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Pharmaceutical Standardization

Development of Random Amplified Polymorphic DNA markers for authentification of *Cissus repanda* vahl.

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Abstract

Cissus repanda Vahl. belongs to the family Vitaceae, commonly known in Hindi as "*Panivel*," is a large climber distributed all over India. The crushed or powder of root is prescribed by tribal people and traditional medical practitioners of Orissa for its healing properties in cases of bone fracture, cuts and wounds, swellings, and so on. In spite of its reputation, its leaves have not been investigated scientifically. The present study deals with pharmacognostical and molecular characterization by Random Amplified Polymorphic DNA (RAPD) markers and their role in laying down standardization and pharmacopoeial parameters. Genomic isolation of DNA from fresh leaves was amplified by RAPD markers. The diagnostic characters are mucilage, calcium oxalate rosette crystals, spiral vessels, and fibers. The unique bands obtained in Polymerase Chain Reaction (PCR) amplification clearly discriminated having, many bright and light bands indicating the genuinity of the plant. RAPD may serve as a complementary tool in quality control of many herbal sources.

Key words: *Cissus repanda*, pharmacognosy, RAPD

Introduction

Cissus repanda Vahl. (Vitaceae) commonly known as "*Panivel*" in Hindi, is an important medicinal plant distributed from Kuman to Arunachal Pradesh, Tripura, Assam, Bihar, Orissa, Madhya Pradesh, and Western Ghats region up to 1350 m.[1] It is a large climber, with soft, very porous wood with corky bark. The stem yields potable water on cutting thus the name *Panivel* (*Pani*-Water, *Vel*-Creeper). The medicinal potential of *C. repanda* has been known to traditional system and widely used in folklore medicine. The roots and leaf powder of *C. rependa* have been traditionally used in the form of paste for cuts, wounds, and bone fractures.[2] Root and stem show anti-inflammatory and analgesic effect.[3] Pharmacognostical evaluation of root and stem were reported.[4,5] In spite of its reputation and studies, its leaves have not yet been investigated scientifically and hence it was thought worthwhile to study in detail. The present manuscript highlights macroscopic, microscopic, and molecular level studies(DNA fingerprints) of the leaves for its authentification and standardization.

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Materials and Methods

Collection and preservation of the plant

Fresh *C. repanda* Vahl. was uprooted from the natural habitat from Orissa. The collected samples were identified, authentified by the Taxonomist. A specimen of the plant was preserved in the departmental herbarium museum vide no. Phm. 6001/2009 for future references. Fresh leaves were collected and fixed immediately using FAA (Formalin: Acetic Acid: Ethyl Alcohol) as fixative agent for anatomical studies.[6,7]

Macroscopy of leaf

Macroscopic characters, such as size, shape, margin, apex, surface, color, odor, taste, nature, and texture were studied for morphological investigation.[8]

Microscopy of leaf

For microscopical studies, free hand transverse sections of leaves were taken and examined. Surface preparation was done and both the surfaces of the leaves were observed. The powder microscopy of dried leaves of the plant was carried out with and without staining. The photomicrographs were taken using Carl–Zeiss binocular microscope.[9,10]

Molecular characterization (DNA fingerprints)

Fresh leaves were used in molecular characterization and DNA fingerprints were obtained by standard and most convenient RAPD method. The RAPD reaction was performed according to the method developed by researchers.[11]

Plant DNA isolation: RAPD analysis

Lysis buffer

100 mM Tris–HCl (pH 8.0), 1.2 M NaCl, 20 mM EDTA, 2% CTAB 2 μL was loaded from both the elutions. DNA was seen in a concentration of 50 ng/μL; and 1 μL of sample was used for PCR [Tables 1–3].

Amplification

Two random primers were used.

Random primer 21: TGC CGA GCT G
Random primer 22: GAA CGG ACT C.

