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Morphological and molecular characterization of plaksha ficus lacor buch ham a medicinal bark

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Abstract
Introduction: *Plaksha (Ficus lacor)* a medicinal bark used in Indian system of medicine. Because of increased use of herbal products, similar looking nature of bark pieces and scarcity of natural products led to adulteration with other bark samples. Authentication parameters like macroscopy, microscopy can establish anatomical and physical features of a raw material. Molecular markers are nucleotide sequences of a plant material, which are unique for a particular species, devoid of its maturity, growth, geographical condition, part used.
Materials and Methods: Bark sample of *Ficus Lacor* collected from its natural habitat, macro-microscopic, powder microscopic features recorded. Fresh bark samples of *Panchavalkala, F. religiosa, F. benghalensis, F. racemosa, Thespesia populnea* and *F. Lacor* were collected from various parts of India. DNA extraction carried out through CTAB method. Templates of all the 5 species were used for RAPD**.** After amplification, Clones were confirmed by amplification using vector primers and sequenced and SCAR obtained.
Results: Macroscopically bark of *F. lacor* appear as rough, flat, curved with rough outer layer and reddish brown, fibrous internal surface. Transverse section of this bark sample shown outer 5-8 layers of cork tissue, secondary cortex with cell inclusion like prismatic crystals of calcium oxalate, elliptical stone cells etc. Powder of test drug showed cork cells, cork cambium, prismatic crystals and stone cells. Primer L gave an amplification product of 700 base pairs (positive) and does not give amplification with other species.

Keywords: *Plaksha* (*Ficus lacor*), Macro- microscopy, RAPD, DNA, SCAR

Introduction

Plaksha a popular bark drug used in Indian system of medicine, botanically identified as *Ficus lacor* Buch Ham. (*F. infectoria*), (*F.lucescens*), (*F. virens*) belonging to family *Moraceae* [1]. It also constitutes one among *Panchavalkala*, five bark drugs used as uterine tonic, styptic, bone healing [2]. Morphologically it is a large deciduous tree, without aerial roots from the branches, epiphytic in early stages, found throughout India, commonly planted as an avenue tree [3]. The bark decoction of this tree is used for menstrual disorder, leucorrhea, mouth ulcer, epistaxis, erysipelas. Ripe fruits used in diabetes, whereas leaves said to be estrogenic [4]. Beta sitosterol, methyl ricinolate, lanosterol, bergenin, triterpenoids, lupeol are few chemical constituents reported from stem bark [5].

Ficus is a genus coming under Family *Moraceae*. It is a large genus of trees or shrubs, often climbers, with milky juice, widely distributed throughout the tropics of both hemispheres, but abundant in south-east Asia and Polynesia. About 65 species occur in India. The genus is remarkable for the large variation in the habits of its species [6]. Bark of *Ficus tsjahela* is also added as source of *Plaksha* by few; whereas bark of *Panchavalkala* like *F. religiosa, F. racemosa, F. benghalensis, Thespesia populnea* are also said to be adulterated at different regions [7].

Increased demand and rapid commercialization of traditional medicines led to adulteration and substitution of authentic drugs [8]. But the therapeutic efficacy of the drugs used greatly depends upon proper and genuine raw materials. Hence assurance of safety, quality and efficacy of medicinal plants and herbal products has now become a key issue [9].

Morphological standardization like macroscopic recordings, microscopic structures form primitive phase in herbal material quality control method [10] Geographical condition, growth, maturity of a plant bring about changes in above said features.

Correct genotype identification of medicinal plant drug remains important for botanical industry [11]. Limitations of chemical and morphological approaches for authentication have generated need for newer methods in quality control of botanicals. Like all living organism, the differences that distinguish one plant from another are encoded in the plants genetic material, the deoxyribonucleic acid (DNA). DNA is the hereditary or genetic material, present in all cells, that carries information for the structure and function of living things [12]. Molecular markers are nucleotide sequences and can be investigated through the polymorphism present between the nucleotide sequences of different individuals [13]. Insertion, deletion, point mutations duplication and translocation are basis of these polymorphisms; however, they do not necessarily affect the activity of genes [14].

A scar marker is a geneomic DNA fragment that is identified by PCR amplification using a pair of specific oligonucleotide primers (Param and Michelmore 1993). SCARs are derived by cloning and sequencing the two ends of RAPD markers that

appeared to be diagnostic for specific purposes. SCARs are advantageous over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions and they can potentially be converted into co dominant markers [15]. Hence with above all discussions it has been decided to develop DNA SCAR marker for *Plaksa (Ficus lacor* Buch Ham.*)* a multiutility therapeutic agent.

