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Research Article

DEVELOPMENT OF RANDOM AMPLIFIED POLYMORPHIC DNA MARKERS FOR AUTHENTIFICATION OF *Blepharispermum subsessile* DC.

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Abstract

Blepharispermum subsessile DC. (Asteraceae), a less explored folklore medicinal plant is found in the forest of Odisha, Karnataka, Madhya pradesh and Maharashtra. The whole plant is used as Rasna, a potent Ayurvedic drug, by traditional practitioners, and is claimed to be beneficial in treatment of rheumatism, ophthalmic disorder, skin diseases, menstrual irregularities, diarrhoea etc. The majority of the information regarding identity, purity and quality of the plant can be obtained from its macroscopic, microscopic and DNA finger prints. The present study details with the Pharmacognostical characters of the stem of *B. subsessile* DC. and the molecular characterization of the plant by Random Amplified Polymorphic DNA (RAPD) technique. Pharmacognostical characters of stem shows pericyclic fibers, centrally located parenchyma along with pitted parenchyma, xylem with xylem fiber, uniserrate to multiserrate medullary rays, stone cells and sclereides. Unique bands obtained in Polymerase Chain Reaction (PCR) amplification clearly discriminated having, many bright and light bands indicating the genuinity of the plant.

Key words: *Blepharispermum subsessile*; DNA finger print; Ethno-medicine; Pharmacognosy; Rasna.

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INTRODUCTION

Blepharispermum subsessile DC. (Asteraceae), is distributed in Odisha, Karnataka, Madhya pradesh and Maharashtra and is marketed with high price in the name of Rasna, known as Rasnajhadi. Its role in ethnopharmacology has been reported for the management of diarrhoea,[1] skin diseases,[2] eye troubles, backache and rheumatism,[3] irregular menstruation.[4] Establishing the standards is an integral part of establishing the correct identity and quality of a crude drug. Randomly amplified polymorphism of DNA (RAPD) analysis is powerful and convenient molecular marker system, widely used for the genetic mapping, taxonomic and polygenic studies of many plants. In spite of its high medical as well as market value the molecular characterization *B. subsessile* and the pharmacognostical characters of its stem, is not reported yet. Hence, in this research paper an attempt has been made to establish the genuinity of the plant through pharmacognostical characters of stem which includes macroscopic, microscopic characters including preliminary physicochemical analysis and molecular characterization through Randomly Amplified Polymorphism of DNA (RAPD) analysis.

MATERIAL AND METHODS

Collection and authentification

Rasnajhadi, was identified by local traditional practitioners and authenticated by expert taxonomist as *Blepharispermum subsessile* DC of family Asteraceae, on the basis of characters given in Flora of Orissa.[5] The fresh sample of stem and leaf was collected by the scholar from its natural habitat Odisha, in the month of October 2011 and voucher specimen has been preserved in the Pharmacognosy laboratory, I.P.G.T. and R.A., vide no.: Phm/36561. The leaves were separated from the stem, washed with running fresh water and few pieces of the stems were stored in solution of AAF (Alcohol: Acetic acid: Formalin) in the ratio of (90:5:5)[6] to utilize them for microscopic studies. Remaining stem pieces were shade dried, pulverized and sieved through mesh no.80# and preserved in an airtight glass container for future physicochemical and phytochemical analysis. The fresh leaves of *Blepharispermum subsessile* DC. were used for RAPD analysis.

Pharmacognostic studies

Morphological characters were studied by observing the stem as such and also with the help of the dissecting microscope. For detailed microscopical observation, free hand thin transverse section were taken and cleared with chloral hydrate and observed as such for the presence of any crystals, then were stained with Phloroglucinol and Hydrochloric acid to notice the lignified elements like fibers, vessels etc.[7] Photographs of the section were taken with the help of Canon digital camera attached to Carl Zeiss trinocular microscope. Powder characters were observed and micrometric evaluation was carried out.