Results and Discussion

Macroscopic characteristics of leaf

Macroscopic study shows that leaves are simple, alternate, petiole 4–5 cm long, broadly cordate to ovate, 12–20 cm long, but attaining 18 × 12.5 cm, bristle–serrate shortly caudate, glabrous or often with few long, yellow hairs on the nerves, 3–5 nerved at base, secondary nerves above base 4–6, strong, gently curved excurvent, membranous, base cordate, margin repeatedly toothed, apex acute, petiole 15–20 cm long, stipules oblong rounded, 5 mm spreading, leaving a persistent base on the falling. Tendrils dichotomous forked externally opposite to leaf [Figures 1–3].

Organoleptic characters

Powder was coarse greenish grey in color, sour in taste, and slightly aromatic in odor.

Surface preparation

Small pieces (2 mm^2) of the leaves were taken in chloral hydrate solution in a test tube, boiled in water bath until transparent material was obtained, mounted in glycerine and the following characters, such as trichomes, rosette crystals of calcium oxalate [Figure 4], epidermal cells [Figure 5], and stomata [Figure 6] were observed under the microscope.

Transverse section of the leaf

Transverse section through midrib of the leaf shows epidermis on both upper and lower surfaces. Single-layered barrel-shaped cells covered with cuticle, some epidermal cells with unicellular simple trichomes and stomata were found at lower surface of the leaf. Mesophyll differentiated into upper two-layered palisade parenchyma and lower spongy parenchyma, and chlorophyllus with intercellular spaces. Some cells are filled with tannin materials and also with rosette crystals of calcium oxalate crystals. The upper side of the midrib shows a prominent ridge at the middle filled with collenchyma cells just beneath the epidermis. The vascular bundles are found at the midrib region around small pith. Out of which the largest one is situated on the upper side and the remaining vascular bundles are smaller in size and they are at the lower side. Each vascular bundle has an outer nonlignified sclerenchyma, a band of middle phloem, and an inner xylem. The rest of the midrib region is covered with parenchyma cells with mucilage sacs with or without acicular crystals of calcium oxalate [Figure 7a and b].

Figure 1: Entire plant of *C. repanda*

Table 1: Cocktail was made with PCR master mix and random primer

	For 1 reaction (μL)	Cocktail (μL)	Notes
Double distilled water	24	48	--
2× PCR master mix	25	50	1× Contains 100 μM each of dATP, dGTP, dCTP, and dTTP. Assay buffer with 15 mM $MgCl_2$, 3μ/reaction Taq Polymerase
Random primer	1	2	100 ng used for each reaction
Total volume	50 × 2	100	--

49 μL of this was aliquoted into 2 different labelled PCR vials and to this 1 μL of different template DNA was added. The PCR was set

Table 2: Polymerase chain reaction conditions

Temperature (°C)	Time	No. of cycles
94	2 min	1
94	30 s	40
45	1 min	
72	1 min 30 s	
72	7 min	1

Min- minutes; s-seconds

Table 3: DNA fingerprints of *Cissus repanda*

Base pair	Sample 1 (*C. repanda*)
1000	--
900	--
800	--
700	--
600	--
500	--
400	Bright band
300	--
200	--
100	Bright band

Details of 100 bp DNA ladder in 1% agarose gel. 1 - Sample (*Cissus repanda* Vahl.) with random primer 21. 2 - Sample with random primer 22

Powder microscopy

The leaves were shade dried and made into coarse powder for the powder microscopy. The diagnostic features are trichomes from epidermal cells, rosette and acicular crystals of calcium oxalate from mesophyll, epidermal cells, spiral and annular vessels of xylem [Figures 8–13].