Materials and Methods

Plant Material

Bark sample of *Ficus Lacor* collected from its natural habitat, authenticated, photographs taken, kept in FAA (Formalin-5ml + Acetic acid-5ml + 70% Ethyl alcohol-90ml) solution for microscopic study. Few pieces shade dried powdered and used for powder microscopy.

Fresh bark samples of *Panchavalkala (F. religiosa, F. benghalensis, F. racemosa, Thespesia populnea* and *F. Lacor*) were collected from various parts of India. Plant samples authenticated, all pieces labelled separately, and frozen at -70°C till further molecular marker study.

Macroscopy: External features of bark pieces were recorded using camera and its colour, shape, size, any particular smell was recorded [16].

Microscopy: The bark sample preserved were cut into thin transverse section using a sharp blade and the sections stained with saffranine. Transverse sections photographed using Zeiss AXIO trinocular microscope attached with Zeiss AxioCam camera under bright field light. Magnifications of the figures are indicated by the scale-bars [17].

Powder Microscopy

A pinch of test drug powder was warmed with drops of chloral hydrate on a microscopic slide and mounted in glycerine. Slide observed, photographed and characteristic features marked [18].

DNA SCAR Marker Development

Preparation of bark powder: Above collected Bark samples were crushed into small pieces using autoclaved pestle and mortar and powdered thoroughly using dry ice intermittently. Separate pestle and mortar were used for each sample and powder was collected in a fresh container till further use.

CTAB method DNA Extraction: Next step will be DNA isolation, attempted with different methods including urea method, DNAzol method, CTAB method and modified CTAB method. CTAB method DNA isolation was found better for RAPD PCR, though the yields were better with other methods. Ethanol and water washed bark powder was homogenized with CTAB buffer thoroughly. Beta Mercapto ethanol was added and incubated for 90 minutes at 60^0C in a water bath. This was centrifuged at 10,000 rpm for 15 minutes and supernatant was decanted the into fresh tubes. Equal volume of 100% chloroform was added and mixed well for 10 minutes by inverting tube. This was, centrifuged at 10,000 rpm for five minutes. Separated aqueous layer was transferred in to fresh tube and added equal volumes of iso-propyl alcohol. Centrifuged at 10,000 rpm for 15 minutes. Discarded supernatant and washed DNA pellets with 0.5 ml of 70% ethanol and centrifuged at 10,000 rpm for 5 minutes. Discarded ethanol and air dried DNA pellet. DNA was suspended in 1X TE buffer. The DNA was further purified using silica membrane based column and quantitated on agarose gel [19].

Rapd PCR: Templates of all the 5 species were used for RAPD**.** PCR was set using master mix consisting of 100µM each of dNTPs, 100mM KCl, 1.5mM MgCl2, 0.1% each of tween 20 and NonidetP40, 15 p moles of random primer and 1u of taq polymerase in 40 µl volume. 100ng of template was used. PCR Cycle conditions were, initial denaturation at 94˚C for 5 minutes, denaturation for 30 seconds, annealing for 1 minute at 45˚C, extension for 90 seconds at 72˚C and final extension for another 7 minutes [20].

After amplification, the samples were run in 2% agarose gel using DNA marker with 0.1, 0.2, 0.3, 0.6, 1.0, 1.5, 2, 2.5, 3 and 3.5 Kb fragments.

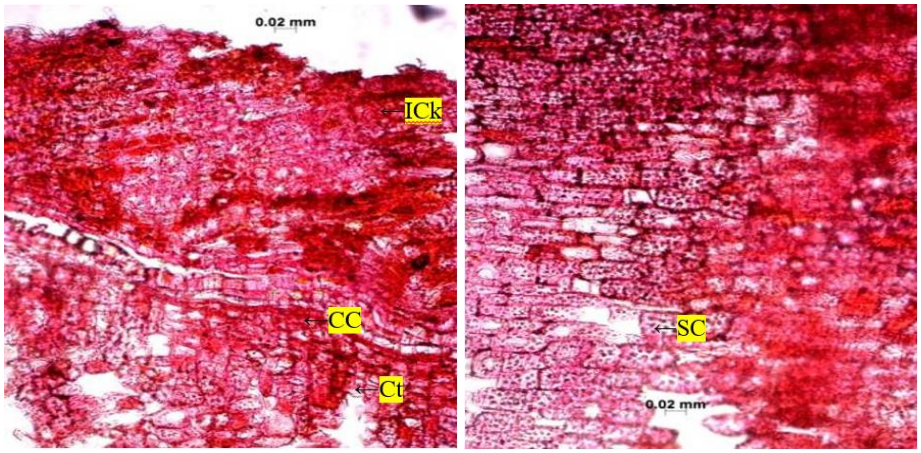
Unique band will be observed for particular species which is not seen in other species. This fragment will be gel purifie, amplified and used for cloning into T vector. Clones will be confirmed by amplification using vector primers and will be sequenced. Sequence obtained will be analysed and primers designed. Randomly selected ficus samples were used for checking the efficacy of the primers designed.