Preliminary phytochemical evaluation

The dried sample was used for the preliminary physicochemical investigations by the standard procedure adopted in API.[8]

Quantitative microscopy

Scientific measurement of individual cells and layers are accurately done, these micrometric values (mean value) are measured by pre loaded software digital camera attached to carl zeiss trinocular microscope.[9]

Molecular characterization (DNA fingerprints)

Fresh leaves were used in molecular characterization and DNA fingerprints were obtained by standard and most convenient RAPD method.

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Plate 1: *Blepharispermum subsessile* DC.

Fig.a Plant in natural habitat

Fig.b Plant in natural habitat

Fig.c Plant in natural habitat

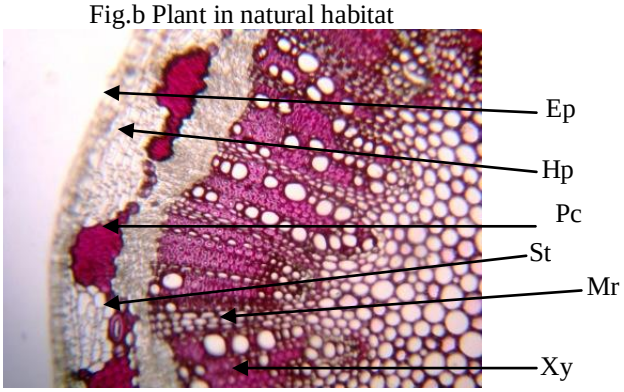

Fig.d T.S. of Stem *B. subsessile* DC.

Various parts of T.S. in enlarged view

Fig.e Epidermis with cuticle

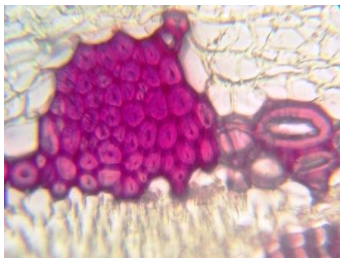

Fig.f Pericyclic fibre

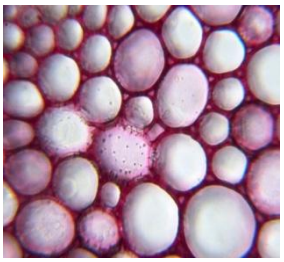

Fig.g Centrally located parenchyma with pitted parenchyma

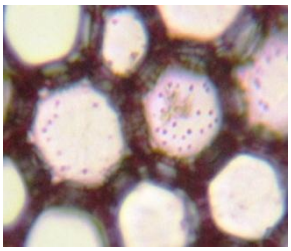

Fig.h Lignified pitted parenchyma

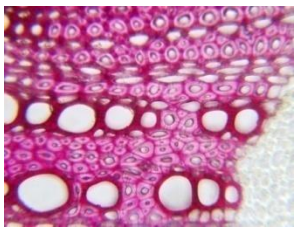

Fig.i Xylem with xylem fibres

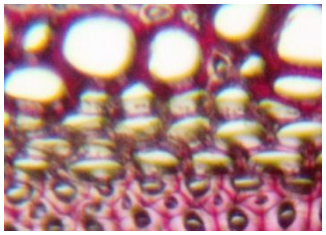

Fig.j Uniseriate to biseriate medullary rays with xylem

Fig.k Stone cells

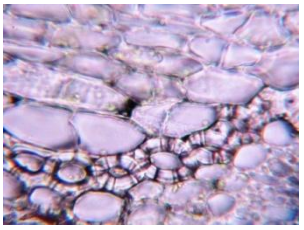

Fig.l Sclereids

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The RAPD reaction was performed following standard procedures at Aristogene Biosciences Pvt. Ltd, Bangalore.[10]

DNA isolation

Tender leaves were selected and crushed to powder using dry ice. 200 mg of this powdered leaves was taken in a centrifuge tube. Equal volumes of Isopropanol and 1/10th volumes of 3M Sodium acetate were added and mixed well and left at room temperature to stand for 5-10 min then centrifuged at 10000 rpm for 10-15 min. The supernatant portion was discarded. The pellet was washed with 1 ml of 70% ethanol. The pellet was air-dried and suspended in 400 µl of 1X Tris-EDTA buffer. Since DNA samples had inhibitors for PCR, the samples were column purified.

