
08	OPS-09	TCCTGGTCCC	27.0
09	OPT-08	AACGGCGACA	25.0
10	OPS-07	TCCGATGCTG	25.0

RAPD: Random amplified polymorphic DNA,
B. montanum: *Baliospermum montanum*

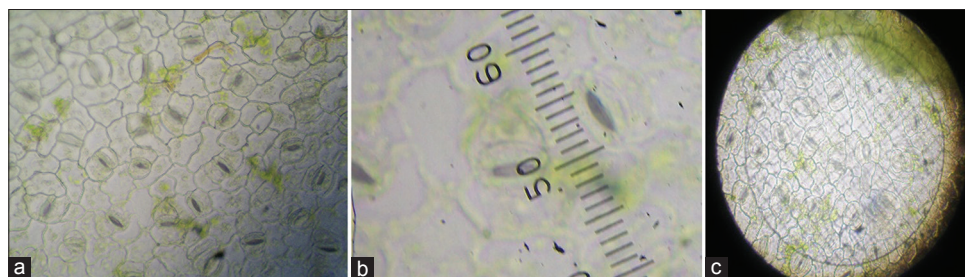


Plate 1: Microscopy study of leaf - upper surface (a) Paracytic stomata, (b) micrometric measurement of stomata with subsidiary cells, (c) stomatal index

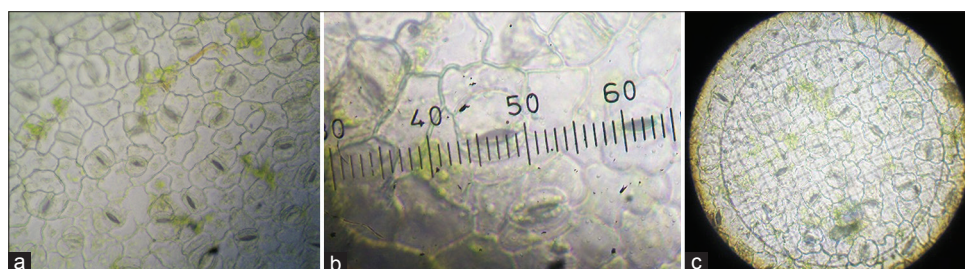


Plate 2: Microscopy study of leaf - Lower surface, (a) Paracytic stomata, (b) micrometric measurement, (c) stomatal index