Results

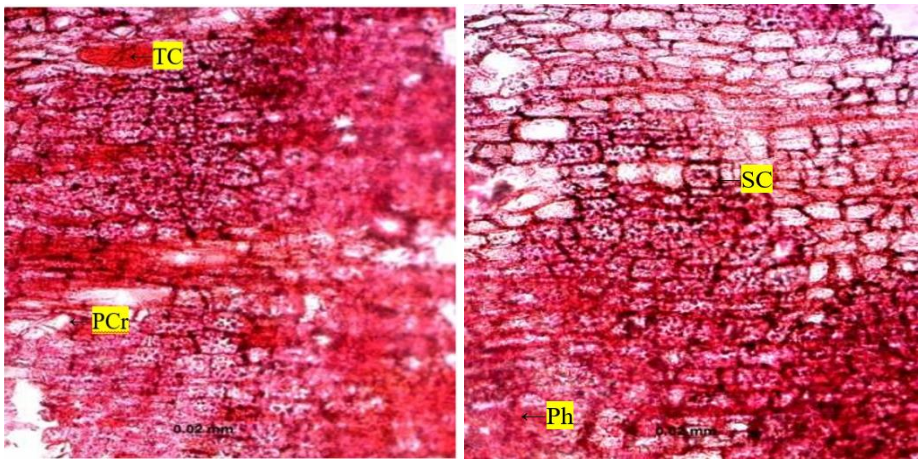
Macroscopy: Rough, flat to curved; external surface ash or whitish-grey; transversely arranged lenticels common, lip-shaped and exfoliating; internal surface rough, fibrous, longitudinally striated, reddish-brown; fracture, fibrous, astringent. (Figure 1)

Microscopy: 5-8 layered cork consisting of thin-walled, rectangular cells, a few external layers exfoliating; secondary cortex very wide consisting of compactly arranged, rectangular, thick-walled, pitted cells, patches of circular to elongated, lignified, elliptical stone cells with striations; a few prismatic crystals of calcium oxalate and reddish-brown contents found scattered throughout the secondary cortex; secondary phloem very wide consisting of mostly stratified layers of collapsed cells forming ceratenchyma, groups of fibres, phloem parenchyma, laticiferous cells, traversed by 2-5 seriatephloem rays. (Figure 2)

Fig 1: Bark *Ficus lacor* Buch.-Ham

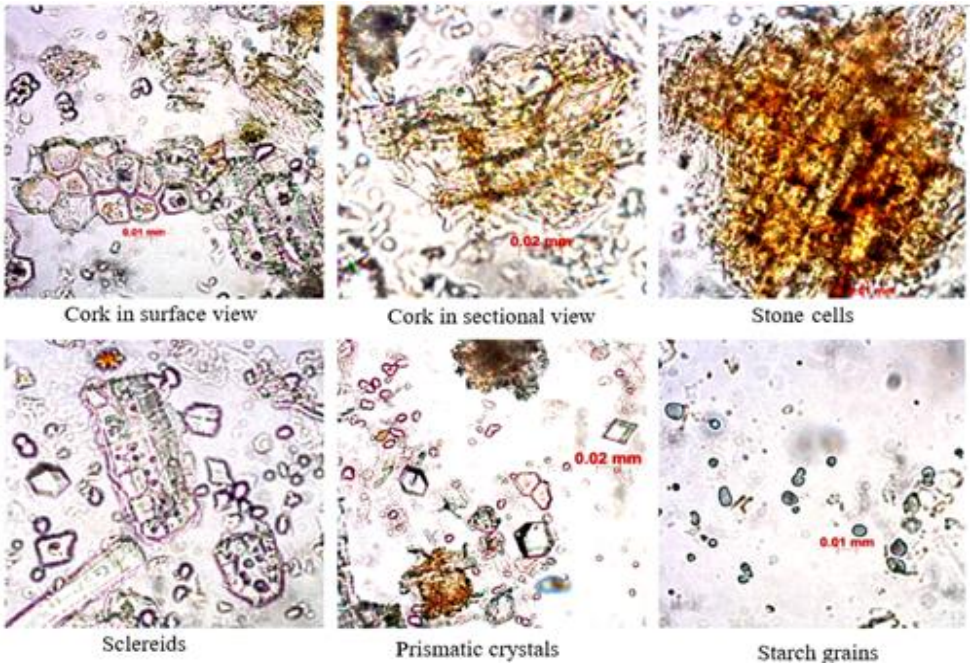


Cork and cortex Sclereids



Sclereids and Crystals Phloem

Fig 2: TS of bark *Ficus lacor* Buch.-Ham



CC - Cork cambium, Ct - Cortex, ICk - Irregular cork, PCr - Prismatic Crystal, Ph - Phloem, SC-Stone cells, TC - Tannin cell.