Column purification

The column was placed in collection tube, 400 µl of equilibration buffer was added to the column and centrifuged at 10000 rpm for 1 min. 500 µl of wash buffer 2 was added, centrifuge at 10000 rpm for 1 min and buffer was collected. The column was centrifuged empty for 2 min with collection tube to completely remove the wash buffer. 50 µl of elution buffer was added to the column placed in new collection tube. Incubated at room temperature for 2 min and centrifuged at 10000 rpm for 1 min and eluted sample was saved (elution 1). Previous step was repeated. (DNA eluted in this fraction also) (elution 2). Quantization of eluted DNA samples was done by loading into the agarose gel. (Table 1, 2)

RESULTS AND DISCUSSION

Morphology

Blepharispermum subsessile DC. is a glabrous erect shrub, about 3-4 ft in height. In fresh condition, stem is greenish, herbaceous, slender, stout, more or less armed or not, with proper nodes and internodes, with some tomentose hairs, gives longitudinal breakage along with fibers. Mature stem is hollow due to reduced central part of the pith. (Plate 1:a-c)

Microscopic description

T.S. of the fresh stem

The diagramatic section is circular in shape, shows outer epidermis followed by cortex along with the pericyclic fibre zone, radially arranged vascular bundles and centrally located large parenchymatous pith. (Plate 1:d) Epidermis single layered, barrel shaped, with thick cuticle. Cortex made up of compactly arranged parenchymatous cells, some of the parenchymatous cells are filled with prismatic crystal of calcium oxalate, simple starch grains with hilum and oil globules. Endodermis single layered somewhat elongated with thin walled parenchymatous cells forming ring like structure. Pericyclic fibres situated above the xylem forming an arc like structure. Two successive pericyclic fibres are connected by a single or two layered lignified pitted stone cells. Medullary rays biseriate to multiseriate, separates the vascular tissues, somewhat longitudinally arranged barrel shaped cells filled with some oil globules and starch grains. In vascular bundles metaxylem towards periphery and protoxylem towards pith, xylem consists of xylem parenchyma and fibres, phloem situated above xylem forming cap like structure with few elements and fibre. Pith covers nearly half portion of the section, made up of thick walled, compactly arranged parenchyma cells with some prismatic crystals and oil globules. Tail region of the vascular bundle consist of thick walled lignified pitted parenchyma cells. (Plate 1: d-l)

Organoleptic characters

Organoleptic evaluation of stem of *B. subsessile* powder revealed its yellowish green colour, aromatic odour and slightly bitter in taste.

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Table 1: A cocktail was made with PCR master mix and respective Random primer

	For 1 reaction	For 12 reactions	Notes
Double Distilled water	19 µl	2280 µl	--
2X PCR master mix	20 µl	240µl	1X Contains 100 µM each of dATP, dGTP, dCTP and dTTP. Assay buffer with 15mM $MgCl_2$, 3U/reaction Taq Polymerase.
Random Prime	1 µl	12 µl	10pM used for each reaction
Total Volume	40 µl x 12	480 µl	--

Diagnostic characters of the stem powder are- presence of fibres, multicellular glandular trichomes, tannin containing cells, pitted sclereides with wide lumen, prismatic crystals and simple starch grains with hilum, spiral and annular vessels. (Plate: 2: f-m)

Quantitative microscopy

Total length from epidermis to pith is 60.83 µm. Surface area of each epidermal cell measures about 216.87 µm². Pericyclic fibres measures about 28.15µm². Phloem area covered measures about 152.11 µm². Lignified pitted parenchyma measures about 21.52 µm². Pitted parenchyma measures about 26.58 µm². Xylem measures about 21.91 µm². Medullary rays measures about 2.70 µm² (in each mean readings are taken by successive 3 intervals.) (Table 3), (Plate: 2:a-e)

Table 2: Development of PCR

Temperature	Time	No. of cycles
94˚C	2 min	1
94˚C	30 sec	40
45˚C	1 min	
72˚C	1min 30 sec	
72˚C	7 min	1

Table 3: Size of pericyclic fibres

Length	Breadth	Circumference
5.27 µm	7.5 µm	31.52 µm²
4.76 µm	6.41 µm	26.38 µm²

Preliminary phytochemical evaluation

The preliminary physico-chemical parameters of the stem viz. foreign matter, loss on drying, total ash, water soluble and alcohol soluble extractive value were found to be 0.00%, 7.58%, 3.72%, 0.30% and 4.92% respectively.