DNA studies

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

Figure 2: Leaf with petiole and dimensions

Figure 3: Leaf of *C. rependa*

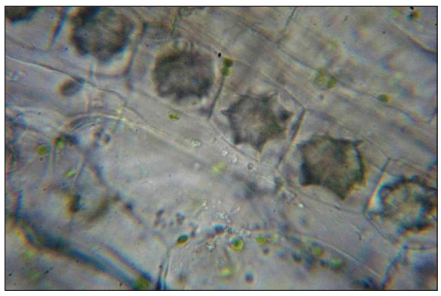


Figure 4: Rosette crystals of calcium oxalate

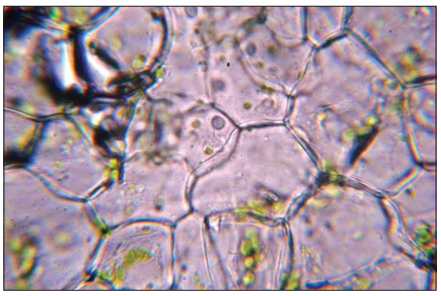
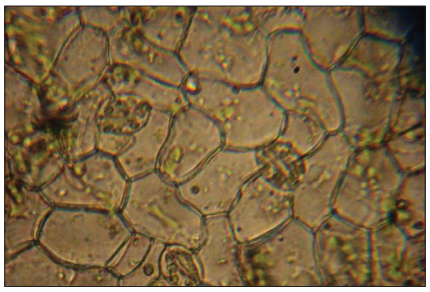


Figure 5: Epidermal cells



Figures 6: Anisocytic stomata

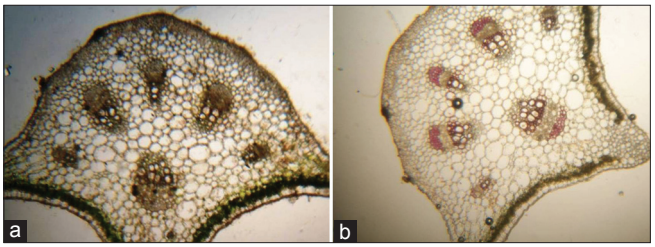


Figure 7: (a) T.S. of Leaf *C. repanda* (unstained) (b) T.S. of Leaf *C. repanda* (stained)

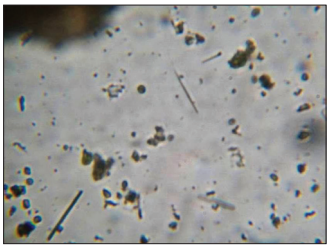


Figure 8: Acicular crystals

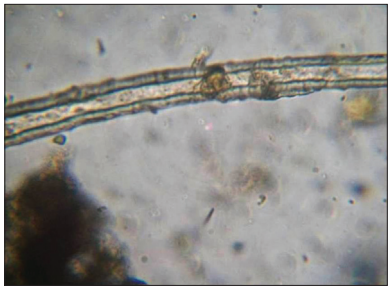


Figure 9: Fibre with lumen

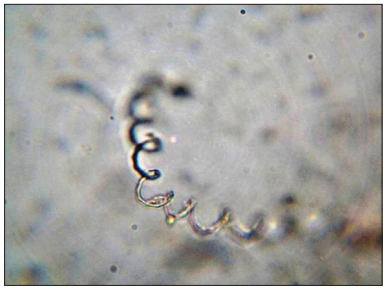


Figure 10: Spiral vessel

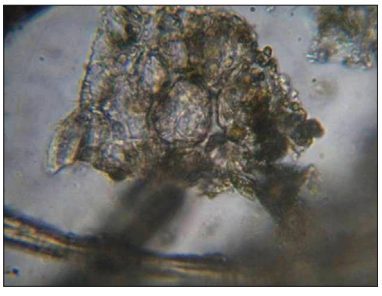


Figure 11: Spongy parenchyma

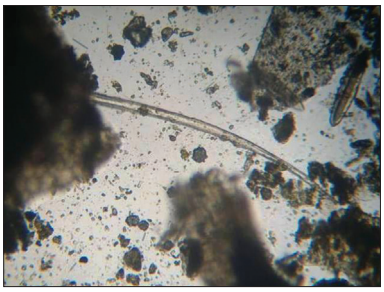


Figure 12: Simple trichome with rosette crystal

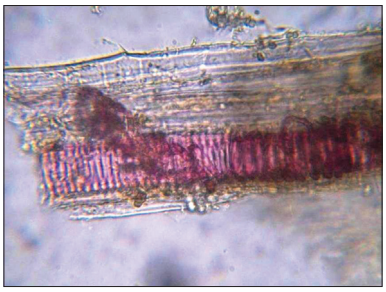


Figure 13: Annular vessel

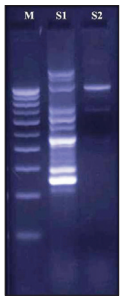


Figure 14: DNA fingerprints of *C. repanda* Note: M-100bo ladder ,S1- Sample *C. repanda* S2- Sample with random Primer