Fig 3: Powder microscopy *F. lacor* Buch.-Ham

Powder Microscopy

Powder shows cork cells, cork cambium, irregular pieces of cork cells. Apart from these prismatic crystals, stone cells, tannin filled cells, few phloem fibers also found. (Figure 3)

Acceptance criteria

Primer L: gives an amplification product of 700 base pairs (positive) and does not give amplification with other species. (Figure 4)

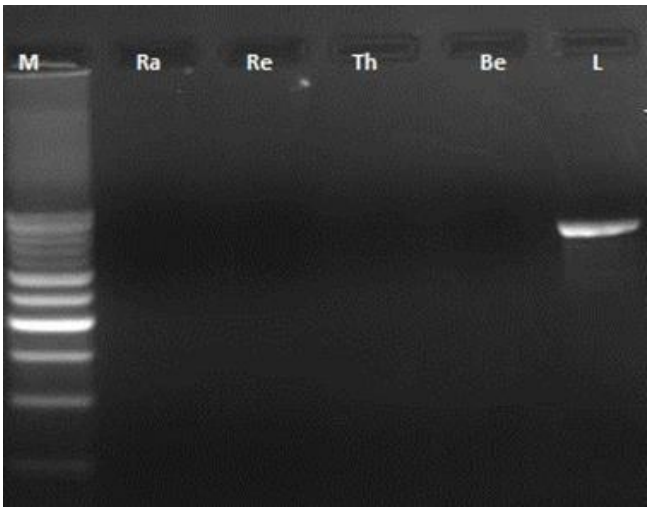


Fig 4: Amplification of *F. racemosa, F. religious, T. populanea and F. lacor* with L primer set.

M-DNA ruler with fragments of 0.1, 0.2, 0.3, 0. 4, 0. 5, 0. 6, 0. 7, 0. 8, 0. 9 & 1Kb.

Discussion

Plaksha (F.lacor) popularly used bark in Indian system of medicine which also constitute one among *Panchavalkala* (five bark drugs). This is a big tree belonging to *Moraceae* family. Bark, fruits, latex, tender foliar buds of this tree are used in medicine in various pathological condition. Because of increased use of herbal products, similar looking nature of bark pieces and scarcity of *Ficus lacor*, led to adulteration with other bark samples of *F. tsjahela*, *F. callosa*, and other drugs of *panchavalkala (F. religiosa, F. racemose, F. benghalensis and Thespesia populnea)*. Genuine herbal products used in treatment can bring about effective treatment [21]. Authentication parameters like macroscopy, microscopy, Physicochemical standards can establish anatomical and physical features of a raw material [22]. Molecular markers are nucleotide sequences of a plant material, which are unique for a particular species, devoid of its maturity, growth, Geographical condition, part used [23]. Hence form as a reliable phase in herbal drug standardization though an expensive one. DNA SCAR marker is a genomic DNA fragment which is identified by PCR using specific primer.

Macroscopically bark of *F. lacor* appear as rough, flat, curved with rough outer layer and reddish brown, fibrous internal surface.

Transverse section of this bark sample shown outer 5-8 layers of cork tissue, secondary cortex with cell inclusion like prismatic crystals of calcium oxalate, elliptical stone cells etc. Wide secondary phloem found towards center with laticiferous cells and phloem parenchyma. Powder of test drug showed cork cells, cork cambium, prismatic crystals and stone cells.

Fresh bark sample of *Panchavalkala* (*F. religiosa, F. racemose, F. benghalensis and Thespesia populnea along with F.lacor*) were collected from different parts of India. Powdered separately and were labelled properly. DNA extraction attempted with CTAB method as yield and quality of DNA found satisfactory. RAPD PCR was set using templates of all five species under standard condition. After amplification, Unique band of will be observed for F. lacor which is not seen in other species. This fragment will be gel purified, amplified and used for cloning into T vector. Clones confirmed by amplification using vector primers and sequenced. Primer L gave an amplification product of 700 base pairs (positive) and does not give amplification with other species.

Conclusion

Non-availability of herbal material, there by spurious adulteration with other herbal products are major threatening issues in herbal drug practice. Morphological standardization though initial authentication procedure, molecular markers will serve ultimate genomic print of herbal material. *F. lacor* morphological and genomic study results presented in this paper will serve as quality marker of this drug.

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