The Percentage of alcohol extractive was more in comparison to water soluble extractive. The pH of aqueous solution was 6.5 (Table 4).

Table 4: Preliminary Physico-chemical analysis

Parameters	Stem
Foreign matter	Nil
Loss on drying (% w/w)	7.58%
Total ash content (% w/w)	3.72%
Water soluble extractive value (% w/w)	0.30%
Alcohol soluble extractive value (% w/w)	4.92%
pH	6.5

DNA finger printing

DNA extracted by regular CTAB (hexadecyltrimethylammonium bromide) method did not show amplification. Plant extracts had to be column purified to get the DNA amplified. RAPD analysis shows that OPA-02 and OPA-13 primers amplified the DNA to produce good polymorphic bands. Differentiation or the DNA finger print is very clear with these primers, shows distinct patterns. DNA markers being environmentally stable and specific, they have gained wide popularity in quality control and standardization of medicinal plant materials. The complement of genes expressed by a cell is very dynamic and responds rapidly to external stimuli. Therefore, analysis of gene expression becomes necessary for providing clues about regulatory mechanisms, biochemical pathways, and broader cellular function.[11]

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Plate 2: Quantitative microscopy

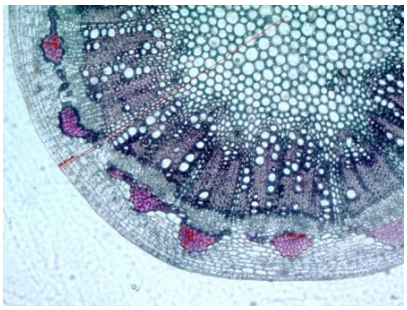

Fig.a. Epidermis to pith

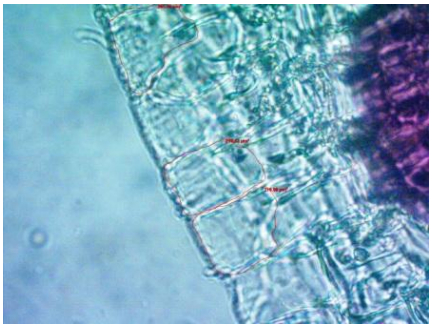

Fig.b. Epidermis

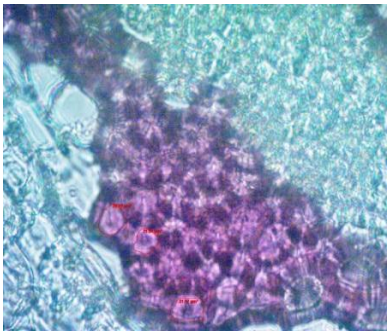

Fig.c. Pericyclic fibre

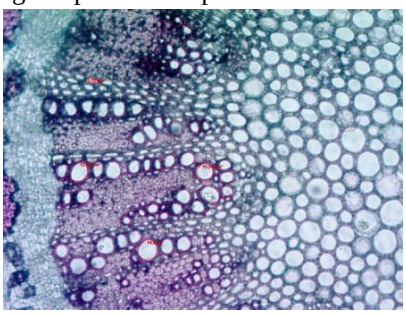

Fig.d. Xylem

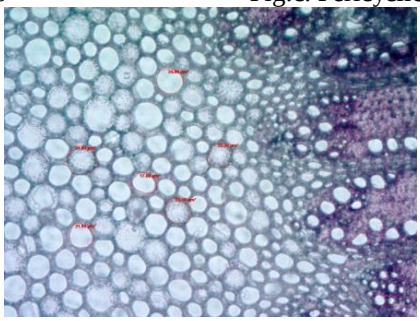

Fig.e. Parenchyma cells

Powder microscopy

Fig.f fibre

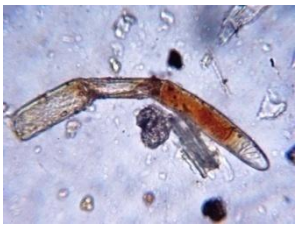

Fig.g multicellular glandular trichome (3)