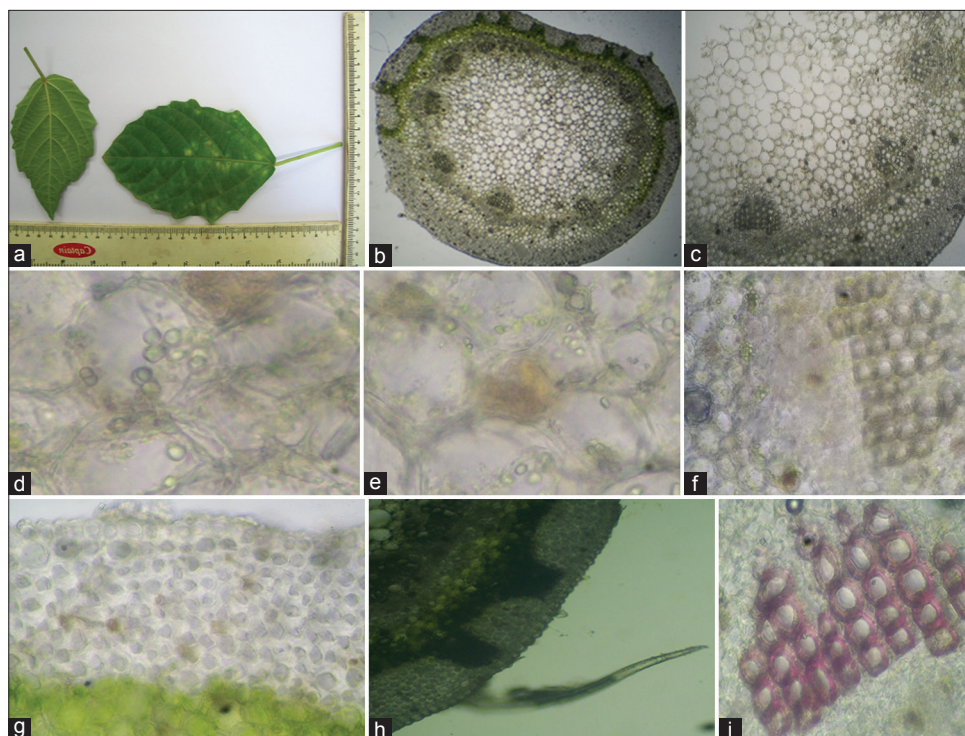


Plate 3: Through petiole, (a) Micrometric measurement of petiole, (b) diagrammatic section, (c) transverse section of petiole with epidermis and hypodermis, (d) parenchyma with starch grains, (e) parenchyma with brown content, (f) cluster crystals with phloem and xylem, (g) epidermis –hypodermis, (h) Warty trichome, (i) phloem with lignified xylem

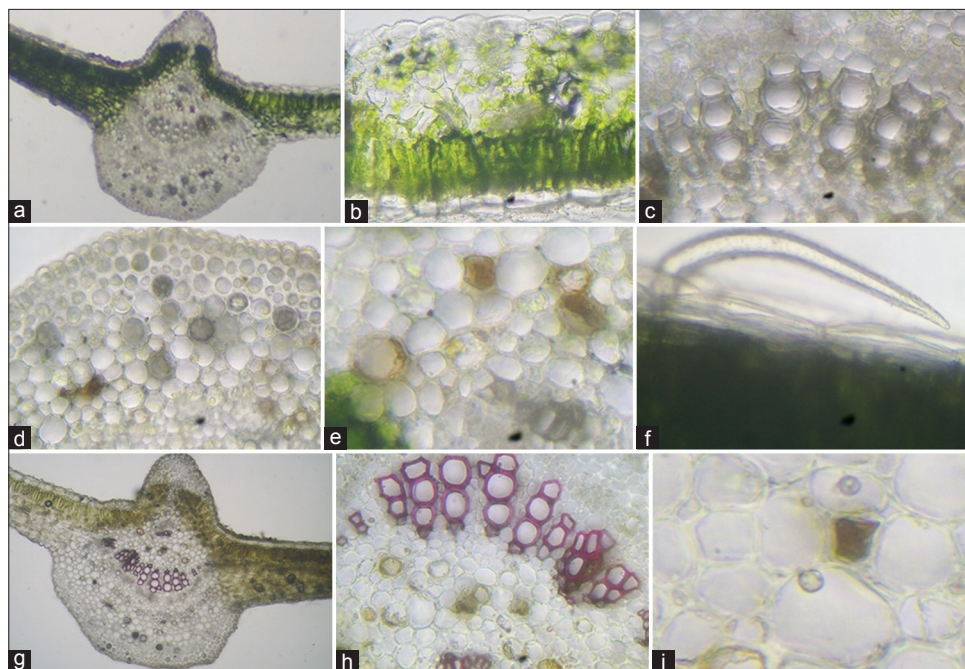


Plate 4: Through mid-rib, (a) Diagrammatic section, (b) mesophyll content, (c) phloem-xylem, (d) lower epidermis with crystals, (e) tannin content, (f) warty trichome, (g) stained section through midrib, (h) phloem with stained xylem, (i) tannin content with oil globule

- Through midrib: Beneath epidermal cells, few layers of collenchymatous cells present on both the sides of the epidermis which gives strength to leaf. A single layer of bundle sheath covers the vascular bundle. Vascular bundle made up of lower phloem and xylem towards the upper epidermis and are radially arranged [Plate 4].