expression becomes necessary for providing clues about regulatory mechanisms, biochemical pathways, and broader cellular function.[12] The DNA fingerprint is generally independent of environment, and is consistent throughout different parts and developmental stages of the plant.

RAPD profile generated from genomic DNA isolated from fresh leaves found identical with 2 random primers. The number of unique bands are specific to genuine as well as other samples with different primers.

RAPD fingerprints of *C. repanda* run on 1% agarose gel with 100 bp ladder, random primer 21 (sample 1 *C. repanda* Vahl.) and random primer 22 (sample 2). The primers 22 discriminate the primer species of *C. repanda* by presence and absence of unique bands, that is, one prominent and two lighter bands, which act as markers for species authentification [Table 3]. The single primer in our study clearly discriminated the genuinity [Figure 14].

Conclusion

Establishing the standards is an integral part of establishing the correct identity and quality of a crude drug. Before any drug included in pharmacopoeia, these standards must be established. The majority of the information on the identity, purity, and quality of the plant material can be obtained from its macroscopy, microscopy, and DNA fingerprints. As there is no record on Pharmacognostical work on leaves of *C. repanda* Vahl, the present work was under taken to produce some pharmacognostical standards and the observations can be considered as reference standards in further studies.

Acknowledgement

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हिन्दी सारांश

सायसस रेपण्डा को सत्यापित करने हेतु रॅण्डम ऑम्प्लिफाईड पॉलीमॉरफिक डी. एन. ए. (आर.ए.पी.डी.) मार्कर्स का विकास

हरिशा सी. आर., रबिनारायण आचार्य, मालतीबेन जी. चौहान

सायसस रेपण्डा वाइटेसी परिवार की वनस्पति, हिन्दी में 'पानीवेल' नाम से जानी जाती है । यह विशाल आरोहणी लता है, जो संपूर्ण भारत में पायी जाती है । उड़ीसा के आदिवासी लोग और पारंपरिक वैद्य इसके रोपण गुण के कारण इसकी मूल के चूर्ण का अस्थि भग्न, व्रण, शोथ आदि में उपयोग करते हैं । इस बहुउपयोगी वनस्पति के पत्तों का अभी तक शास्त्रीय परीक्षण नहीं किया गया था । प्रस्तुत अध्ययन में इसके फार्माकोग्नॉस्टीकल एवं मॉलेकुलर कॅरेक्टरायझेशन, रॅण्डम ऑम्प्लिफाईड पॉलीमॉरफिक डी.एन.ए. (आर.ए.पी.डी.) मार्कर्स का विस्तार से अध्ययन किया गया है और फार्माकोपियल दृष्टिकोण से उसकी उपयोगिता का मानकीकरण किया गया है । आर्द्र पत्तों से डी.एन.ए. का पृथक्करण करके ऑम्प्लिफाय किया, विशेष रुप से इसमें म्युसीलेज, कॅल्सियम ऑक्झालेट रोसेट क्रिस्टल, स्पायरल वेसल्स और अधिक मात्रा में रेशे पाये गये । पी.सी.आर. ऑम्प्लिफिकेशन में विशिष्ट सफेद एवं काले बेण्ड्स पाये गये, उनसे इस वनस्पति की वास्तविकता एवं सत्यता प्रस्थापित होती है । इस प्रकार आर.ए.पी.डी. मार्कर गुणवत्ता नियन्त्रण हेतु उपयोगी मापदण्ड हो सकता है ।