Fig.h tannin containing cell

Fig.i pitted scleride with wide lumen

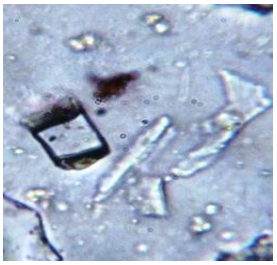

Fig.j prismatic crystal

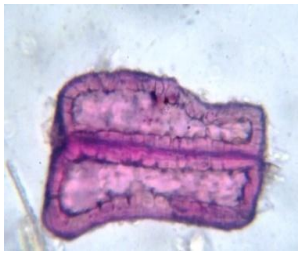

Fig.k sclerides

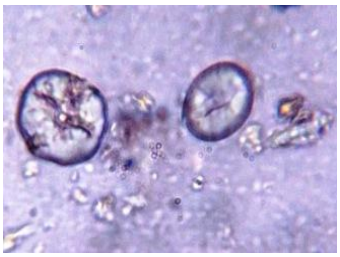

Fig.l simple starch grain with hylem

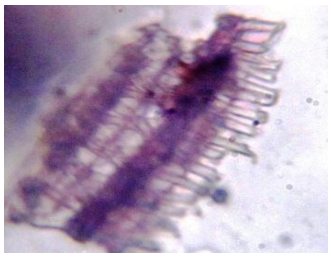

Fig.m spiral and annular vessel

RAPD profile generated from genomic DNA isolated from fresh leaves of *B. subsessile* found identical with 2 random primers. The number of unique bands is specific to genuine as well as other samples with different primers. RAPD fingerprints of *B. subsessile* run on 1% agarose gel with 1000 bp ladder, random primer 21 (sample 1 *B. subsessile*.) and random primer 22 (sample 2).

The primers 22 discriminate the primer species of *B. subsessile* by presence and absence of unique bands, two prominent bright and six lighter bands, which act as markers for species authentification. (Plate: 3) The single primer in this study clearly discriminated the genuinity.

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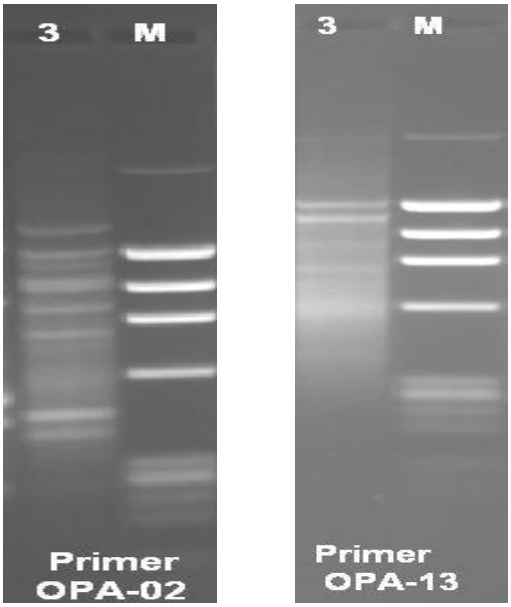
Plate 3: DNA finger printing of *Blepharispermum subsessile* DC



M = 1000 bp ladder; 3 = Sample of *B. subsessile* DC

CONCLUSION

The observed DNA finger prints will provide accuracy in pharmacognostical standards of *Blepharispermum subsessile* DC. (Asteraceae) and the observation can be considered as reference standards in the further studies. Its stem can be identified on the basis of key specific characters like pericyclic fibre zone, radially arranged vascular bundles, biserrete to multiserrate medullary rays separating the vascular tissues and centrally located large parenchymatous pith.

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