Powder microscopy: The organoleptic characters of leaf showed parrot green color, characteristic odor with bitter taste.

The diagnostic characteristics of powder microscopy of leaf showed epidermal cells with stomata and brown content.

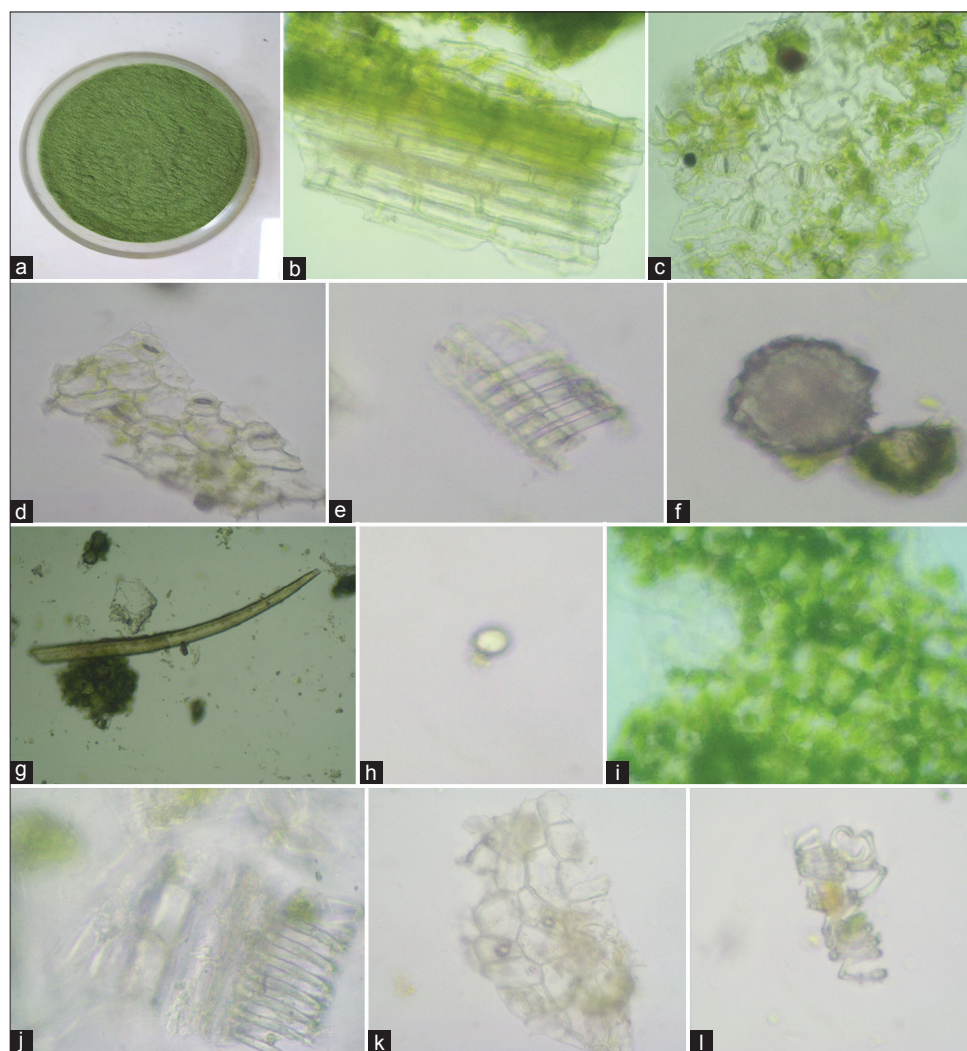


Plate 5: Powder microscopy, (a) Powder of leaf, (b) group of fibres, (c) epidermal cells with stomata, (d) epidermal cell, (e) spiral vessels, (f) cluster crystals, (g) unicellular warty trichomes, (h) oil globules, (i) spongy parenchyma, (j) palisade parenchyma, (k) pericyclic fibres, (l) spiral vessels

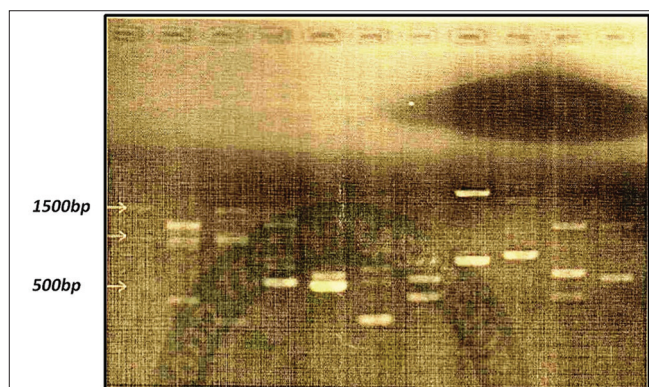


Plate 6: DNA randomly amplified polymorphic DNA of leaf of *Baliospermum montanum*

Powder microscopy was also observed possessing unicellular warty trichome with fragment of spiral vessels, cluster crystals, fragment of pericyclic fiber with oil globules, and group of fibers [Plate 5].

The fingerprinting patterns of *B. montanum* sample seen as vertical columns with horizontal light bands on a dark background have been depicted in the Plate 6 for RAPD. For the analysis of DNA, samples of *B. montanum* 10 primers were used (1-10 RAPD) (primers mentioned in table). Primers have been loaded from left to right. Primer 1 was on the left most side and primer 10 was on the right side [Plate 6].

DISCUSSION

Leaf dorsiventral differentiated into palisade and spongy parenchyma cells T.S. of the petiole showed that many vascular bundles distributed in the ground tissue and centrally located large pith. T.S. through midrib showed that presence of centrally located vascular bundle and palisade ratio is 07 ± 01 and stomatal index is 20.6 ± 6.2 are the distinctive characters of the plant. DNA-based techniques have been widely used for authentication of plant species of medicinal importance.

This is especially useful for substituted or adulterated drugs with other species or varieties that are morphologically and/or phytochemically indistinguishable.^[20] The advantages of RAPD technique include their simplicity, rapidity, and low amount of genomic DNA required. RAPD marker is easily reproducible under a wide variation of amplification conditions as it was clearly visible up to a nailing temperature of 38°C and result was not affected with changes in the origin of the primer. Following this method, various plants have been evaluated.^[21-26] RAPD analysis was done with 10 primers with all primers showed amplification. In primer 1, range of band size was observed from 400 to 1200 bp. More numbers of bright bands were observed in the primers 5, 6, 7, and 8 highlighted the family genetic characters and the species characters. All primers showed amplification. In primer 1, range of band size was observed from 400 to 1200 bp; in primer 2, band size was observed at 250 bp to around 1500 bp; in primer 3, the range of band size was observed from 500 to around 1500 bp; in primer 4, the range of band size was observed from 500 to 1400 bp; in primer 5, range of band size was observed at 300 bp to above 1500 bp; in primer 6, range of band size was observed from 500 to 950 bp; in primer 7, the range of band size was observed from 700 and above to 1500 bp; in primer 8, range of band size was observed at 300-1500 bp; in primer 9, range of band size was observed from 400 to 1100 bp; in primer 10, band size was observed at 300, at 500 bp, and at 1100 bp [Plate 6].

CONCLUSION

Pharmacognostical findings reveal that are scientific observation in identification of the plant. The observed DNA fingerprints will provide accuracy in pharmacognostical standards of *B. montanum*. Leaf of *B. montanum* can be identified with the key microscopical features of unicellular warty trichomes, cluster crystals, rosette crystals, paracytic stomata with oil globules, and group of fibers. The observed characters and findings can be considered as a reference standards for the future study.

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